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I *Acts adopted under the EC Treaty/Euratom Treaty whose publication is obligatory*

REGULATIONS

★ **Council Regulation (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH)** [1] 1

Price: 82,50 EUR

[1] Text with EEA relevance

EN

Acts whose titles are printed in light type are those relating to day-to-day management of agricultural matters, and are generally valid for a limited period.

The titles of all other acts are printed in bold type and preceded by an asterisk.

I

(Acts adopted under the EC Treaty/Euratom Treaty whose publication is obligatory)

REGULATIONS

COUNCIL REGULATION (EC) No 440/2008

of 30 May 2008

laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH)

(Text with EEA relevance)

THE COMMISSION OF THE EUROPEAN COMMUNITIES,

Having regard to the Treaty establishing the European Community,

Having regard to Regulation (EC) No 1907/2006 of 18 December 2006 of the European Parliament and of the Council concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No 793/93 and Commission Regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC ([1]), and in particular Article 13(3)thereof,

Whereas:

(1) Pursuant to Regulation (EC) No 1907/2006, test methods are to be adopted at Community level for the purposes of tests on substances where such tests are required to generate information on intrinsic properties of substances.

(2) Council Directive 67/548/EEC of 27 June 1967 on the approximation of the laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances ([2]), laid down, in Annex V, methods for the determination of the physico-chemical properties, toxicity and ecotoxicity of substances and preparations. Annex V to Directive 67/548/EEC has been deleted by Directive 2006/121/EC of the European Parliament and of the Council with effect from 1 June 2008.

(3) The test methods contained in Annex V to Directive 67/548/EEC should be incorporated into this Regulation.

(4) This Regulation does not exclude the use of other test methods, provided that their use is in accordance with Article 13(3) of Regulation 1907/2006.

(5) The principles of replacement, reduction and refinement of the use of animals in procedures should be fully taken into account in the design of the test methods, in particular when appropriate validated methods become available to replace, reduce or refine animal testing.

(6) The provisions of this Regulation are in accordance with the opinion of the Committee established under Article 133 of Regulation (EC) No 1907/2006,

HAS ADOPTED THIS REGULATION:

Article 1

The test methods to be applied for the purposes of Regulation 1907/2006/EC are set out in the Annex to this Regulation.

Article 2

The Commission shall review, where appropriate, the test methods contained in this Regulation with a view to replacing, reducing or refining testing on vertebrate animals.

([1]) OJ L 396, 30.12.2006, p. 1, as corrected by OJ L 136, 29.5.2007, p. 3.

([2]) OJ 196, 16.8.1967, p. 1. Directive as last amended by Directive 2006/121/CE of the European Parliament and of the Council (OJ L 396, 30.12.2006, p. 850, as corrected by OJ L 136, 29.5.2007, p. 281).

Article 3

All references to Annex V to Directive 67/548/EEC shall be construed as references to this Regulation.

Article 4

This Regulation shall enter into force on the day following its publication in the *Official Journal of the European Union*.

It shall apply from 1 June 2008.

Done at Brussels, 30 May 2008.

For the Commission

Stavros DIMAS

Member of the Commission

ANNEX

PART A: METHODS FOR THE DETERMINATION OF PHYSICO-CHEMICAL PROPERTIES

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A.1. MELTING/FREEZING TEMPERATURE

1. METHOD

The majority of the methods described are based on the OECD Test Guideline (1). The fundamental principles are given in references (2) and (3).

1.1. INTRODUCTION

The methods and devices described are to be applied for the determination of the melting temperature of substances, without any restriction with respect to their degree of purity.

The selection of the method is dependent on the nature of the substance to be tested. In consequence the limiting factor will be according to, whether or not the substance can be pulverised easily, with difficulty, or not at all.

For some substances, the determination of the freezing or solidification temperature is more appropriate and the standards for these determinations have also been included in this method.

Where, due to the particular properties of the substance, none of the above parameters can be conveniently measured, a pour point may be appropriate.

1.2. DEFINITIONS AND UNITS

The melting temperature is defined as the temperature at which the phase transition from solid to liquid state occurs at atmospheric pressure and this temperature ideally corresponds to the freezing temperature.

As the phase transition of many substances takes place over a temperature range, it is often described as the melting range.

Conversion of units (K to °C)

$t = T - 273{,}15$

t: Celsius temperature, degree Celsius (°C)
T: thermodynamic temperature, kelvin (K)

1.3. REFERENCE SUBSTANCES

Reference substances do not need to be employed in all cases when investigating a new substance. They should primarily serve to check the performance of the method from time to time and to allow comparison with results from other methods.

Some calibration substances are listed in the references (4).

1.4. PRINCIPLE OF THE TEST METHOD

The temperature (temperature range) of the phase transition from the solid to the liquid state or from the liquid to the solid state is determined. In practice while heating/cooling a sample of the test substance at atmospheric pressure the temperatures of the initial melting/freezing and the final stage of melting/freezing are determined. Five types of methods are described, namely capillary method, hot stage methods, freezing temperature determinations, methods of thermal analysis, and determination of the pour point (as developed for petroleum oils).

In certain cases, it may be convenient to measure the freezing temperature in place of the melting temperature.

1.4.1. **Capillary method**

1.4.1.1. *Melting temperature devices with liquid bath*

A small amount of the finely ground substance is placed in a capillary tube and packed tightly. The tube is heated, together with a thermometer, and the temperature rise is adjusted to less than about 1 K/min during the actual melting. The initial and final melting temperatures are determined.

1.4.1.2. *Melting temperature devices with metal block*

As described under 1.4.1.1, except that the capillary tube and the thermometer are situated in a heated metal block, and can be observed through holes in the block.

1.4.1.3. *Photocell detection*

The sample in the capillary tube is heated automatically in a metal cylinder. A beam of light is directed through the substance, by way of a hole in the cylinder, to a precisely calibrated photocell. The optical properties of most substances change from opaque to transparent when they are melting. The intensity of light reaching the photocell increases and sends a stop signal to the digital indicator reading out the temperature of a platinum resistance thermometer located in the heating chamber. This method is not suitable for some highly coloured substances.

1.4.2. **Hot stages**

1.4.2.1. *Kofler hot bar*

The Kofler hot bar uses two pieces of metal of different thermal conductivity, heated electrically, with the bar designed so that the temperature gradient is almost linear along its length. The temperature of the hot bar can range from 283 to 573 K with a special temperature-reading device including a runner with a pointer and tab designed for the specific bar. In order to determine a melting temperature, the substance is laid, in a thin layer, directly on the surface of the hot bar. In a few seconds a sharp dividing line between the fluid and solid phase develops. The temperature at the dividing line is read by adjusting the pointer to rest at the line.

1.4.2.2. *Melt microscope*

Several microscope hot stages are in use for the determination of melting temperatures with very small quantities of material. In most of the hot stages the temperature is measured with a sensitive thermocouple but sometimes mercury thermometers are used. A typical microscope hot stage melting temperature apparatus has a heating chamber which contains a metal plate upon which the sample is placed on a slide. The centre of the metal plate contains a hole permitting the entrance of light from the illuminating mirror of the microscope. When in use, the chamber is closed by a glass plate to exclude air from the sample area.

The heating of the sample is regulated by a rheostat. For very precise measurements on optically anisotropic substances, polarised light may be used.

1.4.2.3. *Meniscus method*

This method is specifically used for polyamides.

The temperature at which the displacement of a meniscus of silicone oil, enclosed between a hot stage and a cover-glass supported by the polyamide test specimen, is determined visually.

1.4.3. **Method to determine the freezing temperature**

The sample is placed in a special test tube and placed in an apparatus for the determination of the freezing temperature. The sample is stirred gently and continuously during cooling and the temperature is measured at suitable intervals. As soon as the temperature remains constant for a few readings this temperature (corrected for thermometer error) is recorded as the freezing temperature.

Supercooling must be avoided by maintaining equilibrium between the solid and the liquid phases.

1.4.4. **Thermal analysis**

1.4.4.1 *Differential thermal analysis (DTA)*

This technique records the difference in temperatures between the substance and a reference material as a function of temperature, while the substance and reference material are subjected to the same controlled temperature programme. When the sample undergoes a transition involving a change of enthalpy, that change is indicated by an endothermic (melting) or exothermic (freezing) departure from the base line of the temperature record.

1.4.4.2 *Differential scanning calorimetry (DSC)*

This technique records the difference in energy inputs into a substance and a reference material, as a function of temperature, while the substance and reference material are subjected to the same controlled temperature programme. This energy is the energy necessary to establish zero temperature difference between the substance and the reference material. When the sample undergoes a transition involving a change of enthalpy, that change is indicated by an endothermic (melting) or exothermic (freezing) departure from the base line of the heat flow record.

1.4.5. **Pour point**

This method was developed for use with petroleum oils and is suitable for use with oily substances with low melting temperatures.

After preliminary heating, the sample is cooled at a specific rate and examined at intervals of 3 K for flow characteristics. The lowest temperature at which movement of the substance is observed is recorded as the pour point.

1.5. QUALITY CRITERIA

The applicability and accuracy of the different methods used for the determination of the melting temperature/ melting range are listed in the following table:

TABLE: APPLICABILITY OF THE METHODS

A. **Capillary methods**

Method of measurement	Substances which can be pulverised	Substances which are not readily pulverised	Temperature range	Estimated accuracy [1]	Existing standards
Melting temperature devices with liquid bath	yes	only to a few	273 to 573 K	± 0,3 K	JIS K 0064
Melting temperature with metal block	yes	only to a few	293 to >573 K	± 0,5 K	ISO 1218 (E)
Photocell detection	yes	several with appliance devices	253 to 573 K	± 0,5 K	

[1] Dependent on type of instrument and on degree of purity of the substance.

B. **Hot stages and freezing methods**

Method of measurement	Substances which can be pulverised	Substances which are not readily pulverised	Temperature range	Estimated accuracy (1)	Existing standards
Kofler hot bar	yes	no	283 to > 573 K	± 1K	ANSI/ASTM D 3451-76
Melt microscope	yes	only to a few	273 to > 573 K	± 0,5 K	DIN 53736
Meniscus method	no	specifically for polyamides	293 to > 573 K	± 0,5 K	ISO 1218 (E)
Freezing temperature	yes	yes	223 to 573 K	± 0,5 K	e.g. BS 4695

(1) Dependent on type of instrument and on degree of purity of the substance

C. **Thermal analysis**

Method of measurement	Substances which can be pulverised	Substances which are not readily pulverised	Temperature range	Estimated accuracy (1)	Existing standards
Differential thermal analysis	yes	yes	173 to 1 273 K	up to 600 K ± 0,5 K up to 1 273 K ± 2,0 K	ASTM E 537-76
Differential scanning calorimetry	yes	yes	173 to 1 273 K	up to 600 K ± 0,5 K up to 1 273 K ± 2,0 K	ASTM E 537-76

(1) Dependent on type of instrument and on degree of purity of the substance

D. **Pour point**

Method of measurement	Substances which can be pulverised	Substances which are not readily pulverised	Temperature range	Estimated accuracy (1)	Existing standards
Pour point	for petroleum oils and oily substances	for petroleum oils and oily substances	223 to 323 K	± 0,3 K	ASTM D 97-66

(1) Dependent on type of instrument and on degree of purity of the substance

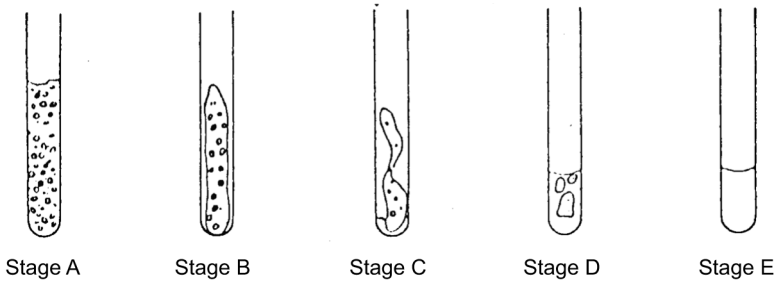
1.6. DESCRIPTION OF THE METHODS

The procedures of nearly all the test methods have been described in international and national standards (see Appendix 1).

1.6.1. **Methods with capillary tube**

When subjected to a slow temperature rise, finely pulverised substances usually show the stages of melting shown in figure 1.

Figure 1



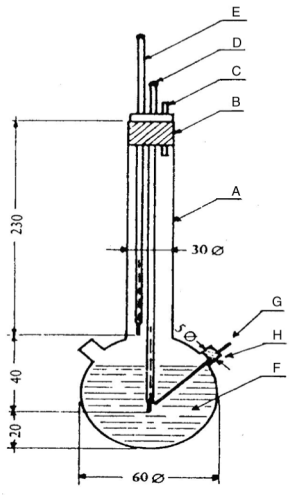
Stage A	(beginning of melting): fine droplets adhere uniformly to the inside wall of the capillary tube
Stage B	a clearance appears between the sample and the inside wall due to shrinkage of the melt
Stage C	the shrunken sample begins to collapse downwards and liquefies
Stage D	a complete meniscus is formed at the surface but an appreciate amount of the sample remains solid
Stage E	(final stage melting): there are no solid particles

During the determination of the melting temperature, the temperatures are recorded at the beginning of the melting and at the final stage.

1.6.1.1. *Melting temperature devices with liquid bath apparatus*

Figure 2 shows a type of standardised melting temperature apparatus made of glass (JIS K 0064); all specifications are in millimeters.

Figure 2



A: Measurement vessel
B: Stopper
C: Vent
D: Thermometer
E: Auxiliary thermometer
F: Bath liquid
G: Capillary tube made of glass, 80 to 100 mm in length, 1,0 ± 0,2 mm inner diameter, 0,2 to 0,3 mm wall thickness
H: Side tube

Bath liquid:

A suitable liquid should be chosen. The choice of the liquid depends upon the melting temperature to be determined, e.g. liquid paraffin for melting temperatures no higher than 473 K, silicone oil for melting temperatures no higher than 573 K.

For melting temperatures above 523 K, a mixture consisting of three parts sulphuric acid and two parts potassium sulphate (in mass ratio) can be used. Suitable precautions should be taken if a mixture such as this is used.

Thermometer:

Only those thermometers should be used which fulfil the requirements of the following or equivalent standards:

ASTM E 1-71, DIN 12770, JIS K 8001.

Procedure:

The dry substance is finely pulverised in a mortar and is put into the capillary tube, fused at one end, so that the filling level is approximately 3 mm after being tightly packed. To obtain a uniform packed sample, the capillary tube should be dropped from a height of approximately 700 mm through a glass tube vertically onto a watch glass.

The filled capillary tube is placed in the bath so that the middle part of the mercury bulb of the thermometer touches the capillary tube at the part where the sample is located. Usually the capillary tube is introduced into the apparatus about 10 K below the melting temperature.

The bath liquid is heated so that the temperature rise is approximately 3 K/min. The liquid should be stirred. At about 10 K below the expected melting temperature the rate of temperature rise is adjusted to a maximum of 1 K/min.

Calculation:

The calculation of the melting temperature is as follows:

$$T = T_D + 0{,}00016\ (T_D - T_E)\ n$$

where:

T = corrected melting temperature in K
T_D = temperature reading of thermometer D in K
T_E = temperature reading of thermometer E in K
n = number of graduations of mercury thread on thermometer D at emergent stem.

1.6.1.2. *Melting temperature devices with metal block*

Apparatus:

This consists of:

— a cylindrical metal block, the upper part of which is hollow and forms a chamber (see figure 3),

— a metal plug, with two or more holes, allowing tubes to be mounted into the metal block,

— a heating system, for the metal block, provided for example by an electrical resistance enclosed in the block,

— a rheostat for regulation of power input, if electric heating is used,

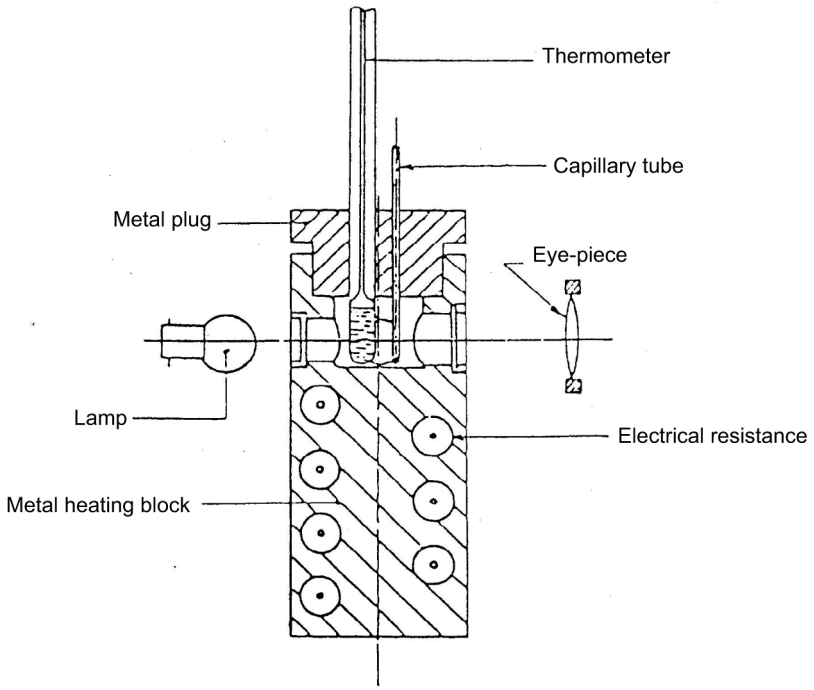
— four windows of heat-resistant glass on the lateral walls of the chamber, diametrically disposed at right-angles to each other. In front of one of these windows is mounted an eye-piece for observing the capillary tube. The other three windows are used for illuminating the inside of the enclosure by means of lamps,

— a capillary tube of heat-resistant glass closed at one end (see 1.6.1.1).

Thermometer:

See standards mentioned in 1.6.1.1. Thermoelectrical measuring devices with comparable accuracy are also applicable.

Figure 3



1.6.1.3. *Photocell detection*

Apparatus and procedure:

The apparatus consists of a metal chamber with automated heating system. Three capillary are filled accordingly to 1.6.1.1 and placed in the oven.

Several linear increases of temperature are available for calibrating the apparatus and the suitable temperature rise is electrically adjusted at a pre-selected constant and linear rate. recorders show the actual oven temperature and the temperature of the substance in the capillary tubes.

1.6.2. **Hot stages**

1.6.2.1. *Kofler hot bar*

See Appendix.

1.6.2.2. *Melt microscope*

See Appendix.

1.6.2.3. *Meniscus method (polyamides)*

See Appendix.

The heating rate through the melting temperature should be less than 1 K/min.

1.6.3. **Methods for the determination of the freezing temperature**

See Appendix.

1.6.4. **Thermal analysis**

1.6.4.1. *Differential thermal analysis*

See Appendix.

1.6.4.2. *Differential scanning calorimetry*

See Appendix.

1.6.5. **Determination of the pour point**

See Appendix.

2. **DATA**

A thermometer correction is necessary in some cases.

3. **REPORTING**

The test report shall, if possible, include the following information:

— method used,

— precise specification of the substance (identity and impurities) and preliminary purification step, if any,

— an estimate of the accuracy.

The mean of at least two measurements which are in the range of the estimated accuracy (see tables) is reported as the melting temperature.

If the difference between the temperature at the beginning and at the final stage of melting is within the limits of the accuracy of the method, the temperature at the final stage of melting is taken as the melting temperature; otherwise the two temperatures are reported.

If the substance decomposes or sublimes before the melting temperature is reached, the temperature at which the effect is observed shall be reported.

All information and remarks relevant for the interpretation of results have to be reported, especially with regard to impurities and physical state of the substance.

4. **REFERENCES**

(1) OECD, Paris, 1981, Test Guideline 102, Decision of the Council C(81) 30 final.

(2) IUPAC, B. Le Neindre, B. Vodar, eds. Experimental thermodynamics, Butterworths, London 1975, vol. II, p. 803-834.

(3) R. Weissberger ed.: Technique of organic Chemistry, Physical Methods of Organic Chemistry, 3rd ed., Interscience Publ., New York, 1959, vol. I, Part I, Chapter VII.

(4) IUPAC, Physicochemical measurements: Catalogue of reference materials from national laboratories, Pure and applied chemistry, 1976, vol. 48, p. 505-515.

Appendix

For additional technical details, the following standards may be consulted for example.

1. **Capillary methods**

1.1. Melting temperature devices with liquid bath

ASTM E 324-69	Standard test method for relative initial and final melting points and the melting range of organic chemicals
BS 4634	Method for the determination of melting point and/or melting range
DIN 53181	Bestimmung des Schmelzintervalles von Harzen nach Kapillarverfarehn
JIS K 00-64	Testing methods for melting point of chemical products

1.2. Melting temperature devices with metal block

DIN 53736	Visuelle Bestimmung der Schmelztemperatur von teilkristallinen Kunststoffen
ISO 1218 (E)	Plastics — polyamides — determination of 'melting point'

2. **Hot stages**

2.1. Kofler hot bar

ANSI/ASTM D 3451-76	Standard recommended practices for testing polymeric powder coatings

2.2. Melt microscope

DIN 53736	Visuelle Bestimmung der Schmelztemperatur von teilkristallinen Kunststoffen

2.3. Meniscus method (polyamides)

ISO 1218 (E)	Plastics — polyamides — determination of 'melting point'
ANSI/ASTM D 2133-66	Standard specification for acetal resin injection moulding and extrusion materials
NF T 51-050	Résines de polyamides. Détermination du 'point de fusion' méthode du ménisque

3. **Methods for the determination of the freezing temperature**

BS 4633	Method for the determination of crystallising point
BS 4695	Method for Determination of Melting Point of petroleum wax (Cooling Curve)
DIN 51421	Bestimmung des Gefrierpunktes von Flugkraftstoffen, Ottokraftstoffen und Motorenbenzolen
ISO 2207	Cires de pétrole: détermination de la température de figeage
DIN 53175	Bestimmung des Erstarrungspunktes von Fettsäuren
NF T 60-114	Point de fusion des paraffines
NF T 20-051	Méthode de détermination du point de cristallisation (point de congélation)
ISO 1392	Method for the determination of the freezing point

4. **Thermal analysis**

4.1. Differential thermal analysis

ASTM E 537-76	Standard method for assessing the thermal stability of chemicals by methods of differential thermal analysis
ASTM E 473-85	Standard definitions of terms relating to thermal analysis
ASTM E 472-86	Standard practice for reporting thermoanalytical data
DIN 51005	Thermische Analyse, Begriffe

4.2. Differential scanning calorimetry

ASTM E 537-76	Standard method for assessing the thermal stability of chemicals by methods of differential thermal analysis
ASTM E 473-85	Standard definitions of terms relating to thermal analysis
ASTM E 472-86	Standard practice for reporting thermoanalytical data
DIN 51005	Thermische Analyse, Begriffe

5. **Determination of the pour point**

NBN 52014	Echantillonnage et analyse des produits du pétrole: Point de trouble et point d'écoulement limite — Monsterneming en ontleding van aardolieproducten: Troebelingspunt en vloeipunt
ASTM D 97-66	Standard test method for pour point of petroleum oils
ISO 3016	Petroleum oils — Determination of pour point

A.2. BOILING TEMPERATURE

1. METHOD

The majority of the methods described are based on the OECD Test Guideline (1). The fundamental principles are given in references (2) and (3).

1.1. INTRODUCTION

The methods and devices described here can be applied to liquid and low melting substances, provided that these do not undergo chemical reaction below the boiling temperature (for example: auto-oxidation, rearrangement, degradation, etc.). The methods can be applied to pure and to impure liquid substances.

Emphasis is put on the methods using photocell detection and thermal analysis, because these methods allow the determination of melting as well as boiling temperatures. Moreover, measurements can be performed automatically.

The 'dynamic method' has the advantage that it can also be applied to the determination of the vapour pressure and it is not necessary to correct the boiling temperature to the normal pressure (101,325 kPa) because the normal pressure can be adjusted during the measurement by a manostat.

Remarks:

The influence of impurities on the determination of the boiling temperature depends greatly upon the nature of the impurity. When there are volatile impurities in the sample, which could affect the results, the substance may be purified.

1.2. DEFINITIONS AND UNITS

The normal boiling temperature is defined as the temperature at which the vapour pressure of a liquid is 101,325 kPa.

If the boiling temperature is not measured at normal atmospheric pressure, the temperature dependence of the vapour pressure can be described by the Clausius-Clapeyron equation:

$$\log p = \frac{\Delta H_v}{2{,}3RT} + const.$$

where:

p = the vapour pressure of the substance in pascals
ΔH_v = its heat of vaporisation in J mol^{-1}
R = the universal molar gas constant = 8,314 J mol^{-1} K^{-1}
T = thermodynamic temperature in K

The boiling temperature is stated with regard to the ambient pressure during the measurement.

Conversions

Pressure (units: kPa)

100 kPa = 1 bar = 0,1 MPa
('bar' is still permissible but not recommended)
133 Pa = 1 mm Hg = 1 Torr
(the units 'mm Hg' and 'Torr' are not permitted)
1 atm = standard atmosphere = 101 325 Pa
(the unit 'atm' is not permitted)

Temperature (units: K)

$t = T - 273{,}15$

t: Celsius temperature, degree Celsius (°C)
T: thermodynamic temperature, kelvin (K)

1.3. REFERENCE SUBSTANCES

Reference substances do not need to be employed in all cases when investigating a new substance. They should primarily serve to check the performance of the method from time to time and to allow comparison with results from other methods.

Some calibration substances can be found in the methods listed in the Appendix.

1.4. PRINCIPLE OF THE TEST METHOD

Five methods for the determination of the boiling temperature (boiling range) are based on the measurement of the boiling temperature, two others are based on thermal analysis.

1.4.1. **Determination by use of the ebulliometer**

Ebulliometers were originally developed for the determination of the molecular weight by boiling temperature elevation, but they are also suited for exact boiling temperature measurements. A very simple apparatus is described in ASTM D 1120-72 (see Appendix). The liquid is heated in this apparatus under equilibrium conditions at atmospheric pressure until it is boiling.

1.4.2. **Dynamic method**

This method involves the measurement of the vapour recondensation temperature by means of an appropriate thermometer in the reflux while boiling. The pressure can be varied in this method.

1.4.3. **Distillation method for boiling temperature**

This method involves distillation of the liquid and measurement of the vapour recondensation temperature and determination of the amount of distillate.

1.4.4. **Method according to Siwoloboff**

A sample is heated in a sample tube, which is immersed in a liquid in a heat-bath. A fused capillary, containing an air bubble in the lower part, is dipped in the sample tube.

1.4.5. **Photocell detection**

Following the principle according to Siwoloboff, automatic photo-electrical measurement is made using rising bubbles.

1.4.6. **Differential thermal analysis**

This technique records the difference in temperatures between the substance and a reference material as a function of temperature, while the substance and reference material are subjected to the same controlled temperature programme. When the sample undergoes a transition involving a change of enthalpy, that change is indicated by an endothermic departure (boiling) from the base line of the temperature record.

1.4.7. **Differential scanning calorimetry**

This technique records the difference in energy inputs into a substance and a reference material as a function of temperature, while the substance and reference material are subjected to the same controlled temperature programme. This energy is the energy necessary to establish zero temperature difference between the substance and the reference material. When the sample undergoes a transition involving a change of enthalpy, that change is indicated by an endothermic departure (boiling) from the base line of the heat flow record.

1.5. QUALITY CRITERIA

The applicability and accuracy of the different methods used for the determination of the boiling temperature/boiling range are listed in table 1.

Table 1:

Comparison of the methods

Method of measurement	Estimated accuracy	Existing standard
Ebulliometer	± 1,4 K (up to 373 K) (1) (2) ± 2,5 K (up to 600 K) (1) (2)	ASTM D 1120-72 (1)
Dynamic method	± 0,5 K (up to 600 K) (2)	
Distillation process (boiling range)	± 0,5 K (up to 600 K)	ISO/R 918, DIN 53171, BS 4591/71
According to Siwoloboff	± 2 K (up to 600 K) (2)	
Photocell detection	± 0,3 K (up to 373 K) (2)	
Differential thermal calorimetry	± 0,5 K (up to 600 K) ± 2,0 K (up to 1 273 K)	ASTM E 537-76
Differential scanning calorimetry	± 0,5 K (up to 600 K) ± 2,0 K (up to 1 273 K)	ASTM E 537-76

(1) This accuracy is only valid for the simple device as for example described in ASTM D 1120-72; it can be improved with more sophisticated ebulliometer devices.
(2) Only valid for pure substances. The use in other circumstances should be justified.

1.6. DESCRIPTION OF THE METHODS

The procedures of some test methods have been described in international and national standards (see Appendix).

1.6.1. **Ebulliometer**

See Appendix.

1.6.2. **Dynamic method**

See test method A.4 for the determination of the vapour pressure.

The boiling temperature observed with an applied pressure of 101,325 kPa is recorded.

1.6.3. **Distillation process (boiling range)**

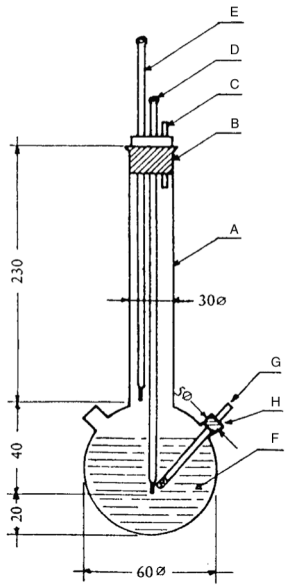
See Appendix.

1.6.4. **Method according to Siwoloboff**

The sample is heated in a melting temperature apparatus in a sample tube, with a diameter of approximately 5 mm (figure 1).

Figure 1 shows a type of standardised melting and boiling temperature apparatus (JIS K 0064) (made of glass, all specifications in millimetres).

Figure 1



A: Measuring vessel
B: Stopper
C: Vent
D: Thermometer
E: Auxiliary thermometer
F: Bath liquid
G: Sample tube, maximum 5 mm outer diameter; containing a capillary tube, approximately 100 mm long, approximately 1 mm long inner diameter and approximately 0,2 to 0,3 mm wall-thickness
H: Side tube

A capillary tube (boiling capillary) which is fused about 1 cm above the lower end is placed in the sample tube. The level to which the test substance is added is such that the fused section of the capillary is below the surface of the liquid. The sample tube containing the boiling capillary is fastened either to the thermometer with a rubber band or is fixed with a support from the side (see figure 2).

Figure 2

Principle according to Siwoloboff

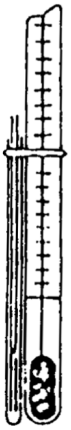
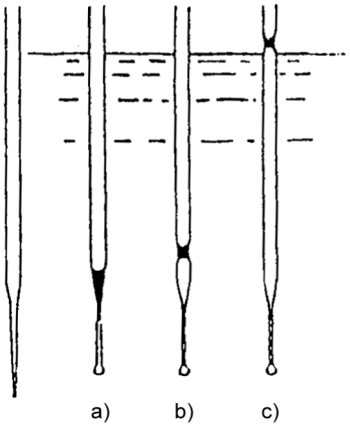


Figure 3

Modified principle



The bath liquid is chosen according to boiling temperature. At temperatures up to 573 K, silicone oil can be used. Liquid paraffin may only be used up to 473 K. The heating of the bath liquid should be adjusted to a temperature rise of 3 K/min at first. The bath liquid must be stirred. At about 10 K below the expected boiling temperature, the heating is reduced so that the rate of temperature rise is less than 1 K/min. Upon approach of the boiling temperature, bubbles begin to emerge rapidly from the boiling capillary.

The boiling temperature is that temperature when, on momentary cooling, the string of bubbles stops and fluid suddenly starts rising in the capillary. The corresponding thermometer reading is the boiling temperature of the substance.

In the modified principle (figure 3) the boiling temperature is determined in a melting temperature capillary. It is stretched to a fine point about 2 cm in length (a) and a small amount of the sample is sucked up. The open end of the fine capillary is closed by melting, so that a small air bubble is located at the end. While heating in the melting temperature apparatus (b), the air bubble expands. The boiling temperature corresponds to the temperature at which the substance plug reaches the level of the surface of the bath liquid (c).

1.6.5. **Photocell detection**

The sample is heated in a capillary tube inside a heated metal block.

A light beam is directed, via suitable holes in the block, through the substance onto a precisely calibrated photocell.

During the increase of the sample temperature, single air bubbles emerge from the boiling capillary. When the boiling temperature is reached the number of bubbles increases greatly. This causes a change in the intensity of light, recorded by a photocell, and gives a stop signal to the indicator reading out the temperature of a platinum resistance thermometer located in the block.

This method is especially useful because it allows determinations below room temperature down to 253,15 K (– 20 °C) without any changes in the apparatus. The instrument merely has to be placed in a cooling bath.

1.6.6. **Thermal analysis**

1.6.6.1. *Differential thermal analysis*

See Appendix.

1.6.6.2. *Differential scanning calorimetry*

See Appendix.

2. **DATA**

At small deviations from the normal pressure (max. ± 5 kPa) the boiling temperatures are normalised to T_n by means of the following number-value equation by Sidney Young:

$T_n = T + (f_T \times \Delta p)$

where:

Δp = (101,325 − p) [note sign]
P = pressure measurement in kPa
f_T = rate of change of boiling temperature with pressure in K/kPa
T = measured boiling temperature in K
T_n = boiling temperature corrected to normal pressure in K

The temperature-correction factors, f_T, and equations for their approximation are included in the international and national standards mentioned above for many substances.

For example, the DIN 53171 method mentions the following rough corrections for solvents included in paints:

Table 2:

Temperature — corrections factors f_T

Temperature T (K)	Correction factor f_T (K/kPa)
323,15	0,26
348,15	0,28
373,15	0,31
398,15	0,33
423,15	0,35
448,15	0,37

Temperature T (K)	Correction factor f_T (K/kPa)
473,15	0,39
498,15	0,41
523,15	0,4
548,15	0,45
573,15	0,47

3. **REPORTING**

The test report shall, if possible, include the following information:

— method used,

— precise specification of the substance (identity and impurities) and preliminary purification step, if any,

— an estimate of the accuracy.

The mean of at least two measurements which are in the range of the estimated accuracy (see table 1) is reported as the boiling temperature.

The measured boiling temperatures and their mean shall be stated and the pressure(s) at which the measurements were made shall be reported in kPa. The pressure should preferably be close to normal atmospheric pressure.

All information and remarks relevant for the interpretation of results have to be reported, especially with regard to impurities and physical state of the substance.

4. **REFERENCES**

(1) OECD, Paris, 1981, Test Guideline 103, Decision of the Council C (81) 30 final.

(2) IUPAC, B. Le Neindre, B. Vodar, editions. Experimental thermodynamics, Butterworths, London, 1975, vol. II.

(3) R. Weissberger edition: Technique of organic chemistry, Physical methods of organic chemistry, Third Edition, Interscience Publications, New York, 1959, vol. I, Part I, Chapter VIII.

Appendix

For additional technical details, the following standards may be consulted for example.

1. **Ebulliometer**

1.1. Melting temperature devices with liquid bath

ASTM D 1120-72	Standard test method for boiling point of engine anti-freezes

2. **Distillation process (boiling range)**

ISO/R 918	Test Method for Distillation (Distillation Yield and Distillation Range)
BS 4349/68	Method for determination of distillation of petroleum products
BS 4591/71	Method for the determination of distillation characteristics
DIN 53171	Losungsmittel für Anstrichstoffe, Bestimmung des Siedeverlaufes
NF T 20-608	Distillation: détermination du rendement et de l'intervalle de distillation

3. **Differential thermal analysis and differential scanning calorimetry**

ASTM E 537-76	Standard method for assessing the thermal stability of chemicals by methods of differential thermal analysis
ASTM E 473-85	Standard definitions of terms relating to thermal analysis
ASTM E 472-86	Standard practice for reporting thermoanalytical data
DIN 51005	Thermische Analyse, Begriffe

A.3. RELATIVE DENSITY

1. METHOD

The methods described are based on the OECD Test Guideline (1). The fundamental principles are given in reference (2).

1.1. INTRODUCTION

The methods for determining relative density described are applicable to solid and to liquid substances, without any restriction in respect to their degree of purity. The various methods to be used are listed in table 1.

1.2. DEFINITIONS AND UNITS

The relative density D^{20}_4 of solids or liquids is the ratio between the mass of a volume of substance to be examined, determined at 20 °C, and the mass of the same volume of water, determined at 4 °C. The relative density has no dimension.

The density, ρ, of a substance is the quotient of the mass, m, and its volume, v.

The density, ρ, is given, in SI units, in kg/m^3.

1.3. REFERENCE SUBSTANCES (1) (3)

Reference substances do not need to be employed in all cases when investigating a new substance. They should primarily serve to check the performance of the method from time to time and to allow comparison with results from other methods.

1.4. PRINCIPLE OF THE METHODS

Four classes of methods are used.

1.4.1. **Buoyancy methods**

1.4.1.1. *Hydrometer* (for liquid substances)

Sufficiently accurate and quick determinations of density may be obtained by the floating hydrometers, which allow the density of a liquid to be deduced from the depth of immersion by reading a graduated scale.

1.4.1.2. *Hydrostatic balance* (for liquid and solid substances)

The difference between the weight of a test sample measured in air and in a suitable liquid (e.g. water) can be employed to determine its density.

For solids, the measured density is only representative of the particular sample employed. For the determination of density of liquids, a body of known volume, v, is weighed first in air and then in the liquid.

1.4.1.3. *Immersed body method* (for liquid substances) (4)

In this method, the density of a liquid is determined from the difference between the results of weighing the liquid before and after immersing a body of known volume in the test liquid.

1.4.2. **Pycnometer methods**

For solids or liquids, pycnometers of various shapes and with known volumes may be employed. The density is calculated from the difference in weight between the full and empty pycnometer and its known volume.

1.4.3. **Air comparison pycnometer** (for solids)

The density of a solid in any form can be measured at room temperature with the gas comparison pycnometer. The volume of a substance is measured in air or in an inert gas in a cylinder of variable calibrated volume. For the calculation of density one mass measurement is taken after concluding the volume measurement.

1.4.4. **Oscillating densitimeter** (5) (6) (7)

The density of a liquid can be measured by an oscillating densitimeter. A mechanical oscillator constructed in the form of a U-tube is vibrated at the resonance frequency of the oscillator which depends on its mass. Introducing a sample changes the resonance frequency of the oscillator. The apparatus has to be calibrated by two liquid substances of known densities. These substances should preferably be chosen such that their densities span the range to be measured.

1.5. QUALITY CRITERIA

The applicability of the different methods used for the determination of the relative density is listed in the table.

1.6. DESCRIPTION OF THE METHODS

The standards given as examples, which are to be consulted for additional technical details, are attached in the Appendix.

The tests have to be run at 20 °C, and at least two measurements performed.

2. **DATA**

See standards.

3. **REPORTING**

The test report shall, if possible, include the following information:

— method used,

— precise specification of the substance (identity and impurities) and preliminary purification step, if any.

The relative density, D_4^{20}, shall be reported as defined in 1.2, along with the physical state of the measured substance.

All information and remarks relevant for the interpretation of results have to be reported, especially with regard to impurities and physical state of the substance.

Table:

Applicability of methods

Method of measurement	Density		Maximum possible dynamic viscosity	Existing Standards
	solid	liquid		
1.4.1.1. Hydrometer		yes	5 Pa s	ISO 387, ISO 649-2, NF T 20-050

Method of measurement	Density		Maximum possible dynamic viscosity	Existing Standards
	solid	liquid		
1.4.1.2. Hydrostatic balance				
(a) solids	yes			ISO 1183 (A)
(b) liquids		yes	5 Pa s	ISO 901 and 758
1.4.1.3. Immersed body method		yes	20 Pa s	DIN 53217
1.4.2. Pycnometer				ISO 3507
(a) solids	yes			ISO 1183(B), NF T 20-053
(b) liquids		yes	500 Pa s	ISO 758
1.4.3. Air comparison pycnometer	yes			DIN 55990 Teil 3, DIN 53243
1.4.4. Oscillating densitimer		yes	5 Pa s	

4. **REFERENCES**

(1) OECD, Paris, 1981, Test Guideline 109, Decision of the Council C(81) 30 final.

(2) R. Weissberger ed., Technique of Organic Chemistry, Physical Methods of Organic Chemistry, 3rd ed., Chapter IV, Interscience Publ., New York, 1959, vol. I, Part 1.

(3) IUPAC, Recommended reference materials for realization of physico-chemical properties, Pure and applied chemistry, 1976, vol. 48, p. 508.

(4) Wagenbreth, H., Die Tauchkugel zur Bestimmung der Dichte von Flüssigkeiten, Technisches Messen tm, 1979, vol. II, p. 427-430.

(5) Leopold, H., Die digitale Messung von Flüssigkeiten, Elektronik, 1970, vol. 19, p. 297-302.

(6) Baumgarten, D., Füllmengenkontrolle bei vorgepackten Erzeugnissen -Verfahren zur Dichtebestimmung bei flüssigen Produkten und ihre praktische Anwendung, Die Pharmazeutische Industrie, 1975, vol. 37, p. 717-726.

(7) Riemann, J., Der Einsatz der digitalen Dichtemessung im Brauereilaboratorium, Brauwissenschaft, 1976, vol. 9, p. 253-255.

Appendix

For additional technical details, the following standards may be consulted for example.

1. **Buoyancy methods**

1.1. Hydrometer

DIN 12790, ISO 387	Hydrometer; general instructions
DIN 12791	Part I: Density hydrometers; construction, adjustment and use
	Part II: Density hydrometers; standardised sizes, designation
	Part III: Use and test
ISO 649-2	Laboratory glassware: Density hydrometers for general purpose
NF T 20-050	Chemical products for industrial use — Determination of density of liquids — Areometric method
DIN 12793	Laboratory glassware: range find hydrometers

1.2. Hydrostatic balance

For solid substances

ISO 1183	Method A: Methods for determining the density and relative density of plastics excluding cellular plastics
NF T 20-049	Chemical products for industrial use — Determination of the density of solids other than powders and cellular products — Hydrostatic balance method
ASTM-D-792	Specific gravity and density of plastics by displacement
DIN 53479	Testing of plastics and elastomers; determination of density

For liquid substances

ISO 901	ISO 758
DIN 51757	Testing of mineral oils and related materials; determination of density
ASTM D 941-55, ASTM D 1296-67 and ASTM D 1481-62	
ASTM D 1298	Density, specific gravity or API gravity of crude petroleum and liquid petroleum products by hydrometer method
BS 4714	Density, specific gravity or API gravity of crude petroleum and liquid petroleum products by hydrometer method

1.3. Immersed body method

DIN 53217	Testing of paints, varnishes and similar coating materials; determination of density; immersed body method

2. **Pycnometer methods**

2.1. For liquid substances

ISO 3507	Pycnometers
ISO 758	Liquid chemical products; determination of density at 20 °C
DIN 12797	Gay-Lussac pycnometer (for non-volatile liquids which are not too viscous)

DIN 12798	Lipkin pycnometer (for liquids with a kinematic viscosity of less than 100 10^{-6} m^2 s^{-1} at 15 °C)
DIN 12800	Sprengel pycnometer (for liquids as DIN 12798)
DIN 12801	Reischauer pycnometer (for liquids with a kinematic viscosity of less than 100. 10^{-6} m^2 s^{-1} at 20 °C, applicable in particular also to hydrocarbons and aqueous solutions as well as to liquids with higher vapour pressure, approximately 1 bar at 90 °C)
DIN 12806	Hubbard pycnometer (for viscous liquids of all types which do not have too high a vapour pressure, in particular also for paints, varnishes and bitumen)
DIN 12807	Bingham pycnometer (for liquids, as in DIN 12801)
DIN 12808	Jaulmes pycnometer (in particular for ethanol — water mixture)
DIN 12809	Pycnometer with ground-in thermometer and capillary side tube (for liquids which are not too viscous)
DIN 53217	Testing of paints, varnishes and similar products; determination of density by pycnometer
DIN 51757	Point 7: Testing of mineral oils and related materials; determination of density
ASTM D 297	Section 15: Rubber products — chemical analysis
ASTM D 2111	Method C: Halogenated organic compounds
BS 4699	Method for determination of specific gravity and density of petroleum products (graduated bicapillary pycnometer method)
BS 5903	Method for determination of relative density and density of petroleum products by the capillary — stoppered pycnometer method
NF T 20-053	Chemical products for industrial use — Determination of density of solids in powder and liquids — Pyknometric method

2.2. For solid substances

ISO 1183	Method B: Methods for determining the density and relative density of plastics excluding cellular plastics
NF T 20-053	Chemical products for industrial use — Determination of density of solids in powder and liquids — Pyknometric method
DIN 19683	Determination of the density of soils

3. **Air comparison pycnometer**

DIN 55990	Part 3: Prüfung von Anstrichstoffen und ähnlichen Beschichtungsstoffen; Pulverlack; Bestimmung der Dichte
DIN 53243	Anstrichstoffe; chlorhaltige Polymere; Prüfung

A.4. VAPOUR PRESSURE

1. METHOD

The majority of the methods described are based on the OECD Test Guideline (1). The fundamental principles are given in references (2) and (3).

1.1. INTRODUCTION

It is useful to have preliminary information on the structure, the melting temperature and the boiling temperature of the substance to perform this test.

There is no single measurement procedure applicable to the entire range of vapour pressures. Therefore, several methods are recommended to be used for the measurement of vapour pressure from < 10^{-4} to 10^5 Pa.

Impurities will usually affect the vapour pressure, and to an extent which depends greatly upon the kind of impurity.

Where there are volatile impurities in the sample, which could affect the result, the substance may be purified. It may also be appropriate to quote the vapour pressure for the technical material.

Some methods described here use apparatus with metallic parts; this should be considered when testing corrosive substances.

1.2. DEFINITIONS AND UNITS

The vapour pressure of a substance is defined as the saturation pressure above a solid or liquid substance. At the thermodynamic equilibrium, the vapour pressure of a pure substance is a function of temperature only.

The SI unit of pressure which should be used is the pascal (Pa).

Units which have been employed historically, together with their conversion factors, are:

1 Torr (≡ 1 mm Hg)	= $1{,}333 \times 10^2$ Pa
1 atmosphere	= $1{,}013 \times 10^5$ Pa
1 bar	= 10^5 Pa

The SI unit of temperature is the kelvin (K).

The universal molar gas constant R is 8,314 J mol^{-1} K^{-1}

The temperature dependence of the vapour pressure is described by the Clausius-Clapeyron equation:

$$\log p = \frac{\Delta H_v}{2{,}3RT} + const.$$

where:

p = the vapour pressure of the substance in pascals
ΔH_v = its heat of vaporisation in $Jmol^{-1}$
R = the universal molar gas constant in $Jmol^{-1}$ K^{-1}
T = thermodynamic temperature in K

1.3. REFERENCE SUBSTANCES

Reference substances do not need to be employed in all cases when investigating a new substance. They should primarily serve to check the performance of the method from time to time and to allow comparison with results from other methods.

1.4. PRINCIPLE OF THE TEST METHODS

For determining the vapour pressure, seven methods are proposed which can be applied in different vapour pressure ranges. For each method, the vapour pressure is determined at various temperatures. In a limited temperature range, the logarithm of the vapour pressure of a pure substance is a linear function of the inverse of the temperature.

1.4.1. **Dynamic method**

In the dynamic method, the boiling temperature which pertains to a specified pressure is measured.

Recommended range:

10^3 up to 10^5 Pa.

This method has also been recommended for the determination of normal boiling temperature and is useful for that purpose up to 600 K.

1.4.2. **Static method**

In the static process, at thermodynamic equilibrium, the vapour pressure established in a closed system is determined at a specified temperature. This method is suitable for one component and multicomponent solids and liquids.

Recommended range:

10 up to 10^5 Pa.

This method can also be used in the range 1 to 10 Pa, providing care is taken.

1.4.3. **Isoteniscope**

This standardised method is also a static method but is usually not suitable for multicomponent systems. Additional information is available in ASTM method D-2879-86.

Recommended range:

from 100 up to 10^5 Pa.

1.4.4. **Effusion method: Vapour pressure balance**

The quantity of substance leaving a cell per unit time through an aperture of known size is determined under vacuum conditions such that return of substance into the cell is negligible (e.g. by measurement of the pulse generated on a sensitive balance by a vapour jet or by measuring the weight loss).

Recommended range:

10^{-3} to 1 Pa.

1.4.5. **Effusion method: By loss of weight or by trapping vaporisate**

The method is based on the estimation of the mass of test substance flowing out per unit of time of a Knudsen cell (4) in the form of vapour, through a micro-orifice under ultra-vacuum conditions. The mass of effused

vapour can be obtained either by determining the loss of mass of the cell or by condensing the vapour at low temperature and determining the amount of volatilised substance using chromatographic analysis. The vapour pressure is calculated by applying the Hertz-Knudsen relation.

Recommended range:

10^{-3} to 1 Pa.

1.4.6. **Gas saturation method**

A stream of inert carrier gas is passed over the substance in such a way that it becomes saturated with its vapour. The amount of material transported by a known amount of carrier gas is measurable either by collection in a suitable trap or by an intrain analytical technique. This is then used to calculate the vapour pressure at a given temperature.

Recommended range:

10^{-4} to 1 Pa.

This method can also be used in the range 1 to 10 Pa providing care is taken.

1.4.7. **Spinning rotor**

In the spinning rotor gauge, the actual measuring element is a small steel ball which is suspended in a magnetic field and rotates with high speed. The gas pressure is deduced from the pressure-dependent slow-down of the steel ball.

Recommended range:

10^{-4} to 0,5 Pa.

1.5. QUALITY CRITERIA

The various methods of determining the vapour pressure are compared as to application, repeatability, reproducibility, measuring range, existing standard. This is done in the following table.

Table:

Quality criteria

Measuring Method	Substances		Estimated Repeatability ([1])	Estimated Reproducibility ([1])	Recommended Range	Existing Standard
	solid	liquid				
1.4.1. Dynamic method	Low melting	yes	Up to 25 %	Up to 25 %	10^3 Pa to 2×10^3 Pa	—
			1 to 5 %	1 to 5 %	2×10^3 Pa to 10^5 Pa	—
1.4.2. Static method	yes	yes	5 to 10 %	5 to 10 %	10 Pa to 10^5 Pa ([2])	NFT 20-048 (5)
1.4.3. Isoteniscope	yes	yes	5 to 10 %	5 to 10 %	10^2 Pa to 10^5 Pa	ASTM-D 2879-86
1.4.4. Effusion method Vap. Pres.balance	yes	yes	5 to 20 %	5 to 50 %	10^{-3} Pa to 1 Pa	NFT 20-047(6)

Measuring Method	Substances		Estimated Repeatability ([1])	Estimated Reproducibility ([1])	Recommended Range	Existing Standard
	solid	liquid				
1.4.5. Effusion method weigt loss	yes	yes	10 to 30 %	—	10^{-3} Pa to 1 Pa	—
1.4.6. Gas saturation method	yes	yes	10 to 30 %	Up to 50 %	10^{-4} Pa to 1 Pa ([2])	—
1.4.7. Spinning rotor method	yes	yes	10 to 20 %	—	10^{-4} Pa to 0,5 Pa	—

([1]) Dependent of the degree of purity.
([2]) These methods can also be used in the range 1 to 10 Pa providing care is taken.

1.6. DESCRIPTION OF THE METHODS

1.6.1. **Dynamic measurement**

1.6.1.1. *Apparatus*

The measuring apparatus typically consists of a boiling vessel with attached cooler made of glass or metal (figure 1), equipment for measuring the temperature, and equipment for regulating and measuring the pressure. A typical measuring apparatus shown in the drawing is made from heat-resistant glass and is composed of five parts:

The large, partially double-walled tube consists of a ground jacket joint, a cooler, a cooling vessel and an inlet.

The glass cylinder, with a Cottrell 'pump', is mounted in the boiling section of the tube and has a rough surface of crushed glass to avoid 'bumping' in the boiling process.

The temperature is measured with a suitable temperature sensor (e.g. resistance thermometer, jacket thermocouple) immersed in the apparatus to the point of measurement (No 5, figure 1) through a suitable inlet (e.g. male ground joint).

The necessary connections are made to the pressure regulation and measuring equipment.

The bulb, which acts as a buffer volume, is connected with the measuring apparatus by means of a capillary tube.

The boiling vessel is heated by a heating element (e.g. cartridge heater) inserted into the glass apparatus from below. The heating current required is set and regulated via a thermocouple.

The necessary vacuum of between 10^2 Pa and approximately 10^5 Pa is produced with a vacuum pump.

A suitable valve is used to meter air or nitrogen for pressure regulation (measuring range approximately 10^2 to 10^5 Pa) and ventilation.

Pressure is measured with a manometer.

1.6.1.2. *Measurement procedure*

The vapour pressure is measured by determining the boiling temperature of the sample at various specified pressures between roughly 10^3 and 10^5 Pa. A steady temperature under constant pressure indicates that the boiling temperature has been reached. Frothing substances cannot be measured using this method.

The substance is placed in the clean, dry sample vessel. Problems may be encountered with non-powder solids but these can sometimes be solved by heating the cooling jacket. Once the vessel has been filled the apparatus is sealed at the flange and the substance degassed. The lowest desired pressure is then set and the heating is switched on. At the same time, the temperature sensor is connected to a recorder.

Equilibrium is reached when a constant boiling temperature is recorded at constant pressure. Particular care must be taken to avoid bumping during boiling. In addition, complete condensation must occur on the cooler. When determining the vapour pressure of low melting solids, care should be taken to avoid the condenser blocking.

After recording this equilibrium point, a higher pressure is set. The process is continued in this manner until 10^5 Pa has been reached (approximately 5 to 10 measuring points in all). As a check, equilibrium points must be repeated at decreasing pressures.

1.6.2. **Static measurement**

1.6.2.1. *Apparatus*

The apparatus comprises a container for the sample, a heating and cooling system to regulate the temperature of the sample and measure the temperature. The apparatus also includes instruments to set and measure the pressure. Figures 2a and 2b illustrate the basic principles involved.

The sample chamber (figure 2a) is bounded on one side by a suitable high-vacuum valve. A U-tube containing a suitable manometer fluid is attached to the other side. One end of the U-tube branches off to the vacuum pump, the nitrogen cylinder or ventilation valve, and a manometer.

A pressure gauge with a pressure indicator can be used instead of a U-tube (figure 2b).

In order to regulate the temperature of the sample, the sample vessel together with valve and U-tube or pressure gauge is placed in a bath which is kept at a constant temperature of ± 0,2 K. The temperature measurements are taken on the outside wall of the vessel containing the sample or in the vessel itself.

A vacuum pump with an upstream cooling trap is used to evacuate the apparatus.

In method 2a the vapour pressure of the substance is measured indirectly using a zero indicator. This takes into account the fact that the density of the fluid in the U-tube alters if the temperature changes greatly.

The following fluids are suitable for use as zero indicators for the U-tube, depending on the pressure range and the chemical behaviour of the test substance: silicone fluids, phthalates. The test substance must not dissolve noticeably in or react with the U-tube fluid.

For the manometer, mercury can be used in the range of normal air pressure to 10^2 Pa, while silicone fluids and phthalates are suitable for use below 10^2 Pa down to 10 Pa. Heatable membrane capacity manometers can even be used at below 10^{-1} Pa. There are also other pressure gauges which can be used below 10^2 Pa.

1.6.2.2. *Measurement procedure*

Before measuring, all components of the apparatus shown in figure 2 must be cleaned and dried thoroughly.

For method 2a, fill the U-tube with the chosen liquid, which must be degassed at an elevated temperature before readings are taken.

The test substance is placed in the apparatus, which is then closed and the temperature is reduced sufficiently for degassing. The temperature must be low enough to ensure that the air is sucked out, but — in the case of multiple component system — it must not alter the composition of the material. If required, equilibrium can be established more quickly by stirring.

The sample can be supercooled with e.g. liquid nitrogen (taking care to avoid condensation of air or pump fluid) or a mixture of ethanol and dry ice. For low-temperature measurements use a temperature-regulated bath connected to an ultra-cryomat.

With the valve over the sample vessel open, suction is applied for several minutes to remove the air. The valve is then closed and the temperature of the sample reduced to the lowest level desired. If necessary, the degassing operation must be repeated several times.

When the sample is heated the vapour pressure increases. This alters the equilibrium of the fluid in the U-tube. To compensate for this, nitrogen or air is admitted to the apparatus via a valve until the pressure indicator fluid is at zero again. The pressure required for this can be read off a precision manometer at room temperature. This pressure corresponds to the vapour pressure of the substance at that particular measuring temperature.

Method 2b is similar but the vapour pressure is read off directly.

The temperature-dependence of vapour pressure is determined at suitably small intervals (approximately 5 to 10 measuring points in all) up to the desired maximum. Low-temperature readings must be repeated as a check.

If the values obtained from the repeated readings do not coincide with the curve obtained for increasing temperature, this may be due to one of the following:

1. the sample still contains air (e.g. high-viscosity materials) or low-boiling substances, which is/are released during heating and can be removed by suction following further supercooling;

2. the cooling temperature is not low enough. In this case liquid nitrogen is used as the cooling agent.

   If either l or 2 is the case, the measurements must be repeated;

3. the substance undergoes a chemical reaction in the temperature range investigated (e.g. decomposition, polymerisation).

1.6.3. **Isoteniscope**

A complete description of this method can be found in reference 7. The principle of the measuring device is shown in figure 3. Similarly to the static method described in 1.6.2, the isoteniscope is appropriate for the investigation of solids or liquids.

In the case of liquids, the substance itself serves as the fluid in the auxiliary manometer. A quantity of the liquid, sufficient to fill the bulb and the short leg of the manometer section, is put in the isoteniscope. The isoteniscope is attached to a vacuum system and evacuated, then filled by nitrogen. The evacuation and purge of the system is repeated twice to remove residual oxygen. The filled isoteniscope is placed in an horizontal position so that the sample spreads out into a thin layer in the sample bulb and manometer section (U-part). The pressure of the system is reduced to 133 Pa and the sample gently warmed until it just boils (removal of dissolved fixed gases). The isoteniscope is then placed so that the sample returns to the bulb and short leg of the manometer, so that both are entirely filled with liquid. The pressure is maintained as for degassing; the drawn-out tip of the sample bulb is heated with a small flame until sample vapour released expands sufficiently to displace part of the sample from the upper part of the bulb and manometer arm into the manometer section of the isoteniscope, creating a vapour-filled, nitrogen-free space.

The isoteniscope is then placed in a constant temperature bath, and the pressure of nitrogen is adjusted until its pressure equals that of the sample. Pressure balance is indicated by the manometer section of the isoteniscope. At the equilibrium, the vapour pressure of nitrogen equals the vapour pressure of the substance.

In the case of solids, depending on the pressure and temperature range, the manometer liquids listed in 1.6.2.1 are used. The degassed manometer liquid is filled into a bulge on the long arm of the isoteniscope. Then the solid to be investigated is placed in the bulb and is degassed at elevated temperature. After that the isoteniscope is inclined so that the manometer liquid can flow into the U-tube. The measurement of vapour pressure as a function of temperature is done according to 1.6.2.

1.6.4. **Effusion method: vapour pressure balance**

1.6.4.1. *Apparatus*

Various versions of the apparatus are described in the literature (1). The apparatus described here illustrates the general principle involved (figure 4). Figure 4 shows the main components of the apparatus, comprising a high-vacuum stainless steel or glass container, equipment to produce and measure a vacuum and built-in components to measure the vapour pressure on a balance. The following built-in components are included in the apparatus:

— an evaporator furnace with flange and rotary inlet. The evaporator furnace is a cylindrical vessel, made of e.g. copper or a chemically resistant alloy with good thermal conductivity. A glass vessel with a copper

wall can also be used. The furnace has a diameter of approximately 3 to 5 cm and is 2 to 5 cm high. There are between one and three openings of different sizes for the vapour stream. The furnace is heated either by a heating spiral around the outside. To prevent heat being dissipated to the base plate, the heater is attached to the base plate by a metal with low thermal conductivity (nickel-silver or chromium-nickel steel), e.g. a nickel-silver pipe attached to a rotary inlet if using a furnace with several openings. This arrangement has the advantage of allowing the introduction of a copper bar. This allows cooling from the outside using a cooling bath,

— if the copper furnace lid has three openings of different diameters at 90° to each other, various vapour pressure ranges within the overall measuring range can be covered (openings between approximately 0,30 and 4,50 mm diameter). Large openings are used for low vapour pressure and vice versa. By rotating the furnace the desired opening or an intermediate position in the vapour stream (furnace opening — shield — balance pan) can be set and the stream of molecules is released or deflected through the furnace opening onto the scale pan. In order to measure the temperature of the substance, a thermocouple or resistance thermometer is placed at a suitable point,

— above the shield is a balance pan belonging to a highly sensitive microbalance (see below). The balance pan is approximately 30 mm in diameter. Gold-plated aluminium is a suitable material,

— the balance pan is surrounded by a cylindrical brass or copper refrigeration box. Depending on the type of balance, it has openings for the balance beam and a shield opening for the stream of molecules and should guarantee complete condensation of the vapour on the balance pan. Heat dissipation to the outside is ensured e.g. by a copper bar connected to the refrigeration box. The bar is routed through the base plate and thermally insulated from it, e.g. with a chromium-nickel steel tube. The bar is immersed in a Dewar flask containing liquid nitrogen under the base plate or liquid nitrogen is circulated through the bar. The refrigeration box is thus kept at approximately − 120 °C. The balance pan is cooled exclusively by radiation and is satisfactory for the pressure range under investigation (cooling approximately 1 hour before the start of measurement),

— the balance is positioned above the refrigeration box. Suitable balances are e.g. a highly sensitive 2-arm electronic microbalance (8) or a highly sensitive moving coil instrument (see OECD Test Guideline 104, Edition 12.05.81),

— the base plate also incorporates electrical connections for thermocouples (or resistance thermometers) and heating coils,

— a vacuum is produced in the vessel using a partial vacuum pump or high-vacuum pump (required vacuum approximately 1 to 2 × 10^{-3} Pa, obtained after 2 h pumping). The pressure is regulated with a suitable ionisation manometer.

1.6.4.2. *Measurement procedure*

The vessel is filled with the test substance and the lid is closed. The shield and refrigeration box are slid across the furnace. The apparatus is closed and the vacuum pumps are switched on. The final pressure before starting to take measurements should be approximately 10^{-4} Pa. Cooling of the refrigeration box starts at 10^{-2} Pa.

Once the required vacuum has been obtained, start the calibration series at the lowest temperature required. The corresponding opening in the lid is set, the vapour stream passes through the shield directly above the opening and strikes the cooled balance pan. The balance pan must be big enough to ensure that the entire stream guided through the shield strikes it. The momentum of the vapour stream acts as a force against the balance pan and the molecules condense on its cool surface.

The momentum and simultaneous condensation produce a signal on the recorder. Evaluation of the signals provides two pieces of information:

1. in the apparatus described here the vapour pressure is determined directly from the momentum on the balance pan (it is not necessary to know the molecular weight for this (2)). Geometrical factors such as the furnace opening and the angle of the molecular stream must be taken into account when evaluating the readings;

2. the mass of the condensate can be measured at the same time and the rate of evaporation can be calculated from this. The vapour pressure can also be calculated from the rate of evaporation and molecular weight using the Hertz equation (2).

$$p = G \sqrt{\frac{2\,\pi RT \times 10^3}{M}}$$

where

G = evaporation rate (kg s^{-1} m^{-2})
M = molar mass (g mol^{-1})
T = temperature (K)
R = universal molar gas constant (Jmol^{-1} K^{-1})
p = vapour pressure (Pa)

After the necessary vacuum is reached, the series of measurements is commenced at the lowest desired measuring temperature.

For further measurements, the temperature is increased by small intervals until the maximum desired temperature value is reached. The sample is then cooled again and a second curve of the vapour pressure may be recorded. If the second run fails to confirm the results of the first run, then it is possible that the substance may be decomposing in the temperature range being measured.

1.6.5. **Effusion method — by loss of weight**

1.6.5.1. *Apparatus*

The effusion apparatus consists of the following basic parts:

— a tank that can be thermostated and evacuated and in which the effusion cells are located,

— a high vacuum pump (e.g. diffusion pump or turbomolecular pump) with vacuum gauge,

— a trap, using liquefied nitrogen or dry ice.

An electrically heated, aluminium vacuum tank with four stainless steel effusion cells is shown in figure 5 for example. The stainless steel foil of about 0,3 mm thickness has an effusion orifice of 0,2 to 1,0 mm diameter and is attached to the effusion cell by a threaded lid.

1.6.5.2. *Measurement procedure*

The reference and test substances are filled into each effusion cell, the metal diaphragm with the orifice is secured by the threaded lid, and each cell is weighed to within an accuracy of 0,1 mg. The cell is placed in the thermostated apparatus, which is then evacuated to below one tenth of the expected pressure. At defined intervals of time ranging from 5 to 30 hours, air is admitted into the apparatus, and the loss in mass of the effusion cell is determined by reweighing.

In order to ensure that the results are not influenced by volatile impurities, the cell is reweighed at defined time intervals to check that the evaporation rate is constant over at least two such intervals of time.

The vapour pressure p in the effusion cell is given by:

$$p = \frac{m}{KAt} \sqrt{\frac{2\,\pi RT}{M}}$$

where

p = vapour pressure (Pa)
m = mass of the substance leaving the cell during time t (kg)
t = time (s)
A = area of the hole (m^2)
K = correction factor
R = universal gas constant (Jmol^{-1} K^{-1})
T = temperature (K)

M = molecular mass (kg mol^{-1})

The correction factor K depends on the ratio of length to radius of the cylindrical orifice:

ratio	0,1	0,2	0,6	1,0	2,0
K	0,952	0,909	0,771	0,672	0,514

The above equation may be written:

$$p = E \frac{m}{t} \sqrt{\frac{T}{M}}$$

where: $E = \frac{1}{KA} \sqrt{2\pi R}$ and is the effusion cell constant.

This effusion cell constant E may be determined with reference substances (2,9), using the following equation:

$$E = \frac{p(r)t}{m} \sqrt{\frac{M(r)}{T}}$$

where:

p(r) = vapour pressure of the reference substance (Pa)
M(r)= molecular mass of the reference substance (kg × mol^{-1})

1.6.6. **Gas saturation method**

1.6.6.1. *Apparatus*

A typical apparatus used to perform this test comprises a number of components given in figure 6a and described below (1).

Inert gas:

The carrier gas must not react chemically with the test substance. Nitrogen is usually sufficient for this purpose but occasionally other gases may be required (10). The gas employed must be dry (see figure 6a, key 4: relative humidity sensor).

Flow control:

A suitable gas-control system is required to ensure a constant and selected flow through the saturator column.

Traps to collect vapour:

These are dependent on the particular sample characteristics and the chosen method of analysis. The vapour should be trapped quantitatively and in a form which permits subsequent analysis. For some test substances, traps containing liquids such as hexane or ethylene glycol will be suitable. For others, solid absorbents may be applicable.

As an alternative to vapour trapping and subsequent analysis, in-train analytical techniques, like chromatography, may be used to determine quantitatively the amount of material transported by a known amount of carrier gas. Furthermore, the loss of mass of the sample can be measured.

Heat exchanger:

For measurements at different temperatures it may be necessary to include a heat-exchanger in the assembly.

Saturator column:

The test substance is deposited from a solution onto a suitable inert support. The coated support is packed into the saturator column, the dimensions of which and the flow rate should be such that complete saturation of the carrier gas is ensured. The saturator column must be thermostated. For measurements above room temperature, the region between the saturator column and the traps should be heated to prevent condensation of the test substance.

In order to lower the mass transport occurring by diffusion, a capillary may be placed after the saturator column (figure 6b).

1.6.6.2. *Measurement procedure*

Preparation of the saturator column:

A solution of the test substance in a highly volatile solvent is added to a suitable amount of support. Sufficient test substance should be added to maintain saturation for the duration of the test. The solvent is totally evaporated in air or in a rotary evaporator, and the thoroughly mixed material is added to the saturator column. After thermostating the sample, dry nitrogen is passed through the apparatus.

Measurement:

The traps or in-train detector are connected to the column effluent line and the time recorded. The flow rate is checked at the beginning and at regular intervals during the experiment, using a bubble meter (or continuously with a mass flow-meter).

The pressure at the outlet to the saturator must be measured. This may be done either:

(a) by including a pressure gauge between the saturator and traps (this may not be satisfactory because this increases the dead space and the adsorptive surface); or

(b) by determining the pressure drops across the particular trapping system used as a function of flow rate in a separate experiment (this may be not very satisfactory for liquid traps).

The time required for collecting the quantity of test substance that is necessary for the different methods of analysis is determined in preliminary runs or by estimates. As an alternative to collecting the substance for further analysis, in-train quantitative analytical technique may be used (e.g. chromatography). Before calculating the vapour pressure at a given temperature, preliminary runs are to be carried out to determine the maximum flow rate that will completely saturate the carrier gas with substance vapour. This is guaranteed if the carrier gas is passed through the saturator sufficiently slowly so that a lower rate gives no greater calculated vapour pressure.

The specific analytical method will be determined by the nature of the substance being tested (e.g. gas chromatography or gravimetry).

The quantity of substance transported by a known volume of carrier gas is determined.

1.6.6.3. *Calculation of vapour pressure*

Vapour pressure is calculated from the vapour density, W/V, through the equation:

$$p = \frac{W}{V} \times \frac{RT}{M}$$

where:

p = vapour pressure (Pa)
W = mass of evaporated test substance (g)
V = volume of saturated gas (m^3)
R = universal molar gas constant ($Jmol^{-1}\ K^{-1}$)
T = temperature (K)
M = molar mass of test substance ($g\ mol^{-1}$)

Measured volumes must be corrected for pressure and temperature differences between the flow meter and the thermostated saturator. If the flow meter is located downstream from the vapour trap, corrections may be necessary to account for any vaporised trap ingredients (1).

1.6.7. **Spinning rotor (8, 11, 13)**

1.6.7.1. *Apparatus*

The spinning rotor technique can be carried out using a spinning rotor viscosity gauge as shown in figure 8. A schematic drawing of the experimental set-up is shown in figure 7.

The measuring apparatus typically consists of a spinning rotor measuring head, placed in a thermostated enclosure (regulated within 0,1 °C). The sample container is placed in a thermostated enclosure (regulated within 0,01 °C), and all other parts of the set-up are kept at a higher temperature to prevent condensation. A high-vacuum pump device is connected to the system by means of high-vacuum valves.

The spinning rotor measuring head consists of a steel ball (4 to 5 mm diameter) in a tube. The ball is suspended and stabilised in a magnetic field, generally using a combination of permanent magnets and control coils.

The ball is made to spin by rotating fields produced by coils. Pick-up coils, measuring the always present low lateral magnetisation of the ball, allow its spinning rate to be measured.

1.6.7.2. *Measurement procedure*

When the ball has reached a given rotational speed v(o) (usually about 400 revolutions per second), further energising is stopped and deceleration takes place, due to gas friction.

The drop of rotational speed is measured as a function of time. As the friction caused by the magnetic suspension is negligible as compared with the gas friction, the gas pressure p is given by:

$$p = \frac{\pi \bar{c} r \rho}{\sigma 10 t} \times \ln \frac{v(t)}{v(o)}$$

where:

$\bar{c}$ = average speed of the gas molecules
r = radius of the ball
ρ = mass density of the ball
σ = coefficient of tangential momentum transfer (ε = 1 for an ideal spherical surface of the ball)
t = time
v(t) = rotational speed after time t
v(o) = initial rotational speed

This equation may also be written:

$$p = \frac{\pi \bar{c} r \rho}{10 \sigma} \times \frac{t_n - t_{n-1}}{t_n \times t_{n-1}}$$

where t_n, t_{n-1} are the times required for a given number N of revolutions. These time intervals t_n and t_{n-1} succeed one another, and $t_n > t_{n-1}$.

The average speed of the gas molecule $\bar{c}$ is given by:

$$\bar{c} = \left(\frac{8RT}{\pi M} \right)^{\frac{1}{2}}$$

where:

T = temperature

R = universal molar gas constant
M = molar mass

2. **DATA**

The vapour pressure from any of the preceding methods should be determined for at least two temperatures. Three or more are preferred in the range 0 to 50 °C, in order to check the linearity of the vapour pressure curve.

3. **REPORTING**

The test report shall, if possible, include the following information:

— method used,

— precise specification of the substance (identity and impurities) and preliminary purification step, if any,

— at least two vapour pressure and temperature values, preferably in the range 0 to 50 °C,

— all of the raw data,

— a log p versus 1/T curve,

— an estimate of the vapour pressure at 20 or 25 °C.

If a transition (change of state, decomposition) is observed, the following information should be noted:

— nature of the change,

— temperature at which the change occurs at atmospheric pressure,

— vapour pressure at 10 and 20 °C below the transition temperature and 10 and 20 °C above this temperature (unless the transition is from solid to gas).

All information and remarks relevant for the interpretation of results have to be reported, especially with regard to impurities and physical state of the substance.

4. **REFERENCES**

(1) OECD, Paris, 1981, Test Guideline 104, Decision of the Council C(81) 30 final.

(2) Ambrose, D. in B. Le Neindre, B. Vodar, (Eds.): Experimental Thermodynamics, Butterworths, London, 1975, vol. II.

(3) R. Weissberger ed.: Technique of Organic Chemistry, Physical Methods of Organic Chemistry, 3rd ed. Chapter IX, Interscience Publ., New York, 1959, vol. I, Part I.

(4) Knudsen, M. Ann. Phys. Lpz., 1909, vol. 29, 1979; 1911, vol. 34, p. 593.

(5) NF T 20-048 AFNOR (September 85) Chemical products for industrial use — Determination of vapour pressure of solids and liquids within range from 10^{-1} to 10^{5} Pa — Static method.

(6) NF T 20-047 AFNOR (September 85) Chemical products for industrial use — Determination of vapour pressure of solids and liquids within range from 10^{-3} to 1 Pa — Vapour pressure balance method.

(7) ASTM D 2879-86, Standard test method for vapour pressure-temperature relationship and initial decomposition temperature of liquids by isoteniscope.

(8) G. Messer, P. Rohl, G. Grosse and W. Jitschin. J. Vac. Sci. Technol.(A), 1987, 'Vol. 5 (4), p. 2440.

(9) Ambrose, D.; Lawrenson, I.J.; Sprake, C.H.S. J. Chem. Thermodynamics 1975, vol. 7, p. 1173.

(10) B.F. Rordorf. Thermochimica Acta, 1985, vol. 85, p. 435.

(11) G. Comsa, J.K. Fremerey and B. Lindenau. J. Vac. Sci. Technol., 1980, vol. 17 (2), p. 642.

(12) G. Reich. J. Vac. Sci. Technol., 1982, vol. 20 (4), p. 1148.

(13) J.K. Fremerey. J. Vac. Sci. Technol.(A), 1985, vol. 3 (3), p. 1715.

Appendix 1

Estimation method

INTRODUCTION

Calculated values of the vapour pressure can be used:

— for deciding which of the experimental methods is appropriate,

— for providing an estimate or limit value in cases where the experimental method cannot be applied due to technical reasons (including where the vapour pressure is very low),

— to help identify those cases where omitting experimental measurement is justified because the vapour pressure is likely to be $< 10^{-5}$ Pa at ambient temperature.

ESTIMATION METHOD

The vapour pressure of liquids and solids can be estimated by use of the modified Watson Correlation (a). The only experimental data required is the normal boiling point. The method is applicable over the pressure range from 10^5 Pa to 10^{-5} Pa.

Detailed information on the method is given in 'Handbook of Chemical Property Estimation Methods' (b).

CALCULATION PROCEDURE

According to (b) the vapour pressure is calculated as follows:

$$\ln P_{vp} \approx \frac{\Delta H_{vb}}{\Delta Z_b R T_b}\left[1 - \frac{\left(3 - 2\frac{T}{T_b}\right)^m}{\frac{T}{T_b}} - 2m\left(3 - 2\frac{T}{T_b}\right)^{m-1} \ln\frac{T}{T_b}\right]$$

where:

T = temperature of interest
T_b = normal boiling point
P_{vp} = vapour pressure at temperature T
ΔH_{vb} = heat of vaporisation
ΔZ_b = compressibility factor (estimated at 0,97)
m = empirical factor depending on the physical state at the temperature of interest

further:

$$\frac{\Delta H_{vb}}{T_b} = K_F(8{,}75 + R \ln T_b)$$

where K_F is an empirical factor considering the polarity of the substance. For several compound types, K_F factors are listed in reference (b).

Quite often, data are available in which a boiling point at reduced pressure is given. In such a case, according to (b), the vapour pressure is calculated as follows:

$$\ln P_{vp} \approx \ln P_1 + \frac{\Delta Hv_1}{\Delta Z_b R T_1}\left[1 - \left(3 - 2\frac{T}{T_1}\right)m\frac{T_1}{T} - 2m\left(3 - 2\frac{T}{T_1}\right)^{m-1} \ln\frac{T}{T_1}\right]$$

where T_1 is the boiling point at the reduced pressure P_1.

REPORT

When using the estimation method, the report shall include a comprehensive documentation of the calculation.

LITERATURE

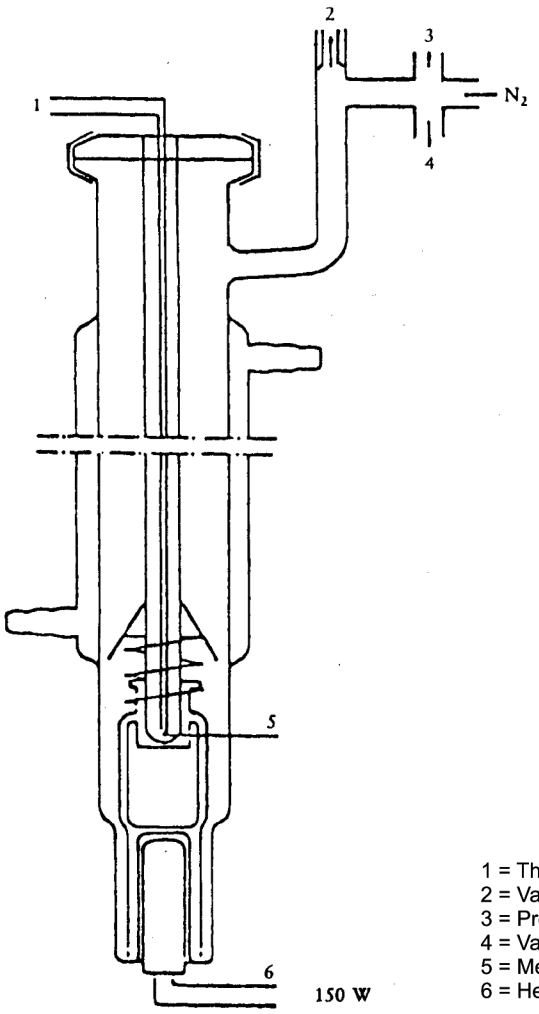
(a) K.M. Watson, Ind. Eng. Chem., 1943, vol. 35, p. 398.

(b) W.J. Lyman, W.F. Reehl, D.H. Rosenblatt. Handbook of Chemical Property Estimation Methods, Mc Graw-Hill, 1982.

Appendix 2

Figure 1

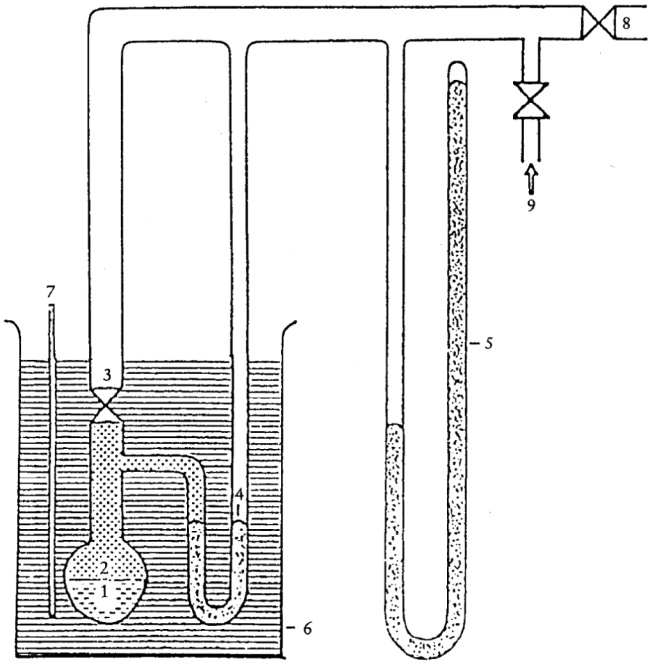
Apparatus for determining the vapour pressure curve according to the dynamic method



1 = Thermocouple
2 = Vacuum buffer volume
3 = Pressure gauge
4 = Vacuum
5 = Measuring point
6 = Heating element *circa* 150 W

Figure 2a

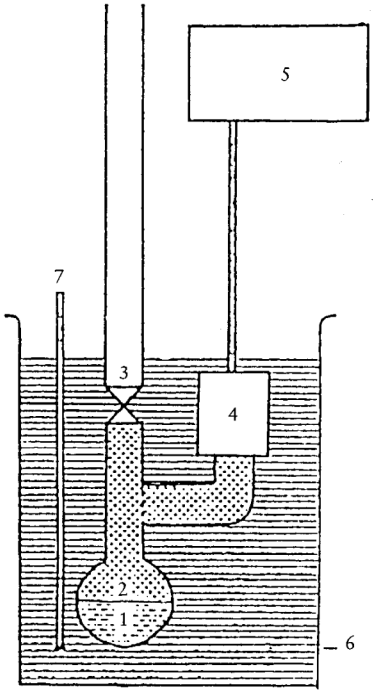
Apparatus for determining the vapour pressure curve according to the static method (using a U-tube manometer)



1. Test substance
2. Vapour phase
3. High vacuum valve
4. U-tube (auxiliary manometer)
5. Manometer
6. Temperature bath
7. Temperature measuring device
8. To vacuum pump
9. Ventilation

Figure 2b

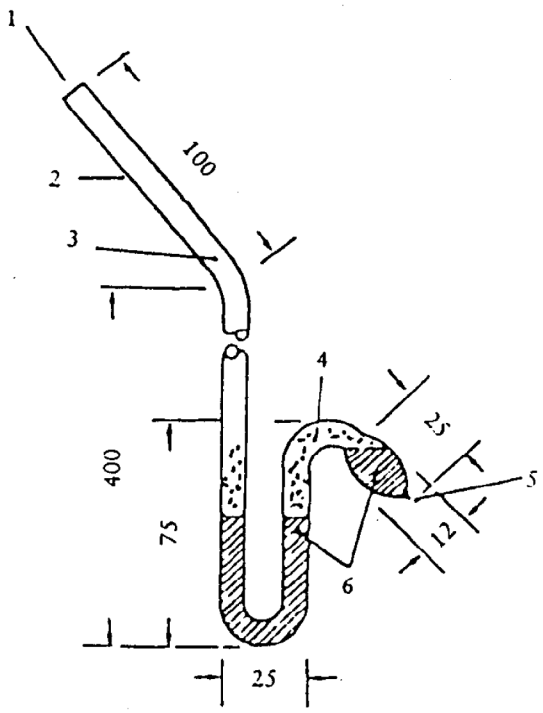
Apparatus for determining the vapour pressure curve according to the static method (using a pressure indicator)



1. Test substance
2. Vapour phase
3. High vacuum valve
4. Pressure gauge
5. Pressure indicator
6. Temperature bath
7. Temperature measuring device

Figure 3

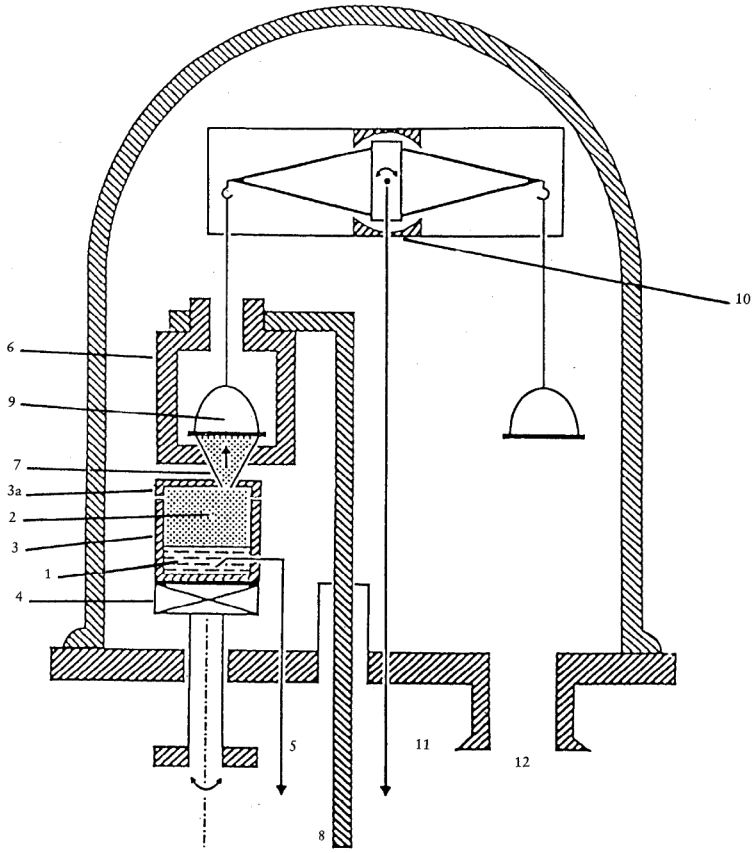
Isoteniscope (see reference 7)



1. To pressure control and measurement system
2. 8 mm OD tube
3. Dry nitrogen in pressure system
4. Sample vapour
5. Small tip
6. Liquid sample

Figure 4

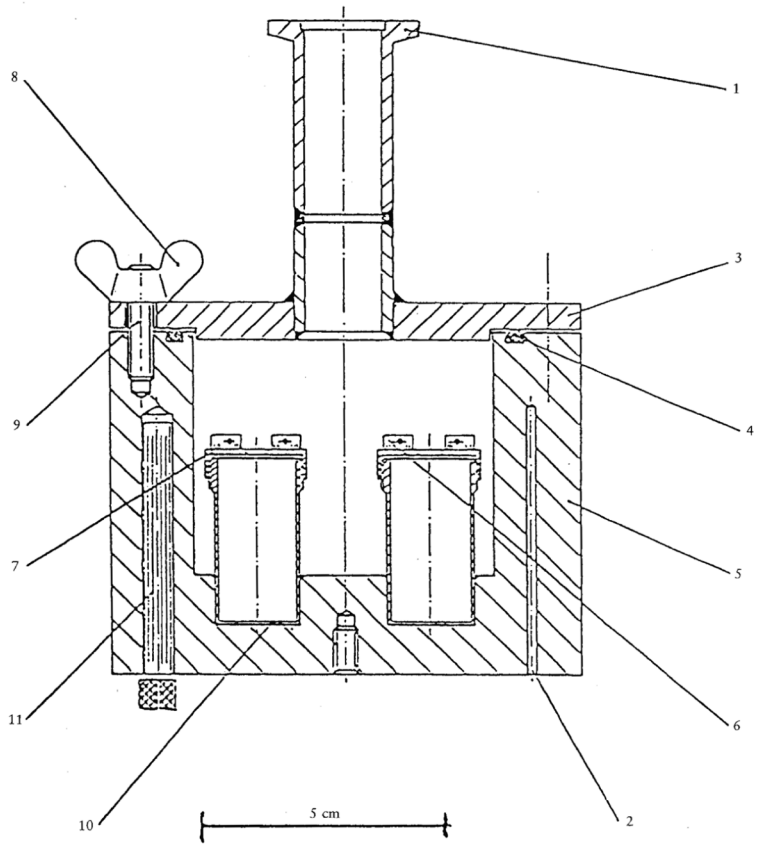
Apparatus for determining the vapour pressure curve according to the vapour pressure balance method



1. Test substance
2. Vapour phase with vapour stream
3. Evaporation furnace with rotary inlet
3a. Furnace lid with opening
4. Furnace heating (refrigeration)
5. Measurement of temperature of sample
6. Refrigeration box
7. Shield
8. Refrigeration bar for refrigeration box
9. Balance pan
10. Microbalance
11. To recorder
12. To high-vacuum pump

Figure 5

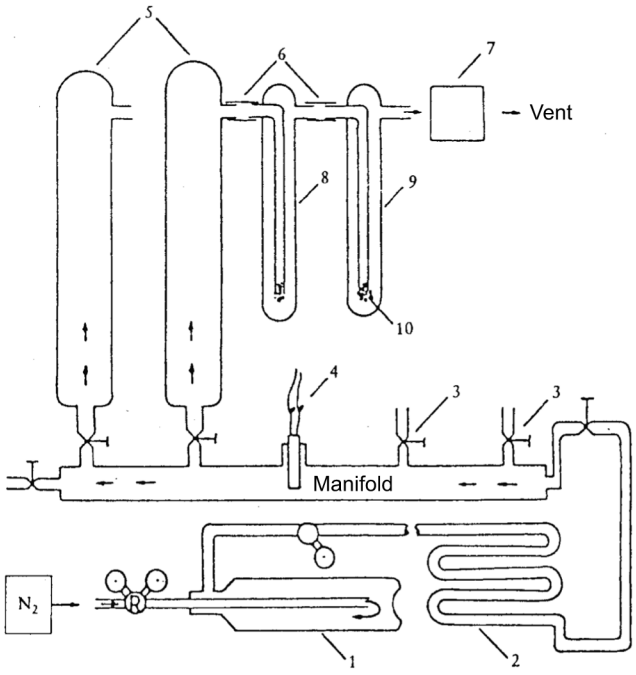
Example of apparatus for evaporation at low pressure by effusion methode, with an effusion cell volume of 8 cm³



1 Connection to vacuum
2 Wells for platinum resistance thermometer or temperature measurement and control (2)
3 Lid for vacuum tank
4 O-ring
5 Aluminium vacuum tank
6 Device for installing and removing the effusion cells
7 Threaded lid
8 Butterfly nuts (6)
9 Bolts (6)
10 Stainless steel effusion cells
11 Heater cartridges (6)

Figure 6a

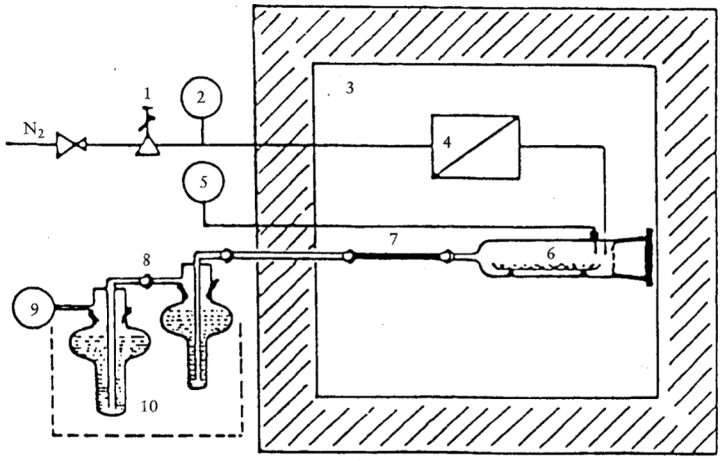
An example of a flow system for the determination of vapour pressure by the gas saturation method



1 = Flow regulator
2 = Heat exchanger
3 = Needle valves
4 = Relative humidity sensor
5 = Saturation columns
6 = PTFE joints
7 = Flow meter
8 = Trap (absorber)
9 = Oil trap
10 = Fritted bubbler

Figure 6b

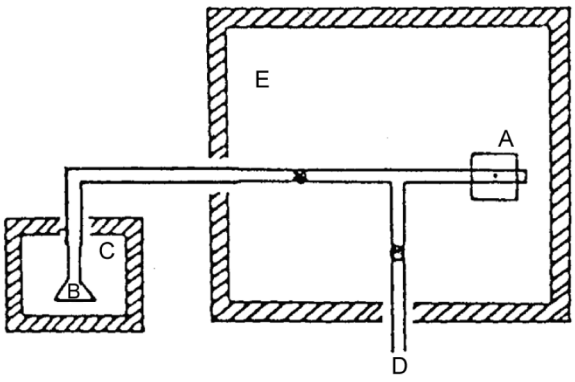
An example of system for the determination of vapour pressure by the gas saturation method, with a capillary placed after the saturation chamber



1. Thermal mass flowmeter
2. Manometer
3. Temperature-controlled chamber
4. Thermostating coil for carrier gas
5. Thermometer (Pt 100)
6. Gas saturation chamber
7. Capillary
8. Absorption vessels
9. Gas meter
10. Cold trap

Figure 7

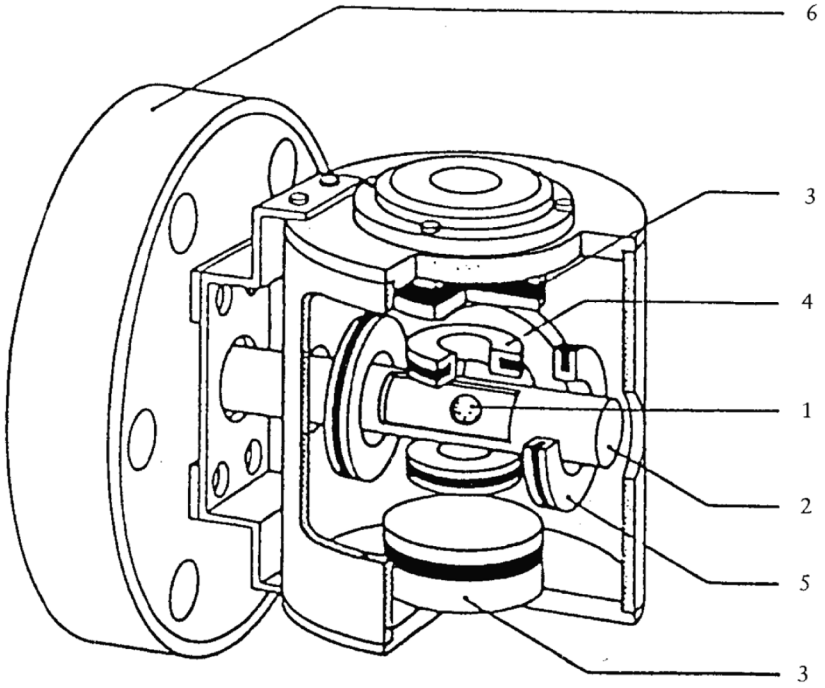
Example of the experimental set-up for spinning rotor method



Vapour pressure apparatus
A. spinning rotor sensor head;
B. sample cell;
C. thermostat;
D. vacuum line (turbo pump);
E. air thermostat.

Figure 8

Example of spinning rotor measuring head



1. Ball;
2. Evacuated tubular extension of 6;
3. Permanent magnets (2);
4. Coils (2) for vertical stabilization;
5. Driving coils (4);
6. Connection flange.

A.5. SURFACE TENSION

1. METHOD

The methods described are based on the OECD Test Guideline (1). The fundamental principles are given in reference (2).

1.1. INTRODUCTION

The described methods are to be applied to the measurement of the surface tension of aqueous solutions.

It is useful to have preliminary information on the water solubility, the structure, the hydrolysis properties and the critical concentration for micelles formation of the substance before performing these tests.

The following methods are applicable to most chemical substances, without any restriction in respect to their degree of purity.

The measurement of the surface tension by the ring tensiometer method is restricted to aqueous solutions with a dynamic viscosity of less than approximately 200 mPa s.

1.2. DEFINITIONS AND UNITS

The free surface enthalpy per unit of surface area is referred to as surface tension.

The surface tension is given as:

N/m (SI unit) or

mN/m (SI sub-unit)

1 N/m = 10^3 dynes/cm

1 mN/m = 1 dyne/cm in the obsolete cgs system

1.3. REFERENCE SUBSTANCES

Reference substances do not need to be employed in all cases when investigating a new substance. They should primarily serve to check the performance of the method from time to time and to allow comparison with results from other methods.

Reference substances which cover a wide range of surface tensions are given in references 1 and 3.

1.4. PRINCIPLE OF THE METHODS

The methods are based on the measurement of the maximum force which is necessary to exert vertically, on a stirrup or a ring in contact with the surface of the liquid being examined placed in a measuring cup, in order to separate it from this surface, or on a plate, with an edge in contact with the surface, in order to draw up the film that has formed.

Substances which are soluble in water at least at a concentration of 1 mg/l are tested in aqueous solution at a single concentration.

1.5. QUALITY CRITERIA

These methods are capable of greater precision than is likely to be required for environmental assessment.

1.6. DESCRIPTION OF THE METHODS

A solution of the substance is prepared in distilled water. The concentration of this solution should be 90 % of the saturation solubility of the substance in water; when this concentration exceeds 1 g/l, a concentration of 1 g/l is used for testing. Substances with water solubility lower than 1 mg/l need not be tested.

1.6.1. **Plate method**

See ISO 304 and NF T 73-060 (Surface active agents — determination of surface tension by drawing up liquid films).

1.6.2. **Stirrup method**

See ISO 304 and NF T 73-060 (Surface active agents — determination of surface tension by drawing up liquid films).

1.6.3. **Ring method**

See ISO 304 and NF T 73-060 (Surface active agents — determination of surface tension by drawing up liquid films).

1.6.4. **OECD harmonised ring method**

1.6.4.1. *Apparatus*

Commercially available tensiometers are adequate for this measurement. They consist of the following elements:

— mobile sample table,

— force measuring system,

— measuring body (ring),

— measurement vessel.

1.6.4.1.1. Mobile sample table

The mobile sample table is used as a support for the temperature-controlled measurement vessel holding the liquid to be tested. Together with the force measuring system, it is mounted on a stand.

1.6.4.1.2. Force measuring system

The force measuring system (see figure) is located above the sample table. The error of the force measurement shall not exceed ± 10^{-6} N, corresponding to an error limit of ± 0,1 mg in a mass measurement. In most cases, the measuring scale of commercially available tensiometers is calibrated in mN/m so that the surface tension can be read directly in mN/m with an accuracy of 0,1 mN/m.

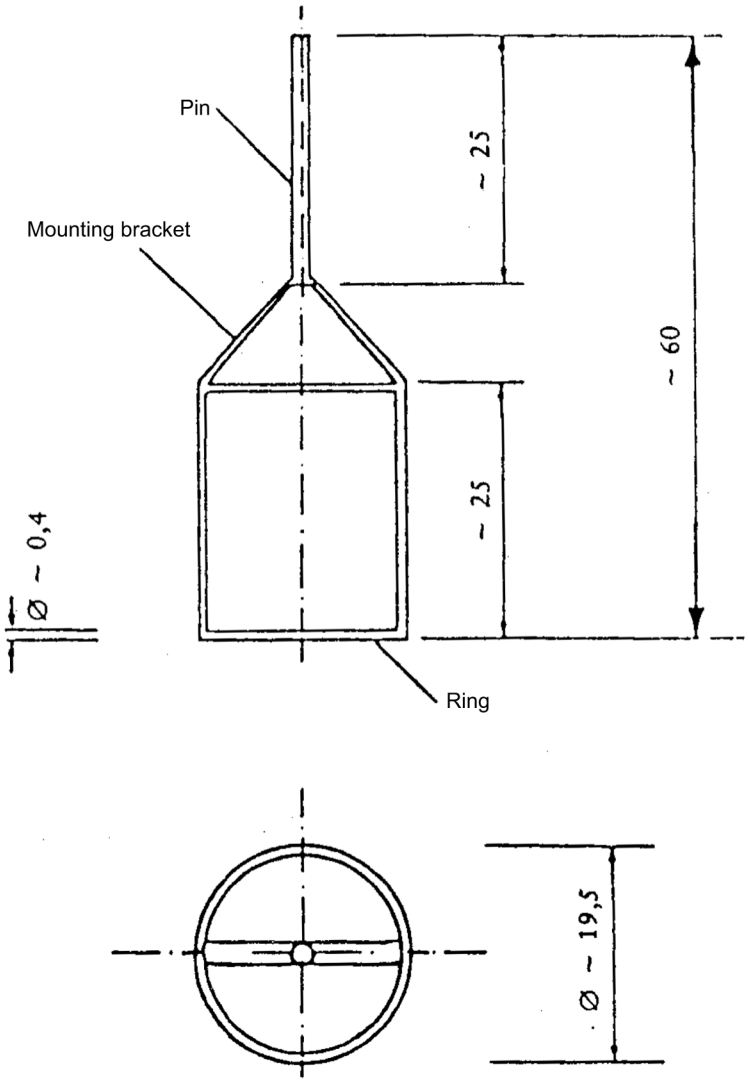
1.6.4.1.3. Measuring body (ring)

The ring is usually made of a platinum-iridium wire of about 0,4 mm thickness and a mean circumference of 60 mm. The wire ring is suspended horizontally from a metal pin and a wire mounting bracket to establish the connection to the force measuring system (see figure).

Figure

Measuring body

(All dimensions expressed in millimetres)



1.6.4.1.4. Measurement vessel

The measurement vessel holding the test solution to be measured shall be a temperature-controlled glass vessel. It shall be designed so that during the measurement the temperature of the test solution liquid and the gas phase above its surface remains constant and that the sample cannot evaporate. Cylindrical glass vessels having an inside diameter of not less than 45 mm are acceptable.

1.6.4.2. *Preparation of the apparatus*

1.6.4.2.1. Cleaning

Glass vessels shall be cleaned carefully. If necessary they shall be washed with hot chromo-sulphuric acid and subsequently with syrupy phosphoric acid (83 to 98 % by weight of H_3PO_4), thoroughly rinsed in tap water and finally washed with double-distilled water until a neutral reaction is obtained and subsequently dried or rinsed with part of the sample liquid to be measured.

The ring shall first be rinsed thoroughly in water to remove any substances which are soluble in water, briefly immersed in chromo-sulphuric acid, washed in double-distilled water until a neutral reaction is obtained and finally heated briefly above a methanol flame.

Note:

Contamination by substances which are not dissolved or destroyed by chromo-sulphuric acid or phosphoric acid, such as silicones, shall be removed by means of a suitable organic solvent.

1.6.4.2.2. Calibration of the apparatus

The validation of the apparatus consists of verifying the zero point and adjusting it so that the indication of the instrument allows reliable determination in mN/m.

Mounting:

The apparatus shall be levelled, for instance by means of a spirit level on the tensiometer base, by adjusting the levelling screws in the base.

Zero point adjustment:

After mounting the ring on the apparatus and prior to immersion in the liquid, the tensiometer indication shall be adjusted to zero and the ring checked for parallelism to the liquid surface. For this purpose, the liquid surface can be used as a mirror.

Calibrations:

The actual test calibration can be accomplished by means of either of two procedures:

(a) Using a mass: procedure using riders of known mass between 0,1 and 1,0 g placed on the ring. The calibration factor, Φ_a by which all the instrument readings must be multiplied, shall be determined according to equation (1).

$$\Phi_a = \frac{\sigma_r}{\sigma_a}$$

where:

$$\sigma_r = \frac{mg}{2b} \text{ (mN/m)}$$

m = mass of the rider (g)
g = gravity acceleration (981 cm s^{-2} at sea level)
b = mean circumference of the ring (cm)
σ_a = reading of the tensiometer after placing the rider on the ring (mN/m).

(b) Using water: procedure using pure water whose surface tension at, for instance, 23 °C is equal to 72,3 mN/m. This procedure is accomplished faster than the weight calibration but there is always the danger that the surface tension of the water is falsified by traces of contamination by surfactants.

The calibration factor, Φ_b by which all the instrument readings shall be multiplied, shall be determined in accordance with the equation (2):

$$\Phi_b = \frac{\sigma_o}{\sigma_g}$$

where:

σ_o = value cited in the literature for the surface tension of water (mN/m)
σ_g = measured value of the surface tension of the water (mN/m) both at the same temperature.

1.6.4.3. *Preparation of samples*

Aqueous solutions shall be prepared of the substances to be tested, using the required concentrations in water, and shall not contain any non-dissolved substances.

The solution must be maintained at a constant temperature (± 0,5 °C). Since the surface tension of a solution in the measurement vessel alters over a period of time, several measurements shall be made at various times and a curve plotted showing surface tension as a function of time. When no further change occurs, a state of equilibrium has been reached.

Dust and gaseous contamination by other substances interfere with the measurement. The work shall therefore be carried out under a protective cover.

1.6.5. **Test conditions**

The measurement shall be made at approximately 20 °C and shall be controlled to within ± 0,5 °C.

1.6.6. **Performance of test**

The solutions to be measured shall be transferred to the carefully cleaned measurement vessel, taking care to avoid foaming, and subsequently the measurement vessel shall be placed onto the table of the test apparatus. The table-top with measurement vessel shall be raised until the ring is immersed below the surface of the solution to be measured. Subsequently, the table-top shall be lowered gradually and evenly (at a rate of approximately 0,5 cm/min) to detach the ring from the surface until the maximum force has been reached. The liquid layer attached to the ring must not separate from the ring. After completing the measurements, the ring shall be immersed below the surface again and the measurements repeated until a constant surface tension value is reached. The time from transferring the solution to the measurement vessel shall be recorded for each determination. Readings shall be taken at the maximum force required to detach the ring from the liquid surface.

2. **DATA**

In order to calculate the surface tension, the value read in mN/m on the apparatus shall be first multiplied by the calibration factor Φ_a or Φ_b (depending on the calibration procedure used). This will yield a value which applies only approximately and therefore requires correction.

Harkins and Jordan (4) have empirically determined correction factors for surface-tension values measured by the ring method which are dependent on ring dimensions, the density of the liquid and its surface tension.

Since it is laborious to determine the correction factor for each individual measurement from the Harkins and Jordan tables, in order to calculate the surface tension for aqueous solutions the simplified procedure of reading the corrected surface-tension values directly from the table may be used. (Interpolation shall be used for readings ranging between the tabular values.)

Table:

Correction of the measured surface tension

Only for aqueous solutions, $\rho = 1\ g/cm^3$

r	= 9,55 mm (average ring radius)
r	= 0,185 mm (ring wire radius)

Experimental Value (mN/m)	Corrected Value (mN/m)	
	Weight calibration (see 1.6.4.2.2(a))	Water calibration (see 1.6.4.2.2(b))
20	16,9	18,1
22	18,7	20,1
24	20,6	22,1

Experimental Value (mN/m)	Corrected Value (mN/m)	
	Weight calibration (see 1.6.4.2.2(a))	Water calibration (see 1.6.4.2.2(b))
26	22,4	24,1
28	24,3	26,1
30	26,2	28,1
32	28,1	30,1
34	29,9	32,1
36	31,8	34,1
38	33,7	36,1
40	35,6	38,2
42	37,6	40,3
44	39,5	42,3
46	41,4	44,4
48	43,4	46,5
50	45,3	48,6
52	47,3	50,7
54	49,3	52,8
56	51,2	54,9
58	53,2	57,0
60	55,2	59,1
62	57,2	61,3
64	59,2	63,4
66	61,2	65,5
68	63,2	67,7
70	65,2	69,9
72	67,2	72,0
74	69,2	—
76	71,2	—
78	73,2	—

This table has been compiled on the basis of the Harkins-Jordan correction. It is similar to that in the DIN Standard (DIN 53914) for water and aqueous solutions (density $\rho = 1\ g/cm^3$ and is for a commercially available ring having the dimensions R = 9,55 mm (mean ring radius) and r = 0,185 mm (ring wire radius). The table provides corrected values for surface-tension measurements taken after calibration with weights or calibration with water.

Alternatively, without the preceding calibration, the surface tension call can be calculated according to the following formula:

$$\sigma = \frac{f \times F}{4\pi R}$$

where:

F = the force measured on the dynamometer at the breakpoint of the film

R = the radius of the ring

f = the correction factor (1)

3. REPORTING

3.1. TEST REPORT

The test report shall, if possible, include the following information:

— method used,

— type of water or solution used,

— precise specification of the substance (identity and impurities),

— measurement results: surface tension (reading) stating both the individual readings and their arithmetic mean as well as the corrected mean (taking into consideration the equipment factor and the correction table),

— concentration of the solution,

— test temperature,

— age of solution used; in particular the time between preparation and measurement of the solution,

— description of time dependence of surface tension after transferring the solution to the measurement vessel,

— all information and remarks relevant for the interpretation of results have to be reported, especially with regard to impurities and physical state of the substance.

3.2. INTERPRETATION OF RESULTS

Considering that distilled water has a surface tension of 72,75 mN/m at 20 °C, substances showing a surface tension lower than 60 mN/m under the conditions of this method should be regarded as being surface-active materials.

4. REFERENCES

(1) OECD, Paris, 1981, Test Guideline 115, Decision of the Council C(81) 30 final.

(2) R. Weissberger ed.: Technique of Organic Chemistry, Physical Methods of Organic Chemistry, 3rd ed., Interscience Publ., New York, 1959, vol. I, Part I, Chapter XIV.

(3) Pure Appl. Chem., 1976, vol. 48, p. 511.

(4) Harkins, W.D., Jordan, H.F., J. Amer. Chem. Soc., 1930, vol. 52, p. 1751.

A.6. WATER SOLUBILITY

1. METHOD

The methods described are based on the OECD Test Guideline (1).

1.1. INTRODUCTION

It is useful to have preliminary information on the structural formula, the vapour pressure, the dissociation constant and the hydrolysis (as a function of pH) of the substance to perform this test.

No single method is available to cover the whole range of solubilities in water.

The two test methods described below cover the whole range of solubilities but are not applicable to volatile substances:

— one which applies to essentially pure substances with low solubilities, (< 10^{-2} grams per litre), and which are stable in water, referred to as the 'column elution method',

— the other which applies to essentially pure substances with higher solubilities (> 10^{-2} grams per litre), and which are stable in water, referred to as the 'flask method'.

The water solubility of the test substance can be considerably affected by the presence of impurities.

1.2. DEFINITION AND UNITS

The solubility in water of a substance is specified by the saturation mass concentration of the substance in water at a given temperature. The solubility in water is specified in units of mass per volume of solution. The SI unit is kg/m^3 (grams per litre may also be used).

1.3. REFERENCE SUBSTANCES

Reference substances do not need to be employed in all cases when investigating a new substance. They should primarily serve to check the performance of the method from time to time and to allow comparison with results from other methods.

1.4. PRINCIPLE OF THE TEST METHOD

The approximate amount of the sample and the time necessary to achieve the saturation mass concentration should be determined in a simple preliminary test.

1.4.1. **Column elution method**

This method is based on the elution of a test substance with water from a micro-column which is charged with an inert support material, such as glass beads or sand, coated with an excess of test substance. The water solubility is determined when the mass concentration of the eluate is constant. This is shown by a concentration plateau as a function of time.

1.4.2. **Flask method**

In this method, the substance (solids must be pulverised) is dissolved in water at a temperature somewhat above the test temperature. When saturation is achieved the mixture is cooled and kept at the test temperature, stirring as long as necessary to reach equilibrium. Alternatively, the measurement can be performed directly at the test temperature, if it is assured by appropriate sampling that the saturation equilibrium is reached. Subsequently, the mass concentration of the substance in the aqueous solution, which must not contain any undissolved particles, is determined by a suitable analytical method.

1.5. QUALITY CRITERIA

1.5.1. **Repeatability**

For the column elution method, < 30 % may be obtainable; for the flask method, < 15 % should be observed.

1.5.2. **Sensitivity**

This depends upon the method of analysis, but mass concentration determinations down to 10^{-6} grams per litre can be determined.

1.6. DESCRIPTION OF THE METHOD

1.6.1. **Test conditions**

The test is preferably run at 20 ± 0,5 °C. If a temperature dependence is suspected in the solubility (> 3 % per °C), two other temperatures at least 10 °C above and below the initially chosen temperature should also be used. In this case, the temperature control should be ± 0,1 °C. The chosen temperature should be kept constant in all relevant parts of the equipment.

1.6.2. **Preliminary test**

To approximately 0,1 g of the sample (solid substances must be pulverised) in a glass-stoppered 10 ml graduated cylinder, increasing volumes of distilled water at room temperature are added according to the steps shown in the table below:

0,1 g soluble in 'x' ml of water	0,1	0,5	1	2	10	100	> 100
Approximative solubility (grams per litre)	> 1 000	1 000 to 200	200 to 100	100 to 50	50 to 10	10 to 1	< 1

After each addition of the indicated amount of water, the mixture is shaken vigorously for 10 minutes and is visually checked for any undissolved parts of the sample. If, after addition of 10 ml of water, the sample or parts of it remain undissolved, the experiment has to be repeated in a 100 ml measuring cylinder with larger volumes of water. At lower solubilities the time required to dissolve a substance can be considerably longer (at least 24 h should be allowed). The approximate solubility is given in the table under that volume of added water in which complete dissolution of the sample occurs. If the substance is still apparently insoluble, more than 24 h should be allowed (96 h maximum), or further dilution should be undertaken to ascertain whether the column elution or flask solubility method should be used.

1.6.3. **Column elution method**

1.6.3.1. *Support material, solvent and eluent*

The support material for the column elution method should be inert. Possible materials which can be employed are glass beads and sand. A suitable volatile solvent of analytical reagent quality should be used to apply the test substance to the support material. Water which has been double distilled in glass or quartz apparatus should be employed as the eluent.

Note:

Water directly from an organic ion exchanger must not be used.

1.6.3.2. *Loading of the support*

Approximately 600 mg of support material is weighed and transferred to a 50 ml round-bottom flask.

A suitable, weighed amount of test substance is dissolved in the chosen solvent. An appropriate amount of this solution is added to the support material. The solvent must be completely evaporated, e.g. in a rotary evaporator; otherwise water saturation of the support is not achieved due to partition effects on the surface of the support material.

The loading of support material may cause problems (erroneous results) if the test substance is deposited as an oil or a different crystal phase. The problem should be examined experimentally and the details reported.

The loaded support material is allowed to soak for about two hours in approximately 5 ml of water, and then the suspension is added to the microcolumn. Alternatively, dry loaded support material may be poured into the microcolumn, which has been filled with water, and then equilibrated for approximately two hours.

Test procedure:

The elution of the substance from the support material can be carried out in one of two different ways:

— recirculating pump (see figure 1),

— levelling vessel (see figure 4).

1.6.3.3. *Column elution method with recirculating pump*

Apparatus

A schematic arrangement of a typical system is presented in figure 1. A suitable microcolumn is shown in figure 2, although any size is acceptable, provided it meets the criteria for reproducibility and sensitivity. The column should provide for a headspace of at least five bed volumes of water and be able to hold a minimum of five samples. Alternatively, the size can be reduced if make-up solvent is employed to replace the initial five bed volumes removed with impurities.

The column should be connected to a recirculating pump capable of controlling flows of approximately 25 ml/h. The pump is connected with polytetrafluoroethylene (P.T.F.E.) and/or glass connections. The column and pump, when assembled, should have provision for sampling the effluent and equilibrating the headspace at atmospheric pressure. The column material is supported with a small (5 mm) plug of glass wool, which also serves to filter out particles. The recirculating pump can be, for example, a peristaltic pump or a membrane pump (care must be taken that no contamination and/or absorption occurs with the tube material).

Measurement procedure

The flow through the column is started. It is recommended that a flow rate of approximately 25 ml/hr be used (this corresponds to 10 bed volumes/hr for the column described). The first five bed volumes (minimum) are discarded to remove water-soluble impurities. Following this, the recirculating pump is allowed to run until equilibration is established, as defined by five successive samples whose concentrations do not differ by more than ± 30 % in a random fashion. These samples should be separated from each other by time intervals corresponding to the passage of at least 10 bed volumes of the eluent.

1.6.3.4. *Column elution method with levelling vessel*

Apparatus (see figures 4 and 3)

Levelling vessel: the connection to the levelling vessel is made by using a ground glass joint which is connected by PTFE tubing. It is recommended that a flow rate of approximately 25 ml/hr be used. Successive eluate fractions should be collected and analysed by the chosen method.

Measurement procedure

Those fractions from the middle eluate range where the concentrations are constant (± 30 %) in at least five consecutive fractions are used to determine the solubility in water.

In both cases (using a recirculating pump or a levelling vessel), a second run is to be performed at half the flow rate of the first. If the results of the two runs are in agreement, the test is satisfactory; if there is a higher apparent solubility with the lower flow rate, then the halving of the flow rate must continue until two successive runs give the same solubility.

In both cases (using a recirculating pump or a levelling vessel) the fractions should be checked for the presence of colloidal matter by examination for the Tyndall effect (light scattering). Presence of such particles invalidates the results, and the test should be repeated with improvements in the filtering action of the column.

The pH of each sample should be recorded. A second run should be performed at the same temperature.

1.6.4. **Flask method**

1.6.4.1. *Apparatus*

For the flask method the following material is needed:

— normal laboratory glassware and instrumentation,

— a device suitable for the agitation of solutions under controlled constant temperatures,

— a centrifuge (preferably thermostated), if required with emulsions, and

— equipment for analytical determination.

1.6.4.2. *Measurement procedure*

The quantity of material necessary to saturate the desired volume of water is estimated from the preliminary test. The volume of water required will depend on the analytical method and the solubility range. About five times the quantity of material determined above is weighed into each of three glass vessels fitted with glass stoppers (e.g. centrifuge tubes, flasks). The chosen volume of water is added to each vessel, and the vessels are tightly stoppered. The closed vessels are then agitated at 30 °C. (A shaking or stirring device capable of operating at constant temperature should be used, e.g. magnetic stirring in a thermostatically controlled water bath). After one day, one of the vessels is removed and re-equilibrated for 24 hours at the test temperature with occasional shaking. The contents of the vessel are then centrifuged at the test temperature, and the concentration of test substance in the clear aqueous phase is determined by a suitable analytical method. The other two flasks are treated similarly after initial equilibration at 30 °C for two and three days, respectively. If the concentration results from at least the last two vessels agree with the required reproducibility, the test is satisfactory. The whole test should be repeated, using longer equilibration times, if the results from vessels 1, 2 and 3 show a tendency to increasing values.

The measurement procedure can also be performed without pre-incubation at 30 °C. In order to estimate the rate of establishment of the saturation equilibrium, samples are taken until the stirring time no longer influences the concentration of the test solution.

The pH of each sample should be recorded.

1.6.5. **Analysis**

A substance-specific analytical method is preferred for these determinations, since small amounts of soluble impurities can cause large errors in the measured solubility. Examples of such methods are: gas or liquid chromatography, titration methods, photometric methods, voltammetric methods.

2. **DATA**

2.1. COLUMN ELUTION METHOD

The mean value from at least five consecutive samples taken from the saturation plateau should be calculated for each run, as should the standard deviation. The results should be given in units of mass per volume of solution.

The means calculated on two tests using different flows are compared and should have a repeatability of less than 30 %.

2.2. FLASK METHOD

The individual results should be given for each of the three flasks and those results deemed to be constant (repeatability of less than 15 %) should be averaged and given in units of mass per volume of solution. This may require the reconversion of mass units to volume units, using the density when the solubility is very high (> 100 grams per litre).

3. **REPORTING**

3.1. COLUMN ELUTION METHOD

The test report shall, if possible, include the following information:

— the results of the preliminary test,

— precise specification of the substance (identity and impurities),

— the individual concentrations, flow rates and pH of each sample,

— the means and standard deviations from at least five samples from the saturation plateau of each run,

— the average of the two successive, acceptable runs,

— the temperature of the water during the saturation process,

— the method of analysis employed,

— the nature of the support material employed,

— loading of support material,

— solvent used,

— evidence of any chemical instability of the substance during the test and the method used,

— all information relevant for the interpretation of the results, especially with regard to impurities and physical state of the substance.

3.2. FLASK METHOD

The test report shall, if possible, include the following information:

— the results of the preliminary test,

— precise specification of the substance (identity and impurities),

— the individual analytical determinations and the average where more than one value was determined for each flask,

— the pH of each sample,

— the average of the value for the different flasks which were in agreement,

— the test temperature,

— the analytical method employed,

— evidence of any chemical instability of the substance during the test and the method used,

— all information relevant for the interpretation of the results, especially with regard to impurities and physical state of the substance.

4. REFERENCES

(1) OECD, Paris, 1981, Test Guideline 105, Decision of the Council C(81) 30 final.

(2) NF T 20-045 (AFNOR) (September 85) Chemical products for industrial use — Determination of water solubility of solids and liquids with low solubility — Column elution method.

(3) NF T 20-046 (AFNOR) (September 85) Chemical products for industrial use — Determination of water solubility of solids and liquids with high solubility — Flask method.

Appendix

Figure 1

Column elution method with recirculating pump

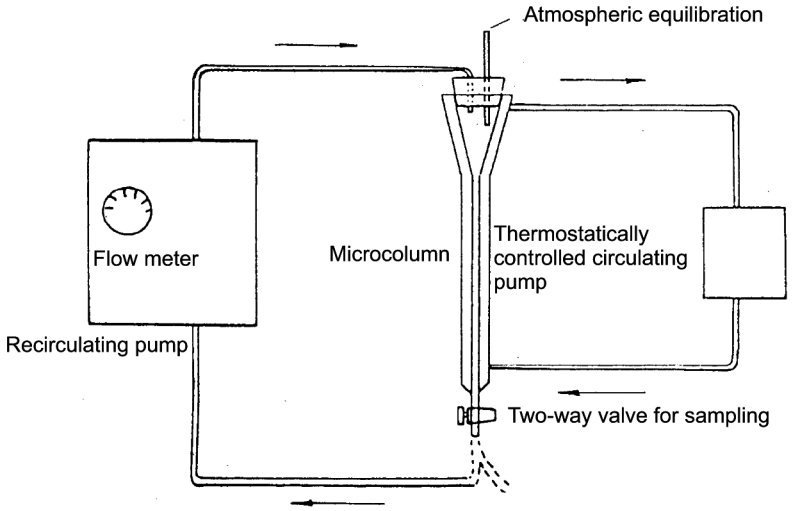


Figure 2

A typical microcolumn

(All dimensions in millimetres)

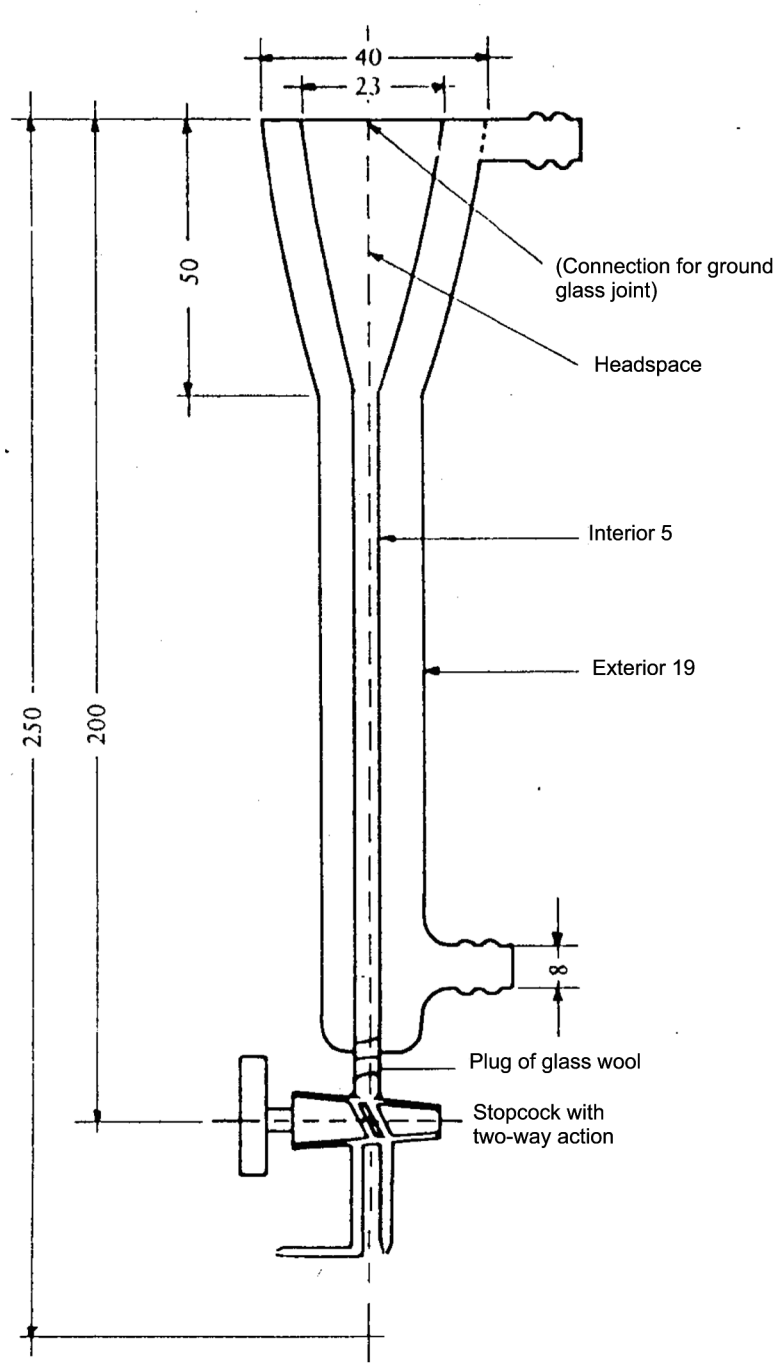


Figure 3

A typical microcolumn

(All dimensions in millimetres)

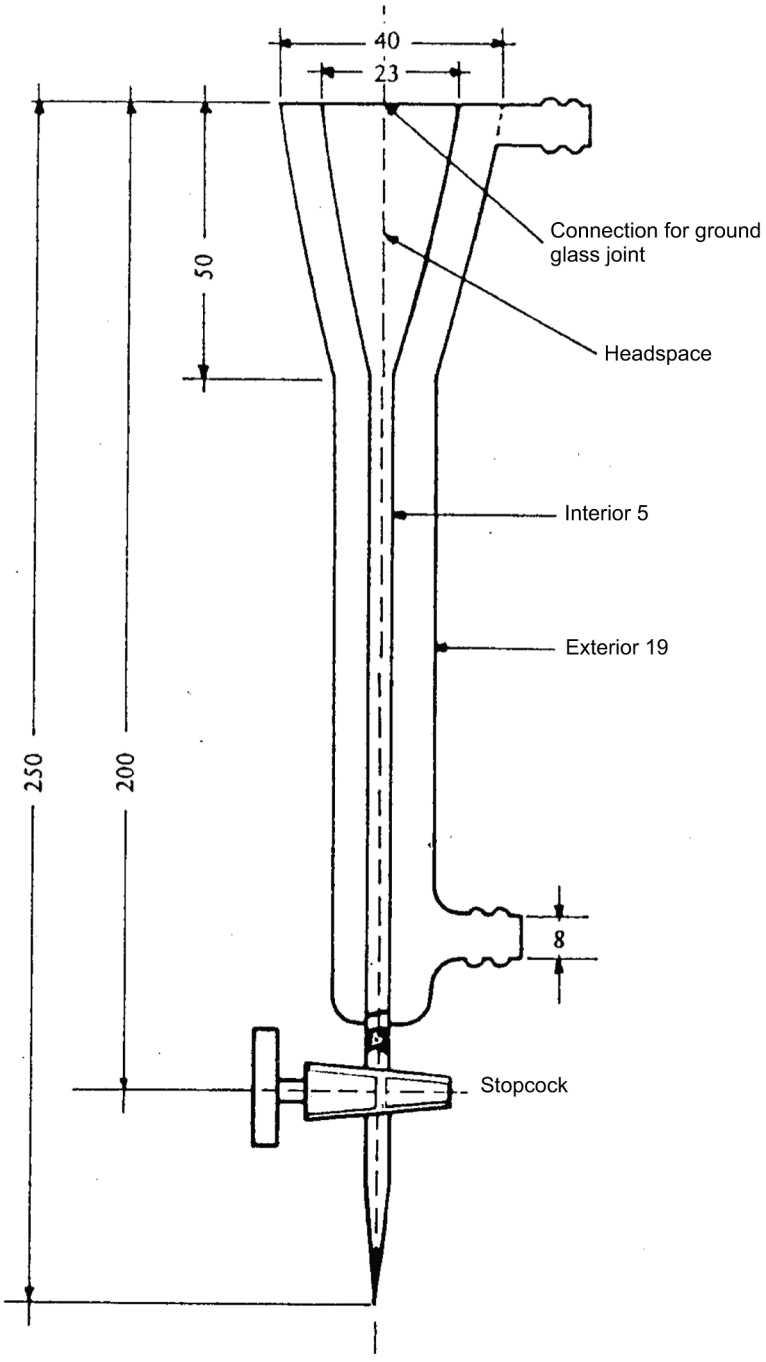
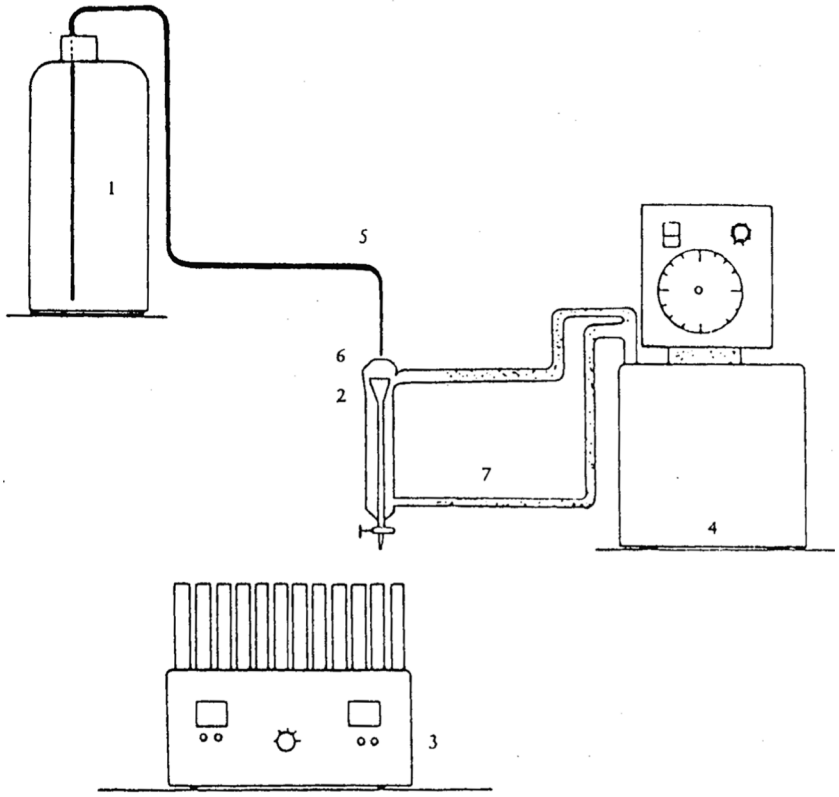


Figure 4

Column elution method with levelling vessel



1 = Levelling vessel (e.g. 2,5 litre flask)
2 = Column (see figure 3)
3 = Fraction collector
4 = Thermostat
5 = Teflon tubing
6 = Ground glass joint
7 = Water line (between thermostat and column, inner diameter: approximately 8 mm)

A.8. PARTITION COEFFICIENT

1. METHOD

The 'shake flask' method described is based on the OECD Test Guideline (1).

1.1. INTRODUCTION

It is useful to have preliminary information on structural formula, dissociation constant, water solubility, hydrolysis, n-octanol solubility and surface tension of the substance to perform this test.

Measurements should be made on ionisable substances only in their non-ionised form (free acid or free base) produced by the use of an appropriate buffer with a pH of at least one pH unit below (free acid) or above (free base) the pK.

This test method includes two separate procedures: the shake flask method and high performance liquid chromatography (HPLC). The former is applicable when the log P_{ow} value (see below for definitions) falls within the range − 2 to 4 and the latter within the range 0 to 6. Before carrying out either of the experimental procedures a preliminary estimate of the partition coefficient should first be obtained.

The shake-flask method applies only to essentially pure substances soluble in water and n-octanol. It is not applicable to surface active materials (for which a calculated value or an estimate based on the individual n-octanol and water solubilities should be provided).

The HPLC method is not applicable to strong acids and bases, metal complexes, surface-active materials or substances which react with the eluent. For these materials, a calculated value or an estimate based on individual n-octanol and water solubilities should be provided.

The HPLC method is less sensitive to the presence of impurities in the test compound than is the shake-flask method. Nevertheless, in some cases impurities can make the interpretation of the results difficult because peak assignment becomes uncertain. For mixtures which give an unresolved band, upper and lower limits of log P should be stated.

1.2. DEFINITION AND UNITS

The partition coefficient (P) is defined as the ratio of the equilibrium concentrations (c_i) of a dissolved substance in a two-phase system consisting of two largely immiscible solvents. In the case n-octanol and water:

$$P_{ow} = \frac{c_{n-octanol}}{c_{water}}$$

The partition coefficient (P) therefore is the quotient of two concentrations and is usually given in the form of its logarithm to base 10 (log P).

1.3. REFERENCE SUBSTANCES

Shake-flask method

Reference substances do not need to be employed in all cases when investigating a new substance. They should primarily serve to check the performance of the method from time to time and to allow comparison with results from other methods.

HPLC method

In order to correlate the measured HPLC data of a compound with its P value, a calibration graph of log P versus chromatographic data using at least six reference points has to be established. It is for the user to select the appropriate reference substances. Whenever possible, at least one reference compound should have a P_{ow} above that of the test substance, and another a P_{ow} below that of the test substance. For log P values less than 4, the calibration can be based on data obtained by the shake-flask method. For log P values greater than 4, the calibration can be based on validated literature values if these are in agreement with calculated values. For better accuracy, it is preferable to choose reference compounds which are structurally related to the test substance.

Extensive lists of values of log P_{ow} for many groups of chemicals are available (2)(3). If data on the partition coefficients of structurally related compounds are not available, then a more general calibration, established with other reference compounds, may be used.

A list of recommended reference substances and their P_{ow} values is given in Appendix 2.

1.4. PRINCIPLE OF THE METHOD

1.4.1. **Shake-flask method**

In order to determine a partition coefficient, equilibrium between all interacting components of the system must be achieved, and the concentrations of the substances dissolved in the two phases must be determined. A study of the literature on this subject indicates that several different techniques can be used to solve this problem, i.e. the thorough mixing of the two phases followed by their separation in order to determine the equilibrium concentration for the substance being examined.

1.4.2. **HPLC method**

HPLC is performed on analytical columns packed with a commercially available solid phase containing long hydrocarbon chains (e.g. C_8, C_{18}) chemically bound onto silica. Chemicals injected onto such a column move along it at different rates because of the different degrees of partitioning between the mobile phase and the hydrocarbon stationary phase. Mixtures of chemicals are eluted in order of their hydrophobicity, with water-soluble chemicals eluted first and oil-soluble chemicals last, in proportion to their hydrocarbon-water partition coefficient. This enables the relationship between the retention time on such a (reverse phase) column and the n-octanol/water partition coefficient to be established. The partition coefficient is deduced from the *capacity factor* k, given by the expression:

$$k = \frac{t_r - t_o}{t_o}$$

in which, t_r = retention time of the test substance, and t_o = average time a solvent molecule needs to pass through the column (dead-time).

Quantitative analytical methods are not required and only the determination of elution times is necessary.

1.5. QUALITY CRITERIA

1.5.1. **Repeatability**

Shake-flask method

In order to assure the accuracy of the partition coefficient, duplicate determinations are to be made under three different test conditions, whereby the quantity of substance specified as well as the ratio of the solvent volumes may be varied. The determined values of the partition coefficient expressed as their common logarithms should fall within a range of ± 0,3 log units.

HPLC method

In order to increase the confidence in the measurement, duplicate determinations must be made. The values of log P derived from individual measurements should fall within a range of ± 0,1 log units.

1.5.2. **Sensitivity**

Shake-flask method

The measuring range of the method is determined by the limit of detection of the analytical procedure. This should permit the assessment of values of log P_{ow} in the range of − 2 to 4 (occasionally when conditions apply, this range may be extended to log P_{ow} up to 5) when the concentration of the solute in either phase is not more than 0,01 mol per litre.

HPLC method

The HPLC method enables partition coefficients to be estimated in the log P_{ow} range 0 to 6.

Normally, the partition coefficient of a compound can be estimated to within ± 1 log unit of the shake-flask value. Typical correlations can be found in the literature (4)(5)(6)(7)(8). Higher accuracy can usually be achieved when correlation plots are based on structurally-related reference compounds (9).

1.5.3. **Specificity**

Shake-flask method

The Nernst Partition Law applies only at constant temperature, pressure and pH for dilute solutions. It strictly applies to a pure substance dispersed between two pure solvents. If several different solutes occur in one or both phases at the same time, this may affect the results.

Dissociation or association of the dissolved molecules result in deviations from the Nernst Partition Law. Such deviations are indicated by the fact that the partition coefficient becomes dependent upon the concentration of the solution.

Because of the multiple equilibria involved, this test method should not be applied to ionisable compounds without applying a correction. The use of buffer solutions in place of water should be considered for such compounds; the pH of the buffer should be at least 1 pH unit from the pKa of the substance and bearing in mind the relevance of this pH for the environment.

1.6. DESCRIPTION OF THE METHOD

1.6.1. **Preliminary estimate of the partition coefficient**

The partition coefficient is estimated preferably by using a calculation method (see Appendix 1), or where appropriate, from the ratio of the solubilities of the test substance ill the pure solvents (10).

1.6.2. **Shake-flask method**

1.6.2.1. *Preparation*

n-Octanol: the determination of the partition coefficient should be carried out with high purity analytical grade reagent.

Water: water distilled or double distilled in glass or quartz apparatus should be employed. For ionisable compounds, buffer solutions in place of water should be used if justified.

Note:

Water taken directly from an ion exchanger should not be used.

1.6.2.1.1. Pre-saturation of the solvents

Before a partition coefficient is determined, the phases of the solvent system are mutually saturated by shaking at the temperature of the experiment. To do this, it is practical to shake two large stock bottles of high purity analytical grade n-octanol or water each with a sufficient quantity of the other solvent for 24 hours on a mechanical shaker and then to let them stand long enough to allow the phases to separate and to achieve a saturation state.

1.6.2.1.2. Preparation for the test

The entire volume of the two-phase system should nearly fill the test vessel. This will help prevent loss of material due to volatilisation. The volume ratio and quantities of substance to be used are fixed by the following:

— the preliminary assessment of the partition coefficient (see above),

— the minimum quantity of test substance required for the analytical procedure, and

— the limitation of a maximum concentration in either phase of 0,01 mol per litre.

Three tests are carried out. In the first, the calculated volume ratio of n-octanol to water is used; in the second, this ratio is divided by two; and in the third, this ratio is multiplied by two (e.g. 1:1, 1:2, 2:1).

1.6.2.1.3. Test substance

A stock solution is prepared in n-octanol pre-saturated with water. The concentration of this stock solution should be precisely determined before it is employed in the determination of the partition coefficient. This solution should be stored under conditions which ensure its stability.

1.6.2.2. *Test conditions*

The test temperature should be kept constant (± 1 °C) and lie in the range of 20 to 25 °C.

1.6.2.3. *Measurement procedure*

1.6.2.3.1. Establishment of the partition equilibrium

Duplicate test vessels containing the required, accurately measured amounts of the two solvents together with the necessary quantity of the stock solution should be prepared for each of the test conditions.

The n-octanol phases should be measured by volume. The test vessels should either be placed in a suitable shaker or shaken by hand. When using a centrifuge tube, a recommended method is to rotate the tube quickly through 180° about its transverse axis so that any trapped air rises through the two phases. Experience has shown that 50 such rotations are usually sufficient for the establishment of the partition equilibrium. To be certain, 100 rotations in five minutes are recommended.

1.6.2.3.2. Phase separation

When necessary, in order to separate the phases, centrifugation of the mixture should be carried out. This should be done in a laboratory centrifuge maintained at room temperature, or, if a non-temperature controlled centrifuge is used, the centrifuge tubes should be kept for equilibration at the test temperature for at least one hour before analysis.

1.6.2.4. *Analysis*

For the determination of the partition coefficient, it is necessary to determine the concentrations of the test substance in both phases. This may be done by taking an aliquot of each of the two phases from each tube for each test condition and analyzing them by the chosen procedure. The total quantity of substance present in both phases should be calculated and compared with the quantity of the substance originally introduced.

The aqueous phase should be sampled by a procedure that minimises the risk of including traces of n-octanol: a glass syringe with a removable needle can be used to sample the water phase. The syringe should initially be partially filled with air. Air should be gently expelled while inserting the needle through the n-octanol layer. An adequate volume of aqueous phase is withdrawn into the syringe. The syringe is quickly removed from the solution and the needle detached. The contents of the syringe may then be used as the aqueous sample. The concentration in the two separated phases should preferably be determined by a substance-specific method. Examples of analytical methods which may be appropriate are:

— photometric methods,

— gas chromatography,

— high-performance liquid chromatography.

1.6.3. **HPLC method**

1.6.3.1. *Preparation*

Apparatus

A liquid chromatograph, fitted with a pulse-free pump and a suitable detection device, is required. The use of an injection valve with injection loops is recommended. The presence of polar groups in the stationary phase may seriously impair the performance of the HPLC column. Therefore, stationary phases should have the minimal percentage of polar groups (11). Commercial microparticulate reverse-phase packings or ready-packed columns can be used. A guard column may be positioned between the injection system and the analytical column.

Mobile phase

HPLC grade methanol and HPLC grade water are used to prepare the eluting solvent, which is degassed before use. Isocratic elution should be employed. Methanol/water ratios with a minimum water content of 25 % should be used. Typically a 3:1 (v/v) methanol-water mixture is satisfactory for eluting compounds of log P 6 within an hour, at a flow rate of 1 ml/min. For compounds of high log P it may be necessary to shorten the elution time (and those of the reference compounds) by decreasing the polarity of the mobile phase or the column length.

Substances with very low solubility in n-octanol tend to give abnormally low log P_{ow} values with the HPLC method; the peaks of such compounds sometimes accompany the solvent front. This is probably due to the fact that the partitioning process is too slow to reach the equilibrium in the time normally taken by an HPLC separation. Decreasing the flow rate and/or lowering the methanol/water ratio may then be effective to arrive at a reliable value.

Test and reference compounds should be soluble in the mobile phase in sufficient concentrations to allow their detection. Only in exceptional cases may additives be used with the methanol-water mixture, since additives will change the properties of the column. For chromatograms with additives it is mandatory to use a separate column of the same type. If methanol-water is not appropriate, other organic solvent-water mixtures call be used, e.g. ethanol-water or acetonitrile-water.

The pH of the eluent is critical for ionisable compounds. It should be within the operating pH range of the column, which is usually between 2 and 8. Buffering is recommended. Care must be taken to avoid salt precipitation and column deterioration which occur with some organic phase/buffer mixtures. HPLC measurements with silica-based stationary phases above pH 8 are not advisable since the use of an alkaline, mobile phase may cause rapid deterioration in the performance of the column.

Solutes

The reference compounds should be the purest available. Compounds to be used for test or calibration purposes are dissolved in the mobile phase if possible.

Test conditions

The temperature during the measurements should not vary by more than ± 2 K.

1.6.3.2. **Measurement**

Calculation of dead time t_o

The dead time to can be determined by using either a homologous series (e.g. n-alkyl methyl ketones) or unretained organic compounds (e.g. thiourea or formamide). For calculating the dead time to by using a homologous series, a set of at least seven members of a homologous series is injected and the respective retention times are determined. The raw retention times $t_{r\ (n_c\ +\ 1)}$ are plotted as a function of $t_{r(n_c)}$ and the intercept a and slope b of the regression equation:

$$t_{r(n_c\ +\ 1)} = a + b\ t_{r(n_c)}$$

are determined (n_c = number of carbon atoms). The dead time to is then given by:

$$t_o = a/(1 - b)$$

Calibration graph

The next step is to construct a correlation plot of log k values versus log p for appropriate reference compounds. In practice, a set of between 5 and 10 standard reference compounds whose log p is around the expected range are injected simultaneously and the retention times are determined, preferably on a recording integrator linked to the detection system. The corresponding logarithms of the capacity factors, log k, are calculated and plotted as a function of the log p determined by the shake-flask method. The calibration is performed at regular intervals, at least once daily, so that possible changes in column performance can be allowed for.

Determination of the capacity factor of the test substance

The test substance is injected in as small a quantity of mobile phase as possible. The retention time is determined (in duplicate), permitting the calculation of the capacity factor k. From the correlation graph of the reference compounds, the partition coefficient of the test substance can be interpolated. For very low and very high partition coefficients, extrapolation is necessary. In those cases particular care has to be taken of the confidence limits of the regression line.

2. **DATA**

Shake-flask method

The reliability of the determined values of P can be tested by comparison of the means of the duplicate determinations with the overall mean.

3. **REPORTING**

The test report shall, if possible, include the following information:

— precise specification of the substance (identity and impurities),

— when the methods are not applicable (e.g. surface active material), a calculated value or an estimate based on the individual n-octanol and water solubilities should be provided,

— all information and remarks relevant for the interpretation of results, especially with regard to impurities and physical state of the substance.

For shake-flask method:

— the result of the preliminary estimation, if any,

— temperature of the determination,

— data on the analytical procedures used in determining concentrations,

— time and speed of centrifugation, if used,

— the measured concentrations in both phases for each determination (this means that a total of 12 concentrations will be reported),

— the weight of the test substance, the volume of each phase employed in each test vessel and the total calculated amount of test substance present in each phase after equilibration,

— the calculated values of the partition coefficient (P) and the mean should be reported for each set of test conditions as should the mean for all determinations. If there is a suggestion of concentration dependency of the partition coefficient, this should be noted in the report,

— the standard deviation of individual P values about their mean should be reported,

— the mean P from all determinations should also be expressed as its logarithm (base 10),

— the calculated theoretical P_{ow} when this value has been determined or when the measured value is > 10^4,

— pH of water used and of the aqueous phase during the experiment,

— if buffers are used, justification for the use of buffers in place of water, composition, concentration and pH of the buffers, pH of the aqueous phase before and after the experiment.

For HPLC method:

— the result of the preliminary estimation, if any,

— test and reference substances, and their purity,

— temperature range of the determinations,

— pH at which the determinations are made,

— details of the analytical and guard column, mobile phase and means of detection,

— retention data and literature log P values for reference compounds used in calibration,

— details of fitted regression line (log k versus log P),

— average retention data and interpolated log P value for the test compound,

— description of equipment and operating conditions,

— elution profiles,

— quantities of test and references substances introduced in the column,

— dead-time and how it was measured.

4. **REFERENCES**

(1) OECD, Paris, 1981, Test Guideline 107, Decision of the Council C(81) 30 final.

(2) C. Hansch and A.J. Leo, Substituent Constants for Correlation Analysis in Chemistry and Biology, John Wiley, New York, 1979.

(3) Log P and Parameter Database, A tool for the quantitative prediction of bioactivity (C. Hansch, chairman, A.J. Leo, dir.) — Available from Pomona College Medical Chemistry Project 1982, Pomona College, Claremont, California 91711.

(4) L. Renberg, G. Sundström and K. Sundh-Nygärd, Chemosphere, 1980, vol. 80, p. 683.

(5) H. Ellgehausen, C. D'Hondt and R. Fuerer, Pestic. Sci., 1981, vol. 12, p. 219.

(6) B. McDuffie, Chemosphere, 1981, vol. 10, p. 73.

(7) W.E. Hammers et al., J. Chromatogr., 1982, vol. 247, p. 1.

(8) J.E. Haky and A.M. Young, J. Liq. Chromat., 1984, vol. 7, p. 675.

(9) S. Fujisawa and E. Masuhara, J. Biomed. Mat. Res., 1981, vol. 15, p. 787.

(10) O. Jubermann, Verteilen und Extrahieren, in Methoden der Organischen Chemie (Houben Weyl), Allgemeine Laboratoriumpraxis (edited by E. Muller), Georg Thieme Verlag, Stuttgart, 1958, Band I/1, p. 223-339.

(11) R.F. Rekker and H.M. de Kort, Euro. J. Med. Chem., 1979, vol. 14, p. 479.

(12) A. Leo, C. Hansch and D. Elkins, Partition coefficients and their uses. Chem. Rev., 1971, vol. 71, p. 525.

(13) R.F. Rekker, The Hydrophobic Fragmental Constant, Elsevier, Amsterdam, 1977.

(14) NF T 20-043 AFNOR (1985). Chemical products for industrial use — Determination of partition coefficient — Flask shaking method.

(15) C.V. Eadsforth and P. Moser, Chemosphere, 1983, vol. 12, p. 1459.

(16) A. Leo, C. Hansch and D. Elkins, Chem. Rev., 1971, vol. 71, p. 525.

(17) C. Hansch, A. Leo, S.H. Unger, K.H. Kim, D. Nikaitani and E.J. Lien, J. Med. Chem., 1973, vol. 16, p. 1207.

(18) W.B. Neely, D.R. Branson and G.E. Blau, Environ. Sci. Technol., 1974, vol. 8, p. 1113.

(19) D.S. Brown and E.W. Flagg, J. Environ. Qual., 1981, vol. 10, p. 382.

(20) J.K. Seydel and K.J. Schaper, Chemische Struktur und biologische Aktivität von Wirkstoffen, Verlag Chemie, Weinheim, New York, 1979.

(21) R. Franke, Theoretical Drug Design Methods, Elsevier, Amsterdam, 1984.

(22) Y.C. Martin, Quantitative Drug Design, Marcel Dekker, New York, Base1, 1978.

(23) N.S. Nirrlees, S.J. Noulton, C.T. Murphy, P.J. Taylor; J. Med. Chem., 1976, vol. 19, p. 615.

Appendix 1

Calculation/estimation methods

INTRODUCTION

A general introduction to calculation methods, data and examples are provided in the Handbook of Chemical Property Estimation Methods (a).

Calculated values of P_{ow} can be used:

— for deciding which of the experimental methods is appropriate (shake-flask range: log P_{ow}: – 2 to 4, HPLC range: log P_{ow}: 0 to 6),

— for selecting the appropriate test conditions (e.g. reference substances for HPLC procedures, volume ratio n-octanol/water for shake flask method),

— as a laboratory internal check on possible experimental errors,

— for providing a P_{ow}-estimate in cases where the experimental methods cannot be applied for technical reasons.

ESTIMATION METHOD

Preliminary estimate of the partition coefficient

The value of the partition coefficient can be estimated by the use of the solubilities of the test substance in the pure solvents: For this:

$$P_{estimate} = \frac{\text{saturation } c_{n-octanol}}{\text{saturation } c_{water}}$$

CALCULATION METHODS

Principle of the calculation methods

All calculation methods are based on the formal fragmentation of the molecule into suitable substructures for which reliable log P_{ow}-increments are known. The log P_{ow} of the whole molecule is then calculated as the sum of its corresponding fragment values plus the sum of correction terms for intramolecular interactions.

Lists of fragment constants and correction terms ate available (b)(c)(d)(e);. Some are regularly updated (b).

Quality criteria

In general, the reliability of the calculation method decreases with increasing complexity of the compound under study. In the case of simple molecules with low molecular weight and one or two functional groups, a deviation of 0,1 to 0,3 log P_{ow} units between the results of the different fragmentation methods and the measured value can be expected. In the case of more complex molecules the margin of error can be greater. This will depend on the reliability and availability of fragment constants, as well as on the ability to recognise intramolecular interactions (e.g. hydrogen bonds) and the correct use of the correction terms (less of a problem with the computer software CLOGP-3) (b). In the case of ionising compounds the correct consideration of the charge or degree of ionisation is important.

Calculation procedures

Hansch π-method

The original hydrophobic substituent constant, π, introduced by Fujira et al. (f) is defined as:

$\pi_x = \log P_{ow}\ (PhX) - \log P_{ow}\ (PhH)$

where P_{ow} (PhX) is the partition coefficient of an aromatic derivative and P_{ow} (PhH) that of the parent compound

(e.g. $\pi_{Cl} = \log P_{ow}\ (C_6H_5Cl) - \log P_{ow}\ (C_6H_6) = 2{,}84 - 2{,}13 = 0{,}71$).

According to its definition the π-method is applicable predominantly for aromatic substitution. π-values for a large number of substituents have been tabulated (b)(c)(d). They are used for the calculation of log P_{ow} for aromatic molecules or substructures.

Rekker method

According to Rekker (g) the log P_{ow} value is calculated as follows:

$$\log P_{ow} = \sum_i a_i f_i + \sum_j (\text{interactious terms})$$

where f_i represents the different molecular fragment constants and a_i the frequency of their occurrence in the molecule under investigation. The correction terms can be expressed as an integral multiple of one single constant C_m (so-called magic constant). The fragment constants f_i and C_m were determined from a list of 1 054 experimental P_{ow} values (825 compounds) using multiple regression analysis (c)(h). The determination of the interaction terms is carried out according to set rules described in the literature (e)(h)(i).

Hansch-Leo method

According to Hansch and Leo (c), the log P_{ow} value is calculated from:

$$\log P_{ow} = \sum_i a_i\ f_i + \sum_j b_j\ F_j$$

where f_i represents the different molecular fragment constants, F_j the correction terms and a_i, b_j the corresponding frequencies of occurrence. Derived from experimental P_{ow} values, a list of atomic and group fragmental values and a list of correction terms F_j (so-called factors) were determined by trial and error. The correction terms have been ordered into several different classes (a)(c). It is relatively complicated and time consuming to take into account all the rules and correction terms. Software packages have been developed (b).

Combined method

The calculation of log P_{ow} of complex molecules can be considerably improved, if the molecule is dissected into larger substructures for which reliable log P_{ow} values are available, either from tables (b)(c) or from one's own measurements. Such fragments (e.g. heterocycles, anthraquinone, azobenzene) can then be combined with the Hansch π-values or with Rekker or Leo fragment constants.

Remarks

(i) The calculation methods can only be applied to partly or fully ionised compounds when it is possible to take the necessary correction factors into account;

(ii) if intramolecular hydrogen bonds can be assumed, the corresponding correction terms (approx. + 0,6 to + 1,0 log P_{ow} units) have to be added (a). Indications for the presence of such bonds can be obtained from stereo models or spectroscopic data of the molecule;

(iii) If several tautomeric forms are possible, the most likely form should be used as the basis of the calculation;

(iv) the revisions of lists of fragment constants should be followed carefully.

Report

When using calculation/estimation methods, the test report shall, if possible, include the following information:

— description of the substance (mixture, impurities, etc.),

— indication of any possible intramolecular hydrogen bonding, dissociation, charge and any other unusual effects (e.g. tautomerism),

— description of the calculation method,

— identification or supply of database,

— peculiarities in the choice of fragments,

— comprehensive documentation of the calculation.

LITERATURE

(a) W.J. Lyman, W.F. Reehl and D.H. Rosenblatt (ed.), Handbook of Chemical Property Estimation Methods, McGraw-Hill, New York, 1983.

(b) Pomona College, Medicinal Chemistry Project, Claremont, California 91711, USA, Log P Database and Med. Chem. Software (Program CLOGP-3).

(c) C. Hansch, A.J. Leo, Substituent Constants for Correlation Analysis in Chemistry and Biology, John Wiley, New York, 1979.

(d) A. Leo, C. Hansch, D. Elkins, Chem. Rev., 1971, vol. 71, p. 525.

(e) R.F. Rekker, H.M. de Kort, Eur. J. Med. Chem. -Chill. Ther. 1979, vol. 14, p. 479.

(f) T. Fujita, J. Iwasa and C. Hansch, J. Amer. Chem. Soc., 1964, vol. 86, p. 5175.

(g) R.F. Rekker, The Hydrophobic Fragmental Constant, Pharmacochemistry Library, Elsevier, New York, 1977, vol. 1.

(h) C.V. Eadsforth, P. Moser, Chemosphere, 1983, vol. 12, p. 1459.

(i) R.A. Scherrer, ACS, American Chemical Society, Washington D.C., 1984, Symposium Series 255, p. 225.

Appendix 2

Recommended Reference Substances for the HLPC Method

No	Reference Substance	log P_{ow}	pKa
1	2-Butanone	0,3	
2	4-Acetylpyridine	0,5	
3	Aniline	0,9	
4	Acetanilide	1,0	
5	Benzylalcohol	1,1	
6	p-Methoxyphenol	1,3	pKa = 10,26
7	Phenoxy acetic acid	1,4	pKa = 3,12
8	Phenol	1,5	pKa = 9,92
9	2,4-Dinitrophenol	1,5	pKa = 3,96
10	Benzonitrile	1,6	
11	Phenylacetonitrile	1,6	
12	4-Methylbenzyl alcohol	1,6	
13	Acetophenone	1,7	
14	2-Nitrophenol	1,8	pKa = 7,17
15	3-Nitrobenzoic acid	1,8	pKa = 3,47
16	4-Chloraniline	1,8	pKa = 4,15
17	Nitrobenzene	1,9	
18	Cinnamic alcohol	1,9	
19	Benzoic acid	1,9	pKa = 4,19
20	p-Cresol	1,9	pKa = 10,17
21	Cinnamic acid	2,1	pKa = 3,89 cis 4,44 trans
22	Anisole	2,1	
23	Methylbenzoate	2,1	
24	Benzene	2,1	
25	3-Methylbenzoic acid	2,4	pKa = 4,27
26	4-Chlorophenol	2,4	pKa = 9,1
27	Trichloroethylene	2,4	
28	Atrazine	2,6	
29	Ethylbenzoate	2,6	
30	2,6-Dichlorobenzonitrile	2,6	
31	3-Chlorobenzoic acid	2,7	pKa = 3,82
32	Toluene	2,7	
33	1-Naphthol	2,7	pKa = 9,34
34	2,3-Dichloroaniline	2,8	
35	Chlorobenzene	2,8	
36	Allyl-phenylether	2,9	
37	Bromobenzene	3,0	
38	Ethylbenzene	3,2	
39	Benzophenone	3,2	
40	4-Phenylphenol	3,2	pKa = 9,54
41	Thymol	3,3	

No	Reference Substance	log P_{ow}	pKa
42	1,4-Dichlorobenzene	3,4	
43	Diphenylamine	3,4	pKa = 0,79
44	Naphthalene	3,6	
45	Phenylbenzoate	3,6	
46	Isopropylbenzene	3,7	
47	2,4,6-Trichlorophenol	3,7	pKa = 6
48	Biphenyl	4,0	
49	Benzylbenzoate	4,0	
50	2,4-Dinitro-6 sec. butyophenol	4,1	
51	1,2,4-Trichlorobenzene	4,2	
52	Dodecanoic acid	4,2	
53	Diphenylether	4,2	
54	n-Butylbenzene	4,5	
55	Phenanthrene	4,5	
56	Fluoranthene	4,7	
57	Dibenzyl	4,8	
58	2,6-Diphenylpyridine	4,9	
59	Triphenylamine	5,7	
60	DDT	6,2	
	Other reference substances of low log P_{ow}		
1	Nicotinic acid	- 0,07	

A.9. **FLASH-POINT**

1. **METHOD**

1.1. INTRODUCTION

It is useful to have preliminary information on the flammability of the substance before performing this test. The test procedure is applicable to liquid substances whose vapours can be ignited by ignition sources. The test methods listed in this text are only reliable for flash-point ranges which are specified in the individual methods.

The possibility of chemical reactions between the substance and the sample holder should be considered when selecting the method to be used.

1.2. DEFINITIONS AND UNITS

The flash-point is the lowest temperature, corrected to a pressure of 101,325 kPa, at which a liquid evolves vapours, under the conditions defined in the test method, in such an amount that a flammable vapour/air mixture is produced in the test vessel.

Units: °C

$t = T - 273{,}15$

(t in °C and T in K)

1.3. REFERENCE SUBSTANCES

Reference substances do not need to be employed in all cases when investigating a new substance. They should primarily serve to check the performance of the method from time to time and to allow comparison with results from other methods.

1.4. PRINCIPLE OF THE METHOD

The substance is placed in a test vessel and heated or cooled to the test temperature according to the procedure described in the individual test method. Ignition trials are carried out in order to ascertain whether or not the sample flashed at the test temperature.

1.5. QUALITY CRITERIA

1.5.1. **Repeatability**

The repeatability varies according to flash-point range and the test method used; maximum 2 °C.

1.5.2. **Sensitivity**

The sensitivity depends on the test method used.

1.5.3. **Specificity**

The specificity of some test methods is limited to certain flash-point ranges and subject to substance-related data (e.g. high viscosity).

1.6. DESCRIPTION OF THE METHOD

1.6.1. **Preparations**

A sample of the test substance is placed in a test apparatus according to 1.6.3.1 and/or 1.6.3.2.

For safety, it is recommended that a method utilising a small sample size, circa 2 cm^3, be used for energetic or toxic substances.

1.6.2. **Test conditions**

The apparatus should, as far as is consistent with safety, be placed in a draught-free position.

1.6.3. **Performance of the test**

1.6.3.1. *Equilibrium method*

See ISO 1516, ISO 3680, ISO 1523, ISO 3679.

1.6.3.2. *Non-equilibrium method*

Abel apparatus:

See BS 2000 part 170, NF M07-011, NF T66-009.

Abel-Pensky apparatus:

See EN 57, DIN 51755 part 1 (for temperatures from 5 to 65 °C), DIN 51755 part 2 (for temperatures below 5 °C), NF M07-036.

Tag apparatus:

See ASTM D 56.

Pensky-Martens apparatus:

See ISO 2719, EN 11, DIN 51758, ASTM D 93, BS 2000-34, NF M07-019.

Remarks:

When the flash-point, determined by a non-equilibrium method in 1.6.3.2, is found to be 0 ± 2 °C, 21 ± 2 °C or 55 ± 2 °C, it should be confirmed by an equilibrium method using the same apparatus.

Only the methods which can give the temperature of the flash-point may be used for a notification.

To determine the flash-point of viscous liquids (paints, gums and similar) containing solvents, only apparatus and test methods suitable for determining the flash-point of viscous liquids may be used.

See ISO 3679, ISO 3680, ISO 1523, DIN 53213 part 1.

2. **DATA**

3. **REPORTING**

The test report shall, if possible, include the following information:

— the precise specification of the substance (identification and impurities),

— the method used should be stated as well as any possible deviations,

— the results and any additional remarks relevant for the interpretation of results.

4. **REFERENCES**

None.

A.10. **FLAMMABILITY (SOLIDS)**

1. **METHOD**

1.1. INTRODUCTION

It is useful to have preliminary information on potentially explosive properties of the substance before performing this test.

This test should only be applied to powdery, granular or paste-like substances.

In order not to include all substances which can be ignited but only those which burn rapidly or those whose burning behaviour is in any way especially dangerous, only substances whose burning velocity exceeds a certain limiting value are considered to be highly flammable.

It can be especially dangerous if incandescence propagates through a metal powder because of the difficulties in extinguishing a fire. Metal powders should be considered highly flammable if they support spread of incandescence throughout the mass within a specified time.

1.2. DEFINITION AND UNITS

Burning time expressed in seconds.

1.3. REFERENCE SUBSTANCES

Not specified.

1.4. PRINCIPLE OF THE METHOD

The substance is formed into an unbroken strip or powder train about 250 mm long and a preliminary screening test performed to determine if, on ignition by a gas flame, propagation by burning with flame or smouldering occurs. If propagation over 200 mm of the train occurs within a specified time then a full test programme to determine the burning rate is carried out.

1.5. QUALITY CRITERIA

Not stated.

1.6. DESCRIPTION OF METHOD

1.6.1. **Preliminary screening test**

The substance is formed into an unbroken strip or powder train about 250 mm long by 20 mm wide by 10 mm high on a non-combustible, non-porous and low heat-conducting base plate. A hot flame from a gas burner (minimum diameter 5 mm) is applied to one end of the powder train until the powder ignites or for a maximum of two minutes (five minutes for powders of metals or metal-alloys). It should be noted whether combustion propagates along 200 mm of the train within the 4 minutes test period (or 40 minutes for metal powders). If the substance does not ignite and propagate combustion either by burning with flame or smouldering along 200 mm of the powder train within the four minutes (or 40 minutes) test period, then the substance should not be considered as highly flammable and no further testing is required. If the substance propagates burning of a 200 mm length of the powder train in less than four minutes, or less than 40 minutes for metal powders, the procedure described below (point 1.6.2. and following) should be carried out.

1.6.2. **Burning rate test**

1.6.2.1. *Preparation*

Powdery or granular substances are loosely filled into a mould 250 mm long with a triangular cross-section of inner height 10 mm and width 20 mm. On both sides of the mould in a longitudinal direction two metal plates are mounted as lateral limitations which project 2 mm beyond the upper edge of the triangular cross section (figure). The mould is then dropped three times from a height of 2 cm onto a solid surface. If necessary the

mould is then filled up again. The lateral limitations are then removed and the excess substance scraped off. A non-combustible, non-porous and low heat-conducting base plate is placed on top of the mould, the apparatus inverted and the mould removed.

Paste-like substances are spread on a non-combustible, non-porous and low heat-conducting base plate in the form of a rope 250 mm in length with a cross section of about 1 cm^2.

1.6.2.2. *Test conditions*

In the case a moisture-sensitive substance, the test should be carried out as quickly as possible after its removal from the container.

1.6.2.3. *Performance of the test*

Arrange the pile across the draught in a fume cupboard.

The air-speed should be sufficient to prevent fumes escaping into the laboratory and should not be varied during the test. A draught screen should be erected around the apparatus.

A hot flame from a gas burner (minimum diameter of 5 mm) is used to ignite the pile at one end. When the pile has burned a distance of 80 mm, the rate of burning over the next 100 mm is measured.

The test is performed six times, using a clean cool plate each time, unless a positive result is observed earlier.

2. DATA

The burning time from the preliminary screening test (1.6.1) and the shortest burning time in up to six tests (1.6.2.3) are relevant for evaluation.

3. REPORTING

3.1. TEST REPORT

The test report shall, if possible, include the following information:

— the precise specification of the substance (identification and impurities),

— a description of the substance to be tested, its physical state including moisture content,

— results from the preliminary screening test and from the burning rate test if performed,

— all additional remarks relevant to the interpretation of results.

3.2. INTERPRETATION OF THE RESULT

Powdery, granular or paste-1ike substances are to be considered as highly flammable when the time of burning in any tests carried out according to the test procedure described in 1.6.2 is less than 45 seconds. Powders of metals or metal-alloys are considered to be highly flammable when they can be ignited and the flame or the zone of reaction spreads over the whole sample in 10 minutes or less.

4. REFERENCES

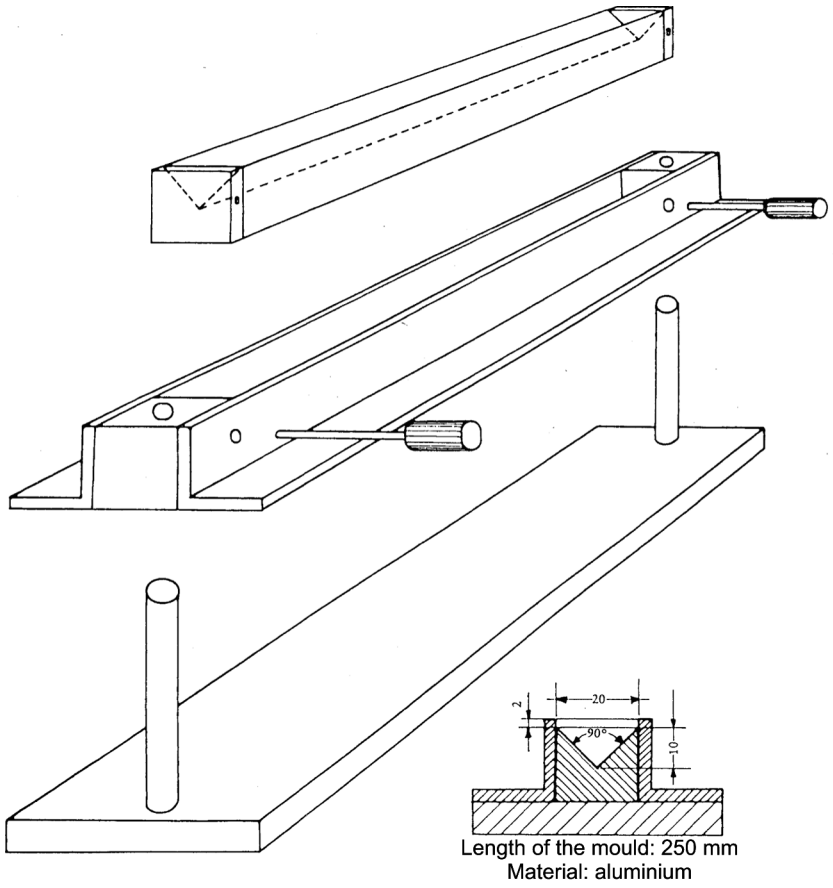
NF T 20-042 (September 85) Chemical products for industrial use. Determination of the flammability of solids.

Appendix

Figure

Mould and accessories for the preparation of the pile

(All dimensions in millimetres)



A.11. FLAMMABILITY (GASES)

1. METHOD

1.1. INTRODUCTION

This method allows a determination of whether gases mixed with air at room temperature (circa 20 °C) and atmospheric pressure are flammable and, if so, over what range of concentrations. Mixtures of increasing concentrations of the test gas with air are exposed to an electrical spark and it is observed whether ignition occurs.

1.2. DEFINITION AND UNITS

The range of flammability is the range of concentration between the lower and the upper explosion limits. The lower and the upper explosion limits are those limits of concentration of the flammable gas in admixture with air at which propagation of a flame does not occur.

1.3. REFERENCE SUBSTANCES

Not specified.

1.4. PRINCIPLE OF THE METHOD

The concentration of gas in air is increased step by step and the mixture is exposed at each stage to an electrical spark.

1.5. QUALITY CRITERIA

Not stated.

1.6. DESCRIPTION OF THE METHOD

1.6.1. **Apparatus**

The test vessel is an upright glass cylinder having a minimum inner diameter of 50 mm and a minimum height of 300 mm. The ignition electrodes are separated by a distance of 3 to 5 mm and are placed 60 mm above the bottom of the cylinder. The cylinder is fitted with a pressure-release opening. The apparatus has to be shielded to restrict any explosion damage.

A standing induction spark of 0,5 sec. duration, which is generated from a high voltage transformer with an output voltage of 10 to 15 kV (maximum of power input 300 W), is used as the ignition source. An example of a suitable apparatus is described in reference (2).

1.6.2. **Test conditions**

The test must be performed at room temperature (circa 20 °C).

1.6.3. **Performance of the test**

Using proportioning pumps, a known concentration of gas in air is introduced into the glass cylinder. A spark is passed through the mixture and it is observed whether or not a flame detaches itself from the ignition source and propagates independently. The gas concentration is varied in steps of 1 % vol. until ignition occurs as described above.

If the chemical structure of the gas indicates that it would be non-flammable and the composition of the stoichiometric mixture with air can be calculated, then only mixtures in the range from 10 % less than the stoichiometric composition to 10 % greater than this composition need be tested in 1 % steps.

2. **DATA**

The occurrence of flame propagation is the only relevant information data for the determination of this property.

3. **REPORTING**

The test report shall, if possible, include the following information:

— the precise specification of the substance (identification and impurities),

— a description, with dimensions, of the apparatus used,

— the temperature at which the test was performed,

— the tested concentrations and the results obtained,

— the result of the test: non-flammable gas or highly flammable gas,

— if it is concluded that the gas is non-flammable then the concentration range over which it was tested in 1 % steps should be stated,

— all information and remarks relevant to the interpretation of results have to be reported.

4. **REFERENCES**

(1) NF T 20-041 (September 85) Chemical products for industrial use. Determination of the flammability of gases.

(2) W. Berthold, D. Conrad, T. Grewer, H. Grosse-Wortmann 'Entwicklung einer Standard-Apparatur zur Messung von Explosionsgrenzen'. Chem.-Ing.- Tech. 1984, vo1. 56, 2, 126-127. , T. Redeker und H. Schacke, p. 126-127.

A.12. FLAMMABILITY (CONTACT WITH WATER)

1. METHOD

1.1. INTRODUCTION

This test method can be used to determine whether the reaction of a substance with water or damp air leads to the development of dangerous amounts of gas or gases which may be highly flammable.

The test method can be applied to both solid and liquid substances. This method is not applicable to substances which spontaneously ignite when in contact with air.

1.2. DEFINITIONS AND UNITS

Highly flammable: substances which, in contact with water or damp air, evolve highly flammable gases in dangerous quantities at a minimum rate of 1 litre/kg per hour.

1.3. PRINCIPLE OF THE METHOD

The substance is tested according to the step by step sequence described below; if ignition occurs at any step, no further testing is necessary. If it is known that the substance does not react violently with water then proceed to step 4 (1.3.4).

1.3.1. **Step 1**

The test substance is placed in a trough containing distilled water at 20 °C and it is noted whether or not the evolved gas ignites.

1.3.2. **Step 2**

The test substance is placed on a filter paper floating on the surface of a dish containing distilled water at 20 °C and it is noted whether or not the evolved gas ignites. The filter paper is merely to keep the substance in one place to increase the chances of ignition.

1.3.3. **Step 3**

The test substance is made into a pile approximately 2 cm high and 3 cm diameter. A few drops of water are added to the pile and it is noted whether or not the evolved gas ignites.

1.3.4. **Step 4**

The test substance is mixed with distilled water at 20 °C and the rate of evolution of gas is measured over a period of seven hours, at one-hour intervals. If the rate of evolution is erratic, or is increasing, after seven hours, the measuring time should be extended to a maximum time of five days. The test may be stopped if the rate at any time exceeds 1 litre/kg per hour.

1.4. REFERENCE SUBSTANCES

Not specified.

1.5. QUALITY CR1TERIA

Not stated.

1.6. DESCRIPTION OF METHODS

1.6.1. **Step 1**

1.6.1.1. *Test conditions*

The test is performed at room temperature (circa 20 °C).

1.6.1.2. *Performance of the test*

A small quantity (approximately 2 mm diameter) of the test substance should be placed in a trough containing distilled water. A note should be made of whether (i) any gas is evolved and (ii) if ignition of the gas occurs. If ignition of the gas occurs then no further testing of the substance is needed because the substance is regarded as hazardous.

1.6.2. **Step 2**

1.6.2.1. *Apparatus*

A filter-paper is floated flat on the surface of distilled water in any suitable vessel, e.g. a 100 mm diameter evaporating dish.

1.6.2.2. *Test conditions*

The test is performed at room temperature (circa 20 °C).

1.6.2.3. *Performance of the test*

A small quantity of the test substance (approximately 2 mm diameter) is placed onto the centre of the filter-paper. A note should be made of whether (i) any gas is evolved and (ii) if ignition of the gas occurs. If ignition of the gas occurs then no further testing of the substance is needed because the substance is regarded as hazardous.

1.6.3. **Step 3**

1.6.3.1. *Test conditions*

The test is performed at room temperature (circa 20 °C).

1.6.3.2. *Performance of the test*

The test substance is made into a pile approximately 2 cm high and 3 cm diameter with an indentation in the top. A few drops of water are added to the hollow and a note is made of whether (i) any gas is evolved and (ii) if ignition of the gas occurs. If ignition of the gas occurs then no further testing of the substance is needed because the substance is regarded as hazardous.

1.6.4. **Step 4**

1.6.4.1. *Apparatus*

The apparatus is set up as shown in the figure.

1.6.4.2. *Test conditions*

Inspect the container of the test substance for any powder < 500 μm (particle size). If the powder constitutes more than 1 % w/w of the total, or if the sample is friable, then the whole of the substance should be ground to a powder before testing to allow for a reduction in particle size during storage and handling; otherwise the substance is to be tested as received. The test should be performed at room temperature (circa 20 °C) and atmospheric pressure.

1.6.4.3. *Performance of the test*

10 to 20 ml of water are put into the dropping funnel of the apparatus and 10 g of substance are put in the conical flask. The volume of gas evolved can be measured by any suitable means. The tap of the dropping funnel is opened to let the water into the conical flask and a stop watch is started. The gas evolution is measured each hour during a seven hour period. If, during this period, the gas evolution is erratic, or if, at the end of this period, the rate of gas evolution is increasing, then measurements should be continued for up to five days. If, at any time of measurement, the rate of gas evolution exceeds 1 litre/kg per hour, the test can be discontinued. This test should be performed in triplicate.

If the chemical identity of the gas is unknown, the gas should be analysed. When the gas contains highly flammable components and it is unknown whether the whole mixture is highly flammable, a mixture of the same composition has to be prepared and tested according to the method A.11.

2. DATA

The substance is considered hazardous if:

— spontaneous ignition takes place in any step of the test procedure,

or

— there is evolution of flammable gas at a rate greater than 1 litre/kg of the substance per hour.

3. REPORTING

The test report shall, if possible, include the following information:

— the precise specification of the substance (identification and impurities),

— details of any initial preparation of the test substance,

— the results of the tests (steps 1, 2, 3 and 4),

— the chemical identity of gas evolved,

— the rate of evolution of gas if step 4 (1.6.4) is performed,

— any additional remarks relevant to the interpretation of the results.

4. REFERENCES

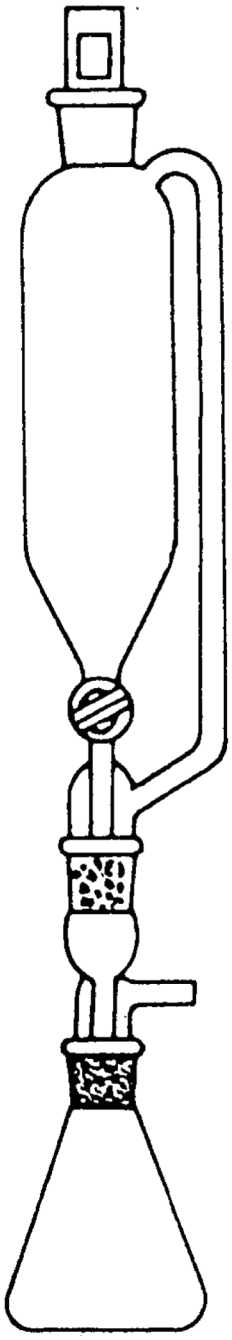
(1) Recommendations on the transport of dangerous goods, test and criteria, 1990, United Nations, New York.

(2) NF T 20-040 (September 85) Chemical products for industrial use. Determination of the flammability of gases formed by the hydrolysis of solid and liquid products.

Appendix

Figure

Apparatus



A.13. PYROPHORIC PROPERTIES OF SOLIDS AND LIQUIDS

1. METHOD

1.1. INTRODUCTION

The test procedure is applicable to solid or liquid substances, which, in small amounts, will ignite spontaneously a short time after coming into contact with air at room temperature (circa 20 °C).

Substances which need to be exposed to air for hours or days at room temperature or at elevated temperatures before ignition occurs are not covered by this test method.

1.2. DEFINITIONS AND UNITS

Substances are considered to have pyrophoric properties if they ignite or cause charring under the conditions described in 1.6.

The auto-flammability of liquids may also need to be tested using method A.15. Auto-ignition temperature (liquids and gases).

1.3. REFERENCE SUBSTANCES

Not specified.

1.4. PRINCIPLE OF THE METHOD

The substance, whether solid or liquid, is added to an inert carrier and brought into contact with air at ambient temperature for a period of five minutes. If liquid substances do not ignite then they are absorbed onto filter paper and exposed to air at ambient temperature (circa 20 °C) for five minutes. If a solid or liquid ignites, or a liquid ignites or chars a filter paper, then the substance is considered to be pyrophoric.

1.5. QUALITY CRITERIA

Repeatability: because of the importance in relation to safety, a single positive result is sufficient for the substance to be considered pyrophoric.

1.6. DESCRIPTION OF THE TEST METHOD

1.6.1. **Apparatus**

A porcelain cup of circa 10 cm diameter is filled with diatomaceous earth to a height of about 5 mm at room temperature (circa 20 °C).

Note:

Diatomaceous earth or any other comparable inert substance which is generally obtainable shall be taken as representative of soil onto which the test substance might be spilled in the event of an accident.

Dry filter paper is required for testing liquids which do not ignite on contact with air when in contact with an inert carrier.

1.6.2. **Performance of the test**

(a) *Powdery solids*

1 to 2 cm^3 of the substance to be tested is poured from circa 1 m height onto a non-combustible surface and it is observed whether the substance ignites during dropping or within five minutes of settling.

The test is performed six times unless ignition occurs;

(b) *liquids*

Circa 5 cm^3 of the liquid to be tested is poured into the prepared porcelain cup and it is observed whether the substance ignites within five minutes.

If no ignition occurs in the six tests, perform the following tests:

A 0,5 ml test sample is delivered from a syringe to an indented filter paper and it is observed whether ignition or charring of the filter paper occurs within five minutes of the liquid being added. The test is performed three times unless ignition or charring occurs.

2. DATA

2.1. TREATMENT OF RESULTS

Testing can be discontinued as soon as a positive result occurs in any of the tests.

2.2. EVALUATION

If the substance ignites within five minutes when added to an inert carrier and exposed to air, or a liquid substance chars or ignites a filter paper within five minutes when added and exposed to air, it is considered to be pyrophoric.

3. REPORTING

The test report shall, if possible, include the following information:

— the precise specification of the substance (identification and impurities),

— the results of the tests,

— any additional remark relevant to the interpretation of the results.

4. REFERENCES

(1) NF T 20-039 (September 85) Chemical products for industrial use. Determination of the spontaneous flammability of solids and liquids.

(2) Recommendations on the Transport of Dangerous Goods, Test and criteria, 1990, United Nations, New York.

A.14. EXPLOSIVE PROPERTIES

1. METHOD

1.1. INTRODUCTION

The method provides a scheme of testing to determine whether a solid or a pasty substance presents a danger of explosion when submitted to the effect of a flame (thermal sensitivity), or to shock or friction (sensitivity to mechanical stimuli), and whether a liquid substance presents a danger of explosion when submitted to the effect of a flame or shock.

The method comprises three parts:

(a) a test of thermal sensitivity (1);

(b) a test of mechanical sensitivity with respect to shock (1);

(c) a test of mechanical sensitivity with respect to friction (1).

The method yields data to assess the likelihood of initiating an explosion by means of certain common stimuli. The method is not intended to ascertain whether a substance is capable of exploding under any conditions.

The method is appropriate for determining whether a substance will present a danger of explosion (thermal and mechanical sensitivity) under the particular conditions specified in the directive. It is based on a number of types of apparatus which are widely used internationally (1) and which usually give meaningful results. It is recognised that the method is not definitive. Alternative apparatus to that specified may be used provided that it is internationally recognised and the results can be adequately correlated with those from the specified apparatus.

The tests need not be performed when available thermodynamic information (e.g. heat of formation, heat of decomposition) and/or absence of certain reactive groups (2) in the structural formula establishes beyond reasonable doubt that the substance is incapable of rapid decomposition with evolution of gases or release of heat (i.e. the material does not present any risk of explosion). A test of mechanical sensitivity with respect to friction is not required for liquids.

1.2. DEFINITIONS AND UNITS

Explosive:

Substances which may explode under the effect of flame or which are sensitive to shock or friction in the specified apparatus (or are more mechanically sensitive than 1,3-dinitrobenzene in alternative apparatus).

1.3. REFERENCE SUBSTANCES

1,3-dinitrobenzene, technical crystalline product sieved to pass 0,5 mm, for the friction and shock methods.

Perhydro-1,3,5-trinitro-1,3,5-triazine (RDX, hexogen, cyclonite — CAS 121-82-4), recrystallised from aqueous cyclohexanone, wet-sieved through a 250 μm and retained on a 150 μm sieve and dried at 103 ± 2 °C (for four hours) for the second series of friction and shock tests.

1.4. PRINCIPLE OF THE METHOD

Preliminary tests are necessary to establish safe conditions for the performance of the three tests of sensitivity.

1.4.1. **Safety-in-handling tests (3)**

For safety reasons, before performing the main tests, very small samples (circa 10 mg) of the substance are subjected to heating without confinement in a gas flame, to shock in any convenient form of apparatus and to friction by the use of a mallet against an anvil or any form of friction machine. The objective is to ascertain if the substance is so sensitive and explosive that the prescribed sensitivity tests, particularly that of thermal sensitivity, should be performed with special precautions so as to avoid injury to the operator.

1.4.2. **Thermal sensitivity**

The method involves heating the substance in a steel tube, closed by orifice plates with differing diameters of hole, to determine whether the substance is liable to explode under conditions of intense heat and defined confinement.

1.4.3. **Mechanical sensitivity (shock)**

The method involves subjecting the substance to the shock from a specified mass dropped from a specified height.

1.4.4. **Mechanical sensitivity (friction)**

The method involves subjecting solid or pasty substances to friction between standard surfaces under specified conditions of load and relative motion.

1.5. QUALITY CRITERIA

Not stated.

1.6. DESCRIPTION OF METHOD

1.6.1. **Thermal sensitivity (effect of a flame)**

1.6.1.1. *Apparatus*

The apparatus consists of a non-reusable steel tube with its re-usable closing device (figure 1), installed in a heating and protective device. Each tube is deep-drawn from sheet steel (see Appendix) and has an internal diameter of 24 mm, a length of 75 mm and wall thickness of 0,5 mm. The tubes are flanged at the open end to enable them to be closed by the orifice plate assembly. This consists of a pressure-resistant orifice plate, with a central hole, secured firmly to a tube using a two-part screw joint (nut and threaded collar). The nut and threaded collar are made from chromium-manganese steel (see Appendix) which is spark-free up to 800 °C. The orifice plates are 6 mm thick, made from heat-resistant steel (see Appendix), and are available with a range of diameters of opening.

1.6.1.2. *Test conditions*

Normally the substance is tested as received although in certain cases, e.g. if pressed, cast or otherwise condensed, it may be necessary to test the substance after crushing.

For solids, the mass of material to be used in each test is determined using a two-stage dry run procedure. A tared tube is filled with 9 cm^3 of substance and the substance tamped with 80 N force applied to the total cross-section of the tube. For reasons of safety or in cases where the physical form of the sample can be changed by compression other filling procedures may be used; e.g. if the substance is very friction sensitive then tamping is not appropriate. If the material is compressible then more is added and tamped until the tube is filled to 55 mm from the top. The total mass used to fill the tube to the 55 mm level is determined and two further increments, each tamped with 80 N force, are added. Material is then either added with tamping, or taken out, as required, to leave the tube filled to a level 15 mm from the top. A second dry run is performed, starting with a tamped quantity of a third of the total mass found in the first dry run. Two more of these increments are added with 80 N tamping and the level of the substance in the tube adjusted to 15 mm from the top by addition or subtraction of material as required. The amount of solid determined in the second dry run is used for each trial; filling being performed in three equal amounts, each compressed to 9 cm^3 by whatever force is necessary. (This may be facilitated by the use of spacing rings).

Liquids and gels are loaded into the tube to a height of 60 mm taking particular care with gels to prevent the formation of voids. The threaded collar is slipped onto the tube from below, the appropriate orifice plate is inserted and the nut tightened after applying some molybdenum disulphide based lubricant. It is essential to check that none of the substance is trapped between the flange and the plate, or in the threads.

Heating is provided by propane taken from an industrial cylinder, fitted with a pressure regulator (60 to 70 mbar), through a meter and evenly distributed (as indicated by visual observation of the flames from the burners) by a manifold to four burners. The burners are located around the test chamber as shown in figure 1. The four burners have a combined consumption of about 3,2 litres of propane per minute. Alternative fuel gases and burners may be used but the heating rate must be as specified in figure 3. For all apparatus, the heating rate must be checked periodically using tubes filled with dibutyl phthalate as indicated in figure 3.

1.6.1.3. *Performance of the tests*

Each test is performed until either the tube is fragmented or the tube has been heated for five minutes. A test resulting in the fragmentation of the tube into three or more pieces, which in some cases may be connected to each other by narrow strips of metal as illustrated in figure 2, is evaluated as giving an explosion. A test resulting in fewer fragments or no fragmentation is regarded as not giving an explosion.

A series of three tests with a 6,0 mm diameter orifice plate is first performed and, if no explosions are obtained, a second series of three tests is performed with a 2,0 mm diameter orifice plate. If an explosion occurs during either test series then no further tests are required.

1.6.1.4. *Evaluation*

The test result is considered positive if an explosion occurs in either of the above series of tests.

1.6.2. **Mechanical sensitivity (shock)**

1.6.2.1. *Apparatus (figure 4)*

The essential parts of a typical fall hammer apparatus are a cast steel block with base, anvil, column, guides, drop weights, release device and a sample holder. The steel anvil 100 mm (diameter) × 70 mm (height) is screwed to the top of a steel block 230 mm (length) × 250 mm (width) × 200 mm (height) with a cast base 450 mm (length) × 450 mm (width) × 60 mm (height). A column, made from seamless drawn steel tube, is secured in a holder screwed on to the back of the steel block. Four screws anchor the apparatus to a solid concrete block 60 × 60 × 60 cm such that the guide rails are absolutely vertical and the drop weight falls freely. 5 and 10 kg weights, made from solid steel, are available for use. The striking head of each weight is of hardened steel, HRC 60 to 63, and has a minimum diameter of 25 mm.

The sample under test is enclosed in a shock device consisting of two coaxial solid steel cylinders, one above the other, in a hollow cylindrical steel guide ring. The solid steel cylinders should be of 10 (- 0,003, - 0,005) mm diameter and 10 mm height and have polished surfaces, rounded edges (radius of curvature 0,5 mm) and a hardness of HRC 58 to 65. The hollow cylinder must have an external diameter of 16 mm, a polished bore of 10 (+ 0,005, + 0,010) mm and a height of 13 mm. The shock device is assembled on an intermediate anvil (26 mm diameter and 26 mm height) made of steel and centred by a ring with perforations to allow escape of fumes.

1.6.2.2. *Test conditions*

The sample volume should be 40 mm^3, or a volume to suit any alternative apparatus. Solid substances should be tested in the dry state and prepared as follows:

(a) powdered substances are sieved (sieve size 0,5 mm); all that has passed through the sieve is used for testing;

(b) pressed, cast or otherwise condensed substances are broken into small pieces and sieved; the sieve fraction from 0,5 to 1 mm diameter is used for testing and should be representative of the original substance.

Substances normally supplied as pastes should be tested in the dry state where possible or, in any case, following removal of the maximum possible amount of diluent. Liquid substances are tested with a 1 mm gap between the upper and lower steel cylinders.

1.6.2.3. *Performance of the tests*

A series of six tests are performed dropping the 10 kg mass from 0,40 m (40 J). If an explosion is obtained during the six tests at 40 J, a further series of six tests, dropping a 5 kg mass from 0,15 m (7,5 J), must be performed. In other apparatus, the sample is compared with the chosen reference substance using an established procedure (e.g. up-and-down technique etc.).

1.6.2.4. *Evaluation*

The test result is considered positive if an explosion (bursting into flame and/or a report is equivalent to explosion) occurs at least once in any of the tests with the specified shock apparatus or the sample is more sensitive than 1,3-dinitrobenzene or RDX in an alternative shock test.

1.6.3. **Mechanical sensitivity (friction)**

1.6.3.1. *Apparatus (figure 5)*

The friction apparatus consists of a cast steel base plate on which is mounted the friction device. This consists of a fixed porcelain peg and moving porcelain plate. The porcelain plate is held in a carriage which runs in two guides. The carriage is connected to an electric motor via a connecting rod, an eccentric cam and suitable gearing such that the porcelain plate is moved, once only, back and forth beneath the porcelain peg for a distance of 10 mm. The porcelain peg may be loaded with, for example, 120 or 360 newtons.

The flat porcelain plates are made from white technical porcelain (roughness 9 to 32 μm) and have the dimensions 25 mm (length) × 25 mm (width) × 5 mm (height). The cylindrical porcelain peg is also made of white technical porcelain and is 15 mm long, has a diameter of 10 mm and roughened spherical end surfaces with a radius of curvature of 10 mm.

1.6.3.2. *Test conditions*

The sample volume should be 10 mm^3 or a volume to suit any alternative apparatus.

Solid substances are tested in the dry state and prepared as follows:

(a) powdered substances are sieved (sieve size 0,5 mm); all that has passed through the sieve is used for testing;

(b) pressed, cast or otherwise condensed substances are broken into small pieces and sieved; the sieve fraction < 0,5 mm diameter is used for testing.

Substances normally supplied as pastes should be tested in the dry state where possible. If the substance cannot be prepared in the dry state, the paste (following removal of the maximum possible amount of diluent) is tested as a 0,5 mm thick, 2 mm wide, 10 mm long film, prepared with a former.

1.6.3.3. *Performance of the tests*

The porcelain peg is brought onto the sample under test and the load applied. When carrying out the test, the sponge marks of the porcelain plate must lie transversely to the direction of the movement. Care must be taken that the peg rests on the sample, that sufficient test material lies under the peg and also that the plate moves correctly under the peg. For pasty substances, a 0,5 mm thick gauge with a 2 × 10 mm slot is used to apply the substance to the plate. The porcelain plate has to move 10 mm forwards and backwards under the porcelain peg in a time of 0,44 seconds. Each part of the surface of the plate and peg must only be used once; the two ends of each peg will serve for two trials and the two surfaces of a plate will each serve for three trials.

A series of six tests are performed with a 360 N loading. If a positive event is obtained during these six tests, a further series of six tests must be performed with a 120 N loading. In other apparatus, the sample is compared with the chosen reference substance using an established procedure (e.g. up-and-down technique, etc.).

1.6.3.4. *Evaluation*

The test result is considered positive if an explosion (crepitation and/or a report or bursting into flame are equivalent to explosion) occurs at least once in any of the tests with the specified friction apparatus or satisfies the equivalent criteria in an alternative friction test.

2. **DATA**

In principle, a substance is considered to present a danger of explosion in the sense of the directive if a positive result is obtained in the thermal, shock or friction sensitivity test.

3. REPORTING

3.1. TEST REPORT

The test report shall, if possible, include the following information:

— identity, composition, purity, moisture content, etc. of the substance tested,

— the physical form of the sample and whether or not it has been crushed, broken and/or sieved,

— observations during the thermal sensitivity tests (e.g. sample mass, number of fragments, etc.),

— observations during the mechanical sensitivity tests (e.g. formation of considerable amounts of smoke or complete decomposition without a report, flames, sparks, report, crepitation, etc.),

— results of each type of test,

— if alternative apparatus has been used, scientific justification as well as evidence of correlation between results obtained with specified apparatus and those obtained with equivalent apparatus must be given,

— any useful comments such as reference to tests with similar products which might be relevant to a proper interpretation of the results,

— all additional remarks relevant for the interpretation of the results.

3.2. INTERPRETATION AND EVALUATION OF RESULTS

The test report should mention any results which are considered false, anomalous or unrepresentative. If any of the results should be discounted, an explanation and the results of any alternative or supplementary testing should be given. Unless an anomalous result can be explained, it must be accepted at face value and used to classify the substance accordingly.

4. REFERENCES

(1) Recommendations on the Transport of Dangerous Goods: Tests and criteria, 1990, United Nations, New York.

(2) Bretherick, L., Handbook of Reactive Chemical Hazards, 4th edition, Butterworths, London, ISBN 0-750-60103-5, 1990.

(3) Koenen, H., Ide, K.H. and Swart, K.H., Explosivstoffe, 1961, vol. 3, 6-13 and 30-42.

(4) NF T 20-038 (September 85) Chemical products for industrial use — Determination of explosion risk.

Appendix

Example of material specification for thermal sensitivity test (see DIN 1623)

(1) Tube: Material specification No 1.0336.505 g

(2) Orifice plate: Material specification No 1.4873

(3) Threaded collar and nut: Material specification No 1.3817

Figure 1

Thermal sensitivity test apparatus

(all dimensions in millimetres)

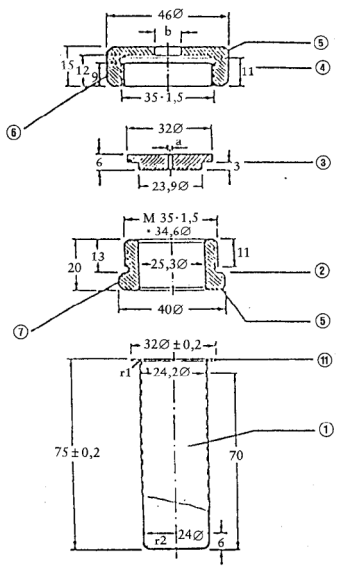


Fig. 1a Steel tube and accessories

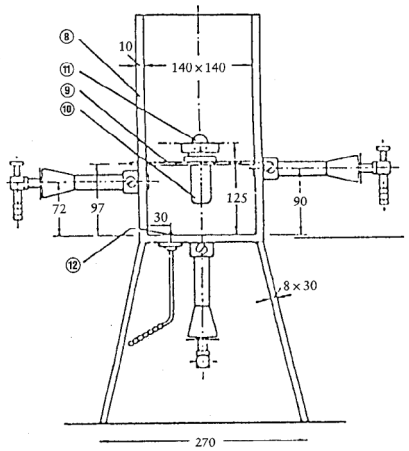


Fig. 1b Heating and protective device

(1) tube
(1a) outer flange
(2) threaded collar; low-friction thread
(3) orifice plate a = 2,0 or 6,0 mm diameter
(4) nut b = 10 mm diameter
(5) chamfered surface
(6) 2 flat for spanner size 41
(7) 2 flat for spanner size 36
(8) splinter-proof box
(9) 2 supporting rods for tube
(10) assembled tube
(11) position for rear burner; the other burners are visible
(12) pilot jet

Figure 2

Thermal sensitivity test

(example of fragmentation)

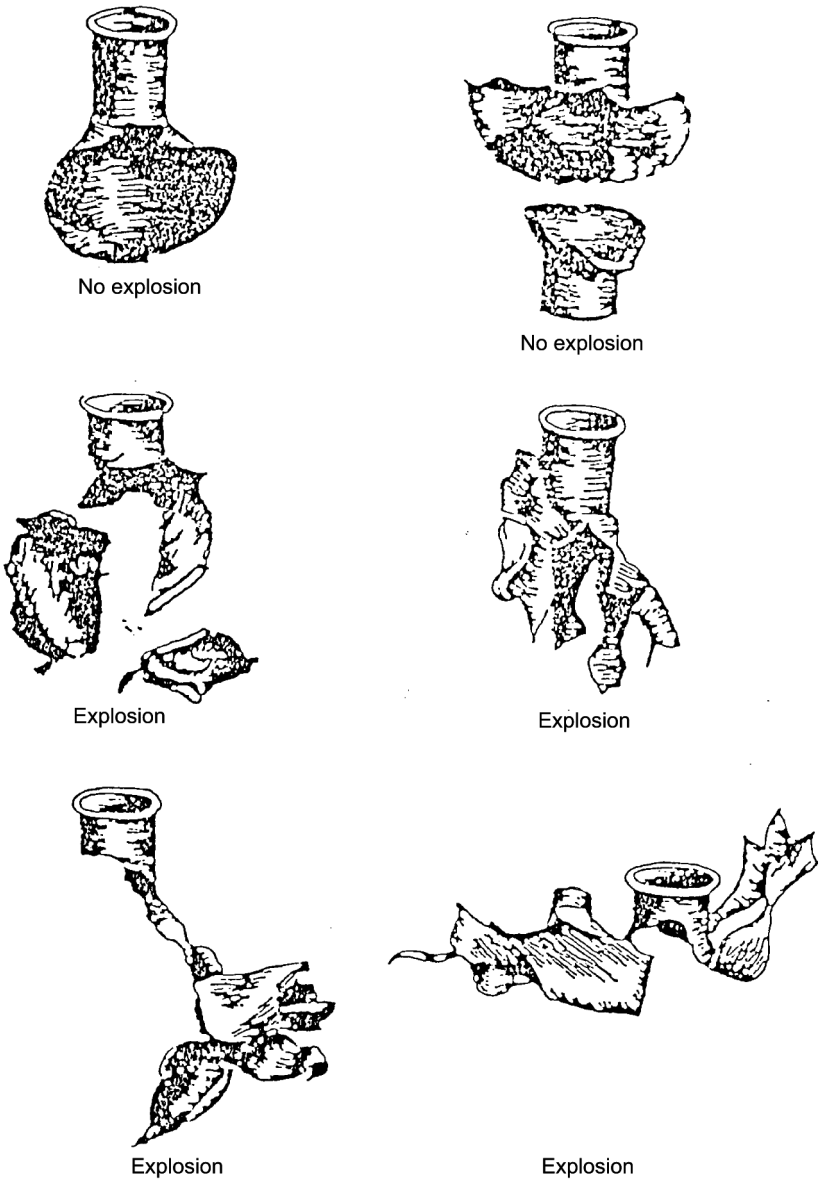
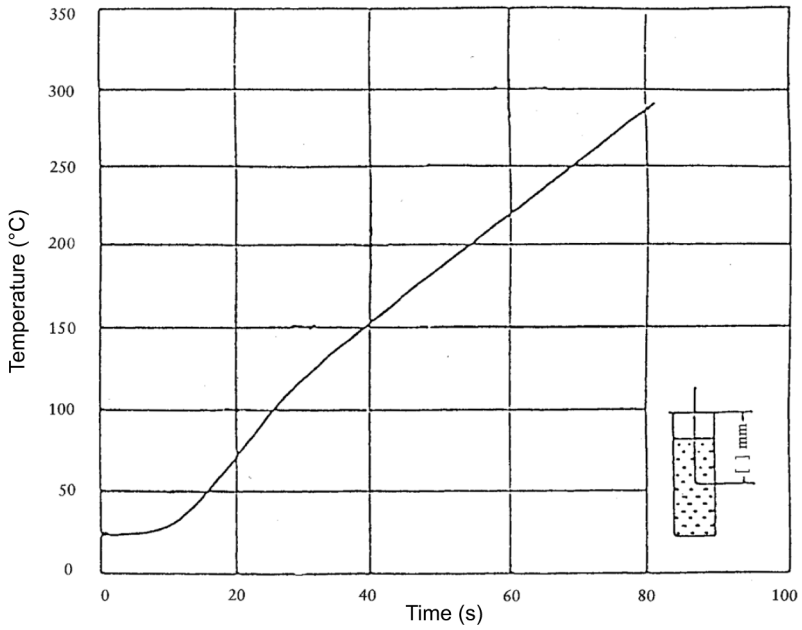


Figure 3

Heating rate calibration for thermal sensitivity test



Temperature/time curve obtained on heating dibutyl phtalate (27 cm^3) in a closed (1,5 mm orifice plate) tube using a propane flow rate of 3,2 litre/minute. The temperature is measured with a 1 mm diameter stainless steel sheathed chromel/alumel thermocouple, placed centrally 43 mm below the rim of the tube. The heating rate between 135 °C and 285 °C should be between 185 and 215 K/minute.

Figure 4

Shock test apparatus

(all dimensions in millimetres)

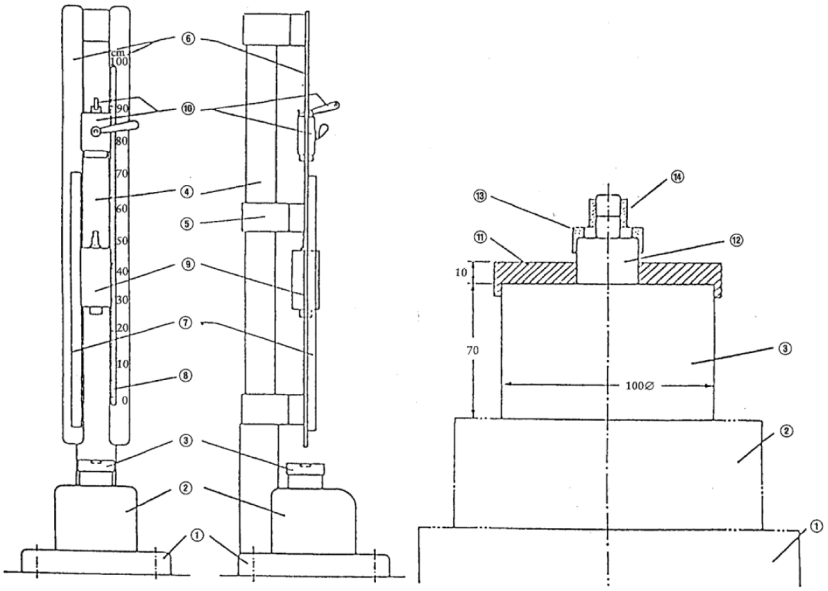


Fig. 4a Fall-hammer, front and side, general view

(1) base, 450 x 450 x 60
(2) steel block, 230 x 250 x 200
(3) anvil, 100 diameter x 70
(4) column
(5) median cross-member
(6) 2 guides
(7) toothed rack

Fig. 4b Fall-hammer, lower part

(8) graduated scale
(9) fall-hammer (drop mass)
(10) holding and releasing device
(11) locating plate
(12) intermediate anvil (interchangeable), 26 diameter x 26
(13) locating ring with orifices
(14) impact device

Figure 4

Continued

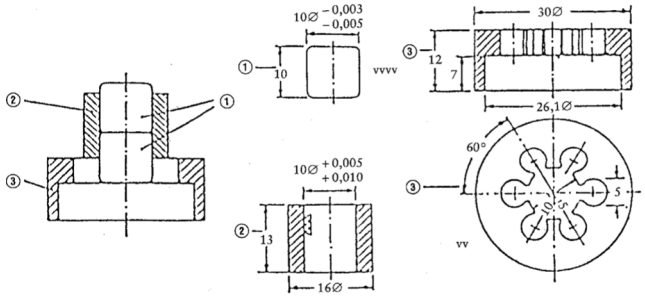
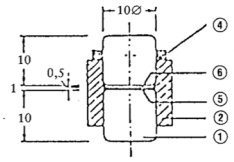


Fig. 4c Shock device for substances in powdered or paste-like form

Fig. 4d Shock device for liquid substances

(1) steel cylinders
(2) guide ring for steel cylinders
(3) locating ring with orifices
(a) vertical section
(b) plan
(4) rubber ring
(5) liquid substance (40 mm^3)
(6) space free from liquid



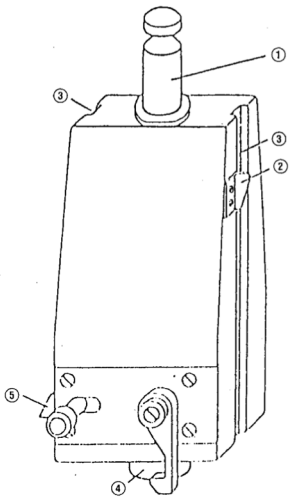


Fig. 4e Hammer (drop mass of 5 kg)

(1) suspension spigot
(2) height marker
(3) positioning groove
(4) cylindrical striking head
(5) rebound catch

Figure 5

Friction sensitivity apparatus

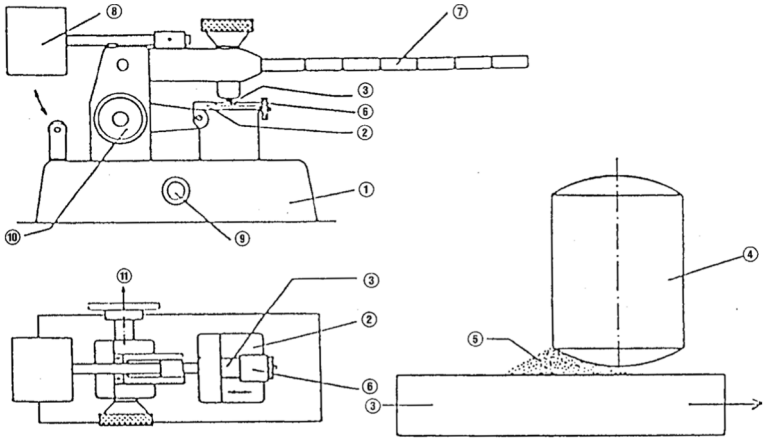


Fig. 5a Friction apparatus; elevation and plan view

Fig. 5b Starting position of peg on sample

(1) steel base
(2) movable carriage
(3) porcelain plate, 25 x 25 x 5 mm, held on carriage
(4) fixed porcelain peg, 10 diameter x 15 mm
(5) sample under test, approximately 10 mm^3
(6) peg-holder
(7) loading arm
(8) counterweight
(9) switch
(10) wheel for setting carriage at starting position
(11) direction to electric drive motor

A.15. AUTO-IGNITION TEMPERATURE (LIQUIDS AND GASES)

1. METHOD

1.1. INTRODUCTION

Explosive substances and substances which ignite spontaneously in contact with air at ambient temperature should not be submitted to this test. The test procedure is applicable to gases, liquids and vapours which, in the presence of air, can be ignited by a hot surface.

The auto-ignition temperature can be considerably reduced by the presence of catalytic impurities, by the surface material or by a higher volume of the test vessel.

1.2. DEFINITIONS AND UNITS

The degree of auto-ignitability is expressed in terms of the auto-ignition temperature. The auto-ignition temperature is the lowest temperature at which the test substance will ignite when mixed with air under the conditions defined in the test method.

1.3. REFERENCE SUBSTANCES

Reference substances are cited in the standards (see 1.6.3). They should primarily serve to check the performance of the method from time to time and to allow comparison with results from other methods.

1.4. PRINCIPLE OF THE METHOD

The method determines the minimum temperature of the inner surface of an enclosure that will result in ignition of a gas, vapour or liquid injected into the enclosure.

1.5. QUALITY CRITERIA

The repeatability varies according to the range of auto-ignition temperatures and the test method used.

The sensitivity and specificity depend on the test method used.

1.6. DESCRIPTION OF THE METHOD

1.6.1. **Apparatus**

The apparatus is described in the method referred to in 1.6.3.

1.6.2. **Test conditions**

A sample of the test substance is tested according to the method referred to in 1.6.3.

1.6.3. **Performance of the test**

See IEC 79-4, DIN 51794, ASTM-E 659-78, BS 4056, NF T 20-037.

2. DATA

Record the test-temperature, atmospheric pressure, quantity of sample used and time-1ag until ignition occurs.

3. **REPORTING**

The test report shall, if possible, include the following information:

— the precise specification of the substance (identification and impurities),

— the quantity of sample used, atmospheric pressure,

— the apparatus used,

— the results of measurements (test temperatures, results concerning ignition, corresponding time-lags),

— all additional remarks relevant to the interpretation of results.

4. **REFERENCES**

None.

A.16. RELATIVE SELF-IGNITION TEMPERATURE FOR SOLIDS

1. METHOD

1.1. INTRODUCTION

Explosive substances and substances which ignite spontaneously in contact with air at ambient temperature should not be submitted to this test.

The purpose of this test is to provide preliminary information on the auto-flammability of solid substances at elevated temperatures.

If the heat developed either by a reaction of the substance with oxygen or by exothermic decomposition is not lost rapidly enough to the surroundings, self-heating leading to self-ignition occurs. Self-ignition therefore occurs when the rate of heat-production exceeds the rate of heat loss.

The test procedure is useful as a preliminary screening test for solid substances. In view of the complex nature of the ignition and combustion of solids, the self-ignition temperature determined according to this test method should be used for comparison purposes only.

1.2. DEFINITIONS AND UNITS

The self-ignition temperature as obtained by this method is the minimum ambient temperature expressed in °C at which a certain volume of a substance will ignite under defined conditions.

1.3. REFERENCE SUBSTANCE

None.

1.4. PRINCIPLE OF THE METHOD

A certain volume of the substance under test is placed in an oven at room temperature; the temperature/time curve relating to conditions in the centre of the sample is recorded while the temperature of the oven is increased to 400 °C, or to the melting point if lower, at a rate of 0,5 °C/min. For the purpose of this test, the temperature of the oven at which the sample temperature reaches 400 °C by self-heating is called the self-ignition temperature.

1.5. QUALITY CRITERIA

None.

1.6. DESCRIPTION OF THE METHOD

1.6.1. **Apparatus**

1.6.1.1. *Oven*

A temperature-programmed laboratory oven (volume about 2 litres) fitted with natural air circulation and explosion relief. In order to avoid a potential explosion risk, any decomposition gases must not be allowed to come into contact with the electric heating elements.

1.6.1.2. *Wire mesh cube*

A piece of stainless steel wire mesh with 0,045 mm openings should be cut according to the pattern in figure 1. The mesh should be folded and secured with wire into an open-topped cube.

1.6.1.3. *Thermocouples*

Suitable thermocouples.

1.6.1.4. *Recorder*

Any two-channel recorder calibrated from 0 to 600 °C or corresponding voltage.

1.6.2. **Test conditions**

Substances are tested as received.

1.6.3. **Performance of the test**

The cube is filled with the substance to be tested and is tapped gently, adding more of the substance until the cube is completely full. The cube is then suspended in the centre of the oven at room temperature. One thermocouple is placed at the centre of the cube and the other between the cube and the oven wall to record the oven temperature.

The temperatures of the oven and sample are continuously recorded while the temperature of the oven is increased to 400 °C, or to the melting point if lower, at a rate of 0,5 °C/min.

When the substance ignites the sample thermocouple will show a very sharp temperature rise above the oven temperature.

2. **DATA**

The temperature of the oven at which the sample temperature reaches 400 °C by self-heating is relevant for evaluation (see figure 2).

3. **REPORTING**

The test report shall, if possible, include the following information:

— a description of the substance to be tested,

— the results of measurement including the temperature/time curve,

— all additional remarks relevant for the interpretation of the results.

4. **REFERENCES**

NF T 20-036 (September 85) Chemical products for industrial use. Determination of the relative temperature of the spontaneous flammability of solids.

Figure 1

Pattern of 20 mm test cube

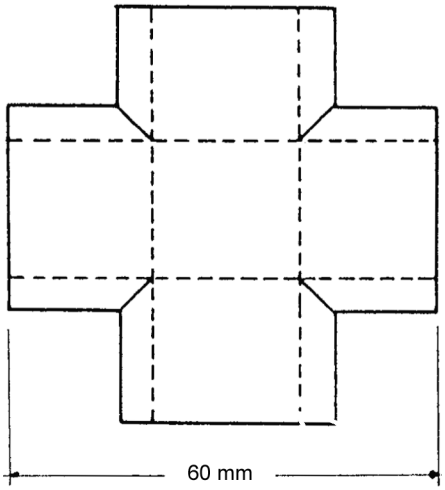
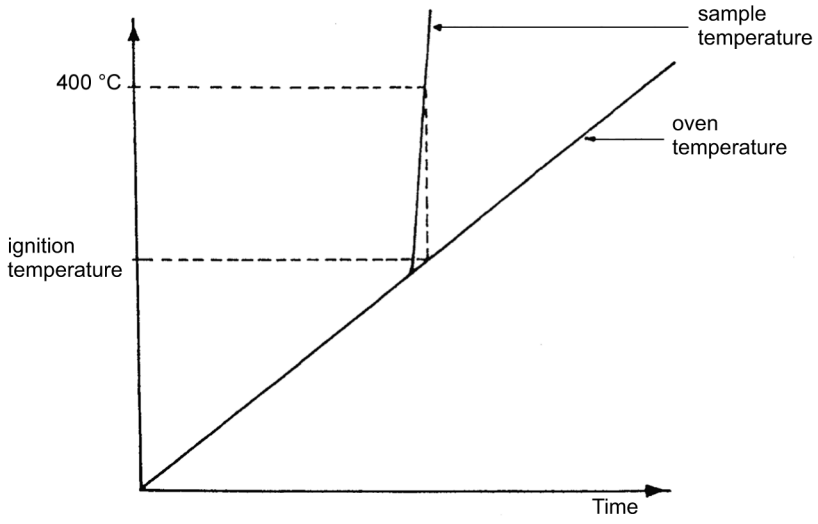


Figure 2

Typical temperature/time curve



A.17. OXIDISING PROPERTIES (SOLIDS)

1. METHOD

1.1. INTRODUCTION

It is useful to have preliminary information on any potentially explosive properties of the substance before performing this test.

This test is not applicable to liquids, gases, explosive or highly flammable substances, or organic peroxides.

This test need not be performed when examination of the structural formula establishes beyond reasonable doubt that the substance is incapable of reacting exothermically with a combustible material.

In order to ascertain if the test should be performed with special precautions, a preliminary test should be performed.

1.2. DEFINITION AND UNITS

Burning time: reaction time, in seconds, taken for the reaction zone to travel along a pile, following the procedure described in 1.6.

Burning rate: expressed in millimetres per second.

Maximum burning rate: the highest value of the burning rates obtained with mixtures containing 10 to 90 % by weight of oxidiser.

1.3. REFERENCE SUBSTANCE

Barium nitrate (analytical grade) is used as reference substance for the test and the preliminary test.

The reference mixture is that mixture of barium nitrate with powdered cellulose, prepared according to 1.6, which has the maximum burning rate (usually a mixture with 60 % barium nitrate by weight).

1.4. PRINCIPLE OF THE METHOD

A preliminary test is carried out in the interests of safety. No further testing is required when the preliminary test clearly indicates that the test substance has oxidising properties. When this is not the case, the substance should then be subject to the full test.

In the full test, the substance to be tested and a defined combustible substance will be mixed in various ratios. Each mixture is then formed into a pile and the pile is ignited at one end. The maximum burning rate determined is compared with the maximum burning rate of the reference mixture.

1.5. QUALITY CRITERIA

If required, any method of grinding and mixing is valid provided that the difference in the maximum rate of burning in the six separate tests differs from the arithmetic mean value by no more than 10 %.

1.6. DESCRIPTION OF THE METHOD

1.6.1. **Preparation**

1.6.1.1. *Test substance*

Reduce the test sample to a particle size < 0,125 mm using the following procedure: sieve the test substance, grind the remaining fraction, repeat the procedure until the whole test portion has passed the sieve.

Any grinding and sieving method satisfying the quality criteria may be used.

Before preparing the mixture the substance is dried at 105 °C, until constant weight is obtained. If the decomposition temperature of the substance to be tested is below 105 °C, the substance has to be dried at a suitable lower temperature.

1.6.1.2. *Combustible substance*

Powdered cellulose is used as a combustible substance. The cellulose should be a type used for thin-layer chromatography or column chromatography. A type with fibre-lengths of more than 85 % between 0,020 and 0,075 mm has proved to be suitable. The cellulose powder is passed through a sieve with a mesh-size of 0,125 mm. The same batch of cellulose is to be used throughout the test.

Before preparing the mixture, the powdered cellulose is dried at 105 °C until constant weight is obtained.

If wood-meal is used in the preliminary test, then prepare a soft-wood wood-meal by collecting the portion which passes through a sieve mesh of 1,6 mm, mix thoroughly, then dry at 105 °C for four hours in a layer not more than 25 mm thick. Cool and store in an air-tight container filled as full as practicable until required, preferably within 24 hours of drying.

1.6.1.3. *Ignition source*

A hot flame from a gas burner (minimum diameter 5 mm) should be used as the ignition source. If another ignition source is used (e.g. when testing in an inert atmosphere), the description and the justification should be reported.

1.6.2. **Performance of the test**

Note:

Mixtures of oxidisers with cellulose or wood-meal must be treated as potentially explosive and handled with due care.

1.6.2.1. *Preliminary test*

The dried substance is thoroughly mixed with the dried cellulose or wood-meal in the proportions 2 of test substance to 1 of cellulose or wood-meal by weight and the mixture is formed into a small cone-shaped pile of dimensions 3,5 cm (diameter of base) × 2,5 cm (height) by filling, without tamping, a cone-shaped former (e.g. a laboratory glass funnel with the stem plugged).

The pile is placed on a cool, non-combustible, non-porous and low heat-conducting base plate. The test should be carried out in a fume cupboard as in 1.6.2.2.

The ignition source is put in contact with the cone. The vigour and duration of the resultant reaction are observed and recorded.

The substance is to be considered as oxidising if the reaction is vigorous.

In any case where the result is open to doubt, it is then necessary to complete the full train test described below.

1.6.2.2. *Train test*

Prepare oxidiser cellulose-mixtures containing 10 to 90 % weight of oxidiser in 10 % increments. For borderline cases, intermediate oxidiser cellulose mixtures should be used to obtain the maximum burning rate more precisely.

The pile is formed by means of a mould. The mould is made of metal, has a length of 250 mm and a triangular cross-section with an inner height of 10 mm and an inner width of 20 mm. On both sides of the mould, in the longitudinal direction, two metal plates are mounted as lateral limitations which project 2 mm beyond the upper edge of the triangular cross-section (figure). This arrangement is loosely filled with a slight excess of mixture. After dropping the mould once from a height of 2 cm onto a solid surface, the remaining excess

substance is scraped off with an obliquely positioned sheet. The lateral limitations are removed and the remaining powder is smoothed, using a roller. A non-combustible, non-porous and low heat-conducting base plate is then placed on the top of the mould, the apparatus inverted and the mould removed.

Arrange the pile across the draught in a fume cupboard.

The air-speed should be sufficient to prevent fumes escaping into the laboratory and should not be varied during the test. A draught screen should be erected around the apparatus.

Due to hygroscopicity of cellulose and of some substances to be tested, the test should be carried out as quickly as possible.

Ignite one end of the pile by touching with the flame.

Measure the time of reaction over a distance of 200 mm after the reaction zone has propagated an initial distance of 30 mm.

The test is performed with the reference substance and at least once with each one of the range of mixtures of the test substance with cellulose.

If the maximum burning rate is found to be significantly greater than that from the reference mixture, the test can be stopped; otherwise the test should be repeated five times for each of the three mixtures giving the fastest burning rate.

If the result is suspected of being a false positive, then the test should be repeated using an inert substance with a similar particle size, such as kieselguhr, in place of cellulose. Alternatively, the test substance cellulose mixture, having the fastest burning rate, should be retested in an inert atmosphere (< 2 % v/v oxygen content).

2. DATA

For safety reasons the maximum burning rate — not the mean value — shall be considered to be the characteristic oxidising property of the substance under test.

The highest value of burning rate within a run of six tests of a given mixture is relevant for evaluation.

Plot a graph of the highest value of burning rate for each mixture versus the oxidiser concentration. From the graph take the maximum burning rate.

The six measured values of burning rate within a run obtained from the mixture with the maximum burning rate must not differ from the arithmetic mean value by more than 10 %; otherwise the methods of grinding and mixing must be improved.

Compare the maximum burning rate obtained with the maximum burning rate of the reference mixture (see 1.3).

If tests are conducted in an inert atmosphere, the maximum reaction rate is compared with that from the reference mixture in an inert atmosphere.

3. REPORT

3.1. TEST REPORT

The test report shall, if possible, include the following information:

— the identity, composition, purity, moisture content etc. of the substance tested,

— any treatment of the test sample (e.g. grinding, drying),

— the ignition source used in the tests,

— the results of measurements,

— the mode of reaction (e.g. flash burning at the surface, burning through the whole mass, any information concerning the combustion products, etc.),

— all additional remarks relevant for the interpretation of results, including a description of the vigour (flaming, sparking, fuming, slow smouldering, etc.) and approximate duration produced in the preliminary safety/screening test for both test and reference substance,

— the results from tests with an inert substance, if any,

— the results from tests in an inert atmosphere, if any.

3.2. INTERPRETATION OF THE RESULT

A substance is to be considered as an oxidising substance when:

(a) in the preliminary test, there is a vigorous reaction;

(b) in the full test, the maximum burning rate of the mixtures tested is higher than or equal to the maximum burning rate of the reference mixture of cellulose and barium nitrate.

In order to avoid a false positive, the results obtained when testing the substance mixed with an inert material and/or when testing under an inert atmosphere should also be considered when interpreting the results.

4. **REFERENCES**

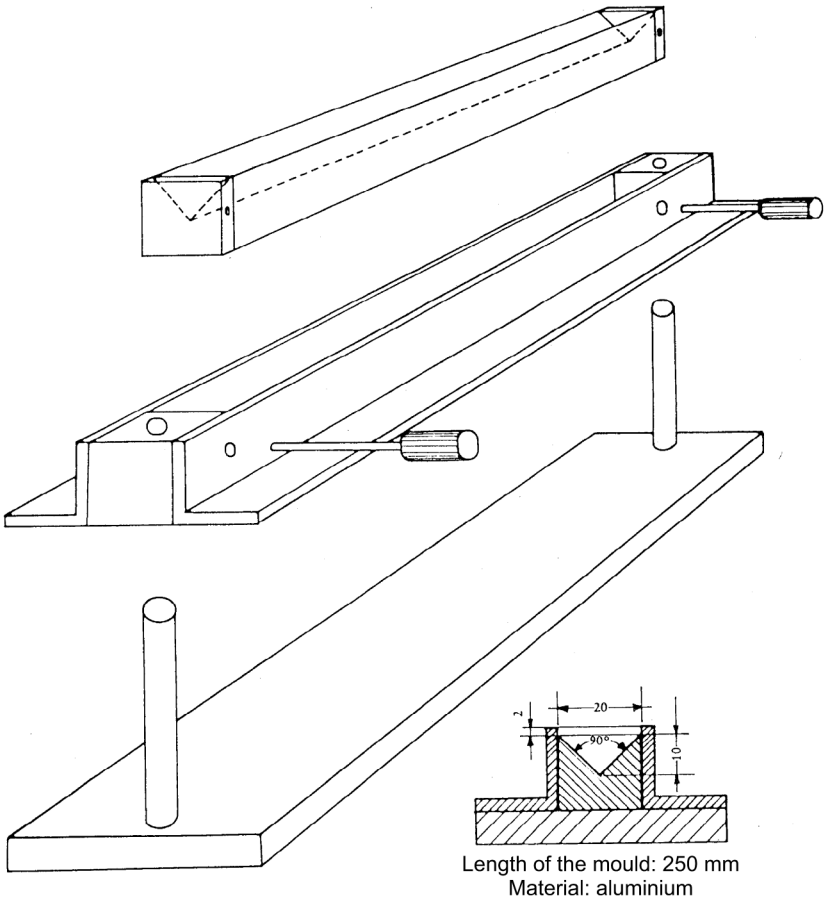
NF T 20-035 (September 85) Chemical products for industrial use. Determination of the oxidising properties of solids.

Appendix

Figure

Mould and accessories for the preparations of the pile

(All dimensions in millimetres)



Length of the mould: 250 mm
Material: aluminium

A.18. NUMBER-AVERAGE MOLECULAR WEIGHT AND MOLECULAR WEIGHT DISTRIBUTION OF POLYMERS

1. METHOD

This Gel Permeation Chromatographic method is a replicate of the OECD TG 118 (1996). The fundamental principles and further technical information are given in reference (1).

1.1. INTRODUCTION

Since the properties of polymers are so varied, it is impossible to describe one single method setting out precisely the conditions for separation and evaluation which cover all eventualities and specificities occurring in the separation of polymers. In particular, complex polymer systems are often not amenable to gel permeation chromatography (GPC). When GPC is not practicable, the molecular weight may be determined by means of other methods (see Appendix). In such cases, full details and justification should be given for the method used.

The method described is based on DIN Standard 55672 (1). Detailed information about how to carry out the experiments and how to evaluate the data can be found in this DIN Standard. In case modifications of the experimental conditions are necessary, these changes must be justified. Other standards may be used, if fully referenced. The method described uses polystyrene samples of known polydispersity for calibration and it may have to be modified to be suitable for certain polymers, e.g. water soluble and long-chain branched polymers.

1.2. DEFINITIONS AND UNITS

The number-average molecular weight M_n and the weight average molecular weight M_w are determined using the following equations:

$$M_n = \frac{\sum_{i=1}^{n} H_i}{\sum_{i=1}^{n} H_i / M_i} \qquad M_w = \frac{\sum_{i=1}^{n} H_i \times M_i}{\sum_{i=1}^{n} H_i}$$

where,

H_i is the level of the detector signal from the baseline for the retention volume V_i,

M_i is the molecular weight of the polymer fraction at the retention volume V_i, and

n is the number of data points.

The breadth of the molecular weight distribution, which is a measure of the dispersity of the system, is given by the ratio M_w/M_n.

1.3. REFERENCE SUBSTANCES

Since GPC is a relative method, calibration must be undertaken. Narrowly distributed, linearly constructed polystyrene standards with known average molecular weights M_n and M_w and a known molecular weight distribution are normally used for this. The calibration curve can only be used in the determination of the molecular weight of the unknown sample if the conditions for the separation of the sample and the standards have been selected in an identical manner.

A determined relationship between the molecular weight and elution volume is only valid under the specific conditions of the particular experiment. The conditions include, above all, the temperature, the solvent (or solvent mixture), the chromatography conditions and the separation column or system of columns.

The molecular weights of the sample determined in this way are relative values and are described as 'polystyrene equivalent molecular weights'. This means that dependent on the structural and chemical differences between the sample and the standards, the molecular weights can deviate from the absolute values to a greater or a lesser degree. If other standards are used, e.g. polyethylene glycol, polyethylene oxide, polymethyl methacrylate, polyacrylic acid, the reason should be stated.

1.4. PRINCIPLE OF THE TEST METHOD

Both the molecular weight distribution of the sample and the average molecular weights (M_n, M_w) can be determined using GPC. GPC is a special type of liquid chromatography in which the sample is separated according to the hydrodynamic volumes of the individual constituents (2).

Separation is effected as the sample passes through a column which is filled with a porous material, typically an organic gel. Small molecules can penetrate the pores whereas large molecules are excluded. The path of the large molecules is thereby shorter and these are eluted first. The medium-sized molecules penetrate some of the pores and are eluted later. The smallest molecules, with a mean hydrodynamic radius smaller than the pores of the gel, can penetrate all of the pores. These are eluted last.

In an ideal situation, the separation is governed entirely by the size of the molecular species, but in practice it is difficult to avoid at least some absorption effects interfering. Uneven column packing and dead volumes can worsen the situation (2).

Detection is effected by, e.g. refractive index or UV-absorption, and yields a simple distribution curve. However, to attribute actual molecular weight values to the curve, it is necessary to calibrate the column by passing down polymers of known molecular weight and, ideally, of broadly similar structure e.g. various polystyrene standards. Typically a Gaussian curve results, sometimes distorted by a small tail to the low molecular weight side, the vertical axis indicating the quantity, by weight, of the various molecular weight species eluted, and the horizontal axis the log molecular weight.

1.5. QUALITY CRITERIA

The repeatability (Relative Standard Deviation: RSD) of the elution volume should be better than 0,3 %. The required repeatability of the analysis has to be ensured by correction via an internal standard if a chromatogram is evaluated time-dependently and does not correspond to the above mentioned criterion (1). The polydispersities are dependent on the molecular weights of the standards. In the case of polystyrene standards typical values are:

$M_p < 2\,000$	$M_w/M_n < 1{,}20$
$2\,000 \leq M_p \leq 10^6$	$M_w/M_n < 1{,}05$
$M_p > 10^6$	$M_w/M_n < 1{,}20$

(M_p is the molecular weight of the standard at the peak maximum)

1.6. DESCRIPTION OF THE TEST METHOD

1.6.1. **Preparation of the standard polystyrene solutions**

The polystyrene standards are dissolved by careful mixing in the chosen eluent. The recommendations of the manufacturer must be taken into account in the preparation of the solutions.

The concentrations of the standards chosen are dependent on various factors, e.g. injection volume, viscosity of the solution and sensitivity of the analytical detector. The maximum injection volume must be adapted to the length of the column, in order to avoid overloading. Typical injection volumes for analytical separations using GPC with a column of 30 cm × 7,8 mm are normally between 40 and 100 µl. Higher volumes are possible, but they should not exceed 250 µl. The optimal ratio between the injection volume and the concentration must be determined prior to the actual calibration of the column.

1.6.2. **Preparation of the sample solution**

In principle, the same requirements apply to the preparation of the sample solutions. The sample is dissolved in a suitable solvent, e.g. tetrahydrofuran (THF), by shaking carefully. Under no circumstances should it be dissolved using an ultrasonic bath. When necessary, the sample solution is purified via a membrane filter with a pore size of between 0,2 and 2 µm.

The presence of undissolved particles must be recorded in the final report as these may be due to high molecular weight species. An appropriate method should be used to determine the percentage by weight of the undissolved particles. The solutions should be used within 24 hours.

1.6.3. **Apparatus**

— solvent reservoir,

— degasser (where appropriate),

— pump,

— pulse dampener (where appropriate),

— injection system,

— chromatography columns,

— detector,

— flowmeter (where appropriate),

— data recorder-processor,

— waste vessel.

It must be ensured that the GPC system is inert with regard to the utilised solvents (e.g. by the use of steel capillaries for THF solvent).

1.6.4. **Injection and solvent delivery system**

A defined volume of the sample solution is loaded onto the column either using an auto-sampler or manually in a sharply defined zone. Withdrawing or depressing the plunger of the syringe too quickly, if done manually, can cause changes in the observed molecular weight distribution. The solvent-delivery system should, as far as possible, be pulsation-free ideally incorporating a pulse dampener. The flow rate is of the order of 1 ml/min.

1.6.5. **Column**

Depending on the sample, the polymer is characterised using either a simple column or several columns connected in sequence. A number of porous column materials with defined properties (e.g. pore size, exclusion limits) are commercially available. Selection of the separation gel or the length of the column is dependent on both the properties of the sample (hydrodynamic volumes, molecular weight distribution) and the specific conditions for separation such as solvent, temperature and flow rate (1)(2)(3).

1.6.6. **Theoretical plates**

The column or the combination of columns used for separation must be characterised by the number of theoretical plates. This involves, in the case of THF as elution solvent, loading a solution of ethyl benzene or other suitable non-polar solute onto a column of known length. The number of theoretical plates is given by the following equation:

$$N = 5{,}54\left(\frac{V_e}{W_{1/2}}\right)^2 \qquad \text{or} \qquad N = 16\left(\frac{V_e}{W}\right)^2$$

where,

N = the number of theoretical plates

V_e = the elution volume at the peak maximum

W = the baseline peak width

$W_{1/2}$ = the peak width at half height

1.6.7. **Separation efficiency**

In addition to the number of theoretical plates, which is a quantity determining the bandwidth, a part is also played by the separation efficiency, this being determined by the steepness of the calibration curve. The separation efficiency of a column is obtained from the following relationship:

$$\frac{V_{e,M_x} - V_{e,(10M_x)}}{\text{cross sectional area of the column}} \geq 6{,}0 \left[\frac{cm^3}{cm^2}\right]$$

where,

$V_{e,\,M_x}$ = the elution volume for polystyrene with the molecular weight M_x
$V_{e,(10.M_x)}$ = the elution volume for polystyrene with a ten times greater molecular weight

The resolution of the system is commonly defined as follows:

$$R_{1,2} = 2 \times \frac{V_{e1} - V_{e2}}{W_1 + W_2} \times \frac{1}{\log_{10}(M_2/M_1)}$$

where,

V_{e1}, V_{e2} = the elution volumes of the two polystyrene standards at the peak maximum
W_1, W_2 = the peak widths at the base-line
M_1, M_2 = the molecular weights at the peak maximum (should differ by a factor of 10)

The R-value for the column system should be greater than 1.7 (4).

1.6.8. **Solvents**

All solvents must be of high purity (for THF purity of 99,5 % is used). The solvent reservoir (if necessary in an inert gas atmosphere) must be sufficiently large for the calibration of the column and several sample analyses. The solvent must be degassed before it is transported to the column via the pump.

1.6.9. **Temperature control**

The temperature of the critical internal components (injection loop, columns, detector and tubing) should be constant and consistent with the choice of solvent.

1.6.10. **Detector**

The purpose of the detector is to record quantitatively the concentration of sample eluted from the column. In order to avoid unnecessary broadening of peaks the cuvette volume of the detector cell must be kept as small as possible. It should not be larger than 10 µl except for light scattering and viscosity detectors. Differential refractometry is usually used for detection. However, if required by the specific properties of the sample or the elution solvent, other types of detectors can be used, e.g. UV/VIS, IR, viscosity detectors, etc.

2. **DATA AND REPORTING**

2.1. DATA

The DIN Standard (1) should be referred to for the detailed evaluation criteria as well as for the requirements relating to the collecting and processing of data.

For each sample, two independent experiments must be carried out. They have to be analysed individually.

M_n, M_w, M_w/M_n and M_p must be provided for every measurement. It is necessary to indicate explicitly that the measured values are relative values equivalent to the molecular weights of the standard used.

After determination of the retention volumes or the retention times (possibly corrected using an internal standard), log M_p values (M_p being the peak maxima of the calibration standard) are plotted against one of those quantities. At least two calibration points are necessary per molecular weight decade, and at least five measurement points are required for the total curve, which should cover the estimated molecular weight of the sample. The low molecular weight end-point of the calibration curve is defined by n-hexyl benzene or another suitable non-polar solute. The number average and the weight-average molecular weights are generally determined by means of electronic data processing, based on the formulas of section 1.2. In case manual digitisation is used, ASTM D 3536-91 can be consulted (3).

The distribution curve must be provided in the form of a table or as figure (differential frequency or sum percentages against log M). In the graphic representation, one molecular weight decade should be normally about 4 cm in width and the peak maximum should be about 8 cm in height. In the case of integral distribution curves the difference in the ordinate between 0 and 100 % should be about 10 cm.

2.2. TEST REPORT

The test report must include the following information:

2.2.1. **Test substance:**

— available information about test substance (identity, additives, impurities),

— description of the treatment of the sample, observations, problems.

2.2.2. **Instrumentation:**

— reservoir of eluent, inert gas, degassing of the eluent, composition of the eluent, impurities,

— pump, pulse dampener, injection system,

— separation columns (manufacturer, all information about the characteristics of the columns, such as pore size, kind of separation material, etc., number, length and order of the columns used),

— number of the theoretical plates of the column (or combination), separation efficiency (resolution of the system),

— information on symmetry of the peaks,

— column temperature, kind of temperature control,

— detector (measurement principle, type, cuvette volume),

— flowmeter if used (manufacturer, measurement principle),

— system to record and process data (hardware and software).

2.2.3. **Calibration of the system:**

— detailed description of the method used to construct the calibration curve,

— information about quality criteria for this method (e.g. correlation coefficient, error sum of squares, etc.),

— information about all extrapolations, assumptions and approximations made during the experimental procedure and the evaluation and processing of data,

— all measurements used for constructing the calibration curve have to be documented in a table which includes the following information for each calibration point:

  — name of the sample,

  — manufacturer of the sample,

  — characteristic values of the standards M_p, M_n, M_w, M_w/M_n, as provided by the manufacturer or derived by subsequent measurements, together with details about the method of determination,

  — injection volume and injection concentration,

  — M_p value used for calibration,

  — elution volume or corrected retention time measured at the peak maxima,

  — M_p calculated at the peak maximum,

  — percentage error of the calculated M_p and the calibration value.

2.2.4. **Evaluation:**

— evaluation on a time basis: methods used to ensure the required reproducibility (method of correction, internal standard, etc.),

— information about whether the evaluation was effected on the basis of the elution volume or the retention time,

— information about the limits of the evaluation if a peak is not completely analysed,

— description of smoothing methods, if used,

— preparation and pre-treatment procedures of the sample,

— the presence of undissolved particles, if any,

— injection volume (µl) and injection concentration (mg/ml),

— observations indicating effects which lead to deviations from the ideal GPC profile,

— detailed description of all modifications in the testing procedures,

— details of the error ranges,

— any other information and observations relevant for the interpretation of the results.

3. **REFERENCES**

(1) DIN 55672(1995) Gelpermeationschromatographie (GPC) mit Tetrahydrofuran (THF) als Elutionsmittel, Teil 1.

(2) Yau, W.W., Kirkland, J.J., and Bly, D.D. eds., (1979) Modern Size Exclusion Liquid Chromatography, J. Wiley and Sons.

(3) ASTM D 3536-91, (1991). Standard Test Method for Molecular Weight Averages and Molecular Weight Distribution by Liquid Exclusion Chromatography (Gel Permeation Chromatography-GPC) American Society for Testing and Materials, Philadelphia, Pennsylvania.

(4) ASTM D 5296-92, (1992) Standard Test Method for Molecular Weight Averages and Molecular Weight Distribution of Polystyrene by High Performance Size-Exclusion Chromatography. American Society for Testing and Materials, Philadelphia, Pennsylvania.

Appendix

Examples of other methods for determination of number average molecular weight (Mn) for polymers

Gel permeation chromatography (GPC) is the preferred method for determination of M_n, especially when a set of standards are available, whose structure are comparable with the polymer structure. However, where there are practical difficulties in using GPC or there is already an expectation that the substance will fail a regulatory M_n criterion (and which needs confirming), alternative methods are available, such as:

1. **Use of colligative properties**

1.1. Ebullioscopy/Cryoscopy

involves measurement of boiling point elevation (ebullioscopy) or freezing point depression (cryoscopy) of a solvent, when the polymer is added. The method relies on the fact that the effect of the dissolved polymer on the boiling/freezing point of the liquid is dependent on the molecular weight of the polymer (1) (2).

Applicability, M_n < 20 000.

1.2. Lowering of vapour pressure

involves the measurement of the vapour pressure of a chosen reference liquid before and after the addition of known quantities of polymer (1) (2).

Applicability, M_n < 20 000 (theoretically; in practice however of limited value).

1.3 Membrane osmometry

relies on the principle of osmosis, i.e. the natural tendency of solvent molecules to pass through a semi-permeable membrane from a dilute to a concentrated solution to achieve equilibrium. In the test, the dilute solution is at zero concentration, whereas the concentrated solution contains the polymer. The effect of drawing solvent through the membrane causes a pressure differential that is dependent on the concentration and the molecular weight of the polymer (1) (3) (4).

Applicability, M_n between 20 000 - 200 000.

1.4 Vapour phase osmometry

involves comparison of the rate of evaporation of a pure solvent aerosol to at least three aerosols containing the polymer at different concentrations (1)(2)(4).

Applicability, M_n < 20 000.

2. **End-group analysis**

To use this method, knowledge of both the overall structure of the polymer and the nature of the chain terminating end groups is needed (which must be distinguishable from the main skeleton by, e.g. NMR or titration/derivatisation). The determination of the molecular concentration of the end groups present on the polymer can lead to a value for the molecular weight (7) (8) (9).

Applicability, M_n up to 50 000 (with decreasing reliability).

3. **References**

(1) Billmeyer, F.W. Jr., (1984) Textbook of Polymer Science, 3rd Edn., John Wiley, New York.

(2) Glover, C.A., (1975) Absolute Colligative Property Methods. Chapter 4. In: Polymer Molecular Weights, Part I P.E. Slade, Jr. ed., Marcel Dekker, New York.

(3) ASTM D 3750-79, (1979) Standard Practice for Determination of Number-Average Molecular Weight of Polymers by Membrane Osmometry. American Society for Testing and Materials, Philadelphia, Pennsylvania.

(4) Coll, H. (1989) Membrane Osmometry. In: Determination of Molecular Weight, A.R. Cooper ed., J. Wiley and Sons, pp. 25-52.

(5) ASTM 3592-77, (1977) Standard Recommended Practice for Determination of Molecular Weight by Vapour Pressure, American Society for Testing and Materials, Philadelphia, Pennsylvania.

(6) Morris, C.E.M., (1989) Vapour Pressure Osmometry. In: Determinationn of Molecular Weight, A.R. Cooper ed., John Wiley and Sons.

(7) Schröder, E., Müller, G., and Arndt, K-F., (1989) Polymer Characterisation, Carl Hanser Verlag, Munich.

(8) Garmon, R.G., (1975) End-Group Determinations, Chapter 3 In: Polymer Molecular Weights, Part I, P.E. Slade, Jr. ed., Marcel Dekker, New York.

(9) Amiya, S., et al. (1990) Pure and Applied Chemistry, 62, 2139-2146.

A.19. LOW MOLECULAR WEIGHT CONTENT OF POLYMERS

1. METHOD

This Gel Permeation Chromatographic method is a replicate of the OECD TG 119 (1996). The fundamental principles and further technical information are given in the references.

1.1. INTRODUCTION

Since the properties of polymers are so varied, it is impossible to describe one single method setting out precisely the conditions for separation and evaluation which cover all eventualities and specificities occurring in the separation of polymers. In particular, complex polymer systems are often not amenable to gel permeation chromatography (GPC). When GPC is not practicable, the molecular weight may be determined by means of other methods (see Appendix). In such cases, full details and justification should be given for the method used.

The method described is based on DIN Standard 55672 (1). Detailed information about how to carry out the experiments and how to evaluate the data can be found in this DIN Standard. In case modifications of the experimental conditions are necessary, these changes must be justified. Other standards may be used, if fully referenced. The method described uses polystyrene samples of known polydispersity for calibration and it may have to be modified to be suitable for certain polymers, e.g. water soluble and long-chain branched polymers.

1.2. DEFINITIONS AND UNITS

Low molecular weight is arbitrarily defined as a molecular weight below 1 000 dalton.

The number-average molecular weight M_n and the weight average molecular weight M_w are determined using the following equations:

$$M_n = \frac{\sum_{i=1}^{n} H_i}{\sum_{i=1}^{n} H_i / M_i} \qquad M_w = \frac{\sum_{i=1}^{n} H_i \times M_i}{\sum_{i=1}^{n} H_i}$$

where,

H_i = the level of the detector signal from the baseline for the retention volume V_i,

M_i = the molecular weight of the polymer fraction at the retention volume V_i, and n is the number of data points

The breadth of the molecular weight distribution, which is a measure of the dispersity of the system, is given by the ratio M_w/M_n.

1.3. REFERENCE SUBSTANCES

Since GPC is a relative method, calibration must be undertaken. Narrowly distributed, linearly constructed polystyrene standards with known average molecular weights M_n and M_w and a known molecular weight distribution are normally used for this. The calibration curve can only be used in the determination of the molecular weight of the unknown sample if the conditions for the separation of the sample and the standards have been selected in an identical manner.

A determined relationship between the molecular weight and elution volume is only valid under the specific conditions of the particular experiment. The conditions include, above all, the temperature, the solvent (or solvent mixture), the chromatography conditions and the separation column or system of columns.

The molecular weights of the sample determined in this way are relative values and are described as 'polystyrene equivalent molecular weights'. This means that dependent on the structural and chemical differences between the sample and the standards, the molecular weights can deviate from the absolute values to a greater or a lesser degree. If other standards are used, e.g. polyethylene glycol, polyethylene oxide, polymethyl methacrylate, polyacrylic acid, the reason should be stated.

1.4. PRINCIPLE OF THE TEST METHOD

Both the molecular weight distribution of the sample and the average molecular weights (M_n, M_w) can be determined using GPC. GPC is a special type of liquid chromatography in which the sample is separated according to the hydrodynamic volumes of the individual constituents (2).

Separation is effected as the sample passes through a column which is filled with a porous material, typically an organic gel. Small molecules can penetrate the pores whereas large molecules are excluded. The path of the large molecules is thereby shorter and these are eluted first. The medium-sized molecules penetrate some of the pores and are eluted later. The smallest molecules, with a mean hydrodynamic radius smaller than the pores of the gel, can penetrate all of the pores. These are eluted last.

In an ideal situation, the separation is governed entirely by the size of the molecular species, but in practice it is difficult to avoid at least some absorption effects interfering. Uneven column packing and dead volumes can worsen the situation (2).

Detection is effected by e.g. refractive index or UV-absorption and yields a simple distribution curve. However, to attribute actual molecular weight values to the curve, it is necessary to calibrate the column by passing down polymers of known molecular weight and, ideally, of broadly similar structure, e.g. various polystyrene standards. Typically a Gaussian curve results, sometimes distorted by a small tail to the low molecular weight side, the vertical axis indicating the quantity, by weight, of the various molecular weight species eluted, and the horizontal axis the log molecular weight.

The low molecular weight content is derived from this curve. The calculation can only be accurate if the low molecular weight species respond equivalently on a per mass basis to the polymer as a whole.

1.5. QUALITY CRITERIA

The repeatability (Relative Standard Deviation: RSD) of the elution volume should be better than 0,3 %. The required repeatability of the analysis has to be ensured by correction via an internal standard if a chromatogram is evaluated time-dependently and does not correspond to the above mentioned criterion (1). The polydispersities are dependent on the molecular weights of the standards. In the case of polystyrene standards typical values are:

$M_p < 2\,000$	$M_w/M_n < 1{,}20$
$2\,000 \leq M_p \leq 10^6$	$M_w/M_n < 1{,}05$
$M_p > 10^6$	$M_w/M_n < 1{,}20$

(M_p is the molecular weight of the standard at the peak maximum)

1.6. DESCRIPTION OF THE TEST METHOD

1.6.1. **Preparation of the standard polystyrene solutions**

The polystyrene standards are dissolved by careful mixing in the chosen eluent. The recommendations of the manufacturer must be taken into account in the preparation of the solutions.

The concentrations of the standards chosen are dependent on various factors, e.g. injection volume, viscosity of the solution and sensitivity of the analytical detector. The maximum injection volume must be adapted to the length of the column, in order to avoid overloading. Typical injection volumes for analytical separations using GPC with a column of 30 cm × 7,8 mm are normally between 40 and 100 µl. Higher volumes are possible, but they should not exceed 250 µl. The optimal ratio between the injection volume and the concentration must be determined prior to the actual calibration of the column.

1.6.2. **Preparation of the sample solution**

In principle, the same requirements apply to the preparation of the sample solutions. The sample is dissolved in a suitable solvent, e.g. tetrahydrofuran (THF), by shaking carefully. Under no circumstances should it be dissolved using an ultrasonic bath. When necessary, the sample solution is purified via a membrane filter with a pore size of between 0,2 and 2 µm.

The presence of undissolved particles must be recorded in the final report as these may be due to high molecular weight species. An appropriate method should be used to determine the percentage by weight of the undissolved particles. The solutions should be used within 24 hours.

1.6.3. **Correction for content of impurities and additives**

Correction of the content of species of M < 1 000 for the contribution from non-polymer specific components present (e.g. impurities and/or additives) is usually necessary, unless the measured content is already < 1 %. This is achieved by direct analysis of the polymer solution or the GPC eluate.

In cases where the eluate, after passage through the column, is too dilute for a further analysis it must be concentrated. It may be necessary to evaporate the eluate to dryness and dissolve it again. Concentration of the eluate must be effected under conditions which ensure that no changes occur in the eluate. The treatment of the eluate after the GPC step is dependent on the analytical method used for the quantitative determination.

1.6.4. **Apparatus**

GPC apparatus comprises the following components:

— solvent reservoir,

— degasser (where appropriate),

— pump,

— pulse dampener (where appropriate),

— injection system,

— chromatography columns,

— detector,

— flowmeter (where appropriate),

— data recorder-processor,

— waste vessel.

It must be ensured that the GPC system is inert with regard to the utilised solvents (e.g. by the use of steel capillaries for THF solvent).

1.6.5. **Injection and solvent delivery system**

A defined volume of the sample solution is loaded onto the column either using an auto-sampler or manually in a sharply defined zone. Withdrawing or depressing the plunger of the syringe too quickly, if done manually, can cause changes in the observed molecular weight distribution. The solvent-delivery system should, as far as possible, be pulsation-free ideally incorporating a pulse dampener. The flow rate is of the order of 1 ml/min.

1.6.6. **Column**

Depending on the sample, the polymer is characterised using either a simple column or several columns connected in sequence. A number of porous column materials with defined properties (e.g. pore size, exclusion limits) are commercially available. Selection of the separation gel or the length of the column is dependent on both the properties of the sample (hydrodynamic volumes, molecular weight distribution) and the specific conditions for separation such as solvent, temperature and flow rate (1) (2) (3).

1.6.7. **Theoretical plates**

The column or the combination of columns used for separation must be characterised by the number of theoretical plates. This involves, in the case of THF as elution solvent, loading a solution of ethyl benzene or other suitable non-polar solute onto a column of known length. The number of theoretical plates is given by the following equation:

$$N = 5{,}54\left(\frac{V_e}{W_{1/2}}\right)^2 \qquad \text{or} \qquad N = 16\left(\frac{V_e}{W}\right)^2$$

where,

N = the number of theoretical plates
V_e = the elution volume at the peak maximum
W = the baseline peak width
$W_{1/2}$ = the peak width at half height

1.6.8. **Separation efficiency**

In addition to the number of theoretical plates, which is a quantity determining the bandwidth, a part is also played by the separation efficiency, this being determined by the steepness of the calibration curve. The separation efficiency of a column is obtained from the following relationship:

$$\frac{V_{e,M_x} - V_{e,(10M_x)}}{\text{cross sectional area of the column}} \geq 6{,}0\left[\frac{cm^3}{cm^2}\right]$$

where,

$V_{e,\,M_x}$ = the elution volume for polystyrene with the molecular weight M_x
$V_{e,(10.M_x)}$ = the elution volume for polystyrene with a ten times greater molecular weight

The resolution of the system is commonly defined as follows:

$$R_{1,2} = 2 \times \frac{V_{e1} - V_{e2}}{W_1 + W_2} \times \frac{1}{\log_{10}(M_2/M_1)}$$

where,

V_{e1}, V_{e2} = the elution volumes of the two polystyrene standards at the peak maximum
W_1, W_2 = the peak widths at the base-1ine
M_1, M_2 = the molecular weights at the peak maximum (should differ by a factor of 10).

The R-value for the column system should be greater than 1,7 (4).

1.6.9. **Solvents**

All solvents must be of high purity (for THF purity of 99,5 % is used). The solvent reservoir (if necessary in an inert gas atmosphere) must be sufficiently large for the calibration of the column and several sample analyses. The solvent must be degassed before it is transported to the column via the pump.

1.6.10. **Temperature control**

The temperature of the critical internal components (injection loop, columns, detector and tubing) should be constant and consistent with the choice of solvent.

1.6.11. **Detector**

The purpose of the detector is to record quantitatively the concentration of sample eluted from the column. In order to avoid unnecessary broadening of peaks the cuvette volume of the detector cell must be kept as small as

possible. It should not be larger than 10 µl except for light scattering and viscosity detectors. Differential refractometry is usually used for detection. However, if required by the specific properties of the sample or the elution solvent, other types of detectors can be used, e.g. UV/VIS, IR, viscosity detectors, etc.

2. DATA AND REPORTING

2.1. DATA

The DIN Standard (1) should be referred to for the detailed evaluation criteria as well as for the requirements relating to the collecting and processing of data.

For each sample, two independent experiments must be carried out. They have to be analysed individually. In all cases it is essential to determine also data from blanks, treated under the same conditions as the sample.

It is necessary to indicate explicitly that the measured values are relative values equivalent to the molecular weights of the standard used.

After determination of the retention volumes or the retention times (possibly corrected using an internal standard), log M_p values (M_p being the peak maxima of the calibration standard) are plotted against one of those quantities. At least two calibration points are necessary per molecular weight decade, and at least five measurement points are required for the total curve, which should cover the estimated molecular weight of the sample. The low molecular weight end-point of the calibration curve is defined by n-hexyl benzene or another suitable non-polar solute. The portion of the curve corresponding to molecular weights below 1 000 is determined and corrected as necessary for impurities and additives. The elution curves are generally evaluated by means of electronic data processing. In case manual digitisation is used, ASTM D 3536-91 can be consulted (3).

If any insoluble polymer is retained on the column, its molecular weight is likely to be higher than that of the soluble fraction, and if not considered would result in an overestimation of the low molecular weight content. Guidance for correcting the low molecular weight content for insoluble polymer is provided in the Appendix.

The distribution curve must be provided in the form of a table or as figure (differential frequency or sum percentages against log M). In the graphic representation, one molecular weight decade should be normally about 4 cm in width and the peak maximum should be about 8 cm in height. In the case of integral distribution curves the difference in the ordinate between 0 and 100 % should be about 10 cm.

2.2. TEST REPORT

The test report must include the following information:

2.2.1. **Test substance:**

— available information about test substance (identity, additives, impurities),

— description of the treatment of the sample, observations, problems.

2.2.2. **Instrumentation:**

— reservoir of eluent, inert gas, degassing of the eluent, composition of the eluent, impurities,

— pump, pulse dampener, injection system,

— separation columns (manufacturer, all information about the characteristics of the columns, such as pore size, kind of separation material, etc., number, length and order of the columns used),

— number of the theoretical plates of the column (or combination), separation efficiency (resolution of the system),

— information on symmetry of the peaks,

— column temperature, kind of temperature control,

— detector (measurement principle, type, cuvette volume),

— flowmeter if used (manufacturer, measurement principle),

— system to record and process data (hardware and software).

2.2.3. **Calibration of the system:**

— detailed description of the method used to construct the calibration curve,

— information about quality criteria for this method (e.g. correlation coefficient, error sum of squares, etc.),

— information about all extrapolations, assumptions and approximations made during the experimental procedure and the evaluation and processing of data,

— all measurements used for constructing the calibration curve have to be documented in a table which includes the following information for each calibration point:

  — name of the sample,

  — manufacturer of the sample,

  — characteristic values of the standards M_p, M_n, M_w, M_w/M_n, as provided by the manufacturer or derived by subsequent measurements, together with details about the method of determination,

  — injection volume and injection concentration,

  — M_p value used for calibration,

  — elution volume or corrected retention time measured at the peak maxima,

  — M_p calculated at the peak maximum,

  — percentage error of the calculated M_p and the calibration value.

2.2.4. **Information on the low molecular weight polymer content:**

— description of the methods used in the analysis and the way in which the experiments were conducted,

— information about the percentage of the low molecular weight species content (w/w) related to the total sample,

— information about impurities, additives and other non-polymer species in percentage by weight related to the total sample.

2.2.5. **Evaluation:**

— evaluation on a time basis: all methods to ensure the required reproducibility (method of correction, internal standard etc.),

— information about whether the evaluation was effected on the basis of the elution volume or the retention time,

— information about the limits of the evaluation if a peak is not completely analysed,

— description of smoothing methods, if used,

— preparation and pre-treatment procedures of the sample,

— the presence of undissolved particles, if any,

— injection volume (µl) and injection concentration (mg/ml),

— observations indicating effects which lead to deviations from the ideal GPC profile,

— detailed description of all modifications in the testing procedures,

— details of the error ranges,

— any other information and observations relevant for the interpretation of the results.

3. **REFERENCES**

(1) DIN 55672 (1995) Gelpermeationschromatographie (GPC) mit Tetrahydrofuran (THF) als Elutionsmittel, Teil 1.

(2) Yau, W.W., Kirkland, J.J., and Bly, D.D. eds. (1979) Modern Size Exclusion Liquid Chromatography, J. Wiley and Sons.

(3) ASTM D 3536-91, (1991) Standard Test method for Molecular Weight Averages and Molecular Weight Distribution by Liquid Exclusion Chromatography (Gel Permeation Chromatography-GPC). American Society for Testing and Materials, Philadelphia, Pennsylvania.

(4) ASTM D 5296-92, (1992) Standard Test method for Molecular Weight Averages and Molecular Weight Distribution of Polystyrene by High Performance Size-Exclusion Chromatography. American Society for Testing and Materials, Philadelphia, Pennsylvania.

Appendix

Guidance for correcting low molecular content for the presence of insoluble polymer

When insoluble polymer is present in a sample, it results in mass loss during the GPC analysis. The insoluble polymer is irreversibly retained on the column or sample filter while the soluble portion of the sample passes through the column. In the case where the refractive index increment (dn/dc) of the polymer can be estimated or measured, one can estimate the sample mass lost on the column. In that case, one makes a correction using an external calibration with standard materials of known concentration and dn/dc to calibrate the response of the refractometer. In the example hereafter a poly(methyl methacrylate) (pMMA) standard is used.

In the external calibration for analysis of acrylic polymers, a pMMA standard of known concentration in tetrahydrofuran, is analysed by GPC and the resulting data are used to find the refractometer constant according to the equation:

$$K = R/(C \times V \times dn/dc)$$

where:

K = the refractometer constant (in microvolt second/ml),
R = the response of the pMMA standard (in microvolt/second),
C = the concentration of the pMMA standard (in mg/ml),
V = the injection volume (in ml), and
dn/dc = the refractive index increment for pMMA in tetrahydrofuran (in ml/mg).

The following data are typical for a pMMA standard:

R = 2 937 891
C = 1,07 mg/ml
V = 0,1 ml
dn/dc = 9×10^{-5} ml/mg

The resulting K value, $3{,}05 \times 10^{11}$ is then used to calculate the theoretical detector response if 100 % of the polymer injected had eluted through the detector.

A.20. SOLUTION/EXTRACTION BEHAVIOUR OF POLYMERS IN WATER

1. METHOD

The method described is a replicate of the revised version of OECD TG 120 (1997). Further technical information is given in reference (1).

1.1. INTRODUCTION

For certain polymers, such as emulsion polymers, initial preparatory work may be necessary before the method set out hereafter can be used. The method is not applicable to liquid polymers and to polymers that react with water under the test conditions.

When the method is not practical or not possible, the solution/extraction behaviour may be investigated by means of other methods. In such cases, full details and justification should be given for the method used.

1.2. REFERENCE SUBSTANCES

None.

1.3. PRINCIPLE OF THE TEST METHOD

The solution/extraction behaviour of polymers in an aqueous medium is determined using the flask method (see A.6 Water Solubility, Flask method) with the modifications described below.

1.4. QUALITY CRITERIA

None.

1.5. DESCRIPTION OF THE TEST METHOD

1.5.1. **Equipment**

The following equipment is required for the method:

— crushing device, e.g. grinder for the production of particles of known size,

— apparatus for shaking with possibility of temperature control,

— membrane filter system,

— appropriate analytical equipment,

— standardised sieves.

1.5.2. **Sample preparation**

A representative sample has first to be reduced to a particle size between 0,125 and 0,25 mm using appropriate sieves. Cooling may be required for the stability of the sample or for the grinding process. Materials of a rubbery nature can be crushed at liquid nitrogen temperature (1).

If the required particle size fraction is not attainable, action should be taken to reduce the particle size as much as possible, and the result reported. In the report, it is necessary to indicate the way in which the crushed sample was stored prior to the test

1.5.3. **Procedure**

Three samples of 10 g of the test substance are weighed into each of three vessels fitted with glass stoppers and 1 000 ml of water is added to each vessel. If handling an amount of 10 g polymer proves impracticable, the next highest amount which can be handled should be used and the volume of water adjusted accordingly.

The vessels are tightly stoppered and then agitated at 20 °C. A shaking or stirring device capable of operating at constant temperature should be used. After a period of 24 hours, the content of each vessel is centrifuged or filtered and the concentration of polymer in the clear aqueous phase is determined by a suitable analytical method. If suitable analytical methods for the aqueous phase are not available, the total solubility/extractivity can be estimated from the dry weight of the filter residue or centrifuged precipitate.

It is usually necessary to differentiate quantitatively between the impurities and additives on the one hand and the low molecular weight species on the other hand. In the case of gravimetric determination, it is also important to perform a blank run using no test substance in order to account for residues arising from the experimental procedure.

The solution/extraction behaviour of polymers in water at 37 °C at pH 2 and pH 9 may be determined in the same way as described for the conduct of the experiment at 20 °C. The pH values can be achieved by the addition of either suitable buffers or appropriate acids or bases such as hydrochloric acid, acetic acid, analytical grade sodium or potassium hydroxide or NH_3.

Depending on the method of analysis used, one or two tests should be performed. When sufficiently specific methods are available for direct analysis of the aqueous phase for the polymer component, one test as described above should suffice. However, when such methods are not available and determination of the solution/extraction behaviour of the polymer is limited to indirect analysis by determining only the total organic carbon content (TOC) of the aqueous extract, an additional test should be conducted. This additional test should also be done in triplicate, using ten times smaller polymer samples and the same amounts of water as those used in the first test.

1.5.4. **Analysis**

1.5.4.1. *Test conducted with one sample size*

Methods may be available for direct analysis of polymer components in the aqueous phase. Alternatively, indirect analysis of dissolved/extracted polymer components, by determining the total content of soluble parts and correcting for non polymer-specific components, could also be considered.

Analysis of the aqueous phase for the total polymeric species is possible:

either by a sufficiently sensitive method, e.g.:

— TOC using persulphate or dichromate digestion to yield CO_2 followed by estimation by IR or chemical analysis,

— Atomic Absorption Spectrometry (AAS) or its Inductively Coupled Plasma (ICP) emission equivalent for silicon or metal containing polymers,

— UV absorption or spectrofluorimetry for aryl polymers,

— LC-MS for low molecular weight samples,

or by vacuum evaporation to dryness of the aqueous extract and spectroscopic (IR, UV, etc.) or AAS/ICP analysis of the residue.

If analysis of the aqueous phase as such is not practicable, the aqueous extract should be extracted with a water-immiscible organic solvent e.g. a chlorinated hydrocarbon. The solvent is then evaporated and the residue analysed as above for the notified polymer content. Any components in this residue which are identified as being impurities or additives are to be subtracted for the purpose of determining the degree of solution/extraction of the polymer itself.

When relatively large quantities of such materials are present, it may be necessary to subject the residue to e.g. HPLC or GC analysis to differentiate the impurities from the monomer and monomer-derived species present so that the true content of the latter can be determined.

In some cases, simple evaporation of the organic solvent to dryness and weighing the dry residue may be sufficient.

1.5.4.2. *Test conducted with two different sample sizes*

All aqueous extracts are analysed for TOC.

A gravimetric determination is performed on the undissolved/not extracted part of the sample. If, after centrifugation or filtering of the content of each vessel, polymer residues remain attached to the wall of the vessel, the vessel should be rinsed with the filtrate until the vessel is cleared from all visible residues. Following which, the filtrate is again centrifuged or filtered. The residues remaining on the filter or in the centrifuge tube are dried at 40 °C under vacuum and weighed. Drying is continued until a constant weight is reached.

2. DATA

2.1. TEST CONDUCTED WITH ONE SAMPLE SIZE

The individual results for each of the three flasks and the average values should be given and expressed in units of mass per volume of the solution (typically mg/l) or mass per mass of polymer sample (typically mg/g). Additionally, the weight loss of the sample (calculated as the weight of the solute divided by the weight of the initial sample) should also be given. The relative standard deviations (RSD) should be calculated. Individual figures should be given for the total substance (polymer + essential additives, etc.) and for the polymer only (i.e. after subtracting the contribution from such additives).

2.2. TEST CONDUCTED WITH TWO DIFFERENT SAMPLE SIZES

The individual TOC values of the aqueous extracts of the two triplicate experiments and the average value for each experiment should be given expressed as units of mass per volume of solution (typically mgC/l), as well as in units of mass per weight of the initial sample (typically mgC/g).

If there is no difference between the results at the high and the low sample/water ratios, this may indicate that all extractable components were indeed extracted. In such a case, direct analysis would normally not be necessary.

The individual weights of the residues should be given and expressed in percentage of the initial weights of the samples. Averages should be calculated per experiment. The differences between 100 and the percentages found represent the percentages of soluble and extractable material in the original sample.

3. REPORTING

3.1. TEST REPORT

The test report must include the following information:

3.1.1. **Test substance:**

— available information about test substance (identity, additives, impurities, content of low molecular weight species).

3.1.2. **Experimental conditions:**

— description of the procedures used and experimental conditions,

— description of the analytical and detection methods.

3.1.3. **Results:**

— results of solubility/extractivity in mg/l; individual and mean values for the extraction tests in the various solutions, broken down in polymer content and impurities, additives, etc.,

— results of solubility/extractivity in mg/g of polymer,

— TOC values of aqueous extracts, weight of the solute and calculated percentages, if measured,

— the pH of each sample,

— information about the blank values,

— where necessary, references to the chemical instability of the test substance, during both the testing process and the analytical process,

— all information which is important for the interpretation of the results.

4. **REFERENCES**

(1) DIN 53733 (1976) Zerkleinerung von Kunststofferzeugnissen für Prüfzwecke.

A.21. OXIDISING PROPERTIES (LIQUIDS)

1. METHOD

1.1. INTRODUCTION

This test method is designed to measure the potential for a liquid substance to increase the burning rate or burning intensity of a combustible substance, or to form a mixture with a combustible substance which spontaneously ignites, when the two are thoroughly mixed. It is based on the UN test for oxidising liquids (1) and is equivalent to it. However, as this method A.21 is primarily designed to satisfy the requirements of Regulation (EC) No 1907/2006, comparison with only one reference substance is required. Testing and comparison to additional reference substances may be necessary when the results of the test are expected to be used for other purposes. ([1])

This test need not be performed when examination of the structural formula establishes beyond reasonable doubt that the substance is incapable of reacting exothermically with a combustible material.

It is useful to have preliminary information on any potential explosive properties of the substance before performing this test.

This test is not applicable to solids, gases, explosive or highly flammable substances, or organic peroxides.

This test may not need to be performed when results for the test substance in the UN test for oxidising liquids (1) are already available.

1.2. DEFINITIONS AND UNITS

Mean pressure rise time is the mean of the measured times for a mixture under test to produce a pressure rise from 690 kPa to 2 070 kPa above atmospheric.

1.3. REFERENCE SUBSTANCE

65 % (w/w) aqueous nitric acid (analytical grade) is required as a reference substance. ([2])

Optionally, if the experimenter foresees that the results of this test may eventually be used for other purposes ([1]), testing of additional reference substances may also be appropriate. ([3])

1.4. PRINCIPLE OF THE TEST METHOD

The liquid to be tested is mixed in a 1 to 1 ratio, by mass, with fibrous cellulose and introduced into a pressure vessel. If during mixing or filling spontaneous ignition occurs, no further testing is necessary.

If spontaneous ignition does not occur the full test is carried out. The mixture is heated in a pressure vessel and the mean time taken for the pressure to rise from 690 kPa to 2 070 kPa above atmospheric is determined. This is compared with the mean pressure rise time for the 1:1 mixture of the reference substance(s) and cellulose.

1.5. QUALITY CRITERIA

In a series of five trials on a single substance no results should differ by more than 30 % from the arithmetic mean. Results that differ by more than 30 % from the mean should be discarded, the mixing and filling procedure improved and the testing repeated.

([1]) As, for example, in the framework of UN transport regulations.

([2]) The acid should be titrated before testing to confirm its concentration.

([3]) E.g.: 50 % (w/w) perchloric acid and 40 % (w/w) sodium chlorate are used in reference 1.

1.6. DESCRIPTION OF THE METHOD

1.6.1. **Preparation**

1.6.1.1. *Combustible substance*

Dried, fibrous cellulose with a fibre length between 50 and 250 μm and a mean diameter of 25 μm ([1]), is used as the combustible material. It is dried to constant weight in a layer not more than 25 mm thick at 105 °C for four hours and kept in a desiccator, with desiccant, until cool and required for use. The water content of the dried cellulose should be less than 0,5 % by dry mass ([2]). If necessary, the drying time should be prolonged to achieve this. ([3]) The same batch of cellulose is to be used throughout the test.

1.6.1.2. *Apparatus*

1.6.1.2.1. Pressure vessel

A pressure vessel is required. The vessel consists of a cylindrical steel pressure vessel 89 mm in length and 60 mm in external diameter (see figure 1). Two flats are machined on opposite sides (reducing the cross-section of the vessel to 50 mm) to facilitate holding whilst fitting up the firing plug and vent plug. The vessel, which has a bore of 20 mm diameter is internally rebated at either end to a depth of 19 mm and threaded to accept 1" British Standard Pipe (BSP) or metric equivalent. A pressure take-off, in the form of a side arm, is screwed into the curved face of the pressure vessel 35 mm from one end and at 90° to the machined flats. The socket for this is bored to a depth of 12 mm and threaded to accept the 1/2" BSP (or metric equivalent) thread on the end of the side-arm. If necessary, an inert seal is fitted to ensure a gas-tight seal. The side-arm extends 55 mm beyond the pressure vessel body and has a bore of 6 mm. The end of the side-arm is rebated and threaded to accept a diaphragm type pressure transducer. Any pressure-measuring device may be used provided that it is not affected by the hot gases or the decomposition products and is capable of responding to rates of pressure rise of 690-2 070 kPa in not more than 5 ms.

The end of the pressure vessel farthest from the side-arm is closed with a firing plug which is fitted with two electrodes, one insulated from, and the other earthed to, the plug body. The other end of the pressure vessel is closed by a bursting disk (bursting pressure approximately 2 200 kPa) held in place with a retaining plug which has a 20 mm bore. If necessary, an inert seal is used with the firing plug to ensure a gas-tight fit. A support stand (figure 2) holds the assembly in the correct attitude during use. This usually comprises a mild steel base plate measuring 235 mm × 184 mm × 6 mm and a 185 mm length of square hollow section (S.H.S.) 70 mm × 70 mm × 4 mm.

A section is cut from each of two opposite sides at one end of the length of S.H.S. so that a structure having two flat sided legs surmounted by 86 mm length of intact box section results. The ends of these flat sides are cut to an angle of 60° to the horizontal and welded to the base plate. A slot measuring 22 mm wide × 46 mm deep is machined in one side of the upper end of the base section such that when the pressure vessel assembly is lowered, firing plug end first, into the box section support, the side-arm is accommodated in the slot. A piece of steel 30 mm wide and 6 mm thick is welded to the lower internal face of the box section to act as a spacer. Two 7 mm thumb screws, tapped into the opposite face, serve to hold the pressure vessel firmly in place. Two 12 mm wide strips of 6 mm thick steel, welded to the side pieces abutting the base of the box section, support the pressure vessel from beneath.

1.6.1.2.2. Ignition system

The ignition system consists of a 25 cm long Ni/Cr wire with a diameter 0,6 mm and a resistance of 3,85 ohm/m. The wire is wound, using a 5 mm diameter rod, in the shape of a coil and is attached to the firing plug electrodes. The coil should have one of the configurations shown in figure 3. The distance between the bottom of the vessel and the underside of the ignition coil should be 20 mm. If the electrodes are not adjustable, the ends of the ignition wire between the coil and the bottom of the vessel should be insulated by a ceramic sheath. The wire is heated by a constant current power supply able to deliver at least 10 A.

1.6.2. **Performance of the test** ([4])

The apparatus, assembled complete with pressure transducer and heating system but without the bursting disk in position, is supported firing plug end down. 2,5 g of the liquid to be tested is mixed with 2,5 g of dried cellulose in a glass beaker using a glass stirring rod ([5]). For safety, the mixing should be performed with a safety shield between the operator and mixture. If the mixture ignites during mixing or filling, no further testing is

([1]) E.g. Whatman Column Chromatographic Cellulose Powder CF 11, catalogue No 4021 050.

([2]) Confirmed by, e.g. Karl-Fisher titration.

([3]) Alternatively, this water content can also be achieved by, e.g. heating at 105 °C under vacuum for 24 h.

([4]) Mixtures of oxidisers with cellulose must be treated as potentially explosive and handled with due care.

([5]) In practice this can be achieved by preparing a 1:1 mixture of the liquid to be tested and cellulose in a greater amount than needed for the trial and transferring 5 ± 0,1 g to the pressure vessel. The mixture is to be freshly prepared for each trial.

necessary. The mixture is added, in small portions with tapping, to the pressure vessel making sure that the mixture is packed around the ignition coil and is in good contact with it. It is important that the coil is not distorted during the packing process as this may lead to erroneous results ([1]). The bursting disk is placed in position and the retaining plug is screwed in tightly. The charged vessel is transferred to the firing support stand, bursting disk uppermost, which should be located in a suitable, armoured fume cupboard or firing cell. The power supply is connected to the external terminals of the firing plug and 10 A applied. The time between the start of mixing and switching on the power should not exceed 10 minutes.

The signal produced by the pressure transducer is recorded on a suitable system which allows both evaluation and the generation of a permanent record of the time pressure profile obtained (e.g. a transient recorder coupled to a chart recorder). The mixture is heated until the bursting disk ruptures or until at least 60 s have elapsed. If the bursting disk does not rupture, the mixture should be allowed to cool before carefully dismantling the apparatus, taking precautions to allow for any pressurisation which may occur. Five trials are performed with the test substance and the reference substance(s). The time taken for the pressure to rise from 690 kPa to 2 070 kPa above atmospheric is noted. The mean pressure rise time is calculated.

In some cases, substances may generate a pressure rise (too high or too low), caused by chemical reactions not characterising the oxidising properties of the substance. In these cases, it may be necessary to repeat the test with an inert substance, e.g. diatomite (kieselguhr), in place of the cellulose in order to clarify the nature of the reaction.

2. DATA

Pressure rise times for both the test substance and the reference substance(s). Pressure rise times for the tests with an inert substance, if performed.

2.1. TREATMENT OF RESULTS

The mean pressure rise times for both the test substance and the reference substances(s) are calculated.

The mean pressure rise time for the tests with an inert substance (if performed) is calculated.

Some examples of results are shown in Table 1.

Table 1

Examples of results ([a])

Substance ([b])	Mean pressure rise time for a 1:1 mixture with celulose (ms)
Ammonium dichromate, saturated aqueous solution	20 800
Calcium nitrate, saturated aqueous solution	6 700
Ferric nitrate, saturated aqueous solution	4 133
Lithium perchlorate, saturated aqueous solution	1 686
Magnesium perchlorate, saturated aqueous solution	777
Nickel nitrate, saturated aqueous solution	6 250
Nitric acid, 65 %	4 767 ([c])
Perchloric acid, 50 %	121 ([c])
Perchloric acid, 55 %	59
Potassium nitrate, 30 % aqueous solution	26 690
Silver nitrate, saturated aqueous solution	([d])
Sodium chlorate, 40 % aqueous solution	2 555 ([c])

([1]) In particular, contact between the adjacent turns of the coil must be avoided.

Substance [b]	Mean pressure rise time for a 1:1 mixture with celulose (ms)
Sodium nitrate, 45 % aqueous solution	4 133
Inert substance	
Water: cellulose	[d]

[a] See reference (1) for classification under the UN transport scheme.
[b] Saturated solutions should be prepared at 20 °C.
[c] Mean value from interlaboratory comparative trials.
[d] Maximum pressure of 2 070 kPa not reached.

3. **REPORT**

3.1. TEST REPORT

The test report should include the following information:

— the identity, composition, purity, etc. of the substance tested,

— the concentration of the test substance,

— the drying procedure of the cellulose used,

— the water content of the cellulose used,

— the results of the measurements,

— the results from tests with an inert substance, if any,

— the calculated mean pressure rise times,

— any deviations from this method and the reasons for them,

— all additional information or remarks relevant to the interpretation of the results.

3.2. INTERPRETATION OF THE RESULTS [1]

The test results are assessed on the basis of:

(a) whether the mixture of test substance and cellulose spontaneously ignites; and

(b) the comparison of the mean time taken for the pressure to rise from 690 kPa to 2 070 kPa with that of the reference substance(s).

A liquid substance is to be considered as an oxidiser when:

(a) a 1:1 mixture, by mass, of the substance and cellulose spontaneously ignites; or

[1] See reference 1 for interpretation of the results under the UN transport regulations using several reference substances.

(b) a 1:1 mixture, by mass, of the substance and cellulose exhibits a mean pressure rise time less than or equal to the mean pressure rise time of a 1:1 mixture, by mass, of 65 % (w/w) aqueous nitric acid and cellulose.

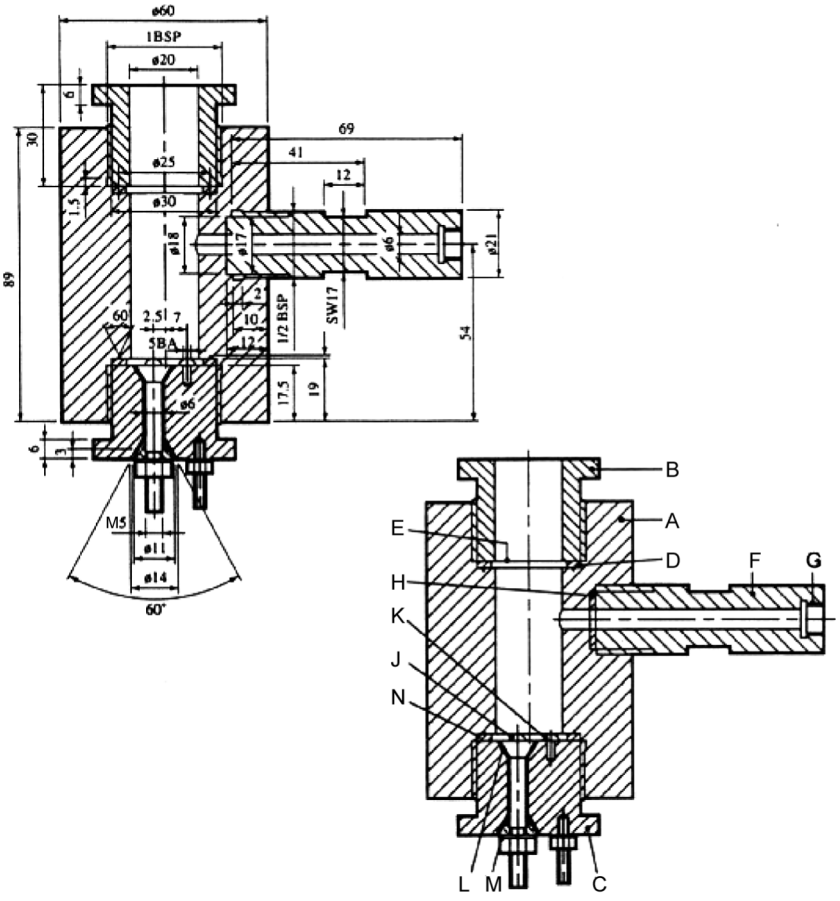
In order to avoid a false positive result, if necessary, the results obtained when testing the substance with an inert material should also be considered when interpreting the results.

4. REFERENCES

(1) Recommendations on the Transport of Dangerous Goods, Manual of Tests and Criteria. 3rd revised edition. UN Publication No: ST/SG/AC.10/11/Rev. 3, 1999, page 342. Test O.2: Test for oxidising liquids.

Figure 1

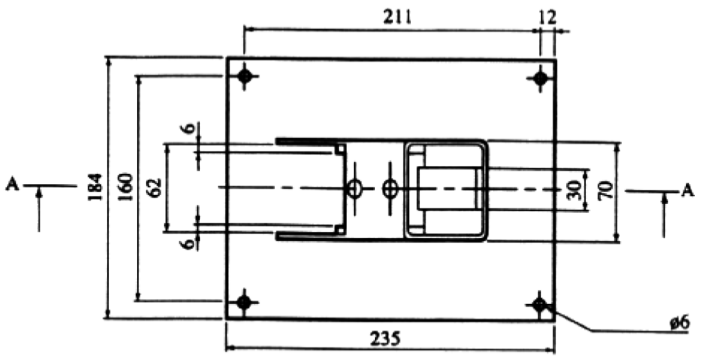
Pressure vessel



(A) Pressure vessel body
(B) Bursting disk retaining plug
(C) Firing plug
(D) Soft lead washer
(E) Bursting disc
(F) Side arm
(G) Pressure transducer head
(H) Washer
(J) Insulated electrode
(K) Earthed electrode
(L) Insulation
(M) Steel cone
(N) Washer distorting groove

Figure 2

Support stand



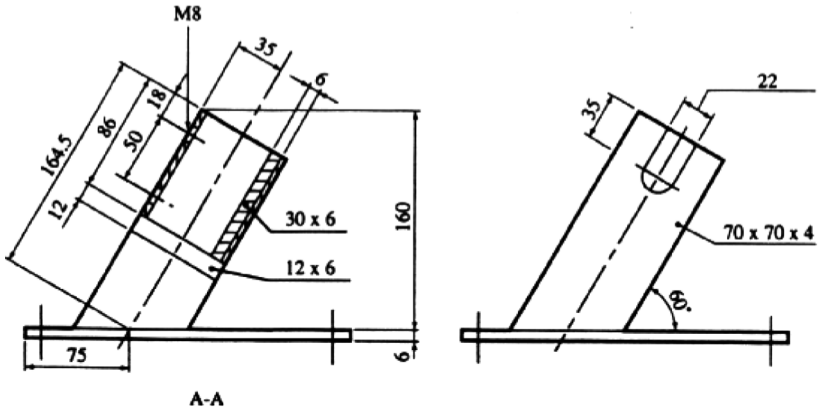
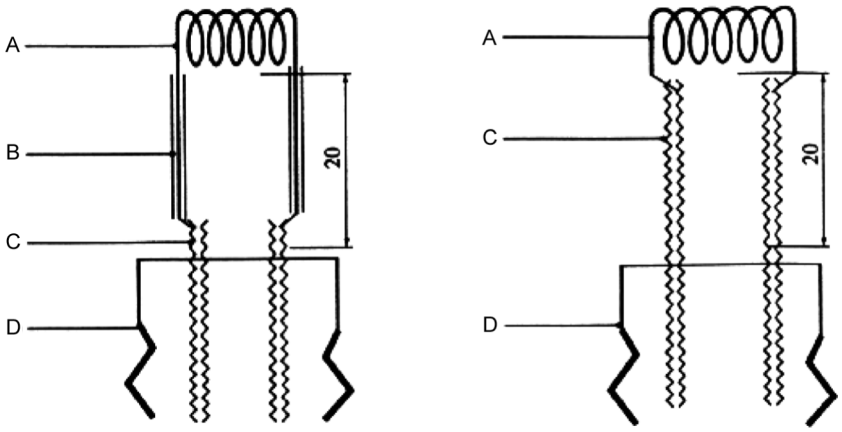


Figure 3

Ignition system

(A) Ignition coil (B) Insulation (C) Electrodes (D) Firing plug



Note: either of these configurations may be used.

PART B: METHODS FOR THE DETERMINATION OF TOXICITY AND OTHER HEALTH EFFECTS

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GENERAL INTRODUCTION

A. CHARACTERISATION OF THE TEST SUBSTANCE

The composition of the test substance, including major impurities, and its relevant physico-chemical properties including stability, should be known prior to the initiation of any toxicity study.

The physico-chemical properties of the test substance provide important information for the selection of the route of administration, the design of each particular study and the handling and storage of the test substance.

The development of an analytical method for qualitative and quantitative determination of the test substance (including major impurities when possible) in the dosing medium and the biological material should precede the initiation of the study.

All information relating to the identification, the physico-chemical properties, the purity, and behaviour of the test substance should be included in the test report.

B. ANIMAL CARE

Stringent control of environmental conditions and proper animal care techniques are essential in toxicity testing.

(i) *Housing conditions*

The environmental conditions in the experimental animal rooms or enclosures should be appropriate to the test species. For rats, mice and guinea pigs, suitable conditions are a room temperature of 22 °C ± 3 °C with a relative humidity of 30 to 70 %; for rabbits the temperature should be 20 ± 3 °C with a relative humidity of 30 to 70 %.

Some experimental techniques are particularly sensitive to temperature effects and, in these cases, details of appropriate conditions are included in the description of the test method. In all investigations of toxic effects, the temperature and humidity should be monitored, recorded, and included in the final report of the study.

Lighting should be artificial, the sequence being 12 hours light, 12 hours dark. Details of the lighting pattern should be recorded and included in the final report of the study.

Unless otherwise specified in the method, animals may be housed individually, or be caged in small groups of the same sex; for group caging, no more than five animals should be housed per cage.

In reports of animal experiments, it is important to indicate the type of caging used and the number of animals housed in each cage both during exposure to the chemical and any subsequent observation period.

(ii) *Feeding conditions*

Diets should meet all the nutritional requirements of the species under test. Where test substances are administered to animals in their diet the nutritional value may be reduced by interaction between the substance and a dietary constituent. The possibility of such a reaction should be considered when interpreting the results of tests. Conventional laboratory diets may be used with an unlimited supply of drinking water. The choice of the diet may be influenced by the need to ensure a suitable admixture of a test substance when administered by this method.

Dietary contaminants which are known to influence the toxicity should not be present in interfering concentrations.

C. ALTERNATIVE TESTING

The European Union is committed to promoting the development and validation of alternative techniques which can provide the same level of information as current animal tests, but which use fewer animals, cause less suffering or avoid the use of animals completely.

Such methods, as they become available, must be considered wherever possible for hazard characterisation and consequent classification and labelling for intrinsic hazards and chemical safety assessment.

D. EVALUATION AND INTERPRETATION

When tests are evaluated and interpreted, limitations in the extent to which the results of animal and *in vitro* studies can be extrapolated directly to man must be considered and therefore, evidence of adverse effects in humans, where available, may be used for confirmation of testing results.

E. LITERATURE REFERENCES

Most of these methods are developed within the framework of the OECD programme for Testing Guidelines, and should be performed in conformity with the principles of Good Laboratory Practice, in order to ensure as wide as possible 'mutual acceptance of data'.

Additional information may be found in the references listed in the OECD guidelines and the relevant literature published elsewhere.

B.1 bis. **ACUTE ORAL TOXICITY — FIXED DOSE PROCEDURE**

1. **METHOD**

This test method is equivalent to OECD TG 420 (2001)

1.1. INTRODUCTION

Traditional methods for assessing acute toxicity use death of animals as an endpoint. In 1984, a new approach to acute toxicity testing was suggested by the British Toxicology Society based on the administration at a series of fixed dose levels (1). The approach avoided using death of animals as an endpoint, and relied instead on the observation of clear signs of toxicity at one of a series of fixed dose levels. Following UK (2) and international (3) *in vivo* validation studies the procedure was adopted as a testing method in 1992. Subsequently, the statistical properties of the Fixed Dose Procedure have been evaluated using mathematical models in a series of studies (4)(5)(6). Together, the *in vivo* and modelling studies have demonstrated that the procedure is reproducible, uses fewer animals and causes less suffering than the traditional methods and is able to rank substances in a similar manner to the other acute toxicity testing methods.

Guidance on the selection of the most appropriate test method for a given purpose can be found in the Guidance Document on Acute Oral Toxicity Testing (7). This guidance document also contains additional information on the conduct and interpretation of Testing Method B.1*bis*.

It is a principle of the method that in the main study only moderately toxic doses are used, and that administration of doses that are expected to be lethal should be avoided. Also, doses that are known to cause marked pain and distress, due to corrosive or severely irritant actions, need not be administered. Moribund animals, or animals obviously in pain or showing signs of severe and enduring distress shall be humanely killed, and are considered in the interpretation of the test results in the same way as animals that died on test. Criteria for making the decision to kill moribund or severely suffering animals, and guidance on the recognition of predictable or impending death, are the subject of a separate Guidance Document (8).

The method provides information on the hazardous properties and allows the substance to be ranked and classified according to the Globally Harmonised System (GHS) for the classification of chemicals which cause acute toxicity (9).

The testing laboratory should consider all available information on the test substance prior to conducting the study. Such information will include the identity and chemical structure of the substance; its physico-chemical properties; the results of any other *in vitro* or *in vivo* toxicity tests on the substance; toxicological data on structurally related substances; and the anticipated use(s) of the substance. This information is necessary to satisfy all concerned that the test is relevant for the protection of human health, and will help in the selection of an appropriate starting dose.

1.2. DEFINITIONS

Acute oral toxicity: refers to those adverse effects occurring following oral administration of a single dose of a substance or multiple doses given within 24 hours.

Delayed death: means that an animal does not die or appear moribund within 48 hours but dies later during the 14-day observation period.

Dose: is the amount of test substance administered. Dose is expressed as weight of test substance per unit weight of test animal (e.g. mg/kg).

Evident toxicity: is a general term describing clear signs of toxicity following the administration of test substance (see (3) for examples) such that at the next highest fixed dose either severe pain and enduring signs of severe distress, moribund status (criteria are presented in the Humane Endpoints Guidance Document (8)), or probable mortality in most animals can be expected.

GHS: Globally Harmonised Classification System for Chemical Substances and Mixtures. A joint activity of OECD (human health and the environment), UN Committee of Experts on Transport of Dangerous Goods (physical-chemical properties) and ILO (hazard communication) and coordinated by the Interorganisation Programme for the Sound Management of Chemicals (IOMC).

Impending death: when moribund state or death is expected prior to the next planned time of observation. Signs indicative of this state in rodents could include convulsions, lateral position, recumbence and tremor. (See the Humane Endpoint Guidance Document (8) for more details).

LD_{50} (median lethal dose): is a statistically derived single dose of a substance that can be expected to cause death in 50 % of animals when administered by the oral route. The LD_{50} value is expressed in terms of weight of test substance per unit weight of test animal (mg/kg).

Limit dose: refers to a dose at an upper limitation on testing (2 000 or 5 000 mg/kg).

Moribund status: being in a state of dying or inability to survive, even if treated. (See the Humane Endpoint Guidance Document (8) for more details).

Predictable death: presence of clinical signs indicative of death at a known time in the future before the planned end of the experiment, for example: inability to reach water or food. (See the Humane Endpoint Guidance Document (8) for more details).

1.3. PRINCIPLE OF THE TEST METHOD

Groups of animals of a single sex are dosed in a stepwise procedure using the fixed doses of 5, 50, 300 and 2 000 mg/kg (exceptionally an additional fixed dose of 5 000 mg/kg may be considered, see Section 1.6.2). The initial dose level is selected on the basis of a sighting study as the dose expected to produce some signs of toxicity without causing severe toxic effects or mortality. Clinical signs and conditions associated with pain, suffering, and impending death, are described in detail in a separate OECD Guidance Document (8). Further groups of animals may be dosed at higher or lower fixed doses, depending on the presence or absence of signs of toxicity or mortality. This procedure continues until the dose causing evident toxicity or no more than one death is identified, or when no effects are seen at the highest dose or when deaths occur at the lowest dose.

1.4. DESCRIPTION OF THE TEST METHOD

1.4.1. **Selection of animal species**

The preferred rodent species is the rat, although other rodent species may be used. Normally females are used (7). This is because literature surveys of conventional LD_{50} tests show that usually there is little difference in sensitivity between the sexes, but in those cases where differences are observed, females are generally slightly more sensitive (10). However, if knowledge of the toxicological or toxicokinetic properties of structurally related chemicals indicates that males are likely to be more sensitive then this sex should be used. When the test is conducted in males, adequate justification should be provided.

Healthy young adult animals of commonly used laboratory strains should be employed. Females should be nulliparous and non-pregnant. Each animal, at the commencement of its dosing, should be between eight and 12 weeks old and its weight should fall in an interval within ± 20 % of the mean weight of any previously dosed animals.

1.4.2. **Housing and feeding conditions**

The temperature of the experimental animal room should be 22 °C (± 3 °C). Although the relative humidity should be at least 30 % and preferably not exceed 70 % other than during room cleaning the aim should be 50-60 %. Lighting should be artificial, the sequence being 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. Animals may be group-caged by dose, but the number of animals per cage must not interfere with clear observations of each animal.

1.4.3. **Preparation of animals**

The animals are randomly selected, marked to permit individual identification, and kept in their cages for at least five days prior to the start of dosing to allow for acclimatisation to the laboratory conditions.

1.4.4. **Preparation of doses**

In general test substances should be administered in a constant volume over the range of doses to be tested by varying the concentration of the dosing preparation. Where a liquid end product or mixture is to be tested however, the use of the undiluted test substance, i.e. at a constant concentration, may be more relevant to the subsequent risk assessment of that substance, and is a requirement of some regulatory authorities. In either case, the maximum dose volume for administration must not be exceeded. The maximum volume of liquid that

can be administered at one time depends on the size of the test animal. In rodents, the volume should not normally exceed 1ml /100 g of body weight: however in the case of aqueous solutions 2 ml/100 g body weight can be considered. With respect to the formulation of the dosing preparation, the use of an aqueous solution/ suspension/emulsion is recommended wherever possible, followed in order of preference by a solution/ suspension/emulsion in oil (e.g. corn oil) and then possibly solution in other vehicles. For vehicles other than water the toxicological characteristics of the vehicle should be known. Doses must be prepared shortly prior to administration unless the stability of the preparation over the period during which it will be used is known and shown to be acceptable.

1.5. PROCEDURE

1.5.1. **Administration of doses**

The test substance is administered in a single dose by gavage using a stomach tube or a suitable intubation canula. In the unusual circumstance that a single dose is not possible, the dose may be given in smaller fractions over a period not exceeding 24 hours.

Animals should be fasted prior to dosing (e.g. with the rat, food but not water should be withheld over-night; with the mouse, food but not water should be withheld for three to four hours). Following the period of fasting, the animals should be weighed and the test substance administered. After the substance has been administered, food may be withheld for a further three to four hours in rats or one to two hours in mice. Where a dose is administered in fractions over a period of time, it may be necessary to provide the animals with food and water depending on the length of the period.

1.5.2. **Sighting study**

The purpose of the sighting study is to allow selection of the appropriate starting dose for the main study. The test substance is administered to single animals in a sequential manner following the flowcharts in Appendix 1. The sighting study is completed when a decision on the starting dose for the main study can be made (or if a death is seen at the lowest fixed dose).

The starting dose for the sighting study is selected from the fixed dose levels of 5, 50, 300 and 2 000 mg/kg as a dose expected to produce evident toxicity based, when possible, on evidence from *in vivo* and *in vitro* data from the same chemical and from structurally related chemicals. In the absence of such information, the starting dose will be 300 mg/kg.

A period of at least 24 hours will be allowed between the dosing of each animal. All animals should be observed for at least 14 days.

Exceptionally, and only when justified by specific regulatory needs, the use of an additional upper fixed dose level of 5 000 mg/kg may be considered (see Appendix 3). For reasons of animal welfare concern, testing of animals in GHS Category 5 ranges (2 000-5 000 mg/kg is discouraged and should only be considered when there is a strong likelihood that the results of such a test have a direct relevance for protecting human or animal health or the environment.

In cases where an animal tested at the lowest fixed dose level (5 mg/kg) in the sighting study dies, the normal procedure is to terminate the study and assign the substance to GHS Category 1 (as shown in Appendix 1). However, if further confirmation of the classification is required, an optional supplementary procedure may be conducted, as follows. A second animal is dosed at 5 mg/kg. If this second animal dies, then GHS Category 1 will be confirmed and the study will be immediately terminated. If the second animal survives, then a maximum of three additional animals will be dosed at 5 mg/kg. Because there will be a high risk of mortality, these animals should be dosed in a sequential manner to protect animal welfare. The time interval between dosing each animal should be sufficient to establish that the previous animal is likely to survive. If a second death occurs, the dosing sequence will be immediately terminated and no further animals will be dosed. Because the occurrence of a second death (irrespective of the number of animals tested at the time of termination) falls into outcome A (two or more deaths), the classification rule of Appendix 2 at the 5 mg/kg fixed dose is followed (Category 1 if there are two or more deaths or Category 2 if there is no more than one death). In addition, Appendix 4 gives guidance on the classification in the EU system until the new GHS is implemented.

1.5.3. **Main study**

1.5.3.1. *Numbers of animals and dose levels*

The action to be taken following testing at the starting dose level is indicated by the flowcharts in Appendix 2. One of three actions will be required; either stop testing and assign the appropriate hazard classification class, test at a higher fixed dose or test at a lower fixed dose. However, to protect animals, a dose level that caused death in the sighting study will not be revisited in the main study (see Appendix 2). Experience has shown that the most likely outcome at the starting dose level will be that the substance can be classified and no further testing will be necessary.

A total of five animals of one sex will normally be used for each dose level investigated. The five animals will be made up of one animal from the sighting study dosed at the selected dose level together with an additional four animals (except, unusually, if a dose level used on the main study was not included in the sighting study).

The time interval between dosing at each level is determined by the onset, duration, and severity of toxic signs. Treatment of animals at the next dose should be delayed until one is confident of survival of the previously dosed animals. A period of three or four days between dosing at each dose level is recommended, if needed, to allow for the observation of delayed toxicity. The time interval may be adjusted as appropriate, e.g. in case of inconclusive response.

When the use of an upper fixed dose of 5 000 mg/kg is considered, the procedure outlined in Appendix 3 should be followed (see also section 1.6.2).

1.5.3.2. *Limit test*

The limit test is primarily used in situations where the experimenter has information indicating that the test material is likely to be nontoxic, i.e., having toxicity only above regulatory limit doses. Information about the toxicity of the test material can be gained from knowledge about similar tested compounds or similar tested mixtures or products, taking into consideration the identity and percentage of components known to be of toxicological significance. In those situations where there is little or no information about its toxicity, or in which the test material is expected to be toxic, the main test should be performed.

Using the normal procedure, a sighting study starting dose of 2 000 mg/kg (or exceptionally 5 000 mg/kg) followed by dosing of a further four animals at this level serves as a limit test for this guideline.

1.6. OBSERVATIONS

Animals are observed individually after dosing at least once during the first 30 minutes, periodically during the first 24 hours, with special attention given during the first four hours, and daily thereafter, for a total of 14 days, except where they need to be removed from the study and humanely killed for animal welfare reasons or are found dead. However, the duration of observation should not be fixed rigidly. It should be determined by the toxic reactions, time of onset and length of recovery period, and may thus be extended when considered necessary. The times at which signs of toxicity appear and disappear are important, especially if there is a tendency for toxic signs to be delayed (11). All observations are systematically recorded, with individual records being maintained for each animal.

Additional observations will be necessary if the animals continue to display signs of toxicity. Observations should include changes in skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous systems, and somatomotor activity and behaviour pattern. Attention should be directed to observations of tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma. The principles and criteria summarised in the Humane Endpoints Guidance Document should be taken into consideration (8). Animals found in a moribund condition and animals showing severe pain or enduring signs of severe distress should be humanely killed. When animals are killed for humane reasons or found dead, the time of death should be recorded as precisely as possible.

1.6.1. **Body weight**

Individual weights of animals should be determined shortly before the test substance is administered and at least weekly thereafter. Weight changes should be calculated and recorded. At the end of the test surviving animals are weighed and then humanely killed.

1.6.2. **Pathology**

All test animals (including those that die during the test or are removed from the study for animal welfare reasons) should be subjected to gross necropsy. All gross pathological changes should be recorded for each animal. Microscopic examination of organs showing evidence of gross pathology in animals surviving 24 or more hours after the initial dosing may also be considered because it may yield useful information.

2. **DATA**

Individual animal data should be provided. Additionally, all data should be summarised in tabular form, showing for each test group the number of animals used, the number of animals displaying signs of toxicity, the number of animals found dead during the test or killed for humane reasons, time of death of individual animals, a description and the time course of toxic effects and reversibility, and necropsy findings.

3. **REPORTING**

3.1. TEST REPORT

The test report must include the following information, as appropriate:

Test substance:

— physical nature, purity, and, where relevant, physico-chemical properties (including isomerisation),

— identification data, including CAS number.

Vehicle (if appropriate):

— justification for choice of vehicle, if other than water.

Test animals:

— species/strain used,

— microbiological status of the animals, when known,

— number, age and sex of animals (including, where appropriate, a rationale for use of males instead of females),

— source, housing conditions, diet, etc.

Test conditions:

— details of test substance formulation, including details of the physical form of the material administered,

— details of the administration of the test substance including dosing volumes and time of dosing,

— details of food and water quality (including diet type/source, water source),

— the rationale for the selection of the starting dose.

Results:

— tabulation of response data and dose level for each animal (i.e. animals showing signs of toxicity including mortality, nature, severity and duration of effects),

— tabulation of body weight and body weight changes,

— individual weights of animals at the day of dosing, in weekly intervals thereafter, and at time of death or sacrifice,

— date and time of death if prior to scheduled sacrifice,

— time course of onset of signs of toxicity and whether these were reversible for each animal,

— necropsy findings and histopathological findings for each animal, if available.

Discussion and interpretation of results.

Conclusions.

4. **REFERENCES**

(1) British Toxicology Society Working Party on Toxicity (1984) Special report: a new approach to the classification of substances and preparations on the basis of their acute toxicity. Human Toxicol., 3, p. 85-92.

(2) Van den Heuvel, M.J., Dayan, A.D. and Shillaker, R.O (1987) Evaluation of the BTS approach to the testing of substances and preparations for their acute toxicity. Human Toxicol., 6, p. 279-291.

(3) Van den Heuvel, M.J., Clark, D.G., Fielder, R.J., Koundakjian, P.P., Oliver, G.J.A., Pelling, D., Tomlinson, N.J. and Walker, A.P (1990) The international validation of a fixed-dose procedure as an alternative to the classical LD_{50} test. Fd. Chem. Toxicol. 28, p. 469-482.

(4) Whitehead, A. and Curnow, R.N (1992) Statistical evaluation of the fixed-dose procedure. Fd. Chem. Toxicol., 30, p. 313-324.

(5) Stallard, N. and Whitehead, A (1995) Reducing numbers in the fixed-dose procedure. Human Exptl. Toxicol. 14, p. 315-323.

(6) Stallard, N., Whitehead, A and Ridgeway, P. (2002) Statistical evaluation of the revised fixed dose procedure. Hum. Exp. Toxicol., 21, p. 183-196.

(7) OECD (2001) Guidance Document on Acute Oral Toxicity Testing. Environmental Health and Safety Monograph Series on Testing and Assessment No 24. Paris

(8) OECD (2000) Guidance Document on the Recognition, Assessment and Use of Clinical Signs as Humane Endpoints for Experimental Animals Used in Safety Evaluation. Environmental Health and Safety Monograph Series on Testing and Assesment No 19.

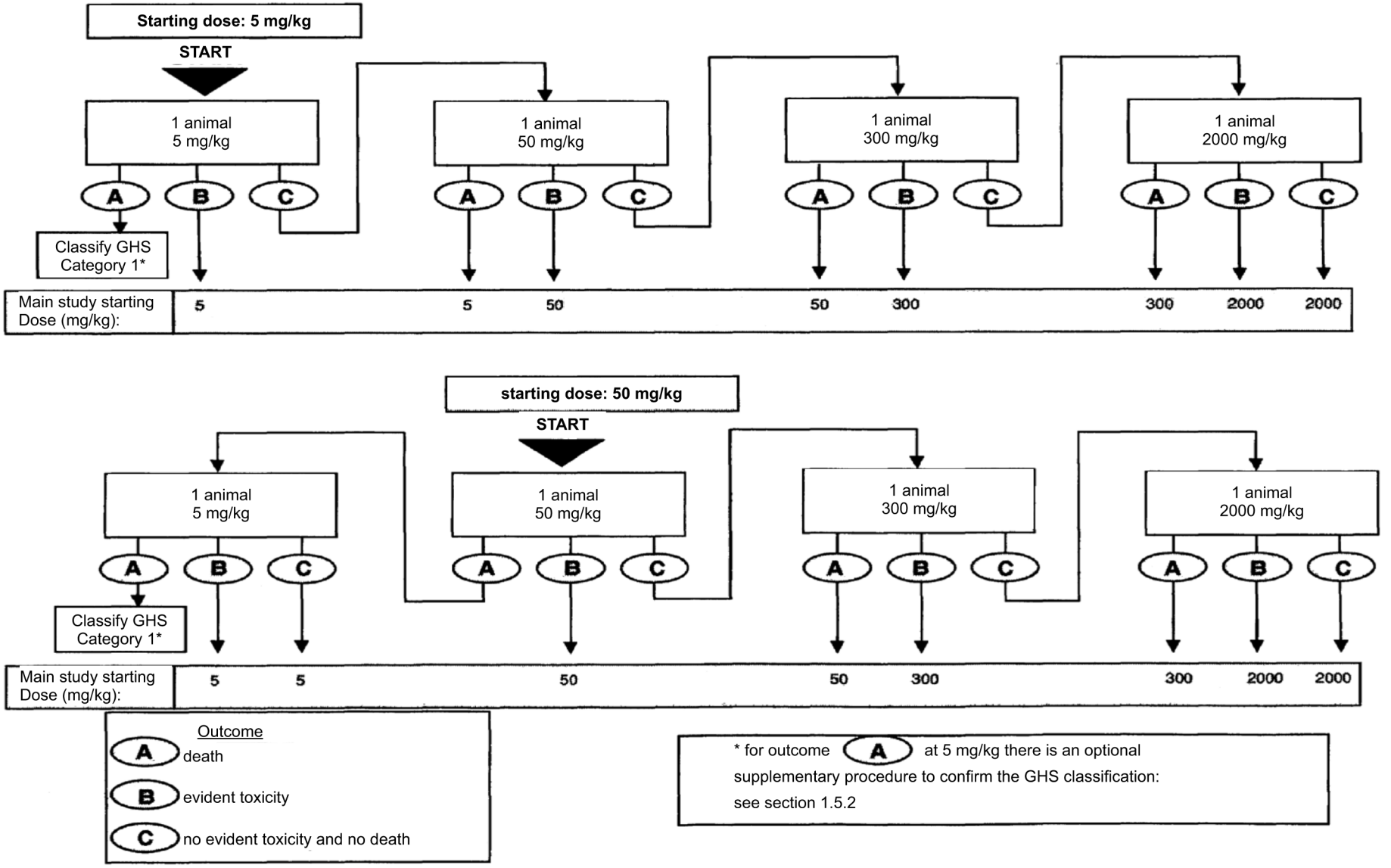
(9) OECD (1998) Harmonised Integrated Hazard Classification for Human Health and Environmental Effects of Chemical Substances as endorsed by the 28th Joint Meeting of the Chemicals Committee and the Working Party on Chemicals in November 1998, Part 2, p. 11 [http://webnet1.oecd.org/oecd/pages/home/displaygeneral/0,3380, EN-documents-521-14-no-24-no-0,FF.html].

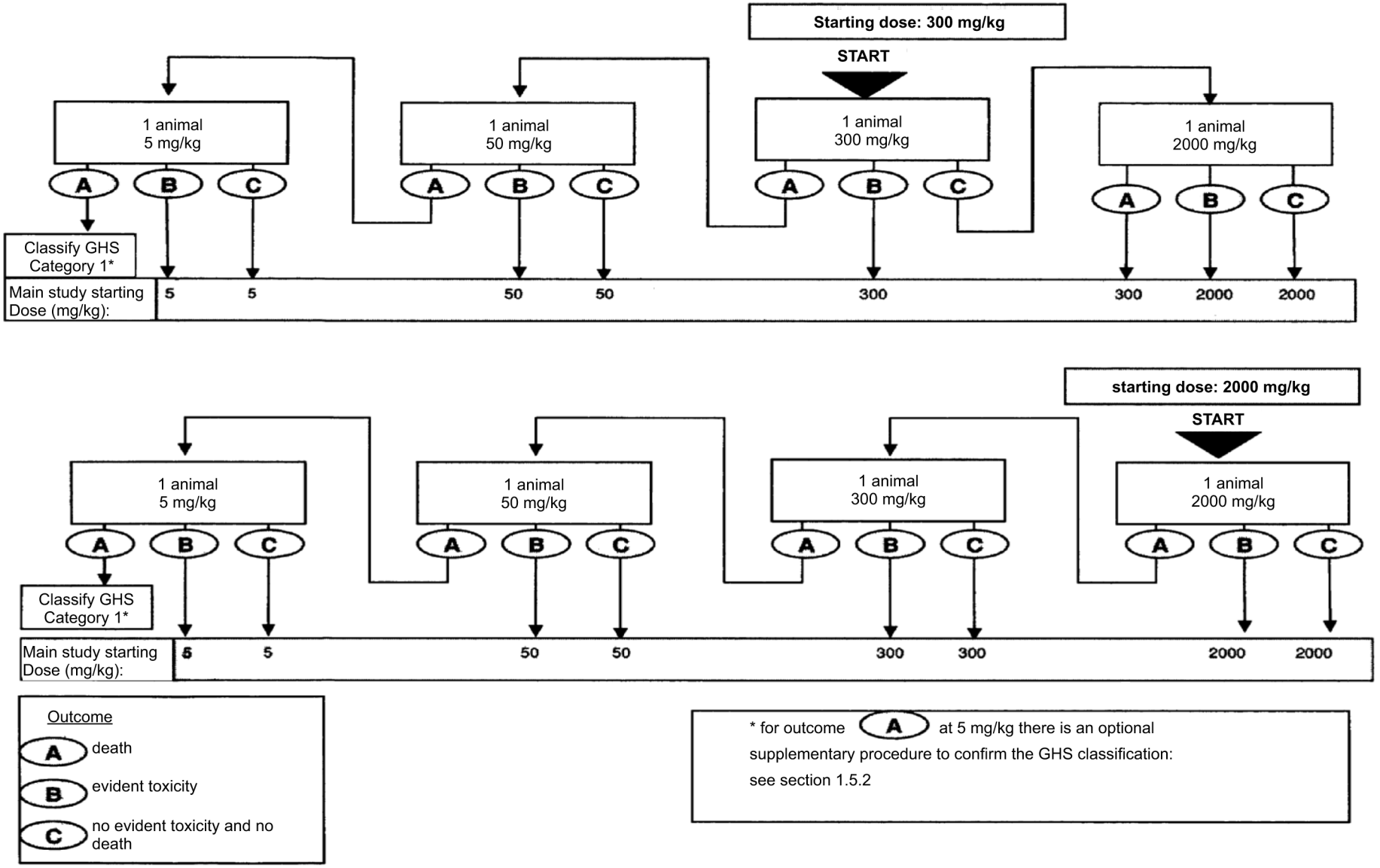
(10) Lipnick, R.L., Cotruvo, J.A., Hill, R.N., Bruce, R.D., Stitzel, K.A., Walker, A.P., Chu, I., Goddard, M., Segal, L., Springer, J.A. and Myers, R.C (1995) Comparison of the Up-and-Down, Conventional LD_{50}, and Fixed-Dose Acute Toxicity Procedures. Fd. Chem. Toxicol. 33, p. 223-231.

(11) Chan P.K and A. W Hayes (1994) Chapter 16 Acute Toxicity and Eye Irritation. In: Principles and Methods of Toxicology. 3rd Edition. A.W. Hayes, Editor. Raven Press Ltd. New York, USA.

Appendix 1

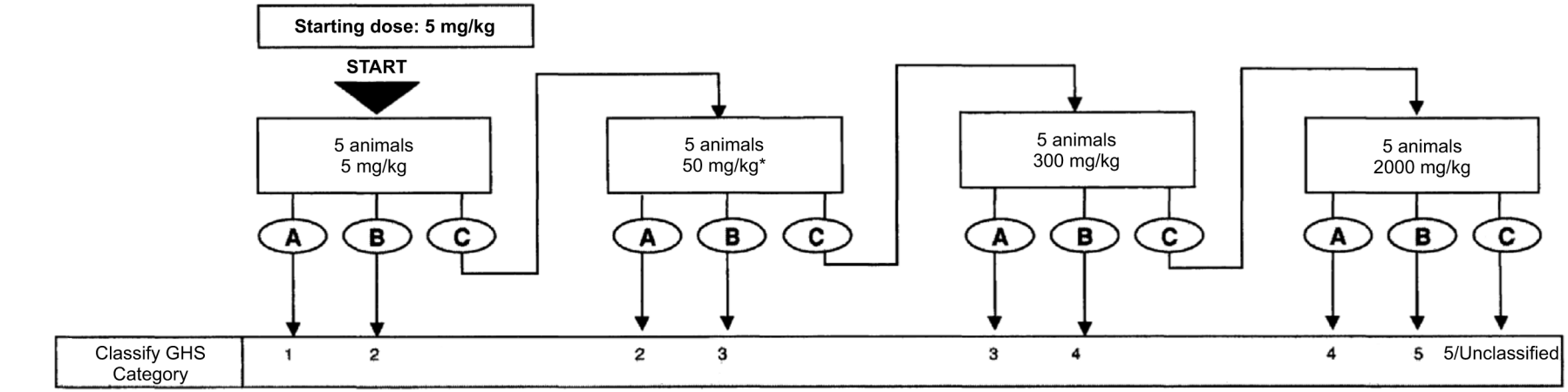
FLOW CHART FOR THE SIGHTING STUDY

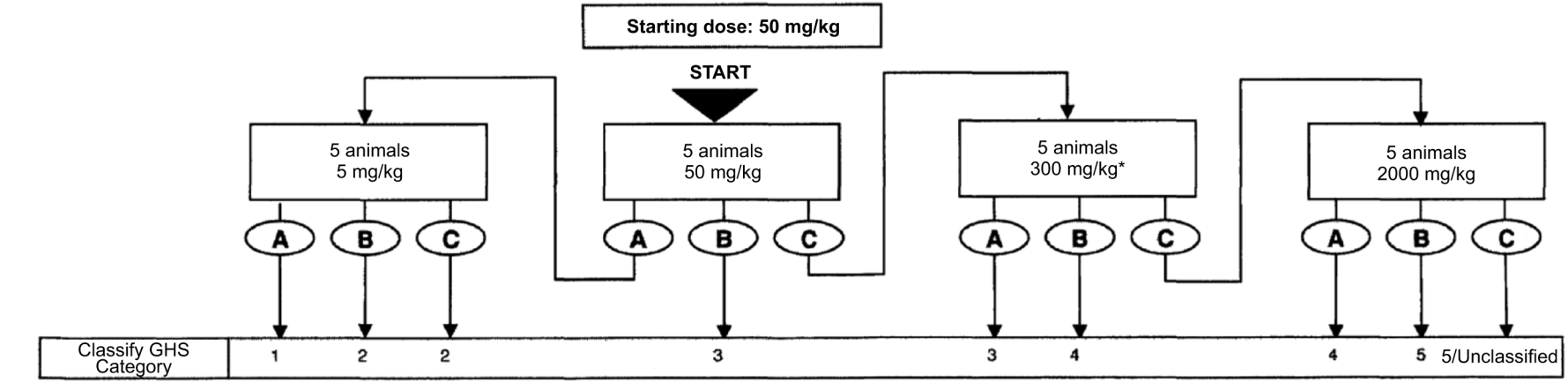



Starting dose: 300 mg/kg
START
1 animal
5 mg/kg
1 animal
50 mg/kg
1 animal
300 mg/kg
1 animal
2000 mg/kg
A
B
C
Classify GHS
Category 1*
Main study starting
Dose (mg/kg):
5
5
50
50
300
300
2000
2000
starting dose: 2000 mg/kg
START
1 animal
5 mg/kg
1 animal
50 mg/kg
1 animal
300 mg/kg
1 animal
2000 mg/kg
Classify GHS
Category 1*
Main study starting
Dose (mg/kg):
5
5
50
50
300
300
2000
2000
Outcome
A death
B evident toxicity
C no evident toxicity and no death
* for outcome A at 5 mg/kg there is an optional supplementary procedure to confirm the GHS classification:
see section 1.5.2

Appendix 2

FLOW CHART FOR THE MAIN STUDY





Outcome

A ≥ 2 deaths

B ≥ 1 with evident toxicity and / or 1 death

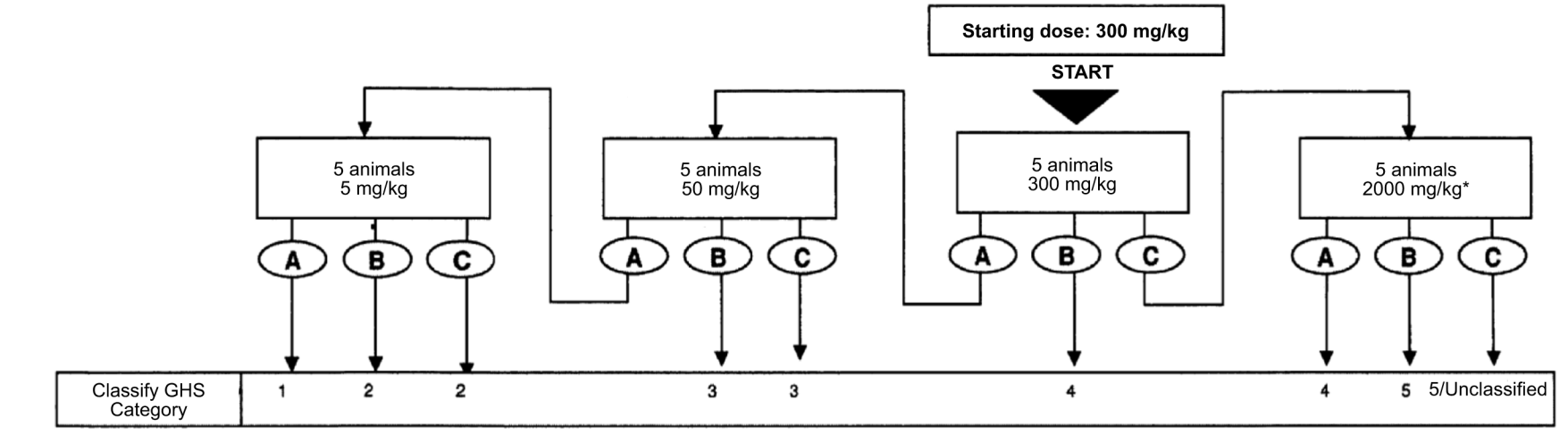
C No evident toxicity and no death

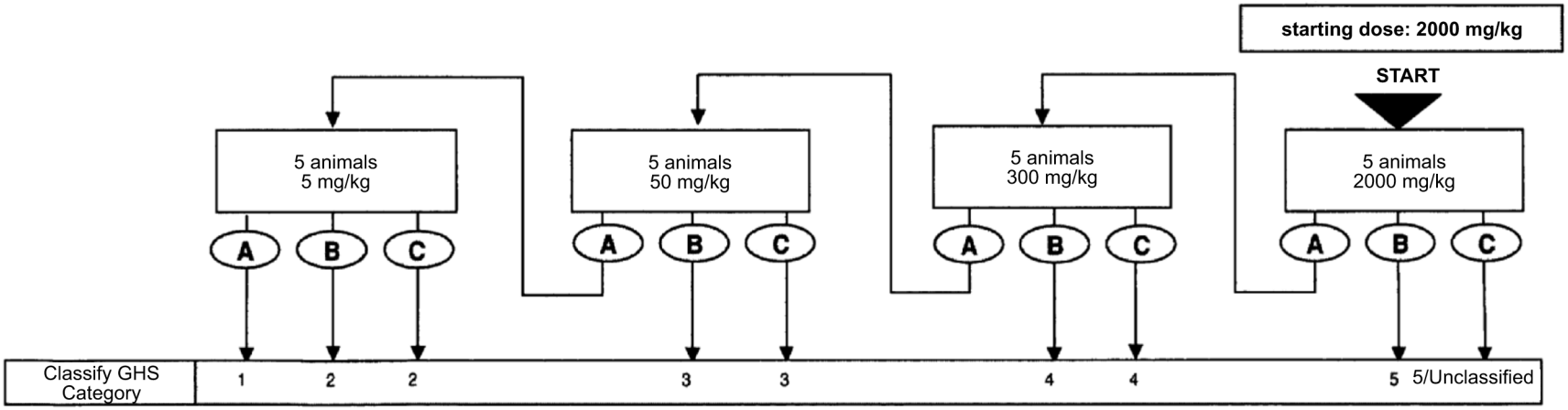
Group size

The 5 animals in each main study group will include any animal tested at that dose level in the sighting study

* Animal welfare override

If this dose level caused death in the sighting study, then no further animals will be tested. Go directly to outcome A





Outcome

A ≥ 2 deaths

B ≥ 1 with evident toxicity and / or 1 death

C No evident toxicity and no death

Group size

The 5 animals in each main study group will include any animal tested at that dose level in the sighting study

* Animal welfare override

If this dose level caused death in the sighting study, then no further animals will be tested. Go directly to outcome A

Appendix 3

CRITERIA FOR CLASSIFICATION OF TEST SUBSTANCES WITH EXPECTED LD_{50} VALUES EXCEEDING 2 000 MG/KG WITHOUT THE NEED FOR TESTING.

Criteria for hazard Category 5 are intended to enable the identification of test substances which are of relatively low acute toxicity hazard but which, under certain circumstances may present a danger to vulnerable populations. These substances are anticipated to have an oral or dermal LD_{50} in the range of 2 000-5 000 mg/kg or equivalent doses for other routes. Test substances could be classified in the hazard category defined by: 2 000 mg/kg < LD_{50} < 5 000 mg/kg (Category 5 in the GHS) in the following cases:

(a) if directed to this category by any of the testing schemes of Appendix 2, based on mortality incidences

(b) if reliable evidence is already available that indicates the LD_{50} to be in the range of Category 5 values; or other animal studies or toxic effects in humans indicate a concern for human health of an acute nature;

(c) through extrapolation, estimation or measurement of data if assignment to a more hazardous class is not warranted; and

— reliable information is available indicating significant toxic effects in humans, or

— any mortality is observed when tested up to Category 4 values by the oral route, or

— where expert judgement confirms significant clinical signs of toxicity, when tested up to Category 4 values, except for diarrhoea, piloerection or an ungroomed appearance, or

— where expert judgement confirms reliable information indicating the potential for significant acute effects from the other animal studies.

TESTING AT DOSES ABOVE 2 000 MG/KG

Exceptionally, and only when justified by specific regulatory needs, the use of an additional upper fixed dose level of 5 000 mg/kg may be considered. Recognising the need to protect animal welfare, testing at 5 000 mg/kg is discouraged and should only be considered when there is a strong likelihood that the results of such a test would have a direct relevance for protecting animal or human health (9).

Sighting study

The decision rules governing the sequential procedure presented in Appendix 1 are extended to include a 5 000 mg/kg dose level. Thus, when a sighting study starting dose of 5 000 mg/kg is used outcome A (death) will require a second animal to be tested at 2 000 mg/kg; outcomes B and C (evident toxicity or no toxicity) will allow the selection of 5 000 mg/kg as the main study starting dose. Similarly, if a starting dose other than 5 000 mg/kg is used then testing will progress to 5 000 mg/kg in the event of outcomes B or C at 2 000 mg/kg; a subsequent 5 000 mg/kg outcome A will dictate a main study starting dose of 2 000 mg/kg and outcomes B and C will dictate a main study starting dose of 5 000 mg/kg.

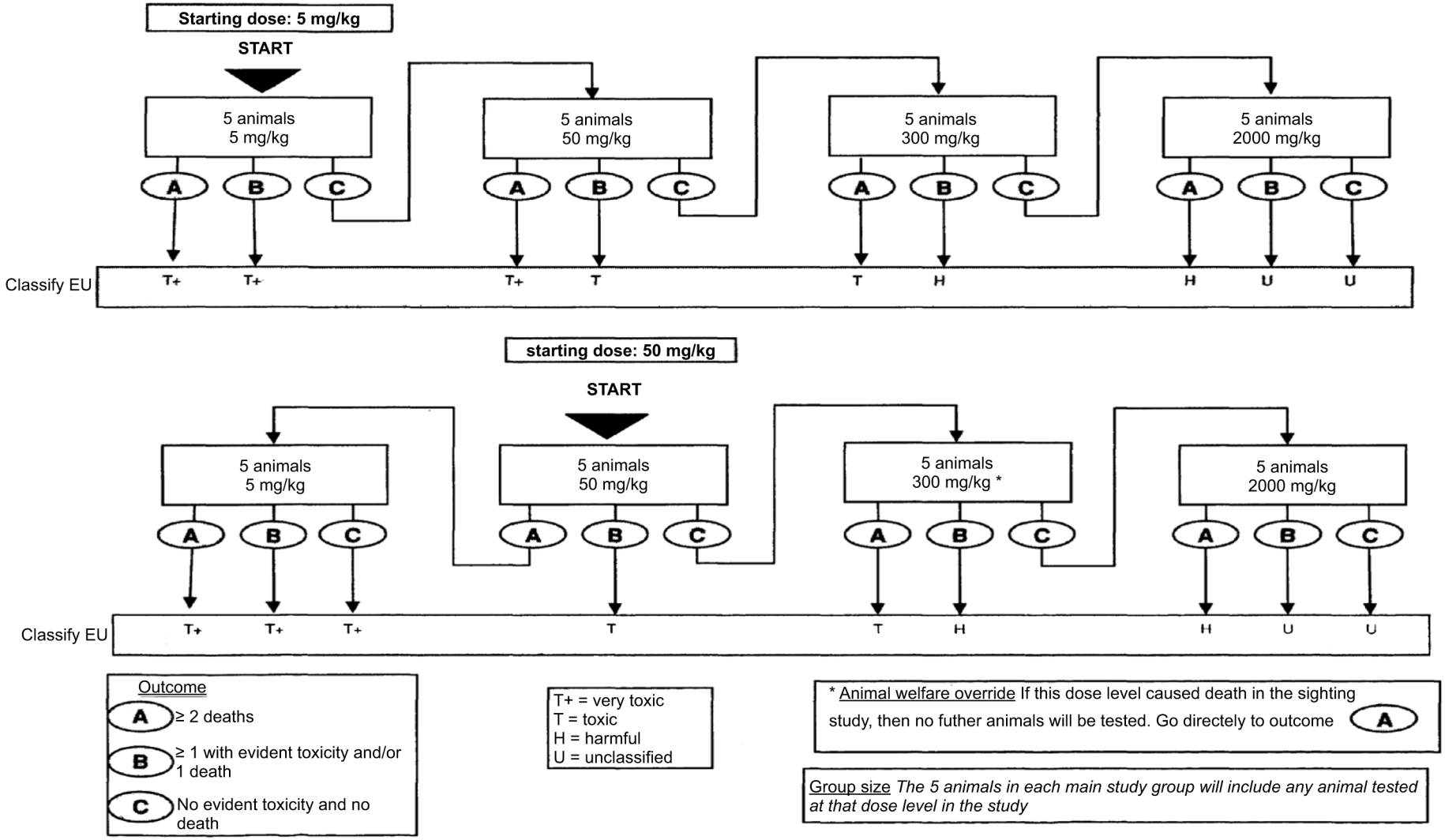
Main study

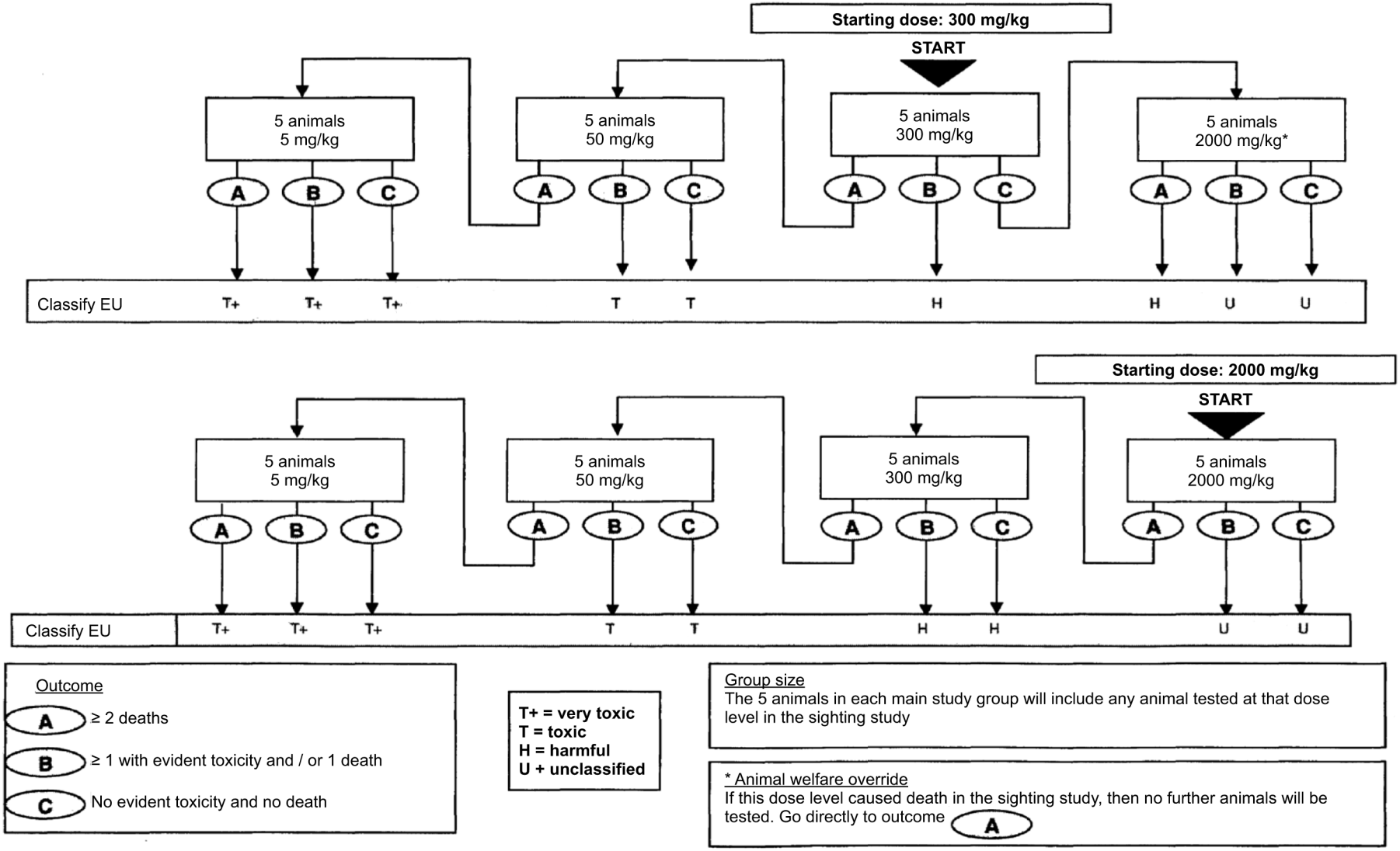
The decision rules governing the sequential procedure presented in Appendix 2 are extended to include a 5 000 mg/kg dose level. Thus, when a main study starting dose of 5 000 mg/kg is used, outcome A (≥ 2 deaths) will require the testing of a second group at 2 000 mg/kg; outcome B (evident toxicity and/or ≤ 1 death) or C (no toxicity) will result in the substance being unclassified according to GHS. Similarly, if a starting dose other than 5 000 mg/kg is used then testing will progress to 5 000 mg/kg in the event of outcome C at 2 000 mg/kg; a subsequent 5 000 mg/kg outcome A will result in the substance being assigned to GHS Category 5 and outcomes B or C will lead to the substance being unclassified.

Appendix 4

TEST METHOD B.1 bis

Guidance on classification according to the EU scheme to cover the transition period until full implementation of the Globally Harmonised Classification System (GHS) (taken from reference (8))




Starting dose: 300 mg/kg
START
5 animals 5 mg/kg
5 animals 50 mg/kg
5 animals 300 mg/kg
5 animals 2000 mg/kg*
A B C
Classify EU
T+ T+ T+ T T H H U U
Starting dose: 2000 mg/kg
START
5 animals 5 mg/kg
5 animals 50 mg/kg
5 animals 300 mg/kg
5 animals 2000 mg/kg
A B C
Classify EU
T+ T+ T+ T T H H U U
Outcome
A ≥ 2 deaths
B ≥ 1 with evident toxicity and / or 1 death
C No evident toxicity and no death
T+ = very toxic
T = toxic
H = harmful
U + unclassified
Group size
The 5 animals in each main study group will include any animal tested at that dose level in the sighting study
* Animal welfare override
If this dose level caused death in the sighting study, then no further animals will be tested. Go directly to outcome A

B.1 tris. **ACUTE ORAL TOXICITY — ACUTE TOXIC CLASS METHOD**

1. **METHOD**

This test method is equivalent to OECD TG 423 (2001)

1.1. INTRODUCTION

The acute toxic class method (1) set out in this test is a stepwise procedure with the use of three animals of a single sex per step. Depending on the mortality and/or the moribund status of the animals, on average two to four steps may be necessary to allow judgement on the acute toxicity of the test substance. This procedure is reproducible, uses very few animals and is able to rank substances in a similar manner to the other acute toxicity testing methods. The acute toxic class method is based on biometric evaluations (2)(3)(4)(5) with fixed doses, adequately separated to enable a substance to be ranked for classification purposes and hazard assessment. The method as adopted in 1996 was extensively validated *in vivo* against LD_{50} data obtained from the literature, both nationally (6) and internationally (7).

Guidance on the selection of the most appropriate test method for a given purpose can be found in the Guidance Document on Acute Oral Toxicity Testing (8). This Guidance Document also contains additional information on the conduct and interpretation of testing method B.1tris.

Test substances, at doses that are known to cause marked pain and distress due to corrosive or severely irritant actions, need not be administered. Moribund animals, or animals obviously in pain or showing signs of severe and enduring distress shall be humanely killed, and are considered in the interpretation of the test results in the same way as animals that died on test. Criteria for making the decision to kill moribund or severely suffering animals, and guidance on the recognition of predictable or impending death, are the subject of a separate Guidance Document (9).

The method uses pre-defined doses and the results allow a substance to be ranked and classified according to the Globally Harmonised System for the classification of chemicals which cause acute toxicity (10).

In principle, the method is not intended to allow the calculation of a precise LD_{50}, but does allow for the determination of defined exposure ranges where lethality is expected since death of a proportion of the animals is still the major endpoint of this test. The method allows for the determination of an LD_{50} value only when at least two doses result in mortality higher than 0 % and lower than 100 %. The use of a selection of pre-defined doses, regardless of test substance, with classification explicitly tied to number of animals observed in different states improves the opportunity for laboratory to laboratory reporting consistency and repeatability.

The testing laboratory should consider all available information on the test substance prior to conducting the study. Such information will include the identity and chemical structure of the substance; its physico-chemical properties; the result of any other *in vivo* or *in vitro* toxicity tests on the substance; toxicological data on the structurally related substances; and the anticipated use(s) of the substance. This information is necessary to satisfy all concerned that the test is relevant for the protection of human health and will help in the selection of the most appropriate starting dose.

1.2. DEFINITIONS

Acute oral toxicity: refers to those adverse effects occurring following oral administration of a single dose of a substance or multiple doses given within 24 hours.

Delayed death: means that an animal does not die or appear moribund within 48 hours but dies later during the 14-day observation period.

Dose: is the amount of test substance administered. Dose is expressed as weight of test substance per unit weight of test animal (e.g. mg/kg).

GHS: Globally Harmonised Classification System for Chemical Substances and Mixtures. A joint activity of OECD (human health and the environment), UN Committee of Experts on Transport of Dangerous Goods (physical-chemical properties) and ILO (hazard communication) and coordinated by the Interorganisation Programme for the Sound Management of Chemicals (IOMC).

Impending death: when moribund state or death is expected prior to the next planned time of observation. Signs indicative of this state in rodents could include convulsions, lateral position, recumbence and tremor (See the Humane Endpoint Guidance Document (9) for more details).

LD_{50} (median lethal oral dose): is a statistically derived single dose of a substance that can be expected to cause death in 50 % of animals when administered by the oral route. The LD_{50} value is expressed in terms of weight of test substance per unit weight of test animal (mg/kg).

Limit dose: refers to a dose at an upper limitation on testing (2 000 or 5 000 mg/kg).

Moribund status: being in a state of dying or inability to survive, even if treated (See the Humane Endpoint Guidance Document (9) for more details).

Predictable death: presence of clinical signs indicative of death at a known time in the future before the planned end of the experiment; for example: inability to reach water or food. (See the Humane Endpoint Guidance Document (9) for more details).

1.3. PRINCIPLE OF THE TEST

It is the principle of the test that, based on a stepwise procedure with the use of a minimum number of animals per step, sufficient information is obtained on the acute toxicity of the test substance to enable its classification. The substance is administered orally to a group of experimental animals at one of the defined doses. The substance is tested using a stepwise procedure, each step using three animals of a single sex (normally females). Absence or presence of compound-related mortality of the animals dosed at one step will determine the next step, i.e.;

— no further testing is needed,

— dosing of three additional animals, with the same dose,

— dosing of three additional animals at the next higher or the next lower dose level.

Details of the test procedure are described in Appendix 1. The method will enable a judgement with respect to classifying the test substance to one of a series of toxicity classes defined by fixed LD_{50} cut-off values.

1.4. DESCRIPTION OF THE METHOD

1.4.1. **Selection of animal species**

The preferred rodent species is the rat, although other rodent species may be used. Normally females are used (9). This is because literature surveys of conventional LD_{50} tests show that, although there is little difference in sensitivity between the sexes, in those cases where differences are observed females are generally slightly more sensitive (11). However if knowledge of the toxicological or toxicokinetic properties of structurally related chemicals indicates that males are likely to be more sensitive, then this sex should be used. When the test is conducted in males, adequate justification should be provided.

Healthy young adult animals of commonly used laboratory strains should be employed. Females should be nulliparous and non-pregnant. Each animal, at the commencement of its dosing, should be between eight and 12 weeks old and its weight should fall in an interval within ± 20 % of the mean weight of any previously dosed animals.

1.4.2. **Housing and feeding conditions**

The temperature in the experimental animal room should be 22 °C (± 3 °C). Although the relative humidity should be at least 30 % and preferably not exceed 70 % other than during room cleaning the aim should be 50-60 %. Lighting should be artificial, the sequence being 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. Animals may be group-caged by dose, but the number of animals per cage must not interfere with clear observations of each animal.

1.4.3. **Preparation of animals**

The animals are randomly selected, marked to permit individual identification, and kept in their cages for at least five days prior to dosing to allow for acclimatisation to the laboratory conditions.

1.4.4. **Preparation of doses**

In general, test substances should be administered in a constant volume over the range of doses to be tested by varying the concentration of the dosing preparation. Where a liquid end product or mixture is to be tested however, the use of the undiluted test substance, i.e. at a constant concentration, may be more relevant to the subsequent risk assessment of that substance, and is a requirement of some regulatory authorities. In either case, the maximum dose volume for administration must not be exceeded. The maximum volume of liquid that can be administered at one time depends on the size of the test animal. In rodents, the volume should not normally exceed 1 ml/100 g of body weight: however in the case of aqueous solutions 2 ml/100 g body weight can be considered. With respect to the formulation of the dosing preparation, the use of an aqueous solution/suspension/emulsion is recommended wherever possible, followed in order of preference by a solution/suspension/emulsion in oil (e.g. corn oil) and then possibly solution in other vehicles. For vehicles other than water the toxicological characteristics of the vehicle should be known. Doses must be prepared shortly prior to administration unless the stability of the preparation over the period during which it will be used is known and shown to be acceptable.

1.5. PROCEDURE

1.5.1. **Administration of doses**

The test substance is administered in a single dose by gavage using a stomach tube or a suitable intubation canula. In the unusual circumstance that a single dose is not possible, the dose may be given in smaller fractions over a period not exceeding 24 hours.

Animals should be fasted prior to dosing (e.g. with the rat, food but not water should be withheld overnight, with the mouse, food but not water should be withheld for three or four hours). Following the period of fasting, the animals should be weighed and the test substance administered. After the substance has been administered, food may be withheld for a further three or fours hours in rats or one or two hours in mice. Where a dose is administered in fractions over a period it may be necessary to provide the animals with food and water depending on the length of the period.

1.5.2. **Number of animals and dose levels**

Three animals are used for each step. The dose level to be used as the starting dose is selected from one of four fixed levels, 5, 50, 300 and 2 000 mg/kg body weight. The starting dose level should be that which is most likely to produce mortality in some of the dosed animals. The flowcharts of Appendix 1 describe the procedure that should be followed for each of the starting doses. In addition, Appendix 4 gives guidance on the classification in the EU system until the new GHS is implemented.

When available information suggests that mortality is unlikely at the highest starting dose level (2 000 mg/kg body weight), then a limit test should be conducted. When there is no information on a substance to be tested, for animal welfare reasons it is recommended to use the starting dose of 300 mg/kg body weight.

The time interval between treatment groups is determined by the onset, duration, and severity of toxic signs. Treatment of animals at the next dose should be delayed until one is confident of survival of the previously dosed animals.

Exceptionally, and only when justified by specific regulatory needs, the use of additional upper dose level of 5 000 mg/kg body weight may be considered (see Appendix 2). For reasons of animal welfare concern, testing of animals in GHS Category 5 ranges (2 000-5 000 mg/kg) is discouraged and should only be considered when there is a strong likelihood that the results of such a test would have a direct relevance for protecting human or animal health or the environment.

1.5.3. **Limit test**

The limit test is primarily used in situations where the experimenter has information indicating that the test material is likely to be non-toxic, i.e., having toxicity only above regulatory limit doses. Information about the toxicity of the test material can be gained from knowledge about similar tested compounds or similar tested mixtures or products, taking into consideration the identity and percentage of components known to be of toxicological significance. In those situations where there is little or no information about its toxicity, or in which the test material is expected to be toxic, the main test should be performed.

A limit test at one dose level of 2 000 mg/kg body weight may be carried out with six animals (three animals per step). Exceptionally a limit test at one dose level of 5 000 mg/kg may be carried out with three animals (see Appendix 2). If test substance-related mortality is produced, further testing at the next lower level may need to be carried out.

1.6. OBSERVATIONS

Animals are observed individually after dosing at least once during the first 30 minutes, periodically during the first 24 hours, with special attention given during the first four hours, and daily thereafter, for a total of 14 days, except where they need to be removed from the study and humanely killed for animal welfare reasons or are found dead. However, the duration of observation should not be fixed rigidly. It should be determined by the toxic reactions, time of onset and length of recovery period, and may thus be extended when considered necessary. The times at which signs of toxicity appear and disappear are important, especially if there is a tendency for toxic signs to be delayed (12). All observations are systematically recorded with individual records being maintained for each animal.

Additional observations will be necessary if the animals continue to display signs of toxicity. Observations should include changes in skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous systems, and somatomotor activity and behaviour pattern. Attention should be directed to observations of tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma. The principles and criteria summarised in the Humane Endpoints Guidance Document (9) should be taken into consideration. Animals found in a moribund condition and animals showing severe pain or enduring signs of severe distress should be humanely killed. When animals are killed for humane reasons or found dead, the time of death should be recorded as precisely as possible.

1.6.1. **Body weight**

Individual weights of animals should be determined shortly before the test substance is administered, and at least weekly thereafter. Weight changes should be calculated and recorded. At the end of the test surviving animals are weighed and humanely killed.

1.6.2. **Pathology**

All test animals (including those that die during the test or are removed from the study for animal welfare reasons) should be subjected to gross necropsy. All gross pathological changes should be recorded for each animal. Microscopic examination of organs showing evidence of gross pathology in animals surviving 24 or more hours may also be considered because it may yield useful information.

2. **DATA**

Individual animal data should be provided. Additionally, all data should be summarised in tabular form, showing for each test group the number of animals used, the number of animals displaying signs of toxicity, the number of animals found dead during the test or killed for humane reasons, time of death of individual animals, a description and the time course of toxic effects and reversibility, and necropsy findings.

3. **REPORTING**

3.1. **Test report**

The test report must include the following information, as appropriate:

Test substance:

— physical nature, purity, and, where relevant, physico-chemical properties (including isomerisation),

— identification data, including CAS number.

Vehicle (if appropriate):

— justification for choice of vehicle, if other than water.

Test animals:

— species/strain used,

— microbiological status of the animals, when known,

— number, age, and sex of animals (including, where appropriate, a rationale for the use of males instead of females),

— source, housing conditions, diet, etc.

Test conditions:

— details of test substance formulation including details of the physical form of the material administered,

— details of the administration of the test substance including dosing volumes and time of dosing,

— details of food and water quality (including diet type/source, water source),

— the rationale for the selection of the starting dose.

Results:

— tabulation of response data and dose level for each animal (i.e. animals showing signs of toxicity including mortality; nature, severity, and duration of effects),

— tabulation of body weight and body weight changes,

— individual weights of animals at the day of dosing, in weekly intervals thereafter, and at the time of death or sacrifice,

— date and time of death if prior to scheduled sacrifice,

— time course of onset of signs of toxicity, and whether these were reversible for each animal,

— necropsy findings and histopathological findings for each animal, if available.

Discussion and interpretation of results.

Conclusions.

4. **REFERENCES**

(1) Roll R., Höfer-Bosse Th. And Kayser D (1986) New Perspectives in Acute Toxicity Testing of Chemicals. Toxicol. Lett., Suppl. 31, p. 86.

(2) Roll R., Riebschläger M., Mischke U. and Kayser D (1989) Neue Wege zur Bestimmung der akuten Toxizität von Chemikalien. Bundesgesundheitsblatt 32, p. 336-341.

(3) Diener W., Sichha L., Mischke U., Kayser D. and Schlede E (1994) The Biometric Evaluation of the Acute-Toxic-Class Method (Oral). Arch. Toxicol. 68, p. 559-610.

(4) Diener W., Mischke U., Kayser D. and Schlede E., (1995) The Biometric Evaluation of the OECD Modified Version of the Acute-Toxic-Class Method (Oral). Arch. Toxicol. 69, p. 729-734.

(5) Diener W., and Schlede E., (1999) Acute Toxicity Class Methods: Alterations to LD/LC_{50} Tests. ALTEX 16, p. 129-134.

(6) Schlede E., Mischke U., Roll R. and Kayser D., (1992). A National Validation Study of the Acute-Toxic-Class Method — An Alternative to the LD_{50} Test. Arch. Toxicol. 66, 455-470.

(7) Schlede E., Mischke U., Diener W. and Kayser D., (1994) The International Validation Study of the Acute-Toxic-Class Method (Oral). Arch. Toxicol. 69, p. 659-670.

(8) OECD, (2001) Guidance Document on Acute Oral Toxicity Testing. Environmental Health and Safety Monograph Series on Testing and Assessment No 24. Paris.

(9) OECD, (2000) Guidance Document on the Recognition, Assessment and Use of Clinical Signs as Humane Endpoints for Experimental Animals Used in Safety Evaluation. Environmental Health and Safety Monograph Series on Testing and Assessment No 19.

(10) OECD, (1998) Harmonised Integrated Hazard Classification System For Human Health And Environmental Effects Of Chemical Substances as endorsed by the 28th Joint Meeting of the Chemicals Committee and the Working Party on Chemicals in November 1998, Part 2, p. 11 [http://webnet1.oecd.org/oecd/pages/home/displaygeneral/0,3380, EN-documents-521-14-no-24-no-0,FF.html].

(11) Lipnick R. L., Cotruvo, J.A., Hill R. N., Bruce R. D., Stitzel K. A., Walker A. P., Chu I.; Goddard M., Segal L., Springer J. A. and Myers R. C. (1995) Comparison of the Up-and Down, Conventional LD_{50}, and Fixed Dose Acute Toxicity Procedures. Fd. Chem. Toxicol 33, p. 223-231.

(12) Chan P.K. and A.W. Hayes. (1994). Chap. 16. Acute Toxicity and Eye Irritancy. *Principles and Methods of Toxicology*. Third Edition. A.W. Hayes, Editor. Raven Press, Ltd., New York, USA.

Appendix 1

PROCEDURE TO BE FOLLOWED FOR EACH OF THE STARTING DOSES

GENERAL REMARKS

For each starting dose, the respective testing schemes as included in this Appendix outline the procedure to be followed.

— Appendix 1 a: starting dose is 5 mg/kg bw,

— Appendix 1 b: starting dose is 50 mg/kg bw,

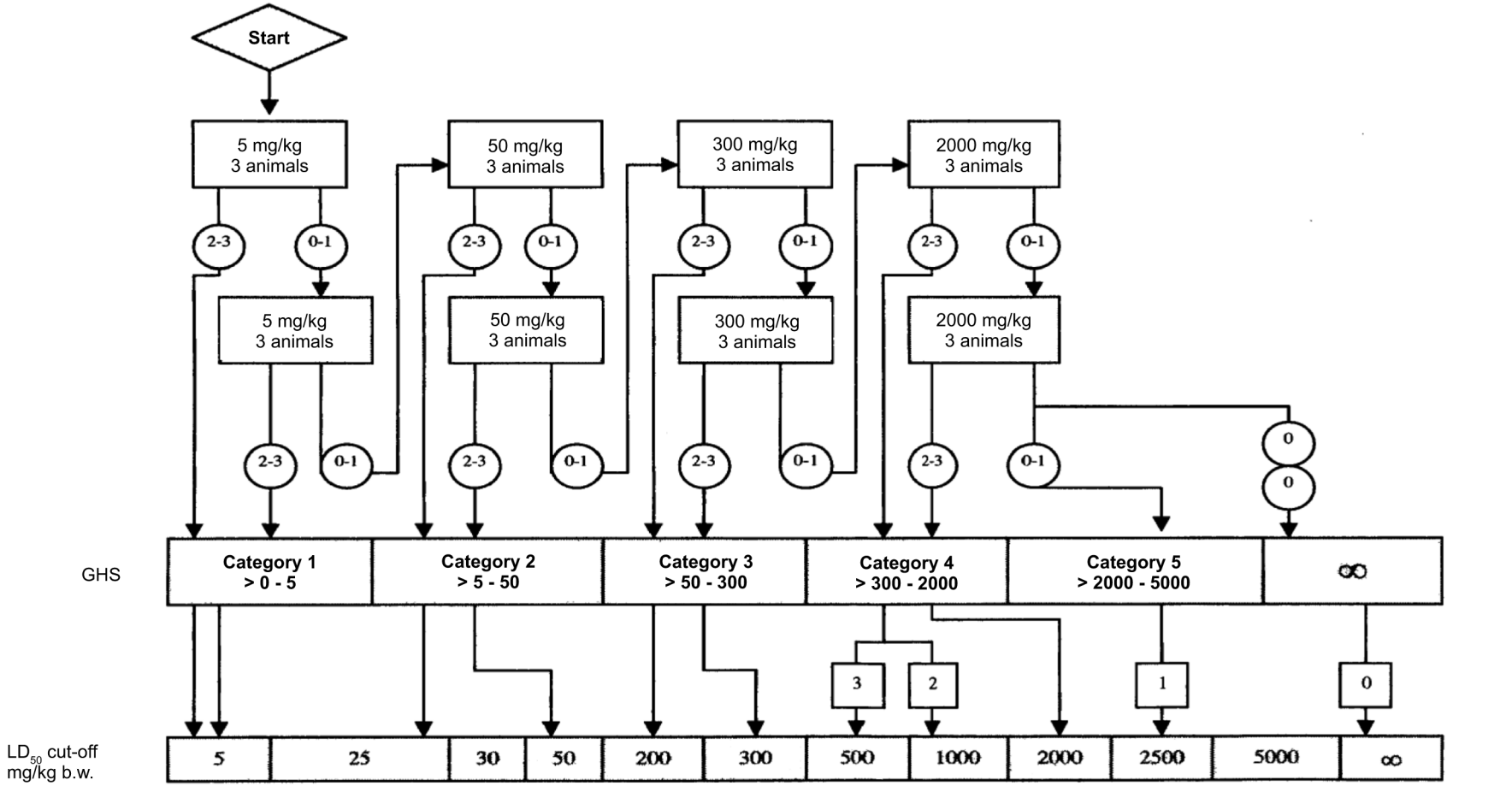
— Appendix 1 c: starting dose is: 300 mg/kg bw,

— Apendix 1 d: starting dose is: 2 000 mg/kg bw.

Depending on the number of humanely killed or dead animals, the test procedure follows the indicated arrows.

Appendix 1A

TEST PROCEDURE WITH A STARTING DOSE OF 5 MG/KG BODY WEIGHT

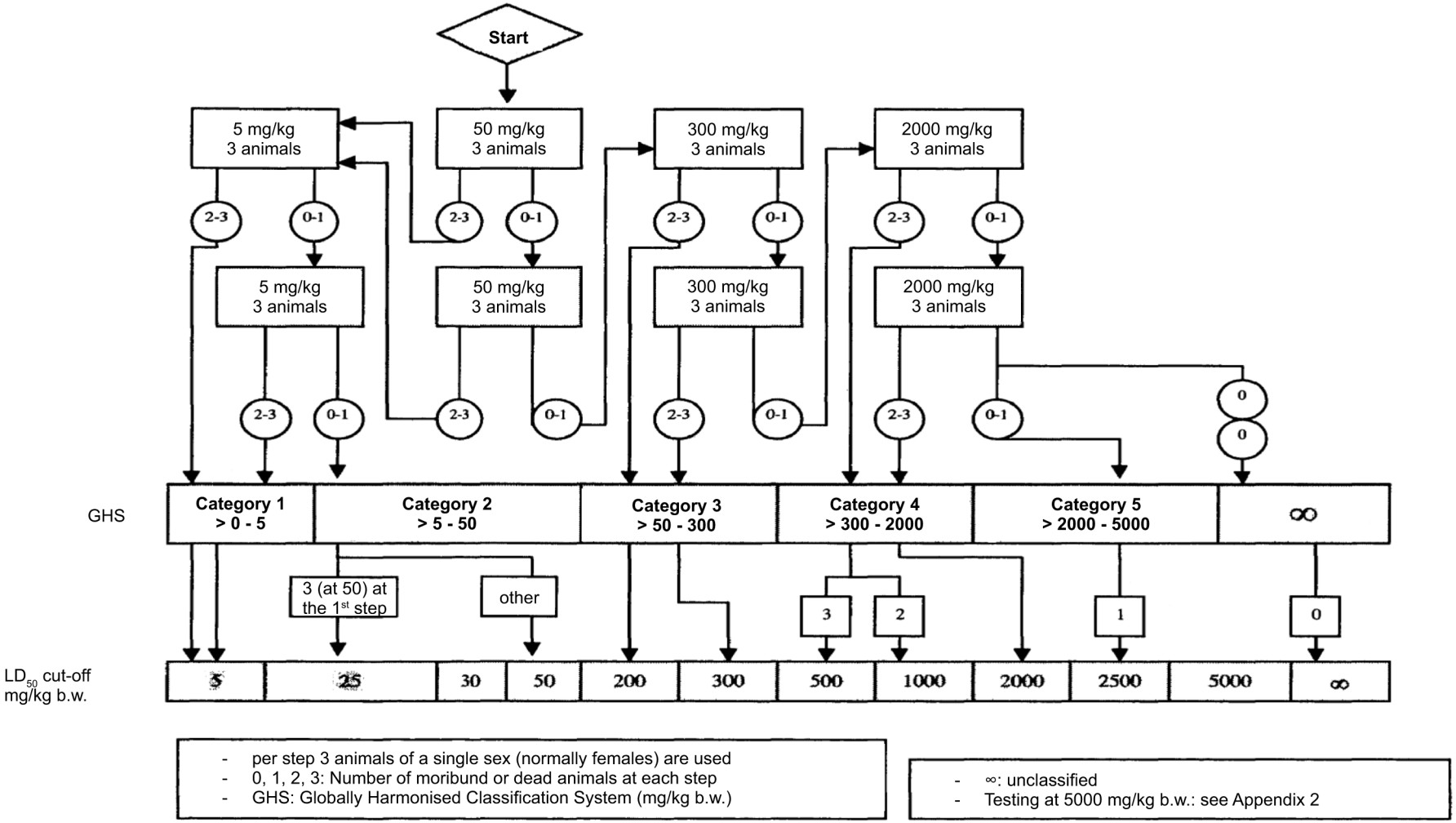


- per step 3 animals of a single sex (normally females) are used
- 0, 1, 2, 3: Number of moribund or dead animals at each step
- GHS: Globally Harmonised Classification System (mg/kg b.w.)

- ∞: unclassified
- Testing at 5000 mg/kg b.w.: see Appendix 2

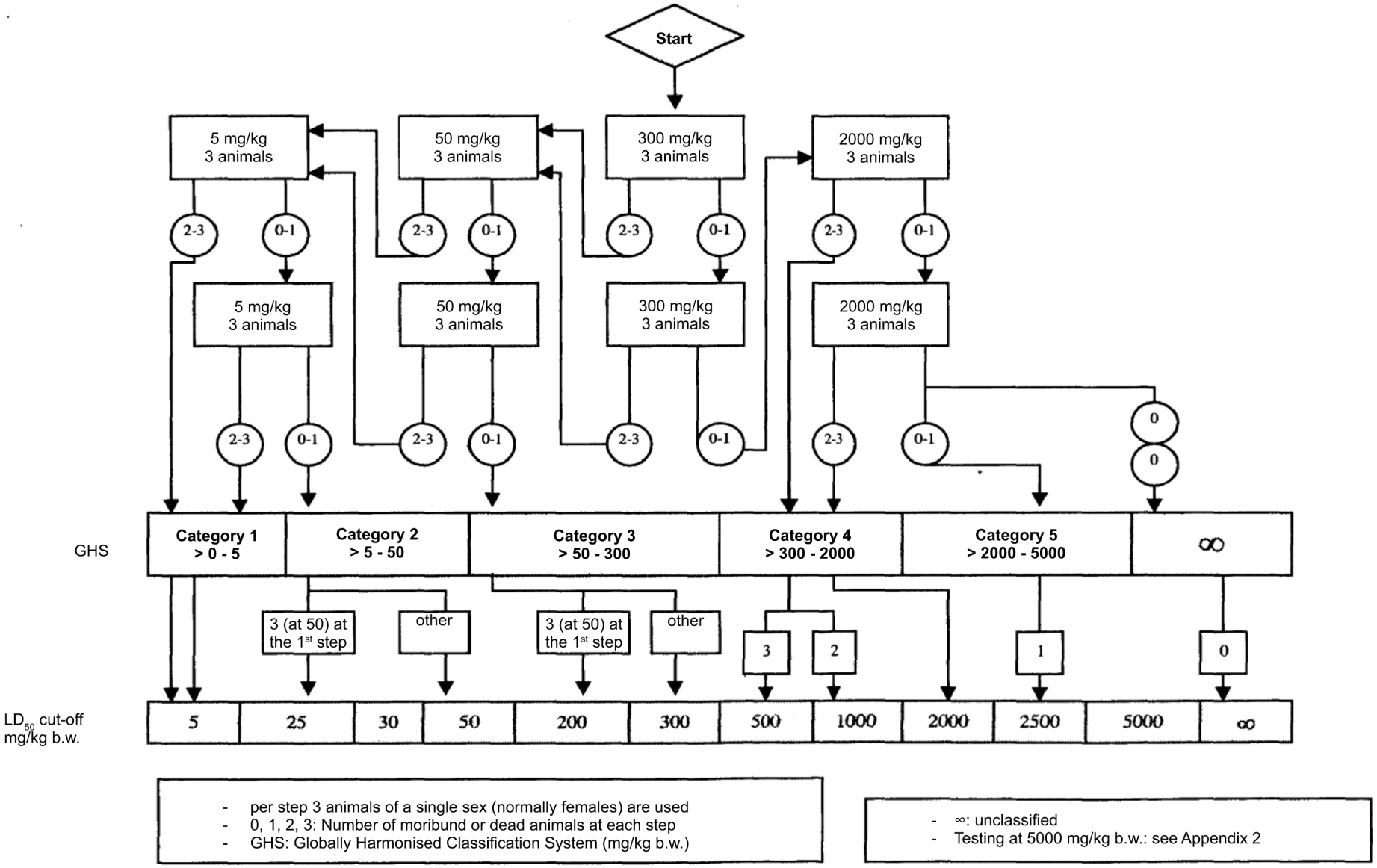
Appendix 1B

TEST PROCEDURE WITH A STARTING DOSE OF 50 MG/KG BODY WEIGHT



Appendix1C

TEST PROCEDURE WITH A STARTING DOSE OF 300 MG/KG BODY WEIGHT

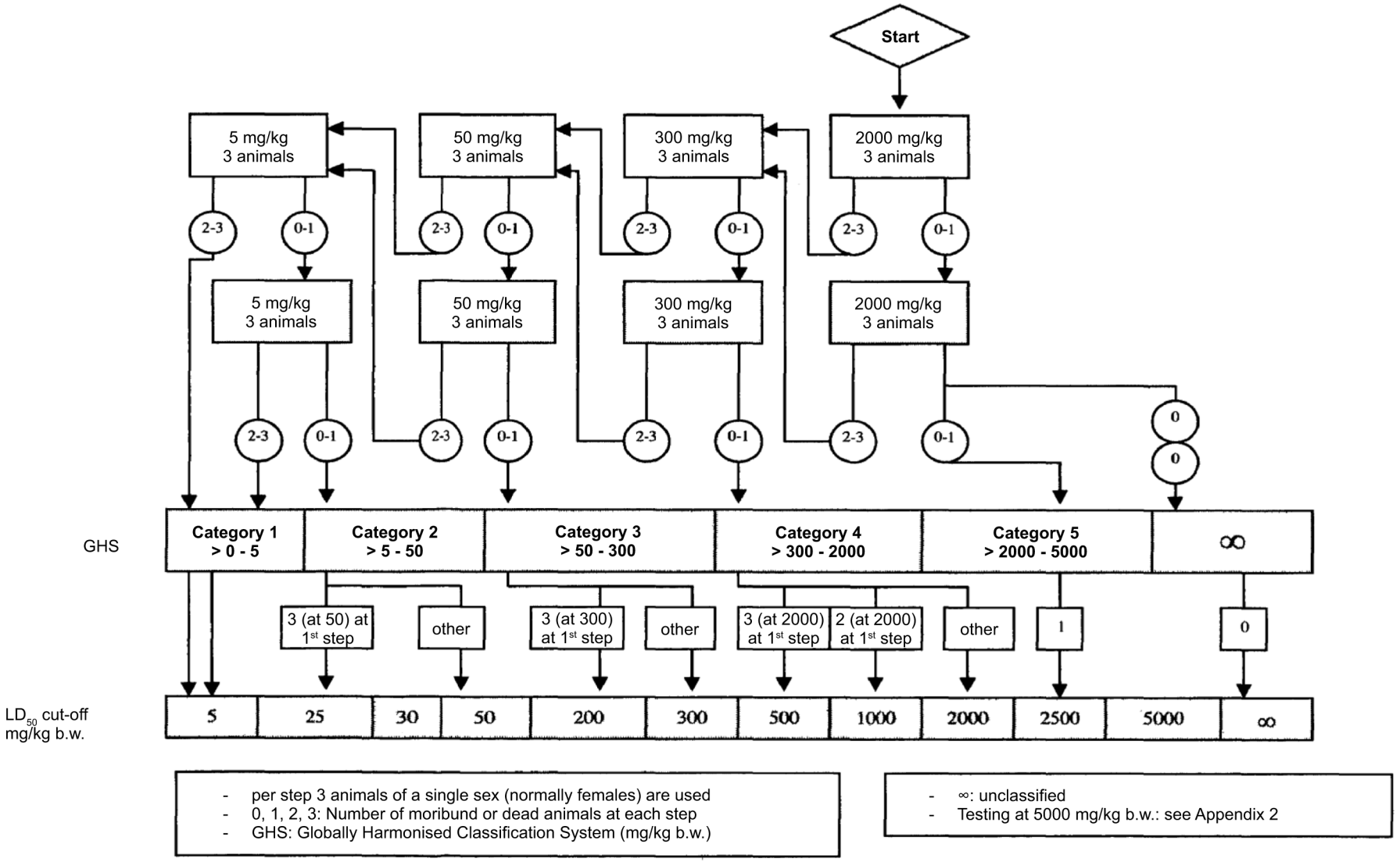


- per step 3 animals of a single sex (normally females) are used
- 0, 1, 2, 3: Number of moribund or dead animals at each step
- GHS: Globally Harmonised Classification System (mg/kg b.w.)

- ∞: unclassified
- Testing at 5000 mg/kg b.w.: see Appendix 2

Appendix 1D

TEST PROCEDURE WITH A STARTING DOSE OF 2 000 MG/KG BODY WEIGHT



Appendix 2

CRITERIA FOR CLASSIFICATION OF TEST SUBSTANCES WITH EXPECTED LD_{50} VALUES EXCEEDING 2 000 MG/KG WITHOUT THE NEED FOR TESTING

Criteria for hazard Category 5 are intended to enable the identification of test substances which are of relatively low acute toxicity hazard but which, under certain circumstances may present a danger to vulnerable populations. These substances are anticipated to have an oral or dermal LD_{50} in the range of 2 000-5 000 mg/kg or equivalent doses for other routes. The test substance should be classified in the hazard category defined by: 2 000 mg/kg < LD_{50} < 5 000 mg/kg (Category 5 in the GHS) in the following cases:

(a) If directed to this category by any of the testing schemes of Appendix 1a-1d, based on mortality incidences;

(b) if reliable evidence is already available that indicates the LD_{50} to be in the range of Category 5 values; or other animal studies or toxic effects in humans indicate a concern for human health of an acute nature;

(c) through extrapolation, estimation or measurement of data if assignment to a more hazardous class is not warranted; and

— reliable information is available indicating significant toxic effects in humans, or

— any mortality is observed when tested up to Category 4 values by the oral route, or

— where expert judgement confirms significant clinical signs of toxicity, when tested up to Category 4 values, except for diarrhoea, piloerection or an ungroomed appearance, or

— where expert judgement confirms reliable information indicating the potential for significant acute effects from the other animal studies.

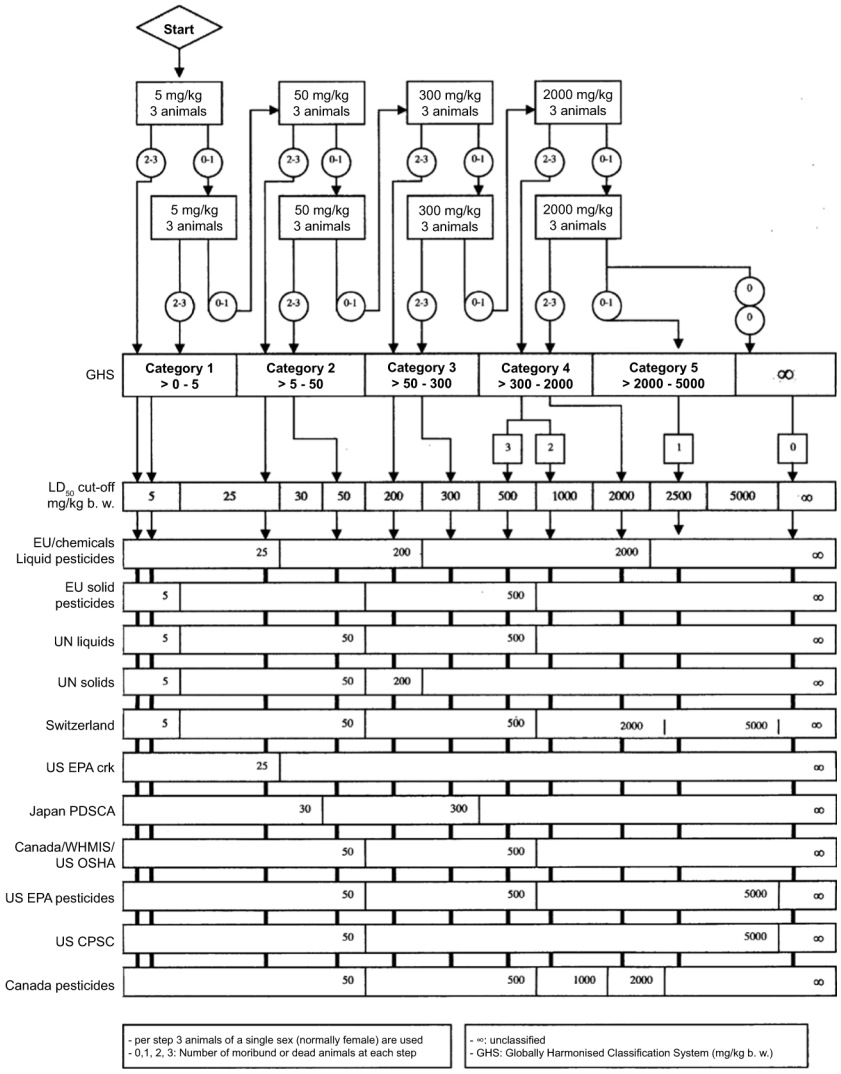
TESTING AT DOSES ABOVE 2 000 MG/KG

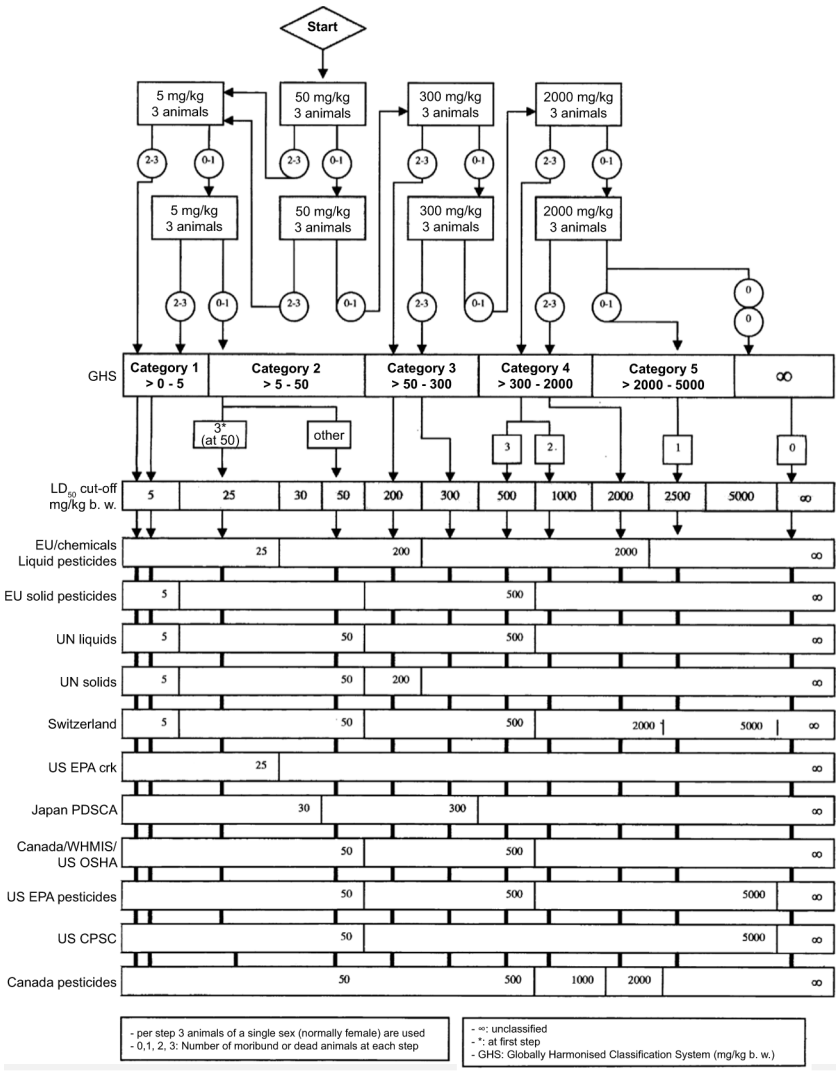
Recognising the need to protect animal welfare, testing of animals in Category 5 (5 000 mg/kg) ranges is discouraged and should only be considered when there is a strong likelihood that results of such a test have a direct relevance for protecting human or animal health (10). No further testing should be conducted at higher dose levels.

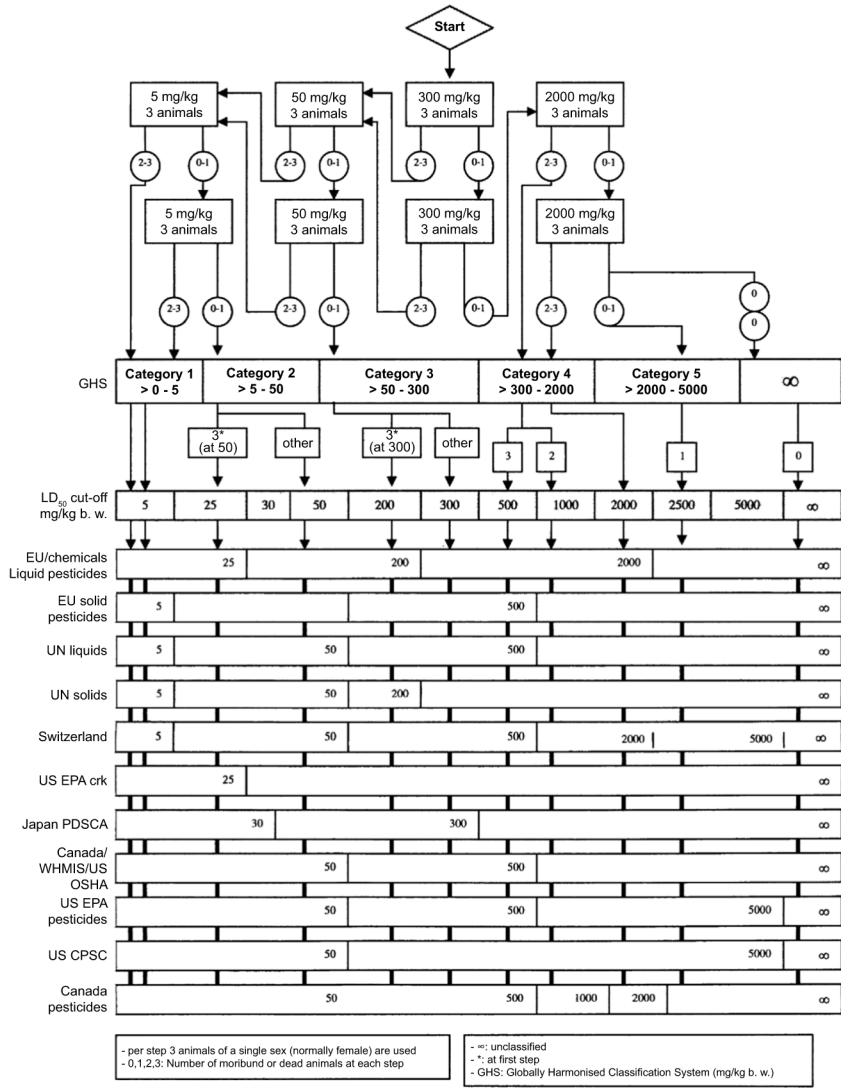
When testing is required a dose of 5 000 mg/kg, only one step (i.e. three animals) is required. If the first animal dosed dies, then dosing proceeds at 2 000 mg/kg in accordance with the flowcharts in Appendix 1. If the first animal survives, two further animals are dosed. If only one of the three animals dies, the LD_{50} value is expected to exceed 5 000 mg/kg. If both animals die, then dosing proceeds at 2 000 mg/kg.

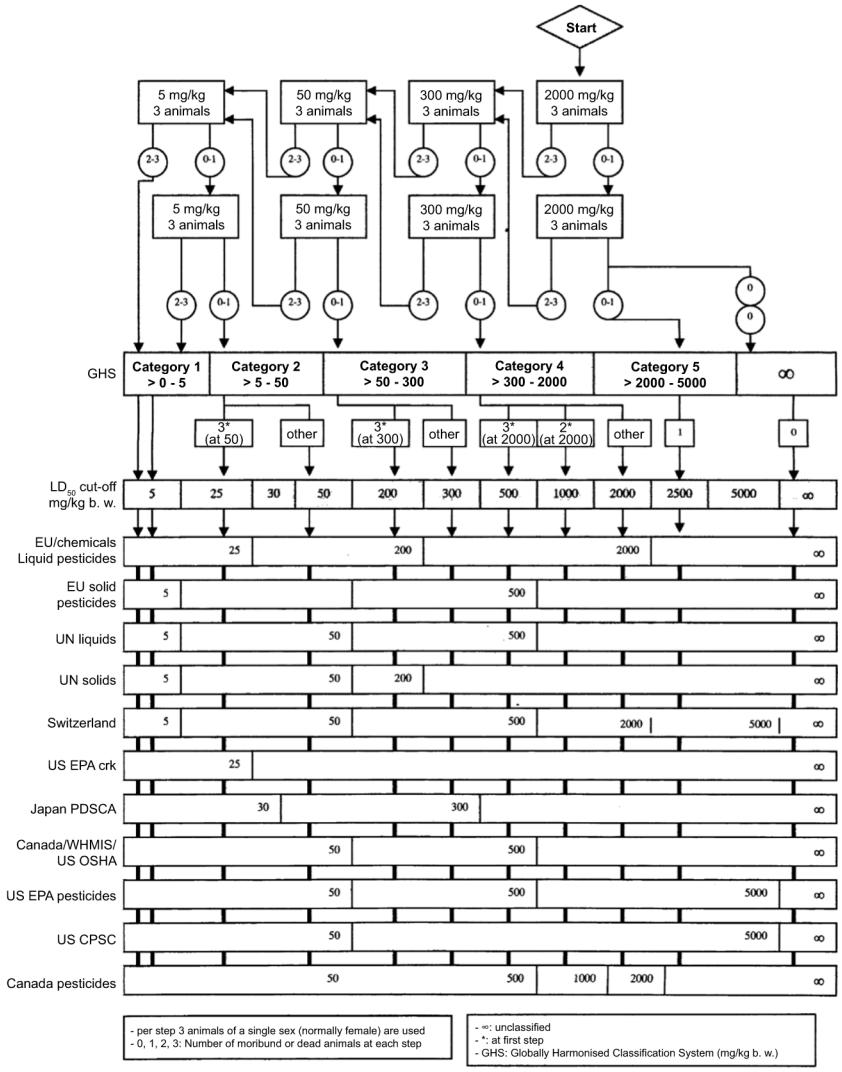
Appendix 3

TEST METHOD B.1 tris: Guidance on classififcation according to EU scheme to cover the transition period until full implementation of the Globally Harmonised Classification System (GHS) (taken from reference (8))




Start
5 mg/kg 3 animals
50 mg/kg 3 animals
300 mg/kg 3 animals
2000 mg/kg 3 animals
2-3
0-1
0
GHS
Category 1 > 0 - 5
Category 2 > 5 - 50
Category 3 > 50 - 300
Category 4 > 300 - 2000
Category 5 > 2000 - 5000
∞
3* (at 50)
other
LD50 cut-off mg/kg b. w.
5 25 30 50 200 300 500 1000 2000 2500 5000 ∞
EU/chemicals Liquid pesticides
EU solid pesticides
UN liquids
UN solids
Switzerland
US EPA crk
Japan PDSCA
Canada/WHMIS/ US OSHA
US EPA pesticides
US CPSC
Canada pesticides
- per step 3 animals of a single sex (normally female) are used
- 0,1, 2, 3: Number of moribund or dead animals at each step
- ∞: unclassified
- *: at first step
- GHS: Globally Harmonised Classification System (mg/kg b. w.)


Start
5 mg/kg 3 animals
50 mg/kg 3 animals
300 mg/kg 3 animals
2000 mg/kg 3 animals
2-3
0-1
0
GHS
Category 1 > 0 - 5
Category 2 > 5 - 50
Category 3 > 50 - 300
Category 4 > 300 - 2000
Category 5 > 2000 - 5000
∞
3* (at 50)
other
3* (at 300)
other
3
2
1
0
LD50 cut-off mg/kg b. w.
5 25 30 50 200 300 500 1000 2000 2500 5000 ∞
EU/chemicals Liquid pesticides
25 200 2000 ∞
EU solid pesticides
5 500 ∞
UN liquids
5 50 500 ∞
UN solids
5 50 200 ∞
Switzerland
5 50 500 2000 5000 ∞
US EPA crk
25 ∞
Japan PDSCA
30 300 ∞
Canada/ WHMIS/US OSHA
50 500 ∞
US EPA pesticides
50 500 5000 ∞
US CPSC
50 5000 ∞
Canada pesticides
50 500 1000 2000 ∞
- per step 3 animals of a single sex (normally female) are used
- 0,1,2,3: Number of moribund or dead animals at each step
- ∞: unclassified
- *: at first step
- GHS: Globally Harmonised Classification System (mg/kg b. w.)


Start
5 mg/kg 3 animals
50 mg/kg 3 animals
300 mg/kg 3 animals
2000 mg/kg 3 animals
2-3
0-1
0
GHS
Category 1 > 0 - 5
Category 2 > 5 - 50
Category 3 > 50 - 300
Category 4 > 300 - 2000
Category 5 > 2000 - 5000
∞
3* (at 50)
other
3* (at 300)
3* (at 2000)
2* (at 2000)
1
LD50 cut-off mg/kg b. w.
5 25 30 50 200 300 500 1000 2000 2500 5000 ∞
EU/chemicals Liquid pesticides
EU solid pesticides
UN liquids
UN solids
Switzerland
US EPA crk
Japan PDSCA
Canada/WHMIS/ US OSHA
US EPA pesticides
US CPSC
Canada pesticides
- per step 3 animals of a single sex (normally female) are used
- 0, 1, 2, 3: Number of moribund or dead animals at each step
- ∞: unclassified
- *: at first step
- GHS: Globally Harmonised Classification System (mg/kg b. w.)

B.2. ACUTE TOXICITY (INHALATION)

1. METHOD

1.1. INTRODUCTION

It is useful to have preliminary information on the particle size distribution, the vapour pressure, the melting point, the boiling point, the flash point and explosivity (if applicable) of the substance.

See also General introduction Part B (A).

1.2. DEFINITIONS

See General introduction Part B (B).

1.3. REFERENCE SUBSTANCES

None.

1.4. PRINCIPLE OF THE TEST METHOD

Several groups of experimental animals are exposed for a defined period to the test substance in graduated concentrations, one concentration being used per group. Subsequently observations of effects and deaths are made. Animals, which die during the test are necropsied and at the conclusion of the test surviving animals are necropsied.

Animals showing severe and enduring signs of distress and pain may need to be humanely killed. Dosing test substances in a way known to cause marked pain and distress due to corrosive or severe irritating properties need not be carried out.

1.5. QUALITY CRITERIA

None.

1.6. DESCRIPTION OF THE TEST METHOD

1.6.1. **Preparations**

The animals are kept under the experimental housing and feeding conditions for at least five days prior to the experiment. Before the test healthy young animals are randomiseds and assigned to the required number of groups. They need not be subjected to simulated exposure unless this is indicated by the type of exposure apparatus being used.

Solid test substances may need to be micronised in order to achieve particles of an appropriate size.

Where necessary a suitable vehicle may be added to the test substance to help generate an appropriate concentration of the test substance in the atmosphere and a vehicle control group should then be used. If a vehicle or other additives are used to facilitate dosing, they should be known not to produce toxic effects. Historical data can be used if appropriate.

1.6.2. **Test conditions**

1.6.2.1. *Experimental animals*

Unless there are contra-indications the rat is the preferred species. Commonly used laboratory strains should be employed. For each sex, at the start of the test the range of weight variation in the animals used should not exceed ± 20 % of the appropriate mean value.

1.6.2.2. *Number and sex*

At least 10 rodents (five female and five male) are used at each concentration level. The females should be nulliparous and non-pregnant.

Note: in acute toxicity tests with animals of a higher order than rodents, the use of smaller numbers should be considered. Doses should be carefully selected, and every effort should be made not to exceed moderately toxic doses. In such tests administration of lethal doses of the test substance should be avoided.

1.6.2.3. *Exposure concentrations*

These should be sufficient in number, at least three, and spaced appropriately to produce test groups with a range of toxic effects and mortality rates. The data should be sufficient to produce a concentration mortality curve and, where possible, permit an acceptable determination of an LC_{50}.1.6.2.4. Limit test

1.6.2.4. *Limit test*

If an exposure of five male and five female test animals to 20 mg per litre of a gas or 5 rug per litre of an aerosol or a particulate for four hours (or where this is not possible due to the physical or chemical, including explosive, properties of the test substance, the maximum attainable concentration) produces no compound related mortality within 14 days further testing may not be considered necessary (18th ATP, dir. 93/21/EEC, Ll10/93)

1.6.2.5. *Exposure time*

The period of exposure should be four hours.

1.6.2.6. *Equipment*

The animals should be tested with inhalation equipment designed to sustain a dynamic airflow of at least 12 air changes per hour, to ensure an adequate oxygen content and an evenly distributed exposure atmosphere. Where a chamber is used its design should minimise crowding of the test animals and maximise their exposure by inhalation to the test substance. As a general rule to ensure stability of a chamber atmosphere the total 'volume' of the test animals should not exceed 5 % of the volume of the test chamber. Oro-nasal, head only, or whole body individual chamber exposure may be used; the first two will help to minimise the uptake of the test substance by other routes.

1.6.2.7. *Observation period*

The observation period should be at least 14 days. However, the duration of observations should not be rigidly fixed. It should be determined by the toxic reactions, their rate of onset and the length of the recovery period; it may thus be extended when considered necessary. The time at which signs of toxicity appear and disappear and the time of death are important, especially if there is a tendency for deaths to be delayed.

1.6.3. **Procedure**

Shortly before exposure, the animals are weighed, and then exposed to the test concentration in the designated apparatus for a period of four hours, after equilibration of the chamber concentration. Time for equilibration should be short. The temperature at which the test is performed should be maintained at 22 ± 3 °C. Ideally the relative humidity should be maintained between 30 % and 70 %, but in certain instances (e.g. tests of some aerosols) this may not be practicable. Maintenance of a slight negative pressure inside the chamber (≥ 5 mm of water) will prevent leakage of the test substance into the surrounding area. Food and water should be withheld during exposure. Suitable systems for the generation and monitoring of the test atmosphere should be used. The system should ensure that stable exposure conditions are achieved as rapidly as possible. The chamber should be designed and operated in such a way that a homogeneous distribution of the test atmosphere within the chamber is maintained.

Measurements or monitoring should be made:

(a) of the rate of air flow (continuously);

(b) of the actual concentration of the test substance measured in the breathing zone at least three times during exposure (some atmospheres, e.g. aerosols at high concentrations, may need more frequent monitoring). During the exposure period the concentration should not vary by more than ± 15 % of the mean value. However in the case of some aerosols, this level of control may not be achievable and a wider range would then be acceptable. For aerosols, particle size analysis should be performed as often as necessary (at least once per test group);

(c) of temperature and humidity, continuously if possible.

During and following exposure, observations are made and recorded systematically; individual records should be maintained for each animal. Observations should be made frequently during the first day. A careful clinical examination should be made at least once each working day, other observations should be made daily with appropriate actions taken to minimise loss of animals from the study, e.g. necropsy or refrigeration of those animals found dead and isolation or sacrifice of weak or moribund animals.

Observations should include changes in the skin and fur, eyes, mucous membranes, respiratory, circulatory, autonomic and central nervous systems, and somatomotor activity and behaviour pattern. Particular attention should be directed to observation of respiratory behaviour, tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma. The time of death should be recorded as precisely as possible. Individual weights of animals should be determined weekly after exposure, and at death.

Animals that die during the test and those surviving at the termination of the test are subjected to necropsy with particular reference to any changes in the upper and lower respiratory tract. All gross pathological changes should be recorded. Where indicated, tissues should be taken for histopathological examination.

2. DATA

Data should be summarised in tabular form showing for each test group the number of animals at the start of the test, time of death of individual animals, number of animals displaying other signs of toxicity, description of toxic effects and necropsy findings. Changes in weight must be calculated and recorded when survival exceeds one day. Animals, which are humanely killed due to compound-related distress and pain are recorded as compound-related deaths. The LC_{50} should be determined by a recognised method. Data evaluation should include the relationship, if any, between the animal's exposure to the test substance and the incidence and severity of all abnormalities, including behavioural and clinical abnormalities, gross lesions, body weight changes, mortality and any other toxic effects.

3. REPORTING

3.1. TEST REPORT

The test report shall, if possible, include the following information:

— species, strain, source, environmental conditions, diet, etc.,

— test conditions: description of exposure apparatus, including design, type, dimensions, source of air, system for generating aerosols, method of conditioning air and the method of housing animals in a test chamber when this is used. The equipment for measuring temperature, humidity, and aerosol concentrations and particle size distribution should be described.

Exposure data

These should be tabulated and presented with mean values and a measure of variability (e.g. standard deviation) and shall, if possible, include:

(a) airflow rates through the inhalation equipment;

(b) temperature and humidity of the air;

(c) nominal concentrations (total amount of test substance fed into the inhalation equipment divided by volume of air);

(d) nature of vehicle, if used;

(e) actual concentrations in test breathing zone;

(f) The mass median aerodynamic diameter (MMAD) and the geometric standard deviation (GSD);

(g) equilibration period;

(h) exposure period;

— tabulation of response data by sex and exposure level (i.e. number of animals that died or were killed during the test, number of animals showing signs of toxicity, number of animals exposed),

— time of death during or following exposure, reasons and criteria used for humane killing of animals,

— all observations,

— LC50 value for each sex determined at the end of the observation period (with method of calculation specified),

— 95 % confidence interval for the LC50 (where this can be provided),

— dose/mortality curve and slope (where permitted by the method of determination),

— necropsy findings,

— any histopathological findings,

— discussions of the results (particular attention should be given to the effect that humane killing of animals during the test may have on the calculated LC50 value),

— interpretation of the results.

3.2. EVALUATION AND INTERPRETATION

See General introduction Part B (D).

4. **REFERENCES**

See General introduction Part B (E).

B.3. **ACUTE TOXICITY (DERMAL)**

1. **METHOD**

1.1. INTRODUCTION

See General introduction Part B (A).

1.2. DEFINITION

See General introduction Part B (B).

1.3. REFERENCE SUBSTANCES

None.

1.4. PRINCIPLE OF THE TEST METHOD

The test substance is applied to the skin in graduated doses to several groups of experimental animals, one dose being used per group. Subsequently, observations of effects and deaths are made. Animals, which die during the test are necropsied and at the conclusion of the test surviving animals are necropsied.

Animals showing severe and enduring signs of distress and pain may need to be humanely killed. Dosing test substances in a way known to cause marked pain and distress due to corrosive or irritating properties need not be carried out.

1.5. QUALITY CRITERIA

None.

1.6. DESCRIPTION OF THE TEST METHOD

1.6.1. **Preparations**

The animals are kept in their experimental cages under the experimental housing and feeding conditions for at least five days prior to the experiment. Before the test, healthy young adult animals are randomised and assigned to the treatment groups. Approximately 24 hours before the test, fur should be removed by clipping or shaving from the dorsal area of the trunk of the animals. When clipping or shaving the fur, care must be taken to avoid abrading the skin which could alter its permeability. Not less than 10 % of the body surface should be clear for the application of the test substance. When testing solids, which may be pulverised if appropriate, the test substance should be moistened sufficiently with water or, where necessary, a suitable vehicle to ensure good contact with the skin. When a vehicle is used, the influence of the vehicle on penetration of skin by the test substance should be taken into account. Liquid test substances are generally used undiluted.

1.6.2. **Test conditions**

1.6.2.1. *Experimental animals*

The adult rat or rabbit may be used. Other species may be used but their use would require justification. Commonly used laboratory strains should be employed. For each sex, at the start of the test the range of weight variation in the animals used should not exceed ± 20 % of the appropriate mean value.

1.6.2.2. *Number and sex*

At least five animals are used at each dose level. They should all be of the same sex. If females are used, they should be nulliparous and non-pregnant. Where information is available demonstrating that a sex is markedly more sensitive, animals of this sex should be dosed.

Note: in acute toxicity tests with animals of a higher order than rodents, the use of smaller numbers should be considered. Doses should be carefully selected, and every effort should be made not to exceed moderately toxic doses. In such tests, administration of lethal doses of the test substance should be avoided.

1.6.2.3. *Dose levels*

These should be sufficient in number, at least three, and spaced appropriately to produce test groups with a range of toxic effects and mortality rates. Any irritant or corrosive effects should be taken into account when deciding on dose levels. The data should be sufficient to produce a dose/response curve and, where possible, permit an acceptable determination of the LD_{50}.

1.6.2.4. *Limit test*

A limit test at one dose level of at least 2 000 mg/kg bodyweight may be carried out in a group of five male and five female animals, using the procedures described above. If compound-related mortality is produced, a full study may need to be considered.

1.6.2.5. *Observation period*

The observation period should be at least 14 days. However, the duration of observation should not be rigidly fixed. It should be determined by the toxic reactions, their rate of onset and the length of the recovery period; it may thus be extended when considered necessary. The time at which signs of toxicity appear and disappear, their duration and the time of death are important, especially if there is a tendency for deaths to be delayed.

1.6.3. **Procedure**

Animals should be caged individually. The test substance should be applied uniformly over an area, which is approximately 10 % of the total body surface area. With highly toxic substances the surface area covered may be less but as much of the area should be covered with a layer as thin and uniform as possible.

Test substances should be held in contact with the skin with a porous gauze dressing and non-irritating tape throughout a 24-hour exposure period. The test site should be further covered in a suitable manner to retain the gauze dressing and test substance and ensure that the animals cannot ingest the test substance. Restrainers may be used to prevent the ingestion of the test substance but complete immobilisation is not a recommended method.

At the end of the exposure period, residual test substance should be removed, where practicable, using water or some other appropriate method of cleansing the skin.

Observations should be recorded systematically as they are made. Individual records should be maintained for each animal. Observations should be made frequently during the first day. A careful clinical examination should be made at least once each working day, other observations should be made daily with appropriate actions taken to minimise loss of animals to the study, e.g. necropsy or refrigeration of those animals found dead and isolation or sacrifice of weak or moribund animals.

Observations should include changes in fur, treated skin, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous systems, and somatomotor activity and behaviour pattern. Particular attention should be directed to observations of tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma. The time of death must be recorded as precisely as possible. Animals that die during the test and those surviving at the termination of the test are subjected to necropsy. All gross pathological changes should be recorded. Where indicated, tissues should be taken for histopathological examination.

Assessment of toxicity in the other sex

After completion of the study in one sex, at least one group of five animals of the other sex is dosed to establish that animals of this sex are not markedly more sensitive to the test substance. The use of fewer animals may be justified in individual circumstances. Where adequate information is available to demonstrate that animals of the sex tested are markedly more sensitive, testing in animals of the other sex may be dispensed with.

2. **DATA**

Data should be summarised in tabular form, showing for each test group the number of animals at the start of the test, time of death of individual animals, number of animals displaying other signs of toxicity, description of toxic effects and necropsy findings. Individual weights of animals should be determined and recorded shortly before the test substance is applied, weekly thereafter, and at death; changes in weight should be calculated and recorded when survival exceeds one day. Animals, which are humanely killed due to compound-related distress and pain are recorded as compound-related deaths. The LD_{50} should be determined by a recognised method.

Data evaluation should include an evaluation of relationships, if any, between the animal's exposure to the test substance and the incidence and severity of all abnormalities, including behavioural and clinical abnormalities, gross lesions, body weight changes, mortality, and any other toxicological effects.

3. **REPORTING**

3.1. TEST REPORT

The test report shall, if possible, include the following information:

— species, strain, source, environmental conditions, diet, etc.,

— test conditions (including method of skin cleansing and type of dressing: occlusive or not occlusive),

— dose levels (with vehicle, if used, and concentrations),

— sex of animals dosed,

— tabulation of response data by sex and dose level (i.e. number of animals that died or were killed during the test, number of animals showing signs of toxicity, number of animals exposed),

— time of death after dosing, reasons and criteria used for humane killing of animals,

— all observations,

— LD_{50} value for the sex subjected to a full study, determined at 14 days with the method of determination specified,

— 95 % confidence interval for the LD_{50} (where this can be provided),

— dose/mortality curve and slope where permitted by the method of determination,

— necropsy findings,

— any histopathological findings,

— results of any test on the other sex,

— discussion of results (particular attention should be given to the effect that humane killing of animals during the test may have on the calculated LD_{50} value),

— interpretation of the results.

3.2. EVALUATION AND INTERPRETATION

See General introduction Part B (D).

4. **REFERENCES**

See General introduction Part B (E).

B.4. ACUTE TOXICITY: DERMAL IRRITATION/CORROSION

1. METHOD

This method is equivalent to the OECD TG 404 (2002).

1.1. INTRODUCTION

In the preparation of this updated method special attention was given to possible improvements in relation to animal welfare concerns and to the evaluation of all existing information on the test substance in order to avoid unnecessary testing in laboratory animals. This method includes the recommendation that prior to undertaking the described *in vivo* test for corrosion/irritation of the substance, a weight-of-the-evidence analysis be performed on the existing relevant data. Where insufficient data are available, they can be developed through application of sequential testing (1). The testing strategy includes the performance of validated and accepted *in vitro* tests and is provided as an Appendix to this method. In addition, where appropriate, the successive, instead of simultaneous, application of the three test patches to the animal in the initial *in vivo* test is recommended.

In the interest of both sound science and animal welfare, *in vivo* testing should not be undertaken until all available data relevant to the potential dermal corrosivity/irritation of the substance have been evaluated in a weight-of-the-evidence analysis. Such data will include evidence from existing studies in humans and/or laboratory animals, evidence of corrosivity/irritation of one or more structurally related substances or mixtures of such substances, data demonstrating strong acidity or alkalinity of the substance (2)(3), and results from validated and accepted *in vitro* or *ex vivo* tests (4)(5)(5a). This analysis should decrease the need for *in vivo* testing for dermal corrosivity/irritation of substances for which sufficient evidence already exists from other studies as to those two endpoints.

A preferred sequential testing strategy, which includes the performance of validated and accepted *in vitro* or *ex vivo* tests for corrosion/irritation, is included as an Appendix to this Method. The strategy was developed at, and unanimously recommended by the participants of, an OECD workshop (6), and has been adopted as the recommended testing strategy in the Globally Harmonised System for the Classification of Chemical Substances (GHS) (7). It is recommended that this testing strategy be followed prior to undertaking *in vivo* testing. For new substances it is the recommended a stepwise testing approach for developing scientifically sound data on the corrosivity/irritation of the substance. For existing substances with insufficient data on dermal corrosion/irritation, the strategy should be used to fill missing data gaps. The use of a different testing strategy or procedure, or a decision not to use a stepwise testing approach, should be justified.

If a determination of corrosivity or irritation cannot be made using a weight-of-the-evidence analysis, consistent with the sequential testing strategy, an *in vivo* test should be considered (see Appendix).

1.2. DEFINITIONS

Dermal irritation: is the production of reversible damage of the skin following the application of a test substance for up to four hours.

Dermal corrosion: is the production of irreversible damage of the skin; namely, visible necrosis through the epidermis and into the dermis, following the application of a test substance for up to four hours. Corrosive reactions are typified by ulcers, bleeding, bloody scabs, and, by the end of observation at 14 days, by discoloration due to blanching of the skin, complete areas of alopecia, and scars. Histopathology should be considered to evaluate questionable lesions.

1.3. PRINCIPLE OF THE TEST METHOD

The substance to be tested is applied in a single dose to the skin of an experimental animal; untreated skin areas of the test animal serve as the control. The degree of irritation/corrosion is read and scored at specified intervals and is further described in order to provide a complete evaluation of the effects. The duration of the study should be sufficient to evaluate the reversibility or irreversibility of the effects observed.

Animals showing continuing signs of severe distress and/or pain at any stage of the test should be humanely killed, and the substance assessed accordingly. Criteria for making the decision to humanely kill moribund and severely suffering animals can be found in reference (8).

1.4. DESCRIPTION OF THE TEST METHOD

1.4.1. **Preparation for the *in vivo* test**

1.4.1.1. *Selection of animal species*

The albino rabbit is the preferable laboratory animal and healthy young adult rabbits are used. A rationale for using other species should be provided.

1.4.1.2. *Preparation of the animals*

Approximately 24 hours before the test, fur should be removed by closely clipping the dorsal area of the trunk of the animals. Care should be taken to avoid abrading the skin, and only animals with healthy, intact skin should be used.

Some strains of rabbit have dense patches of hair that are more prominent at certain times of the year. Such areas of dense hair growth should not be used as test sites.

1.4.1.3. *Housing and feeding conditions*

Animals should be individually housed. The temperature of the experimental animal room should be 20 °C (± 3 °C) for rabbits. Although the relative humidity should be at least 30 % and preferably not exceed 70 %, other than during room cleaning, the aim should be 50-60 %. Lighting should be artificial, the sequence being 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unrestricted supply of drinking water.

1.4.2. **Test procedure**

1.4.2.1. *Application of the test substance*

The test substance should be applied to a small area (approximately 6 cm^2) of skin and covered with a gauze patch, which is held in place with non-irritating tape. In cases in which direct application is not possible (e.g. liquids or some pastes), the test substance should first be applied to the gauze patch, which is then applied to the skin. The patch should be loosely held in contact with the skin by means of a suitable semi-occlusive dressing for the duration of the exposure period. If the test substance is applied to the patch, it should be attached to the skin in such a manner that there is good contact and uniform distribution of the substance on the skin. Access by the animal to the patch and ingestion or inhalation of the test substance should be prevented.

Liquid test substances are generally used undiluted. When testing solids (which may be pulverised, if considered necessary), the test substance should be moistened with the smallest amount of water (or, where necessary, of another suitable vehicle) sufficient to ensure good skin contact. When vehicles other than water are used, the potential influence of the vehicle on irritation of the skin by the test substance should be minimal, if any.

At the end of the exposure period, which is normally four hours, residual test substance should be removed, where practicable, using water or an appropriate solvent without altering the existing response or the integrity of the epidermis.

1.4.2.2. *Dose level*

A dose of 0,5 ml. of liquid or 0,5 g of solid or paste is applied to the test site.

1.4.2.3. *Initial test* (in vivo *dermal irritation/corrosion test using one animal*)

It is strongly recommended that the *in vivo* test be performed initially using one animal, especially when the substance is suspected to have corrosion potential. This is in accordance with the sequential testing strategy (see Appendix 1).

When a substance has been judged to be corrosive on the basis of a weight-of-the-evidence analysis, no further animal testing is needed. For most substances suspected of being corrosive, further *in vivo* testing is normally not necessary. However, in those cases where additional data are felt warranted because of insufficient evidence, limited animal testing may be carried out using the following approach: up to three tests patches are applied

sequentially to the animal. The first patch is removed after three minutes. If no serious skin reaction is observed, a second patch is applied and removed after one hour. If the observations at this stage indicate that exposure can humanely be allowed to extend to four hours, a third patch is applied and removed after four hours, and the response is graded.

If a corrosive effect is observed after any of the three sequential exposures, the test is immediately terminated. If a corrosive effect is not observed after the last patch is removed, the animal is observed for 14 days, unless corrosion develops at an earlier time point.

In those cases in which the test substance is not expected to produce corrosion but may be irritating, a single patch should be applied to one animal for four hours.

1.4.2.4. *Confirmatory test (*in vivo *dermal irritation test with additional animals)*

If a corrosive effect is not observed in the initial test, the irritant or negative response should be confirmed using up to two additional animals, each with one patch, for an exposure period of four hours. If an irritant effect is observed in the initial test, the confirmatory test may be conducted in a sequential manner, or by exposing two additional animals simultaneously. In the exceptional case, in which the initial test is not conducted, two or three animals may be treated with a single patch, which is removed after four hours. When two animals are used, if both exhibit the same response, no further testing is needed. Otherwise, the third animal is also tested. Equivocal responses may need to be evaluated using additional animals.

1.4.2.5. *Observation period*

The duration of the observation period should be sufficient to evaluate fully the reversibility of the effects observed. However, the experiment should be terminated at any time that the animal shows continuing signs of severe pain or distress. To determine the reversibility of effects, the animals should be observed up to 14 days after removal of the patches. If reversibility is seen before 14 days, the experiment should be terminated at that time.

1.4.2.6. *Clinical observations and grading of skin reactions*

All animals should be examined for signs of erythema and oedema, and the responses scored at 60 minutes, and then at 24, 48 and 72 hours after patch removal. For the initial test in one animal, the test site is also examined immediately after the patch has been removed. Dermal reactions are graded and recorded according to the grades in the Table below. If there is damage to skin which cannot be identified as irritation or corrosion at 72 hours, observations may be needed until day 14 to determine the reversibility of the effects. In addition to the observation of irritation, all local toxic effects, such as defatting of the skin, and any systemic adverse effects (e.g. effects on clinical signs of toxicity and body weight), should be fully described and recorded. Histopathological examination should be considered to clarify equivocal responses.

The grading of skin responses is necessarily subjective. To promote harmonisation in grading of skin response and to assist testing laboratories and those involved in making and interpreting the observations, the personnel performing the observations need to be adequately trained in the scoring system used (see Table below). An illustrated guide for grading skin irritation and other lesions could be helpful (9).

2. **DATA**

2.1. PRESENTATION OF RESULTS

Study results should be summarised in tabular form in the final test report and should cover all items listed in section 3.1.

2.2. EVALUATION OF RESULTS

The dermal irritation scores should be evaluated in conjunction with the nature and severity of lesions, and their reversibility or lack of reversibility. The individual scores do not represent an absolute standard for the irritant properties of a material, as other effects of the test material are also evaluated. Instead, individual scores should be viewed as reference values, which need to be evaluated in combination with all other observations from the study.

Reversibility of dermal lesions should be considered in evaluating irritant responses. When responses such as alopecia (limited area), hyperkeratosis, hyperplasia and scaling, persist to the end of the 14-day observation period, the test substance should be considered an irritant.

3. **REPORTING**

3.1. TEST REPORT

The test report must include the following information:

Rationale for *in vivo* testing: weight-of-evidence analysis of pre-existing test data, including results from sequential testing strategy:

— description of relevant data available from prior testing,

— data derived at each stage of testing strategy,

— description of *in vitro* tests performed, including details of procedures, results obtained with test/reference substances,

— weight-of-the-evidence analysis for performing *in vivo* study.

Test substance:

— identification data (e.g. CAS number, source, purity, known impurities, lot number),

— physical nature and physicochemical properties (e.g. pH, volatility, solubility, stability),

— if mixture, composition and relative percentages of components.

Vehicle:

— identification, concentration (where appropriate), volume used,

— justification for choice of vehicle.

Test animals:

— species/strain used, rationale for using animals other than albino rabbit,

— number of animals of each sex,

— individual animal weights at start and conclusion of test,

— age at start of study,

— source of animals, housing conditions, diet, etc.

Test conditions:

— technique of patch site preparation,

— details of patch materials used and patching technique,

— details of test substance preparation, application, and removal.

Results:

— tabulation of irritation/corrosion response scores for each animal at all time points measured,

— descriptions of all lesions observed,

— narrative description of nature and degree of irritation or corrosion observed, and any histopathological findings,

— description of other adverse local (e.g. defatting of skin) and systemic effects in addition to dermal irritation or corrosion.

— Discussion of results

4. **REFERENCES**

(1) Barratt, M.D., Castell, J.V., Chamberlain, M., Combes, R.D., Dearden, J.C., Fentem, J.H., Gerner, I., Giuliani, A., Gray, T.J.B., Livingston, D.J., Provan, W.M., Rutten, F.A.J.J.L., Verhaar, H.J.M., Zbinden, P. (1995) The Integrated Use of Alternative Approaches for Predicting Toxic Hazard. ECVAM Workshop Report 8. ATLA 23, p. 410-429.

(2) Young, J.R., How, M.J., Walker, A.P., Worth W.M.H. (1988) Classification as Corrosive or Irritant to Skin of Preparations Containing Acidic or Alkaline Substance Without Testing on Animals. Toxicollogy *In Vitro*, 2, p. 19-26.

(3) Worth, A.P., Fentem, J.H., Balls, M., Botham, P.A., Curren, R.D., Earl, L.K., Esdaile, D.J., Liebsch, M. (1998) Evaluation of the proposed OECD Testing Strategy for skin corrosion. ATLA 26, p. 709-720.

(4) ECETOC (1990) Monograph No 15, 'Skin Irritation', European Chemical Industry, Ecology and Toxicology Centre, Brussels.

(5) Fentem, J.H., Archer, G.E.B., Balls, M., Botham, P.A., Curren, R.D., Earl, L.K., Edsail, D.J., Holzhutter, H.G. and Liebsch, M. (1998) The ECVAM international validation study on in vitro tests for skin corrosivity. 2. Results and evaluation by the Management Team. Toxicology *In Vitro* 12, p. 483-524.

(5a) Testing Method B.40 Skin Corrosion.

(6) OECD (1996) OECD Test Guidelines Programme: Final Report of the OECD Workshop on Harmonisation of Validation and Acceptance Criteria for Alternative Toxicological Test Methods. Held in Solna, Sweden, 22-24 January 1996 (http://www.oecd1.org/ehs/test/background.htm).

(7) OECD (1998) Harmonised Integrated Hazard Classification System for Human Health and Environmental Effects of Chemical Substances, as endorsed by the 28th Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, November 1998 (http://www.oecd1.org/ehs/Class/HCL6.htm).

(8) OECD (2000). Guidance Document on the Recognition, Assessment and Use of Clinical Signs as Humane Endpoints for Experimental Animals Used in Safety Evaluation. OECD Environmental Health and Safety Publications. Series on Testing and Assessment No 19 (http://www.oecd1.org/ehs/test/monos.htm).

(9) EPA (1990). Atlas of Dermal Lesions, *(20T-2004)*. United States Environmental Protection Agency, Office of Pesticides and Toxic Substances, Washington, DC, August 1990.

[Available from OECD Secretariat upon request].

Table I

GRADING OF SKIN REACTIONS

Erythema and Eschar formation

No erythema	0
Very slight erythema (barely perceptible)	1
Well defined erythema	2
Moderate to severe erythema	3
Severe erythema (beef redness) to eschar formation preventing grading of erythema	4

Maximum possible: 4

Oedema formation

No oedema	0
Very slight oedema (barely perceptible)	1
Slight oedema (edges of area well defined by definite raising)	2
Moderate oedema (raised approximately 1 mm)	3
Severe oedema (raised more than 1 mm and extending beyond area of exposure)	4

Maximum possible: 4

Histopathological examination may be carried out to clarify equivocal responses.

Appendix

A Sequential Testing Strategy for Dermal Irritation and Corrosion

GENERAL CONSIDERATIONS

In the interest of sound science and animal welfare, it is important to avoid the unnecessary use of animals and to minimise any testing that is likely to produce severe responses in animals. All information on a substance relevant to its potential skin corrosivity/irritancy should be evaluated prior to considering *in vivo* testing. Sufficient evidence may already exist to classify a test substance as to its dermal corrosion or irritation potential without the need to conduct testing in laboratory animals. Therefore, utilising a weight-of-the-evidence analysis and a sequential testing strategy, will minimise the need for *in vivo* testing, especially if the substance is likely to produce severe reactions.

It is recommended that a weight-of-the-evidence analysis be used to evaluate existing information regarding the skin irritation and corrosion of substances to determine whether additional studies, other than *in vivo* dermal studies, should be performed to help characterise such potential. Where further studies are needed, it is recommended that the sequential testing strategy be utilised to develop the relevant experimental data. For substances which have no testing history, the sequential testing strategy should be utilised to develop the data set needed to evaluate its dermal corrosion/irritation potential. The testing strategy described in this Appendix was developed at an OECD workshop (1) and was later affirmed and expanded in the Harmonised Integrated Hazard Classification System for Human Health and Environmental Effects of Chemical Substances, as endorsed by the 28th Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, in November 1998 (2).

Although this sequential testing strategy is not an integral part of testing method B.4, it expresses the recommended approach for the determination of skin irritation/corrosion characteristics. This approach represents both best practice and an ethical benchmark for *in vivo* testing for skin irritation/corrosion. The testing method provides guidance for the conduct of the *in vivo* test and summarises the factors that should be addressed before initiating such a test. The strategy provides an approach for the evaluation of existing data on the skin irritation/corrosion properties of test substances and a tiered approach for the generation of relevant data on substances for which additional studies are needed, or for which no studies have been performed. It also recommends the performance of validated and accepted *in vitro* or *ex vivo* tests for skin corrosion/irritation under specific circumstances.

DESCRIPTION OF THE EVALUATION AND TESTING STRATEGY

Prior to undertaking tests as part of the sequential testing strategy (Figure), all available information should be evaluated to determine the need for *in vivo* skin testing. Although significant information might be gained from the evaluation of single parameters (e.g. extreme pH), the totality of existing information should be considered. All relevant data on the effects of the substance in question, or its analogues, should be evaluated in making a weight-of-the-evidence decision, and a rationale for the decision should be presented. Primary emphasis should be placed upon existing human and animal data on the substance, followed by the outcome of *in vitro* or *ex vivo* testing. *In vivo* studies of corrosive substances should be avoided whenever possible. The factors considered in the testing strategy include:

Evaluation of existing human and animal data (Step 1). Existing human data, e.g. clinical or occupational studies and case reports, and/or animal test data, e.g. from single or repeated dermal exposure toxicity studies, should be considered first, because they provide information directly related to effects on the skin. Substances with known irritancy or corrosivity, and those with clear evidence of non-corrosivity or non-irritancy, need not be tested in *in vivo* studies.

Analysis of structure activity relationships (SAR) (Step 2). The results of testing of structurally related substances should be considered, if available. When sufficient human and/or animal data are available on structurally related substances or mixtures of such substances to indicate their skin corrosion/irritancy potential, it can be presumed that the test substance being evaluated will produce the same responses. In those cases, the test substance may not need to be tested. Negative data from studies of structurally related substances or mixtures of such substances do not constitute sufficient evidence of non-corrosivity/non-irritancy of a substance under the sequential testing strategy. Validated and accepted SAR approaches should be used to identify both dermal corrosion and irritation potential.

Physicochemical properties and chemical reactivity (Step 3). Substances exhibiting pH extremes such as ≤ 2,0 and ≥ 11,5 may have strong local effects. If extreme pH is the basis for identifying a substance as corrosive to skin, then its acid/alkali reserve (or buffering capacity) may also be taken into consideration (3)(4). If the buffering capacity suggests that a substance may not be corrosive to the skin, then further testing should be undertaken to confirm this, preferably by the use of a validated and accepted *in vitro* or *ex vivo* test (see steps 5 and 6).

Dermal toxicity (Step 4). If a chemical has proven to be very toxic by the dermal route, an *in vivo* dermal irritation/corrosion study may not be practicable because the amount of test substance normally applied could exceed the very toxic dose and, consequently result in the death or severe suffering of the animals. In addition, when dermal toxicity studies utilising albino rabbits have already been performed up to the limit dose level of 2 000 mg/kg body weight or higher, and no dermal irritation or corrosion has been seen, additional testing for skin irritation/corrosion may not be needed. A number of considerations should be borne in mind when evaluating acute dermal toxicity in previously performed studies. For example, reported information on dermal lesions may be incomplete. Testing and observations may have been made on a species other than the rabbit, and species may differ widely in sensitivity of their responses. Also the form of test substance applied to animals may not have been suitable for assessment of skin irritation/corrosion (e.g., dilution of substances for testing dermal toxicity (5). However, in those cases in which well-designed and conducted dermal toxicity studies have been performed in rabbits, negative findings may be considered sufficient evidence that the substance is not corrosive or irritating.

Results from in vitro or ex vivo tests (Steps 5 and 6). Substances that have demonstrated corrosive or severe irritant properties in a validated and accepted *in vitro* or *ex vivo* test (6)(7) designed for the assessment of these specific effects, need not be tested in animals. It can be presumed that such substances will produce similar severe effects *in vivo*.

In vivo test in rabbits (Steps 7 and 8). Should a weight-of the-evidence decision be made to conduct *in vivo* testing, it should begin with an initial test using one animal. If the results of this test indicate the substance to be corrosive to the skin, further testing should not be performed. If a corrosive effect is not observed in the initial test, the irritant or negative response should be confirmed using up to two additional animals for an exposure period of four hours. If an irritant effect is observed in the initial test, the confirmatory test may be conducted in a sequential manner, or by exposing the two additional animals simultaneously.

REFERENCES

(1) OECD, (1996) Test Guidelines Programme: Final Report on the OECD Workshop on Harmonisation of Validation and Acceptance Criteria for Alternative Toxicological Test Methods. Held on Solna, Sweden, 22-24 January 1996 (http://www1.oecd.org/ehs/test/background.htm).

(2) OECD, (1998) Harmonised Integrated Hazard Classification System for Human Health and Environmental Effects of Chemical Substances, as endorsed by the 28th Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, November 1998 (http://www1.oecd.org/ehs/Class/HCL6.htm).

(3) Worth, A.P., Fentem J.H., Balls M., Botham P.A., Curren R.D., Earl L.K., Esdaile D.J., Liebsch M., (1998). An Evaluation of the Proposed OECD Testing Strategy for Skin Corrosion. ATLA 26, p. 709-720.

(4) Young, J.R., How, M.J., Walker, A.P., Worth, W.M.H., (1988). Classification as Corrosive or Irritant to Skin of Preparations Containing Acidic or Alkaline Substances, Without Testing on Animals. Toxicology *In Vitro*, 2(1), p. 19-26.

(5) Patil, S.M., Patrick, E., Maibach, H.I. (1996) Animal, Human, and In Vitro Test Methods for Predicting Skin Irritation, in: Francis N. Marzulli and Howard I. Maibach (editors): Dermatotoxicology. Fifth Edition ISBN 1-56032-356-6, Chapter 31, p. 411-436.

(6) Testing Method B.40.

(7) Fentem, J.H., Archer, G.E.B., Balls, M., Botham, P.A., Curren, R.D., Earl, L.K., Esdaile, D.J., Holzhutter, H.G. and Liebsch, M. (1998) The ECVAM international validation study on in vitro tests for skin corrosivity. 2. Results and evaluation by the Management Team. Toxicology *In Vitro* 12, p. 483-524.

Figure

TESTING AND EVALUATION STRATEGY FOR DERMAL IRRITATION/CORROSION

	Activity	Finding	Conclusion
1	Existing human and/or animal data showing effects on skin or mucous membranes	Corrosive	Apical endpoint; considered corrosive. No testing is needed.
		Irritating	Apical endpoint; considered to be an irritant. No testing is needed.
		Not corrosive/not irritating	Apical endpoint; considered not corrosive or irritating. No testing required.
	↓ *No information available, or available information is not conclusive*		
2	Perform SAR evaluation for skin corrosion/irritation	Predict severe damage to skin	Considered corrosive. No testing is needed.
		Predict irritation to skin	Considered an irritant. No testing is needed.
	↓ *No predictions can be made, or predictions are not conclusive or negative*		
3	Measure pH (consider buffering capacity, if relevant)	pH ≤ 2 or ≥ 11.5 (with high buffering capacity, if relevant)	Assume corrosivity. No testing is needed.
	↓ *2 < pH < 11.5, or pH ≤ 2.0 or ≥ 11.5 with low/no buffering capacity, if relevant*		
4	Evaluate systemic toxicity data via dermal route (1)	Very toxic	No further testing is needed.
		Not corrosive or irritating when tested to limit dose of 2000 mg/kg body weight or higher, using rabbits	Assume not corrosive or irritating. No further testing is needed.
	↓ *Such information is not available or is non-conclusive*		
5	Perform validated and accepted *in vitro* or *ex vivo* test for skin corrosion	Corrosive response	Assume corrosivity *in vivo*. No further testing is needed.
	↓ *Substance is not corrosive*		
6	Perform validated and accepted *in vitro* or *ex vivo* test for skin irritation	Irritant response	Assume irritancy *in vivo*. No further testing is needed.
	↓ *Validated in vitro or ex vivo testing methods for skin irritation are not yet available or substance is not an irritant*		
7	Perform initial *in vivo* rabbit test using one animal	Severe damage to skin	Considered corrosive. No further testing is needed.
	↓ *No severe damage*		
8	Perform confirmatory test using one or two additional animals	Corrosive or irritating	Considered corrosive or irritating. No further testing is needed
		Not corrosive or irritating	Considered not corrosive or irritating. No further testing needed

(1) can be considered before Steps 2 and 3.

B.5. ACUTE TOXICITY: EYE IRRITATION/CORROSION

1. METHOD

This method is equivalent to the OECD TG 405 (2002)

1.1. INTRODUCTION

In the preparation of this updated method special attention was given to possible improvements through the evaluation of all existing information on the test substance in order to avoid unnecessary testing in laboratory animals and thereby address animal welfare concerns. This method includes the recommendation that prior to undertaking the described *in vivo* test for acute eye irritation/corrosion, a weight-of-the-evidence analysis be performed (1) on the existing relevant data. Where insufficient data are available, it is recommended that they be developed through application of sequential testing (2)(3). The testing strategy includes the performance of validated and accepted *in vitro* tests and is provided as an Appendix to the testing method. In addition, the use of an *in vivo* dermal irritation/corrosion test to predict eye corrosion prior to consideration of an *in vivo* eye test is recommended.

In the interest of both sound science and animal welfare, *in vivo* testing should not be considered until all available data relevant to the potential eye corrosivity/irritation of the substance has been evaluated in a weight-of-the-evidence analysis. Such data will include evidence from existing studies in humans and/or laboratory animals, evidence of corrosivity/irritation of one or more structurally related substances or mixtures of such substances, data demonstrating high acidity or alkalinity of the substance (4)(5), and results from validated and accepted *in vitro* or *ex vivo* tests for skin corrosion and irritation (6)(6a). The studies may have been conducted prior to, or as a result of, a weight-of-the-evidence analysis.

For certain substances, such an analysis may indicate the need for *in vivo* studies of the ocular corrosion/irritation potential of the substance. In all such cases, before considering the use of the *in vivo* eye test, preferably a study of the *in vivo* dermal effects of the substance should be conducted first and evaluated in accordance with testing method B.4 (7). The application of a weight-of-the-evidence analysis and the sequential testing strategy should decrease the need for *in vivo* testing for eye corrosivity/irritation of substances for which sufficient evidence already exists from other studies. If a determination of eye corrosion or irritation potential cannot be made using the sequential testing strategy, even after the performance of an *in vivo* study of dermal corrosion and irritation, an *in vivo* eye corrosion/irritation test may be performed.

A preferred sequential testing strategy, which includes the performance of validated *in vitro* or *ex vivo* tests for corrosion/irritation, is included in the Appendix to this testing method. The strategy was developed at, and unanimously recommended by the participants of, an OECD workshop (8), and has been adopted as the recommended testing strategy in the Globally Harmonised System for the Classification of Chemical Substances (GHS) (9). It is recommended that this testing strategy be followed prior to undertaking *in vivo* testing. For new substances it is the recommended stepwise testing approach for developing scientifically sound data on the corrosivity/irritation of the substance. For existing substances with insufficient data on skin and eye corrosion/irritation, the strategy should be used to fill missing data gaps. The use of a different testing strategy or procedure, or the decision not to use a stepwise testing approach, should be justified.

1.2. DEFINITIONS

Eye irritation: is the production of changes in the eye following the application of a test substance to the anterior surface of the eye, which are fully reversible within 21 days of application.

Eye corrosion: is the production of tissue damage in the eye, or serious physical decay of vision, following application of a test substance to the anterior surface of the eye, which is not fully reversible within 21 days of application.

1.3. PRINCIPLE OF THE TEST METHOD

The substance to be tested is applied in a single dose to one of the eyes of the experimental animal; the untreated eye serves as the control. The degree of eye irritation/corrosion is evaluated by scoring lesions of conjunctiva, cornea, and iris, at specific intervals. Other effects in the eye and adverse systemic effects are also described to provide a complete evaluation of the effects. The duration of the study should be sufficient to evaluate the reversibility or irreversibility of the effects.

Animals showing continuing signs of severe distress and/or pain at any stage of the test should be humanely killed, and the substance assessed accordingly. Criteria for making the decision to humanely kill moribund and severely suffering animals can be found in reference (10).

1.4. DESCRIPTION OF THE TEST METHOD

1.4.1. **Preparation for the *in vivo* test**

1.4.1.1. *Selection of species*

The albino rabbit is the preferable laboratory animal, and healthy young adult animals are used. A rationale for using other strains or species should be provided.

1.4.1.2. *Preparation of animals*

Both eyes of each experimental animal provisionally selected for testing should be examined within 24 hours before testing starts. Animals showing eye irritation, ocular defects, or pre-existing corneal injury should not be used.

1.4.1.3. *Housing and feeding conditions*

Animals should be individually housed. The temperature of the experimental animal room should be 20 °C (± 3 °C) for rabbits. Although the relative humidity should be at least 30 % and preferably not exceed 70 %, other than during room cleaning, the aim should be 50-60 %. Lighting should be artificial, the sequence being 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unrestricted supply of drinking water.

1.4.2. **Test procedure**

1.4.2.1. *Application of the test substance*

The test substance should be placed in the conjunctival sac of one eye of each animal after gently pulling the lower lid away from the eyeball. The lids are then gently held together for about one second in order to prevent loss of the material. The other eye, which remains untreated, serves as a control.

1.4.2.2. *Irrigation*

The eyes of the test animals should not be washed for at least 24 hours following instillation of the test substance, except for solids (see Section 1.4.2.3.2), and in case of immediate corrosive or irritating effects. At 24 hours a washout may be used if considered appropriate.

Use of a satellite group of animals to investigate the influence of washing is not recommended unless it is scientifically justified. If a satellite group is needed, two rabbits should be used. Conditions of washing should be carefully documented, e.g. time of washing; composition and temperature of wash solution; duration, volume, and velocity of application.

1.4.2.3. *Dose level*

1.4.2.3.1. *Testing of liquids*

For testing liquids, a dose of 0,1 ml is used. Pump sprays should not be used for instilling the substance directly into the eye. The liquid spray should be expelled and collected in a container prior to instilling 0,1 ml into the eye.

1.4.2.3.2. *Testing of solids*

When testing solids, pastes, and particulate substances, the amount used should have a volume of 0,1 ml or a weight of not more than 100 mg. The test material should be ground to a fine dust. The volume of solid material should be measured after gently compacting it, e.g. by tapping the measuring container. If the solid test substance has not been removed from the eye of the test animal by physiological mechanisms at the first observation time point of one hour after treatment, the eye may be rinsed with saline or distilled water.

1.4.2.3.3. *Testing of aerosols*

It is recommended that all pump sprays and aerosols be collected prior to instillation into the eye. The one exception is for substances in pressurised aerosol containers, which cannot be collected due to vaporisation. In such cases, the eye should be held open, and the test substance administered to the eye in a simple burst of about one second, from a distance of 10 cm directly in front of the eye. This distance may vary depending on the pressure of the spray and its contents. Care should be taken not to damage the eye from the pressure of the spray. In appropriate cases, there may be a need to evaluate the potential for 'mechanical' damage to the eye from the force of the spray.

An estimate of the dose from an aerosol can be made by simulating the test as follows: the substance is sprayed on to weighing paper through an opening the size of a rabbit eye placed directly before the paper. The weight increase of the paper is used to approximate the amount sprayed into the eye. For volatile substances, the dose may be estimated by weighing a receiving container before and after removal of the test material.

1.4.2.4. *Initial test (*in vivo *eye irritation/corrosion test using one animal)*

As articulated in the sequential testing strategy (see Appendix 1), it is strongly recommended that the *in vivo* test be performed initially using one animal.

If the results of this test indicate the substance to be corrosive or a severe irritant to the eye using the procedure described, further testing for ocular irritancy should not be performed.

1.4.2.5. *Local anaesthetics*

Local anaesthetics may be used on a case-by-case basis. If the weight-of-the-evidence analysis indicates that the substance has the potential to cause pain, or initial testing shows that a painful reaction will occur, a local anaesthetic may be used prior to instillation of the test substance. The type, concentration, and dose of the local anaesthetic should be carefully selected to ensure that differences in reaction to the test substance will not result from its use. The control eye should be similarly anaesthetised.

1.4.2.6. *Confirmatory test (*in vivo *eye irritation test with additional animals)*

If a corrosive effect is not observed in the initial test, the irritant or negative response should be confirmed using up to two additional animals. If a severe irritant effect is observed in the initial test indicating a possible strong (irreversible) effect in the confirmatory testing, it is recommended that the confirmatory test be conducted in a sequential manner in one animal at a time, rather than exposing the two additional animals simultaneously. If the second animal reveals corrosive or severe irritant effects, the test is not continued. Additional animals may be needed to confirm weak or moderate irritant responses.

1.4.2.7. *Observation period*

The duration of the observation period should be sufficient to evaluate fully the magnitude and reversibility of the effects observed. However, the experiment should be terminated at any time that the animal shows continuing signs of severe pain or distress (9). To determine reversibility of effects, the animals should be observed normally for 21 days post administration of the test substance. If reversibility is seen before 21 days, the experiment should be terminated at that time.

1.4.2.7.1. *Clinical observations and grading of eye reactions*

The eyes should be examined at one, 24, 48, and 72 hours after test substance application. Animals should be kept on test no longer than necessary once definitive information has been obtained. Animals showing continuing severe pain or distress should be humanely killed without delay, and the substance assessed accordingly. Animals with the following eye lesions post-instillation should be humanely killed: corneal perforation or significant corneal ulceration including staphyloma; blood in the anterior chamber of the eye; grade 4 corneal opacity which persists for 48 hours; absence of a light reflex (iridial response grade 2) which persists for 72 hours; ulceration of the conjunctival membrane; necrosis of the conjuctivae or nictitating membrane; or sloughing. This is because such lesions generally are not reversible

Animals that do not develop ocular lesions may be terminated not earlier than three days post instillation. Animals with mild to moderate lesions should be observed until the lesions clear, or for 21 days, at which time the study is terminated. Observations should be performed at seven, 14, and 21 days in order to determine the status of the lesions, and their reversibility or irreversibility.

The grades of ocular reaction (conjunctivae, cornea and iris) should be recorded at each examination (Table I). Any other lesions in the eye (e.g. pannus, staining) or adverse systemic effects should also be reported.

Examination of reactions can be facilitated by use of a binocular loupe, hand slit-lamp, biomicroscope, or other suitable device. After recording the observations at 24 hours, the eyes may be further examined with the aid of fluorescein.

The grading of ocular responses is necessarily subjective. To promote harmonisation of grading of ocular response and to assist testing laboratories and those involved in making and interpreting the observations, the personnel performing the observations need to be adequately trained in the scoring system used.

2. DATA

2.2. EVALUATION OF RESULTS

The ocular irritation scores should be evaluated in conjunction with the nature and severity of lesions, and their reversibility or lack of reversibility. The individual scores do not represent an absolute standard for the irritant properties of a material, as other effects of the test material are also evaluated. Instead, individual scores should be viewed as reference values and are only meaningful when supported by a full description and evaluation of all observations.

3. REPORTING

3.1. TEST REPORT

The test report must include the following information:

Rationale for *in vivo* testing: weight-of-the-evidence analysis of pre-existing test data, including results from sequential testing strategy

— description of relevant data available from prior testing,

— data derived in each step of testing strategy,

— description of *in vitro* tests performed, including details of procedures, results obtained with test/reference substances,

— description of *in vivo* dermal irritation/corrosion study performed, including results obtained,

— weight-of-the-evidence analysis for performing *in vivo* study.

Test substance:

— identification data (e.g. CAS number, source, purity, known impurities, lot number),

— physical nature and physicochemical properties (e.g. pH, volatility, solubility, stability, reactivity with water),

— in case of a mixture, composition and relative percentages of components,

— if local anaesthetic is used, identification, purity, type, dose, and potential interaction with test substance.

Vehicle:

— identification, concentration (where appropriate), volume used,

— justification for choice of vehicle.

Test animals:

— species/strain used, rationale for using animals other than albino rabbit,

— age of each animal at start of study,

— number of animals of each sex in test and control groups (if required),

— individual animal weights at start and conclusion of test,

— source, housing conditions, diet, etc.

Results:

— description of method used to score irritation at each observation time (e.g. hand slitlamp, biomicroscope, fluorescein),

— tabulation of irritant/corrosive response data for each animal at each observation time up to removal of each animal from the test,

— narrative description of the degree and nature of irritation or corrosion observed,

— description of any other lesions observed in the eye (e.g. vascularisation, pannus formation, adhesions, staining),

— description of non-ocular local and systemic adverse effects, and histopathological findings, if any.

Discussion of results.

3.2. INTERPRETATION OF THE RESULTS

Extrapolation of the results of eye irritation studies in laboratory animals to humans is valid only to a limited degree. In many cases the albino rabbit is more sensitive than humans to ocular irritants or corrosives.

Care should be taken in the interpretation of data to exclude irritation resulting from secondary infection.

4. **REFERENCES**

(1) Barratt, M.D., Castell, J.V., Chamberlain, M., Combes, R.D., Dearden, J.C., Fentem, J.H., Gerner, I., Giuliani, A., Gray, T.J.B., Livingston, D.J., Provan, W.M., Rutten, F.A.J.J.L., Verhaar, H.J.M., Zbinden, P. (1995) The Integrated Use of Alternative Approaches for Predicting Toxic Hazard. ECVAM Workshop Report 8. ATLA 23, p. 410-429.

(2) de Silva, O., Cottin, M., Dami, N., Roguet, R., Catroux, P., Toufic, A., Sicard, C., Dossou, K.G., Gerner, I., Schlede, E., Spielmann, H., Gupta, K.C., Hill, R.N., (1997) Evaluation of Eye Irritation Potential: Statistical Analysis and Tier Testing Strategies. Food Chem. Toxicol 35, p. 159-164.

(3) Worth A.P. and Fentem J.H., (1999) A general approach for evaluating stepwise testing strategies ATLA 27, p. 161-177

(4) Young, J.R., How, M.J., Walker, A.P., Worth W.M.H., (1988) Classification as Corrosive or Irritant to Skin of Preparations Containing Acidic or Alkaline Substance Without Testing on Animals. Toxicollogy *In Vitro*, 2, p. 19-26.

(5) Neun, D.J. (1993) Effects of Alkalinity on the Eye Irritation Potential of Solutions Prepared at a Single pH. J. Toxicol. Cut. Ocular Toxicol. 12, p. 227-231.

(6) Fentem, J.H., Archer, G.E.B., Balls, M., Botham, P.A., Curren, R.D., Earl, L.K., Edsaile, D.J., Holzhutter, H.G. and Liebsch, M. (1998) The ECVAM international validation study on in vitro tests for skin corrosivity. 2. Results and evaluation by the Management Team. Toxicology *In Vitro* 12, p. 483-524.

(6a) Testing Method B.40 Skin Corrosion.

(7) Testing method B.4. Acute toxicity: dermal irritation/corrosion.

(8) OECD, (1996) OECD Test Guidelines Programme: Final Report of the OECD Workshop on Harmonisation of Validation and Acceptance Criteria for Alternative Toxicological Test Methods. Held in Solna, Sweden, 22-24 January 1996 (http://www.oecd.org/ehs/test/background.htm).

(9) OECD, (1998) Harmonised Integrated Hazard Classification System for Human Health and Environmental Effects of Chemical Substances, as endorsed by the 28th Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, November 1998 (http://www.oecd.org/ehs/Class/HCL6.htm).

(10) OECD, (2000) Guidance Document on the Recognition, Assessment and Use of Clinical Signs as Humane Endpoints for Experimental Animals Used in Safety Evaluation. OECD Environmental Health and Safety Publications. Series on Testing and Assessment No 19 (http://www.oecd.org/ehs/test/monos.htm).

Table I

GRADING OF OCULAR LESIONS

Cornea

Opacity: degree of density (readings should be taken from most dense area) (*)

No ulceration or opacity	0
Scattered or diffuse areas of opacity (other than slight dulling of normal lustre); details of iris clearly visible	1
Easily discernible translucent area; details of iris slightly obscured	2
Nacrous area; no details of iris visible; size of pupil barely discernible	3
Opaque cornea; iris not discernible through the opacity	4

Maximum possible: 4

NOTES
(*) The area of corneal opacity should be noted

Iris

Normal	0
Markedly deepened rugae, congestion, swelling, moderate circumcorneal hyperaemia; or injection; iris reactive to light (a sluggish reaction is considered to be an effect)	1
Hemorrhage, gross destruction, or no reaction to light	2

Maximum possible: 2

Conjunctivae

Redness (refers to palpebral and bulbar conjunctivae; excluding cornea and iris)

Normal	0
Some blood vessels hyperaemic (injected)	1
Diffuse, crimson colour; individual vessels not easily discernible	2
Diffuse beefy red	3

Maximum possible: 3

Chemosis

Swelling (refers to lids and/or nictating membranes)

Normal	0
Some swelling above normal	1
Obvious swelling, with partial eversion of lids	2
Swelling, with lids about half closed	3
Swelling, with lids more than half closed	4

Maximum possible: 4

Appendix

A Sequential Testing Strategy for Eye Irritation and Corrosion

GENERAL CONSIDERATIONS

In the interests of sound science and animal welfare, it is important to avoid the unnecessary use of animals, and to minimise testing that is likely to produce severe responses in animals. All information on a substance relevant to its potential ocular irritation/corrosivity should be evaluated prior to considering *in vivo* testing. Sufficient evidence may already exist to classify a test substance as to its eye irritation or corrosion potential without the need to conduct testing in laboratory animals. Therefore, utilising a weight-of-the-evidence analysis and sequential testing strategy will minimise the need for *in vivo* testing, especially if the substance is likely to produce severe reactions.

It is recommended that a weight-of-the-evidence analysis be used to evaluate existing information pertaining to eye irritation and corrosion of substances and to determine whether additional studies, other than *in vivo* eye studies, should be performed to help characterise such potential. Where further studies are needed, it is recommended that the sequential testing strategy be utilised to develop the relevant experimental data. For substances which have no testing history, the sequential testing strategy should be utilised to develop the data needed to evaluate its eye corrosion/irritation. The testing strategy described in this Appendix was developed at an OECD workshop (1). It was subsequently affirmed and expanded in the Harmonised Integrated Hazard Classification System for Human Health and Environmental Effects of Chemical Substances, as endorsed by the 28th Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, in November 1998 (2).

Although this testing strategy is not an integrated part of testing method B.5, it expresses the recommended approach for the determination of eye irritation/corrosion properties. This approach represents both best practice and an ethical benchmark for *in vivo* testing for eye irritation/corrosion. The testing method provides guidance for the conduct of the *in vivo* test and summarises the factors that should be addressed before considering such a test. The sequential testing strategy provides a weight-of-the-evidence approach for the evaluation of existing data on the eye irritation/corrosion properties of substances and a tiered approach for the generation of relevant data on substances for which additional studies are needed or for which no studies have been performed. The strategy includes the performance first of validated and accepted *in vitro* or *ex vivo* tests and then of testing method B.4 skin irritation/corrosion studies under specific circumstances (3)(4).

DESCRIPTION OF THE STEPWISE TESTING STRATEGY

Prior to undertaking tests as part of the sequential testing strategy (Figure), all available information should be evaluated to determine the need for *in vivo* eye testing. Although significant information might be gained from the evaluation of single parameters (e.g., extreme pH), the totality of existing information should be assessed. All relevant data on the effects of the substance in question, and its structural analogues, should be evaluated in making a weight-of-the-evidence decision, and a rationale for the decision should be presented. Primary emphasis should be placed upon existing human and animal data on the substance, followed by the outcome of *in vitro* or *ex vivo* testing. *In vivo* studies of corrosive substances should be avoided whenever possible. The factors considered in the testing strategy include:

Evaluation of existing human and animal data (Step 1). Existing human data, e.g. clinical and occupational studies, and case reports, and/or animal test data from ocular studies should be considered first, because they provide information directly related to effects on the eyes. Thereafter, available data from human and/or animal studies investigating dermal corrosion/irritation should be evaluated. Substances with known corrosivity or severe irritancy to the eye should not be instilled into the eyes of animals, nor should substances showing corrosive or irritant effects to the skin; such substances should be considered to be corrosive and/or irritating to the eyes as well. Substances with sufficient evidence of non-corrosivity and non-irritancy from previously performed ocular studies should also not be tested in *in vivo* eye studies.

Analysis of structure activity relationships (SAR) (Step 2). The results of testing of structurally related chemicals should be considered, if available. When sufficient human and/or animal data are available on structurally related substances or mixtures of such substances to indicate their eye corrrosion/irritancy potential, it can be presumed that the test substance will produce the same responses. In those cases, the substance may not need to be tested. Negative data from studies of structurally related substances or mixtures of such substances do not constitute sufficient evidence of non-corrosivity/non-irritancy of a substance under the sequential testing strategy. Validated and accepted SAR approaches should be used to identify the corrosion and irritation potential for both dermal and ocular effects.

Physicochemical properties and chemical reactivity (Step 3). Substances exhibiting pH extremes such as ≤ 2,0 or ≥ 11,5 may have strong local effects. If extreme pH is the basis for identifying a substance as corrosive or irritant to the eye, then its acid/alkaline reserve (buffering capacity) may also be taken into consideration (5)(6). If the buffering capacity suggests that a substance may not be corrosive to the eye, then further testing should be undertaken to confirm this, preferably by the use of a validated and accepted *in vitro* or *ex vivo* test (see Section step 5 and 6).

Consideration of other existing information (Step 4). All available information on systemic toxicity via the dermal route should be evaluated at this stage. The acute dermal toxicity of the test substance should also be considered. If the test substance has been shown to be very toxic by the dermal route, it may not need to be tested in the eye. Although there is not necessarily a relationship between acute dermal toxicity and eye irritation/corrosion, it can be assumed that if an agent is very toxic via the dermal route, it will also exhibit high toxicity when instilled into the eye. Such data may also be considered between Steps 2 and 3.

Results from in vitro or ex vivo tests (Steps 5 and 6). Substances that have demonstrated corrosive or severe irritant properties in an *in vitro* or *ex vivo* test (7)(8) that has been validated and accepted for the assessment specifically of eye or skin corrosivity/irritation, need not be tested in animals. It can be presumed that such substances will produce similar severe effects *in vivo*. If validated and accepted *in vitro/ex vivo* tests are not available, one should bypass Steps 5 and 6 and proceed directly to Step 7.

Assessment of in vivo dermal irritancy or corrosivity of the substance (Step 7). When insufficient evidence exists with which to perform a conclusive weight-of-the-evidence analysis of the potential eye irritation/corrosivity of a substance based upon data from the studies listed above, the *in vivo* skin irritation/corrosion potential should be evaluated first, using testing method B.4 (4) and its accompanying Appendix (9). If the substance is shown to produce corrosion or severe skin irritation, it should be considered to be a corrosive eye irritant unless other information supports an alternative conclusion. Thus, an *in vivo* eye test would not need to be performed. If the substance is not corrosive or severely irritating to the skin, an *in vivo* eye test should be performed.

In vivo test in rabbits (Steps 8 and 9): *in vivo* ocular testing should begin with an initial test using one animal. If the results of this test indicate the substance to be a severe irritant or corrosive to the eyes, further testing should not be performed. If that test does not reveal any corrosive or severe irritant effects, a confirmatory test is conducted with two additional animals.

REFERENCES

(1) OECD, (1996) OECD Test Guidelines Programme: Final Report of the OECD Workshop on Harmonisation of Validation and Acceptance Criteria for Alternative Toxicological Test Methods. Held in Solna, Sweden, 22-24 January 1996 (http://www.oecd.org/ehs/test/background.htm).

(2) OECD, (1998) Harmonised Integrated Hazard Classification System for Human Health and Environmental Effects of Chemical Substances, as endorsed by the 28th Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, November 1998 (http://www.oecd.org/ehs/Class/HCL6.htm).

(3) Worth, A.P. and Fentem J.H., (1999). A General Approach for Evaluating Stepwise Testing Strategies. ATLA 27, p. 161-177.

(4) Testing method B.4. Acute Toxicity: dermal irritation/corrosion.

(5) Young, J.R., How, M.J., Walker, A.P., Worth W.M.H., (1988) Classification as Corrosive or Irritant to Skin of Preparations Containing Acidic or Alkaline Substance Without Testing on Animals. Toxicolohy *In Vitro*, 27, p. 19-26.

(6) Neun, D.J., (1993) Effects of Alkalinity on the Eye Irritation Potential of Solutions Prepared at a Single pH. J. Toxicol. Cut. Ocular Toxicol. 12, p. 227-231.

(7) Fentem, J.H., Archer, G.E.B., Balls, M., Botham, P.A., Curren, R.D., Earl, L.K., Edsail, D.J., Holzhutter, H.G. and Liebsch, M., (1998) The ECVAM international validation study on *in vitro* tests for skin corrosivity. 2. Results and evaluation by the Management Team. Toxicology *In Vitro* 12, p. 483-524.

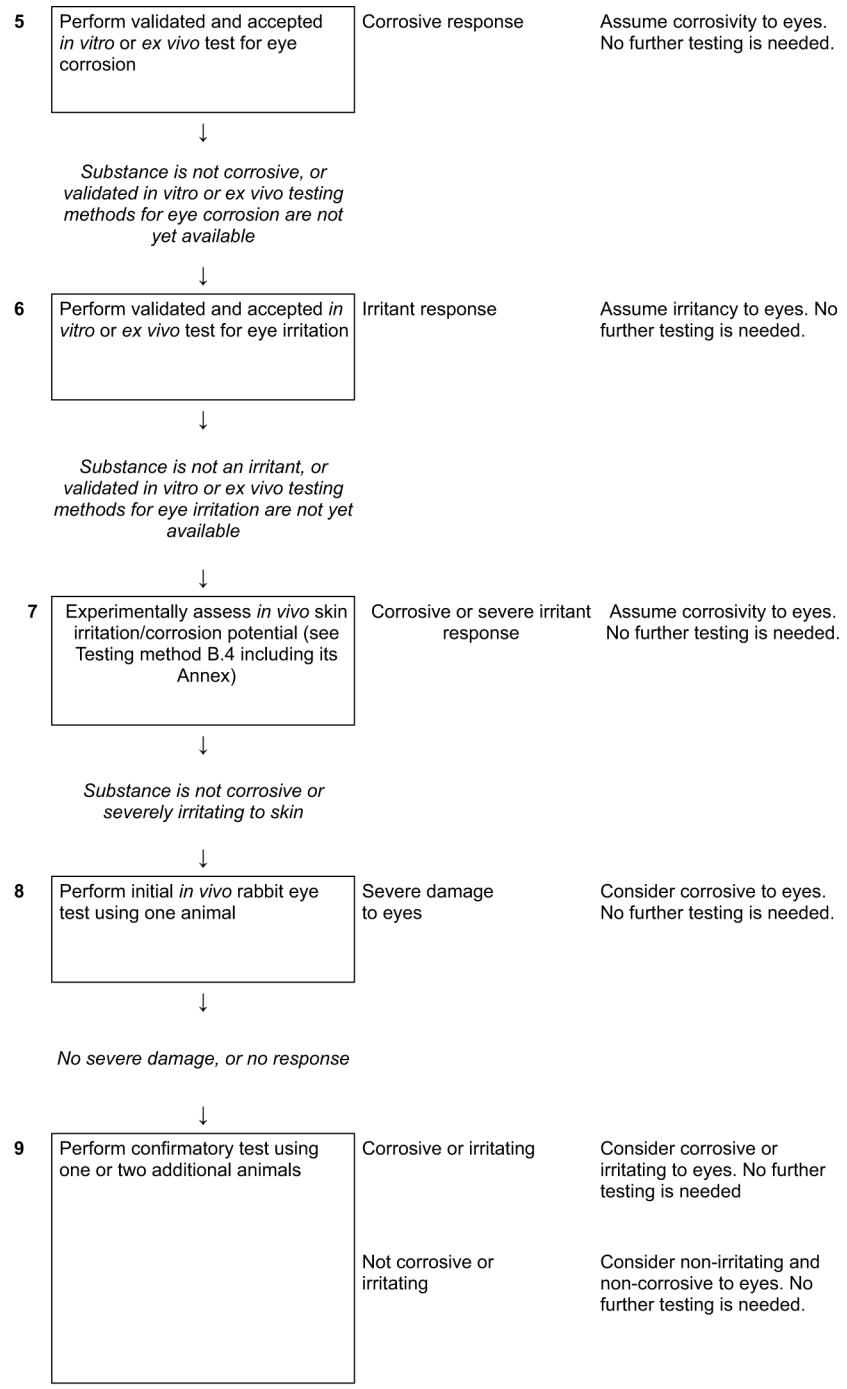
(8) Testing Method B.40 Skin Corrosion.

(9) Appendix to Testing method B.4: A Sequential Testing Strategy for Skin Irritation and Corrosion.

Figure

TESTING AND EVALUATION STRATEGY FOR EYE IRRITATION/CORROSION

	Activity	Finding	Conclusion
1	Existing human and/or animal data showing effects on eyes	Severe damage to eyes	Apical endpoint; consider corrosive to eyes. No testing is needed.
		Eye irritant	Apical endpoint; consider irritating to eyes. No testing is needed.
		Not corrosive/not irritating to eyes	Apical endpoint; considered non-corrosive and non-irritating to eyes. No testing required.
	Existing human and/or animal data showing corrosive effects on skin	Skin corrosive	Assume corrosivity to eyes. No testing is needed.
	Existing human and/or animal data showing severe irritant effects on skin	Severe skin irritant	Assume irritating to eyes. No testing is needed.
	↓		
	no information available, or available information is not conclusive		
	↓		
2	Perform SAR for eye corrosion/ irritation	Predict severe damage to eyes	Assume corrosivity to eyes. No testing is needed.
		Predict irritation to eyes	Assume irritating to eyes. No testing is needed.
	Perform SAR for skin corrosion	Predict skin corrosivity	Assume corrosivity to eyes. No testing is needed.
	↓		
	No predictions can be made, or predictions are not conclusive or negative		
	↓		
3	Measure pH (buffering capacity, if relevant)	pH ≤ 2 or ≥ 11.5 (with high buffering capacity, if relevant)	Assume corrosivity to eyes. No testing is needed.
	↓		
	2< pH < 11.5, or pH ≤ 2.0 or ≥ 11.5 with low/no buffering capacity, if relevant		
	↓		
4	Evaluate systemic toxicity via the dermal route	Very toxic at concentrations that would be tested in the eye	Substance would be too toxic for testing. No testing is needed.
	↓		
	Such information is not available, or substance is not very toxic		
	↓		


5
Perform validated and accepted *in vitro* or *ex vivo* test for eye corrosion
Corrosive response
Assume corrosivity to eyes. No further testing is needed.
Substance is not corrosive, or validated in vitro or ex vivo testing methods for eye corrosion are not yet available
6
Perform validated and accepted *in vitro* or *ex vivo* test for eye irritation
Irritant response
Assume irritancy to eyes. No further testing is needed.
Substance is not an irritant, or validated in vitro or ex vivo testing methods for eye irritation are not yet available
7
Experimentally assess *in vivo* skin irritation/corrosion potential (see Testing method B.4 including its Annex)
Corrosive or severe irritant response
Assume corrosivity to eyes. No further testing is needed.
Substance is not corrosive or severely irritating to skin
8
Perform initial *in vivo* rabbit eye test using one animal
Severe damage to eyes
Consider corrosive to eyes. No further testing is needed.
No severe damage, or no response
9
Perform confirmatory test using one or two additional animals
Corrosive or irritating
Consider corrosive or irritating to eyes. No further testing is needed
Not corrosive or irritating
Consider non-irritating and non-corrosive to eyes. No further testing is needed.

B.6. **SKIN SENSITISATION**

1. **METHOD**

1.1. INTRODUCTION

Remarks:

The sensitivity and ability of tests to detect potential human skin sensitisers are considered important in a classification system for toxicity relevant to public health.

There is no single test method which will adequately identify all substances with a potential for sensitising human skin and which is relevant for all substances.

Factors such as the physical characteristics of a substance, including its ability to penetrate the skin, must be considered in the selection of a test.

Two types of tests using guinea pigs have been developed: the adjuvant-type tests, in which an allergic state is potentiated by dissolving or suspending the test substance in Freunds Complete Adjuvant (FCA), and the non-adjuvant tests.

Adjuvant-type tests are likely to be more accurate in predicting a probable skin sensitising effect of a substance in humans than those methods not employing Freunds Complete Adjuvant and are thus the preferred methods.

The Guinea-Pig Maximisation Test (GPMT) is a widely used adjuvant-type test. Although several other methods can be used to detect the potential of a substance to provoke skin sensitisation reaction, the GPMT is considered to be the preferred adjuvant technique.

With many chemical classes, non-adjuvant tests (the preferred one being the Buehler test) are considered to be less sensitive.

In certain cases there may be good reasons for choosing the Buehler test involving topical application rather than the intradermal injection used in the Guinea-Pig Maximisation Test. Scientific justification should be given when the Buehler test is used.

The Guinea-Pig Maximisation Test (GPMT) and the Buehler test are described in this method. Other methods may be used provided that they are well-validated and scientific justification is given.

If a positive result is seen in a recognised screening test, a test substance may be designated as a potential sensitiser, and it may not be necessary to conduct a further guinea pig test. However, if a negative result is seen in such a test, the guinea pig test must be conducted using the procedure described in this tes method.

See also General introduction Part B.

1.2. DEFINITIONS

Skin sensitisation: (allergic contact dermatitis) is an immunologically mediated cutaneous reaction to a substance. In the human, the responses may be characterised by pruritis, erythema, oedema, papules, vesicles, bullae or a combination of these. In other species the reactions may differ and only erythema and oedema may be seen.

Induction exposure: an experimental exposure of a subject to a test substance with the intention of inducing a hypersensitive state.

Induction period: a period of at least one week following an induction exposure during which a hypersensitive state may be developed.

Challenge exposure: an experimental exposure of a previously treated subject to a test substance following an induction period, to determine if the subject reacts in a hypersensitive manner.

1.3. REFERENCE SUBSTANCES

The sensitivity and reliability of the experimental technique used should be assessed every six months by use of substances, which are known to have mild-to-moderate skin sensitisation properties.

In a properly conducted test, a response of at least 30 % in an adjuvant test and at least 15 % in a non-adjuvant test should be expected for mild/moderate sensitisers.

The following substances are preferred.

CAS numbers	EINECS numbers	EINECS names	Common names
101-86-0	202-983-3	α-hexylcinnamaldehyde	α-hexylcinnamaldehyde
149-30-4	205-736-8	Benzothiazole-2-thiol (mercapto-benzothiazole)	kaptax
94-09-7	202-303-5	Benzocaine	norcaine

There may be circumstances where, given adequate justification other control substances meeting the above criteria may be used.

1.4. PRINCIPLE OF THE TEST METHOD

The test animals are initially exposed to the test substance by intradermal injections and/or epidermal application (induction exposure). Following a rest period of 10 to 14 days (induction period), during which an immune response may develop, the animals are exposed to a challenge dose. The extent and degree of skin reaction to the challenge exposure in the test animals is compared with that demonstrated by control animals which undergo sham treatment during induction and receive the challenge exposure.

1.5. DESCRIPTION OF THE TEST METHODS

If removal of the test substance is considered necessary, this should be achieved using water or an appropriate solvent without altering the existing response or the integrity of the epidermis.

1.5.1. *Guinea-Pig Maximisation Test (GPMT)*

1.5.1.1. Preparations

Healthy young adult albino guinea pigs are acclimatised to the laboratory conditions for at least five days prior to the test. Before the test, animals are randomised and assigned to the treatment groups. Removal of hair is by clipping, shaving or possibly by chemical depilation, depending on the test method used. Care should be taken to avoid abrading the skin. The animals are weighed before the test commences and at the end of the test.

1.5.1.2. Test conditions

1.5.1.2.1. Test animals

Commonly used laboratory strains of albino guinea-pigs are used.

1.5.1.2.2. Number and sex

Male and/or female animals can be used. If females are used, they should be nulliparous and non-pregnant.

A minimum of 10 animals is used in the treatment group and at least five animals in the control group. When fewer than 20 test and 10 control guinea pigs have been used, and it is not possible to conclude that the test substance is a sensitiser, testing in additional animals to give a total of at least 20 test and 10 control animals is strongly recommended.

1.5.1.2.3. Dose levels

The concentration of the test substance used for each induction exposure should be well-tolerated systemically and should be the highest to cause mild-to-moderate skin irritation. The concentration used for the challenge exposure should be the highest non-irritant dose. The appropriate concentrations should be determined from a pilot study using two or three animals, if other information is not available. Consideration should be given to the use of FCA-treated animals for this purpose.

1.5.1.3. Procedure

1.5.1.3.1. Induction

Day 0-treated group

Three pairs of intradermal injections of 0,1 ml volume are given in the shoulder region which is cleared of hair so that one of each pair lies on each side of the midline.

Injection 1: a 1:1 mixture (v/v) FCA/water or physiological saline.

Injection 2: the test substance in an appropriate vehicle at the selected concentration.

Injection 3: the test substance at the selected concentration formulated in a 1:1 mixture (v/v) FCA/water or physiological saline.

In injection 3, water soluble substances are dissolved in the aqueous phase prior to mixing with FCA. Liposoluble or insoluble substances are suspended in FCA prior to combining with the aqueous phase. The final concentration of test substance shall be equal to that used in injection 2.

Injections 1 and 2 are given close to each other and nearest the head, while 3 is given towards the caudal part of the test area.

Day 0-control group

Three pairs of intradermal injections of 0,1 ml volume are given in the same sites as in the treated animals.

Injection 1: a 1:1 mixture (v/v) FCA/water or physiological saline.

Injection 2: the undiluted vehicle.

Injection 3: a 50 % w/v formulation of the vehicle in a 1:1 mixture (v/v) FCA/water or physiological saline.

Day 5-7-treated and control groups

Approximately 24 hours before the topical induction application, if the substance is not a skin irritant, the test area, after close-clipping and/or shaving is treated with 0,5 ml of 10 % sodium lauryl sulphate in vaseline, in order to create a local irritation.

Day 6-8-treated group

The test area is again cleared of hair. A filter paper (2 × 4 cm) is fully-loaded with test substance in a suitable vehicle and applied to the test area and held in contact by an occlusive dressing for 48 hours. The choice of the vehicle should be justified. Solids are finely pulverised and incorporated in a suitable vehicle. Liquids can be applied undiluted, if appropriate.

Day 6-8-control group

The test area is again cleared of hair. The vehicle only is applied in a similar manner to the test area and held in contact by an occlusive dressing for 48 hours.

1.5.1.3.2. Challenge

Day 20-22-treated and control groups

The flanks of treated and control animals are cleared of hair. A patch or chamber loaded with the test substance is applied to one flank of the animals and, when relevant, a patch or chamber loaded with the vehicle only may also be applied to the other flank. The patches are held in contact by an occlusive dressing for 24 hours.

1.5.1.3.3. Observation and Grading: treated and control groups

— approximately 21 hours after removing the patch the challenge area is cleaned and closely-clipped and/or shaved and depilated if necessary;

— approximately three hours later (approximately 48 hours from the start of the challenge application) the skin reaction is observed and recorded according to the grades shown in the Appendix;

— approximately 24 hours after this observation a second observation (72 hours) is made and once again recorded.

Blind reading of test and control animals is encouraged.

If it is necessary to clarify the results obtained in the first challenge, a second challenge (i.e. a rechallenge), where appropriate with a new control group, should be considered approximately one week after the first one. A rechallenge may also be performed on the original control group.

All skin reactions and any unusual findings, including systemic reactions, resulting from induction and challenge procedures should be observed and recorded according to the grading scale of Magnusson/Kligman (See Appendix). Other procedures, e.g. histopathological examination, the measurement of skin fold thickness, may be carried out to clarify doubtful reactions.

1.5.2. *Buehler test*

1.5.2.1. Preparations

Healthy young adult albino guinea-pigs are acclimatised to the laboratory conditions for at least five days prior to the test. Before the test, animals are randomised and assigned to the treatment groups. Removal of hair is by clipping, shaving or possibly by chemical depilation, depending on the test method used. Care should be taken to avoid abrading the skin. The animals are weighed before the test commences and at the end of the test.

1.5.2.2. Test conditions

1.5.2.2.1. Test animals

Commonly used laboratory strains of albino guinea-pigs are used.

1.5.2.2.2. Number and sex

Male and/or female animals can be used. If females are used, they should be nulliparous and non-pregnant.

A minimum of 20 animals is used in the treatment group and at least 10 animals in the control group.

1.5.2.2.3. Dose levels

The concentration of test substance used for each induction exposure should be the highest possible to produce a mild but not excessive irritation. The concentration used for the challenge exposure should be the highest

non-irritating dose. If necessary, the appropriate concentration can be determined from a pilot study using two or three animals.

For water soluble test materials, it is appropriate to use water or a dilute non-irritating solution of surfactant as the vehicle. For other test materials 80 % ethanol/water is preferred for induction and acetone for challenge.

1.5.2.3. Procedure

1.5.2.3.1. Induction

Day 0-treated group

One flank is cleared of hair (closely-clipped). The test patch system should be fully loaded with test substance in a suitable vehicle (the choice of the vehicle should be justified; liquid test substances can be applied undiluted, if appropriate).

The test patch system is applied to the test area and held in contact with the skin by an occlusive patch or chamber and a suitable dressing for six hours.

The test patch system must be occlusive. A cotton pad is appropriate and can be circular or square, but should approximate 4-6 cm^2. Restraint using an appropriate restrainer is preferred to assure occlusion. If wrapping is used, additional exposures may be required.

Day 0-control group

One flank is cleared of hair (closely-clipped). The vehicle only is applied in a similar manner to that used for the treated group. The test patch system is held in contact with the skin by an occlusive patch or chamber and a suitable dressing for six hours. If it can be demonstrated that a sham control group is not necessary, a naive control group may be used.

Days 6-8 and 13-15-treated and control group

The same application as on day 0 is carried out on the same test area (cleared of hair if necessary) of the same flank on day 6-8, and again on day 13-15.

1.5.2.3.2. Challenge

Day 27-29-treated and control group

The untreated flank of treated and control animals is cleared of hair (closely-clipped). An occlusive patch or chamber containing the appropriate amount of test substance is applied, at the maximum non-irritant concentration, to the posterior untreated flank of treated and control animals.

When relevant, an occlusive patch or chamber with vehicle only is also applied to the anterior untreated flank of both treated and control animals. The patches or chambers are held in contact by a suitable dressing for six hours.

1.5.2.3.3. Observation and grading

— approximately 21 hours after removing the patch the challenge area is cleared of hair,

— approximately three hours later (approximately 30 hours after application of the challenge patch) the skin reactions are observed and recorded according to the grades shown in the Appendix,

— approximately 24 hours after the 30 hour observation (approximately 54 hours after application of the challenge patch) skin reactions are again observed and recorded.

Blind reading of the test and control animals is encouraged.

If it is necessary to clarify the results obtained in the first challenge, a second challenge (i.e. a rechallenge), where appropriate with a new control group, should be considered approximately one week after the first one. A rechallenge may also be performed on the original control group.

All skin reactions and any unusual findings, including systemic reactions, resulting from induction and challenge procedures should be observed and recorded according to the Magnusson/Kligman grading scale (See Appendix). Other procedures, e.g. histopathological examination, the measurement of skin fold thickness, may be carried out to clarify doubtful reactions.

2. **DATA (GPMT and Buehler test)**

Data should be summarised in tabular form, showing for each animal the skin reactions at each observation.

3. **REPORTING (GPMT and Buehler test)**

If a screening assay is performed before the guinea pig test the description or reference of the test (e.g. Mouse Ear Swelling Test (MEST)), including details of the procedure, must be given together with results obtained with the test and reference substances.

Test report (GMPT and Buehler test)

The test report shall, if possible, include the following information:

Test animals:

— strain of guinea-pig used,

— number, age and sex of animals,

— source, housing conditions, diet, etc.,

— individual weights of animals at the start of the test.

Test conditions:

— technique of patch site preparation,

— details of patch materials used and patching technique,

— result of pilot study with conclusion on induction and challenge concentrations to be used in the test,

— details of test substance preparation, application and removal,

— justification for choice of vehicle,

— vehicle and test substance concentrations used for induction and challenge exposures and the total amount of substance applied for induction and challenge.

Results:

— a summary of the results of the latest sensitivity and reliability check (see 1.3) including information on substance, concentration and vehicle used,

— on each animal including grading system,

— narrative description of the nature and degree effects observed,

— any histopathological findings.

Discussion of results.

Conclusions.

4. **REFERENCES**

This method is analogous to OECD TG 406.

Appendix

TABLE

Magnusson/Kligman grading scale for the evaluation of challenge patch test reactions

0 = no visible change

1 = discrete or patchy erythema

2 = moderate and confluent erythema

3 = intense erythema and swelling

B.7. **REPEATED DOSE (28 DAYS) TOXICITY (ORAL)**

1. **METHOD**

1.1. INTRODUCTION

See General introduction Part B.

1.2. DEFINITIONS

See General introduction Part B.

1.3. PRINCIPLE OF THE TEST METHOD

The test substance is orally administered daily in graduated doses to several groups of experimental animals, one dose level per group for a period of 28 days. During the period of administration the animals are observed closely, each day for signs of toxicity. Animals which die or are killed during the test are necropsied and at the conclusion of the test surviving animals are killed and necropsied.

This method places more emphasis on neurological effects as a specific endpoint, and the need for careful clinical observations of the animals, so as to obtain as much information as possible, is stressed. The method should identify chemicals with neurotoxic potential, which may warrant further indepth investigation of this aspect. In addition, the method may give an indication of immunological effects and reproductive organ toxicity.

1.4. DESCRIPTION OF THE TEST METHOD

1.4.1. **Preparations**

Healthy young adult animals are randomly assigned to the control and treatment groups. Cages should be arranged in such a way that possible effects due to cage placement are minimised. The animals are identified uniquely and kept in their cages for at least five days prior to the start of the study to allow for acclimatisation to the laboratory conditions.

The test substance is administered by gavage or via the diet or drinking water. The method of oral administration is dependent on the purpose of the study, and the physical/chemical properties of the substance.

Where necessary, the test substance is dissolved or suspended in a suitable vehicle. It is recommended that, wherever possible, the use of an aqueous solution/suspension be considered first, followed by consideration of a solution/emulsion in oil (e.g. corn oil) and then by possible solution in other vehicles. For vehicles other than water the toxic characteristics of the vehicle must be known. The stability of the test substance in the vehicle should be determined.

1.4.2. **Test conditions**

1.4.2.1. *Test animals*

The preferred rodent species is the rat, although other rodent species may be used. Commonly used laboratory strains of young healthy adult animals should be employed. The females should be nulliparous and non-pregnant. Dosing should begin as soon as possible after weaning and, in any case, before the animals are nine weeks old.

At the commencement of the study the weight variation of animals used should be minimal and not exceed ± 20 % of the mean weight of each sex.

Where a repeated dose oral study is conducted as a preliminary to a long term-study, preferably animals from the same strain and source should be used in both studies.

1.4.2.2. *Number and sex*

At least 10 animals (five female and five male) should be used at each dose level. If interim kills are planned, the number should be increased by the number of animals scheduled to be killed before the completion of the study.

In addition, a satellite group of 10 animals (five animals per sex) may be treated with the high dose level for 28 days and observed for reversibility, persistence, or delayed occurrence of toxic effects for 14 days post-treatment. A satellite group of 10 control animals (five animals per sex) is also used.

1.4.2.3. *Dose levels*

Generally, at least three test groups and a control group should be used. Except for treatment with the test substance, animals in the control group should be handled in an identical manner to the test group subjects. If a vehicle is used in administering the test substance, the control group should receive the vehicle in the highest volume used.

If from assessment of other data, no effects would be expected at a dose of 1 000 mg/kg bw/d, a limit test may be performed. If there are no suitable data available, a range finding study may be performed to aid the determination of the doses to be used.

Dose levels should be selected taking into account any existing toxicity and (toxico-) kinetic data available for the test substance or related materials. The highest dose level should be chosen with the aim of inducing toxic effects but not death or severe suffering. Thereafter, a descending sequence of dose levels should be selected with a view to demonstrating any dosage related response and no-observed-adverse effects at the lowest dose level (NOAEL). Two to four fold intervals are frequently optimal for setting the descending dose levels and addition of a fourth test group is often preferable to using very large intervals (e.g. more than a factor of 10) between dosages.

For substances administered via the diet or drinking water it is important to ensure that the quantities of the test substance involved do not interfere with normal nutrition or water balance. When the test substance is administered in the diet either a constant dietary concentration (ppm) or a constant dose level in terms of the animals' body weight may be used; the alternative used must be specified. For a substance administered by gavage, the dose should be given at similar times each day, and adjusted as necessary to maintain a constant dose level in terms of animal body weight.

Where a repeated dose study is used as a preliminary to a long term-study, a similar diet should be used in both studies.

1.4.2.4. *Limit test*

If a test at one dose level of at least 1 000 mg/kg body weight/day or, for dietary or drinking water administration, an equivalent percentage in the diet or drinking water (based upon body weight determinations), using the procedures described for this study, produces no observable toxic effects and if toxicity would not be expected based upon data from structurally related substances, then a full study using three dose levels may not be considered necessary. The limit test applies except when human exposure indicates the need for a higher dose level to be used.

1.4.2.5. *Observation period*

The observation period should be 28 days. Animals in a satellite group scheduled for follow-up observations should be kept for at least a further 14 days without treatment to detect delayed occurrence, or persistence of, or recovery from toxic effects.

1.4.3. **Procedure**

The animals are dosed with the test substance daily seven days each week for a period of 28 days; use of a five-day per week dosing regime needs to be justified. When the test substance is administered by gavage, this should be done in a single dose to the animals using a stomach tube or a suitable intubation cannula. The maximum volume of liquid that can be administered at one time depends on the size of the test animal. The volume should not exceed 1 ml/100 g body weight, except in the case of aqueous solutions where 2 ml/100 g body weight may be used. Except for irritating or corrosive substances, which will normally reveal exacerbated effects with higher concentrations, variability in test volume should be minimised by adjusting the concentration to ensure a constant volume at all dose levels.

1.4.3.1. *General observations*

General clinical observations should be made at least once a day, preferably at the same time(s) each day and considering the peak period of anticipated effects after dosing. The health condition of the animals should be recorded. At least twice daily, all animals are observed for morbidity and mortality. Moribund animals and animals in severe distress or pain should be removed when noticed, humanely killed and necropsied.

Once before the first exposure (to allow for within-subject comparisons), and at least once a week thereafter, detailed clinical observations should be made in all animals. These observations should be made outside the home cage in a standard arena and preferably at the same time, each time. They should be carefully recorded, preferably using scoring systems, explicitly defined by the testing laboratory. Effort should be made to ensure that variations in the test conditions are minimal and that observations are preferably conducted by observers unaware of the treatment. Signs noted should include, but not be limited to, changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions and autonomic activity (e.g. lacrimation, piloerection, pupil size, unusual respiratory pattern). Changes in gait, posture and response to handling as well as the presence of clonic or tonic movements, stereotypes (e.g. excessive grooming, repetitive circling) or bizarre behaviour (e.g. self-mutilation, walking backwards) should also be recorded.

In the fourth exposure week sensory reactivity to stimuli of different types (e.g. auditory, visual and proprioceptive stimuli), assessment of grip strength and motor activity assessment should be conducted. Further details of the procedures that could be followed are given in the literature (see General introduction Part B).

Functional observations conducted in the fourth exposure week may be omitted when the study is conducted as a preliminary study to a subsequent subchronic (90-day) study. In that case, the functional observations should be included in this follow-up study. On the other hand, the availability of data on functional observations from the repeated dose study may enhance the ability to select dose levels for a subsequent subchronic study.

Exceptionally, functional observations may also be omitted for groups that otherwise reveal signs of toxicity to an extent that would significantly interfere with the functional test performance.

1.4.3.2. *Body weight and food/water consumption*

All animals should be weighed at least once a week. Measurements of food and water consumption should be made at least weekly. If the test substance is administered via the drinking water, water consumption should also be measured at least weekly.

1.4.3.3. *Haematology*

The following haematological examinations should be made at the end of the test period: haematocrit, haemoglobin concentration, erythrocyte count, total and differential leucocyte count, platelet count and a measure of blood clotting time/potential.

Blood samples should be taken from a named site just prior to or as part of the procedure for killing the animals, and stored under appropriate conditions.

1.4.3.4. *Clinical biochemistry*

Clinical biochemistry determinations to investigate major toxic effects in tissues and, specifically, effects on kidney and liver, should be performed on blood samples obtained of all animals just prior to or as part of the procedure for killing the animals (apart from those found moribund and/or intercurrently killed). Overnight fasting of the animals prior to blood sampling is recommended ([1]). Investigations of plasma or serum shall include sodium, potassium, glucose, total cholesterol, urea, creatinine, total protein and albumin, at least two enzymes indicative of hepatocellular effects (such as alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, gamma glutamyl transpeptidase, and sorbitol dehydrogenase). Measurements of additional enzymes (of hepatic or other origin) and bile acids may provide useful information under certain circumstances.

([1]) For a number of measurements in serum and plasma, most notably for glucose, overnight fasting would be preferable. The major reason for this preference is that the increased variability which would inevitably result from non-fasting, would tend to mask more subtle effects and make interpretation difficult. On the other hand, however, overnight fasting may interfere with the general metabolism of the animals and, particularly in feeding studies, may disturb the daily exposure to the test substance. If overnight fasting is adopted, clinical biochemical determinations should be performed after the conduct of functional observations in week 4 of the study.

Optionally, the following urine analysis determinations could be performed during the last week of the study using timed urine volume collection; appearance, volume, osmolality or specific gravity, pH, protein, glucose and blood/blood cells.

In addition, studies to investigate serum markers of general tissue damage should be considered. Other determinations that should be carried out if the known properties of the test substance may, or are suspected to, affect related metabolic profiles include calcium, phosphate, fasting triglycerides, specific hormones, methaemoglobin and cholinesterase. These need to be identified for substances in certain classes or on a case-by-case basis.

Overall, there is a need for a flexible approach, depending on the species and the observed and/or expected effect with a given substance.

If historical baseline data are inadequate, consideration should be given to determination of haematological and clinical biochemistry variables before dosing commences.

1.4.3.5. *Gross necropsy*

All animals in the study shall be subjected to a full, detailed gross necropsy, which includes careful examination of the external surface of the body, all orifices, and the cranial, thoracic and abdominal cavities and their contents. The liver, kidneys, adrenals, testes, epididymides, thymus, spleen, brain and heart of all animals should be trimmed of any adherent tissue, as appropriate, and their wet weight taken as soon as possible after dissection to avoid drying.

The following tissues should be preserved in the most appropriate fixation medium for both the type of tissue and the intended subsequent histopathological examination: all gross lesions, brain (representative regions including cerebrum, cerebellum and pons), spinal cord, stomach, small and large intestines (including Peyer's patches), liver, kidneys, adrenals, spleen, heart, thymus, thyroid, trachea and lungs (preserved by inflation with fixative and then immersion), gonads, accessory sex organs (e.g. uterus, prostate), urinary bladder, lymph nodes (preferably one lymph node covering the route of administration and another one distant from the route of administration to cover systemic effects), peripheral nerve (sciatic or tibial) preferably in close proximity to the muscle, and a section of bone marrow (or, alternatively, a fresh mounted bone marrow aspirate). The clinical and other findings may suggest the need to examine additional tissues. Also any organs considered likely to be target organs based on the known properties of the test substance should be preserved.

1.4.3.6. *Histopathological examination*

Full histopathology should be carried out on the preserved organs and tissues of all animals in the control and high dose groups. These examinations should be extended to animals of all other dosage groups, if treatment-related changes are observed in the high dose group.

All gross lesions shall be examined.

When a satellite group is used, histopathology should be performed on tissues and organs identified as showing effects in the treated groups.

2. **DATA**

Individual data should be provided. Additionally, all data should be summarised in tabular form showing for each test group the number of animals at the start of the test, the number of animals found dead during the test or killed for humane reasons and the time of any death or humane kill, the number showing signs of toxicity, a description of the signs of toxicity observed, including time of onset, duration, and severity of any toxic effects, the number of animals showing lesions, the type of lesions and the percentage of animals displaying each type of lesion.

When possible, numerical results should be evaluated by an appropriate and generally acceptable statistical method. The statistical methods should be selected during the design of the study.

3. **REPORTING**

TEST REPORT

The test report shall, if possible, include the following information:

Test animals:

— species/strain used,

— number, age and sex of animals,

— source, housing conditions, diet, etc.,

— individual weights of animals at the start of the test in weekly intervals thereafter and at the end of the test.

Test conditions:

— justification for choice of vehicle, if other than water,

— rationale for dose level selection,

— details of test substance formulation/diet preparation, achieved concentration, stability and homogeneity of the preparation,

— details of the administration of the test substance,

— conversion from diet/drinking water test substance concentration (ppm) to the actual dose (mg/kg body weight/day), if applicable,

— details of food and water quality.

Results:

— body weight/body weight changes,

— food consumption, and water consumption, if applicable,

— toxic response data by sex and dose level, including signs of toxicity,

— nature, severity and duration of clinic observations (whether reversible or not),

— sensory activity, grip strength and motor activity assessments,

— haematological tests with relevant base-line values,

— clinical biochemistry tests with relevant base-line values,

— body weight at killing and organ weight data,

— necropsy findings,

— a detailed description of all histopathological findings,

— absorption data if available,

— statistical treatment of results, where appropriate.

Discussion of results.

Conclusions.

4. **REFERENCES**

This method is analogous to OECD TG 407.

B.8. REPEATED DOSE (28 DAYS) TOXICITY (INHALATION)

1. METHOD

1.1. INTRODUCTION

It is useful to have preliminary information on the particle size distribution, the vapour pressure, the melting point, the boiling point, the flash point and explosivity (if applicable) of the substance.

See also General introduction Part B (A).

1.2. DEFINITION

See General introduction Part B (B).

1.3. REFERENCE SUBSTANCES

None.

1.4. PRINCIPLE OF THE TEST METHOD

Several groups of experimental animals are exposed daily for a defined period to the test substance in graduated concentrations, one concentration being used per group, for a period of 28 days. Where a vehicle is used to help generate an appropriate concentration of the test substance in the atmosphere, a vehicle control group should be used. During the period of administration the animals are observed daily to detect signs of toxicity. Animals, which die during the test are necropsied and at the conclusion of the test surviving animals are necropsied.

1.5. QUALITY CRITERIA

None.

1.6. DESCRIPTION OF THE TEST METHOD

1.6.1. **Preparations**

The animals are kept under the experimental housing and feeding conditions for at least five days prior to the experiment. Before the test, healthy young animals are randomised and assigned to the required number of groups. Where necessary, a suitable vehicle may be added to the test substance to help generate an appropriate concentration of the substance in the atmosphere. If a vehicle or other additive is used to facilitate dosing, it should be known not to produce toxic effects. Historical data can be used if appropriate.

1.6.2. **Test conditions**

1.6.2.1. *Experimental animals*

Unless there are contra-indications, the rat is the preferred species. Commonly used laboratory strains of young healthy animals should be employed.

At the commencement of the study the range of weight variation in the animals used should not exceed ± 20 % of the appropriate mean value.

1.6.2.2. *Number and sex*

At least 10 animals (five female and five male) should be used for each test group. The females should be nulliparous and non-pregnant. If interim sacrifices are planned, the numbers should be increased by the number of animals scheduled to be sacrificed before the completion of the study. In addition, a satellite group of 10 animals (five animals per sex) may be treated with the high concentration level for 28 days and observed for reversibility, persistence, or delayed occurrence of toxic effects for 14 days post-treatment. A satellite group of 10 control animals (five animals per sex) is also used.

1.6.2.3. *Exposure concentration*

At least three concentrations are required, with a control or a vehicle control (corresponding to the concentration of vehicle at the highest level) if a vehicle is used. Except for treatment with the test substance, animals in the control group should be handled in an identical manner to the test-group animals. The highest concentration should result in toxic effects but no, or few, fatalities. The lowest concentration should not produce any evidence of toxicity. Where there is a usable estimation of human exposure, the lowest concentration should exceed this. Ideally, the intermediate concentration should produce minimal observable toxic effects. If more than one intermediate concentration is used the concentrations should be spaced to produce a gradation of toxic effects. In the low and intermediate groups and in the controls, the incidence of fatalities should be low to permit a meaningful evaluation of the results.

1.6.2.4. *Exposure time*

The duration of daily exposure should be six hours but other periods may be needed to meet specific requirements.

1.6.2.5. *Equipment*

The animals should be tested in inhalation equipment designed to sustain a dynamic airflow of at least 12 air changes per hour to ensure an adequate oxygen content and an evenly distributed exposure atmosphere. Where a chamber is used its design should minimise crowding of the test animals and maximise their exposure by inhalation of the test substance. As a general rule to ensure stability of a chamber atmosphere the total 'volume' of the test animals should not exceed 5 % of the volume of the test chamber. Oro-nasal, head only, or individual whole body chamber exposure may be used; the first two will minimise uptake by other routes.

1.6.2.6. *Observation period*

The experimental animals should be observed daily for signs of toxicity during the entire treatment and recovery period. The time of death and the time at which signs of toxicity appear and disappear should be recorded.

1.6.3. **Procedure**

The animals are exposed to the test substance daily, five to seven days per week, for a period of 28 days. Animals in any satellite groups scheduled for follow-up observations should be kept for a further 14 days without treatment to detect recovery from, or persistence of toxic effects. The temperature at which the test is performed should be maintained at 22 ± 3 °C.

Ideally, the relative humidity should be maintained between 30 and 70 %, but in certain instances (e.g. tests of some aerosols) this may not be practicable. Maintenance of a slight negative pressure inside the chamber (≤ 5 mm of water) will prevent leakage of the test substance into the surrounding area. Food and water should be withheld during exposure.

A dynamic inhalation system with a suitable analytical concentration control system should be used. To establish suitable exposure concentrations a trial test is recommended. The airflow should be adjusted to ensure that conditions throughout the exposure chamber are homogeneous. The system should ensure that stable exposure conditions are achieved as rapidly as possible.

Measurements or monitoring should be made:

(a) of the rate of airflow (continuously),

(b) of the actual concentration of the test substance measured in the breathing zone. During the daily exposure period the concentration should not vary by more than ± 15 % of the mean value. However, in the case of some aerosols, this level of control may not be achievable and a wider range would then be acceptable. During the total duration of the study, the day-to-day concentrations should be held as constant as practicable. For aerosols, at least one particle size analysis should be performed per test group weekly,

(c) of temperature and humidity, continuously if possible.

During and following exposure observations are made and recorded systematically; individual records should be maintained for each animal. All the animals should be observed daily and signs of toxicity recorded including the time of onset, their degree and duration. Observations should include changes in the skin and fur, eyes, mucous membranes, respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behaviour pattern. Measurements should be made weekly of the animals' weight. It is also recommended that food consumption is measured weekly. Regular observation of the animals is necessary to ensure that animals are not lost from the study due to causes such as cannibalism, autolysis of tissues or misplacement. At the end of the study period, all survivors in the non-satellite treatment groups are necropsied. Moribund animals and animals in severe distress or pain should be removed when noticed, humanely killed and necropsied.

The following examinations shall be made at the end of the test on all animals including the controls:

(i) haematology, including at least haematocrit, haemoglobin concentration, erythrocyte count, total and differential leucocyte count and a measure of clotting potential;

(ii) clinical blood biochemistry including at least one parameter of liver and kidney function: serum alanine aminotransferase (formerly known as glutamic pyruvic transaminase), serum aspartate aminotransferase (formerly known as glutamic oxaloacetic transaminase), urea nitrogen, albumin, blood creatinine, total bilirubin and total serum protein measurements;

Other determinations, which may be necessary for an adequate toxicological evaluation include calcium, phosphorus, chloride, sodium, potassium, fasting glucose analysis of lipids, hormones, acid/base balance, methaemoglobin and cholinesterase activity.

Additional clinical biochemistry may be employed, where necessary, to extend the investigation of observed toxic effects.

1.6.3.1. *Gross necropsy*

All animals in the study should be subjected to a full gross necropsy. At least the liver, kidneys, adrenals, lungs, and testes should be weighed wet as soon as possible after dissection to avoid drying. Organs and tissues (the respiratory tract, liver, kidneys, spleen, testes, adrenals, heart, and any organs showing gross lesions or changes in size) should be preserved in a suitable medium for possible future histopathological examination. The lungs should be removed intact, weighed and treated with a suitable fixative to ensure that lung structure is maintained.

1.6.3.2. *Histopathological examination*

In the high-concentration group and in the control(s), histological examination should be performed on preserved organs and tissues. Organs and tissues showing defects attributable to the test substance at the highest dosage level should be examined in all lower-dosage groups. Animals in any satellite groups should be examined histologically with particular emphasis on those organs and tissues identified as showing effects in the other treated groups.

2. DATA

Data should be summarised in tabular form, showing for each test group the number of animals at the start of the test and the number of animals displaying each type of lesion.

All observed results should be evaluated by an appropriate statistical method. Any recognised statistical method may be used.

3. REPORTING

3.1. TEST REPORT

The test report shall, if possible, include the following information:

— species, strain, source, environmental conditions, diet, etc.,

— test conditions.

Description of exposure apparatus including design, type, dimensions, source of air, system for generating aerosols, method of conditioning air, treatment of exhaust air and the method of housing animals in a test chamber when this is used. The equipment for measuring temperature, humidity and, where appropriate, stability of aerosol concentrations or particle size distribution, should be described.

Exposure data:

These should be tabulated and presented with mean values and a measure of variability (e.g. standard deviation) and shall, if possible, include:

(a) airflow rates through the inhalation equipment;

(b) temperature and humidity of air;

(c) nominal concentrations (total amount of test substance fed into the inhalation equipment divided by the volume of air);

(d) nature of vehicle, if used;

(e) actual concentrations in test breathing zone;

(f) the mass median aerodynamic diameter (MMAD) and the geometric standard deviation (GSD);

— toxic response data by sex and concentration,

— time of death during the study or whether animals survived to termination,

— description of toxic or other effects, no-effect level,

— the time of observation of each abnormal sign and its subsequent course,

— food and body-weight data,

— haematological tests employed and results,

— clinical biochemistry tests employed and results,

— necropsy findings,

— a detailed description of all histopathological findings,

— a statistical treatment of results where possible,

— discussion of the results,

— interpretation of results.

3.2. EVALUATION AND INTERPRETATION

See General introduction Part B (D).

4. **REFERENCES**

See General introduction Part B (E).

B.9. **REPEATED DOSE (28 DAYS) TOXICITY (DERMAL)**

1. **METHOD**

1.1. INTRODUCTION

See General introduction Part B (A).

1.2. DEFINITIONS

See General introduction Part B (B).

1.3. REFERENCE SUBSTANCES

None.

1.4. PRINCIPLE OF THE TEST METHOD

The test substance is applied daily to the skin in graduated doses to several groups of experimental animals, one dose per group, for a period of 28 days. During the period of application, the animals are observed daily to detect signs of toxicity. Animals, which die during the test, are necropsied and at the conclusion of the test surviving animals are necropsied.

1.5. QUALITY CRITERIA

None.

1.6. DESCRIPTION OF THE TEST METHOD

1.6.1. **Preparations**

The animals are kept under the experimental housing and feeding conditions for at least five days prior to the test. Before the test, healthy young animals are randomised and assigned to the treatment and control groups. Shortly before testing, fur is clipped from the dorsal area of the trunk of the test animals. Shaving may be employed but it should be carried out approximately 24 hours before the test. Repeat clipping or shaving is usually needed at approximately weekly intervals. When clipping or shaving the fur, care must be taken to avoid abrading the skin. Not less than 10 % of the body surface area should be clear for the application of the test substance. The weight of the animal should be taken into account when deciding on the area to be cleared and on the dimensions of the covering. When testing solids, which may be pulverised if appropriate, the test substance should be moistened sufficiently with water or, where necessary, a suitable vehicle to ensure good contact with the skin. Liquid test substances are generally used undiluted. Daily application on a five to seven-day per week basis is used.

1.6.2. **Test conditions**

1.6.2.1. *Experimental animals*

The adult rat, rabbit or guinea-pig may be used. Other species may be used but their use would require justification.

At the commencement of the study, the range of weight variation in the animals used should not exceed ± 20 % of the appropriate mean value.

1.6.2.2. *Number and sex*

At least 10 animals (five female and five male) with healthy skin should be used at each dose level. The females should be nulliparous and non-pregnant. If interim sacrifices are planned, the numbers should be increased by the number of animals scheduled to be sacrificed before the completion of the study. In addition, a satellite group of 10 animals (five animals per sex) may be treated with the high dose level for 28 days and observed for reversibility, persistence, or delayed occurrence of toxic effects for 14 days post-treatment. A satellite group of 10 control animals (five animals per sex) is also used.

1.6.2.3. *Dose levels*

At least three dose levels are required with a control or a vehicle control if a vehicle is used. The exposure period should be at least six hours per day. The application of the test substance should be made at similar times each day, and adjusted at intervals (weekly or bi-weekly) to maintain a constant dose level in terms of animal body-weight. Except for treatment with the test substance, animals in the control group should be handled in an identical manner to the test group subjects. Where a vehicle is used to facilitate dosing, the vehicle control group should be dosed in the same way as the treated groups, and receive the same amount as that received by the highest dose level group. The highest dose level should result in toxic effects but produce no, or few, fatalities. The lowest dose level should not produce any evidence or toxicity. Where there is a usable estimation of human exposure, the lowest level should exceed this. Ideally, the intermediate dose level should produce minimal observable toxic effects. If more than one intermediate dose is used the dose levels should be spaced to produce a gradation of toxic effects. In the low and intermediate groups and in the controls, the incidence of fatalities should be low in order to permit a meaningful evaluation of the results.

If application of the test substance produces severe skin irritation, the concentrations should be reduced and this may result in a reduction in, or absence of, other toxic effects at the high dose level. Moreover if the skin has been badly damaged it may be necessary to terminate the study and undertake a new study at lower concentrations.

1.6.2.4. *Limit test*

If a preliminary study at a dose level of 1 000 mg/kg, or a higher dose level related to possible human exposure where this is known, produces no toxic effects, further testing may not be considered necessary.

1.6.2.5. *Observation period*

The experimental animals should be observed daily for signs of toxicity. The time of death and the time at which signs of toxicity appear and disappear should be recorded.

1.6.3. **Procedure**

Animals should be caged individually. The animals are treated with the test substance, ideally on seven days per week, for a period of 28 days. Animals in any satellite groups scheduled for follow-up observations should be kept for a further 14 days without treatment to detect recovery from or persistence of toxic effects. Exposure time should be at least six hours per day.

The test substance should be applied uniformly over an area, which is approximately 10 % of the total body surface area. With highly toxic substances, the surface area covered may be less but as much of the area as possible should be covered with as thin and uniform a layer as possible.

During exposure the test substance is held in contact with the skin with porous gauze dressing and non-irritating tape. The test site should be further covered in a suitable manner to retain the gauze dressing and test substance and ensure that the animals cannot ingest the test substance. Restrainers may be used to prevent the ingestion of the test substance but complete immobilisation is not a recommended method. As an alternative a 'collar protective device' may be used.

At the end of the exposure period, residual test substance should be removed, where practicable, using water or some other appropriate method of cleansing the skin.

All the animals should be observed daily and signs of toxicity recorded including the time of onset, their degree and duration. Observations should include changes in skin and fur, eyes and mucous membranes as well as respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behaviour pattern. Measurements should be made weekly of the animals' weight. It is also recommended that food consumption is measured weekly. Regular observation of the animals is necessary to ensure that animals are not lost from the study due to causes such as cannibalism, autolysis of tissues or misplacement. At the end of the study period, all survivors in the non-satellite treatment groups are necropsied. Moribund animals and animals in severe distress or pain should be removed when noticed, humanely killed and necropsied.

The following examinations shall be made at the end of the test on all animals including the controls:

(1) haematology, including at least haematocrit, haemoglobin concentration, erythrocyte count, total and differential leucocyte count, and a measure of clotting potential;

(2) clinical blood biochemistry including at least one parameter of liver and kidney function: serum alanine aminotransferase (formerly known as glutamic pyruvic transaminase), serum aspartate aminotransferase (formerly known as glutamic oxaloacetic transaminase), urea nitrogen, albumin, blood creatinine, total bilirubin and total serum protein;

Other determinations which may be necessary for an adequate toxicological evaluation include calcium, phosphorus, chloride, sodium, potassium, fasting glucose, analysis of lipids, hormones, acid/base balance, methaemoglobin and cholinesterase activity.

Additional clinical biochemistry may be employed, where necessary, to extend the investigation of observed effects.

1.6.4. **Gross necropsy**

All animals in the study should be subjected to a full gross necropsy. At least the liver, kidneys, adrenals, and testes should be weighed wet as soon as possible after dissection, to avoid drying. Organs and tissues, i.e. normal and treated skin, liver, kidney, spleen, testes, adrenals, heart, and target organs (that is those organs showing gross lesions or changes in size) should be preserved in a suitable medium for possible future histopathological examination.

1.6.5. **Histopathological examination**

In the high dose group and in the control group, histological examination should be performed on the preserved organs and tissues. Organs and tissues showing defects attributable to the test substance at the highest dosage level should be examined in all lower-dosage groups. Animals in the satellite group should be examined histologically with particular emphasis on those organs and tissues identified as showing effects in the other treated groups.

2. **DATA**

Data should be summarised in tabular form, showing for each test group the number of animals at the start of the test and the number of animals displaying each type of lesion.

All observed results should be evaluated by an appropriate statistical method. Any recognised statistical method may be used.

3. **REPORTING**

3.1. TEST REPORT

The test report shall, if possible, include the following information:

— animal data (species, strain, source, environmental conditions, diet, etc.),

— test conditions (including the type of dressing: occlusive or not-occlusive),

— dose levels (including vehicle, if used) and concentrations,

— no-effect level, where possible,

— toxic response data by sex and dose,

— time of death during the study or whether animals survived to termination,

— toxic or other effects,

— the time of observation of each abnormal sign and its subsequent course,

— food and body-weight data,

— haematological tests employed and results,

— clinical biochemistry tests employed and results,

— necropsy findings,

— a detailed description of all histopathological findings,

— statistical treatment of results where possible,

— discussion of the results,

— interpretation of the results.

3.2. EVALUATION AND INTERPRETATION

See General introduction Part B (D).

4. **REFERENCES**

See General introduction Part B (E).

B.10. **MUTAGENICITY — *IN VITRO* MAMMALIAN CHROMOSOME ABERRATION TEST**

1. **METHOD**

This method is a replicate of the OECD TG 473, *In Vitro* Mammalian Chromosome Aberration Test (1997).

1.1. INTRODUCTION

The purpose of the *in vitro* chromosomal aberration test is to identify agents that cause structural chromosome aberrations in cultured mammalian cells (1)(2)(3). Structural aberrations may be of two types, chromosome or chromatid. With the majority of chemical mutagens, induced aberrations are of the chromatid type, but chromosome-type aberrations also occur. An increase in polyploidy may indicate that a chemical has the potential to induce numerical aberrations. However, this method is not designed to measure numerical aberrations and is not routinely used for that purpose. Chromosome mutations and related events are the cause of many human genetic diseases and there is substantial evidence that chromosome mutations and related events causing alterations in oncogenes and tumour-suppressor genes of somatic cells are involved in cancer induction in humans and experimental animals.

The *in vitro* chromosome aberration test may employ cultures of established cell lines, cell strains or primary cell cultures. The cells used are selected on the basis of growth ability in culture, stability of the karyotype, chromosome number, chromosome diversity and spontaneous frequency of chromosome aberrations.

Tests conducted *in vitro* generally require the use of an exogenous source of metabolic activation. This metabolic activation system cannot mimic entirely the mammalian *in vivo* conditions. Care should be taken to avoid conditions which would lead to positive results which do not reflect intrinsic mutagenicity and may arise from changes in pH, osmolality or high levels of cytotoxicity (4)(5).

This test is used to screen for possible mammalian mutagens and carcinogens. Many compounds that are positive in this test are mammalian carcinogens; however, there is not a perfect correlation between this test and carcinogenicity. Correlation is dependent on chemical class and there is increasing evidence that there are carcinogens that are not detected by this test because they appear to act through mechanisms other than direct DNA damage.

See also General introduction Part B.

1.2. DEFINITIONS

Chromatid-type aberration: structural chromosome damage expressed as breakage of single chromatids or breakage and reunion between chromatids.

Chromosome-type aberration: structural chromosome damage expressed as breakage, or breakage and reunion, of both chromatids at an identical site.

Endoreduplication: a process in which after an S period of DNA replication, the nucleus does not go into mitosis but starts another S period. The result is chromosomes with four, eight, 16, …chromatids.

Gap: an achromatic lesion smaller than the width of one chromatid, and with minimum misalignment of the chromatids).

Mitotic index: the ratio of cells in metaphase divided by the total number of cells observed in a population of cells; an indication of the degree of proliferation of that population.

Numerical aberration: a change in the number of chromosomes from the normal number characteristic of the cells utilised.

Polyploidy: a multiple of the haploid chromosome number (*n*) other than the diploid number (i.e. 3*n*, 4*n* and so on).

Structural aberration: a change in chromosome structure detectable by microscopic examination of the metaphase stage of cell division, observed as deletions and fragments, intrachanges or interchanges.

1.3. PRINCIPLE OF THE TEST METHOD

Cell cultures are exposed to the test substance both with and without metabolic activation. At predetermined intervals after exposure of cell cultures to the test substance, they are treated with a metaphase-arresting substance (e.g. Colcemid® or colchicine), harvested, stained and metaphase cells are analysed microscopically for the presence of chromosome aberrations.

1.4. DESCRIPTION OF THE TEST METHOD

1.4.1. **Preparations**

1.4.1.1. *Cells*

A variety of cell lines, strains or primary cell cultures, including human cells, may be used (e.g. Chinese hamster fibroblasts, human or other mammalian peripheral blood lymphocytes).

1.4.1.2. *Media and culture conditions*

Appropriate culture media and incubation conditions (culture vessels, CO_2 concentration, temperature and humidity) should be used in maintaining cultures. Established cell lines and strains should be checked routinely for stability in the modal chromosome number and the absence of mycoplasma contamination and should not be used if contaminated. The normal cell cycle time for the cells and culture conditions used should be known.

1.4.1.3. *Preparation of cultures*

Established cell lines and strains: cells are propagated from stock cultures, seeded in culture medium at a density such that the cultures will not reach confluency before the time of harvest, and incubated at 37 °C.

Lymphocytes: whole blood treated with an anti-coagulant (e.g. heparin) or separated lymphocytes obtained from healthy subjects are added to the culture medium containing a mitogen (e.g. phytohaemagglutinin) and incubated at 37 °C.

1.4.1.4. *Metabolic activation*

Cells should be exposed to the test substance both in the presence and absence of an appropriate metabolic activation system. The most commonly used system is a cofactor-supplemented post-mitochondrial fraction (S9) prepared from the livers of rodents treated with enzyme-inducing agents such as: Aroclor 1254 (6)(7)(8)(9), or a mixture of phenobarbitone and ß–naphthoflavone (10)(11)(12).

The post-mitochondrial fraction is usually used at concentrations in the range from 1-10 % v/v in the final test medium. The condition of a metabolic activation system may depend upon the class of chemical being tested. In some cases it may be appropriate to utilise more than one concentration of post-mitochondrial fraction.

A number of developments, including the construction of genetically engineered cell lines expressing specific activating enzymes, may provide the potential for endogenous activation. The choice of the cell lines used should be scientifically justified (e.g. by the relevance of the cytochrome P450 isoenzyme for the metabolism of the test substance).

1.4.1.5. *Test substance/Preparation*

Solid test substances should be dissolved or suspended in appropriate solvents or vehicles and diluted if appropriate prior to treatment of the cells. Liquid test substances may be added directly to the test systems and/or diluted prior to treatment. Fresh preparations of the test substance should be employed unless stability data demonstrate the acceptability of storage.

1.4.2. **Test conditions**

1.4.2.1. *Solvent/vehicle*

The solvent/vehicle should not be suspected of chemical reaction with the test substance and should be compatible with the survival of the cells and the S9 activity. If other than well-known solvent/vehicles are used, their inclusion should be supported by data indicating their compatibility. It is recommended that wherever possible, the use of an aqueous solvent/vehicle be considered first. When testing water-unstable substances, the organic solvents used should be free of water. Water can be removed by adding a molecular sieve.

1.4.2.2. *Exposure concentrations*

Among the criteria to be considered when determining the highest concentration are cytotoxicity, solubility in the test system and changes in pH or osmolality.

Cytotoxicity should be determined with and without metabolic activation in the main experiment using an appropriate indication of cell integrity and growth, such as degree of confluency, viable cell counts, or mitotic index. It may be useful to determine cytotoxicity and solubility in a preliminary experiment.

At least three analysable concentrations should be used. Where cytotoxicity occurs, these concentrations should cover a range from the maximum to little or no toxicity; this will usually mean that the concentrations should be separated by no more than a factor between 2 and $\sqrt{10}$. At the time of harvesting, the highest concentration should show a significant reduction in degree of confluency, cell count or mitotic index, (all greater than 50 %). The mitotic index is only an indirect measure of cytotoxic/cytostatic effects and depends on the time after treatment. However, the mitotic index is acceptable for suspension cultures in which other toxicity measurements may be cumbersome and impractical. Information on cell cycle kinetics, such as average generation time (AGT), could be used as supplementary information. AGT, however, is an overall average that does not always reveal the existence of delayed subpopulations, and even slight increases in average generation time can be associated with very substantial delay in the time of optimal yield of aberrations.

For relatively non-cytotoxic substances, the maximum test concentration should be 5 µl/ml, 5 mg/ml or 0,01 M, whichever is the lowest.

For relatively insoluble substances that are not toxic at concentrations lower than the insoluble concentration, the highest dose used should be a concentration above the limit of solubility in the final culture medium at the end of the treatment period. In some cases (e.g. when toxicity occurs only at higher than the lowest insoluble concentration) it is advisable to test at more than one concentration with visible precipitation. It may be useful to assess solubility at the beginning and the end of the treatment, as solubility can change during the course of exposure in the test system due to presence of cells, S9, serum etc. Insolubility can be detected by using the unaided eye. The precipitate should not interfere with the scoring.

1.4.2.3. *Negative and positive controls*

Concurrent positive and negative (solvent or vehicle) controls, both with and without metabolic activation, should be included in each experiment. When metabolic activation is used, the positive control chemical should be the one that requires activation to give a mutagenic response.

Positive controls should employ a known clastogen at exposure levels expected to give a reproducible and detectable increase over background, which demonstrates the sensitivity of the test system.

Positive control concentrations should be chosen so that the effects are clear but do not immediately reveal the identity of the coded slides to the reader. Examples of positive control substances include:

Metabolic Activation condition	Substance	CAS No	EINECS No
Absence of exogenous metabolic Activation	Methyl methanesulphonate	66-27-3	200-625-0
	Ethyl methanesulphonate	62-50-0	200-536-7
	Ethyl nitrosourea	759-73-9	212-072-2
	Mitomycin C	50-07-7	200-008-6
	4-Nitroquinoline-*N*-oxide	56-57-5	200-281-1
Presence of exogenous metabolic Activation	Benzo[*a*]pyrene	50-32-8	200-028-5
	Cyclophosphamide Cyclophosphamide monohydrate	50-18-0 6055-19-2	200-015-4

Other appropriate positive control substances may be used. The use of chemical class-related positive control chemicals should be considered, when available.

Negative controls, consisting of solvent or vehicle alone in the treatment medium, and treated in the same way as the treatment cultures, should be included for every harvest time. In addition, untreated controls should also be used unless there are historical control data demonstrating that no deleterious or mutagenic effects are induced by the chosen solvent.

1.4.3. **Procedure**

1.4.3.1. *Treatment with the test substance*

Proliferating cells are treated with the test substance in the presence and absence of a metabolic activation system. Treatment of lymphocytes should commence at about 48 hours after mitogenic stimulation.

1.4.3.2. Duplicate cultures should normally be used at each concentration, and are strongly recommended for negative/solvent control cultures. Where minimal variation between duplicate cultures can be demonstrated (13)(14), from historical data, it may be acceptable for single cultures to be used at each concentration.

Gaseous or volatile substances should be tested by appropriate methods, such as in sealed culture vessels (15)(16).

1.4.3.3. *Culture harvest time*

In the first experiment, cells should be exposed to the test substance, both with and without metabolic activation, for three to six hours, and sampled at a time equivalent to about 1,5 normal cell cycle length after the beginning of treatment (12). If this protocol gives negative results both with and without activation, an additional experiment without activation should be done, with continuous treatment until sampling at a time equivalent to about 1,5 normal cell cycle lengths. Certain chemicals may be more readily detected by treatment/sampling times longer than 1,5 cycle lengths. Negative results with metabolic activation need to be confirmed on a case-by-case basis. In those cases where confirmation of negative results is not considered necessary, justification should be provided.

1.4.3.4. *Chromosome preparation*

Cell cultures are treated with Colcemid® or colchicine usually for one to three hours prior to harvesting. Each cell culture is harvested and processed separately for the preparation of chromosomes. Chromosome preparation involves hypotonic treatment of the cells, fixation and staining.

1.4.3.5. *Analysis*

All slides, including those of positive and negative controls, should be independently coded before microscopic analysis. Since fixation procedures often result in the breakage of a proportion of metaphase cells with loss of chromosomes, the cells scored should therefore contain a number of centromeres equal to the modal number ± 2 for all cell types. At least 200 well spread metaphases should be scored per concentration and control, equally divided amongst the duplicates, if applicable. This number can be reduced when high number of aberrations is observed.

Though the purpose of the test is to detect structural chromosome aberrations, it is important to record polyploidy and endoreduplication when these events are seen.

2. **DATA**

2.1. TREATMENT OF RESULTS

The experimental unit is the cell, and therefore the percentage of cells with structural chromosome aberration(s) should be evaluated. Different types of structural chromosome aberrations should be listed with their numbers and frequencies for experimental and control cultures. Gaps are recorded separately and reported but generally not included in the total aberration frequency.

Concurrent measures of cytotoxicity for all treated and negative control cultures in the main aberration experiments should also be recorded.

Individual culture data should be provided. Additionally, all data should be summarised in tabular form.

There is no requirement for verification of a clear positive response. Equivocal results should be clarified by further testing preferably using modification of experimental conditions. The need to confirm negative results has been discussed in 1.4.3.3. Modification of study parameters to extend the range of conditions assessed should be considered in follow-up experiments. Study parameters that might be modified include the concentration spacing and the metabolic activation conditions.

2.2. EVALUATION AND INTERPRETATION OF RESULTS

There are several criteria for determining a positive result, such as a concentration-related increase or a reproducible increase in the number of cells with chromosome aberrations. Biological relevance of the results should be considered first. Statistical methods may be used as an aid in evaluating the test results (3)(13). Statistical significance should not be the only determining factor for a positive response.

An increase in the number of polyploid cells may indicate that the test substance has the potential to inhibit mitotic processes and to induce numerical chromosome aberrations. An increase in the number of cells with endoreduplicated chromosomes may indicate that the test substance has the potential to inhibit cell cycle progression (17)(18).

A test substance for which the results do not meet the above criteria is considered non-mutagenic in this system.

Although most experiments will give clearly positive or negative results, in rare cases the data set will preclude making a definite judgement about the activity of the test substance. Results may remain equivocal or questionable regardless of the number of times the experiment is repeated.

Positive results from the *in vitro* chromosome aberration test indicate that the test substance induces structural chromosome aberrations in cultured mammalian somatic cells. Negative results indicate that, under the test conditions, the test substance does not induce chromosome aberrations in cultured mammalian somatic cells.

3. **REPORTING**

TEST REPORT

The test report must include the following information:

Solvent/Vehicle:

— justification for choice of vehicle,

— solubility and stability of the test substance in solvent/vehicle, if known.

Cells:

— type and source of cells,

— karyotype features and suitability of the cell type used,

— absence of mycoplasma, if applicable,

— information on cell cycle length,

— sex of blood donors, whole blood or separated lymphocytes, mitogen used,

— number of passages, if applicable,

— methods for maintenance of cell culture, if applicable,

— modal number of chromosomes.

Test conditions:

— identity of metaphase arresting substance, its concentration and duration of cell exposure,

— rationale for selection of concentrations and number of cultures including, e.g. cytotoxicity data and solubility limitations, if available,

— composition of media, CO_2 concentration if applicable,

— concentration of test substance,

— volume of vehicle and test substance added,

— incubation temperature,

— incubation time,

— duration of treatment,

— cell density at seeding, if appropriate,

— type and composition of metabolic activation system, including acceptability criteria,

— positive and negative controls,

— methods of slide preparation,

— criteria for scoring aberrations,

— number of metaphases analysed,

— methods for the measurements of toxicity,

— criteria for considering studies as positive, negative or equivocal.

Results:

— signs of toxicity, e.g. degree of confluency, cell cycle data, cell counts, mitotic index,

— signs of precipitation,

— data on pH and osmolality of the treatment medium, if determined,

— definition for aberrations, including gaps,

— number of cells with chromosome aberrations and type of chromosome aberrations given separately for each treated and control culture,

— changes in ploidy if seen,

— dose-response relationship, where possible,

— statistical analyses, if any,

— concurrent negative (solvent/vehicle) and positive control data,

— historical negative (solvent/vehicle) and positive control data, with ranges, means and standard deviations.

Discussion of results.

Conclusions.

4. **REFERENCES**

(1) Evans, H. J., (1976) Cytological Methods for Detecting Chemical Mutagens. In: Chemical mutagens, Principles and Methods for their Detection, Vol. 4, Hollaender, A. (ed) Plenum Press, New York and London, p. 1-29.

(2) Ishidate, M. Jr. and Sofuni, T., (1985). The *In Vitro* Chromosomal Aberration Test Using Chinese Hamster Lung (CHL) Fibroblast Cells in Culture. In: Progress in Mutation Research, Vol. 5, Ashby, J. et al., (Eds) Elsevier Science Publishers, Amsterdam-New York-Oxford, p. 427-432.

(3) Galloway, S.M., Armstrong, M.J., Reuben, C., Colman, S., Brown, B., Cannon, C., Bloom, A.D., Nakamura, F., Ahmed, M., Duk, S., Rimpo, J., Margolin, G.H., Resnick, MA, Anderson, G. and Zeiger, E., (1978). Chromosome aberration and sister chromatic exchanges in Chinese hamster ovary cells: Evaluation of 108 chemicals. Environs. Molec. Mutagen 10 (suppl. 10), p. 1-175.

(4) Scott, D., Galloway, S.M., Marshall, R.R., Ishidate, M.Jr., Brusick, D., Ashby, J. and Myhr, B.C., (1991) Genotoxicity under Extreme Culture Conditions. A report from ICPEMC Task Group 9. Mutation Res, 257, p. 147-204.

(5) Morita, T., Nagaki, T., Fukuda, I. and Okumura, K., (1992). Clastogenicity of low pH to Various Cultured Mammalian Cells. Mutation Res., 268, p. 297-305.

(6) Ames, B.N., McCann, J. and Yamasaki, E., (1975). Methods for Detecting Carcinogens and Mutagens with the Salmonella/Mammalian Microsome Mutagenicity Test. Mutation Res., 31, p. 347-364.

(7) Maron, D.M. and Ames, B.N., (1983) Revised Methods for the Salmonella Mutagenicity Test. Mutation Res., 113, p. 173-215.

(8) Natarajan, A.T., Tates, A.D., van Buul, P.P.W., Meijers, M. and de Vogel, N., (1976) Cytogenetic Effects of Mutagen/Carcinogens after Activation in a Microsomal System in Vitro, I. Induction of Chromosome Aberrations and Sister Chromatid Exchange by Diethylnitrosamine (DEN) and Dimethylnitrosamine (DMN) in CHO Cells in the Presence of Rat-Liver Microsomes. Mutation Res., 37, p. 83-90.

(9) Matsuoka, A., Hayashi, M. and Ishidate, M. Jr., (1979) Chromosomal Aberration Tests on 29 Chemicals Combined with S9 Mix *In Vitro*. Mutation Res., 66, p. 277-290.

(10) Elliot, B.M., Combes, R.D., Elcombe, C.R., Gatehouse, D.G., Gibson, G.G., Mackay, J.M. and Wolf, R.C., (1992). Report of UK Environmental Mutagen Society Working Party. Alternatives to Aroclor 1254-induced S9 in *In Vitro* Genotoxicity Assays. Mutagenesis, 7, p. 175-177.

(11) Matsushima, T., Sawamura, M., Hara, K. and Sugimura, T., (1976) A Safe Substitute for Polychlorinated Biphenyls as an Inducer of Metabolic Activation Systems. In: de Serres, F.J., Fouts, J.R., Bend, J.R. and Philpot, R.M. (eds) *In Vitro* Metabolic Activation in Mutagenesis Testing, Elsevier, North-Holland, p. 85-88.

(12) Galloway, S.M., Aardema, M.J., Ishidate, M.Jr., Ivett, J.L., Kirkland, D.J., Morita, T., Mosesso, P., Sofuni, T., (1994) Report from Working Group on in *In Vitro* Tests for Chromosomal Aberrations. Mutation Res., 312, p. 241-261.

(13) Richardson, C., Williams, D.A., Allen, J.A., Amphlett, G., Chanter, D.O. and Phillips, B., (1989) Analysis of Data from *In Vitro* Cytogenetic Assays. In: Statistical Evaluation of Mutagenicity Test Data. Kirkland, D.J., (ed) Cambridge University Press, Cambridge, p. 141-154.

(14) Soper, K.A. and Galloway, S.M., (1994) Replicate Flasks are not Necessary for *In Vitro* Chromosome Aberration Assays in CHO Cells. Mutation Res., 312, p. 139-149.

(15) Krahn, D.F., Barsky, F.C. and McCooey, K.T., (1982) CHO/HGPRT Mutation Assay: Evaluation of Gases and Volatile Liquids. In: Tice, R.R., Costa, D.L., Schaich, K.M. (eds). Genotoxic Effects of Airborne Agents. New York, Plenum, p. 91-103.

(16) Zamora, P.O., Benson, J.M., Li, A.P. and Brooks, A.L., (1983) Evaluation of an Exposure System Using Cells Grown on Collagen Gels for Detecting Highly Volatile Mutagens in the CHO/HGPRT Mutation Assay. Environmental Mutagenesis, 5, p. 795-801.

(17) Locke-Huhle, C., (1983) Endoreduplication in Chinese hamster cells during alpha-radiation induced G2 arrest. Mutation Res., 119, p. 403-413.

(18) Huang, Y., Change, C. and Trosko, J.E., (1983) Aphidicolin-induced endoreduplication in Chinese hamster cells. Cancer Res., 43, p. 1362-1364.

B.11. **MUTAGENICITY — *IN VIVO* MAMMALIAN BONE MARROW CHROMOSOME ABERRATION TEST**

1. **METHOD**

This method is a replicate of the OECD TG 475, Mammalian Bone Marrow Chromosome Aberration Test (1997).

1.1. INTRODUCTION

The mammalian *in vivo* chromosome aberration test is used for the detection of structural chromosome aberrations induced by the test substance to the bone marrow cells of animals, usually rodents (1)(2)(3)(4). Structural chromosome aberrations may be of two types, chromosome or chromatid. An increase in polyploidy may indicate that a chemical has the potential to induce numerical aberrations. With the majority of chemical mutagens, induced aberrations are of the chromatid-type, but chromosome-type aberrations also occur. Chromosome mutations and related events are the cause of many human genetic diseases and there is substantial evidence that chromosome mutations and related events causing alterations in oncogenes and tumour-suppressor genes are involved in cancer in humans and experimental systems.

Rodents are routinely used in this test. Bone marrow is the target tissue in this test, since it is a highly vascularised tissue, and it contains a population of rapidly cycling cells that can be readily isolated and processed. Other species and target tissues are not the subject of this method.

This chromosome aberration test is especially relevant to assessing mutagenic hazard in that it allows consideration of factors of *in vivo* metabolism, pharmacokinetics and DNA-repair processes although these may vary among species and among tissues. An *in vivo* test is also useful for further investigation of a mutagenic effect detected by *in vitro* test.

If there is evidence that the test substance, or a reactive metabolite, will not reach the target tissue, it is not appropriate to use this test.

See also General introduction Part B.

1.2. DEFINITIONS

Chromatid-type aberration: structural chromosome damage expressed as breakage of single chromatids or breakage and reunion between chromatids.

Chromosome-type aberration: structural chromosome damage expressed as breakage, or breakage and reunion, of both chromatids at an identical site.

Endoreduplication: a process in which after an S period of DNA replication, the nucleus does not go into mitosis but starts another S period. The result is chromosomes with four, eight, 16, ...chromatids.

Gap: an achromatic lesion smaller than the width of one chromatid, and with minimum misalignment of the chromatid(s).

Numerical aberration: a change in the number of chromosomes from the normal number characteristic of the cells utilised.

Polyploidy: a multiple of the haploid chromosome number (*n*) other than the diploid number (i.e. 3*n*, 4*n* and so on).

Structural aberration: a change in chromosome structure detectable by microscopic examination of the metaphase stage of cell division, observed as deletions and fragments, intrachanges or interchanges.

1.3. PRINCIPLE OF THE TEST METHOD

Animals are exposed to the test substance by an appropriate route of exposure and are sacrificed at appropriate times after treatment. Prior to sacrifice, animals are treated with a metaphase arresting agent (e.g. Colcemid® or colchicine). Chromosome preparations are then made from the bone marrow cells and stained, and metaphase cells are analysed for chromosome aberrations.

1.4. DESCRIPTION OF THE TEST METHOD

1.4.1. **Preparations**

1.4.1.1. *Selection of animal species*

Rats, mice and Chinese hamsters are commonly used, although any appropriate mammalian species may be used. Commonly used laboratory strains of young healthy adult animals should be employed. At the commencement of the study, the weight variation of animals should be minimal and not exceed ± 20 % of the mean weight of each sex.

1.4.1.2. *Housing and feeding conditions*

General conditions referred in the General introduction to Part B are applied although the aim for humidity should be 50-60 %.

1.4.1.3. *Preparation of the animals*

Healthy young adult animals are randomly assigned to the control and treatment groups. Cages should be arranged in such a way that possible effects due to cage placement are minimised. The animals are identified uniquely. The animals are acclimated to the laboratory conditions for at least five days.

1.4.1.4. *Preparation of doses*

Solid test substances should be dissolved or suspended in appropriate solvents or vehicles and diluted, if appropriate, prior to dosing of the animals. Liquid test substances may be dosed directly or diluted prior to dosing. Fresh preparations of the test substance should be employed unless stability data demonstrate the acceptability of storage.

1.4.2. **Test conditions**

1.4.2.1. *Solvent/Vehicle*

The solvent/vehicle should not produce toxic effects at the dose levels used, and should not be suspected of chemical reaction with the test substance. If other than well-known solvents/vehicles are used, their inclusion should be supported with data indicating their compatibility. It is recommended that wherever possible, the use of an aqueous solvent/vehicle should be considered first.

1.4.2.2. *Controls*

Concurrent positive and negative (solvent/vehicle) controls should be included for each sex in each test. Except for treatment with the test substance, animals in the control groups should be handled in an identical manner to the animals in the treated groups.

Positive controls should produce structural aberrations *in vivo* at exposure levels expected to give a detectable increase over background. Positive control doses should be chosen so that the effects are clear but do not immediately reveal the identity of the coded slides to the reader. It is acceptable that the positive control be administered by a route different from the test substance and sampled at only a single time. The use of chemical class related positive control chemicals may be considered, when available. Examples of positive control substances include:

Substance	CAS No	EINECS No
Ethyl methanesulphonate	62-50-0	200-536-7
Ethyl nitrosourea	759-73-9	212-072-2
Mitomycin C	50-07-7	200-008-6
Cyclophosphamide Cyclophosphamide monohydrate	50-18-0 6055-19-2	200-015-4
Triethylenemelamine	51-18-3	200-083-5

Negative controls, treated with solvent or vehicle alone, and otherwise treated in the same way as the treatment groups, should be included for every sampling time, unless acceptable inter-animal variability and frequencies of cells with chromosome aberrations are available from historical control data. If single sampling is applied for negative controls, the most appropriate time is the first sampling time. In addition, untreated controls should also be used unless there are historical or published control data demonstrating that no deleterious or mutagenic effects are induced by the chosen solvent/vehicle.

1.5. PROCEDURE

1.5.1. **Number and sex of animals**

Each treated and control group must include at least five analysable animals per sex. If at the time of the study there are data available from studies in the same species and using the same route of exposure that demonstrate that there are no substantial differences in toxicity between sexes, then testing in a single sex will be sufficient. Where human exposure to chemicals may be sex-specific, as for example with some pharmaceutical agents, the test should be performed with animals of the appropriate sex.

1.5.2. **Treatment schedule**

Test substances are preferably administered as a single treatment. Test substances may also be administered as a split dose, i.e. two treatments on the same day separated by no more than a few hours, to facilitate administering a large volume of material. Other dose regimens should be scientifically justified.

Samples should be taken at two separate times following treatment on one day. For rodents, the first sampling interval is 1,5 normal cell cycle length (the latter being normally 12-18 hr) following treatment. Since the time required for uptake and metabolism of the test substance as well as its effect on cell cycle kinetics can affect the optimum time for chromosome aberration detection, a later sample collection 24 hr after the first sample time is recommended. If dose regimens of more than one day are used, one sampling time at 1,5 normal cell cycle lengths after the final treatment should be used.

Prior to sacrifice, animals are injected intraperitoneally with an appropriate dose of a metaphase arresting agent (e.g. Colcemid® or colchicine). Animals are sampled at an appropriate interval thereafter. For mice this interval is approximately three to five hours; for Chinese hamsters this interval is approximately four to five hours. Cells are harvested from the bone marrow and analysed for chromosome aberrations.

1.5.3. **Dose levels**

If a range finding study is performed because there are no suitable data available, it should be performed in the same laboratory, using the same species, strain, sex and treatment regimen to be used in the main study (5). If there is toxicity, three dose levels are used for the first sampling time. These dose levels should cover a range from the maximum to little or no toxicity. At the later sampling time only the highest dose needs to be used. The highest dose is defined as the dose producing signs of toxicity such that higher dose levels, based on the same dosing regimen, would be expected to produce lethality. Substances with specific biological activities at low non-toxic doses (such as hormones and mitogens) may be exceptions to the dose-setting criteria and should be evaluated on a case-by-case basis. The highest dose may also be defined as a dose that produces some indication of toxicity in the bone marrow (e.g. greater than 50 % reduction in mitotic index).

1.5.4. **Limit test**

If a test at one dose level of at least 2 000 mg/kg body weight using a single treatment, or as two treatments on the same day, produces no observable toxic effects, and if genotoxicity would not be expected based on data from structurally related substances, then a full study using three dose levels may not be considered necessary. For studies of a longer duration, the limit dose is 2 000 mg/kg/body weight/day for treatment up to 14 days, and 1 000 mg/kg/body weight/day for treatment longer than 14 days. Expected human exposure may indicate the need for a higher dose level to be used in the limit test.

1.5.5. **Administration of doses**

The test substance is usually administered by gavage using a stomach tube or a suitable intubation cannula, or by intraperitoneal injection. Other routes of exposure may be acceptable where they can be justified. The maximum volume of liquid that can be administered by gavage or injection at one time depends on the size of the test animal. The volume should not exceed 2 ml/100 g body weight. The use of volumes higher than these

must be justified. Except for irritating or corrosive substances which will normally reveal exacerbated effects with higher concentrations, variability in test volume should be minimised by adjusting the concentration to ensure a constant volume at all dose levels.

1.5.6. **Chromosome preparation**

Immediately after sacrifice, bone marrow is obtained, exposed to hypotonic solution and fixed. The cells are then spread on slides and stained.

1.5.7. **Analysis**

The mitotic index should be determined as a measure of cytotoxicity in at least 1 000 cells per animal for all treated animals (including positive controls) and untreated negative control animals.

At least 100 cells should be analysed for each animal. This number could be reduced when high numbers of aberrations are observed. All slides, including those of positive and negative controls, should be independently coded before microscopic analysis. Since slide preparation procedures often result in the breakage of a proportion of metaphases with loss of chromosomes, the cells scored should therefore contain a number of centromeres equal to the number 2n ± 2.

2. **DATA**

2.1. TREATMENT OF RESULTS

Individual animal data should be presented in tabular form. The experimental unit is the animal. For each animal the number of cells scored, the number of aberrations per cell and the percentage of cells with structural chromosome aberration(s) should be evaluated. Different types of structural chromosome aberrations should be listed with their numbers and frequencies for treated and control groups. Gaps are recorded separately and reported but generally not included in the total aberration frequency. If there is no evidence for a difference in response between the sexes, the data from both sexes may be combined for statistical analysis.

2.2. EVALUATION AND INTERPRETATION OF RESULTS

There are several criteria for determining a positive result, such as a dose-related increase in the relative number of cells with chromosome aberrations or a clear increase in the number of cells with aberrations in a single dose group at a single sampling time. Biological relevance of the results should be considered first. Statistical methods may be used as an aid in evaluating the test results (6). Statistical significance should not be the only determining factor for a positive response. Equivocal results should be clarified by further testing preferably using a modification of experimental conditions.

An increase in polyploidy may indicate that the test substance has the potential to induce numerical chromosome aberrations. An increase in endoreduplication may indicate that the test substance has the potential to inhibit cell cycle progression (7)(8).

A test substance for which the results do not meet the above criteria is considered non-mutagenic in this test.

Although most experiments will give clearly positive or negative results, in rare cases the data set will preclude making a definite judgement about the activity of the test substance. Results may remain equivocal or questionable regardless of the number of experiments performed.

Positive results from the *in vivo* chromosome aberration test indicate that a substance induces chromosome aberrations in the bone marrow of the species tested. Negative results indicate that, under the test conditions, the test substance does not induce chromosome aberrations in the bone marrow of the species tested.

The likelihood that the test substance or its metabolites reach the general circulation or specifically the target tissue (e.g. systemic toxicity) should be discussed.

3. **REPORTING**

3.1. TEST REPORT

The test report must include the following information:

Solvent/Vehicle:

— justification for choice of vehicle,

— solubility and stability of the test substance in solvent/vehicle, if known,

Test animals:

— species/strain used,

— number, age and sex of animals,

— source, housing conditions, diet, etc.,

— individual weight of the animals at the start of the test, including body weight range, mean and standard deviation for each group,

Test conditions:

— positive and negative (vehicle/solvent) controls,

— data from range-finding study, if conducted,

— rationale for dose level selection,

— details of test substance preparation,

— details of the administration of the test substance,

— rationale for route of administration,

— methods for verifying that the test substance reached the general circulation or target tissue, if applicable,

— conversion from diet/drinking water test substance concentration (ppm) to the actual dose (mg/kg body weight/day), if applicable,

— details of food and water quality,

— detailed description of treatment and sampling schedules,

— methods for measurements of toxicity,

— identity of metaphase arresting substance, its concentration and duration of treatment,

— methods of slide preparation,

— criteria for scoring aberrations,

— number of cells analysed per animal,

— criteria for considering studies as positive, negative or equivocal.

Results:

— signs of toxicity,

— mitotic index,

— type and number of aberrations, given separately for each animal,

— total number of aberrations per group with means and standard deviations,

— number of cells with aberrations per group with means and standard deviations,

— changes in ploidy, if seen,

— dose-response relationship, where possible,

— statistical analyses, if any,

— concurrent negative control data,

— historical negative control data with ranges, means and standard deviations,

— concurrent positive control data.

Discussion of the results.

Conclusions.

4. **REFERENCES**

(1) Adler, I.D., (1984) Cytogenetic Tests in Mammals. In: Mutagenicity Testing: a Practical Approach. S. Venitt and J.M. Parry (Eds). IRL Press, Oxford, Washington D.C., p. 275-306.

(2) Preston, R.J., Dean, B.J., Galloway, S., Holden, H., McFee, A.F. and Shelby, M. (1987). Mammalian *In Vivo* Cytogenetic Assays: Analysis of Chromosome Aberrations in Bone Marrow Cells. Mutation Res., 189, p. 157-165.

(3) Richold, M., Chandley, A., Ashby, J., Gatehouse, D.G., Bootman, J. and Henderson, L., (1990) *In Vivo* Cytogenetic Assays. In: D.J. Kirkland (Ed.) Basic Mutagenicity Tests, UKEMS Recommended Procedures. UKEMS Sub-Committee on Guidelines for Mutagenicity Testing. Report. Part I revised. Cambridge University Press, Cambridge, New York, Port Chester, Melbourne, Sydney, p. 115-141.

(4) Tice, R.R., Hayashi, M., MacGregor, J.T., Anderson, D., Blakey, D.H., Holden, H.E., Kirsch-Volders, M., Oleson Jr., F.B., Pacchierotti, F., Preston, R.J., Romagna, F., Shimada, H., Sutou, S. and Vannier, B., (1994) Report from the Working Group on the *In Vivo* Mammalian Bone Marrow Chromosomal Aberration Test. Mutation Res., 312, p. 305-312.

(5) Fielder, R.J., Allen, J.A., Boobis, A.R., Botham, P.A., Doe, J., Esdaile, D.J., Gatehouse, D.G., Hodson-Walker, G., Morton, D.B., Kirkland, D.J. and Richold, M. (1992). Report of British Toxicology Society/UK Environmental Mutagen Society Working Group: Dose setting in *In vivo* Mutagenicity Assays. Mutagenesis, 7, p. 313-319.

(6) Lovell, D.P., Anderson, D., Albanese, R., Amphlett, G.E., Clare, G., Ferguson, R., Richold, M., Papworth, D.G. and Savage, J.R.K. (1989). Statistical Analysis of *In Vivo* Cytogenetic Assays. In: UKEMS Sub-Committee on Guidelines for Mutagenicity Testing. Report Part III. Statistical Evaluation of Mutagenicity Test Data. D.J. Kirkland, (Ed.) Cambridge University Press, Cambridge. p. 184-232.

(7) Locke-Huhle, C., (1983) Endoreduplication in Chinese hamster cells during alpha-radiation induced G2 arrest. Mutation Res. 119, p. 403-413.

(8) Huang, Y., Change, C. and Trosko, J.E., (1983) Aphidicolin-induced endoreduplication in Chinese hamster cells. Cancer Res., 43, p. 1362-1364.

B.12. MUTAGENICITY — *IN VIVO* MAMMALIAN ERYTHROCYTE MICRONUCLEUS TEST

1. METHOD

This method is a replicate of the OECD TG 474, Mammalian Erythrocyte Micronucleus Test (1997).

1.1. INTRODUCTION

The mammalian *in vivo* micronucleus test is used for the detection of damage induced by the test substance to the chromosomes or the mitotic apparatus of erythroblasts by analysis of erythrocytes as sampled in bone marrow and/or peripheral blood cells of animals, usually rodents.

The purpose of the micronucleus test is to identify substances that cause cytogenetic damage, which results in the formation of micronuclei containing lagging chromosome fragments or whole chromosomes.

When a bone marrow erythroblast develops into a polychromatic erythrocyte, the main nucleus is extruded; any micronucleus that has been formed may remain behind in the otherwise anucleated cytoplasm. Visualisation of micronuclei is facilitated in these cells because they lack a main nucleus. An increase in the frequency of micronucleated polychromatic erythrocytes in treated animals is an indication of induced chromosome damage.

The bone marrow of rodents is routinely used in this test since polychromatic erythrocytes are produced in that tissue. The measurement of micronucleated immature (polychromatic) erythrocytes in peripheral blood is equally acceptable in any species in which the inability of the spleen to remove micronucleated erythrocytes has been demonstrated, or which has shown an adequate sensitivity to detect agents that cause structural or numerical chromosome aberrations. Micronuclei can be distinguished by a number of criteria. These include identification of the presence or absence of a kinetochore or centromeric DNA in the micronuclei. The frequency of micronucleated immature (polychromatic) erythrocytes is the principal endpoint. The number of mature (normochromatic) erythrocytes in the peripheral blood that contain micronuclei among a given number of mature erythrocytes can also be used as the endpoint of the assay when animals are treated continuously for four weeks or more.

This mammalian *in vivo* micronucleus test is especially relevant to assessing mutagenic hazard in that it allows consideration of factors of *in vivo* metabolism, pharmacokinetics and DNA-repair processes although these may vary among species, among tissues and among genetic endpoints. An *in vivo* assay is also useful for further investigation of a mutagenic effect detected by an *in vitro* system.

If there is evidence that the test substance, or a reactive metabolite, will not reach the target tissue, it is not appropriate to use this test.

See also General introduction Part B.

1.2. DEFINITIONS

Centromere (Kinetochore): region(s) of a chromosome with which spindle fibers are associated during cell division, allowing orderly movement of daughter chromosomes to the poles of the daughter cells.

Micronuclei: small nuclei, separate from and additional to the main nuclei of cells, produced during telophase of mitosis (meiosis) by lagging chromosome fragments or whole chromosomes.

Normochromatic erythrocyte: mature erythrocyte that lacks ribosomes and can be distinguished from immature, polychromatic erythrocytes by stains selective for ribosomes.

Polychromatic erythrocyte: immature erythrocyte, in an intermediate stage of development, that still contains ribosomes and therefore can be distinguished from mature, normochromatic erythrocytes by stains selective for ribosomes.

1.3. PRINCIPLE OF THE TEST METHOD

Animals are exposed to the test substance by an appropriate route. If bone marrow is used, the animals are sacrificed at appropriate times after treatment, the bone marrow extracted, and preparations made and stained (1)(2)(3)(4)(5)(6)(7). When peripheral blood is used, the blood is collected at appropriate times after treatment and smear preparations are made and stained (4)(8)(9)(10). For studies with peripheral blood, as little time as possible should elapse between the last exposure and cell harvest. Preparations are analysed for the presence of micronuclei.

1.4. DESCRIPTION OF THE TEST METHOD

1.4.1. **Preparations**

1.4.1.1. *Selection of animal species*

Mice or rats are recommended if bone marrow is used, although any appropriate mammalian species may be used. When peripheral blood is used, mice are recommended. However, any appropriate mammalian species may be used provided it is a species in which the spleen does not remove micronucleated erythrocytes or a species, which has shown an adequate sensitivity to detect agents that cause structural or numerical chromosome aberrations. Commonly used laboratory strains of young healthy animals should be employed. At the commencement of the study, the weight variation of animals should be minimal and not exceed ±20 % of the mean weight of each sex.

1.4.1.2. *Housing and feeding conditions*

General conditions referred in the General introduction to Part B are applied although the aim for humidity should be 50-60 %.

1.4.1.3. *Preparation of the animals*

Healthy young adult animals are randomly assigned to the control and treatment groups. The animals are identified uniquely. The animals are acclimated to the laboratory conditions for at least five days. Cages should be arranged in such a way that possible effects due to cage placement are minimised.

1.4.1.4. *Preparation of doses*

Solid test substances should be dissolved or suspended in appropriate solvents or vehicles and diluted, if appropriate, prior to dosing of the animals. Liquid test substances may be dosed directly or diluted prior to dosing. Fresh preparations of the test substance should be employed unless stability data demonstrate the acceptability of storage.

1.4.2. **Test conditions**

1.4.2.1. *Solvent/Vehicle*

The solvent/vehicle should not produce toxic effects at the dose levels used, and should not be suspected of chemical reaction with the test substance. If other than well-known solvents/vehicles are used, their inclusion should be supported with reference data indicating their compatibility. It is recommended that wherever possible, the use of an aqueous solvent/vehicle should be considered first.

1.4.2.2. *Controls*

Concurrent positive and negative (solvent/vehicle) controls should be included for each sex in each test. Except for treatment with the test substance, animals in the control groups should be handled in an identical manner to animals of the treatment groups.

Positive controls should produce micronuclei *in vivo* at exposure levels expected to give a detectable increase over background. Positive control doses should be chosen so that the effects are clear but do not immediately reveal the identity of the coded slides to the reader. It is acceptable that the positive control be administered by a route different from the test substance and sampled at only a single time. In addition, the use of chemical class-related positive control chemicals may be considered, when available. Examples of positive control substances include:

Substance	CAS No	EINECS No
Ethyl methanesulphonate	62-50-0	200-536-7
N-ethyl-N-nitrosourea	759-73-9	212-072-2
Mitomycin C	50-07-7	200-008-6
Cyclophosphamide Cyclophosphamide monohydrate	50-18-0 6055-19-2	200-015-4
Triethylenemelamine	51-18-3	200-083-5

Negative controls, treated with solvent or vehicle alone, and otherwise treated in the same way as the treatment groups should be included for every sampling time, unless acceptable inter-animal variability and frequencies of cells with micronuclei are demonstrated by historical control data. If single sampling is applied for negative controls, the most appropriate time is the first sampling time. In addition, untreated controls should also be used unless there are historical or published control data demonstrating that no deleterious or mutagenic effects are induced by the chosen solvent/vehicle.

If peripheral blood is used, a pre-treatment sample may also be acceptable as a concurrent negative control, but only in the short peripheral blood studies (e.g. 1-3 treatment(s)) when the resulting data are in the expected range for the historical control.

1.5. PROCEDURE

1.5.1. **Number and sex of animals**

Each treated and control group must include at least five analysable animals per sex (11). If at the time of the study there are data available from studies in the same species and using the same route of exposure that demonstrate that there are no substantial differences between sexes in toxicity, then testing in a single sex will be sufficient. Where human exposure to chemicals may be sex-specific, as for example with some pharmaceutical agents, the test should be performed with animals of the appropriate sex.

1.5.2. **Treatment schedule**

No standard treatment schedule (i.e. one, two or more treatments at 24 h intervals) can be recommended. The samples from extended dose regimens are acceptable as long as a positive effect has been demonstrated for this study or, for a negative study, as long as toxicity has been demonstrated or the limit dose has been used, and dosing continued until the time of sampling. Test substances may also be administered as a split dose, i.e., two treatments on the same day separated by no more than a few hours, to facilitate administering a large volume of material.

The test may be performed in two ways:

(a) animals are treated with the test substance once. Samples of bone marrow are taken at least twice, starting not earlier than 24 hours after treatment, but not extending beyond 48 hours after treatment with appropriate intervals between samples. The use of sampling times earlier than 24 hours after treatment should be justified. Samples of peripheral blood are taken at least twice, starting not earlier than 36 hours after treatment, with appropriate intervals following the first sample, but not extending beyond 72 hours. When a positive response is recognised at one sampling time, additional sampling is not required.

(b) if two or more daily treatments are used (e.g. two or more treatments at 24 hour intervals), samples should be collected once between 18 and 24 hours following the final treatment for the bone marrow and once between 36 and 48 hours following the final treatment for the peripheral blood (12);

Other sampling times may be used in addition, when relevant.

1.5.3. **Dose levels**

If a range finding study is performed because there are no suitable data available, it should be performed in the same laboratory, using the same species, strain, sex, and treatment regimen to be used in the main study (13). If there is toxicity, three dose levels are used for the first sampling time. These dose levels should cover a range from the maximum to little or no toxicity. At the later sampling time only the highest dose needs to be used. The highest dose is defined as the dose producing signs of toxicity such that higher dose levels, based on the same dosing regimen, would be expected to produce lethality. Substances with specific biological activities at low non-toxic doses (such as hormones and mitogens) may be exceptions to the dose-setting criteria and should be evaluated on a case-by-case basis. The highest dose may also be defined as a dose that produces some indication of toxicity in the bone marrow (e.g. a reduction in the proportion of immature erythrocytes among total erythrocytes in the bone marrow or peripheral blood).

1.5.4. **Limit test**

If a test at one dose level of at least 2 000 mg/kg body weight using a single treatment, or as two treatments on the same day, produces no observable toxic effects, and if genotoxicity would not be expected based upon data from structurally related substances, then a full study using three dose levels may not be considered necessary. For studies of a longer duration, the limit dose is 2 000 mg/kg/body weight/day for treatment up to 14 days, and 1 000 mg/kg/body weight/day for treatment longer than 14 days. Expected human exposure may indicate the need for a higher dose level to be used in the limit test.

1.5.5. **Administration of doses**

The test substance is usually administered by gavage using a stomach tube or a suitable intubation cannula, or by intraperitoneal injection. Other routes of exposure may be acceptable where they can be justified. The maximum volume of liquid that can be administered by gavage or injection at one time depends on the size of the test animal. The volume should not exceed 2 ml/100 g body weight. The use of volumes higher than these must be justified. Except for irritating or corrosive substances, which will normally reveal exacerbated effects with higher concentrations, variability in test volume should be minimised by adjusting the concentration to ensure a constant volume at all dose levels.

1.5.6. **Bone marrow/blood preparation**

Bone marrow cells are usually obtained from the femurs or tibias immediately following sacrifice. Commonly, cells are removed from femurs or tibias, prepared and stained using established methods. Peripheral blood is obtained from the tail vein or other appropriate blood vessel. Blood cells are immediately stained supravitally (8)(9)(10) or smear preparations are made and then stained. The use of a DNA specific stain (e.g. acridine orange (14) or Hoechst 33258 plus pyronin-Y (15)) can eliminate some of the artifacts associated with using a non DNA specific stain. This advantage does not preclude the use of conventional stains (e.g. Giemsa). Additional systems (e.g. cellulose columns to remove nucleated cells (16)) can also be used provided that these systems have been shown to adequately work for micronucleus preparation in the laboratory.

1.5.7. **Analysis**

The proportion of immature among total (immature + mature) erythrocytes is determined for each animal by counting a total of at least 200 erythrocytes for bone marrow and 1 000 erythrocytes for peripheral blood (17). All slides, including those of positive and negative controls, should be independently coded before microscopic analysis. At least 2 000 immature erythrocytes per animal are scored for the incidence of micronucleated immature erythrocytes. Additional information may be obtained by scoring mature erythrocytes for micronuclei. When analysing slides, the proportion of immature erythrocytes among total erythrocytes should not be less than 20 % of the control value. When animals are treated continuously for four weeks or more, at least 2 000 mature erythrocytes per animal can also be scored for the incidence of micronuclei. Systems for automated analysis (image analysis and cell suspensions flow cytometry) are acceptable alternatives to manual evaluation if appropriately justified and validated.

2. DATA

2.1. TREATMENT OF RESULTS

Individual animal data should be presented in tabular form. The experimental unit is the animal. The number of immature erythrocytes scored, the number of micronucleated immature erythrocytes, and the number of immature among total erythrocytes should be listed separately for each animal analysed. When animals are treated continuously for four weeks or more, the data on mature erythrocytes should also be given if it is collected. The proportion of immature among total erythrocytes and, if considered applicable, the percentage of micronucleated erythrocytes is given for each animal. If there is no evidence for a difference in response between the sexes, the data from both sexes may be combined for statistical analysis.

2.2. EVALUATION AND INTERPRETATION OF RESULTS

There are several criteria for determining a positive result, such as a dose-related increase in the number of micronucleated cells or a clear increase in the number of micronucleated cells in a single dose group at a single sampling time. Biological relevance of the results should be considered first. Statistical methods may be used as an aid in evaluating the test results (18)(19). Statistical significance should not be the only determining factor for a positive response. Equivocal results should be clarified by further testing preferably using a modification of experimental conditions.

A test substance for which the results do not meet the above criteria is considered non-mutagenic in this test.

Although most experiments will give clearly positive or negative results, in rare cases the data set will preclude making a definite judgement about the activity of the test substance. Results may remain equivocal or questionable regardless of the number of times the experiment is repeated.

Positive results in the micronucleus test indicate that the substance induces micronuclei which are the result of chromosomal damage or damage to the mitotic apparatus in the erythroblasts of the test species. Negative results indicate that, under the test conditions, the test substance does not produce micronuclei in the immature erythrocytes of the test species.

The likelihood that the test substance or its metabolites reach the general circulation or specifically the target tissue (e.g. systemic toxicity) should be discussed.

3. REPORTING

TEST REPORT

The test report should include the following information:

Solvent/Vehicle:

— justification for choice of vehicle,

— solubility and stability of the test substance in solvent/vehicle, if known.

Test animals:

— species/strain used,

— number, age and sex of animals,

— source, housing conditions, diet, etc.,

— individual weight of the animals at the start of the test, including body weight range, mean and standard deviation for each group.

Test conditions:

— positive and negative (vehicle/solvent) control data,

— data from range-finding study, if conducted,

— rationale for dose level selection,

— details of test substance preparation,

— details of the administration of the test substance,

— rationale for route of administration,

— methods for verifying that the test substance reached the general circulation or target tissue, if applicable,

— conversion from diet/drinking water test substance concentration (ppm) to the actual dose (mg/kg body weight/day), if applicable,

— details of food and water quality,

— detailed description of treatment and sampling schedules,

— methods of slide preparation,

— methods for measurements of toxicity,

— criteria for scoring micronucleated immature erythrocytes,

— number of cells analysed per animal,

— criteria for considering studies as positive, negative or equivocal.

Results:

— signs of toxicity,

— proportion of immature erythrocytes among total erythrocytes,

— number of micronucleated immature erythrocytes, given separately for each animal,

— mean ± standard deviation of micronucleated immature erythrocytes per group,

— dose-response relationship, where possible,

— statistical analyses and methods applied,

— concurrent and historical negative control data,

— concurrent positive control data.

— Discussion of the results.

— Conclusions.

4. **REFERENCES**

(1) Heddle, J.A., (1973) A Rapid *In Vivo* Test for Chromosomal Damage, Mutation Res., 18, p. 187-190.

(2) Schmid, W., (1975) The Micronucleus Test, Mutation Res., 31, p. 9-15.

(3) Heddle, J.A., Salamone, M.F., Hite, M., Kirkhart, B., Mavournin, K., MacGregor, J.G. and Newell, G.W. (1983). The Induction of Micronuclei as a Measure of Genotoxicity. Mutation Res., 123, p. 61-118.

(4) Mavournin, K.H., Blakey, D.H., Cimino, M.C., Salamone, M.F. and Heddle, J.A., (1990) The *In Vivo* Micronucleus Assay in Mammalian Bone Marrow and Peripheral Blood. A report of the U.S. Environmental Protection Agency Gene-Tox Program, Mutation Res., 239, p. 29-80.

(5) MacGregor, J.T., Schlegel, R. Choy, W.N., and Wehr, C.M., (1983) Micronuclei in Circulating Erythrocytes: A Rapid Screen for Chromosomal Damage During Routine Toxicity Testing in Mice. Iin: 'Developments in Science and Practice of Toxicology', Ed. A.W. Hayes, R.C. Schnell and T.S. Miya, Elsevier, Amsterdam, p. 555-558.

(6) MacGregor, J.T., Heddle, J.A. Hite, M., Margolin, G.H., Ramel, C., Salamone, M.F., Tice, R.R., and Wild, D., (1987) Guidelines for the Conduct of Micronucleus Assays in Mammalian Bone Marrow Erytrocytes. Mutation Res., 189, p. 103-112.

(7) MacGregor, J.T., Wehr, C.M., Henika, P.R., and Shelby, M.E., (1990) The *in vivo* Erythrocyte Micronucleus Test: Measurement at Steady State Increases Assay Efficiency and Permits Integration with Toxicity Studies. Fund. Appl. Toxicol., 14, p. 513-522.

(8) Hayashi, M., Morita, T., Kodama, Y., Sofuni, T. and Ishidate, M. Jr., (1990) The Micronucleus Assay with Mouse Peripheral Blood Reticulocytes Using Acridine Orange-Coated Slides. Mutation Res., 245, p. 245-249.

(9) The Collaborative Study Group for the Micronucleus Test, (1992) Micronucleus Test with Mouse Peripheral Blood Erythrocytes by Acridine Orange Supravital Staining: The Summary Report of the 5th Collaborative Study by CSGMT/JEMMS. MMS. Mutation Res., p. 278, 83-98.

(10) The Collaborative Study Group for the Micronucleus Test (CSGMT/JEMMS. MMS: The Mammalian Mutagenesis Study Group of the Environmental Mutagen Society of Japan), (1995) Protocol recommended for the short-term mouse peripheral blood micronucleus test. Mutagenesis, 10, p. 153-159.

(11) Hayashi, M., Tice, R.R., MacGregor, J.T., Anderson, D., Blackey, D.H., Kirsch-Volders, M., Oleson, Jr. F. B., Pacchierotti, F., Romagna, F., Shimada, H., Sutou, S. and Vannier, B., (1994) *In Vivo* Rodent Erythrocyte Micronucleus Assay. Mutation Res., 312, p. 293-304.

(12) Higashikuni, N. and Sutou, S., (1995) An optimal, generalised sampling time of 30 ± 6 h after double dosing in the mouse peripheral blood micronucleus test. Mutagenesis, 10, p. 313-319.

(13) Fielder, R.J., Allen, J.A., Boobis, A.R., Botham, P.A., Doe, J., Esdaile, D.J., Gatehouse, D.G., Hodson-Walker, G., Morton, D.B., Kirkland, D.J. and Rochold, M., (1992) Report of British Toxicology Society/UK Environmental Mutagen Society Working Group: Dose Setting in *In Vivo* Mutagenicity Assays. Mutagenesis, 7, p. 313-319.

(14) Hayashi, M., Sofuni, T. and Ishidate, M. Jr., (1983) An Application of Acridine Orange Fluorescent Staining to the Micronucleus Test. Mutation Res., 120, p. 241-247.

(15) MacGregor, J.T., Wehr, C.M. and Langlois, R.G., (1983) A Simple Fluorescent Staining Procedure for Micronuclei and RNA in Erythrocytes Using Hoechst 33258 and Pyronin Y. Mutation Res., 120, p. 269-275.

(16) Romagna, F. and Staniforth, C.D., (1989) The automated bone marrow micronucleus test. Mutation Res., 213, p. 91-104.

(17) Gollapudi, B. and McFadden, L.G., (1995) Sample size for the estimation of polychromatic to normochromatic erythrocyte ratio in the bone marrow micronucleus test. Mutation Res., 347, p. 97-99.

(18) Richold, M., Ashby, J., Bootman, J., Chandley, A., Gatehouse, D.G. and Henderson, L., (1990) *In Vivo* Cytogenetics Assay. In: D.J. Kirkland (Ed.) Basic Mutagenicity tests. UKEMS Recommended Procedures. UKEMS Sub-Committee on Guidelines for Mutagenicity Testing. Report, Part I, revised. Cambridge University Press, Cambridge, New York, Port Chester, Melbourne, Sydney, p. 115-141.

(19) Lovell, D.P., Anderson, D., Albanese, R., Amphlett, G.E., Clare, G., Ferguson, R., Richold, M., Papworth, D.G. and Savage, J.R.K., (1989) Statistical Analysis of *In Vivo* Cytogenetic Assays. In: D.J. Kirkland (Ed.) Statistical Evaluation of Mutagenicity Test Data. UKEMS Sub-Committee on Guidelines for Mutagenicity Testing. Report, Part III. Cambridge University Press, Cambridge, New York, Port Chester, Melbourne, Sydney, p. 184-232.

B.13/14. **MUTAGENICITY: REVERSE MUTATION TEST USING BACTERIA**

1. **METHOD**

This method is a replicate of the OECD TG 471, Bacterial Reverse Mutation Test (1997).

1.1. INTRODUCTION

The bacterial reverse mutation test uses amino-acid requiring strains of *Salmonella typhimurium* and *Escherichia coli* to detect point mutations, which involve substitution, addition or deletion of one or a few DNA base pairs (1)(2)(3). The principle of this bacterial reverse mutation test is that it detects mutations, which revert mutations present in the test strains and restore the functional capability of the bacteria to synthesise an essential amino acid. The revertant bacteria are detected by their ability to grow in the absence of the amino-acid required by the parent test strain.

Point mutations are the cause of many human genetic diseases and there is substantial evidence that point mutations in oncogenes and tumour-suppressor genes of somatic cells are involved in tumour formation in humans and experimental animals. The bacterial reverse mutation test is rapid, inexpensive and relatively easy to perform. Many of the test strains have several features that make them more sensitive for the detection of mutations including responsive DNA sequences at the reversion sites, increased cell permeability to large molecules and elimination of DNA repair systems or enhancement of error-prone DNA repair processes. The specificity of the test strains can provide some useful information on the types of mutations that are induced by genotoxic agents. A very large data base of results for a wide variety of structures is available for bacterial reverse mutation tests and well-established methodologies have been developed for testing chemicals with different physico-chemical properties, including volatile compounds.

See also General introduction Part B.

1.2. DEFINITIONS

A reverse mutation test in either *Salmonella typhimurium* or *Escherichia coli* detects mutation in an amino-acid requiring strain (histidine or tryptophan, respectively) to produce a strain independent of an outside supply of amino-acid.

Base pair substitution mutagens are agents that cause a base change in DNA. In a reversion test this change may occur at the site of the original mutation, or at a second site in the bacterial genome.

Frameshift mutagens are agents that cause the addition or deletion of one or more base pairs in the DNA, thus changing the reading frame in the RNA.

1.3. INITIAL CONSIDERATIONS

The bacterial reverse mutation test utilises prokaryotic cells, which differ from mammalian cells in such factors as uptake, metabolism, chromosome structure and DNA repair processes. Tests conducted *in vitro* generally require the use of an exogenous source of metabolic activation. *In vitro* metabolic activation systems cannot mimic entirely the mammalian *in vivo* conditions. The test therefore does not provide direct information on the mutagenic and carcinogenic potency of a substance in mammals.

The bacterial reverse mutation test is commonly employed as an initial screen for genotoxic activity and, in particular, for point mutation-inducing activity. An extensive database has demonstrated that many chemicals that are positive in this test also exhibit mutagenic activity in other tests. There are examples of mutagenic agents, which are not detected by this test; reasons for these shortcomings can be ascribed to the specific nature of the endpoint detected, differences in metabolic activation, or differences in bioavailability. On the other hand, factors, which enhance the sensitivity of the bacterial reverse mutation test can lead to an overestimation of mutagenic activity.

The bacterial reverse mutation test may not be appropriate for the evaluation of certain classes of chemicals, for example highly bactericidal compounds (e.g. certain antibiotics) and those which are thought (or known) to interfere specifically with the mammalian cell replication system (e.g. some topoisomerase inhibitors and some nucleoside analogues). In such cases, mammalian mutation tests may be more appropriate.

Although many compounds that are positive in this test are mammalian carcinogens, the correlation is not absolute. It is dependent on chemical class and there are carcinogens that are not detected by this test because they act through other, non-genotoxic, mechanisms or mechanisms absent in bacterial cells.

1.4. PRINCIPLE OF THE TEST METHOD

Suspensions of bacterial cells are exposed to the test substance in the presence and in the absence of an exogenous metabolic activation system. In the plate incorporation method, these suspensions are mixed with an overlay agar and plated immediately onto minimal medium. In the preincubation method, the treatment mixture is incubated and then mixed with an overlay agar before plating onto minimal medium. For both techniques, after two or three days of incubation, revertant colonies are counted and compared to the number of spontaneous revertant colonies on solvent control plates.

Several procedures for performing the bacterial reverse mutation test have been described. Among those commonly used are the plate incorporation method (1)(2)(3)(4), the preincubation method (2)(3)(5)(6)(7)(8), the fluctuation method (9)(10), and the suspension method (11). Modifications for the testing of gases or vapours have been described (12).

The procedures described in the method pertain primarily to the plate incorporation and preincubation methods. Either of them is acceptable for conducting experiments both with and without metabolic activation. Some substances may be detected more efficiently using the preincubation method. These substances belong to chemical classes that include short chain aliphatic nitrosamines, divalent metals, aldehydes, azo-dyes and diazo compounds, pyrollizidine alkaloids, allyl compounds and nitro compounds (3). It is also recognised that certain classes of mutagens are not always detected using standard procedures such as the plate incorporation method or preincubation method. These should be regarded as 'special cases' and it is strongly recommended that alternative procedures should be used for their detection. The following 'special cases' could be identified (together with examples of procedures that could be used for their detection): azo-dyes and diazo compounds (3)(5)(6)(13), gases and volatile chemicals (12)(14)(15)(16) and glycosides (17)(18). A deviation from the standard procedure needs to be scientifically justified.

1.5. DESCRIPTION OF THE TEST METHOD

1.5.1. **Preparations**

1.5.1.1. *Bacteria*

Fresh cultures of bacteria should be grown up to the late exponential or early stationary phase of growth (approximately 10^9 cells per ml). Cultures in late stationary phase should not be used. It is essential that the cultures used in the experiment contain a high titre of viable bacteria. The titre may be demonstrated either from historical control data on growth curves, or in each assay through the determination of viable cell numbers by a plating experiment.

The recommended incubation temperature is 37 °C.

At least five strains of bacteria should be used. These should include four strains of *S. typhimurium* (TA 1535; TA 1537 or TA97a or TA97; TA98; and TA100) that have been shown to be reliable and reproducibly responsive between laboratories. These four *S. typhimurium* strains have GC base pairs at the primary reversion site and it is known that may not detect certain oxidising mutagens, cross-linking agents and hydrazines. Such substances may be detected by *E. coli* WP2 strains or *S. typhimurium* TA102 (19), which have an AT base pair at the primary reversion site. Therefore the recommended combination of strains is:

— *S. typhimurium* TA1535, and

— *S. typhimurium* TA1537 or TA97 or TA97a, and

— *S. typhimurium* TA98, and

— *S. typhimurium* TA100, and

— *E. coli* WP2 uvrA, or *E. coli* WP2 uvrA (pKM101), or *S. typhimurium* TA102.

In order to detect cross-linking mutagens it may be preferable to include TA102 or to add a DNA repair-proficient strain of *E. coli* [e.g. *E. coli* WP2 or *E. coli* WP2 (pKM101)]

Established procedures for stock culture preparation, marker verification and storage should be used. The amino-acid requirement for growth should be demonstrated for each frozen stock culture preparation (histidine for *S. typhimurium* strains, and tryptophan for *E. coli* strains). Other phenotypic characteristics should be similarly checked, namely: the presence or absence of R-factor plasmids where appropriate [i.e. ampicillin resistance in strains TA98, TA100 and TA97a or TA97, WP2 uvrA and WP2 uvrA (pKM101), and ampicillin + tetracycline resistance in strain TA102]; the presence of characteristic mutations (i.e. rfa mutation in *S. typhimurium* through sensitivity to crystal violet, and uvrA mutation in *E. coli* or uvrB mutation in *S. typhimurium,* through sensitivity to ultraviolet light) (2)(3). The strains should also yield spontaneous revertant colony plate counts within the frequency ranges expected from the laboratory's historical control data and preferably within the range reported in the literature.

1.5.1.2. *Medium*

An appropriate minimal agar (e.g. containing Vogel-Bonner minimal medium E and glucose), and an overlay agar containing histidine and biotin or tryptophan to allow for a few cell divisions, is used (1)(2)(9).

1.5.1.3. *Metabolic activation*

Bacteria should be exposed to the test substance both in the presence and absence of an appropriate metabolic activation system. The most commonly used system is a cofactor-supplemented post-mitochondrial fraction (S9) prepared from the livers of rodents treated with enzyme-inducing agents such as Aroclor 1254 (1)(2) or a combination of Phenobarbitone and ß-naphthoflavone (18)(20)(21). The post-mitochondrial fraction is usually used at concentrations in the range from 5 to 30 % v/v in the S9-mix. The choice and condition of a metabolic activation system may depend upon the class of chemical being tested. In some cases, it may be appropriate to utilise more than one concentration of post-mitochondrial fraction. For azo-dyes and diazo-compounds, using a reductive metabolic activation system may be more appropriate (6)(13).

1.5.1.4. *Test substance/Preparation*

Solid test substances should be dissolved or suspended in appropriate solvents or vehicles and diluted if appropriate prior to treatment of the bacteria. Liquid test substances may be added directly to the test systems and/or diluted prior to treatment. Fresh preparations should be employed unless stability data demonstrate the acceptability of storage.

The solvent/vehicle should not be suspected of chemical reaction with the test substance and should be compatible with the survival of the bacteria and the S9 activity (22). If other than well-known solvent/vehicles are used, their inclusion should be supported by data indicating their compatibility. It is recommended that wherever possible, the use of an aqueous solvent/vehicle be considered first. When testing water-unstable substances, the organic solvents used should be free of water.

1.5.2. **Test conditions**

1.5.2.1. *Test strains (see 1.5.1.1)*

1.5.2.2. *Exposure concentration*

Amongst the criteria to be taken into consideration when determining the highest amount of the test substance to be used are the cytotoxicity and the solubility in the final treatment mixture.

It may be useful to determine toxicity and insolubility in a preliminary experiment. Cytotoxicity may be detected by a reduction in the number of revertant colonies, a clearing or diminution of the background lawn, or the degree of survival of treated cultures. The cytotoxicity of a substance may be altered in the presence of metabolic activation systems. Insolubility should be assessed as precipitation in the final mixture under the actual test conditions and evident to the unaided eye.

The recommended maximum test concentration for soluble non-cytotoxic substances is 5 mg/plate or 5 µl/plate. For non-cytotoxic substances that are not soluble at 5 mg/plate or 5 µl/plate, one or more concentrations tested should be insoluble in the final treatment mixture. Test substances that are cytotoxic already below 5 mg/plate or 5 µl/plate should be tested up to a cytotoxic concentration. The precipitate should not interfere with the scoring.

At least five different analysable concentrations of the test substance should be used with approximately half log (i.e. √10) intervals between test points for an initial experiment. Smaller intervals may be appropriate when a concentration-response is being investigated. Testing above the concentration of 5 mg/plate or 5 µl/plate may be considered when evaluating substances containing substantial amounts of potentially mutagenic impurities.

1.5.2.3. *Negative and positive controls*

Concurrent strain-specific positive and negative (solvent or vehicle) controls, both with and without metabolic activation, should be included in each assay. Positive control concentrations that demonstrate the effective performance of each assay should be selected.

For assays employing a metabolic activation system, the positive control reference substance(s) should be selected on the basis of the type of bacteria strains used.

The following substances are examples of suitable positive controls for assays with metabolic activation:

CA numbers	EINECS numbers	Names
781-43-1	212-308-4	9,10-dimethylanthracene
57-97-6	200-359-5	7,12-dimethylbenz[*a*]anthracene
50-32-8	200-028-5	benzo[a]pyrene
613-13-8	210-330-9	2-aminoanthracene
50-18-0		cyclophosphamide
6055-19-2	200-015-4	cyclophosphamide monohydrate

The following substance is a suitable positive control for the reductive metabolic activation method:

CA numbers	EINECS numbers	Names
573-58-0	209-358-4	Congo Red

2-Aminoanthracene should not be used as the sole indicator of the efficacy of the S9-mix. If 2-aminoanthracene is used, each batch of S9 should also be characterised with a mutagen that requires metabolic activation by microsomal enzymes, e.g. benzo[a]pyrene, dimethylbenzanthracene.

The following substances are examples of strain-specific positive controls for assays performed without exogenous metabolic activation system:

CAS numbers	EINECS numbers	Names	Strain
26628-22-8	247-852-1	Sodium azide	TA 1535 and TA 100
607-57-8	210-138-5	2-nitrofluorene	TA 98
90-45-9	201-995-6	9-aminoacridine	TA 1537, TA 97 and TA 97a
17070-45-0	241-129-4	ICR 191	TA 1537, TA 97 and TA 97a
80-15-9	201-254-7	Cumene hydroperoxide	TA 102
50-07-7	200-008-6	Mitomycin C	WP2 uvrA and TA102

CAS numbers	EINECS numbers	Names	Strain
70-25-7	200-730-1	N-ethyl-N-nitro-N-nitrosoguanidine	WP2, WP2uvrA and WP2uvrA(pKM101)
56-57-5	200-281-1	4-nitroquinoline-1-oxide	WP2, WP2uvrA and WP2uvrA(pKM101)
3688-53-7		Furylfuramide (AF2)	plasmid-containing strains

Other appropriate positive control reference substances may be used. The use of chemical class-related positive control chemicals should be considered, when available.

Negative controls, consisting of solvent or vehicle alone, without test substance, and otherwise treated in the same way as the treatment groups, should be included. In addition, untreated controls should also be used unless there are historical control data demonstrating that no deleterious or mutagenic effects are induced by the chosen solvent.

1.5.3. **Procedure**

For the plate incorporation method (1)(2)(3)(4), without metabolic activation, usually 0,05 ml or 0,1 ml of the test solutions, 0,1 ml of fresh bacterial culture (containing approximately 10^8 viable cells) and 0,5 ml of sterile buffer are mixed with 2,0 ml of overlay agar. For the assay with metabolic activation, usually 0,5 ml of metabolic activation mixture containing an adequate amount of post-mitochondrial fraction (in the range from 5 to 30 % v/v in the metabolic activation mixture) are mixed with the overlay agar (2,0 ml), together with the bacteria and test substance/test solution. The contents of each tube are mixed and poured over the surface of a minimal agar plate. The overlay agar is allowed to solidify before incubation.

For the preincubation method (2)(3)(5)(6), the test substance/test solution is preincubated with the test strain (containing approximately 10^8 viable cells) and sterile buffer or the metabolic activation system (0,5 ml) usually for 20 min. or more at 30-37 °C prior to mixing with the overlay agar and pouring onto the surface of a minimal agar plate. Usually, 0,05 or 0,1 ml of test substance/test solution, 0,1 ml of bacteria, and 0,5 ml of S9-mix or sterile buffer are mixed with 2,0 ml of overlay agar. Tubes should be aerated during pre-incubation by using a shaker.

For an adequate estimate of variation, triplicate plating should be used at each dose level. The use of duplicate plating is acceptable when scientifically justified. The occasional loss of a plate does not necessarily invalidate the assay.

Gaseous or volatile substances should be tested by appropriate methods, such as in sealed vessels (12)(14)(15)(16).

1.5.4. **Incubation**

All plates in a given assay should be incubated at 37 °C for 48-72 hours. After the incubation period, the number of revertant colonies per plate is counted.

2. **DATA**

2.1. TREATMENT OF RESULTS

Data should be presented as the number of revertant colonies per plate. The number of revertant colonies on both negative (solvent control, and untreated control if used) and positive control plates should also be given. Individual plate counts, the mean number of revertant colonies per plate and the standard deviation should be presented for the test substance and positive and negative (untreated and/or solvent) controls.

There is no requirement for verification of a clear positive response. Equivocal results should be clarified by further testing preferably using a modification of experimental conditions. Negative results need to be confirmed on a case-by-case basis. In those cases where confirmation of negative results is not considered

necessary, justification should be provided. Modification of study parameters to extend the range of conditions assessed should be considered in follow-up experiments. Study parameters that might be modified include the concentration spacing, the method of treatment (plate-incorporation or liquid pre-incubation), and metabolic activation conditions.

2.2. EVALUATION AND INTERPRETATION OF RESULTS

There are several criteria for determining a positive result, such as a concentration-related increase over the range tested and/or a reproducible increase at one or more concentrations in the number of revertant colonies per plate in at least one strain with or without metabolic activation system (23). Biological relevance of the results should be considered first. Statistical methods may be used as an aid in evaluating the test results (24). However, statistical significance should not be the only determining factor for a positive response.

A test substance for which the results do not meet the above criteria is considered non-mutagenic in this test.

Although most experiments will give clearly positive or negative results, in rare cases the data set will preclude making a definite judgement about the activity of the test substance. Results may remain equivocal or questionable regardless of the number of times the experiment is repeated.

Positive results from the bacterial reverse mutation test indicate that the substance induces point mutations by base substitutions or frameshifts in the genome of either *Salmonella typhimurium* and/or *Escherichia coli*. Negative results indicate that under the test conditions, the test substance is not mutagenic in the tested species.

3. **REPORTING**

TEST REPORT

The test report must include the following information:

Solvent/Vehicle:

— justification for choice of solvent/vehicle,

— solubility and stability of the test substance in solvent/vehicle, if known.

Strains:

— strains used,

— number of cells per culture,

— strain characteristics.

Test conditions:

— amount of test substance per plate (mg/plate or µl/plate) with rationale for selection of dose and number of plates per concentration,

— media used,

— type and composition of metabolic activation system, including acceptability criteria,

— treatment procedures.

Results:

— signs of toxicity,

— signs of precipitation,

— individual plate counts,

— the mean number of revertant colonies per plate and standard deviation,

— dose-response relationship, where possible,

— statistical analyses, if any,

— concurrent negative (solvent/vehicle) and positive control data, with ranges, means and standard deviations,

— historical negative (solvent/vehicle) and positive control data with ranges, means and standard deviations.

Discussion of results.

Conclusions.

4. **REFERENCES**

(1) Ames, B.N., McCann, J. and Yamasaki E., (1975) Methods for Detecting Carcinogens and Mutagens with the Salmonella/Mammalian-Microsome Mutagenicity Test. Mutation Res., 31, p. 347-364.

(2) Maron, D.M. and Ames, B.N., (1983) Revised Methods for the Salmonella Mutagenicity Test. Mutation Res., 113, p. 173-215.

(3) Gatehouse, D., Haworth, S., Cebula, T., Gocke, E., Kier, L., Matsushima, T., Melcion, C., Nohmi, T., Venitt, S. and Zeiger, E., (1994) Recommendations for the Performance of Bacterial Mutation Assays. Mutation Res., 312, p. 217-233.

(4) Kier, L.D., Brusick D.J., Auletta, A.E., Von Halle, E.S., Brown, M.M., Simmon, V.F., Dunkel, V., McCann, J., Mortelmans, K., Prival, M., Rao, T.K. and Ray V., (1986) The *Salmonella typhimurium*/Mammalian Microsomal Assay: A Report of the U.S. Environmental Protection Agency Gene-Tox Program. Mutation Res., 168, p. 69-240.

(5) Yahagi, T., Degawa, M., Seino, Y.Y., Matsushima, T., Nagao, M., Sugimura, T. and Hashimoto, Y., (1975) Mutagenicity of Carcinogen Azo Dyes and their Derivatives, Cancer Letters, 1, p. 91-96.

(6) Matsushima, M., Sugimura, T., Nagao, M., Yahagi, T., Shirai, A. and Sawamura, M., (1980) Factors Modulating Mutagenicity Microbial Tests. In: Short-term Test Systems for Detecting Carcinogens. Ed. Norpoth K.H. and Garner, R.C., Springer, Berlin-Heidelberg-New York. p. 273-285.

(7) Gatehouse, D.G., Rowland, I.R., Wilcox, P., Callender, R.D. and Foster, R., (1980) Bacterial Mutation Assays. In: Basic Mutagenicity Tests: UKEMS Part 1 Revised. Ed. D.J. Kirkland, Cambridge University Press, p. 13-61.

(8) Aeschacher, H.U., Wolleb, U. and Porchet, L., (1987) Liquid Preincubation Mutagenicity Test for Foods. J. Food Safety, 8, p. 167-177.

(9) Green, M.H.L., Muriel, W.J. and Bridges, B.A., (1976) Use of a simplified fluctuation test to detect low levels of mutagens. Mutation Res., 38, p. 33-42.

(10) Hubbard, S.A., Green, M.H.L., Gatehouse, D. and J.W. Bridges (1984) The Fluctuation Test in Bacteria. In: Handbook of Mutagenicity Test Procedures. 2nd Edition. Ed. Kilbey, B.J., Legator, M., Nichols, W. and Ramel, C., Elsevier, Amsterdam-New York-Oxford, p. 141-161.

(11) Thompson, E.D. and Melampy, P.J., (1981) An Examination of the Quantitative Suspension Assay for Mutagenesis with Strains of *Salmonella typhimurium*. Environmental Mutagenesis, 3, p. 453-465.

(12) Araki, A., Noguchi, T., Kato, F. and T. Matsushima (1994) Improved Method for Mutagenicity Testing of Gaseous Compounds by Using a Gas Sampling Bag. Mutation Res., 307, p. 335-344.

(13) Prival, M.J., Bell, S.J., Mitchell, V.D., Reipert, M.D. and Vaughn, V.L., (1984) Mutagenicity of Benzidine and Benzidine-Congener Dyes and Selected Monoazo Dyes in a Modified Salmonella Assay. Mutation Res., 136, p. 33-47.

(14) Zeiger, E., Anderson, B.E., Haworth, S., Lawlor, T. and Mortelmans, K., (1992) Salmonella Mutagenicity Tests. V. Results from the Testing of 311 Chemicals. Environ. Mol. Mutagen., 19, p. 2-141.

(15) Simmon, V., Kauhanen, K. and Tardiff, R.G., (1977) Mutagenic Activity of Chemicals Identified in Drinking Water. In Progress in Genetic Toxicology, D. Scott, B. Bridges and F. Sobels (Eds.) Elsevier, Amsterdam, p. 249-258.

(16) Hughes, T.J., Simmons, D.M., Monteith, I.G. and Claxton, L.D., (1987) Vaporisation Technique to Measure Mutagenic Activity of Volatile Organic Chemicals in the Ames/Salmonella Assay. Environmental Mutagenesis, 9, p. 421-441.

(17) Matsushima, T., Matsumoto, A., Shirai, M., Sawamura, M., and Sugimura, T., (1979) Mutagenicity of the Naturally Occurring Carcinogen Cycasin and Synthetic Methylazoxy Methane Conjugates in *Salmonella typhimurium*. Cancer Res., 39, p. 3780-3782.

(18) Tamura, G., Gold, C., Ferro-Luzzi, A. and Ames, B.N., (1980) Fecalase: A Model for Activation of Dietary Glycosides to Mutagens by Intestinal Flora. Proc. Natl. Acad. Sci. USA, 77, p. 4961-4965.

(19) Wilcox, P., Naidoo, A., Wedd, D.J. and Gatehouse, D.G., (1990) Comparison of *Salmonella typhimurium* TA 102 with *Escherichia coli* WP2 Tester strains. Mutagenesis, 5, p. 285-291.

(20) Matsushima, T., Sawamura, M., Hara, K. and Sugimura, T., (1976) A Safe Substitute for Polychlorinated Biphenyls as an Inducer or Metabolic Activation Systems. In: '*In vitro* metabolic Activation in Mutagenesis Testing' Eds. F.J. de Serres et al. Elsevier, North Holland, p. 85-88.

(21) Elliot, B.M., Combes, R.D., Elcombe, C.R., Gatehouse, D.G., Gibson, G.G., Mackay, J.M. and Wolf, R.C., (1992) Alternatives to Aroclor 1254-induced S9 in *in vitro* Genotoxicity Assays. Mutagenesis, 7, p. 175-177.

(22) Maron, D., Katzenellenbogen, J. and Ames, B.N., (1981) Compatibility of Organic Solvents with the Salmonella/Microsome Test. Mutation Res., p. 88343-350.

(23) Claxton, L.D., Allen, J., Auletta, A., Mortelmans, K., Nestmann, E. and Zeiger, E., (1987) Guide for the *Salmonella typhimurium*/Mammalian Microsome Tests for Bacterial Mutagenicity. Mutation Res. 189, p. 83-91.

(24) Mahon, G.A.T., Green, M.H.L., Middleton, B., Mitchell, I., Robinson, W.D. and Tweats, D.J., (1989) Analysis of Data from Microbial Colony Assays. In: UKEMS Sub-Committee on Guidelines for Mutagenicity Testing. Part II. Statistical Evaluation of Mutagenicity Test Data. Ed. Kirkland, D.J., Cambridge University Press, p. 28-65.

B.15. **MUTAGENICITY TESTING AND SCREENING FOR CARCINOGENICITY GENE MUTATION — *SACCHAROMYCES CEREVISIAE***

1. **METHOD**

1.1. INTRODUCTION

See General introduction Part B.

1.2. DEFINITION

See General introduction Part B.

1.3. REFERENCE SUBSTANCES

None.

1.4. PRINCIPLE OF THE TEST METHOD

A variety of haploid and diploid strains of the yeast *Saccharomyces cerevisiae* can be used to measure the production of gene mutations induced by chemical agents with and without metabolic activation.

Forward mutation systems in haploid strains, such as the measurement of mutation from red, adenine-requiring mutants (*ade*-1, *ade*-2) to double adenine-requiring white mutants and selective systems such as the induction of resistance to canavnaine and cycloheximide, have been utilised.

The most extensively validated reverse mutation system involves the use of the haploid strain XV 185-14C which carries the ochre nonsense mutations *ade* 2-1, *arg* 4-17, *lys* 1-1 and *trp* 5-48, which are reversible by base substitution mutagens that induce site specific mutations or ochre suppressor mutations. XV 185-14C also carries the *his* 1-7 marker, a missense mutation reverted mainly by second site mutations, and the marker *hom* 3-10 which is reverted by frameshift mutagens.

In diploid strains of *S. cerevisiae* the only extensively used strain is D_7 which is homozygous for *ilv* 1-92.

1.5. QUALITY CRITERIA

None.

1.6. DESCRIPTION OF THE TEST METHOD

Preparations

Solutions of test chemicals and control should be prepared just prior to testing, using an appropriate vehicle. In the case of organic compounds, which are not water soluble, not more than a 2 % solution v/v of organic solvents such as ethanol, acetone or dimethylsulphoxide (DMSO) should be used. The final concentration of the vehicle should not significantly affect cell viability and growth characertistics.

Metabolic activation

Cells should be exposed to test chemicals both in the presence and absence of an appropriate exogenous metabolic activation system.

The most commonly used system is a co-factor supplemented post-mitochondrial fraction from the livers of rodents pre-treated with enzyme inducing agents. The use of other species, tissues, post-mitochondrial fractions, or procedures may also be appropriate for metabolic activation.

Test conditions

Tester strains

The haploid strain XV 185-14C and the diploid strain D_7 are the most used in gene mutation studies. Other strains may also be appropriate.

Media

Appropriate culture media are used for the determination of survival and mutant numbers.

Use of negative and positive controls

Positive, untreated and solvent controls should be performed concurrently. Appropriate positive control chemicals should be used for each specific mutational endpoint.

Exposure concentration

At least five adequately spaced concentrations of the test substance should be used. For toxic substances, the highest concentration tested should not reduce survival below 5 to 10 % . Relatively water-insoluble substances should be tested up to their limit of solubility, using appropriate procedures. For freely water-soluble non-toxic substances, the upper concentration should be determined on a case by case basis.

Incubation conditions

The plates are incubated four to seven days at 28 to 30 °C in the dark.

Spontaneous mutation frequencies

Sub-cultures should be used with spontaneous mutation frequencies within the accepted normal range.

Number of replicates

At least three replicate plates should be used per concentration for the assay of prototrophs produced by gene mutation and for cell viability. In the case of experiments using markers such as *hom* 3-10 with a low mutation rate, the number of plates used must be increased to provide statistically relevant data.

Procedure

Treatment of *S. cerevisiae* strains is usually performed in a liquid test procedure involving either stationary or growing cells. Initial experiments should be carried out on growing cells: $1\text{-}5 \times 10^7$ cells/ml are exposed to the test chemical for up to 18 hours at 28 to 37 °C with shaking; an adequate amount of metabolic activation system is added during treatment when appropriate. At the end of the treatment, cells are centrifuged, washed and seeded upon an appropriate culture medium. After incubation, plates are scored for survival and the induction of gene mutation. If the first experiment is negative, then a second experiment should be carried out using stationary phase cells. If the first experiment is positive it is confirmed in an appropriate independent experiment.

2. **DATA**

Data should be presented in tabular form indicating the number of colonies counted, number of mutants, survival and mutant frequency. All results should be confirmed in an independent experiment. The dara should be evaluated using appropriate statistical methods.

3. **REPORTING**

3.1. TEST REPORT

The test report shall, if possible, contain the following information:

— strain used,

— test conditions: stationary phase or growing cells, compositions of media, incubation temperature and duration, metabolic activation system,

— treatment conditions: exposure levels, procedure and duration of treatment, treatment temperature, positive and negative controls,

— number of colonies counted, number of mutants, survival and mutant frequency, dose/response relationship if applicable, statistical evaluation of data,

— discussion of results,

— interpretation of results.

3.2. EVALUATION AND INTERPRETATION

See General introduction Part B.

4. **REFERENCES**

See General introduction Part B.

B.16. MITOTIC RECOMBINATION — *SACCHAROMYCES CEREVISIAE*

1. METHOD

1.1. INTRODUCTION

See General introduction Part B.

1.2. DEFINITION

See General introduction Part B.

1.3. REFERENCE SUBSTANCES

None.

1.4. PRINCIPLE OF THE TEST METHOD

Mitotic recombination in *Saccharomyces cerevisiae* can be detected between genes (or more generally between a gene and its centromere) and within genes. The former event is called mitotic crossing-over and generates reciprocal products whereas the latter event is most frequently non-reciprocal and is called gene conversion. Crossing-over is generally assayed by the production of recessive homozygous colonies or sectors produced in a heterozygous strain, whereas gene conversion is assayed by the production of prototrophic revertants produced in an auxotrophic heteroallelic strain carrying two different defective alleles of the same gene. The most commonly used strains for the detection of mitotic gene conversion are D_4 (heteroallelic at *ade* 2 and *trp* 5) D_7 (heteroallelic at *trp* 5) BZ_{34} (heteroallelic at *arg* 4) and JDl (heteroallelic at *his* 4 and *trp* 5). Mitotic crossing-over producing red and pink homozygous sectors can be assayed in D_5 or in D_7 (which also measures mitotic gene conversion and reverse mutation at *ilv* 1-92) both strains being heteroallelic for complementing alleles of *ade* 2.

1.5. QUALITY CRITERIA

None.

1.6. DESCRIPTION OF THE TEST METHOD

Preparations

Solutions of test chemicals and control or reference compounds should be prepared just prior to testing, using an appropriate vehicle. With organic compounds that are water insoluble not more than a 2 % solution v/v of organic solvents such as ethanol, acetone or dimethylsulphoxide (DMSO) should be used. The final concentration of the vehicle should not significantly affect cell viability and growth characteristics.

Metabolic activation

Cells should be exposed to test chemicals both in the presence and absence of an appropriate exogenous metabolic activation system. The system most commonly used is a co-factor supplemented post-mitochondrial fraction from the livers of rodents pre-treated with enzyme inducing agents. The use of other species, tissues, post-mitochondrial fractions, or procedures may also be appropriate for metabolic activation.

Test conditions

Tester strains

The most frequently used strains are the diploids D_4, D_5, D_7 and JD1. The use of other strains may be appropriate.

Media

Appropriate culture media are used for the determination of survival and the frequency of mitotic recombination.

Use of negative and positive controls

Positive, untreated and solvent controls should be performed concurrently. Appropriate positive control chemicals should be used for each specific recombination endpoint.

Exposure concentrations

At least five adequately spaced concentrations of the test substance should be used. Among the factors to be taken into consideration are cytotoxicity and solubility. The lowest concentration must have no effect on celf viability. For toxic chemicals, the highest concentration tested should not reduce survival below 5 to 10 %. Relatively water-insoluble chemicals should be tested up to the limit of solubility using appropriate procedures. For freely water-soluble non-toxic substances the upper concentration should be determined on a case by case basis.

Cells may be exposed to test chemicals in either the stationary phase or during growth for periods of up to 18 hours. However, for long treatment times cultures should be microscopically inspected for spore formation, the presence of which invalidates the test.

Incubation conditions

The plates are incubated in the dark for four to seven days at 28 to 30 °C. Plates used for the assay of red and pink homozygous sectors produced by mitotic crossing-over should be kept in a refrigerator (about 4 °C) for a further one to two days before scoring to allow for the development of the appropriate pigmented colonies.

Spontaneous mitotic recombination frequencies

Sub-cultures should be used with spontaneous mitotic recombination mutation frequencies within the accepted normal range.

Number of replicates

A minimum of three replicate plates should be used per concentration for the assay of prototrophs produced by mitotic gene conversion and for viability. In the case of the assay of recessive homozygosis produced by mitotic crossing-over, the plate number should be increased to provide an adequate number of colonies.

Procedures

Treatment of *S. cerevisiae* strains is usually performed in a liquid test procedure involving either stationary or growing cells. Initial experiments should be done on growing cells. 1-5 × 10^7 celles/ml are exposed to the test chemical for up to 18 hours at 28 to 37 °C with shaking; an adequate amount of metabolic activation system is added during treatment when appropriate.

At the end of the treatment, cells are centrifuged, washed and seeded upon appropriate culutre medium. After incubation plates are scored for survival and the induction of mitotic recombination.

If the first experiment is negative, then a second experiment should be carried out using stationary phase cells. If the first experiment is positive it is confirmed in an independent experiment.

2. **DATA**

Data should be presented in tabular form indicating the number of colonies counted, the number of recombinants, survival and the frequency of recombinants.

Results should be confirmed in an independent experiment.

The data should be evaluated using appropriate statistical methods.

3. REPORTING

3.1. TEST REPORT

The test report shall, if possible, contain the following information:

— strain used,

— test conditions: stationary phase or growing cells, composition of media, incubation temperature and duration, metabolic activation system,

— treatment conditions: exposure concentration, procedure and duration of treatment, treatment temperature, positive and negative controls,

— number of colonies counted, number of recombinants; survival and recombination frequency, dose/response relationship if applicable, statistical evaluation of data,

— discussion of the results,

— interpretation of the results.

3.2. EVALUATION AND INTERPRETATION

See General introduction Part B.

4. REFERENCES

See General introduction Part B.

B.17. **MUTAGENICITY — *IN VITRO* MAMMALIAN CELL GENE MUTATION TEST**

1. **METHOD**

This method is a replicate of the OECD TG 476, *In Vitro* Mammalian Cell Gene Mutation Test (1997).

1.1. INTRODUCTION

The *in vitro* mammalian cell gene mutation test can be used to detect gene mutations induced by chemical substances. Suitable cell lines include L5178Y mouse lymphoma cells, the CHO, CHO-AS52 and V79 lines of Chinese hamster cells, and TK6 human lymphoblastoid cells (1). In these cell lines the most commonly-used genetic endpoints measure mutation at thymidine kinase (TK) and hypoxanthine-guanine phosphoribosyl transferase (HPRT), and a transgene of xanthine-guanine phosphoribosyl transferase (XPRT). The TK, HPRT and XPRT mutation tests detect different spectra of genetic events. The autosomal location of TK and XPRT may allow the detection of genetic events (e.g. large deletions) not detected at the HPRT locus on X-chromosomes (2)(3)(4)(5)(6).

In the *in vitro* mammalian cell gene mutation test, cultures of established cell lines or cell strains can be used. The cells used are selected on the basis of growth ability in culture and stability of the spontaneous mutation frequency.

Tests conducted *in vitro* generally require the use of an exogenous source of metabolic activation. This metabolic activation system cannot mimic entirely the mammalian *in vivo* conditions. Care should be taken to avoid conditions, which would lead to results not reflecting intrinsic mutagenicity. Positive results, which do not reflect intrinsic mutagenicity may arise from changes in pH, osmolality or high levels of cytotoxicity (7).

This test is used to screen for possible mammalian mutagens and carcinogens. Many compounds that are positive in this test are mammalian carcinogens; however, there is not a perfect correlation between this test and carcinogenicity. Correlation is dependent on chemical class and there is increasing evidence that there are carcinogens that are not detected by this test because they appear to act through other, non genotoxic mechanisms or mechanisms absent in bacterial cells (6).

See also General introduction Part B.

1.2. DEFINITIONS

Forward mutation: a gene mutation from the parental type to the mutant form which gives rise to an alteration or a loss of the enzymatic activity of the function of the encoded protein.

Base pair substitution mutagens: substances, which cause substitution of one or several base pairs in the DNA.

Frameshift mutagens: Substances, which cause the addition or deletion of single or multiple base pairs in the DNA molecule.

Phenotypic expression time: a period during which unaltered gene products are depleted from newly mutated cells.

Mutant frequency: the number of mutant cells observed divided by the number of viable cells.

Relative total growth: increase in cell number over time compared to a control population of cells; calculated as the product of suspension growth relative to the negative control times cloning efficiency relative to negative control.

Relative suspension growth: increase in cell number over the expression period relative to the negative control.

Viability: the cloning efficiency of the treated cells at the time of plating in selective conditions after the expression period.

Survival: the cloning efficiency of the treated cells when plated at the end of the treatment period; survival is usually expressed in relation to the survival of the control cell population.

1.3. PRINCIPLE OF THE TEST METHOD

Cells deficient in thymidine kinase (TK) due to the mutation $TK^{+/-}$ -> $TK^{-/-}$ are resistant to the cytotoxic effects of the pyrimidine analogue trifluorothymidine (TFT). Thymidine kinase proficient cells are sensitive to TFT, which causes the inhibition of cellular metabolism and halts further cell division. Thus mutant cells are able to proliferate in the presence of TFT, whereas normal cells, which contain thymidine kinase, are not. Similarly, cells deficient in HPRT or XPRT are selected by resistance to 6-thioguanine (TG) or 8-azaguanine (AG). The properties of the test substance should be considered carefully if a base analogue or a compound related to the selective agent is tested in any of the mammalian cell gene mutation tests. For example, any suspected selective toxicity by the test substance for mutant and non-mutant cells should be investigated. Thus, performance of the selection system/agent must be confirmed when testing chemicals structurally related to the selective agent (8).

Cells in suspension or monolayer culture are exposed to the test substance, both with and without metabolic activation, for a suitable period of time and subcultured to determine cytotoxicity and to allow phenotypic expression prior to mutant selection (9)(10)(11)(12)(13). Cytotoxicity is usually determined by measuring the relative cloning efficiency (survival) or relative total growth of the cultures after the treatment period. The treated cultures are maintained in growth medium for a sufficient period of time, characteristic of each selected locus and cell type, to allow near-optimal phenotypic expression of induced mutations. Mutant frequency is determined by seeding known numbers of cells in medium containing the selective agent to detect mutant cells and in medium without selective agent to determine the cloning efficiency (viability). After a suitable incubation time, colonies are counted. The mutant frequency is derived from the number of mutant colonies in selective medium and the number of colonies in non-selective medium.

1.4. DESCRIPTION OF THE TEST METHOD

1.4.1. **Preparations**

1.4.1.1. *Cells*

A variety of cell types are available for use in this test including subclones of L5178Y, CHO, CHO-AS52, V79 or TK6 cells. Cell types used in this test should have a demonstrated sensitivity to chemical mutagens, a high cloning efficiency and a stable spontaneous mutant frequency. Cells should be checked for mycoplasma contamination and should not be used if contaminated.

The test should be designed to have a predetermined sensitivity and power. The number of cells, cultures and concentrations of test substance used should reflect these defined parameters (14). The minimal number of viable cells surviving treatment and used at each stage in the test should be based on the spontaneous mutation frequency. A general guide is to use a cell number, which is at least 10 times the inverse of the spontaneous mutation frequency. However, it is recommended to utilise at least 10^6 cells. Adequate historical data on the cell system used should be available to indicate consistent performance of the test.

1.4.1.2. *Media and culture conditions*

Appropriate culture media, and incubation conditions (culture vessels, temperature, CO_2 concentration, and humidity) should be used. Media should be chosen according to the selective systems and cell type used in the test. It is particularly important that culture conditions should be chosen that ensure optimal growth of cells during the expression period and colony forming ability of both mutant and non-mutant cells.

1.4.1.3. *Preparation of cultures*

Cell are propagated from stock cultures, seeded in culture medium and incubated at 37 °C. Prior to use in this test, cultures may need to be cleansed of pre-existing mutant cells.

1.4.1.4. *Metabolic activation*

Cells should be exposed to the test substance both in the presence and absence of an appropriate metabolic activation system. The most commonly used system is a cofactor-supplemented post-mitochondrial fraction (S9) prepared from the livers of rodents treated with enzyme-inducing agents such as Aroclor 1254 (15)(16)(17)(18) or a combination of phenobarbitone and ß–naphthoflavone (19)(20).

The post-mitochondrial fraction is usually used at concentrations in the range from 1-10 % v/v in the final test medium. The choice and condition of a metabolic activation system may depend upon the class of chemical being tested. In some cases it may be appropriate to utilise more than one concentration of post-mitochondrial fraction.

A number of developments, including the construction of genetically engineered cell lines expressing specific activating enzymes, may provide the potential for endogenous activation. The choice of the cell lines used should be scientifically justified (e.g. by the relevance of the cytochrome P450 isoenzyme for the metabolism of the test substance).

1.4.1.5. *Test substance/Preparation*

Solid test substances should be dissolved or suspended in appropriate solvents or vehicles and diluted if appropriate prior to treatment of the cells. Liquid test substances may be added directly to the test systems and/or diluted prior to treatment. Fresh preparations of the test substance should be employed unless stability data demonstrate the acceptability of storage.

1.4.2. **Test conditions**

1.4.2.1. *Solvent/Vehicle*

The solvent/vehicle should not be suspected of chemical reaction with the test substance and should be compatible with the survival of the cells and the S9 activity. If other than well-known solvent/vehicles are used, their inclusion should be supported by data indicating their compatibility. It is recommended that wherever possible, the use of an aqueous solvent/vehicle be considered first. When testing water-unstable substances, the organic solvents used should be free of water. Water can be removed by adding a molecular sieve.

1.4.2.2. *Exposure concentrations*

Among the criteria to be considered when determining the highest concentration are cytotoxicity, solubility in the test system and changes in pH or osmolality.

Cytotoxicity should be determined with and without metabolic activation in the main experiment using an appropriate indication of cell integrity and growth, such as relative cloning efficiency (survival) or relative total growth. It may be useful to determine cytotoxicity and solubility in a preliminary experiment.

At least four analysable concentrations should be used. Where there is cytotoxicity, these concentrations should cover a range from the maximum to little or no toxicity; this will usually mean that the concentration levels should be separated by no more than a factor between 2 and √10. If the maximum concentration is based on cytotoxicity then it should result in approximately 10-20 % (but not less than 10 %) relative survival (relative cloning efficiency) or relative total growth. For relatively non-cytotoxic substances, the maximum test concentration should be 5 mg/ml 5 µl/ml, or 0,01 M, whichever is the lowest.

Relatively insoluble substances should be tested up to or beyond their limit of solubility under culture conditions. Evidence of insolubility should be determined in the final treatment medium to which cells are exposed. It may be useful to assess solubility at the beginning and the end of the treatment, as solubility can change during the course of exposure in the test system due to presence of cells, S9, serum, etc. Insolubility can be detected by using the unaided eye. The precipitate should not interfere with the scoring.

1.4.2.3. *Controls*

Concurrent positive and negative (solvent or vehicle) controls, both with and without metabolic activation should be included in each experiment. When metabolic activation is used, the positive control chemical should be the one that requires activation to give a mutagenic response.

Examples of positive control substances include:

Metabolic activation condition	Locus	Substance	CAS No	EINECS No
Absence of exogenous metabolic activation	HPRT	Ethyl methanesulphonate	62-50-0	200-536-7
		Ethyl nitrosourea	759-73-9	212-072-2
	TK (small and large colonies)	Methyl methanesulphonate	66-27-3	200-625-0
	XPRT	Ethyl methanesulphonate	62-50-0	200-536-7
		Ethyl nitrosourea	759-73-9	212-072-2
Presence of exogenous metabolic activation	HPRT	3-Methylcholanthrene	56-49-5	200-276-4
		N-Nitrosodimethylamine	62-75-9	200-549-8
		7,12-Dimethylbenzanthracene	57-97-6	200-359-5
	TK (small and large colonies)	Cyclophosphamide	50-18-0	200-015-4
		Cyclophosphamide monohydrate	6055-19-2	
		Benzo[*a*]pyrene	50-32-8	200-028-5
		3-Methylcholanthrene	56-49-5	200-276-5
	XPRT	N-Nitrosodimethylamine (for high levels of S-9)	62-75-9	200-549-8
		Benzo[*a*]pyrene	50-32-8	200-028-5

Other appropriate positive control reference substances may be used, e.g. if a laboratory has a historical data base on 5-Bromo 2'-deoxyuridine (CAS n. 59-14-3, EINECS n. 200-415-9), this reference substance could be used as well. The use of chemical class-related positive control chemicals should be considered, when available.

Negative controls, consisting of solvent or vehicle alone in the treatment medium, and treated in the same way as the treatment groups, should be included. In addition, untreated controls should also be used unless there are historical control data demonstrating that no deleterious or mutagenic effects are induced by the chosen solvent.

1.4.3. **Procedure**

1.4.3.1. *Treatment with the test substance*

Proliferating cells should be exposed to the test substance both with and without metabolic activation. Exposure should be for a suitable period of time (usually three to six hours is effective). Exposure time may be extended over one or more cell cycles.

Either duplicate or single treated cultures may be used at each concentration tested. When single cultures are used, the number of concentrations should be increased to ensure an adequate number of cultures for analysis (e.g. at least eight analysable concentrations). Duplicate negative (solvent) control cultures should be used.

Gaseous or volatile substances should be tested by appropriate methods, such as in sealed culture vessels (21) (22).

1.4.3.2. *Measurement of survival, viability and mutant frequency*

At the end of the exposure period, cells are washed and cultured to determine survival and to allow for expression of the mutant phenotype. Measurement of cytotoxicity by determining the relative cloning efficiency (survival) or relative total growth of the cultures is usually initiated after the treatment period.

Each locus has a defined minimum time requirement to allow near optimal phenotypic expression of newly induced mutants (HPRT and XPRT require at least six to eight days, and TK at least two days). Cells are grown in medium with and without selective agent(s) for determination of numbers of mutants and cloning efficiency, respectively. The measurement of viability (used to calculate mutant frequency) is initiated at the end of the expression time by plating in non-selective medium.

If the test substance is positive in the L5178Y $TK^{+/-}$ test, colony sizing should be performed on at least one of the test cultures (the highest positive concentration) and on the negative and positive controls. If the test substance is negative in the L5178Y $TK^{+/-}$ test, colony sizing should be performed on the negative and positive controls. In studies using TK6$TK^{+/-}$, colony sizing may also be performed.

2. DATA

2.1. TREATMENT OF RESULTS

Data should include cytotoxicity and viability determination, colony counts and mutant frequencies for the treated and control cultures. In the case of a positive response in the L5178Y $TK^{+/-}$ test, colonies are scored using the criteria of small and large colonies on at least one concentration of the test substance (highest positive concentration) and on the negative and positive control. The molecular and cytogenetic nature of both large and small colony mutants has been explored in detail (23)(24). In the $TK^{+/-}$ test, colonies are scored using the criteria of normal growth (large) and slow growth (small) colonies (25). Mutant cells that have suffered the most extensive genetic damage have prolonged doubling times and thus form small colonies. This damage typically ranges in scale from the losses of the entire gene to karyotypically visible chromosome aberrations. The induction of small colony mutants has been associated with chemicals that induce gross chromosome aberrations (26). Less seriously affected mutant cells grow at rates similar to the parental cells and form large colonies.

Survival (relative cloning efficiencies) or relative total growth should be given. Mutant frequency should be expressed as number of mutant cells per number of surviving cells.

Individual culture data should be provided. Additionally, all data should be summarised in tabular form.

There is no requirement for verification of a clear positive response. Equivocal results should be clarified by further testing preferably using modification of experimental conditions. Negative results need to be confirmed on a case-by-case basis. In those cases where confirmation of negative results is not considered necessary, justification should be provided. Modification of study parameters to extend the range of conditions assessed should be considered in follow-up experiments for either equivocal or negative results. Study parameters that might be modified include the concentration spacing and the metabolic activation conditions.

2.2. EVALUATION AND INTERPRETATION OF RESULTS

There are several criteria for determining a positive result, such as a concentration-related increase or a reproducible increase in mutant frequency. Biological relevance of the results should be considered first. Statistical methods may be used as an aid in evaluating the test results. Statistical significance should not be the only determining factor for a positive response.

A test substance for which the results do not meet the above criteria is considered non-mutagenic in this system.

Although most studies will give clearly positive or negative results, in rare cases the data set will preclude making a definite judgement about the activity of the test substance. Results may remain equivocal or questionable regardless of the number of times the experiment is repeated.

Positive results from the *in vitro* mammalian cell gene mutation test indicate that the test substance induces gene mutations in the cultured mammalian cells used. A positive concentration response that is reproducible is most meaningful. Negative results indicate that, under the test conditions, the test substance does not induce gene mutations in the cultured mammalian cells used.

3. **REPORTING**

TEST REPORT

The test report must include the following information:

Solvent/Vehicle:

— justification for choice of vehicle/solvent,

— solubility and stability of the test substance in solvent/vehicle, if known,

Cells:

— type and source of cells,

— number of cell cultures,

— number of cell passages, if applicable,

— methods for maintenance of cell culture, if applicable,

— absence of mycoplasma.

Test conditions:

— rationale for selection of concentrations and number of cultures including, e.g. cytotoxicity data and solubility limitations, if available,

— composition of media, CO_2 concentration,

— concentration of test substance,

— volume of vehicle and test substance added,

— incubation temperature,

— incubation time,

— duration of treatment,

— cell density during treatment,

— type and composition of metabolic activation system, including acceptability criteria,

— positive and negative controls,

— length of expression period (including number of cells seeded, and subcultures and feeding schedules, if appropriate),

— selective agents,

— criteria for considering tests as positive, negative or equivocal,

— methods used to enumerate numbers of viable and mutant cells.

— definition of colonies of which size and type are considered (including criteria for 'small' and 'large' colonies, as appropriate).

Results:

— signs of toxicity,

— signs of precipitation,

— data on pH and osmolality during the exposure to the test substance, if determined,

— colony size if scored for at least negative and positive controls,

— laboratory's adequacy to detect small colony mutants with the L5178Y TK+/− system, where appropriate,

— dose-response relationship, where possible,

— statistical analyses, if any,

— concurrent negative (solvent/vehicle) and positive control data,

— historical negative (solvent/vehicle) and positive control data with ranges, means and standard deviations,

— mutant frequency.

Discussion of results.

Conclusions.

4. **REFERENCES**

(1) Moore, M.M., DeMarini, D.M., DeSerres, F.J. and Tindall, K.R. (Eds.), (1987) Banbury Report 28: Mammalian Cell Mutagenesis, Cold Spring Harbor Laboratory, New York.

(2) Chu, E.H.Y. and Malling, H.V., (1968) Mammalian Cell Genetics. II. Chemical Induction of Specific Locus Mutations in Chinese Hamster Cells *In Vitro*, Proc. Natl. Acad. Sci., USA, 61, p. 1306-1312.

(3) Liber, H.L. and Thilly, W.G., (1982) Mutation Assay at the Thymidine Kinase Locus in Diploid Human Lymphoblasts. Mutation Res., 94, p. 467-485.

(4) Moore, M.M., Harington-Brock, K., Doerr, C.L. and Dearfield, K.L., (1989) Differential Mutant Quantitation at the Mouse Lymphoma TK and CHO HGPRT Loci. Mutagenesis, 4, p. 394-403.

(5) Aaron, C.S. and Stankowski, Jr.L.F., (1989) Comparison of the AS52/XPRT and the CHO/HPRT Assays: Evaluation of Six Drug Candidates. Mutation Res., 223, p. 121-128.

(6) Aaron, C.S., Bolcsfoldi, G., Glatt, H.R., Moore, M., Nishi, Y., Stankowski, Jr.L.F., Theiss, J. and Thompson, E., (1994) Mammalian Cell Gene Mutation Assays Working Group Report. Report of the International Workshop on Standardisation of Genotoxicity Test Procedures. Mutation Res., 312, p. 235-239.

(7) Scott, D., Galloway, S.M., Marshall, R.R., Ishidate, M., Brusick, D., Ashby, J. and Myhr, B.C., (1991) Genotoxicity Under Extreme Culture Conditions. A report from ICPEMC Task Group 9. Mutation Res., 257, p. 147-204.

(8) Clive, D., McCuen, R., Spector, J.F.S., Piper, C. and Mavournin, K.H., (1983) Specific Gene Mutations in L5178Y Cells in Culture. A Report of the U.S. Environmental Protection Agency Gene-Tox Program. Mutation Res., 115, p. 225-251.

(9) Li, A.P., Gupta, R.S., Heflich, R.H. and Wasson, J.S., (1988) A Review and Analysis of the Chinese Hamster Ovary/Hypoxanthine Guanine Phosphoribosyl Transferase System to Determine the Mutagenicity of Chemical Agents: A Report of Phase III of the U.S. Environmental Protection Agency Gene-Tox Program. Mutation Res., 196, p. 17-36.

(10) Li, A.P., Carver, J.H., Choy, W.N., Hsie, A.W., Gupta, R.S., Loveday, K.S., O'Neill, J.P., Riddle, J.C., Stankowski, L.F. Jr. and Yang, L.L., (1987) A Guide for the Performance of the Chinese Hamster Ovary Cell/Hypoxanthine-Guanine Phosphoribosyl Transferase Gene Mutation Assay. Mutation Res., 189, p. 135-141.

(11) Liber, H.L., Yandell, D.W and Little, J.B., (1989) A Comparison of Mutation Induction at the TK and HPRT Loci in Human Lymphoblastoid Cells: Quantitative Differences are Due to an Additional Class of Mutations at the Autosomal TK Locus. Mutation Res., 216, p. 9-17.

(12) Stankowski, L.F. Jr., Tindall, K.R. and Hsie, A.W., (1986) Quantitative and Molecular Analyses of Ethyl Methanosulphonate -and ICR 191- Induced Molecular Analyses of Ethyl Methanosulphonate -and ICR 191-Induced Mutation in AS52 Cells. Mutation Res., 160, p. 133-147.

(13) Turner, N.T., Batson, A.G. and Clive, D., (1984) Procedures for the L5178Y/$TK^{+/-}$ — $TK^{+/-}$ Mouse Lymphoma Cell Mutagenicity Assay. In: Kilbey, B.J. et al (eds.) Handbook of Mutagenicity Test Procedures, Elsevier Science Publishers, New York, p. 239-268.

(14) Arlett, C.F., Smith, D.M., Clarke, G.M., Green, M.H.L., Cole, J., McGregor, D.B. and Asquith, J.C., (1989) Mammalian Cell Gene Mutation Assays Based upon Colony Formation. In: Statistical Evaluation of Mutagenicity Test Data, Kirkland, D.J., Ed., Cambridge University Press, p. 66-101.

(15) Abbondandolo, A., Bonatti, S., Corti, G., Fiorio, R., Loprieno, N. and Mazzaccaro, A., (1977) Induction of 6-Thioguanine-Resistant Mutants in V79 Chinese Hamster Cells by Mouse-Liver Microsome-Activated Dimethylnitrosamine. Mutation Res., 46, p. 365-373.

(16) Ames, B.N., McCann, J. and Yamasaki, E., (1975) Methods for Detecting Carcinogens and Mutagens with the Salmonella/Mammalian-Microsome Mutagenicity Test. Mutation Res., 31, p. 347-364.

(17) Clive, D., Johnson, K.O., Spector, J.F.S., Batson, A.G. and Brown M.M.M., (1979) Validation and Characterisation of the L5178Y/$TK^{+/-}$ Mouse Lymphoma Mutagen Assay System. Mutat. Res., 59, p. 61-108.

(18) Maron, D.M. and Ames, B.N., (1983) Revised Methods for the Salmonella Mutagenicity Test. Mutation Res., 113, p. 173-215.

(19) Elliott, B.M., Combes, R.D., Elcombe, C.R., Gatehouse, D.G., Gibson, G.G., Mackay, J.M. and Wolf, R.C., (1992) Alternatives to Aroclor 1254-Induced S9 in *In Vitro* Genotoxicity Assays. Mutagenesis, 7, p. 175-177.

(20) Matsushima, T., Sawamura, M., Hara, K. and Sugimura, T., (1976) A Safe Substitute for Polychlorinated Biphenyls as an Inducer of Metabolic Activation Systems. In: *In vitro* Metabolic Activation in Mutagenesis Testing, de Serres, F.J., Fouts, J.R., Bend, J.R. and Philpot, R.M. (eds), Elsevier, North-Holland, p. 85-88.

(21) Krahn, D.F., Barsky, F.C. and McCooey, K.T., (1982) CHO/HGPRT Mutation Assay: Evaluation of Gases and Volatile Liquids. In: Tice, R.R., Costa, D.L., Schaich, K.M. (eds). Genotoxic Effects of Airborne Agents. New York, Plenum, p. 91-103.

(22) Zamora, P.O., Benson, J.M., Li, A.P. and Brooks, A.L., (1983) Evaluation of an Exposure System Using Cells Grown on Collagen Gels for Detecting Highly Volatile Mutagens in the CHO/HGPRT Mutation

Assay. Environmental Mutagenesis, 5, p. 795-801.

(23) Applegate, M.L., Moore, M.M., Broder, C.B., Burrell, A. and Hozier, J.C., (1990) Molecular Dissection of Mutations at the Heterozygous Thymidine Kinase Locus in Mouse Lymphoma Cells. Proc. Natl. Acad. Sci. USA, 87, p. 51-55.

(24) Moore, M.M., Clive, D., Hozier, J.C., Howard, B.E., Batson, A.G., Turner, N.T. and Sawyer, J., (1985) Analysis of Trifluorothymidine-Resistant (TFT^r) Mutants of $L5178Y/TK^{+/-}$ Mouse Lymphoma Cells. Mutation Res., 151, p. 161-174.

(25) Yandell, D.W., Dryja, T.P. and Little, J.B., (1990) Molecular Genetic Analysis of Recessive Mutations at a Heterozygous Autosomal Locus in Human Cells. Mutation Res., 229, p. 89-102.

(26) Moore, M.M. and Doerr, C.L., (1990) Comparison of Chromosome Aberration Frequency and Small-Colony TK-Deficient Mutant Frequency in $L5178Y/TK^{+/-}$ - 3.7.2C Mouse Lymphoma Cells. Mutagenesis, 5, p. 609-614.

B.18. **DNA DAMAGE AND REPAIR — UNSCHEDULED DNA SYNTHESIS — MAMMALIAN CELLS *IN VITRO***

1. **METHOD**

1.1. INTRODUCTION

See General introduction Part B.

1.2. DEFINITION

See General introduction Part B.

1.3. REFERENCE SUBSTANCES

None.

1.4. PRINCIPLE OF THE TEST METHOD

The Unscheduled DNA Synthesis (UDS) test measures the DNA repair synthesis after excision and removal of a stretch of DNA containing the region of damage induced by chemical and physical agents. The test is based on the incorporation of tritium labelled thymidine (^{3}H-TdR) into the DNA of mammalian cells, which are not in the S phase of the cell cycle. The uptake of ^{3}H-TdR may be determined by autoradiography or by liquid scintillation counting (LSC) of DNA from the treated cells. Mammalian cells in culture, unless primary rat hepatocytes are used, are treated with the test agent with and without an exogenous metabolic activation system. UDS may also be measured in *in vivo* systems.

1.5. QUALITY CRITERIA

None.

1.6. DESCRIPTION OF THE TEST METHOD

Preparations

Test chemicals and control or reference substances should be prepared in growth medium or dissolved or suspended in appropriate vehicles and then further diluted in growth medium for use in the assay. The final concentration of the vehicle should not affect cell viability.

Primary cultures of rat hepatocytes, human lymphocytes or established cell lines (e.g. human diploid fibroblasts) may be used in the assay.

Cells should be exposed to the test chemical both in the presence and absence of an appropriate metabolic activation system.

Test conditions

Number of cultures

At least two cell cultures for autoradiography and six cultures (or less if scientifically justified) for LSC UDS determinations are necessary for each experimental point.

Use of negative and positive controls

Concurrent positive and negative (untreated and/or vehicle) controls with and without metabolic activation should be included in each experiment.

Examples of positive controls for the rat hepatocyte assay include 7,12- dimethylbenzathracene (7,12- DMBA) or 2-acetylaminofluorene (2-AAF). In the case of established cell lines 4-nitroquinoline-N-oxide (4-NQO) is an example of a positive control for both the autoradiographic and LSC assays performed without metabolic activation; N-dimethylnitrosamine is an example of a positive control compound when metabolic activation systems are used.

Exposure concentrations

Multiple concentrations of the test substance over a range adequate to define the response should be used. The highest concentration should elicit some cytotoxic effects. Relatively water-insoluble compounds should be tested up to the limit of solubility. For freely water-soluble non-toxic chemicals, the upper test chemical concentration should be determined on a case-by-case basis.

Cells

Appropriate growth media, CO_2 concentration, temperature and humidity should be used in maintaining cultures. Established cell lines should be periodically checked for *Mycoplasma* contamination.

Metabolic activation

A metabolic activation system is not used with primary hepatocyte cultures. Established cell lines and lymphocytes are exposed to test substance both in the presence and absence of an appropriate metabolic activation system.

Procedure

Preparation of cultures

Established cell lines are generated from stock cultures (e.g. by trypsinisation or by shaking off), seeded in culture vessels at appropriate density, and incubated at 37 °C.

Short-term cultures of rat hepatocytes are established by allowing freshly dissociated hepatocytes in an appropriate medium to attach themselves to the growing surface.

Human lymphocyte cultures are set up using appropriate techniques.

Treatment of the cultures with the test substance

Primary rat hepatocytes

Freshly isolated rat hepatocytes are treated with the test substance in a medium containing ^{3}H-TdR for an appropriate length of time. At the end of the treatment period, medium should be drained off the cells, which are then rinsed fixed and dried. Slides should be dipped in autoradiographic emulsion (alternative stripping film may be used), exposed, developed, stained and counted.

Established cell lines and lymphocytes

Autoradiographic techniques: cell cultures are exposed to the test substance for appropriate durations followed by treatment with ^{3}H-TdR. The times will be governed by the nature of the substance, the activity of metabolising systems and the type of cells. To detect the peak of UDS, ^{3}H-TdR should be added either simultaneously with the test subsrance or within a few minutes after exposure to the test substance. The choice between these two procedures will be influenced by possible interactions between test substance and ^{3}H-TdR. In order to discriminate between UDS and semi-conservative DNA replication, the latter can be inhibited, for example, by the use of an arginine-deficient medium, low serum content or by hydroxyurea in the culture medium.

LSC measurements of UDS: prior to treatment with test substance, entry of cells into S-phase should be blocked as described above; cells should then be exposed to test chemical as described for autoradiography. At the end of the incubation period, DNA should be extracted from the cells and the total DNA content, and the extent of ^{3}H-TdR, incorporation determined.

It should be noted that, where human lymphocytes are used in the above techniques, the suppression of semi-conservative DNA replication is unnecessary in unstimulated cultures.

Analysis

Autoradiographic determinations

In determining UDS in cells in culture, S-phase nuclei are not counted. At least 50 cells per concentration should be counted. Slides should be coded before counting. Several widely separated random fields should be counted on each slide. The amount of ^{3}H-TdR incorporation in the cytoplasm should be determined by counting three nucleus-sized areas in the cyptoplasm of each cell counted.

LSC determinations

An adequate number of cultures should be used at each concentration and in the controls in LSC UDS determinations.

All results should be confirmed in an independent experiment.

2. DATA

Data should be presented in tabular form.

2.1. AUTORADIOGRAPHIC DETERMINATIONS

The extent of ^{3}H-TdR incorporation in the cytoplasm and the number of grains found over the cell nucleus should be recorded separately.

Mean, median and mode may be used to describe the distribution of the extent of ^{3}H-TdR incorporation in the cytoplasm and the number of grains per nucleus.

2.2. LSC DETERMINATIONS

For LSC determinations, ^{3}H-TdR incorporation should be reported as dpm/μg DNA. The mean dpm/μg DNA with standard deviation may be used to describe the distribution of incorporation.

Data should be evaluated using appropriate statistical methods.

3. REPORTING

3.1. TEST REPORT

The test report shall, if possible, contain the following information:

— cells used, density and passage number at time of treatment, number of cell cultures,

— methods used for maintenance of cell cultures including medium, temperature and CO_2 concentration,

— test substance, vehicle, concentrations and rationale for selection of concentrations used in the assay,

— details of metabolic activation systems,

— treatment schedule,

— positive and negative controls,

— autoradiographic technique used,

— procedures used to block entry of cells into S-phase,

— procedures used for DNA extraction and determination of total DNA content in LSC determination,

— dose/response relationship, where possible,

— statistical evaluation,

— discussion of results,

— interpretation of results.

3.2. VALUATION AND INTERPRETATION

See General introduction Part B.

4. **REFERENCES**

See General introduction Part B.

B.19. SISTER CHROMATID EXCHANGE ASSAY *IN VITRO*

1. METHOD

1.1. INTRODUCTION

See General introduction Part B.

1.2. DEFINITION

See General introduction Part B.

1.3. REFERENCE SUBSTANCES

None.

1.4. PRINCIPLE OF THE TEST METHOD

The Sister Chromatid Exchange (SCE) assay is a short-term test for the detection of reciprocal exchanges of DNA between two sister chromatids of a duplicating chromosome. SCEs represent the interchange of DNA replication products at apparently homologous loci. The exchange process presumably involves DNA breakage and reunion, although little is known about its molecular basis. Detection of SCEs requires some means of differentially labelling sister chromatids and this can be achieved by incorporation of bromodeoxyuridine (BrdU) into chromosomal DNA for two cell cycles.

Mammalian cells *in vitro* are exposed to the test chemical with and without a mammalian exogenous metabolic activation system, if appropriate, and cultured for two rounds of replication in BrdU-containing medium. After treatment with a spindle inhibitor (e.g. colchicine) to accumulate cells in a metaphase-like stage of mitosis (c-metaphase), cells are harvested and chromosome preparations are made.

1.5. QUALITY CRITERIA

None.

1.6. DESCRIPTION OF THE TEST METHOD

1.6.1. *Preparations*

— Primary cultures, (human lymphocytes) or established cell lines (e.g. Chinese hamster ovary cells) may be used in the assay. Cell lines should be checked for *Mycoplasma* contamination,

— appropriate culture media and incubation conditions (e.g. temperature, culture vessels, CO_2 concentration and humidity) should be used,

— test substances may be prepared in culture media or dissolved or suspended in appropriate vehicles prior to treatment of the cells. The final concentration of a vehicle in the culture system should not significantly affect, cell viability or growth rate and effects on SCE frequency should be monitored by a solvent control,

— cells should be exposed to the test substance both in the presence and absence of an exogenous mammalian metabolic activation system. Alternatively, where cell types with intrinsic metabolic activity are used, the rate and nature of the activity should be appropriate to the chemical class being tested.

1.6.2. *Test conditions*

Number of cultures

At least duplicate cultures should be used for each experimental point.

Use of negative and positive controls

Positive controls, using both a direct acting compound and a compound requiring metabolic activation should be included in each experiment; a vehicle control should also be used.

The following are examples of substances which might be used as positive controls:

— direct acting compound:

  — ethylmethanesulphonate,

— indirect acting compound:

  — cyclophosphamide.

When appropriate, an additional positive control of the same chemical class as the chemical under test may be included.

Exposure concentrations

At least three adequately spaced concentrations of the test substance should be used. The highest concentration should give rise to a significant toxic effect but must still allow adequate cell replication to occur. Relatively water-insoluble substances should be tested up to the limit of solubility using appropriate procedures. For freely water-soluble non-toxic substances the upper test substance concentration should be determined on a case-by-case basis.

1.6.3. *Procedure*

Preparation of cultures

Established cell lines are generated from stock cultures (e.g. by trypsinisation or by shaking off), seeded in culture vessels at appropriate density and incubated at 37 °C. For monolayer cultures, the number of cells per culture vessel should be adjusted so that the cultures are not much more than 50 % confluent at the time of harvest. Alternatively, cells may be used in suspension culture. Human lymphocyte cultures are set up from heparinized blood, using appropriate techniques, and incubated at 37 °C.

Treatment

Cells in an exponential stage of growth are exposed to the test substance for a suitable period of time; in most cases one to two hours may be effective, but the treatment time may be extended up to two complete cell cycles in certain cases. Cells without sufficient instrinsic metabolic activity should be exposed to the test chemical in the presence and absence of an appropriate metabolic activation system. At the end of the exposure period, cells are washed free of test substance and cultured for two rounds of replication in the presence of BrdU. As an alternative procedure cells may be exposed simultaneously to the test chemical and BrdU for the complete culture time of two cell cycles.

Human lymphocyte cultures are treated while they are in a semisynchronous condition.

Cells are analysed in their second post-treatment division, ensuring that the most sensitive cell cycle stages have been exposed to the chemical. All cultures to which BrdU is added should be handled in darkness or in dim light from incandescent lamps up to the harvesting of cells in order to minimise photolysis of BrdU-containing DNA.

Harvesting of cells

Cell cultures are treated with a spindle inhibitor (e.g. colchicine) one to four hours prior to harvesting. Each culture is harvested and processed separately for the preparation of chromosomes.

Chromosome preparation and staining

Chromosome preparations are made by standard cytogenetic techniques. Staining of slides to show SCEs can be performed by several techniques, (e.g. the fluorescence plus Giemsa method).

Analysis

The number of cells analysed should be based on the spontaneous control frequency of SCE. Usually, at least 25 well-spread metaphases per culture are analysed for SCEs. Slides are coded before analysis. In human lymphocytes only metaphases containing 46 centromeres are analysed. In established cell lines only metaphases containing ± 2 centromeres of the modal number are analysed. It should be stated whether or not centromeric switch of label is, scored as an SCE. The results should be confirmed in an independent experiment.

2. DATA

Data should be presented in tabular form. The number of SCEs for each metaphase and the number of SCEs per chromosome for each metaphase should be listed separately for all treated and control cultures.

The data should be evaluated using appropriate statistical methods.

3. REPORTING

3.1. TEST REPORT

The test report shall, if possible, contain the following information:

— cells used, methods of maintenance of cell culture,

— test conditions: composition of media, CO_2 concentration, concentration of test substance, vehicle used, incubation temperature, treatment time, spindle inhibitor used, its concentration and the duration of treatment with it, type of mammalian activation system used, positive and negative controls,

— number of cell cultures per experimental point,

— details of the technique used for slide preparation,

— number of metaphases analysed (data given separately for each culture),

— mean number of SCE per cell and per chromosome (data given separately for each culture),

— criteria for scoring SCE,

— rationale for dose selection,

— dose-response relationship, if applicable,

— statistical evaluation,

— discussion of results,

— interpretation of results.

3.2. EVALUATION AND INTERPRETATION

See General introduction Part B.

4. **REFERENCES**

See General introduction Part B.

B.20. **SEX-LINKED RECESSIVE LETHAL TEST IN *DROSOPHILA MELANOGASTER***

1. **METHOD**

1.1. INTRODUCTION

See General introduction Part B.

1.2. DEFINITION

See General introduction Part B.

1.3. REFERENCE SUBSTANCES

None.

1.4. PRINCIPLES OF THE TEST METHOD

The sex-linked recessive lethal (SLRL) test using *Drosophila melanogaster* detects the occurrence of mutations, both point mutations and small deletions, in the germ line of the insect. This test is a forward mutation assay capable of screening for mutations at about 800 loci on the X-chromosome; this represents about 80 % of all X-chromosal loci. The X-chromosome represents approximately one-fifth of the entire haploid genome.

Mutations in the X-chromosome of *Drosophila melanogaster* are phenotypically expressed in males carrying the mutant gene. When the mutation is lethal in the hemizygous condition, its presence is inferred from the absence of one class of male offspring out of the two that are normally produced by a heterozygous female. The SLRL test takes advantage of these facts by means of specially marked and arranged chromosomes.

1.5. QUALITY CRITERIA

None.

1.6. DESCRIPTION OF THE TEST METHOD

Preparations

Stocks

Males of a well-defined wild-type stock and females of the Muller-5 stock may be used. Other appropriately marked female stocks with multiple inverted X-chromosomes may also be used.

Test substance

Test substances should be dissolved in water. Substances, which are insoluble in water may be dissolved or suspended in appropriate vehicles (e.g. a mixture of ethanol and Tween-60 or 80), then diluted in water or saline prior to administration. Dimethylsulphoxide (DMSO) should be avoided as a vehicle.

Number of animals

The test should be designed with a predetermined sensitivity and power. The spontaneous mutant frequency observed in the appropriate control will influence strongly the number of treated chromosomes that must be analysed.

Route of administration

Exposure may be oral, by injection or by exposure to gases or vapours. Feeding of the test substance may be done in sugar solution. When necessary, substances may be dissolved in a 0,7 % NaCl solution and injected into the thorax or abdomen.

Use of negative and positive controls

Negative (vehicle) and positive controls should be included. However, if appropriate laboratory historical control data are available, no concurrent controls are needed.

Exposure levels

Three exposure levels should be used. For a preliminary assessment one exposure level of the test substance may be used, that exposure level being either the maximum tolerated concentration or that producing some indication of toxicity. For non-toxic substances exposure to the maximum practicable concentration should be used.

Procedure

Wild-type males (three to five days old) are treated with the test substance and mated individually to an excess of virgin females from the Muller-5 stock or from another appropriately marked (with multiple inverted X-chromosomes) stock. The females are replaced with fresh virgins every two to three days to cover the entire germ cell cycle. The offspring of these females are scored for lethal effects corresponding to the effects on mature sperm, mid or late-stage spermatids, early spermatids, spermatocytes and spermatogonia at the time of treatment.

Heterozygous F_1 females from the above crosses are allowed to mate individually (i.e. one female per vial) with their brothers. In the F_2 generation, each culture is scored for the absence of wild-type males. If a culture appears to have arisen from an F_1 female carrying a lethal in the parental X-chromosome (i.e. no males with the treated chromosome are observed) daughters of that female with the same genotype should be tested to ascertain whether the lethality is repeated in the next generation.

2. DATA

Data should be tabulated to show the number of X-chromosomes tested, the number of non-fertile males and the number of lethal chromosomes at each exposure concentration and for each mating period for each male treated. Numbers of clusters of different sizes per male should be reported. These results should be confirmed in a separate experiment.

Appropriate statistical methods should be used in evaluation sex-linked recessive lethal tests. Clustering of recessive lethals originating from one male should be considered and evaluated in an appropriate statistical manner.

3. REPORTING

3.1. TEST REPORT

The test report shall, if possible, contain the following information:

— stock: Drosophila stocks or strains used, age of insects, number of males treated, number of sterile males, number of F2 cultures established, number of F2 cultures without progeny, number of chromosomes carrying a lethal detected at each germ cell stage,

— criteria for establishing the size of treated groups,

— test conditions. detailed description of treatment and sampling schedule, exposure levels, toxicity data, negative (solvent) and positive controls, if appropriate,

— criteria for scoring lethal mutations,

— exposure/effect relationship where possible,

— evaluation of data,

— discussion of results,

— interpretation of results.

3.2. EVALUATION AND INTERPRETATION

See General introduction Part B.

4. **REFERENCES**

See General introduction Part B.

B.21. *IN VITRO* MAMMALIAN CELL TRANSFORMATION TESTS

1. METHOD

1.1. INTRODUCTION

See General introduction Part B.

1.2. DEFINITION

See General introduction Part B.

1.3. REFERENCE SUBSTANCES

None.

1.4. PRINCIPLE OF THE TEST METHOD

Mammalian cell culture systems may be used to detect phenotypic changes in vitro induced by chemical substances associated with malignant transformation *in vivo*. Widely used cells include C3H10$T_{1/2}$, 3T3, SHE, Fischer rat and the tests rely on changes in cell morphology, focus formation or changes in anchorage dependence in semi-solid agar. Less widely used systems exist which detect other physiological or morphological changes in cells following exposure to carcinogenic chemicals. None of the *in vitro* test endpoints has an established mechanistic link with cancer. Some of the test systems are capable of detecting tumour promotors. Cytotoxicity may be determined by measuring the effect of the test material on colony-forming abilities (cloning efficiency) or growth rates of the cultures. The measurement of cytotoxicity is to establish that exposure to the test chemical has been toxicologically relevant but cannot be used to calculate transformation: frequency in all assays since some may involve prolonged incubation and/or replating.

1.5. QUALITY CRITERIA

None.

1.6. DESCRIPTION OF THE TEST METHOD

Preparations

Cells

A variety of cell lines or primary cells are available depending on the transformation test being used. The investigator must ensure that the cells in the test being performed exhibit the appropriate phenotypic change after exposure to known carcinogens and that the test, in the investigator's laboratory, is of proven and documented validity and reliability.

Medium

Media and experimental conditions should be used that are appropriate to the transformation assay in use.

Test substance

Test substances may be prepared in culture media or dissolved or suspended in appropriate vehicles prior to treatment of the cells. The final concentration of the vehicle in the culture system should not affect cell viability, growth rate or transformation incidence.

Metabolic activation

Cells should be exposed to the test substance both in the presence and absence of an appropriate metabolic activation system. Alternatively, when cell types are used that possess intrinsic metabolic activity, the nature of the activity should be known to be appropriate to the chemical class being tested.

Test conditions

Use of negative and positive controls

Positive controls, using both a direct-acting compound and a compound requiring metabolic activation should be included in each experiment; a negative (vehicle) control should also be used.

The following are examples of substances, which might be used as positive controls:

— Direct-acting chemicals:

  — Ethylmethanesulphonate,

  — β-propiolactone,

— Compounds requiring metabolic activation:

  — 2-acetylaminofluorene,

  — 4-dimethylaminoazobenzene,

  — 7,12-dimethylbenzanthracene.

When appropriate, an additional positive control of the same chemical class as the compound under test should be included.

Exposure concentrations

Several concentrations of the test substance should be used. These concentrations should yield a concentration-related toxic effect, the highest concentration producing a low level of survival and the survival in the lowest concentration being approximately the same as that in the negative control. Relatively water-insoluble substances should be tested up to the limit of solubility using appropriate procedures. For freely water-soluble non-toxic substances the upper test substance concentration should be determined on a case-by-case basis.

Procedure

Cells should be exposed for a suitable period of time depending on the test system in use, and this may involve re-dosing accompanied by a change of medium (and if necessary, fresh metabolic activation mixture) if exposure is prolonged. Cells without sufficient intrinsic metabolic activity should be exposed to the test substance in the presence and absence of an appropriate metabolic activation system. At the end of the exposure period, cells are washed free of test substance and cultured under conditions appropriate for the appearance of the transformed phenotype being monitored and the incidence of transformation determined. All results are confirmed in an independent experiment.

2. DATA

Data should be presented in tabular form and may take a variety of forms according to the assay being used e.g. plate counts, positive plates or numbers of transformed cells. Where appropriate, survival should be expressed as a percentage of control levels and transformation frequency expressed as the number of transformants per number of survivors. Data should be evaluated using appropriate statistical methods.

3. REPORTING

3.1. TEST REPORT

The test report shall, if possible, contain the following information:

— cell type used, number of cell cultures, methods for maintenance of cell cultures,

— test conditions: concentration of test substance, vehicle used, incubation time, duration and frequency of treatment, cell density during treatment, type of exogenous metabolic activation system used, positive and negative controls, specification of phenotype being monitored, selective system used (if appropriate), rational for dose selection,

— method used to enumerate viable and transformed cells,

— statistical evaluation,

— discussion of results,

— interpretation of results.

3.2. EVALUATION AND INTERPRETATION

See General introduction Part B.

4. **REFERENCES**

See General introduction Part B.

B.22. RODENT DOMINANT LETHAL TEST

1. METHOD

1.1. INTRODUCTION

See General introduction Part B.

1.2. DEFINITION

See General introduction Part B.

1.3. REFERENCE SUBSTANCES

None.

1.4. PRINCIPLE OF THE TEST METHOD

Dominant lethal effects cause embryonic or foetal death. Induction of dominant lethals by exposure to a chemical substance indicates that the substance has affected germinal tissue of the test species. It is generally accepted that dominant lethals are due to chromosomal damage (structural and numerical anomalies). Embryonic death if females are treated may also be the result of toxic effects.

Generally, male animals are exposed to the test compound and mated to untreated virgin females. The various germ cell stages can be tested separately by the use of sequential mating intervals. The increase of dead implants per female in the treated group over the dead implants per female in the control group reflects the post-implantational loss. Pre-implantational loss can be estimated based on corpora lutea counts or by comparing the total implants per female in treated and control groups. The total dominant lethal effect is the sum of pre- and post-implantational loss. The calculation of the total dominant lethal effect is based on comparison of the live implants per female in the test group to the live implants per female in the control group. A reduction in the number of implants at certain intervals may be the result of cell killing (i.e. of spermatocytes and/or spermatogonia).

1.5. QUALITY CRITERIA

None.

1.6. DESCRIPTION OF THE TEST METHOD

Preparations

When possible, test substances should be dissolved or suspended in isotonic saline. Chemicals insoluble in water may be dissolved or suspended in appropriate vehicles. The vehicle used should neither interfere with the test chemical nor produce toxic effects. Fresh preparations of the test chemical should be employed.

Test conditions

Route of administration

The test compound should generally be administered only once. Based on toxicological information a repeated treatment schedule can be employed. The usual routes of administration are oral intubation or intraperitoneal injection. Other routes of administration may be appropriate.

Experimental animals

Rats or mice are recommended as the test species. Healthy fully sexually mature animals are randomised and assigned to treatment and control groups.

Number and sex

An adequate number of treated males should be used, taking into account the spontaneous variation of the biological character being evaluated. The number chosen should be based on the pre-determined sensitivity of detection and power of significance. For example in a typical test, the number of males in each dose group should be sufficient to provide between 30 and 50 pregnant females per mating interval.

Use of negative and positive controls

Generally concurrent positive and negative (vehicle) controls should be included in each experiment. When acceptable positive control results are available from experiments conducted recently in the same laboratory these results can be used instead of a concurrent positive control. Positive control substances should be used at an appropriate low dose (e.g. MMS, intraperitoneally, at 10 mg/kilogram) to demonstrate the test sensitivity.

Dose levels

Normally, three dose levels should be used. The high dose should produce signs of toxicity or reduced fertility in the treated animals. In certain cases a single high dose level may be sufficient.

Limit test

Non-toxic substances should be tested at 5 g/kilogram on a single administration or at 1 g/kilogram/day on repeated administration.

Procedure

Several treatment schedules are available. Single administration of the test substance is the most widely used. Other treatment schedules may be used.

Individual males are mated sequentially to one or two untreated virgin females at appropriate intervals after treatment. Females should be left with the males for at least the duration of one oestrous cycle or until mating has occurred as determined by the presence of sperm in the vagina or by the presence of a vaginal plug.

The number of matings following treatment is governed by the treatment schedule and should ensure that all germ cell stages are sampled after treatment.

Females are sacrificed in the second half of pregnancy and uterine contents are examined to determine the number of dead and live implants. The ovaries may be examined to determine the number of corpora lutea.

2. **DATA**

Data should be tabulated to show the number of males, the number of pregnant females, and the number of non-pregnant females. Results of each mating, including the identity of each male and female, should be reported individually. For each female, week of mating, dose level received by the males, the frequencies of live implants and of dead implants should be recorded.

The calculation of the total dominant lethal effect is based on comparison of the live implants per female in the test group to the live implants per female in the control group. The ratio of dead to live implants from the treated group compared to the same ratio from the control group is analysed to indicate the post-implantation loss.

If the data are recorded as early and late deaths, the tables should make that clear. If pre-implantation loss is estimated, it should be reported. Pre-implantation loss can be calculated as a discrepancy between the number of corpora lutea and the number of implants or as a reduction in the average number of implants per uterus in comparison with control matings.

Data are evaluated using appropriate statistical methods.

3. **REPORTING**

3.1. TEST REPORT

The test report shall, if possible, contain the following information:

— species, strain, age and weights of animals used, number of animals of each sex in experimental and control groups,

— test substance, vehicle, dose levels tested and rationale for dose selection, negarive and positive controls, toxicity data,

— route and treatment schedule,

— mating schedule,

— method used to determine that mating has occurred,

— time of sacrifice,

— criteria for scoring dominant lethals,

— dose/response relationship, if applicable,

— statistical evaluation,

— discussion of results,

— interpretation of results.

3.2. EVALUATION AND INTERPRETATION

See General introduction Part B.

4. **REFERENCES**

See General introduction Part B.

B.23. MAMMALIAN SPERMATOGONIAL CHROMOSOME ABERRATION TEST

1. METHOD

This method is a replicate of the OECD TG 483, Mammalian Spermatogonial Chromosome Aberration Test (1997).

1.1. INTRODUCTION

The purpose of the *in vivo* mammalian spermatogonial chromosome aberration test is to identify those substances that cause structural chromosome aberrations in mammalian spermatogonial cells (1)(2)(3)(4)(5). Structural aberrations may be of two types, chromosome or chromatid. With the majority of chemical mutagens, induced aberrations are of the chromatid type, but chromosome-type aberrations also occur. This method is not designed to measure numerical aberrations and is not routinely used for that purpose. Chromosome mutations and related events are the cause of many human genetic diseases.

This test measures chromosome events in spermatogonial germ cells and is, therefore, expected to be predictive of induction of inheritable mutations in germ cells.

Rodents are routinely used in this test. This *in vivo* cytogenetic test detects chromosome aberrations in spermatogonial mitoses. Other target cells are not the subject of this method.

To detect chromatid-type aberrations in spermatogonial cells, the first mitotic cell division following treatment should be examined before these lesions are lost in subsequent cell divisions. Additional information from treated spermatogonial stem cells can be obtained by meiotic chromosome analysis for chromosome-type aberrations at diakinesis-metaphase I when the treated cells become spermatocytes.

This *in vivo* test is designed to investigate whether somatic cell mutagens are also active in germ cells. In addition, the spermatogonial test is relevant to assessing mutagenicity hazard in that it allows consideration of factors of *in vivo* metabolism, pharmacokinetics and DNA-repair processes.

A number of generations of spermatogonia are present in the testis with a spectrum of sensitivity to chemical treatment. Thus, the aberrations detected represent an aggregate response of treated spermatogonial cell populations, with the more numerous differentiated spermatogonial cells predominating. Depending on their position within the testis, different generations of spermatogonia may or may not be exposed to the general circulation, because of the physical and physiological Sertoli cell barrier and the blood-testis barrier.

If there is evidence that the test substance, or a reactive metabolite, will not reach the target tissue, it is not appropriate to use this test.

See also General introduction Part B.

1.2. DEFINITIONS

Chromatid-type aberration: structural chromosome damage expressed as breakage of single chromatids or breakage and reunion between chromatids.

Chromosome-type aberration: structural chromosome damage expressed as breakage, or breakage and reunion, of both chromatids at an identical site.

Gap: an achromatic lesion smaller than the width of one chromatid, and with minimum misalignment of the chromatids.

Numerical aberration: a change in the number of chromosomes from the normal number characteristic of the animals utilised.

Polyploidy: a multiple of the haploid chromosome number (*n*) other than the diploid number (i.e. 3*n*, 4*n* and so on).

Structural aberration: a change in chromosome structure detectable by microscopic examination of the metaphase stage of cell division, observed as deletions, intrachanges or interchanges.

1.3. PRINCIPLE OF THE TEST METHOD

Animals are exposed to the test substance by an appropriate route of exposure and are sacrificed at appropriate times after treatment. Prior to sacrifice, animals are treated with a metaphase-arresting substance (e.g. Colcemid® or colchicine). Chromosome preparations are then made from germ cells and stained, and metaphase cells are analysed for chromosome aberrations.

1.4. DESCRIPTION OF THE TEST METHOD

1.4.1. **Preparations**

1.4.1.1. *Selection of animal species*

Male Chinese hamsters and mice are commonly used. However, males of other appropriate mammalian species may be used. Commonly used laboratory strains of young healthy adult animals should be employed. At the commencement of the study the weight variation of animals should be minimal and not exceed ± 20 % of the mean weight.

1.4.1.2. *Housing and feeding conditions*

General conditions referred in the General introduction to Part B are applied although the aim for humidity should be 50-60 %.

1.4.1.3. *Preparation of the animals*

Healthy young adult males are randomly assigned to the control and treatment groups. Cages should be arranged in such a way that possible effects due to cage placement are minimised. The animals are identified uniquely. The animals are acclimated to the laboratory conditions for at least five days prior to the start of the study.

1.4.1.4. *Preparation of doses*

Solid test substances should be dissolved or suspended in appropriate solvents or vehicles and diluted, if appropriate, prior to dosing of the animals. Liquid test substances may be dosed directly or diluted prior to dosing. Fresh preparations of the test substance should be employed unless stability data demonstrate the acceptability of storage.

1.4.2. **Test conditions**

1.4.2.1. *Solvent/Vehicle*

The solvent/vehicle should not produce toxic effects at the dose levels used and should not be suspected of chemical reaction with the test substance. If other than well-known solvents/vehicles are used, their inclusion should be supported by data indicating their compatibility. It is recommended that wherever possible, the use of an aqueous solvent/vehicle should be considered first.

1.4.2.2. *Controls*

Concurrent positive and negative (solvent/vehicle) controls should be included in each test. Except for treatment with the test substance, animals in the control groups should be handled in an identical manner to animals in the treated groups.

Positive controls should produce structural chromosome aberrations *in vivo* in spermatogonial cells when administered at exposure levels expected to give a detectable increase over background.

Positive control doses should be chosen so that the effects are clear but do not immediately reveal the identity of the coded slides to the reader. It is acceptable that the positive control be administered by a route different from the test substance and sampled at only a single time. In addition, the use of chemical class-related positive control chemicals may be considered, when available. Examples of positive control substances include:

Substance	CAS No	Einecs No
Cyclophosphamide Cyclophosphamide monohydrate	50-18-0 6055-19-2	200-015-4
Cyclohexylamine	108-91-8	203-629-0
Mitomycin C	50-07-7	200-008-6
Monomeric acrylamide	79-06-1	201-173-7
Triethylenemelamine	51-18-3	200-083-5

Negative controls, treated with solvent or vehicle alone, and otherwise treated in the same way as the treatment groups, should be included for every sampling time, unless acceptable inter-animal variability and frequency of cells with chromosome aberrations are demonstrated by historical control data. In addition, untreated controls should also be used unless there are historical or published control data demonstrating that no deleterious or mutagenic effects are induced by the chosen solvent/vehicle.

1.5. PROCEDURE

1.5.1. **Number of animals**

Each treated and control group must include at least five analysable males.

1.5.2. **Treatment schedule**

Test substances are preferably administered once or twice (i.e. as a single treatment or as two treatments). Test substances may also be administered as a split dose, i.e. two treatments on the same day separated by no more than a few hours, to facilitate administering a large volume of material. Other dose regimens should be scientifically justified.

In the highest dose group two sampling times after treatment are used. Since cell cycle kinetics can be influenced by the test substance, one early and one late sampling time are used around 24 and 48 hours after treatment. For doses other than the highest dose, a sampling time of 24 hours or 1,5 cell cycle length after treatment should be taken, unless another sampling time is known to be more appropriate for detection of effects (6).

In addition, other sampling times may be used. For example in the case of chemicals, which may induce chromosome lagging, or may exert S-independent effects, earlier sampling times may be appropriate (1).

The appropriateness of a repeated treatment schedule needs to be identified on a case-by-case basis. Following a repeated treatment schedule the animals should then be sacrificed 24 hours (1,5 cell cycle length) after the last treatment. Additional sampling times may be used where appropriate.

Prior to sacrifice, animals are injected intraperitoneally with an appropriate dose of a metaphase arresting substance (e.g. Colcemid® or colchicine). Animals are sampled at an appropriate interval thereafter. For mice this interval is approximately three to five hours, for Chinese hamsters this interval is approximately four to five hours.

1.5.3. **Dose levels**

If a range finding study is performed because there are no suitable data available, it should be performed in the same laboratory, using the same species, strain and treatment regimen to be used in the main study (7). If there is toxicity, three dose levels are used for the first sampling time. These dose levels should cover a range from the maximum to little or no toxicity. At the later sampling time only the highest dose needs to be used. The highest dose is defined as the dose producing signs of toxicity such that higher dose levels, based on the same dosing regimen, would be expected to produce lethality.

Substances with specific biological activities at low non-toxic doses (such as hormones and mitogens) may be exceptions to the dose-setting criteria and should be evaluated on a case-by-case basis. The highest dose may also be defined as a dose that produces some indication of toxicity in the spermatogonial cells (e.g. a reduction in the ratio of spermatogonial mitoses to first and second meiotic metaphases; this reduction should not exceed 50 %).

1.5.4. **Limit test**

If a test at one dose level of at least 2000 mg/kg body weight/day using a single treatment, or as two treatments on the same day, produces no observable toxic effects, and if genotoxicity would not be expected based upon data from structurally related substances, then a full study using three dose levels may not be considered necessary. Expected human exposure may indicate the need for a higher dose level to be used in the limit test.

1.5.5. **Administration of doses**

The test substance is usually administered by gavage using a stomach tube or a suitable intubation cannula, or by intraperitoneal injection. Other routes of exposure may be acceptable where they can be justified. The maximum volume of liquid that can be administered by gavage or injection at one time depends on the size of the test animal. The volume should not exceed 2 ml/100 g body weight. The use of volumes higher than these must be justified. Except for irritating or corrosive substances, which will normally reveal exacerbated effects with higher concentrations, variability in test volume should be minimised by adjusting the concentration to ensure a constant volume at all dose levels.

1.5.6. **Chromosome preparation**

Immediately after sacrifice, cell suspensions are obtained from one or both testes, exposed to hypotonic solution and fixed. The cells are then spread on slides and stained.

1.5.7. **Analysis**

For each animal at least 100 well-spread metaphase should be analysed (i.e. a minimum of 500 metaphases per group). This number could be reduced when high numbers of aberrations are observed. All slides, including those of positive and negative controls, should be independently coded before microscopic analysis. Since fixation procedures often result in the breakage of a proportion of metaphases with loss of chromosomes, the cells scored should contain a number of centromeres equal to the number $2n \pm 2$.

2. **DATA**

2.1. TREATMENT OF RESULTS

Individual animal data should be presented in a tabular form. The experimental unit is the animal. For each individual animal the number of cells with structural chromosome aberrations and the number of chromosome aberrations per cell should be evaluated. Different types of structural chromosome aberrations should be listed with their numbers and frequencies for treated and control groups. Gaps are recorded separately and reported but generally not included in the total aberration frequency.

If mitosis as well as meiosis is observed, the ratio of spermatogonial mitoses to first and second meiotic metaphases should be determined as a measure of cytotoxicity for all treated and negative control animals in a total sample of 100 dividing cells per animal to establish a possible cytotoxic effect. If only mitosis is observed, the mitosis index should be determined in at least 1 000 cells for each animal.

2.2. EVALUATION AND INTERPRETATION OF RESULTS

There are several criteria for determining a positive result, such as a dose-related increase in the relative number of cells with chromosome aberrations or a clear increase in the number of cells with aberrations in a single dose at a single sampling time. Biological relevance of the results should be considered first. Statistical methods may be used as an aid in evaluating the test results (8). Statistical significance should not be the only determining factor for a positive response. Equivocal results should be clarified by further testing preferably using a modification of experimental conditions.

A test substance for which the results do not meet the above criteria is considered non-mutagenic in this test.

Although most experiments will give clearly positive or negative results, in rare cases the data set will preclude making a definite judgement about the activity of the test substance. Results may remain equivocal or questionable regardless of the number of times the experiment is repeated.

Positive results from the *in vivo* spermatogonial chromosome aberration test indicate that the test substance induces structural chromosome aberrations in the germ cells of the species tested. Negative results indicate that, under the test conditions, the test substance does not induce chromosome aberrations in the germ cells of the species tested.

The likelihood that the test substance or its metabolites reach the target tissue should be discussed.

3. **REPORTING**

TEST REPORT

The test report must include the following information:

Solvent/Vehicle:

— justification for choice of vehicle,

— solubility and stability of the test substance in solvent/vehicle, if known.

Test animals:

— species/strain used,

— number and age of animals,

— source, housing conditions, diet, etc.,

— individual weight of the animals at the start of the test, including body weight range, mean and standard deviation for each group.

Test conditions:

— data from range finding study, if conducted,

— rationale for dose level selection,

— rationale for route of administration,

— details of test substance preparation,

— details of the administration of the test substance,

— rationale for sacrifice times,

— conversion from diet/drinking water test substance concentration (ppm) to the actual dose (mg/kg body weight/day), if applicable.

— details of food and water quality,

— detailed description of treatment and sampling schedules,

— methods for measurement of toxicity,

— identity of metaphase arresting substance, its concentration and duration of treatment,

— methods of slide preparation,

— criteria for scoring aberrations,

— number of cells analysed per animal,

— criteria for considering studies as positive, negative or equivocal.

Results:

— signs of toxicity,

— mitotic index,

— ratio of spermatogonial mitoses cells to first and second meiotic metaphases,

— type and number of aberrations, given separately for each animal,

— total number of aberrations per group,

— number of cells with aberrations per group,

— dose-response relationship, if possible,

— statistical analyses, if any,

— concurrent negative control data,

— historical negative control data with ranges, means and standard deviations,

— concurrent positive control data,

— changes in ploidy, if seen.

Discussion of results.

Conclusions.

4. **REFERENCES**

(1) Adler, I.D., (1986) Clastogenic Potential in Mouse Spermatogonia of Chemical Mutagens Related to their Cell-Cycle Specifications. In: Genetic Toxicology of Environmental Chemicals, Part B: Genetic Effects and Applied Mutagenesis, Ramel, C., Lambert, B. and Magnusson, J. (Eds.) Liss, New York, p. 477-484.

(2) Adler, I.D., (1984) Cytogenetic tests in Mammals. In: Mutagenicity Testing: a Practical Approach. Ed. S. Venitt and J.M. Parry. IRL Press, Oxford, Washington DC, p. 275-306.

(3) Evans, E.P., Breckon, G. and Ford, C.E., (1964) An Air-Drying Method for Meiotic Preparations from Mammalian Testes. Cytogenetics and Cell Genetics, 3, p. 289-294.

(4) Richold, M., Ashby, J., Chandley, A., Gatehouse, D.G. and Henderson, L., (1990) *In Vivo* Cytogenetic Assays, In: D.J. Kirkland (Ed.) Basic Mutagenicity Tests, UKEMS Recommended Procedures. UKEMS Subcommittee on Guidelines for Mutagenicity Testing. Report. Part I revised. Cambridge University Press, Cambridge, New York, Port Chester, Melbourne, Sydney, p. 115-141.

(5) Yamamoto, K. and Kikuchi, Y., (1978) A New Method for Preparation of Mammalian Spermatogonial Chromosomes. Mutation Res., 52, p. 207-209.

(6) Adler, I.D., Shelby M.D., Bootman, J., Favor, J., Generoso, W., Pacchierotti, F., Shibuya, T. and Tanaka N., (1994) International Workshop on Standardisation of Genotoxicity Test Procedures. Summary Report of the Working Group on Mammalian Germ Cell Tests. Mutation Res., 312, p. 313-318.

(7) Fielder, R.J., Allen, J.A., Boobis, A.R., Botham, P.A., Doe, J., Esdaile, D.J., Gatehouse, D.G., Hodson-Walker, G., Morton, D.B., Kirkland, D.J. and Richold, M., (1992) Report of British Toxicology Society/UK Environmental Mutagen Society Working group: Dose setting in *In Vivo* Mutagenicity Assays. Mutagenesis, 7, p. 313-319.

(8) Lovell, D.P., Anderson, D., Albanese, R., Amphlett, G.E., Clare, G., Ferguson, R., Richold, M., Papworth, D.G. and Savage, J.R.K., (1989) Statistical Analysis of *In Vivo* Cytogenetic Assays In: D.J. Kirkland (Ed.) Statistical Evaluation of Mutagenicity Test Data. UKEMS Sub-Committee on Guidelines for Mutagenicity Testing, report, Part III. Cambridge University Press, Cambridge, New York, Port Chester, Melbourne, Sydney, p. 184-232.

B.24. MOUSE SPOT TEST

1. METHOD

1.1. INTRODUCTION

See General introduction Part B.

1.2. DEFINITION

See General introduction Part B.

1.3. REFERENCE SUBSTANCES

None.

1.4. PRINCIPLE OF THE TEST METHOD

This is an *in vivo* test in mice in which developing embryos are exposed to the chemicals. The target cells in the developing embryos are melanoblasts, and the target genes are those which control the pigmentation of the coat hairs. The developing embryos are heterozygous for a number of these coat colour genes. A mutation in, or loss of (by a variety of genetic events), the dominant allele of such a gene in a melanoblast results in the expression of the recessive phenotype in its descendant cells, constituting a spot of changed colour in the coat of the resulting mouse. The number of offspring with these spots, mutations, are scored and their frequency is compared with that among offspring resulting from embryos treated with the solvent only. The mouse spot test detects presumed somatic mutations in foetal cells.

1.5. QUALITY CRITERIA

None.

1.6. DESCRIPTION OF THE TEST METHOD

Preparations

When possible, test substances are dissolved or suspended in isotonic saline. Chemicals insoluble in water are dissolved or suspended in appropriate vehicles. The vehicle used should neither interfere with the test chemical nor produce toxic effects. Fresh preparations of the test chemical should be used.

Experimental animals

Mice of the T strain (nonagouti, a/a; chinchilla, pink eye, $c^{ch}p/c^{ch}p$; brown, b/b; dilute, short ear, d se/d se; piebald spotting, s/s) are mated either with the HT strain (pallid, nonagouti, brachypody, pa a bp/pa a bp; leaden fuzzy, ln fz/ln fz; pearl pe/pe) or with C57BL (nonagouti, a/a). Other appropriate crosses such as between NMRI (nonagouti, a/a; albino, c/c) and DBA (nonagouti, a/a; brown, b/b; dilute d/d) may be used provided they produce nonagouti offspring.

Number and sex

Sufficient pregnant females are treated to provide an appropriate number of surviving offspring for each dose level used. The appropriate sample size in governed by the number of spots observed in the treated mice and the scale of the control data. A negative result is acceptable only when at least 300 offspring from females treated with the highest dose have been scored.

Use of negative and positive controls

Concurrent control data from mice treated with the vehicle only (negative controls) should be available. Historical control data from the same laboratory may be pooled to increase the sensitivity of the test provided they are homogeneous. Positive control data recently obtained in the same laboratory from treatment with a chemical known to show mutagenicity by this test should be available if no mutagenicity of the test chemical is detected.

Route of administration

The usual routes of administration are oral intubation or intraperitoneal injection of the pregnant females. Treatment by inhalation or other routes of administration are used when appropriate.

Dose levels

At least two dose levels are used including one showing signs of toxicity or reduced litter size. For non-toxic chemicals exposure to the maximum practicable dose should be used.

Procedure

A single treatment is normally given on day 8,9 or 10 of pregnancy, counting as day 1 the day on which the vaginal plug is first dbserved. These days correspond to 7,25, 8,25 and 9,25 days after conception. Successive treatments over these days may be used.

Analysis

The offspring are coded and scored for spots between three and four weeks after birth. Three classes of spots are distinguished:

(a) white spots within 5 mm of the mid-ventralline which are presumed to result from cell killing (WMVS);

(b) yellow, agouti-like, spots associated with mammae, genitalia, throat, axillary and inguinal areas and on the mid-forehead, which are presumed to result from misdifferentiation (MDS); and

(c) pigmented and white spots randomly distributed on the coat which are presumed to result from somatic mutations (RS).

All three classes are scored but only the last, RS, is of genetic relevance. Problems of distinguishing between MDS and RS may be solved by fluorescence microscopy of sample hairs.

Obvious gross morphological abnormalities of the offspring should be noted.

2. **DATA**

The data are presented as the total number of offspring scored and the number having one or more presumed somatic mutation spots. Treatment and negative control data are compared by appropriate methods. Data are also presented on a per-litter basis.

3. **REPORTING**

3.1. TEST REPORT

The test report shall, if possible, contain the following information:

— the strains used in the cross,

— the number of pregnant females in the experimental and control groups,

— the average litter size in the experimental and control groups at birth and at weaning,

— the dose level(s) of the test chemical,

— the solvent used,

— the day of pregnancy of which treatment was given,

— the route of treatment,

— the total number of offspring scored, and the number with WMVS, MDS and RS in the experimental and control groups,

— gross morphological abnormalities,

— dose/response relationship of RS when possible,

— statistical evaluation,

— discussion of results,

— interpretation of results.

3.2. EVALUATION AND INTERPRETATION

See General introduction Part B.

4. **REFERENCES**

See General introduction Part B.

B.25. MOUSE HERITABLE TRANSLOCATION

1. METHOD

1.1. INTRODUCTION

See General introduction Part B.

1.2. DEFINITION

See General introduction Part B.

1.3. REFERENCE SUBSTANCES

None.

1.4. PRINCIPLE OF THE TEST METHOD

The mouse heritable translocation test detects structural and numerical chromosome changes in mammalian germ cells as recovered in first generation progeny. The types of chromosome changes detected are reciprocal translocations and, if female progeny are included, X-chromosome loss. Carriers of translocations and XO-females show reduced fertility which is used to select F_1 progeny for cytogenetic analysis. Complete sterility is caused by certain types of translocations (X-autosome and c-t type). Translocations are cytogenetically observed in meiotic cells at diakinesis- metaphase I of male individuals, either F_1 males or male offspring of F_1 females. The XO-females are cytogenetically identified by the presence of only 39 chromosomes in bone marrow mitoses.

1.5. QUALITY CRITERIA

None.

1.6. DESCRIPTION OF THE TEST METHOD

Preparations

The test chemicals are dissolved in isotonic saline. If insoluble they are dissolved or suspended in appropriate vehicles. Freshly prepared solutions of the test compound are employed. If a vehicle is used to facilitate dosing, it must not interfere with the test compound or produce toxic effects.

Route of administration

Routes of administration are usually oral intubation or intraperitoneal injection. Other routes of administration may be appropriate.

Experimental animals

For the ease of breeding and cytological verification these experiments are performed with mice. No specific mouse strain is required. However, the average litter-size of the strain should be greater than eight and be relatively constant.

Healthy sexually mature animals are used.

Number of animals

The number of animals necessary depends upon the spontaneous translocation frequency and the minimal rate of induction required for a positive result.

The test is usually performed by analyses of male F_1 progeny. At least 500 male F_1 progeny should be tested per dose group. If female F_1 progeny are included, 300 males and 300 females are required.

Use of negative and positive controls

Adequate control data, derived from concurrent and historic control should be available. When acceptable positive control results are available from experiments conducted recently in the same laboratory these results can be used instead of a concurrent positive control.

Dose levels

One dose level is tested, usually the highest dose associated with the production of minimal toxic effects, but without affecting reproductive behaviour or survival. To establish a dose/response relationship two additional lower doses are required. For non-toxic chemicals exposure to the maximum practicable dose should be used.

Procedure

Treatment and mating

Two treatment schedules are available. Single administration of the test substance is most widely used. Administration of the test substance on seven days per week for 35 days may also be used. The number of matings following treatment is governed by the treatment schedule and should ensure that all treated germ cell stages are sampled. At the end of the mating period females are caged individually. When females give birth, the date, litter size and sex of progeny are recorded. All male progeny are weaned and all female progeny are discarded unless they are included in the experiment.

Testing for translocation heterozygosity

One of two possible methods is used:

— fertility testing of F_1 progeny and subsequent verification of possible translocation carriers by cytogenetic analysis,

— cytogenetic analysis of all male F_1 progeny without prior selection by fertility testing.

(a) Fertility testing

Reduced fertility of an F_1 individual can be established by litter size observation and/or analysis of uterine contents of female mates.

Criteria for determining normal and reduced fertility must be established for the mouse strain used.

Litter size observation: F_1 males to be tested are caged individually with females either from the same experiment or from the colony. Cages are inspected daily beginning 18 days after mating. Litter size and sex of the F_2 progeny are recorded at birth and litters are discarded thereafter. If female F_1 progeny are tested the F_2 progeny of small litters are kept for further testing. Female translocation carriers are verified by cytogenetic analysis of a translocation in any of their male offspring. XO-females are recognised by the change in sex ratio among their progeny from 1:1 to 1:2 males *versus* females. In a sequential procedure, normal F_1 animals are eliminated from further testing if the first F_2 litter reaches or exceeds a predetermined normal value, otherwise a second or third F_2 litter is observed.

F_1 animals that cannot be classified as normal after observation of up to three F_2 litters are either tested further by analysis of uterine contents of female mates or directly subjected to cytogenetic analysis.

Analysis of uterine contents: the reduction in litter size of translocation carriers is due to embryonic death so that a high number of dead implants is indicative of the presence of a translocation in the animal under test. F_1 males to be tested are mated to two to three females each. Conception is established by daily inspection for vaginal plugs in the morning. Females are sacrificed 14 to 16 days later and living and dead implants in their uteri are recorded.

(b) Cytogenetic analysis

Testes preparations are made by the air-drying technique. Translocation carriers are identified by the presence of multivalent configurations at diakinesis-metaphase I in primary spermatocytes. Observation of at least two cells with multivalent association constitutes the required evidence that the tested animal is a translocation carrier.

If no breeding selection has been performed all F_1 males are inspected cytogenetically. A minimum of 25 diakinesis-metaphase I cells per male must be scored microscopically. Examination of mitotic metaphases, in spermatogonia or bone-marrow, is required in F_1 males with small testes and meiotic breakdown before diakinesis or from F_1 female XO suspects. The presence of an unusually long and/or short chromosome in each of 10 cells is evidence for a particular male sterile translocation (c-t type). Some X-autosome translocations that cause male sterility may only be identified by banding analysis of mitotic chromosomes. The presence of 39 chromosomes in all of 10 mitoses is evidence for an XO condition in a female.

2. DATA

Data are presented in tabular form.

The mean litter size and sex ratio from parental matings at birth and weaning are reported for each mating interval.

For fertility assessment of F_1 animals, the mean litter size of all normal matings and the individual litter sizes of F_1 translocation carriers are presented. For analysis of uterine contents, the mean number of living and dead implants of normal matings and the individual numbers of living and dead implants for each mating of F_1 translocation carriers are reported.

For cytogenetic analysis of diakinesis-metaphase I, the numbers of types of multivalent configurations and the total number of cells are listed for each translocation carrier.

For sterile F_1 individuals, the total number of matings and the duration of the mating period are reported. Testes weights and cytogenetic analysis details are given.

For XO females, the mean litter size, sex ratio of F_1 progeny and cytogenetic analysis results are reported.

Where possible F_1 translocation carriers are preselected by fertility tests, the tables have to include information on how many of these were confirmed translocation heterozygotes.

Data from negative controls and the positive control experiments are reported.

3. REPORTING

3.1. TEST REPORT

The test report shall, if possible, contain the following information:

— strain of mice, age of animals, weights of treated animals,

— numbers of parental animals of each sex in experimental and control groups,

— test conditions, detailed description of treatment, dose levels, solvents, mating schedule,

— number and sex of offspring per female, number and sex of offspring raised for translocation analysis,

— time and criteria of translocation analysis,

— number and detailed description of translocation carriers, including breeding data and uterine content data, if applicable;

— cytogenetic procedures and details of microscopic analysis, preferably with pictures,

— statistical evaluation,

— discussion of results,

— interpretation of results.

3.2. EVALUATION AND INTERPRETATION

See General introduction Part B.

4. **REFERENCES**

See General introduction Part B.

B.26. **SUB-CHRONIC ORAL TOXICITY TESTREPEATED DOSE 90 – DAY ORAL TOXICITY STUDY IN RODENTS**

1. **METHOD**

This sub-chronic oral toxicity test method is a replicate of the OECD TG 408 (1998).

1.1. INTRODUCTION

In the assessment and evaluation of the toxic characteristics of a chemical, the determination of sub-chronic oral toxicity using repeated doses may be carried out after initial information on toxicity has been obtained from acute or repeated dose 28-day toxicity tests. The 90-day study provides information on the possible health hazards likely to arise from repeated exposure over a prolonged period of time covering post-weaning maturation and growth well into adulthood. The study will provide information on the major toxic effects, indicate target organs and the possibility of accumulation, and can provide an estimate of a no-observed-adverse-effect level of exposure which can be used in selecting dose levels for chronic studies and for establishing safety criteria for human exposure.

The method places additional emphasis on neurological endpoints and gives an indication of immunological and reproductive effects. The need for careful clinical observations of the animals, so as to obtain as much information as possible, is also stressed. This study should allow for the identification of chemicals with the potential to cause neurotoxic, immunological or reproductive organ effects, which may warrant further in-depth investigation.

See also General introduction Part B.

1.2. DEFINITIONS

Dose: is the amount of test substance administered. Dose is expressed as weight (g, mg) or as weight of test substance per unit weight of test animal (e.g. mg/kg), or as constant dietary concentrations (ppm).

Dosage: is a general term comprising of dose, its frequency and the duration of dosing.

NOAEL: is the abbreviation for no-observed-adverse-effect level and is the highest dose level where no adverse treatment-related findings are observed.

1.3. PRINCIPLE OF THE TEST METHOD

The test substance is orally administered daily in graduated doses to several groups of experimental animals, one dose level per group for a period of 90 days. During the period of administration the animals are observed closely for signs of toxicity. Animals, which die or are killed during the test are necropsied and, at the conclusion of the test, surviving animals are also killed and necropsied.

1.4. DESCRIPTION OF THE TEST METHOD

1.4.1. **Preparations of animals**

Healthy animals, which have been acclimated to laboratory conditions for at least five days and have not been subjected to previous experimental procedures, should be used. The test animals should be characterised as to species, strain, source, sex, weight and/or age. Animals should be randomly assigned to the control and treatment groups. Cages should be arranged in such a way that possible effects due to cage placement are minimised. Each animal should be assigned a unique identification number.

1.4.2. **Preparations of doses**

The test substance is administered by gavage or via the diet or drinking water. The method of oral administration is dependent on the purpose of the study, and the physical/chemical properties of the test material.

Where necessary, the test substance is dissolved or suspended in a suitable vehicle. It is recommended that, wherever possible, the use of an aqueous solution/suspension be considered first, followed by consideration of a solution/emulsion in oil (e.g. corn oil) and then by possible solution in other vehicles. For vehicles other than water the toxic characteristics of the vehicle must be known. The stability of the test substance under the conditions of administration should be determined.

1.4.3. **Test conditions**

1.4.3.1. *Experimental animals*

The preferred species is the rat, although other rodent species, e.g. the mouse, may be used. Commonly used laboratory strains of young healthy adult animals should be employed. The females should be nulliparous and non-pregnant. Dosing should begin as soon as possible after weaning and, in any case, before the animals are nine weeks old. At the commencement of the study the weight variation of animals used should be minimal and not exceed ± 20 % of the mean weight of each sex. Where the study is conducted as a preliminary to a long term chronic toxicity study, animals from the same strain and source should be used in both studies.

1.4.3.2. *Number and sex*

At least 20 animals (10 female and 10 male) should be used at each dose level. If interim kills are planned, the number should be increased by the number of animals scheduled to be killed before the completion of the study. Based on previous knowledge of the chemical or a close analogue, consideration should be given to including an additional satellite group of ten animals (five per sex) in the control and in the top dose group for observation, after the treatment period, of reversibility or persistence of any toxic effects. The duration of this post-treatment period should be fixed appropriately with regard to the effects observed.

1.4.3.3. *Dose levels*

At least three dose levels and a concurrent control shall be used, except where a limit test is conducted (see 1.4.3.4). Dose levels may be based on the results of repeated dose or range finding studies and should take into account any existing toxicological and toxicokinetic data available for the test substance or related materials. Unless limited by the physical-chemical nature or biological effects of the test substance, the highest dose level should be chosen with the aim to induce toxicity but not death or severe suffering. A descending sequence of dose levels should be selected with a view to demonstrating any dosage related response and a no-observed-adverse-effect level (NOAEL) at the lowest dose level. Two to four-fold intervals are frequently optimal for setting the descending dose levels and addition of a fourth test group is often preferable to using very large intervals (e.g. more than a factor of about 6-10) between dosages.

The control group shall be an untreated group or a vehicle-control group if a vehicle is used in administering the test substance. Except for treatment with the test substance, animals in the control group should be handled in an identical manner to those in the test groups. If a vehicle is used, the control group shall receive the vehicle in the highest volume used. If a test substance is administered in the diet, and causes reduced dietary intake, then a pair-fed control group may be useful in distinguishing between reductions due to palatability or toxicological alterations in the test model.

Consideration should be given to the following characteristics of the vehicle and other additives, as appropriate: effects on the absorption, distribution, metabolism, or retention of the test substance; effects on the chemical properties of the test substance which may alter its toxic characteristics; and effects on the food or water consumption or the nutritional status of the animals.

1.4.3.4. *Limit test*

If a test at one dose level, equivalent to at least 1 000 mg/kg body weight/day, using the procedures described for this study, produces no-observed-adverse-effects and if toxicity would not be expected based upon data from structurally related substances, then a full study using three dose levels may not be considered necessary. The limit test applies except when human exposure indicates the need for a higher dose level to be used.

1.5. PROCEDURE

1.5.1. **Administration of doses**

The animals are dosed with the test substance daily seven days each week for a period of 90 days. Any other dosing regime, e.g. five days per week, needs to be justified. When the test substance is administered by gavage, this should be done in a single dose to the animals using a stomach tube or a suitable intubation cannula. The maximum volume of liquid that can be administered at one time depends on the size of the test animal. The

volume should not exceed 1 ml/100 g body weight, except in the case of aqueous solutions where 2 ml/100 g body weight may be used. Except for irritating or corrosive substances, which will normally reveal exacerbated effects with higher concentrations, variability in test volume should be minimised by adjusting the concentration to ensure a constant volume at all dose levels.

For substances administered via the diet or drinking water it is important to ensure that the quantities of the test substance involved do not interfere with normal nutrition or water balance. When the test substance is administered in the diet either a constant dietary concentration (ppm) or a constant dose level in terms of the animal's body weight may be used; the alternative used must be specified. For a substance administered by gavage, the dose should be given at similar times each day, and adjusted as necessary to maintain a constant dose level in terms of animal body weight. Where a 90-day study is used as a preliminary to a long term chronic toxicity study, a similar diet should be used in both studies.

1.5.2. **Observations**

The observation period should be at least 90 days. Animals in a satellite group scheduled for follow-up observations should be kept for an appropriate period without treatment to detect persistence of, or recovery from toxic effects.

General clinical observations should be made at least once a day, preferably at the same time(s) each day, taking into consideration the peak period of anticipated effects after dosing. The clinical condition of the animals should be recorded. At least twice daily, usually at the beginning and end of each day, all animals are inspected for signs of morbidity and mortality.

At least once prior to the first exposure (to allow for within-subject comparisons), and once a week thereafter, detailed clinical observations should be made in all animals. These observations should be made outside the home cage, preferably in a standard arena and at similar times on each occasion. They should be carefully recorded, preferably using scoring systems, explicitly defined by the testing laboratory. Effort should be made to ensure that variations in the observation conditions are minimal. Signs noted should include, but not be limited to, changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions and autonomic activity (e.g. lacrimation, pilo-erection, pupil size, unusual respiratory pattern). Changes in gait, posture and response to handling as well as the presence of clonic or tonic movements, stereotypes (e.g. excessive grooming, repetitive circling) or bizarre behaviour (e.g. self-mutilation, walking backwards) should also be recorded (1).

Ophthalmological examination, using an ophthalmoscope or equivalent suitable equipment, should be made prior to the administration of the test substance and at the termination of the study, preferably in all animals but at least in the high dose and control groups. If changes in the eyes are detected all animals should be examined.

Towards the end of the exposure period and in any case not earlier than in week 11, sensory reactivity to stimuli of different types (1) (e.g. auditory, visual and proprioceptive stimuli) (2), (3), (4), assessment of grip strength (5) and motor activity assessment (6) should be conducted. Further details of the procedures that could be followed are given in the respective references. However, alternative procedures than those referenced could also be used.

Functional observations conducted towards the end of the study may be omitted when data on functional observations are available from other studies and the daily clinical observations did not reveal any functional deficits.

Exceptionally, functional observations may also be omitted for groups that otherwise reveal signs of toxicity to an extent that would significantly interfere with the functional test performance.

1.5.2.1. *Body weight and food/water consumption*

All animals should be weighed at least once a week. Measurements of food consumption should be made at least weekly. If the test substance is administered via the drinking water, water consumption should also be measured at least weekly. Water consumption may also be considered for dietary or gavage studies during which drinking activity may be altered.

1.5.2.2. *Haematology and clinical biochemistry*

Blood samples should be taken from a named site and stored, if applicable, under appropriate conditions. At the end of the test period, samples are collected just prior to or as part of the procedure for killing the animals.

The following haematological examinations should be made at the end of the test period and when any interim blood samples may have been collected: haematocrit, haemoglobin concentration, erythrocyte count, total and differential leukocyte count, platelet count and a measure of blood clotting time/potential.

Clinical biochemistry determinations to investigate major toxic effects in tissues and, specifically, effects on kidney and liver, should be performed on blood samples obtained from each animal just prior to or as part of the procedure for killing the animals (apart from those found moribund and/or intercurrently killed). In a similar manner to haematological investigations, interim sampling for clinical biochemical tests may be performed. Overnight fasting of the animals prior to blood sampling is recommended ([1]). Determinations in plasma or serum should include sodium, potassium, glucose, total cholesterol, urea, blood urea nitrogen, creatinine, total protein and albumin, and more than two enzymes indicative of hepatocellular effects (such as alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, gamma glutamyl transpeptidase, and sorbitol dehydrogenase). Measurements of additional enzymes (of hepatic or other origin) and bile acids, which may provide useful information under certain circumstances, may also be included.

Optionally, the following urinalysis determinations could be performed during the last week of the study using timed urine volume collection: appearance, volume, osmolality or specific gravity, pH, protein, glucose and blood/blood cells.

In addition, studies to investigate serum markers of general tissue damage should be considered. Other determinations that should be carried out if the known properties of the test substance may, or are suspected to, affect related metabolic profiles include calcium, phosphorus, fasting triglycerides, specific hormones, methaemoglobin and cholinesterase. These need to be identified for chemicals in certain classes or on a case-by-case basis.

Overall, there is a need for a flexible approach, depending on the species and the observed and/or expected effect from a given substance.

If historical baseline data are inadequate, consideration should be given as to whether haematological and clinical biochemistry variables need to be determined before dosing commences; it is generally not recommended that this data be generated before treatment (7).

1.5.2.3. *Gross necropsy*

All animals in the study shall be subjected to a full, detailed gross necropsy which includes careful examination of the external surface of the body, all orifices, and the cranial, thoracic and abdominal cavities and their contents. The liver, kidneys, adrenals, testes, epididymides, uterus, ovaries, thymus, spleen, brain and heart of all animals (apart from those found moribund and/or intercurrently killed) should be trimmed of any adherent tissue, as appropriate, and their wet weight taken as soon as possible after dissection to avoid drying.

The following tissues should be preserved in the most appropriate fixation medium for both the type of tissue and the intended subsequent histopathological examination: all gross lesions, brain (representative regions including cerebrum, cerebellum and medulla/pons), spinal cord (at three levels: cervical, mid-thoracic and lumbar), pituitary, thyroid, parathyroid, thymus, oesophagus, salivary glands, stomach, small and large intestines (including Peyer's patches), liver, pancreas, kidneys, adrenals, spleen, heart, trachea and lungs (preserved by inflation with fixative and then immersion), aorta, gonads, uterus, accessory sex organs, female mammary gland, prostate, urinary bladder, gall bladder (mouse), lymph nodes (preferably one lymph node covering the route of administration and another one distant from the route of administration to cover systemic effects), peripheral nerve (sciatic or tibial) preferably in close proximity to the muscle, a section of bone marrow (and/or a fresh bone marrow aspirate), skin and eyes (if changes were observed during ophthalmological examinations). The clinical and other findings may suggest the need to examine additional tissues. Also any organs considered likely to be target organs based on the known properties of the test substance should be preserved.

1.5.2.4. *Histopathology*

Full histopathology should be carried out on the preserved organs and tissues of all animals in the control and high dose groups. These examinations should be extended to animals of all other dosage groups, if treatment-related changes are observed in the high dose group.

All gross lesions should be examined.

([1]) For a number of measurements in serum and plasma, most notably for glucose, overnight fasting would be preferable. The major reason for this preference is that the increased variability which would inevitably result from non-fasting, would tend to mask more subtle effects and make interpretation difficult. On the other hand, however, overnight fasting may interfere with the general metabolism of the animals and, particularly in feeding studies, may disturb the daily exposure to the test substance. If overnight fasting is adopted, clinical biochemical determinations should be performed after the conduct of functional observations of the study.

When a satellite group is used, histopathology should be performed on tissues and organs identified as showing effects in the treated groups.

2. DATA AND REPORTING

2.1. DATA

Individual data should be provided. Additionally, all data should be summarised in tabular form showing for each test group the number of animals at the start of the test, the number of animals found dead during the test or killed for humane reasons and the time of any death or humane kill, the number showing signs of toxicity, a description of the signs of toxicity observed, including time of onset, duration, and severity of any toxic effects, the number of animals showing lesions, the type of lesions and the percentage of animals displaying each type of lesion.

When applicable, numerical results should be evaluated by an appropriate and generally acceptable statistical method. The statistical methods and the data to be analysed should be selected during the design of the study.

2.2. TEST REPORT

The test report must include the following information:

2.2.1. **Test substance:**

— physical nature, purity and physico-chemical properties,

— identification data,

— vehicle (if appropriate): justification for choice of vehicle, if other than water.

2.2.2. **Test species:**

— species and strain used,

— number, age and sex of animals,

— source, housing conditions, diet etc.,

— individual weights of animals at the start of the test.

2.2.3. **Test conditions:**

— rationale for dose level selection,

— details of test substance formulation/diet preparation, achieved concentration, stability and homogeneity of the preparation,

— details of the administration of the test substance,

— actual doses (mg/kg body weight/day), and conversion factor from diet/drinking water test substance concentration (ppm) to the actual dose, if applicable,

— details of food and water quality.

2.2.4. **Results:**

— body weight and body weight changes,

— food consumption, and water consumption, if applicable,

— toxic response data by sex and dose level, including signs of toxicity,

— nature, severity and duration of clinical observations (whether reversible or not),

— results of ophthalmological examination,

— sensory activity, grip strength and motor activity assessments (when available),

— haematological tests with relevant base-line values,

— clinical biochemistry tests with relevant base-line values,

— terminal body weight, organ weights and organ/body weight ratios,

— necropsy findings,

— a detailed description of all histopathological findings,

— absorption data if available,

— statistical treatment of results, where appropriate,

Discussion of results.

Conclusions.

3. **REFERENCES**

(1) IPCS (1986) Principles and Methods for the Assessment of Neurotoxicity Associated with Exposure to Chemicals. Environmental Health Criteria Document No 60.

(2) Tupper, D.E., Wallace, R.B., (1980) Utility of the Neurologic Examination in Rats. Acta Neurobiol. Exp., 40, p. 999-1003.

(3) Gad, S.C., (1982) A Neuromuscular Screen for Use in Industrial Toxicology. J.Toxicol. Environ. Health, 9, p. 691-704.

(4) Moser, V.C., Mc Daniel, K.M., Phillips, P.M., (1991) Rat Strain and Stock Comparisons Using a Functional Observational Battery: Baseline Values and Effects of Amitraz. Toxicol. Appl. Pharmacol., 108, p. 267-283.

(5) Meyer O.A., Tilson H.A., Byrd W.C., Riley M.T., (1979) A Method for the Routine Assesment of Fore- and Hind- limb grip Strength of Rats and Mice. Neurobehav. Toxivol., 1, p. 233-236.

(6) Crofton K.M., Howard J.L., Moser V.C., Gill M.W., Reiter L.W., Tilson H.A., MacPhail R.C., (1991) Interlaboratory Comparison of Motor Activity Experiments: Implication for Neurotoxicological Assessments. Neurotoxicol. Teratol., 13, p. 599-609.

(7) Weingand K., Brown G., Hall R. *et al*., (1996) 'Harmonisation of Animal Clinic Pathology Testing in Toxicity and Safety Studies', Fundam. & Appl. Toxicol., 29, p. 198-201.

B.27. **SUB-CHRONIC ORAL TOXICITY TEST REPEATED DOSE 90-DAY ORAL TOXICITY STUDY IN NON-RODENTS**

1. **METHOD**

This sub-chronic oral toxicity test method is a replicate of the OECD TG 409 (1998).

1.1. INTRODUCTION

In the assessment and evaluation of the toxic characteristics of a chemical, the determination of sub-chronic oral toxicity using repeated doses may be carried out after initial information on toxicity has been obtained from acute or repeated dose 28-day toxicity tests. The 90-day study provides information on the possible health hazards likely to arise from repeated exposure over a period of rapid growth and into young adulthood. The study will provide information on the major toxic effects, indicate target organs and the possibility of accumulation, and can provide an estimate of a no-observed-adverse-effect level of exposure which can be used in selecting dose levels for chronic studies and for establishing safety criteria for human exposure.

The test method allows for the identification in non-rodent species of adverse effects of chemical exposure and should only be used:

— where effects observed in other studies indicate a need for clarification/characterisation in a second, non-rodent species, or

— where toxicokinetic studies indicate that the use of a specific non-rodent species is the most relevant choice of laboratory animal, or

— where other specific reasons justify the use of a non-rodent species.

See also General introduction Part B.

1.2. DEFINITIONS

Dose: is the amount of test substance administered. Dose is expressed as weight (g, mg) or as weight of test substance per unit weight of test animal (e.g. mg/kg), or as constant dietary concentrations (ppm).

Dosage: is a general term comprising of dose, its frequency and the duration of dosing.

NOAEL: is the abbreviation for no-observed-adverse-effect level and is the highest dose level where no adverse treatment-related findings are observed.

1.3. PRINCIPLE OF THE TEST METHOD

The test substance is orally administered daily in graduated doses to several groups of experimental animals, one dose level per group for a period of 90 days. During the period of administration the animals are observed closely for signs of toxicity. Animals, which die or are killed during the test are necropsied and at the conclusion of the test surviving animals are also killed and necropsied.

1.4. DESCRIPTION OF THE TEST METHOD

1.4.1. **Selection of animal species**

The commonly used non-rodent species is the dog, which should be of a defined breed; the beagle is frequently used. Other species, e.g. swine, mini-pigs, may also be used. Primates are not recommended and their use should be justified. Young, healthy animals should be employed, and in the case of the dog, dosing should begin preferably at four to six months and not later than nine months of age. Where the study is conducted as a preliminary to a long-term chronic toxicity study, the same species/breed should be used in both studies.

1.4.2. **Preparation of animals**

Healthy young animals, which have been acclimated to laboratory conditions and have not been subjected to previous experimental procedures, should be used. The duration of acclimatisation will depend upon the selected test species and their source. At least five days for dogs or purpose bred swine from a resident colony and at least two weeks for these animals if from external sources are recommended. The test animals should be characterised as to species, strain, source, sex, weight and/or age. Animals should be randomly assigned to the control and treatment groups. Cages should be arranged in such a way that possible effects due to cage placement are minimised. Each animal should be assigned a unique identification number.

1.4.3. **Preparations of doses**

The test substance may be administered in the diet or in the drinking water, by gavage or in capsules. The method of oral administration is dependent on the purpose of the study, and the physical-chemical properties of the test material.

Where necessary, the test substance is dissolved or suspended in a suitable vehicle. It is recommended that, wherever possible, the use of an aqueous solution/suspension be considered first, followed by consideration of a solution/emulsion in oil (e.g. corn oil) and then by possible solution in other vehicles. For vehicles other than water the toxic characteristics of the vehicle must be known. The stability of the test substance under the conditions of administration should be determined.

1.5. PROCEDURE

1.5.1. **Number and sex of animals**

At least eight animals (four female and four male) should be used at each dose level. If interim kills are planned, the number should be increased by the number of animals scheduled to be killed before the completion of the study. The number of animals at the termination of the study must be adequate for a meaningful evaluation of toxic effects. Based on previous knowledge of the substance or a close analogue, consideration should be given to including an additional satellite group of eight animals (four per sex) in control and in top dose group for observation after the treatment period of reversibility or persistence of any toxic effects. The duration of this post-treatment period should be fixed appropriately with regard to the effects observed.

1.5.2. **Dosage**

At least three dose levels and a concurrent control shall be used, except where a limit test is conducted (see 1.5.3). Dose levels may be based on the results of repeated dose or range finding studies and should take into account any existing toxicological and toxicokinetic data available for the test compound or related materials. Unless limited by the physical-chemical nature or biological effects of the test substance, the highest dose level should be chosen with the aim to induce toxicity but not death or severe suffering. A descending sequence of dose levels should be selected with a view to demonstrating any dosage related response and a no-observed-adverse-effect level (NOAEL) at the lowest dose level. Two to fourfold intervals are frequently optimal for setting the descending dose levels and addition of a fourth test group is often preferable to using very large intervals (e.g. more than a factor of about 6–10) between dosages.

The control group shall be an untreated group or a vehicle-control group if a vehicle is used in administering the test substance. Except for treatment with the test substance, animals in the control group should be handled in an identical manner to those in the test groups. If a vehicle is used, the control group shall receive the vehicle in the highest volume used. If a test substance is administered in the diet, and causes reduced dietary intake, then a pair-fed control group may be useful in distinguishing between reductions due to palatability or toxicological alterations in the test model.

Consideration should be given to the following characteristics of the vehicle and other additives, as appropriate: effects on the absorption, distribution, metabolism, or retention of the test substance; effects on the chemical properties of the test substance which may alter its toxic characteristics; and effects on the food or water consumption or the nutritional status of the animals.

1.5.3. **Limit test**

If a test at one dose level, equivalent to at least 1 000 mg/kg body weight/day, using the procedures described for this study, produces no-observed-adverse-effects and if toxicity would not be expected based upon data from structurally related substances, then a full study using three dose levels may not be considered necessary. The limit test applies except when human exposure indicates the need for a higher dose level to be used.

1.5.4. **Administration of doses**

The animals are dosed with the test substance daily seven days each week for a period of 90 days. Any other dosing regime, e.g. five days per week, needs to be justified. When the test substance is administered by gavage, this should be done in a single dose to the animals using a stomach tube or a suitable intubation cannula. The maximum volume of liquid that can be administered at one time depends on the size of the test animal. Normally the volume should be kept as low as possible. Except for irritating or corrosive substances which will normally reveal exacerbated effects with higher concentrations, variability in test volume should be minimised by adjusting the concentration to ensure a constant volume at all dose levels.

For substances administered via the diet or drinking water it is important to ensure that the quantities of the test substance involved do not interfere with normal nutrition or water balance. When the test substance is administered in the diet either a constant dietary concentration (ppm) or a constant dose level in terms of the animal's body weight may be used; any alternative used must be specified. For a substance administered by gavage or by capsule, the dose should be given at similar times each day, and adjusted as necessary to maintain a constant dose level in terms of animal body weight. Where the 90 day study is used as a preliminary to a long term chronic toxicity study, a similar diet should be used in both studies.

1.5.5. **Observations**

The observation period should be at least 90 days. Animals in a satellite group scheduled for follow-up observations should be kept for an appropriate period without treatment to detect persistence of, or recovery from toxic effects.

General clinical observations should be made at least once a day, preferably at the same time(s) each day, taking into consideration the peak period of anticipated effects after dosing. The clinical condition of the animals should be recorded. At least twice daily, usually at the beginning and end of each day, all animals should be inspected for signs of morbidity and mortality.

At least once prior to the first exposure (to allow for within-subject comparisons), and once a week thereafter, detailed clinical observations should be made in all animals. These observations should be made, where practical outside the home cage in a standard arena and preferably at similar times on each occasion. Effort should be made to ensure that variations in the observation conditions are minimal. Signs of toxicity should be carefully recorded, including time of onset, degree and duration. Observations should include, but not be limited to, changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions and autonomic activity (e.g. lacrimation, pilo-erection, pupil size, unusual respiratory pattern). Changes in gait, posture and response to handling as well as the presence of clonic or tonic movements, stereotypes (e.g. excessive grooming, repetitive circling) or any bizarre behaviour should also be recorded.

Ophthalmological examination, using an ophthalmoscope or equivalent suitable equipment, should be made prior to the administration of the test substance and at the termination of the study, preferably in all animals but at least in the high dose and control groups. If treatment related changes in the eyes are detected all animals should be examined.

1.5.5.1. *Body weight and food/water consumption*

All animals should be weighed at least once a week. Measurements of food consumption should be made at least weekly. If the test substance is administered via the drinking water, water consumption should also be measured at least weekly. Water consumption may also be considered for dietary or gavage studies during which drinking activity may be altered.

1.5.5.2. *Haematology and clinical biochemistry*

Blood samples should be taken from a named site and stored, if applicable, under appropriate conditions. At the end of the test period, samples are collected just prior to or as part of the procedure for killing the animals.

Haematology, including haematocrit, haemoglobin concentration, erythrocyte count, total and differential leukocyte count, platelet count and a measure of clotting potential such as clotting time, prothrombin time, or thromboplastin time should be investigated at the start of the study, then either at monthly intervals or midway through the test period and finally at the end of the test period.

Clinical biochemistry determinations to investigate major toxic effects in tissues and, specifically, effects on kidney and liver, should be performed on blood samples obtained from all animals at the start, then either at monthly intervals or midway through the test and finally at the end of the test period. Test areas, which should be considered are electrolyte balance, carbohydrate metabolism, and liver and kidney function. The selection of specific tests will be influenced by observations on the mode of action of the test substance. Animals should be

fasted for a period appropriate to the species prior to blood sampling. Suggested determinations include calcium, phosphorus, chloride, sodium, potassium, fasting glucose, alanine aminotransferase, aspartate aminotransferase, ornithine decarboxylase, gamma glutamyl transpeptidase, urea nitrogen, albumin, blood creatinine, total bilirubin and total serum protein measurements.

Urinalysis determinations should be performed at least at the start, then midway and finally at the end of the study using timed urine volume collection. Urinalysis determinations include appearance, volume, osmolality or specific gravity, pH, protein, glucose and blood/blood cells. Additional parameters may be employed where necessary to extend the investigation of observed effect(s).

In addition, studies to investigate markers of general tissue damage should be considered. Other determinations, which may be necessary for an adequate toxicological evaluation include analyses of lipids, hormones, acid/base balance, methaemoglobin, and cholinesterase inhibition. Additional clinical biochemistry may be employed where necessary to extend the investigation of observed effects. These need to be identified for chemicals in certain classes or on a case-by-case basis.

Overall, there is a need for a flexible approach, depending on the species and the observed and/or expected effect from a given substance.

1.5.5.3. *Gross necropsy*

All animals in the study shall be subjected to a full, detailed gross necropsy which includes careful examination of the external surface of the body, all orifices, and the cranial, thoracic and abdominal cavities and their contents. The liver with gall bladder, kidneys, adrenals, testes, epididymides, ovaries, uterus, thyroid (with parathyroids), thymus, spleen, brain and heart of all animals (apart from those found moribund and/or intercurrently killed) should be trimmed of any adherent tissue, as appropriate, and their wet weight taken as soon as possible after dissection to avoid drying.

The following tissues should be preserved in the most appropriate fixation medium for both the type of tissue and the intended subsequent histopathological examination: all gross lesions, brain (representative regions including cerebrum, cerebellum and medulla/pons), spinal cord (at three levels: cervical, mid-thoracic and lumbar), pituitary, eyes, thyroid, parathyroid, thymus, oesophagus, salivary glands, stomach, small and large intestines (including Peyer's patches), liver, gall bladder, pancreas, kidneys, adrenals, spleen, heart, trachea and lungs, aorta, gonads, uterus, accessory sex organs, female mammary gland, prostate, urinary bladder, lymph nodes (preferably one lymph node covering the route of administration and another one distant from the route of administration to cover systemic effects), peripheral nerve (sciatic or tibial) preferably in close proximity to the muscle, a section of bone marrow (and/or a fresh bone marrow aspirate) and skin. The clinical and other findings may suggest the need to examine additional tissues. Also any organs considered likely to be target organs based on the known properties of the test substance should be preserved.

1.5.5.4. *Histopathology*

Full histopathology should be carried out on the preserved organs and tissues in at least all animals in control and high dose group. The examination should be extended to animals of all other dosage groups, if treatment-related changes are observed in the high dose group.

All gross lesions should be examined.

When a satellite group is used, histopathology should be performed on tissues and organs identified as showing effects in the treated groups.

2. DATA AND REPORTING

2.1. DATA

Individual data should be provided. Additionally, all data should be summarised in tabular form showing for each test group the number of animals at the start of the test, the number of animals found dead during the test or killed for humane reasons and the time of any death or humane kill, the number showing signs of toxicity, a description of the signs of toxicity observed, including time of onset, duration, and severity of any toxic effects, the number of animals showing lesions, the type of lesions and the percentage of animals displaying each type of lesion.

When applicable, numerical results should be evaluated by an appropriate and generally acceptable statistical method. The statistical methods and the data to be analysed should be selected during the design of the study.

2.2. TEST REPORT

The test report must include the following information:

2.2.1. **Test substance:**

— physical nature, purity and physico-chemical properties,

— identification data,

— vehicle (if appropriate): justification for choice of vehicle, if other than water.

2.2.2. **Test species:**

— species and strain used,

— number, age and sex of animals,

— source, housing conditions, diet etc.,

— individual weights of animals at the start of the test.

2.2.3. **Test conditions:**

— rationale for dose level selection,

— details of test substance formulation/diet preparation, achieved concentration, stability and homogeneity of the preparation,

— details of the administration of the test substance,

— actual doses (mg/kg body weight/day), and conversion factor from diet/drinking water test substance concentration (ppm) to the actual dose, if applicable,

— details of food and water quality.

2.2.4. **Results:**

— body weight/body weight changes,

— food consumption, and water consumption, if applicable,

— toxic response data by sex and dose level, including signs of toxicity,

— nature, severity and duration of clinical observations (whether reversible or not),

— ophthalmological examination,

— haematological tests with relevant base-line values,

— clinical biochemistry tests with relevant base-line values,

— terminal body weight, organ weights and organ/body weight ratios,

— necropsy findings,

— a detailed description of all histopathological findings,

— absorption data if available,

— statistical treatment of results, where appropriate.

Discussion of results.

Conclusions.

B.28. **SUB-CHRONIC DERMAL TOXICITY STUDY 90-DAY REPEATED DERMAL DOSE STUDY USING RODENT SPECIES**

1. **METHOD**

1.1. INTRODUCTION

See General introduction Part B.

1.2. DEFINITIONS

See General introduction Part B.

1.3. REFERENCE SUBSTANCES

None.

1.4. PRINCIPLE OF THE TEST METHOD

The test substance is applied daily to the skin in graduated doses to several groups of experimental animals, one dose per group for a period of 90 days. During the period of application the animals are observed daily to detect signs of toxicity. Animals, which die during the test are necropsied, and at the conclusion of the test surviving animals are necropsied.

1.5. QUALITY CRITERIA

None.

1.6. DESCRIPTION OF THE TEST METHOD

1.6.1. **Preparations**

The animals are kept under the experimental housing and feeding conditions for at least five days prior to the test. Before the test healthy young animals are randomised and assigned to the treated and control groups. Shortly before testing fur is clipped from the dorsal area of the trunk of the test animals. Shaving may be employed but it should be carried out approximately 24 hours before the test. Repeat clipping or shaving is usually needed at approximately weekly intervals. When clipping or shaving the fur, care must be taken to avoid abrading the skin. Not less than 10 % of the body surface area should be clear for the application of the test substance. The weight of the animal should be taken into account when deciding on the area to be cleared and on the dimensions of the covering. When testing solids, which may be pulverised if appropriate, the test substance should be moistened sufficiently with water or, where necessary, a suitable vehicle to ensure good contact with the skin. Liquid test substances are generally used undiluted. Daily application on a five to seven-day per week basis is used.

1.6.2. *Test conditions*

1.6.2.1. Experimental animals

The adult rat, rabbit or guinea pig may be used. Other species may be used but their use would require justification. At the commencement of the test the range of the weight variation should be ± 20 % of the mean weight. Where a sub-chronic dermal study is conducted as a preliminary to a long-term study, the same species and strain should be used in both studies.

1.6.2.2. Number and sex

At least 20 animals (10 female and 10 male) with healthy skin should be used at each dose level. The females should be nulliparous and non-pregnant. If interim sacrifices are planned the number should be increased by the number of animals scheduled to be sacrificed before the completion of the study. In addition, a satellite group of 20 animals (10 animals per sex) may be treated with the high-dose level for 90 days and observed for reversibility, persistence, or delayed occurrence of toxic effects for 28 days post-treatment.

1.6.2.3. Dose levels

At least three dose levels are required with a controle or a vehicle control if a vehicle is used. The exposure period should be at least six hours per day. The application of the test substance should be made at similar times each day, and the amount of substance applied adjusted at intervals (weekly or bi-weekly) to maintain a constant dose level in terms of animal body weight. Except for treatment with the test substance, animals in the control group should be handled in an identical manner to the test group subjects. Where a vehicle is used to facilitate dosing, the vehicle control group should be dosed in the same way as the treated groups, and receive the same amount as that received by the highest dose level group. The highest dose level should result in toxic effects but produce no, or few, fatalities. The lowest dose level should not produce any evidence of toxicity. Where there is a usable estimation of human exposure the lowest level should exceed this. Ideally, the intermediate dose level should produce minimal observable toxic effects. If more than one intermediate dose is used, the dose levels should be spaced to produce a gradation of toxic effects. In the low and intermediate groups, and in the controls, the incidence of fatalities should be low, in order to permit a meaningful evaluation of the results.

If application of the test substance produces severe skin irritation the concentrations should be reduced and this may result in a reduction in, or absence of, other toxic effects al: the high-dose level. If the skin has been badly damaged it may be necessary to terminate the study and undertake a new study at lower concentrations.

1.6.3. Limit test

If a preliminary study at a dose level of 1 000 mg/kilograms, or a higher dose level related to possible human exposure where this is known, produces no toxic effects, further testing may not be considered necessary.

1.6.4. Observation period

The experimental animals should be observed daily for signs of toxicity. The time of death and the time at which signs of toxicity appear and disappear should be recorded.

1.6.5. Procedure

Animals should be caged individually. The animals are treated with the test substance, ideally on seven days per week, for a period of 90 days.

Animals in any satellite groups scheduled for follow-up observations should be kept for a further 28 days without treatment to detect recovery from, or persistence of, toxic effects. Exposure time should be six hours per day.

The test substance should be applied uniformly over an area, which is approximately 10 % of the total body surface area. With highly toxic substances, the surface area covered may be less but as much of the area should be covered with as thin and uniform a film as possible.

During exposure the test substance is held in contact with the skin with a porous gauze dressing and non-irritating tape. The test site should be further covered in a suitable manner to retain the gauze dressing and test substance and ensure that the animals cannot ingest the test substance. Restrainers may be used to prevent the ingestion of the test substance but complete immobilisation is not a recommended method.

At the end of the exposure period, residual test substance should be removed where practicable using water or some other appropriate method of cleansing the skin.

All the animals should be observed daily and signs of toxicity recorded, including the time of onset, their degree and duration. Cageside observations should include changes in skin and fur, eyes and mucous membranes, as well as respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behavior pattern. Measurements should be made of food consumption weekly and the animals weighed weekly. Regular observations of the animals are necessary to ensure that animals are not lost from the study due to causes such as cannibalism, autolysis of tissues or misplacement. At the end of the study period all survivors in the non-satellite treatment groups are necropsied. Moribund animals should be removed and necropsied when noticed.

The following examinations are customarily made on all animals including the controls:

(a) ophthalmological examination, using an ophthalmoscope or equivalent suitable equipment, should be made prior to exposure to the test substance and at the termination of the study, preferably in all animals but at least in the high-dose and control groups. If changes in the eyes are detected all animals should be examined.

(b) haematology, including haematocrit, haemoglobin concentration, erythrocyte count, total and differential leucocyte count, and a measure of clotting potential, such as clotting time, prothrombin time, thromboplastin time, or platelet count, should be investigated at the end of the test period.

(c) clinical biochemistry determination on blood should be carried out at the end of the test period. Test areas, which are considered appropriate to all studies are electrolyte balance, carbohydrate metabolism, liver and kidney function. The selection of specific tests will be influenced by observations on the mode of action of the substance. Suggested determinations are calcium, phosphorus, chloride, sodium, potassium, fasting glucose (with period of fasting appropriate to the species), sercum glutamic pyruvic transaminase ([1]), serum glutamic oxaloacetic transaminase ([2]), ornithine decarboxylase, gamma glutamyl transpeptidase, urea nitrogen, albumin, blood creatinine, total bilirubin and total serum protein measurements. Other determinations which may be necessary for an adequate toxicological evaluation include analyses of lipids, hormones, acid/base balance, methaemoglobin and choliensterase activity. Additional clinical biochemistry may be employed, where necessary, to extend the investigation of observed effects.

(d) urinalysis is not required on a routine basis but only when there is an indication based on expected or observed toxicity.

If historical baseline data are inadequate, consideration should be given to determination of haem a to logical and clinical biochemistry parameters before dosing commences.

Gross necropsy

All animals should be subjected to a full gross necropsy which includes examination of the external surface of the body, all orifices, and the cranial, thoracic and abdominal cavities and their contents. The liver, kidneys, adrenals and testes must be weighed wet as soon as possible after dissection to avoid drying. The following organs and tissues should be preserved in a suitable medium for possible future histopathological examination: all gross lesions, brain — including sections of medulla/pons, cerebellar cortex and cerebral cortex, pituitary, thyroid/parathyroid, any thymic tissue, (trachea), lungs, heart, aorta, salivary glands, liver, spleen, kidneys, adrenals, pancreas, gonads, uterus, accessory genital organs, gall bladder (if present), oesophagus, stomach, duodenum, jejunum, ileum, caecum, colon, rectum, urinary bladder, representative lymph node, (female mammary gland), (thigh musculature), peripheral nerve, (eyes), (sternum with bone marrow), (femur — including articular surface), (spinal cord at three levels — cervical, mid-thoracic and lumbar), and (exorbital lachrymal glands). The tissues mentioned between brackets need only be examined if indicated by signs of toxicity or target organ involvement.

Histopathological examination

(a) Full histopathology should be carried out on normal and treated skin and on organs and tissues of animals in the control and high-dose groups.

(b) all gross lesions should be examined.

(c) target organs in other dose groups should be examined.

(d) where rats are used, lungs of animals in the low- and intermediate-dose groups should be subjected to histopathological examination for evidence of infection, since this provides a convenient assessment of the state of health of the animals. Further histopathological examination may not be required routinely on the animals in these groups, but must always be carried out in organs, which show evidence of lesions in the high-dose group.

(e) when a satellite group is used, histopathology should be performed on tissues and organs identified as showing effects in the other treated groups.

2. **DATA**

Data should be summarised in tabular form, showing for each test group the number of animals at the start of the test, the number of animals showing lesions, the type of lesions and the percentage of animals displaying each type of lesion. Results should be evaluated by an appropriate statistical method. Any recognised statistical method may be used.

([1]) Now known as serum alanine aminotransferase.
([2]) Now known as serum aspartate aminotransferase.

3. **REPORTING**

3.1. TEST REPORT

The test report shall, if possible, contain the following information:

— species, strain, source, environmental conditions, diet,

— test conditions,

— dose levels (including vehicle, if used) and concentrations,

— toxic response data by sex and dose,

— no-effect level, where possible,

— time of death during the study or whether animals survived to termination,

— description of toxic or other effects,

— the time of observation of each abnormal sign and its subsequent course,

— food and bodyweight data,

— ophthalmological findings,

— haematological tests employed and all results,

— clinical biochemistry tests employed and all results (including results of any urinalysis),

— necropsy findings,

— a detailed description of all histopathological findings,

— statistical treatment of results where possible,

— discussion of the results,

— interpretation of the results.

3.2. EVALUATION AND INTERPRETATION

See General introduction Part B.

4. **REFERENCES**

See General introduction Part B.

B.29. **SUB-CHRONIC INHALATION TOXICITY STUDY 90-DAY REPEATED INHALATION DOSE STUDY USING RODENT SPECIES**

1. **METHOD**

1.1. INTRODUCTION

See General introduction Part B.

1.2. DEFINITIONS

See General introduction Part B.

1.3. REFERENCE SUBSTANCES

None.

1.4. PRINCIPLE OF THE TEST METHOD

Several groups of experimental animals are exponed daily for a defined period to the test substance in graduated concentrations, one concentration being used per group, for a period of 90 days. Where a vehicle is used to help generate an appropriate concentration of the test substance in the atmosphere, a vehicle control group should be used. During the period of administration the animals are observed daily to detect signs of toxicity. Animals, which die during the test are necropsied and at the conclusion of the test surviving animals are necropsied.

1.5. QUALITY CRITERIA

None.

1.6. DESCRIPTION OF THE TEST METHOD

1.6.1. Preparations

The animals are kept under the experimental housing and feeding conditions for at least five days prior to the experiment. Before the test, healthy young aimals are randomised and assigned to the treatment and control groups. Where necessary, a suitable vehicle may be added to the test substance to help generate an appropriate concentration of the substance in the atmosphere. If a vehicle or other additives are used to facilitate dosing, they should be known not to produce toxic effects. Historical data can be used if appropriate.

1.6.2. Test conditions

1.6.2.1. Experimental animals

Unless there are contra-indications, the rat is the preferred species. Commonly used laboratory strains of young healthy animals should be employed. At the commencement of the study the range of weight variation of animals used should not exceed ± 20 % of the appropriate mean value. Where a subchronic inhalation study is conducted as a preliminary to a long-term study, the same species and strain should be used in both studies.

1.6.2.2. Number and sex

At least 20 animals (10 female and 10 male) should be used for each exposure concentration. The females should be nulliparous and non-pregnant. If interim sacrifices are planned the number should be increased by the number of animals scheduled to be sacrified before the completion of the study. In addition, a satellite group of 20 animals (10 animals per sex) may be treated with the high concentration level for 90 days and observed for reversibility, persistence, or delayed occurrence of toxic effects for 28 days post treatment.

1.6.2.3. Exposure concentrations

At least three concentrations are required, with a control or a vehicle control (corresponding to the concentration of vehicle at the highest level) if a vehicle is used. Except for treatment with the test substance, animals in the control group should be handled in an identical manner to the test group subjects. The highest concentration should result in toxic effects but no, or few, fatalities. Where there is a usable estimation of human exposure the lowest level should exceed this. Ideally, the intermediate concentration should produce minimal observable toxic effects. If more than one intermediate concentration is used the concentrations should be spaced to produce a gradation of toxic effects. In the low and intermediate groups, and in the controls, the incidence of fatalities should be low to permit a meaningful evaluation of the results.

1.6.2.4. Exposure time

The duration of daily exposure should be six hours after equilibration of the chamber concentrations. Other durations may be used to meet specific requirements.

1.6.2.5. Equipment

The animals should be tested in inhalation equipment designed to sustain a dynamic air flow of at least 12 air changes per hour to ensure an adequate oxygen content and an evenly distributed exposure atmosphere. Where a chamber is used its design should minimise crowding of the test animals and maximise their exposure by inhalation to the test substance. As a general rule, to ensure stability of a chamber atmosphere the total volume of the test animals should not exceed 5 % of the volume of the test chamber. Oro-nasal, head only, or whole body individual chamber exposure may be used; the first two will minimise uptake by other routes.

1.6.2.6. Observation period

The experimental animals should be observed daily for signs of toxicity during the entire treatment and recovery period. The time of death and the time at which signs of toxicity appear and disappear should be recorded.

1.6.3. Procedure

The animals are exposed to the test substance daily, five to seven days per week, for a period of 90 days. Animals in any satellite groups scheduled for follow-up observations should be kept for a further 28 days without treatment to detect recovery from, or persistence of, toxic effects. The temperature at which the test is performed should be maintained at 22 ± 3 °C. Ideally, the relative humidity should be maintained between 30 % and 70 %, but in certain instances (e.g. tests of aerosols) this may not be practicable. Food and water should be withheld during exposure.

A dynamic inhalation system with a suitable analytical concentration control system should be used. To establish suitable exposure concentrations a trial test is recommended. The air flow should be adjusted to ensure that conditions throughout the exposure chamber are homogeneous. The system should ensure that stable exposure conditions are achieved as rapidly as possible.

Measurements or monitoring should be made of:

(a) the rare of air flow (continuously);

(b) the actual concentration of the test substance measured in the breathing zone. During the daily exposure period the concentration should not vary by more than ± 15 % of the mean value. However, in the case of dusts and aerosols, this level of control may not be achievable and a wider range would then be acceptable. During the total duration of the study, the day-to-day concentrations should be held as constant as practicable. During the development of the generating system, particle-size analysis should be performed to establish the stability of aerosol concentrations. During exposure, analysis should be conducted as often as necessary to determine the consistency of particle-size distribution;

(c) temperature and humidity;

(d) during and following exposure, observations are made and recorded systematically; individual records should be maintained for each animal. All the animals should be observed daily and signs of toxicity recorded including the time of onset, their degree and duration. Cageside observations should include: changes in the skin and fur, eyes, mucous membranes, respiratory, circulatory, autonomic and central nervous systems; somatomotor activity and behaviour pattern. Measurements should be made of food consumption weekly and the animals weighed weekly. Regular observation of the animals is necessary to

ensure that animals are not lost from the study due to causes such as cannibalism, autolysis of tissues or misplacement. At the end of the exposure period all surviving animals are necropsied. Moribund animals should be removed and necropsied when noticed.

The following examinations are customarily made on all animals including the controls:

(a) ophthalmological examination, using an ophthalmoscope or equivalent suitable equipment, should be made prior to the exposure to the test substance and at the termination of the study, preferably in all animals but at least in the high-dose and control groups. If changes in the eyes are detected all animals should be examined;

(b) haematology, including haematocrit, haemoglobin concentration, erythrocyte count, total and differential leucocyte count, and a measure of clotting potential, such as clotting time, prothrombin time, thromboplastin time, or platelet count, should be investigated at the end of the test period;

(c) clinical biochemistry determination on blood should be carried out at the end of the test period. Test areas, which are considered appropriate to all studies are electrolyte balance, carbohydrate metabolism, liver and kidney function. The selection of specific tests will be influenced by observations on the mode of action of the substance. Suggested determinations are calcium, phosphorus, chloride, sodium, potassium, fasting glucose (with period of fasting appropriate to the species), serum glutamic pyruvic transaminase ([1]), serum glutamic oxaloacetic transaminase ([2]), ornithine decarboxylase, gamma glutamyl transpeptidase, urea nitrogen, albumin, blood creatinine, total bilirubin and total serum protein measurements. Other determinations, which may be necessary for an adequate toxicological evaluation include analyses of lipids, hormones, acid/base balance, methaemoglobin and cholinesterase activity. Additional clinical biochemistry may be employed where necessary to extend the investigation of observed effects;

(d) urinalysis is not required on a routine basis but only when there is an indication based on expected or observed toxicity.

If historical baseline data are inadequate, consideration should be given to determination of haematological and clinical biochemistry parameters before dosing commences.

Gross necropsy

All animals should be subjected to a full gross necropsy which includes examination of the external surface of the body, all orifices, and the cranial, thoracic and abdominal cavities and their contents. The liver, kidneys, adrenals and testes should be weighed wet as soon as possible after dissection to avoid drying. The following organs and tissues should be preserved in a suitable medium for possible future histopathological examination: all gross lesions, lungs —which should be removed intact, weighed and treated with a suitable fixative to ensure that lung structure is maintained (perfusion with the fixative is considered to be an effective procedure), nasopharyngeal tissues, brain —including sections of medulla/pons, cerebellar cortex and cerebral cortex, pituitary, thyroid/parathyroid, any thymic tissue, trachea, lungs, heart, aorta, salivary glands, liver, spleen, kidneys, adrenals, pancreas, gonads, uterus (accessory genital organs), (skin), gall bladder (if present), oesophagus, stomach, duodenum, jejunum, ileum, caecum, colon, rectum, urinary bladder, representative lymph node, (female mammary gland), (thigh musculature), peripheral nerve, (eyes), sternum with bone marrow, (femur, including articular surface), and (spinal cord at three levels — cervical, mid-thoracic and lumbar). The tissues mentioned between brackets need only be examined if indicated by signs of toxicity, or target organ involvement.

Histopathological examination

(a) Full histopathology should be carried out on the respiratory tract and other organs and tissues of all animals in the control and high-dose groups.

(b) all gross lesions should be examined.

(c) target organs in other dose groups should be examined.

(d) lungs of animals in the low – and intermediate-dose group should also be subjected to histopathological examination; since this can provide a convenient assessment of the state of health of the animals. Further histopathological examination may not be required routinely on the animals in these groups but must

([1]) Now known as serum alanine aminotransferase.
([2]) Now known as serum aspartate aminotransferase.

always be carried out on organs, which show evidence of lesions in the high-dose group.

(e) when a satellite group is used, histopathology should be performed on tissues and organs identified as showing effects in other treated groups.

2. **DATA**

Data should be summarised in tabular form, showing for each test group the number of animals at the start of the test, the number of animals showing lesions, the types of lesions and the percentage of animals displaying each type of lesion. Results should be evaluated by an appropriate statistical method. Any recognised statistical method may be used.

3. **REPORTING**

3.1. TEST REPORT

The test report shall, if possible, contain the following information:

— species, strain, source, environmental conditions, diet,

— test conditions:

description of exposure apparatus: including design, type, dimensions, source of air, system for generating particulates and aerosols, method of conditioning air, treatment of exhaust air and the method of housing animals in a test chamber then this is used. The equipment for measuring temperature, humidity and, where appropriate, stability of aerosol concentrations or particle size, should be described.

Exposure data: these should be tabulated and presented with mean values and a measure of variability (e. g. standard deviation) and should include:

(a) air flow rates through the inhalation equipment;

(b) temperature and humidity of air;

(c) nominal concentrations (total amount of test substance fed into the inhalation equipment divided by the volume of air);

(d) nature of vehicle, if used;

(e) actual concentrations in test breathing zone;

(f) median particle sizes (where appropriate):

— toxic response data by sex and concentration,

— no-effect level when possible,

— time of death during the study or whether animals surived to termination,

— description of toxic or other effects,

— the time of observation of each abnormal sign and its subsequent course,

— food and bodyweight data,

— ophthalmological findings,

— haematological tests employed and results,

— clinical biochemistry tests employed and results (including results of any urinalysis),

— necropsy findings,

— a detailed description of all histopathological findings,

— statistical treatment of results where appropriate,

— discussion of the results,

— interpretation of the results.

3.2. EVALUATION AND INTERPRETATION

See General introduction Part B.

4. **REFERENCES**

See General introduction Part B.

B.30. **CHRONIC TOXICITY TEST**

1. **METHOD**

1.1. INTRODUCTION

See General introduction Part B.

1.2. DEFINITIONS

See General introduction Part B.

1.3. REFERENCE SUBSTANCES

None.

1.4. PRINCIPLE OF THE TEST METHOD

The test substance is administered normally seven days per week, by an appropriate route, to several groups of experimental animals, one dose per group, for a major portion of their life span. During and after exposure to the test substance, the experimental animals are observed daily to detect signs of toxicity.

1.5. QUALITY CRITERIA

None.

1.6. DESCRIPTION OF THE TEST METHOD

1.6.1. *Preparations*

The animals are kept under the experimental housing and feeding conditions for at least five days prior to the test. Before the test healthy young animals are randomised and assigned to the treated and control groups.

1.6.2. *Test conditions*

1.6.2.1. Experimental animals

The preferred species is the rat.

Based upon the results of previously conducted studies other species (rodent or non-rodent) may be used. Commonly used laboratory strains of young healthy animals should be employed and dosing should begin as soon as possible after weaning.

At the commencement of the study the weight variation in the animals used should not exceed ± 20 % of the mean value. Where a sub-chronic oral study is conducted as a preliminary to a long-term study, the same species/breed and strain should be used in both studies.

1.6.2.2. Number and sex

For rodents at least 40 animals (20 female and 20 male) should be used at each dose level and concurrent control group. The females should be nulliparous and non-pregnant. If interim sacrifices are planned, the number should be increased by the number of animals scheduled to be sacrificed before the completion of the study.

For non-rodents a smaller number of animals, but at least four per sex per group, is acceptable.

1.6.2.3. Dose levels and frequency of exposure

At least three dose levels should be used in addition to the concurrent control group. The highest dose level should elicit definite signs of toxicity without causing excessive lethality.

The lowest dose level should not produce and evidence of toxicity.

The intermediate dose(s) should be established in a mid-range between the high and low doses.

The selection of dose levels should take into account data from preceding toxicity tests and studies.

Frequency of exposure is normally daily. If the chemical is administered in the drinking water or mixed in the diet it should be continuously available.

1.6.2.4. Controls

A concurrent control group which is identical in every respect to the treated groups, except for exposure to the test substance, should be used.

In special circumstances, such as in inhalation studies involving aerosols or the use of an emulsifier of uncharacterised biological activity in oral studies, a concurrent negative control group should also be used. The negative control group is treated in the same manner as the test groups except that the animals are not exposed to the test substance or any vehicle.

1.6.2.5. Route of administration

The two main routes of administration are oral and inhalation. The choice of the route of administration depends upon the physical and chemical characteristics of the test substance and the likely route of exposure in humans.

The use of the dermal route presents considerable practical problems. Chronic systemic toxicity resulting from percutaneous absorption can normally be inferred form the results of another oral test and a knowledge of the extent of percutaneous absorption derived from preceding percutaneous toxicity tests.

1.6.2.6. Oral studies

Where the test substance is absorbed from the gastrointestinal tract, and if the ingestion route is one by which humans may be exposed, the oral route of administration is preferred unless there are contra-indications. The animals may receive the test substance in the diet, dissolved in drinking water or given by capsule. Ideally, daily dosing on a seven-day per week basis should be used because dosing on a five-day per week basis may permit recovery or withdrawal toxicity in the non-dosing period and thus affect the result and subsequent evaluation. However, based primarily on practical considerations, dosing on a five-day per week basis is considered to be acceptable.

1.6.2.7. Inhalation studies

Because inhalation studies present technical problems of greater complexity than the other routes of administration, more detailed guidance on this mode of administration is given here. It should also be noted that intratracheal instillation may constitute a valid alternative in specific situations.

Long-term exposures are usually patterned on projected human exposure, giving the animals either a daily exposure of six hours after equilibration of chamber concentrations, for five days a week (intermittent exposure), or, relevant to possible environmental exposure, 22 to 24 hours of exposure per day for seven days a week (continuous exposure), with about an hour for feeding the animals daily at a similar time and maintaining the chamber.

In both cases, the animals are usually exposed to fixed concentrations of test substance. A major difference between intermittent and continuous exposure is that with the former there is a 17 to 18 hour period in which animals may recover from the effects of each daily exposure, with an even longer recovery period during weekends.

The choice of intermittent or continuous exposure depends on the objectives of the study and on the human exposure that is to be simulated. However, certain technical difficulties must be considered. For example, the

advantages of continuous exposure for simulating environmental conditions may be offset by the necessity for watering and feeding during exposure, and by the need for more complicated (and reliable) aerosol and vapour, generation and monitoring techniques.

1.6.2.8. Exposure chambers

The animals should be tested in inhalation chambers designed to sustain a dynamic flow of at least 12 air changes per hour to assure adequate oxygen content and an evenly distributed exposure atmosphere. Control and exposure chambers should be identical in construction and design to ensure exposure conditions comparable in all respects except for exposure to the test substances. Slight negative pressure inside the chamber is generally maintained to prevent leakage of the test substance into the surrounding area. The chambers should minimise the crowding of test animals. As a general rule, to ensure the stability of the chamber atmosphere, the total volume of the test animals should not exceed 5 % of the volume of the chamber.

Measurements or monitoring should be made of:

(i) air flow: the rate of air flow through the chamber should preferably be monitored continuously;

(ii) concentration: during the daily exposure period the concentration of the test substance should not vary more than ± 15 % of the mean value;

(iii) temperature and humidity: for rodents, the temperature should be maintained at 22 ± 2 °C, and the humidity within the chamber at 30 to 70 %, except when water is used to suspend the test substance in the chamber atmosphere. Preferably both should be monitored continuously;

(iv) particle size measurements: particle-size distribution should be determined in chamber atmospheres involving liquid or solid aerosols. The aerosol particles should be of respirable size for the test animal used. Samples of the chamber atmospheres should be taken in the breathing zone of the animals. The air sample should be representative of the distribution of the particles to which the animals are exposed and should account, on a gravimetric basis, for all the suspended aerosol even when much of the aerosol is not respirable. Particle size analyses should be carried out frequently during the development of the generating system to ensure the stability of the aerosol and thereafter as often as necessary during the exposures to determine adequately the consistency of the particle distribution to which the animals have been exposed.

1.6.2.9. Duration of study

The duration of the period of administration should be at least 12 months.

1.6.3. *Procedure*

Observations

A careful clinical examination should be made at least once each day. Additional observations should be made daily with appropriate actions taken to minimise loss of animals to the study, for example necropsy or refrigeration of those animals found dead and isolation or sacrifice of weak or moribund animals. Careful observations should be made to detect onset and progression of all toxic effects as well as to minimise loss due to disease, autolysis or cannibalism.

Clinical signs, including neurological and ocular changes as well as mortality, should be recorded for all animals. Time of onset and progression of toxic conditions, including suspected tumours, should be recorded.

Bodyweights should be recorded individually for all animals once a week during the first 13 weeks of the test period and at least once every four weeks thereafter. Food intake should be determined weekly during the first 13 weeks of the study, and then at approximately three-monthly intervals unless health status or body weight changes dictate otherwise.

Haematological examination

Haematological examination (e.g. haemoglobin content; packet cell volume, total red blood cells, total white blood cells, platelets or other measures of clotting potential) should be performed at three months, six months, and thereafter at approximately six-month intervals and at termination on blood samples collected form all non-rodents and from 10 rats/sex of all groups. If possible, samples should be from the same rats at each interval. In addition, a pre-test sample should be collected from non-rodents.

If clinical observations suggest a deterioration in the health of the animals during the study, a differential blood count of the affected animals should be performed.

A differential blood count is performed on samples from the animals in the highest dose group and the controls. Differential blood counts are performed for the next lower group(s) only if there is a major discrepancy between the highest group and the controls, or if indicated by pathological findings.

Urinalysis

Urine samples from all non-rodents and from 10 rats/sex of all groups, if possible from the same rats at the same intervals as haematological examination, should be collected for analysis. The following determinations should be made for either individual animals or on a pooled sample/sex/group for rodents:

— appearance: volume and density for individual animals,

— protein, glucose, ketones, occult blood (semi-quantitatively),

— microscopy of sediment (semi-quantitatively).

Clinical chemistry

At approximately six-monthly intervals and at termination, blood samples are drawn for clinical chemistry measurements from all non-rodents and 10 rats/sex of all groups, if possible, from the same rats at each interval. In addition, a pre-test sample should be collected from non-rodents. Plasma is prepared from these samples and the following determinations are made:

— total protein concentration,

— albumin concentration,

— liver function tests (such as alkaline phosphatase activity, glutamic pyruvic transaminase ([1]) activity and glutamic oxaloacetic transaminase ([2]) activity), gamma glutamyl transpeptldase, ornithine decarboxylase,

— carbohydrate metabolism such as fasting blood glucose,

— kidney function tests such as blood urea nitrogen.

Gross necropsy

Full gross necropsy should be performed on all animals, including those which died during the experiment or were killed having been found in a moribund condition. Prior to sacrifice, samples of blood should be collected from all animals, for differential blood counts. All grossly visible lesions, tumours or lesions suspected of being tumours should be preserved. An attempt should be made to correlate gross observations with the microscopic findings.

All organs and tissues should be preserved for histopathological examination. This usually concerns the following organs and tissues: brain ([3]) (medullaipons, cerebellar correx, cerebral cortex), pituitary, thyroid (including parathyroid), thymus, lungs (including trachea), heart, aorta, salivary glands, liver ([3]), spleen, kidneys ([3]), adrenals ([3]), oesophagus, stomach, duodenum, jejunum, ileum, caecum, colon, rectum, uterus, urinary bladder, lymph nodes, pancreas, gonads ([3]), accessory genital organs, female mammary gland, skin, musculature, peripheral, nerve, spinal cord (cervical, thoracic, lumbar), sternum with bone marrow and femur (including joint) and eyes. Inflation of lungs and urinary bladder with a fixative is the optimal way to preserve these tissues; inflation of the lungs in inhalation studies is essential for appropriate histopathological examination. In special studies such as inhalation studies, the entire respiratory tract should be studied, including nose, pharynx and larynx.

([1]) Now known as serum alanine aminotransferase.

([2]) Now known as serum aspartate aminotransferase.

([3]) These organs, from 10 animals per sex per group for rodents and all non-rodents, plus thyroid (with parathyroids) for all non-rodents, should be weighed.

If other clinical examinations are carried out, the information obtained from these procedures should be available before microscopic examination, because it may give significant guidance to the pathologist.

Histopathology

All visible changes, particularly tumours and other lesions occurring in any organ should be examined microscopically. In addition, the following procedures are recommended:

(a) microscopic examination of all preserved organs and tissues with complete description of all lesions found in:

1. all animals that died or were killed during the study;

2. all of the high-dose group and controls;

(b) organs or tissues showing abnormalities caused, or possibly caused, by the test substance are also examined in the lower-dose groups;

(c) where the result of the test gives evidence of substantial reduction of the animals' normal lifespan or the induction of effects that might affect a toxic response, the next-lower dose level should be examined as described above;

(d) information on the incidence of lesions normally occurring in the strain of animals used, under the same laboratory conditions, i.e. historical control data, is indispensable for correctly assessing the significance of changes observed in treated animals.

2. DATA

Data should be summarised in tabular form, showing for each test group the number of animals at the start of the test, the number of animals showing lesions and the percentage of animals displaying each type of lesion. Results should be evaluated by an appropriate statistical method. Any recognised statistical method may be used.

3. REPORTING

TEST REPORT

The test report shall, if possible, contain the following information:

— species, strain, source, environmental conditions, diet,

— test conditions:

Description of exposure apparatus:

Including design, type, dimensions, source of air, system for generating particulates and aerosols, method of conditioning air, treatment of exhaust air and the method of housing animals in a test chamber when this is used. The equipment for measuring temperature, humidity and, where appropriate, stability of aerosol concentration or particle size, should be described.

Exposure data:

These should be tabulated and presented with mean values and a measure of variability (e.g. standard deviation) and should include:

(a) air flow rates through the inhalation equipment;

(b) temperature and humidity of air;

(c) nominal concentrations (total amount of test substance fed into the inhalation equipment divided by the volume of air);

(d) nature of vehicle, if used;

(e) actual concentrations in test breathing zone;

(f) median particle sizes (where appropriate):

— dose levels (including vehicle, if used) and concentrations,

— toxic response data by sex and dose,

— no-effect level,

— time of death during the study or whether animals survived to termination,

— description of toxic and other effects,

— the time of observation of each abnormal sign and its subsequent course,

— food and bodyweight data,

— ophthalmological findings,

— haematological tests employed and all results,

— clinical biochemistry tests employed and all results (including results of any urinalysis),

— necropsy findings,

— a detailed description of all histopathological findings,

— statistical treatment of results where possible,

— discussion of the results,

— interpretation of the results.

3.2. EVALUATION AND INTERPRETATION

See General introduction Part B.

4. **REFERENCES**

See General introduction Part B.

B.31. PRENATAL DEVELOPMENTAL TOXICITY STUDY

1. METHOD

This method is a replicate of OECD TG 414 (2001).

1.1. INTRODUCTION

This method for developmental toxicity testing is designed to provide general information concerning the effects of prenatal exposure on the pregnant test animal and on the developing organism in utero; this may include assessment of maternal effects as well as death, structural abnormalities, or altered growth in the foetus. Functional deficits, although an important part of development, are not an integral part of this test method. They may be tested for in a separate study or as an adjunct to this study using the test method for developmental neurotoxicity. For information on testing for functional deficiencies and other postnatal effects the test method for the two-generation reproductive toxicity study and the developmental neurotoxicity study should be consulted as appropriate.

This test method may require specific adaptation in individual cases on the basis of specific knowledge on e.g. physicochemical or toxicological properties of the test substance. Such adaptation is acceptable, when convincing scientific evidence suggests that the adaptation will lead to a more informative test. In such a case, this scientific evidence should be carefully documented in the study report.

1.2. DEFINITIONS

Developmental toxicology: the study of adverse effects on the developing organism that may result from exposure prior to conception, during prenatal development, or postnatally to the time of sexual maturation. The major manifestations of developmental toxicity include 1) death of the organism, 2) structural abnormality, 3) altered growth, and 4) functional deficiency. Developmental toxicology was formerly often referred to as teratology.

Adverse effect: any treatment-related alteration from baseline that diminishes an organism's ability to survive, reproduce or adapt to the environment. Concerning developmental toxicology, taken in its widest sense it includes any effect which interferes with normal development of the conceptus, both before and after birth.

Altered growth: an alteration in offspring organ or body weight or size.

Alterations (anomalies): structural alterations in development that include both malformations and variations (28).

Malformation/Major abnormality: structural change considered detrimental to the animal (may also be lethal) and is usually rare.

Variation/Minor abnormality: structural change considered to have little or no detrimental effect on the animal; may be transient and may occur relatively frequently in the control population.

Conceptus: the sum of derivatives of a fertilised ovum at any stage of development from fertilisation until birth including the extra-embryonic membranes as well as the embryo or foetus.

Implantation (nidation): attachment of the blastocyst to the epithelial lining of the uterus, including its penetration through the uterine epithelium, and its embedding in the endometrium.

Embryo: the early or developing stage of any organism, especially the developing product of fertilisation of an egg after the long axis appears and until all major structures are present.

Embryotoxicity: detrimental to the normal structure, development, growth, and/or viability of an embryo.

Foetus: the unborn offspring in the post-embryonic period.

Foetotoxicity: detrimental to the normal structure, development, growth, and/or viability of a foetus.

Abortion: the premature expulsion from the uterus of the products of conception: of the embryo or of a nonviable foetus.

Resorption: a conceptus which, having implanted in the uterus, subsequently died and is being, or has been resorbed.

Early resorption: evidence of implantation without recognisable embryo/foetus

Late resorption: dead embryo or foetus with external degenerative changes

NOAEL: abbreviation for no-observed-adverse-effect level and is the highest dose or exposure level where no adverse treatment-related findings are observed.

1.3. REFERENCE SUBSTANCE

None.

1.4. PRINCIPLE OF THE TEST METHOD

Normally, the test substance is administered to pregnant animals at least from implantation to one day prior to the day of scheduled kill, which should be as close as possible to the normal day of delivery without risking loss of data resulting from early delivery. The test method is not intended to examine solely the period of organogenesis, (e.g. days 5-15 in the rodent, and days 6-18 in the rabbit) but also effects from preimplantation, when appropriate, through the entire period of gestation to the day before caesarean section. Shortly before caesarean section, the females are killed, the uterine contents are examined, and the foetuses are evaluated for externally visible anomalies and for soft tissue and skeletal changes.

1.5. DESCRIPTION OF THE TEST METHOD

1.5.1. **Selection of animal species**

It is recommended that testing be performed in the most relevant species, and that laboratory species and strains which are commonly used in prenatal developmental toxicity testing be employed. The preferred rodent species is the rat and the preferred non-rodent species is the rabbit. Justification should be provided if another species is used.

1.5.2. **Housing and feeding conditions**

The temperature in the experimental animal room should be 22 °C (± 3°) for rodents and 18 °C (± 3°) for rabbits. Although the relative humidity should be at least 30 % and preferably not exceed 70 % other than during room cleaning, the aim should be 50-60 %. Lighting should be artificial, the sequence being 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water.

Mating procedures should be carried out in cages suitable for the purpose. While individual housing of mated animals is preferred, group housing in small numbers is also acceptable.

1.5.3. **Preparation of the animals**

Healthy animals, which have been acclimated to laboratory conditions for at least five days and have not been subjected to previous experimental procedures, should be used. The test animals should be characterised as to species, strain, source, sex, weight and/or age. The animals of all test groups should, as nearly as practicable, be of uniform weight and age. Young adult nulliparous female animals should be used at each dose level. The females should be mated with males of the same species and strain, and the mating of siblings should be avoided. For rodents day 0 of gestation is the day on which a vaginal plug and/or sperm are observed; for rabbits day 0 is usually the day of coitus or of artificial insemination, if this technique is used. Mated females should be assigned in an unbiased manner to the control and treatment groups. Cages should be arranged in such a way that possible effects due to cage placement are minimised. Each animal should be assigned a unique identification number. Mated females should be assigned in an unbiased manner to the control and treatment groups, and if the females are mated in batches, the animals in each batch should be evenly distributed across the groups. Similarly, females inseminated by the same male should be evenly distributed across the groups.

1.6. PROCEDURE

1.6.1. **Number and sex of animals**

Each test and control group should contain a sufficient number of females to result in approximately 20 female animals with implantation sites at necropsy. Groups with fewer than 16 animals with implantation sites may be inappropriate. Maternal mortality does not necessarily invalidate the study providing it does not exceed approximately 10 %.

1.6.2. **Preparation of doses**

If a vehicle or other additive is used to facilitate dosing, consideration should be given to the following characteristics: effects on the absorption, distribution, metabolism, and retention or excretion of the test substance; effects on the chemical properties of the test substance which may alter its toxic characteristics; and effects on the food or water consumption or the nutritional status of the animals. The vehicle should neither be developmentally toxic nor have effects on reproduction.

1.6.3. **Dosage**

Normally, the test substance should be administered daily from implantation (e.g. day 5 post mating) to the day prior to scheduled caesarean section. If preliminary studies, when available, do not indicate a high potential for preimplantation loss, treatment may be extended to include the entire period of gestation, from mating to the day prior to scheduled kill. It is well known that inappropriate handling or stress during pregnancy can result in prenatal loss. To guard against prenatal loss from factors which are not treatment related, unnecessary handling of pregnant animals as well as stress from outside factors such as noise should be avoided.

At least three dose levels and a concurrent control should be used. Healthy animals should be assigned in an unbiased manner to the control and treatment groups. The dose levels should be spaced to produce a gradation of toxic effects. Unless limited by the physical/chemical nature or biological properties of the test substance, the highest dose should be chosen with the aim to induce some developmental and/or maternal toxicity (clinical signs or a decrease in body weight) but not death or severe suffering. At least one intermediate dose level should produce minimal observable toxic effects. The lowest dose level should not produce any evidence of either maternal or developmental toxicity. A descending sequence of dose levels should be selected with a view to demonstrating any dosage-related response and no-observed-adverse-effect level (NOAEL). Two- to four-fold intervals are frequently optimal for setting the descending dose levels, and the addition of a fourth test group is often preferable to using very large intervals (e.g. more than a factor of 10) between dosages. Although establishment of a maternal NOAEL is the goal, studies which do not establish such a level may also be acceptable (1).

Dose levels should be selected taking into account any existing toxicity data as well as additional information on metabolism and toxicokinetics of the test substance or related materials. This information will also assist in demonstrating the adequacy of the dosing regimen.

A concurrent control group should be used. This group should be a sham-treated control group or a vehicle-control group if a vehicle is used in administering the test substance. All groups should be administered the same volume of either test substance or vehicle. Animals in the control group(s) should be handled in an identical manner to test group animals. Vehicle control groups should receive the vehicle in the highest amount used (as in the lowest treatment group).

1.6.4. **Limit test**

If a test at one dose level of at least 1 000 mg/kg body weight/day by oral administration, using the procedures described for this study, produces no observable toxicity in either pregnant animals or their progeny and if an effect would not be expected based upon existing data (e.g. from structurally and/or metabolically related compounds), then a full study using three dose levels may not be considered necessary. Expected human exposure may indicate the need for a higher oral dose level to be used in the limit test. For other types of administration, such as inhalation or dermal application, the physico-chemical properties of the test substance often may indicate and limit the maximum attainable level of exposure (for example, dermal application should not cause severe local toxicity).

1.6.5. **Administration of doses**

The test substance or vehicle is usually administered orally by intubation. If another route of administration is used, the tester should provide justification and reasoning for its selection, and appropriate modifications may be necessary (2)(3)(4). The test substance should be administered at approximately the same time each day.

The dose to individual animals should normally be based on the most recent individual body weight determination. However, caution should be exercised when adjusting the dose during the last trimester of pregnancy. Existing data should be used for dose selection to prevent excess maternal toxicity. However, if excess toxicity is noted in the treated dams, those animals should be humanely killed. If several pregnant animals show signs of excess toxicity, consideration should be given to terminating that dose group. When the substance is administered by gavage, this should preferably be given as a single dose to the animals using a stomach tube or a suitable intubation canula. The maximum volume of liquid that can be administered at one time depends on the size of the test animal. The volume should not exceed 1 ml/100 g body weight, except in the case of aqueous solutions where 2 ml/100 g body weight may be used. When corn oil is used as a vehicle, the volume should not exceed 0.4 ml/100 g body weight. Variability in test volume should be minimised by adjusting the concentrations to ensure a constant volume across all dose levels.

1.6.6. **Observations of the dams**

Clinical observations should be made and recorded at least once a day, preferably at the same time(s) each day taking into consideration the peak period of anticipated effects after dosing. The condition of the animals should be recorded including mortality, moribundity, pertinent behavioural changes, and all signs of overt toxicity.

1.6.7. **Body weight and food consumption**

Animals should be weighed on day 0 of gestation or no later than day 3 of gestation if time-mated animals are supplied by an outside breeder, on the first day of dosing, at least every three days during the dosing period and on the day of scheduled kill.

Food consumption should be recorded at three-day intervals and should coincide with days of body weight determination.

1.6.8. **Post-mortem examination**

Females should be killed one day prior to the expected day of delivery. Females showing signs of abortion or premature delivery prior to scheduled kill should be killed and subjected to a thorough macroscopic examination.

At the time of termination or death during the study, the dam should be examined macroscopically for any structural abnormalities or pathological changes. Evaluation of the dams during caesarean section and subsequent foetal analyses should be conducted preferably without knowledge of treatment group in order to minimise bias.

1.6.9. **Examination of uterine contents**

Immediately after termination or as soon as possible after death, the uteri should be removed and the pregnancy status of the animals ascertained. Uteri that appear non gravid should be further examined (e.g. by ammonium sulphide staining for rodents and Salewski staining or a suitable alternative method for rabbits) to confirm the non-pregnant status (5).

Gravid uteri including the cervix should be weighed. Gravid uterine weights should not be obtained from animals found dead during the study.

The number of corpora lutea should be determined for pregnant animals.

The uterine contents should be examined for numbers of embryonic or foetal deaths and viable foetuses. The degree of resorption should be described in order to estimate the relative time of death of the conceptus (see Section 1.2).

1.6.10. **Examination of foetuses**

The sex and body weight of each foetus should be determined.

Each foetus should be examined for external alterations (6).

Foetuses should be examined for skeletal and soft tissue alterations (e.g. variations and malformations or anomalies) (7)(8)(9)(10)(11)(12)(13)(14)(15)(16)(17)(18)(19)(20)(21)(22)(23)(24). Categorisation of foetal

alterations is preferable but not required. When categorisation is done, the criteria for defining each category should be clearly stated. Particular attention should be paid to the reproductive tract which should be examined for signs of altered development.

For rodents, approximately one-half of each litter should be prepared and examined for skeletal alterations. The remainder should be prepared and examined for soft tissue alterations, using accepted or appropriate serial sectioning methods or careful gross dissection techniques.

For non-rodents, e.g. rabbits, all foetuses should be examined for both soft tissue and skeletal alterations. The bodies of these foetuses are evaluated by careful dissection for soft tissue alterations, which may include procedures to further evaluate internal cardiac structure (25). The heads of one-half of the foetuses examined in this manner should be removed and processed for evaluation of soft tissue alterations (including eyes, brain, nasal passages and tongue), using standard serial sectioning methods (26) or an equally sensitive method. The bodies of these foetuses and the remaining intact foetuses should be processed and examined for skeletal alterations, utilising the same methods as described for rodents.

2. DATA

2.1. TREATMENT OF RESULTS

Data shall be reported individually for the dams as well as for their offspring and summarised in tabular form, showing for each test group and each generation the number of animals at the start of the test, the number of animals found dead during the test or killed for humane reasons, the time of any death or humane kill, the number of pregnant females, the number of animals showing signs of toxicity, a description of the signs of toxicity observed, including time of onset, duration, and severity of any toxic effects, the types of embryo/foetal observations, and all relevant litter data.

Numerical results should be evaluated by an appropriate statistical method using the litter as the unit for data analysis. A generally accepted statistical method should be used; the statistical methods should be selected as part of the design of the study and should be justified. Data from animals that do not survive to the scheduled kill should also be reported. These data may be included in group means where relevant. Relevance of the data obtained from such animals, and therefore inclusion or exclusion from any group mean(s), should be justified and judged on an individual basis.

2.2. EVALUATION OF RESULTS

The findings of the Prenatal Developmental Toxicity Study should be evaluated in terms of the observed effects. The evaluation will include the following information:

— maternal and embryo/foetal test results, including the evaluation of the relationship, or lack thereof, between the exposure of the animals to the test substance and the incidence and severity of all findings,

— criteria used for categorising foetal external, soft tissue, and skeletal alterations if categorisation has been done,

— when appropriate, historical control data to enhance interpretation of study results,

— the numbers used in calculating all percentages or indices,

— adequate statistical analysis of the study findings, when appropriate, which should include sufficient information on the method of analysis, so that an independent reviewer/statistician can re-evaluate and reconstruct the analysis.

In any study which demonstrates the absence of any toxic effects, further investigations to establish absorption and bioavailability of the test substance should be considered.

2.3. INTERPRETATION OF RESULTS

A prenatal developmental toxicity study will provide information on the effects of repeated exposure to a substance during pregnancy on the dams and on the intrauterine development of their progeny. The results of the study should be interpreted in conjunction with the findings from subchronic, reproduction, toxicokinetic

and other studies. Since emphasis is placed both on general toxicity in terms of maternal toxicity and on developmental toxicity endpoints, the results of the study will allow to a certain extent for the discrimination between developmental effects occurring in the absence of general toxicity and those which are only induced at levels that are also toxic to the maternal animal (27).

3. **REPORTING**

3.1. TEST REPORT

The test report must include the following specific information:

Test substance:

— physical nature and, where relevant, physiochemical properties,

— identification including CAS number if known/established,

— purity.

Vehicle (if appropriate):

— justification for choice of vehicle, if other than water.

Test animals:

— species and strain used,

— number and age of animals,

— source, housing conditions, diet, etc.,

— individual weights of animals at the start of the test.

Test conditions:

— rationale for dose level selection,

— details of test substance formulation/diet preparation, achieved concentration, stability and homogeneity of the preparation,

— details of the administration of the test substance,

— conversion from diet/drinking water test substance concentration (ppm) to the actual dose (mg/kg body weight/day), if applicable,

— environmental conditions,

— details of food and water quality.

Results:

Maternal toxic response data by dose, including but not limited to:

— the number of animals at the start of the test, the number of animals surviving, the number pregnant, and the number aborting, number of animals delivering early,

— day of death during the study or whether animals survived to termination,

— data from animals that do not survive to the scheduled kill should be reported but not included in the inter-group statistical comparisons,

— day of observation of each abnormal clinical sign and its subsequent course,

— body weight, body weight change and gravid uterine weight, including, optionally, body weight change corrected for gravid uterine weight,

— food consumption and, if measured, water consumption,

— necropsy findings, including uterine weight,

— NOAEL values for maternal and developmental effects should be reported.

Developmental endpoints by dose for litters with implants, including:

— number of corpora lutea,

— number of implantations, number and percent of live and dead foetuses and resorptions,

— number and percent of pre- and post-implantation losses.

Developmental endpoints by dose for litters with live foetuses, including:

— number and percent of live offspring,

— sex ratio,

— foetal body weight, preferably by sex and with sexes combined,

— external, soft tissue, and skeletal malformations and other relevant alterations,

— criteria for categorisation if appropriate,

— total number and percent of foetuses and litters with any external, soft tissue, or skeletal alteration, as well as the types and incidences of individual anomalies and other relevant alterations.

Discussion of results.

Conclusions.

4. **REFERENCES**

(1) Kavlock R.J. et al., (1996) A Simulation Study of the Influence of Study Design on the Estimation of Benchmark Doses for Developmental Toxicity. *Risk Analysis* 16; p. 399-410.

(2) Kimmel, C.A. and Francis, E.Z., (1990) Proceedings of the Workshop on the Acceptability and Interpretation of Dermal Developmental Toxicity Studies. *Fundamental and Applied Toxicology* 14; p. 386-398.

(3) Wong, B.A., et al., (1997) Developing Specialised Inhalation Exposure Systems to Address Toxicological Problems. *CIIT Activities* 17; p. 1-8.

(4) US Environmental Protection Agency, (1985) Subpart E-Specific Organ/Tissue Toxicity, 40 CFR 798.4350: Inhalation Developmental Toxicity Study.

(5) Salewski, E., (1964) Faerbermethode zum Makroskopischen Nachweis von Implantations Stellen am Uterusder Ratte. *Naunyn-Schmeidebergs Archiv fur Pharmakologie und Experimentelle Pathologie*, 247, 367.

(6) Edwards, J.A., (1968) The external Development of the Rabbit and Rat Embryo. In *Advances in Teratology*. D.H.M. Woolam (ed.) Vol. 3. Academic Press, NY.

(7) Inouye, M. (1976) Differential Staining of Cartilage and Bone in Fetal Mouse Skeleton by Alcian Blue and Alizarin Red S. *Congenital Anomalies* 16, p. 171-173.

(8) Igarashi, E. et al., (1992) Frequency Of Spontaneous Axial Skeletal Variations Detected by the Double Staining Techniquefor Ossified and Cartilaginous Skeleton in Rat Foetuses. *Congenital Anomalies* 32, p. 381-391.

(9) Kimmel, C.A. et al. (1993) Skeletal Development Following Heat Exposure in the Rat. *Teratology*, 47 p.229-242.

(10) Marr, M.C. et al. (1988) Comparison of Single and Double Staining for Evaluation of Skeletal Development: The Effects of Ethylene Glycol (EG) in CD Rats. *Teratology* 37; 476.

(11) Barrow, M.V. and Taylor, W.J. (1969) A Rapid Method for Detecting Malformations in Rat Foetuses. *Journal of Morphology*, 127, p. 291-306.

(12) Fritz, H. (1974) Prenatal Ossification in Rabbits ss Indicative of Foetal Maturity. *Teratology*, 11 p. 313-320.

(13) Gibson, J.P. et al. (1966) Use of the Rabbit in Teratogenicity Studies. *Toxicology and Applied Pharmacology*, 9, p. 398-408.

(14) Kimmel, C.A. and Wilson, J.G. (1973) Skeletal Deviation in Rats: Malformations or Variations? *Teratology*, 8, p. 309-316.

(15) Marr, M.C. et al. (1992) Developmental Stages of the CD (Sprague-Dawley) Rat Skeleton after Maternal Exposure to Ethylene Glycol. *Teratology*, 46, p. 169-181.

(16) Monie, I.W. et al. (1965) Dissection Procedures for Rat Foetuses Permitting Alizarin Red Staining of Skeleton and Histological Study of Viscera. *Supplement to Teratology Workshop Manual*, p. 163-173.

(17) Spark, C. and Dawson, A.B. (1928) The Order and Time of appearance of Centers of Ossification in the Fore and Hind Limbs of the Albino Rat, with Special Reference to the Possible Influence of the Sex Factor. *American Journal of Anatomy*, 41, p. 411-445.

(18) Staples, R.E. and Schnell, V.L. (1964) Refinements in Rapid Clearing Technique in the KOH-Alizarin Red S Method for Fetal Bone. *Stain Technology*, 39, p. 61-63.

(19) Strong, R.M. (1928) The Order Time and Rate of Ossification of the Albino Rat (Mus Norvegicus Albinus) Skeleton. *American Journal of Anatomy*, 36, p. 313-355.

(20) Stuckhardt, J.L. and Poppe, S.M. (1984) Fresh Visceral Examination of Rat and Rabbit Foetuses Used in Teratogenicity Testing. *Teratogenesis, Carcinogenesis, and Mutagenesis*, 4, p. 181-188.

(21) Walker, D.G. and Wirtschafter, Z.T. (1957) *The Genesis of the Rat Skeleton*. Thomas, Springfield, IL.

(22) Wilson, J.G. (1965) Embryological Considerations in Teratology. In *Teratology: Principles and Techniques*, Wilson J.G. and Warkany J. (eds). University of Chicago, Chicago, IL, p. 251-277.

(23) Wilson, J.G. and Fraser, F.C. (eds). (1977) *Handbook of Teratology*, Vol. 4. Plenum, NY.

(24) Varnagy, L. (1980) Use of Recent Fetal Bone Staining Techniques in the Evaluation of Pesticide Teratogenicity. *Acta Vet. Acad. Sci. Hung*, 28, p. 233-239.

(25) Staples, R.E. (1974) Detection of visceral Alterations in Mammalian Foetuses. *Teratology*, 9, p. 37-38.

(26) Van Julsingha, E.B. and C.G. Bennett (1977) A Dissecting Procedure for the Detection of Anomalies in the Rabbit Foetal Head. In: *Methods in Prenatal Toxicology* Neubert, D., Merker, H.J. and Kwasigroch, T.E. (eds.). University of Chicago, Chicago, IL, p. 126-144.

(27) US Environmental Protection Agency (1991) Guidelines for Developmental Toxicity Risk Assessment. *Federal Register*, 56, p. 63798-63826.

(28) Wise, D.L. et al. (1997) Terminology of Developmental Abnormalities in Common Laboratory Mammals (Version 1) Teratology, 55, p. 249-292.

B.32. CARCINOGENICITY TEST

1. METHOD

1.1. INTRODUCTION

See General introduction Part B.

1.2. DEFINITIONS

See General introduction Part B.

1.3. REFERENCE SUBSTANCES

None.

1.4. PRINCIPLE OF THE TEST METHOD

The test substance is administered normally seven days per week, by an appropriate route, to several groups of experimental animals, one dose per group, for a major portion of their lifespan. During and after exposure to the test substance, the experimental animals are observed daily to detect signs of toxicity, particularly the development of tumours.

1.5. QUALITY CRITERIA

None.

1.6. DESCRIPTION OF THE TEST METHOD

The animals are kept under the experimental housing and feeding conditions for at least five days prior to the test. Before the test healthy young animals are randomised and assigned to the treated and control groups.

1.6.1. Experimental animals

Based upon the results of previously conducted studies other species (rodent or non-rodent) may be used. Commonly used laboratory strains of young healthy animals should be employed and dosing should begin as soon as possible after weaning.

At the commencement of the study the weight variation in the animals used should not exceed ± 20 % of the mean value. Where a sub-chronic oral study is conducted as a preliminary to a long-term study, the same species/breed and strain should be used in both studies.

1.6.2. Number and sex

For rodents at least 100 animals (50 female and 50 male) should be used at each dose level and concurrent control group. The females should be nulliparous and non-pregnant. If interim sacrifices are planned the number should be increased by the number of animals scheduled to be sacrificed before the completion of the study.

1.6.3. Dose levels and frequency of exposure

At least three dose levels should be used in addition to the concurrent control group. The highest dose level should elicit signs of minimal toxicity, such as a slight depression of bodyweight gain (less than 10 %), without substantially altering the normallifespan due to effects other than tumours.

The lowest dose level should not interfere with normal growth, development and longevity of the animal or produce any indication of toxicity. In general, this should not be lower than 10 % of the high dose.

The intermediate dose(s) should be established in a mid-range between the high and low doses.

The selection of dose levels should take into account data from preceding toxicity tests and studies.

Frequency of exposure is normally daily.

If the chemical is administered in the drinking water or mixed in the diet it should be continuously available.

1.6.4. Controls

A concurrent control group which is identical in every respect to the treated groups, except for exposure to the test substance, should be used.

In special circumstances, such as in inhalation studies involving aerosols or the use of an emulsifier of uncharacterised biological activity in oral studies, an additional control group which is not exposed to the vehicle should be used.

1.6.5. Route of administration

The three main routes of administration are oral, dermal and inhalation. The choice of the route of administration depends upon the physical and chemical characteristics of the test substance and the likely route of exposure in humans.

1.6.5.1. *Oral studies*

Where the test substance is absorbed from the gastro-intestinal tract, and if the ingestion route is one by which humans may be exposed, the oral route of administration is preferred, unless there are contra-indications. The animals may receive the test substance in their diet, dissolved in drinking water or given by capsule.

Ideally, daily dosing on a seven-day per week basis should be used because dosing on a five-day per week basis may permit recovery or withdrawal toxicity in the non-dosing period and thus affect the result and subsequent evaluation. However, based primarily on practical considerations, dosing on a five-day per week basis is considered to be acceptable.

1.6.5.2. *Dermal studies*

Cutaneous exposure by skin painting may be selected to simulate a main route of human exposure and as a model system for induction of skin lesions.

1.6.5.3. *Inhalation studies*

Because inhalation studies present technical problems of greater complexity than the other routes of administration, more detailed guidance on this mode of administration is given here. It should be noted that intratracheal instillation may constitute a valid alternative in specific situations.

Long-term exposures are usually patterned on projected human exposure, giving the animals either a daily exposure of six hours after equilibration of chamber concentrations, for five days a week (intermittent exposure), or, relevant to possible environmental exposure, 22 to 24 hours of exposure per day for seven days a week (continuous exposure), with about an hour for feeding the animals daily at a similar time and maintaining the chambers. In both cases, the animals are usually exposed to fixed concentrations of test substance. A major difference between intermittent and continuous exposure is that with the former there is a 17 to 18 hour period in which animals may recover from the effects of each daily exposure with an even longer recovery period during weekends.

The choice of intermittent or continuous exposure depends on the objectives of the study and on the human exposure that is to be simulated. However, certain technical difficulties must be considered. For example, the advantages of continuous exposure for simulating environmental conditions may be offset by the necessity for watering and feeding during exposure and by the need for more complicated (and reliable) aerosol and vapour generation and monitoring techniques.

1.6.6. Exposure chambers

The animals should be tested in inhalation chambers designed to sustain a dynamic flow of at least 12 air changes per hour to assure adequate oxygen content and an evenly distributed exposure atmosphere. Control and exposure chambers should be identical in construction and design to ensure exposure conditions comparable in all respects except for exposure to the test substances. Slight negative pressure inside the chamber is generally maintained to prevent leakage of the test substance into the surrounding area. The chambers should minimise the crowding of test animals. As a general rule, to ensure the stability of the chamber atmosphere, the total volume of the test animals should not exceed 5 % of the volume of the chamber.

Measurements or monitoring should be made of:

(i) air flow: the rate of air flow through the chamber should preferably be monitored continuously;

(ii) concentration: during the daily exposure period the concentration of the test substance should not vary more than ± 15 % of the mean value. During the total duration of this study, the day-to-day concentrations should be held as constant as practicable;

(iii) temperature and humidity: for rodents, the temperature should be maintained at 22 ± 2 °C and the humidity within the chamber at 30 to 70 %, except when water is used to suspend the test substance in the chamber atmosphere. Preferably both should be monitored continuously;

(iv) particle size measurements: particle size distribution should be determined in chamber atmospheres involving liquid or solid aerosols. The aerosol particles should be of respirable size for the test animal used. Samples of the chamber atmospheres should be taken in the breathing zone of the animals. The air sample should be representative of the distribution of the particles to which the animals are exposed and should account, on a gravimetric basis, for all of the suspended aerosol even when much of the aerosol is not respirable. Particle size analyses should be carried out frequently during the development of the generating system to ensure the stability of the aerosol and thereafter as often as necessary during the exposures to determine adequately the consistency of the particle distribution to which the animals have been exposed.

1.6.7. Duration of study

The duration of a carcinogenicity test comprises the major portion of the normal lifespan of the test animals. The termination of the test should be at 18 months for mice and hamsters and 24 months for rats; however, for certain strains of animals with greater longevity and/or low spontaneous tumour rate, termination should be at 24 months for mice and hamsters and at 30 months for rats. Alternatively, termination of such an extended study is acceptable when the number of survivors in the lowest dose or control group reaches 25 %. When terminating a test in which there is an apparent sex difference in response, each sex should be considered separately. Where only the high-dose group dies prematurely for obvious reasons of toxicity, this need not trigger termination providing toxic manifestations are not causing problems in the other groups. For a negative test result to be acceptable, not more than 10 % of any group may be lost from the experiment due to autolysis, cannibalism or management problems and the survival of all groups is not less than 50 % at 18 months for mice and hamsters and at 24 months for rats.

1.6.8. *Procedure*

1.6.8.1. Observations

Daily cageside observations should include changes in skin and fur, eyes and mucous membranes as well as respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behaviour pattern.

Regular observations of the animals is necessary to ensure that, as far as possible, animals are not lost from the study due to causes such as cannibalism, autolysis of tissues or misplacement. Moribund animals should be removed and necropsied when noticed.

Clinical signs and mortality should be recorded for all animals. Special attention must be paid to tumour development: the time of onset; location, dimensions, appearance and progression of each grossly visible or palpable tumour should be recorded.

Measurements should be made of food consumption (and water consumption when the test substance is administered in the drinking water) weekly during the first 13 weeks of the study and then at approximately

three-month intervals unless health status or body weight changes dictate otherwise.

Bodyweights should be recorded individually for all animals once a week during the first 13 weeks of the test period and at least once every four weeks thereafter.

1.6.8.2. *Clinical examinations*

Haematology

If cage side observations suggest a deterioration in health of the animals during the study, a differential blood count of the affected animals should be performed.

At 12 months, 18 months, and prior to sacrifice, a blood smear is obtained from the animals. A differential blood count is performed on samples from the animals in the high-dose group and the controls. If these data, particularly those obtained prior to sacrifice, or data from the pathological examination indicate a need, differential blood counts should be performed on the next-lower group(s) as well.

Gross necropsy

Full gross necropsy should be performed on all animals, including those which died during the experiment or were sacrificed having been found in a moribund condition. All grossly visible tumours or lesions, or lesions suspected of being tumours, should be preserved.

The following organs and tissues should be preserved in suitable media for possible future histopathological examination: brain (including sections of medulla/pons, cerebellar cortex, cerebral cortex), pituitary, thyroid/parathyroid, any thymic tissue, trachea and lungs, heart, aorta, salivary glands, liver, spleen, kidneys, adrenals, pancreas, gonads, uterus, accessory genital organs, skin, oesophagus, stomach, duodenum, jejunum, ileum, caecum, colon, rectum, urinary bladder, representative lymph node, female mammary gland, thigh musculature, peripheral nerve, sternum with bone marrow, femur (including joint), spinal cord at three levels (cervical, mid-thoracic and lumbar) and eyes.

Inflation of lungs and urinary bladder with a fixative is the optimal way to preserve these tissues; inflation of the lungs in inhalation studies is essential for appropriate histopathological examination. In inhalation studies, the entire respiratory tract should be preserved, including nasal cavity, pharynx and larynx.

Histopathology

(a) Full histopatology should be carried out on the organs and tissues of all animals that died or were sacrificed during the test and all animals in the control and high-dose groups;

(b) all grossly visible tumours or lesions suspected of being tumours should be examined microscopically in all groups,

(c) if there is a significant difference in the incidence of neoplastic lesions in the high-dose and control groups, histopathology should be carried out on that particular organ or tissue in the other groups,

(d) if the survival of the high-dose group is substantially less than the control then the next-lower dose group should be examined fully,

(e) if there is evidence in the high-dose group of the induction of toxic or other effects that might affect a neoplastic response, the next-lower dose level should be examined fully.

2. **DATA**

Data should be summarised in tabular form, showing for each test group the number of animals at the start of the test, the number of animals showing tumours detected during the test, the time of detection and the number of animals found to have tumours following sacrifice. Results should be evaluated by an appropriate statistical method. Any recognised statistical method may be used.

3. **REPORTING**

3.1. TEST REPORT

The test report shall, if possible, contain the following information:

— species, strain, source, environmental conditions, diet,

— test conditions:

3.1.1. Description of exposure apparatus:

including design, type, dimensions, source of air, system for generating particulates and aerosols, method of conditioning air, treatment of exhaust air and the method of housing animals in a test chamber when this is used. The equipment for measuring temperature, humidity and, where appropriate, stability of aerosol) concentration or particle size, should be described.

3.1.2. Exposure data:

these should be tabulated and presented with mean values and a measure of variability (e.g. standard deviation) and should include:

(a) air flow rates through the inhalation equipment;

(b) temperature and humidity of air;

(c) nominal concentrations (total amount of test substance fed into the inhalation equipment divided by the volume of air);

(d) nature of vehicle, if used;

(e) actual concentrations in test breathing zone;

(f) median particle sizes (where appropriate),

— dose levels (including vehicle, if used) and concentrations,

— tumour incidence data by sex, dose and tumour type,

— time of death during the study or whether animals survived to termination,

— toxic response data by sex and dose,

— description of toxic or other effects,

— the time of observation of each abnormal sign and its subsequent course,

— food and bodyweight data,

— haematological tests employed and all results,

— necropsy findings,

— a detailed description of all histopathological findings,

— statistical treatment of results with a description of the methods used,

— discussion of the results,

— interpretation of the result.

3.2. EVALUATION AND INTERPRETATION

See General introduction Part B.

4. **REFERENCES**

See General introduction Part B.

B.33. COMBINED CHRONIC TOXICITY/CARCINOGENICITY TEST

1. METHOD

1.1. INTRODUCTION

See General introduction Part B.

1.2. DEFINITIONS

See General introduction Part B.

1.3. REFERENCE SUBSTANCES

None.

1.4. PRINCIPLE OF THE TEST METHOD

The objective of a combined chronic toxicity carcinogenicity test is to determine the chronic and carcinogenic effects of a substance in a mammalian species following prolonged exposure.

To this end a carcinogenicity test is supplemented with a least one treated satellite group and a control satellite group. The dose used for the high-dose satellite group may be higher than that used for the high-dose group in the carcinogenicity test. The animals in the carcinogenicity test are examined for general toxicity as well as for carcinogenic response. The animals in the treated satellite group are examined for general toxicity.

The test substance is administered normally seven days per week, by an appropriate route, to several groups of experimental animals, one dose per group, for a major portion of their lifespan. During and after exposure to the test substance, the experimental animals are observed daily to detect signs of toxicity and the development of tumours.

1.5. QUALITY CRITERIA

None.

1.6. DESCRIPTION OF THE TEST METHOD

The animals are kept under the experimental housing and feeding conditions for at least five days prior to the test. Before the test healthy young animals are randomised and assigned to the treated and control groups.

1.6.1. Experimental animals

The preferred species is the rat. Based upon the results of previously conducted tests other species (rodent or non-rodent) may be used. Commonly used laboratory strains of young healthy animals should be employed and dosing should begin as soon as possible after weaning.

At the commencement of the test the weight variation in the animals used should not exceed ± 20 % of the mean value. Where a sub-chronic oral test is conducted as a preliminary to a long-term test, the same species and breed/strain should be used in both studies.

1.6.2. Number and sex

For rodents, at least 100 animals (50 female and 50 male) should be used at each dose level and concurrent control group. The females should be nulliparous and non-pregnant. If interim sacrifices are planned, the number should be increased by the number of animals scheduled to be sacrificed before the completion of the study.

The treated satellite group(s) for the evaluation of pathology other than tumours should contain 20 animals of each sex, while the satellite control group should contain 10 animals of each sex.

1.6.3. Dose levels and frequency of exposure

For carcinogenicity testing purposes, at least three dose levels should be used in addition to the concurrent control group. The highest dose level should elicit signs of minimal toxicity, such as a slight depression of body weight gain (less than 10 %), without substantially altering the normal lifespan due to effects other than tumours.

The lowest dose level should not interfere with normal growth, development and longevity of the animal or produce any indication of toxicity. In general, this should not be lower than 10 % of the high dose.

The intermediate dose(s) should be established in a mid-range between the high and low doses.

The selection of dose levels should take into account data from preceding toxicity tests and studies.

For chronic toxicity testing purposes, additional treated groups and a concurrent control satellite group are included in the test. The high dose for treated satellite animals should produce definite signs of toxicity.

Frequency of exposure is normally daily.

If the chemical is administered in the drinking water or mixed in the diet, it should be continuously available.

1.6.4. Controls

A concurrent group which is identical in every respect to the treated groups, except for exposure to the test substance should be used.

In special circumstances, such as in inhalation studies involving aerosols or the use of an emulsifier of uncharacterized biological activity in oral studies, an additional control group which is not exposed to the vehicle should be utilised.

1.6.5. Route of administration

The three main routes of administration are oral, dermal and inhalation. The choice of the route of administration depends upon the physical and chemical characteristics of the test substance and the likely route of exposure in humans.

1.6.5.1. *Oral tests*

Where the test substance is absorbed from the gastro-intestinal tract and the ingestion route is one by which humans may be exposed, the oral route of administration is preferred, unless there are contra-indications. The animals may receive the test substance in their diet, dissolved in drinking water or given by capsule.

Ideally, daily dosing on a seven-day per week basis should be used because dosing on a five-day per week basis may permit recovery or withdrawal toxicity in the non-dosing period and thus affect the result and subsequent evaluation. However, based primarily on practical considerations, dosing on a five-day per week basis is considered to be acceptable.

1.6.5.2. *Dermal tests*

Cutaneous exposure by skin painting may be selected to simulate a main route of human exposure and as a model system for induction of skin lesions.

1.6.5.3. *Inhalation tests*

Because inhalation tests present technical problems of greater complexity than the other routes of administration, more detailed guidance on this mode of administration is given here. It should be noted that intratracheal instillation may constitute a valid alternative in specific situations.

Long-term exposures are usually patterned on projected human exposure, giving the animals either a daily exposure of six hours after equilibration of chamber concentrations, for five days a week (intermittent exposure), or, relevant to possible environmental exposure, 22 to 24 hours of exposure per day for seven days a week (continuous exposure), with about an hour for feeding the animals daily at a similar time and maintaining the chambers. In both cases, the animals are usually exposed to fixed concentrations of test substance. A major difference between intermittent and continuous exposure is that, with the former, there is a 17 to 18 hour period in which animals may recover from the effects of each daily exposure, with an even longer recovery period during weekends.

The choice of intermittent or continuous exposure depends on the objectives of the test and on the human exposure that is to be simulated. However, certain technical difficulties must be considered. For example, the advantages of continuous exposure for simulating environmental conditions may be offset by the necessity for watering and feeding during exposure and by the need for more complicated (and reliable) aerosol and vapour generation and monitoring techniques.

1.6.6. Exposure chambers

The animals should be tested in inhalation chambers designed to sustain a dynamic flow of at least 12 air changes per hour to assure adequate oxygen content and an evenly distributed exposure atmosphere. Control and exposure chambers should be identical in construction and design to ensure exposure conditions comparable in all respects except for exposure to the test substances. Slight negative pressure inside the chamber is generally maintained to prevent leakage of the test substance into the surrounding area. The chambers should minimise the crowding of test animals. As a general rule, to ensure the stability of the chamber atmosphere, the total volume of the test animals should not exceed 5 % of the volume of the chamber.

Measurements or monitoring should be made of:

(i) air flow: the rate of air flow through the chamber should preferably be monitored continuously;

(ii) concentration: during the daily exposure period the concentration should not vary more than ± 15 % of the mean value. During the total duration of this study, the day-to-day concentrations should be held as constant as practicable;

(iii) temperature and humidity: for rodents, the temperature should be maintained at 22 ± 2 °C, and the humidity within the chamber at 30 to 70 %, except when water is used to suspend the test substance in the chamber atmosphere. Preferably both should be monitored continuously;

(iv) particle size measurements: particle size distribution should be determined in chamber atmospheres involving liquid or solid aerosols. The aerosol particles should be of respirable size for the test animal used. Samples of the chamber atmospheres should be taken in the breathing zone of the animals. The air sample should be representative of the distribution of the particles to which the animals are exposed and should account, on a gravimetric basis, for all of the suspended aerosol even when much of the aerosol is not respirable. Particle size analyses should be carried out frequently during the development of the generating system to ensure the stability of the aerosol and thereafter as often as necessary during the exposures to determine adequately the consistency of the particle distribution to which the animals have been exposed.

1.6.7. Duration of test

The duration of the carcinogenicity part of the test comprises the major portion of the normal life span of the test animals. The termination of the test should be at 18 months for mice and hamsters and 24 months for rats; however, for certain strains of animals with greater longevity and/or low spontaneous tumour rate, termination should be at 24 months for mice and hamsters and at 30 months for rats. Alternatively, termination of such an extended test is acceptable when the number of survivors in the lowest dose or control group reaches 25 %. When terminating a test in which there is an apparent sex difference in response, each sex should be considered separately. Where only the high-dose group dies prematurely for obvious reasons of toxicity, this need not trigger termination providing toxic manifestations are not causing problems in the other groups. For a negative test result to be acceptable not more than 10 % of any group may be lost from the experiment due to autolysis, cannibalism or management problems, and the survival of all groups is not less than 50 at 18 months for mice

and hamsters and at 24 months for rats.

The satellite groups of 20 dosed animals per sex and 10 associated control animals per sex used for chronic toxicity testing should be retained in the test for at least 12 months. These animals should be scheduled for sacrifice for an examination of test-substance-related pathology uncomplicated by gerontological changes.

1.6.8. *Procedure*

1.6.8.1. Observations

Daily cageside observations should be made and should include changes in skin and fur, eyes and mucous membranes as well as respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behaviour pattern.

Clinical examination should be performed at appropriate intervals on animals in the treated satellite group(s).

Regular observations of the animals is necessary to ensure, as far as possible, that animals are not lost from the test due to causes such as cannibalism, autolysis of tissues or misplacment. Moribund animals should be removed and necropsied when noticed.

Clinical signs, including neurological and ocular changes as well as mortality should be recorded for all animals. Special attention must be paid to tumour development: the time of onset, location, dimensions, appearance and progression of each grossly visible or palpable tumour should be recorded; the time of onset and progression of toxic conditions should be recorded.

Measurements should be made of food consumption (and water consumption when the test substance is administered in the drinking water) weekly during the first 13 weeks of the study and then at approximately three-month intervals unless health status or body weight changes dictate otherwise.

Bodyweights should be recorded individually for all animals once a week during the first 13 weeks of the test period and at least once every four weeks thereafter.

1.6.8.2. *Clinical examinations*

Haematology

Haematological examination (e.g. haemoglobin content, packed cell volume, total red blood cells, total white blood cells, platelets, or other measures of clotting potential) should be performed at three months, six months and at approximately six-month intervals thereafter, and at termination on blood samples collected from 10 rats/sex of all groups. If possible, samples should be from the same rats at each interval.

If cageside observations suggest a deterioration in the health of the animals during the study, a differential blood count of the affected animals should be performed. A differential blood count is performed on samples of those animals in the highest dose group and the controls. Differential blood counts are performed for the next lower group(s) only if there is a major discrepancy between the highest group and the controls, or if indicated by pathological findings.

Urinalysis

Urine samples from 10 rats/sex of all groups, if possible from the same rats at the same intervals as haematological examination, should be collected for analysis. The following determinations should be made from either individual animals or on a pooled sample/sex/group of rodents:

— appearance: volume and density for individual animals,

— protein, glucose, ketones, occult blood (semi-quantitatively),

— microscopy of sediment (semi-quantitatively).

Clinical chemistry

At approximately six-monthly intervals, and at termination, blood samples are drawn for clinical chemistry measurements from all non-rodents and 10 rats/sex of all groups, if possible, from the same rats at each interval. In addition, a pre-test sample should be collected from non-rodents. Plasma is prepared from these samples and the following determinations are made:

— total protein concentration,

— albumin concentration,

— liver function tests (such as alkaline phosphatase activity, glutamic pyruvic transaminase ([1]) activity and glutamic oxaloacetic transaminase ([2]) activity), gamma glutamyl transpeptidase, ornithine decarboxylase,

— carbohydrate metabolism such as fasting blood glucose,

— kidney function tests such as blood urea nitrogen.

Gross necropsy

Full gross necropsy should be performed in all animals, including those which died during the experiment or were sacrificed having been found in a moribund condition. Prior to sacrifice, samples of blood should be collected from all animals for differential blood counts. All grossly visible tumours or lesions suspected of being tumours should be preserved. An attempt should be made to correlate gross observations with the microsopic findings.

All organs and tissues should be preserved for histopathological examination. This usually concerns the following organs and tissues: brain ([3]) (medulla/pons, cerebellar cortex, cerebral cortex); pituitary, thyroid (including parathyroid), thymus, lungs (including trachea), heart, aorta, salivary glands, liver ([3]), spleen, kidneys ([3]), adrenals ([3]), oesophagus, stomach, duodenum, jejunum, ileum, caecum, colon, rectum, urinary bladder, lymph nodes, pancreas, gonads ([3]), accessory genital organs; female mammary gland, skin, musculature, peripheral nerve, spinal cord (cervical, thoracic, lumbar), sternum with bone marrow and femur (including joint) and eyes.

Although inflation of lungs and urinary bladder with a fixative is the optimal way to preserve these tissues, inflation of the lungs in inhalation studies is a necessary requirement for appropriate histopathological examination. In special studies such as inhalation studies, the entire respiratory tract should be studied, including nose, pharynx and larynx.

If other clinical examinations are carried out, the information obtained from these procedures should be available before microsopic examination, because it may give significant guidance to the pathologist.

Histopathology

For the chronic toxicity testing portion:

Detailed examination should be made of all preserved organs of all animals of the satellite high-dose and control groups. Where test-substance-related pathology is found in the high-dose satellite group, target organs of all other animals in any other treated satellite group should be subjected to full and detailed histological examination as well as those of the heated groups in the carcinogenicity testing portion of the study at its termination.

For the carcinogenicity testing portion:

(a) full histopathology should be carried out on the organs and tissues of all animals that died or were sacrificed during the test, and of all animals in the control and high-dose groups;

([1]) Now known as serum alanine aminotransferase.
([2]) Now known as serum aspartate aminotransferase.
([3]) These organs, from 10 animals per sex per groups for rodents, should be weighed.

(b) all grossly visible tumours or lesions suspected of being tumours in all groups occurring in any organ should be examined microscopically;

(c) if there is a significant difference in the incidence of neoplastic lesions in the high-dose and control groups, histopathology should be carried out on that particular organ or tissue in the other groups;

(d) if the survival of the high-dose group is substantially less than the control then the next-lower dose group should be examined fully;

(e) If there is evidence in the high-dose group of the induction of toxic or other effects that might affect a neoplastic response, the next-lower dose level should be examined fully.

2. **DATA**

Data should be summarised in tabular form, showing for each test group the number of animals at the start of the test, the number of animals showing tumours or toxic effects detected during the test, the time of detection and the number of animals found to have tumours following sacrifice. Results should be evaluated by an appropriate statistical method. Any recognised statistical method may be used.

3. **REPORTING**

3.1. TEST REPORT

The test report shall, if possible, contain the following information:

— species, strain source, environmental conditions, diet,

— test conditions:

3.1.1. Description of exposure apparatus:

including design, type, dimensions, source of air, system for generating particulates and aerosols, method of conditioning air, treatment of exhaust air and the method of housing animals in a test chamber when this is used. The equipment for measuring temperature, humidity and, where appropriate, stability of aerosol concentration or particle size, should be described.

3.1.2. Exposure data:

these should be tabulated and presented with mean values and a measure of variability (e.g. standard deviation), and should include:

(a) air flow rates through the inhalation equipment;

(b) temperature and humidity of air;

(c) nominal concentrations (total amount of test substance fed into the inhalation equipment divided by the volume of air);

(d) nature of vehicle, if used;

(e) actual concentrations in test breathing zone;

(f) median particle sizes (where appropriate):

— dose levels (including vehicle, if used) and concentrations,

— tumour incidence data by sex, dose and tumour type,

— time of death during the study or whether animals survived to termination, including satellite group,

— toxic response data by sex and dose,

— description of toxic or other effects,

— the time of observation of each abnormal sign and its subsequent course,

— ophthalmological findings,

— food and bodyweight data,

— haematological tests employed and all results,

— clinical biochemistry test employed and all results (including any urinalysis),

— necropsy findings,

— a detailed description of all histopathological findings,

— statistical treatment of results with a description of the methods used,

— discussion of the results,

— interpretation of the results.

3.2. EVALUATION AND INTERPRETATION

See General introduction Part B.

4. **REFERENCES**

See General introduction Part B.

B.34. ONE-GENERATION REPRODUCTION TOXICITY TEST

1. METHOD

1.1. INTRODUCTION

See General introduction Part B.

1.2. DEFINITIONS

See General introduction Part B.

1.3. REFERENCE SUBSTANCES

None.

1.4. PRINCIPLE OF THE TEST METHOD

The test substance is administered in graduated doses to several groups of males and females. Males should be dosed during growth and for at least one complete spermatogenic cycle (approximately 56 days in the mouse and 70 days in the rat) in order to elicit any adverse effects on spermatogenesis by the test substance.

Females of the parental (P) generation should be dosed for at least two complete oestrous cycles in order to e adverse effects on oestrus by the test substance. The animals are then mated. The test substance is administered to both sexes during the mating period and thereafter only to females during pregnancy and for the duration of the nursing period. For administration by inhalation the method will require modification.

1.5. QUALITY CRITERIA

None.

1.6. DESCRIPTION OF THE TEST METHOD

1.6.1. *Preparations*

Before the test, healthy young adult animals are randomised and assigned to the treated and control groups. The animals are kept under the experimental housing and feeding conditions for at least five days prior to the test. It is recommended that the test substance be administered in the diet or drinking water. Other routes of administration are also acceptable. All animals should be dosed by the same method during the appropriate experimental period. If a vehicle or other additives are used to facilitate dosing, they should be known not to produce toxic effects. Dosing should be on a seven-day per week basis.

1.6.2. Experimental animals

Selection of species

The rat or mouse are the preferred species. Healthy animals, not subjected to previous experimental procedures, should be used. Strains with low fecundity should not be used. The test animals should be characterized as to species, strain, sex, weight and/or age.

For an adequate assessment of fertility, both males and females should be studied. All test and control animals should be weaned before dosing begins.

Number and sex

Each treated and control group should contain a sufficient number of animals to yield about 20 pregnant females at or near term.

The objective is to produce enough pregnancies and offspring to assure a meaningful evaluation of the potential of the substance to affect fertility, pregnancy and maternal behaviour in P generation animals and suckling, growth and development of the F_1 offspring from conception to weaning.

1.6.3. Test conditions

Food and water should be provided ad libitum. Near parturition, pregnant females should be caged separately in delivery or maternity cages and may be provided with nesting materials.

1.6.3.1. Dose levels

At least three treated groups and a control group should be used. If a vehicle is used in administering the test substance, the control group should receive the vehicle in the highest volume used. If a test substance causes reduced dietary intake or utilisation, then the use of a paired fed control group may be considered necessary. Ideally, unless limited by the physical/chemical nature or biological effects of the test substance, the highest dose level should induce toxicity but not mortality in the parental (P) animals. The intermediate dose(s) should induce minimal toxic effects attributable to the test substance, and the low dose should not induce any observable adverse effects on the parents or offspring. When administered by gavage or capsule the dosage given to each animal should be based on the individual animal's body weight and adjusted weekly for changes in body weight. For females during pregnancy, dosages may be based on the body weight at day 0 or 6 of the pregnancy, if desired.

1.6.3.2. Limit test

In the case of substances of low toxicity, if a dose level of at least 1 000 mg/kilogram produces no evidence of interference with reproductive performance, studies at other dose levels may not be considered necessary. If a preliminary study at the high-dose level, with definite evidence of maternal toxicity, shows no adverse effects on fertility, studies at other dose levels may not be considered necessary.

1.6.3.3. *Performance of the test*

Experimental schedules

Daily dosing of the parental (P) males should begin when they are about five to nine weeks of age, after they have been weaned and acclimatised for at least five days. In rats, dosing is continued for 10 weeks prior to the mating period (for mice, eight weeks). Males should be killed and examined either at the end of the mating period or, alternatively, males may be retained on the test diet for the possible production of a second litter and should be killed and examined at some time before the end of the study. For parental (P) females dosing should begin after at least five days of acclimatisation and continue for at least two weeks prior to mating. Daily dosing of the p females should continue throughout the three-week mating period, pregnancy and up to the weaning of the F_l offspring. Consideration should be given to modification of the dosing schedule based on other available information on the test substance, such as induction of metabolism or bioaccumulation.

Mating procedure

Either 1:1 (one male to one female) or 1:2 (one male to two females) mating may be used in reproduction toxicity studies.

Based on 1:1 mating, one female should be placed with the same male until pregnancy occurs or three weeks have elapsed. Each morning the females should be examined for presence of sperm or vaginal plugs. Day 0 of pregnancy is defined as the day a vaginal plug or sperm is found.

Those pairs that fail to mate should be evaluated to determine the cause of the apparent infertility.

This may involve such procedures as providing additional opportunities to mate with other proven sires or dams, microscopic examination of the reproductive organs, and examination of the oestrous cycle or spermatogenesis.

Litter sizes

Animals dosed during the fertility study are allowed to litter normally and rear their progency to the stage of weaning without standardisation of litters.

Where standardisation is done, the following procedure is suggested. Between day 1 and day 4 after birth, the size of each litter may be adjusted by eliminating extra pups by selection to yield, as nearly as possible, four males and four females per litter.

Whenever the number of male or female pups prevents having four of each sex per litter, partial adjustment (for example, five males and three females) is acceptable. Adjustments are not applicable for litters of less than eight pups.

1.6.4. Observations

Throughout the test period, each animal should be observed at least once daily. Pertinent behavioural changes, signs of difficult or prolonged parturition, and all signs of toxicity, including mortality, should be recorded. During pre-mating and mating periods, food consumption may be measured daily. After parturition and during lactation, food consumption measurements (and water consumption measurements when the test substance is administered in the drinking water) should be made on the same day as the weighing of the litter. P males and females should be weighed on the first day of dosing and weekly thereafter. These observations should be reported individually for each adult animal.

The duration of gestation should be calculated from day 0 of pregnancy. Each litter should be examined as soon as possible after delivery to establish the number and sex of pups, still births, live births and the presence of gross anomalies.

Dead pups and pups sacrificed at day 4 should be preserved and studied for possible defects. Live pups should be counted and litters weighed on the morning after birth and on days 4 and 7 and weekly thereafter until the termination of the study, when animals should be weighed individually.

Physical or behavioural abnormalities observed in the dams or offspring should be recorded.

1.6.5. Pathology

1.6.5.1. Necropsy

At the time of sacrifice or death during the study the animals of the P generation should be examined macroscopically for any structural abnormalities or pathological changes, with special attention being paid to the organs of the reproductive system. Dead or moribund pups should be examined for defects.

1.6.5.2. Histopathology

The ovaries, uterus, cervix, vagina, testes, epididymes, seminal vesicles, prostate, coagulating gland, pituitary gland and target organ(s) of all P animals should be preserved for microscopic examination. In the event that these organs have not been examined in other multiple-dose studies, they should be microscopically examined in all high-dose and control animals and animals which die during the study where practicable.

Organs showing abnormalities in these animals should then be examined in all other P animals. In these instances, microscopic examination should be made of all tissues showing gross pathological changes. As suggested under mating procedures, reproductive organs of animals suspected of infertility may be subjected to microscopic examination.

2. **DATA**

Data may be summarised in tabular form, showing for each test group the number of animals at the start of the test, the number of fertile males, the number of pregnant females, the types of changes and the percentage of animals displaying each type of change.

When possible, numerical results should be evaluated by an appropriate statistical method. Any generally accepted statistical method may be used.

3. **REPORTING**

TEST REPORT

The test report shall, if possible, contain the following information:

— species/strain used,

— toxic response data by sex and dose, including fertility, gestation and viability,

— time of death during the study or whether animals survived to time of scheduled sacrifice or to termination of the study,

— table presenting the weights of each litter, the mean: pup weights and the individual weights of the pups at termination,

— toxic or other effects on reproduction, offspring and postnatal growth,

— the day of observation of each abnormal sign and its subsequent course,

— bodyweight data for P animals,

— necropsy findings,

— a detailed description of all microscopic findings,

— statistical treatment of results, where appropriate,

— discussion of the results,

— interpretation of the results.

3.2. EVALUATION AND INTERPRETATION

See General introduction Part B.

4. **REFERENCES**

See General introduction Part B.

B.35. TWO-GENERATION REPRODUCTION TOXICITY STUDY

1. METHOD

This method is a replicate of the OECD TG 416 (2001).

1.1. INTRODUCTION

This method for two-generation reproduction testing is designed to provide general information concerning the effects of a test substance on the integrity and performance of the male and female reproductive systems, including gonadal function, the oestrus cycle, mating behaviour, conception, gestation, parturition, lactation, and weaning, and the growth and development of the offspring. The study may also provide information about the effects of the test substance on neonatal morbidity, mortality, and preliminary data on prenatal and postnatal developmental toxicity and serve as a guide for subsequent tests. In addition to studying growth and development of the F1 generation, this test method is also intended to assess the integrity and performance of the male and female reproductive systems as well as growth and development of the F2 generation. For further information on developmental toxicity and functional deficiencies, either additional study segments can be incorporated into this protocol, consulting the methods for developmental toxicity and/or developmental neurotoxicity as appropriate, or these endpoints could be studied in separate studies, using the appropriate test methods.

1.2. PRINCIPLE OF THE TEST METHOD

The test substance is administered in graduated doses to several groups of males and females. Males of the P generation should be dosed during growth and for at least one complete spermatogenetic cycle (approximately 56 days in the mouse and 70 days in the rat) in order to elicit any adverse effects on spermatogenesis. Effects on sperm are determined by a number of sperm parameters (e.g. sperm morphology and motility) and in tissue preparation and detailed histopathology. If data on spermatogenesis are available from a previous repeated dose study of sufficient duration, e.g. a 90-day study, males of the P generation need not be included in the evaluation. It is recommended, however, that samples or digital recordings of sperm of the P generation are saved, to enable later evaluation. Females of the P generation should be dosed during growth and for several complete oestrus cycles in order to detect any adverse effects on oestrus cycle normality by the test substance. The test substance is administered to parental (P) animals during their mating, during the resulting pregnancies, and through the weaning of their F1 offspring. At weaning the administration of the substance is continued to F1 offspring during their growth into adulthood, mating and production of an F2 generation, until the F2 generation is weaned.

Clinical observations and pathological examinations are performed on all animals for signs of toxicity with special emphasis on effects on the integrity and performance of the male and female reproductive systems and on the growth and development of the offspring.

1.3. DESCRIPTION OF THE TEST METHOD

1.3.1. Selection of animal species

The rat is the preferred species for testing. If other species are used, justification should be given and appropriate modifications will be necessary. Strains with low fecundity or well-known high incidence of developmental defects should not be used. At the commencement of the study, the weight variation of animals used should be minimal and not exceed 20 % of the mean weight of each sex.

1.3.2. Housing and feeding conditions

The temperature in the experimental animal room should be 22 °C (± 3°). Although the relative humidity should be at least 30 % and preferably not exceed 70 % other than during room cleaning, the aim should be 50-60 %. Lighting should be artificial, the sequence being 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. The choice of diet may be influenced by the need to ensure a suitable admixture of a test substance when administered by this method.

Animals may be housed individually or be caged in small groups of the same sex. Mating procedures should be carried out in cages suitable for the purpose. After evidence of copulation, mated females shall be single-caged in delivery or maternity cages. Mated rats may also be kept in small groups and separated one or two days prior to parturition. Mated animals shall be provided with appropriate and defined nesting materials when parturition is near.

1.3.3. **Preparation of animals**

Healthy young animals, which have been acclimated to laboratory conditions for at least five days and have not been subjected to previous experimental procedures, should be used. The test animals should be characterised as to species, strain, source, sex, weight and/or age. Any sibling relationships among the animals should be known so that mating of siblings is avoided. The animals should be randomly assigned to the control and treated groups (stratification by body weight is recommended). Cages should be arranged in such a way that possible effects due to cage placement are minimised. Each animal should be assigned a unique identification number. For the P generation, this should be done before dosing starts. For the F1 generation, this should be done at weaning for animals selected for mating. Records indicating the litter of origin should be maintained for all selected F1 animals. In addition, individual identification of pups as soon after birth as possible is recommended when individual weighing of pups or any functional tests are considered.

Parental (P) animals shall be about five to nine weeks old at the start of dosing. The animals of all test groups shall, as nearly as practicable, be of uniform weight and age.

1.4. PROCEDURE

1.4.1. **Number and sex of animals**

Each test and control group should contain a sufficient number of animals to yield preferably not less than 20 pregnant females at or near parturition. For substances that cause undesirable treatment related effects (e.g. sterility, excessive toxicity at the high dose) this may not be possible. The objective is to produce enough pregnancies to assure a meaningful evaluation of the potential of the substance to affect fertility, pregnancy and maternal behaviour and suckling, growth and development of the F1 offspring from conception to maturity, and the development of their offspring (F2) to weaning. Therefore, failure to achieve the desired number of pregnant animals (i.e. 20) does not necessarily invalidate the study and should be evaluated on a case-by-case basis.

1.4.2. **Preparation of doses**

It is recommended that the test substance be administered orally (by diet, drinking water or gavage) unless another route of administration (e.g. dermal or inhalation) is considered more appropriate.

Where necessary, the test substance is dissolved or suspended in a suitable vehicle. It is recommended that, wherever possible, the use of an aqueous solution/suspension be considered first, followed by consideration of a solution/emulsion in oil (e.g. corn oil) and then by possible solution in other vehicles. For vehicles other than water, the toxic characteristics of the vehicle must be known. The stability of the test substance in the vehicle should be determined.

1.4.3. **Dosage**

At least three dose levels and a concurrent control shall be used. Unless limited by the physical-chemical nature or biological effects of the test substance, the highest dose level should be chosen with the aim to induce toxicity but not death or severe suffering. In case of unexpected mortality, studies with a mortality rate of less than approximately 10 % in the parental (P) animals would normally still be acceptable. A descending sequence of dose levels should be selected with a view to demonstrating any dosage related effect and no-observed-adverse-effects levels (NOAEL). Two to four fold intervals are frequently optimal for setting the descending dose levels and addition of a fourth test group is often preferable to using very large intervals (e.g. more than a factor of 10) between dosages. For the dietary studies the dose interval should be not more than three fold. Dose levels should be selected taking into account any existing toxicity data, especially results from repeated dose studies. Any available information on metabolism and kinetics of the test compound or related materials should also be considered. In addition, this information will also assist in demonstrating the adequacy of the dosing regimen.

The control group shall be an untreated group or a vehicle-control group if a vehicle is used in administering the test substance. Except for treatment with the test substance, animals in the control group should be handled in an identical manner to the test group subjects. If a vehicle is used, the control group shall receive the vehicle in the highest volume used. If a test substance is administered in the diet, and causes reduced dietary intake or utilisation, then the use of a pair-fed control group may be considered necessary. Alternatively data from controlled studies designed to evaluate the effects of decreased food consumption on reproductive parameters may be used in lieu of a concurrent pair-fed control group.

Consideration should be given to the following characteristics of vehicle and other additives: effects on the absorption, distribution, metabolism, or retention of the test substance; effects on the chemical properties of the test substance which may alter its toxic characteristics; and effects on the food or water consumption or the nutritional status of the animals.

1.4.4. **Limit test**

If an oral study at one dose level of at least 1 000 mg/kg body weight/day or, for dietary or drinking water administration, an equivalent percentage in the diet or drinking water using the procedures described for this study, produces no observable toxic effects in either parental animals or their offspring and if toxicity would not be expected based upon data from structurally and/or metabolically related compounds, then a full study using several dose levels may not be considered necessary. The limit test applies except when human exposure indicates the need for a higher oral dose level to be used. For other types of administration, such as inhalation or dermal application, the physical-chemical properties of the test substance, such as solubility, often may indicate and limit the maximum attainable level of exposure.

1.4.5. **Administration of doses**

The animals should be dosed with the test substance on a 7-days per week basis. The oral route of administration (diet, drinking water, or gavage) is preferred. If another route of administration is used, justification shall be provided, and appropriate modifications may be necessary. All animals shall be dosed by the same method during the appropriate experimental period. When the test substance is administered by gavage, this should be done using a stomach tube. The volume of liquid administered at one time should not exceed 1 ml/100 g body weight (0,4 ml/100 g body weight is the maximum for corn oil), except in the case of aqueous solutions where 2 ml/100 g body weight may be used. Except for irritant or corrosive substances, which will normally reveal exacerbated effects with higher concentrations, variability in test volume should be minimised by adjusting the concentration to ensure a constant volume at all dose levels. In gavage studies, the pups will normally only receive test substance indirectly through the milk, until direct dosing commences for them at weaning. In diet or drinking water studies, the pups will additionally receive test substance directly when they commence eating for themselves during the last week of the lactation period.

For substances administered via the diet or drinking water, it is important to ensure that the quantities of the test substance involved do not interfere with normal nutrition or water balance. When the test substance is administered in the diet either a constant dietary concentration (ppm) or a constant dose level in terms of the body weight of the animal may be used; the alternative used must be specified. For a substance administered by gavage, the dose should be given at similar times each day, and adjusted at least weekly to maintain a constant dose level in terms of animal body weight. Information regarding placental distribution should be considered when adjusting the gavage dose based on weight.

1.4.6. **Experimental schedules**

Daily dosing of the parental (P) males and females shall begin when they are five to nine weeks old. Daily dosing of the F1 males and females shall begin at weaning; it should be kept in mind that in cases of test substance administration via diet or drinking water, direct exposure of the F1 pups to the test substance may already occur during the lactation period. For both sexes (P and F1), dosing shall be continued for at least 10 weeks before the mating period. Dosing is continued in both sexes during the two week mating period. Males should be humanely killed and examined when they are no longer needed for assessment of reproductive effects. For parental (P) females, dosing should continue throughout pregnancy and up to the weaning of the F1 offspring. Consideration should be given to modifications in the dosing schedule based on available information on the test substance, including existing toxicity data, induction of metabolism or bioaccumulation. The dose to each animal should normally be based on the most recent individual body weight determination. However, caution should be exercised when adjusting the dose during the last trimester of pregnancy.

Treatment of the P and F1 males and females shall continue until termination. All P and F1 adult males and females should be humanely killed when they are no longer needed for assessment of reproductive effects. F1 offspring not selected for mating and all F2 offspring should be humanely killed after weaning.

1.4.7. **Mating procedure**

1.4.7.1. *Parental (P) mating*

For each mating, each female shall be placed with a single male from the same dose level (1:1 mating) until copulation occurs or twq weeks have elapsed. Each day, the females shall be examined for presence of sperm or

vaginal plugs. Day 0 of pregnancy is defined as the day a vaginal plug or sperm are found. In case pairing is unsuccessful, re-mating of females with proven males of the same group could be considered. Mating pairs should be clearly identified in the data. Mating of siblings should be avoided.

1.4.7.2. *F1 mating*

For mating the F1 offspring, at least one male and one female should be selected at weaning from each litter for mating with other pups of the same dose level but different litter, to produce the F2 generation. Selection of pups from each litter should be random when no significant differences are observed in body weight or appearance between the litter mates. In case these differences are observed, the best representatives of each litter should be selected. Pragmatically, this is best done on a body weight basis but it may be more appropriate on the basis of appearance. The F1 offspring should not be mated until they have attained full sexual maturity.

Pairs without progeny should be evaluated to determine the apparent cause of the infertility. This may involve such procedures as additional opportunities to mate with other proven sires or dams, microscopic examination of the reproductive organs, and examination of the oestrous cycles or spermatogenesis.

1.4.7.3. *Second mating*

In certain instances, such as treatment-related alterations in litter size or the observation of an equivocal effect in the first mating, it is recommended that the P or F1 adults be remated to produce a second litter. It is recommended to remate females or males, which have not produced a litter with proven breeders of the opposite sex. If production of a second litter is deemed necessary in either generation, animals should be remated approximately one week after weaning of the last litter.

1.4.7.4. *Litter size*

Animals shall be allowed to litter normally and rear their offspring to weaning. Standardisation of litter sizes is optional. When standardisation is done, the method used should be described in detail.

1.5. OBSERVATIONS

1.5.1. **Clinical observations**

A general clinical observation should be made each day and, and in the case of gavage dosing its timing should take into account the anticipated peak period of effects after dosing. Behavioural changes, signs of difficult or prolonged parturition and all signs of toxicity should be recorded. An additional, more detailed examination of each animal should be conducted on at least a weekly basis and could conveniently be performed on an occasion when the animal is weighed. Twice daily, during the weekend once daily when appropriate, all animals should be observed for morbidity and mortality.

1.5.2. **Body weight and food/water consumption of parent animals**

Parental animals (P and Fl) shall be weighed on the first day of dosing and at least weekly thereafter. Parental females (P and F1) shall be weighed at a minimum on gestation days 0, 7, 14, and 20 or 21, and during lactation on the same days as the weighing of litters and on the day the animals are killed. These observations should be reported individually for each adult animal. During the premating and gestation periods food consumption shall be measured weekly at a minimum. Water consumption shall be measured weekly at a minimum if the test substance is administered in the water.

1.5.3. **Oestrus cycle**

Estrous cycle length and normality are evaluated in P and F1 females by vaginal smears prior to mating, and optionally during mating, until evidence of mating is found. When obtaining vaginal/cervical cells, care should be taken to avoid disturbance of mucosa and subsequently, the induction of pseudopregnancy (1).

1.5.4. **Sperm parameters**

For all P and F1 males at termination, testis and epididymis weight shall be recorded and one of each organ reserved for histopathological examination (see Section 1.5.7, 1.5.8.1). Of a subset of at least 10 males of each group of P and F1 males, the remaining testes and epididymides should be used for enumeration of

homogenisation-resistant spermatids and cauda epididymal sperm reserves, respectively. For this same subset of males, sperm from the cauda epididymides or vas deferens should be collected for evaluation of sperm motility and sperm morphology. If treatment-related effects are observed or when there is evidence from other studies of possible effects on spermatogenesis, sperm evaluation should be conducted in all males in each dose group; otherwise enumeration may be restricted to control and high-dose P and F1 males.

The total number of homogenisation-resistant testicular spermatids and cauda epididymal sperm should be enumerated (2)(3). Cauda sperm reserves can be derived from the concentration and volume of sperm in the suspension used to complete the qualitative evaluations, and the number of sperm recovered by subsequent mincing and/or homogenising of the remaining cauda tissue. Enumeration should be performed on the selected subset of males of all dose groups immediately after killing the animals unless video or digital recordings are made, or unless the specimens are freezed and analysed later. In these instances, the controls and high dose group may be analysed first. If no treatment-related effects (e.g. effects on sperm count, motility, or morphology) are seen the other dose groups need not be analysed. When treatment-related effects are noted in the high-dose group, then the lower dose groups should also be evaluated.

Epididymal (or ductus deferens) sperm motility should be evaluated or video taped immediately after sacrifice. Sperm should be recovered while minimising damage, and diluted for motility analysis using acceptable methods (4). The percentage of progressively motile sperm should be determined either subjectively of objectively. When computer-assisted motion analysis is performed (5)(6)(7)(8)(9)(10) the derivation of progressive motility relies on user-defined thresholds for average path velocity and straightness or linear index. If samples are videotaped (11) or the images are otherwise recorded at the time of necropsy, subsequent analysis of only control and high-dose P and F1 males may be performed unless treatment-related effects are observed; in that case, the lower dose groups should also be evaluated. In the absence of a video or digital image, all samples in all treatment groups should be analysed at necropsy.

A morphological evaluation of an epididymal (or *vas deferens*) sperm sample should be performed. Sperm (at least 200 per sample) should be examined as fixed, wet preparations (12) and classified as either normal or abnormal. Examples of morphologic sperm abnormalities would include fusion, isolated heads, and misshapen heads and/or tails. Evaluation should be performed on the selected subset of males of all dose groups either immediately after killing the animals, or, based on the video or digital recordings, at a later time. Smears, once fixed, can also be read at a later time. In these instances, the controls and high dose group may be analysed first. If no treatment-related effects (e.g. effects on sperm morphology) are seen the other dose groups need not be analysed. When treatment-related effects are noted in the high-dose group, then the lower dose groups should also be evaluated.

If any of the above sperm evaluation parameters have already been examined as part of a systemic toxicity study of at least 90 days, they need not necessarily be repeated in the two-generation study. It is recommended, however, that samples or digital recordings of sperm of the P generation are saved, to enable later evaluation, if necessary.

1.5.5. **Offspring**

Each litter should be examined as soon as possible after delivery (lactation day 0) to establish the number and sex of pups, stillbirths, live births, and the presence of gross anomalies. Pups found dead on day 0, if not macerated, should preferably be examined for possible defects and cause of death and preserved. Live pups should be counted and weighed individually at birth (lactation day 0) or on day 1, and on regular weigh days thereafter, e.g. on days 4, 7, 14, and 21 of lactation. Physical or behavioural abnormalities observed in the dams or offspring should be recorded.

Physical development of the offspring should be recorded mainly by body weight gain. Other physical parameters (e.g. ear and eye opening, tooth eruption, hair growth) may give supplementary information, but these data should preferably be evaluated in the context of data on sexual maturation (e.g. age and body weight at vaginal opening or balano-preputial separation) (13). Functional investigations (e.g. motor activity, sensory function, reflex ontogeny) of the F1 offspring before and/or after weaning, particularly those related to sexual maturation, are recommended if such investigations are not included in separate studies. The age of vaginal opening and preputial separation should be determined for F1 weanlings selected for mating. Anogenital distance should be measured at postnatal day 0 in F2 pups if triggered by alterations in F1 sex ratio or timing of sexual maturation.

Functional observations may be omitted in groups that otherwise reveal clear signs of adverse effects (e.g. significant decrease in weight gain, etc.). If functional investigations are made, they should not be done on pups selected for mating.

1.5.6. **Gross necropsy**

At the time of termination or death during the study, all parental animals (P and F1), all pups with external abnormalities or clinical signs, as well as one randomly selected pup/sex/litter from both the F1 and F2 generation, shall be examined macroscopically for any structural abnormalities or pathological changes. Special attention should be paid to the organs of the reproductive system. Pups that are humanely killed in a moribund condition and dead pups, when not macerated, should be examined for possible defects and/or cause of death and preserved.

The uteri of all primiparous females should be examined, in a manner which does not compromise histopathological evaluation, for the presence and number of implantation sites.

1.5.7. **Organ weights**

At the time of termination, body weight and the weight of the following organs of all P and F1 parental animals shall be determined (paired organs should be weighed individually):

— uterus, ovaries,

— testes, epididymides (total and cauda),

— prostate,

— seminal vesicles with coagulating glands and their fluids and prostate (as one unit),

— brain, liver, kidneys, spleen, pituitary, thyroid and adrenal glands and known target organs.

Terminal body weights should be determined for F1 and F2 pups that are selected for necropsy. The following organs from the one randomly selected pup/sex/litter (see Section 1.5.6) shall be weighed: Brain, spleen and thymus.

Gross necropsy and organ weight results should be assessed in context with observations made in other repeated dose studies, when feasible.

1.5.8. **Histopathology**

1.5.8.1. *Parental animals*

The following organs and tissues of parental (P and F1) animals, or representative samples thereof, shall be fixed and stored in a suitable medium for histopathological examination.

— Vagina, uterus with cervix, and ovaries (preserved in appropriate fixative),

— one testis (preserved in Bouin's or comparable fixative), one epididymis, seminal vesicles, prostate, and coagulating gland,

— previously identified target organ(s) from all P and F1 animals selected for mating.

Full histopathology of the preserved organs and tissues listed above should be performed for all high dose and control P and F1 animals selected for mating. Examination of the ovaries of the P animals is optional. Organs demonstrating treatment-related changes should also be examined in the low- and mid-dose groups to aid in the elucidation of the NOAEL. Additionally, reproductive organs of the low-and mid-dose animals suspected of reduced fertility, e.g. those that failed to mate, conceive, sire, or deliver healthy offspring, or for which oestrus cyclicity or sperm number, motility, or morphology were affected, should be subjected to histopathological evaluation. All gross lesions such as atrophy or tumours shall be examined.

Detailed testicular histopathological examination (e.g. using Bouin's fixative, paraffin embedding and transverse sections of 4-5 μm thickness) should be conducted in order to identify treatment-related effects such as retained spermatids, missing germ cell layers or types, multinucleated giant cells or sloughing of spermatogenic cells

into the lumen (14). Examination of the intact epididymis should include the caput, corpus, and cauda, which can be accomplished by evaluation of a longitudinal section. The epididymis should be evaluated for leukocyte infiltration, change in prevalence of cell types, aberrant cell types, and phagocytosis of sperm. PAS and haematoxylin staining may be used for examination of the male reproductive organs.

The postlactational ovary should contain primordial and growing follicles as well as the large corpora lutea of lactation. Histopathological examination should detect qualitative depletion of the primordial follicle population. A quantitative evaluation of primordial follicles should be conducted for F1 females; the number of animals, ovarian section selection, and section sample size should be statistically appropriate for the evaluation procedure used. Examination should include enumeration of the number of primordial follicles, which can be combined with small growing follicles, for comparison of treated and control ovaries (15)(16)(17)(18)(19).

1.5.8.2. *Weanlings*

Grossly abnormal tissue and target organs from all pups with external abnormalities or clinical signs, as well as from the one randomly selected pup/sex/litter from both the F1 and F2 generation which have not been selected for mating, shall be fixed and stored in a suitable medium for histopathological examination. Full histopathological characterisation of preserved tissue should be performed with special emphasis on the organs of the reproductive system.

2. DATA

2.1. TREATMENT OF RESULTS

Data shall be reported individually and summarised in tabular form, showing for each test group and each generation the number of animals at the start of the test, the number of animals found dead during the test or killed for humane reasons, the time of any death or humane kill, the number of fertile animals, the number of pregnant females, the number of animals showing signs of toxicity, a description of the signs of toxicity observed, including time of onset, duration, and severity of any toxic effects, the types of parental and offspring observations, the types of histopathological changes, and all relevant litter data.

Numerical results should be evaluated by an appropriate, generally accepted statistical method; the statistical methods should be selected as part of the design of the study and should be justified. Dose-response statistical models may be useful for analysing data. The report should include sufficient information on the method of analysis and the computer program employed, so that an independent reviewer/statistician can re-evaluate and reconstruct the analysis.

2.2. EVALUATION OF RESULTS

The findings of this two-generation reproduction toxicity study should be evaluated in terms of the observed effects including necropsy and microscopic findings. The evaluation will include the relationship, or lack thereof, between the dose of the test substance and the presence or absence, incidence and severity of abnormalities, including gross lesions, identified target organs, affected fertility, clinical abnormalities, affected reproductive and litter performance, body weight changes, effects on mortality and any other toxic effects. The physico-chemical properties of the test substance, and when available, toxicokinetics data should be taken into consideration when evaluating test results.

A properly conducted reproduction toxicity test should provide a satisfactory estimation of a no-effect level and an understanding of adverse effects on reproduction, parturition, lactation, postnatal development including growth and sexual development.

2.3. INTERPRETATION OF RESULTS

A two-generation reproduction toxicity study will provide information on the effects of repeated exposure to a substance during all phases of the reproductive cycle. In particular, the study provides information on the reproductive parameters, and on development, growth, maturation and survival of offspring. The results of the study should be interpreted in conjunction with the findings from subchronic, prenatal developmental and toxicokinetic and other available studies. The results of this study can be used in assessing the need for further testing of a chemical. Extrapolation of the results of the study to man is valid to a limited degree. They are best used to provide information on no-effect-levels and permissible human exposure (20)(21)(22)(23).

3. **REPORTING**

3.1. TEST REPORT

The test report must include the following information:

Test substance:

— physical nature and, where relevant, physicochemical properties,

— identification data,

— purity.

Vehicle (if appropriate):

— ustification for choice of vehicle if other than water.

Test animals:

— species/strain used,

— number, age and sex of animals,

— source, housing conditions, diet, nesting materials, etc.,

— individual weights of animals at the start of the test.

Test conditions:

— rationale for dose level selection,

— details of test substance formulation/diet preparation, achieved concentrations,

— stability and homogeneity of the preparation,

— details of the administration of the test substance,

— conversion from diet/drinking water test substance concentration (ppm) to the achieved dose (mg/kg body weight/day), if applicable,

— details of food and water quality.

Results:

— food consumption, and water consumption if available, food efficiency (body weight gain per gram of food consumed), and test material consumption for P and F1 animals, except for the period of cohabitation and for at least the last third of lactation,

— absorption data (if available),

— body weight data for P and F1 animals selected for mating,

— litter and pup weight data,

— body weight at sacrifice and absolute and relative organ weight data for the parental animals,

— nature, severity and duration of clinical observations (whether reversible or not),

— time of death during the study or whether animals survived to termination,

— toxic response data by sex and dose, including indices of mating, fertility, gestation, birth, viability, and lactation; the report should indicate the numbers used in calculating these indices,

— toxic or other effects on reproduction, offspring, post-natal growth, etc.,

— necropsy findings,

— detailed description of all histopathological findings,

— number of P and F1 females cycling normally and cycle length,

— total cauda epididymal sperm number, percent progressively motile sperm, percent morphologically normal sperm, and percent of sperm with each identified abnormality,

— time-to-mating, including the number of days until mating,

— gestation length,

— number of implantations, corpora lutea, litter size,

— number of live births and post-implantation loss,

— number of pups with grossly visible abnormalities, if determined the number of runts should be reported,

— data on physical landmarks in pups and other post natal developmental data, physical landmarks evaluated should be justified,

— data on functional observations in pups and adults, as applicable,

— statistical treatment of results, where appropriate.

Discussion of results.

Conclusions, including NOAEL values for maternal and offspring effects.

4. **REFERENCES**

(1) Sadleir, R.M.F.S., (1979) Cycles and Seasons, In: *Reproduction in Mammals:* I. Germ Cells and Fertilisation, C.R. Auston and R.V. Short (eds.), Cambridge, New York.

(2) Gray, L.E. et al., (1989) A Dose-Response Analysis of Methoxychlor-Induced Alterations of Reproductive Development and Function in the Rat. *Fundamental and Applied Toxicology*, 12, p. 92-108.

(3) Robb, G.W. et al., (1978). Daily Sperm Production and Epididymal Sperm Reserves of Pubertal and Adult Rats. *Journal of Reproduction and Fertility* 54:103-107.

(4) Klinefelter, G.R. et al., (1991) The Method of Sperm Collection Significantly Influences Sperm Motion Parameters Following Ethane Dimethanesulfonate Administration in the Rat. *Reproductive Toxicology*, 5, p. 39-44.

(5) Seed, J. et al., (1996). Methods for Assessing Sperm Motility, Morphology, and Counts in the Rat, Rabbit, and Dog: a Consensus Report. *Reproductive Toxicology,* 10(3), p. 237-244.

(6) Chapin, R.E. et al., (1992) Methods for Assessing Rat Sperm Motility. *Reproductive Toxicology,* 6, p. 267-273

(7) Klinefelter, G.R. et al., (1992) Direct Effects of Ethane Dimethanesulphonate on Epididymal Function in Adult Rats: an *In Vitro* Demonstration. *Journal of Andrology,* 13, p. 409-421.

(8) Slott, V.L. et al., (1991) Rat Sperm Motility Analysis: Methodologic Considerations. *Reproductive Toxicology,* 5, p. 449-458.

(9) Slott, V.L. and Perreault, S.D., (1993) Computer-Assisted Sperm Analysis of Rodent Epididymal Sperm Motility Using the Hamilton-Thorn Motility Analyzer. In: *Methods in Toxicology*, Part A., Academic, Orlando, Florida, p. 319-333.

(10) Toth, G.P. et al., (1989) The Automated Analysis of Rat Sperm Motility Following Subchronic Epichlorhydrin Administration: Methodologic and Statistical Considerations. Journal of Andrology, 10, p. 401-415.

(11) Working, P.K. and M. Hurtt, (1987) Computerised Videomicrographic Analysis of Rat Sperm Motility. Journal of Andrology, 8, p. 330-337.

(12) Linder, R.E. et al., (1992) Endpoints of Spermatoxicity in the Rat After Short Duration Exposures to Fourteen Reproductive Toxicants. Reproductive Toxicology, 6, p. 491-505.

(13) Korenbrot, C.C. et al., (1977) Preputial Separation as an External Sign of Pubertal Development in the Male Rat. Biological Reproduction, 17, p. 298-303.

(14) Russell, L.D. et al., (1990) Histological and Histopathological Evaluation of the Testis, Cache River Press, Clearwater, Florida.

(15) Heindel, J.J. and R.E. Chapin, (eds.) (1993) Part B. Female Reproductive Systems, Methods in Toxicology, Academic, Orlando, Florida.

(16) Heindel, J.J. et al., (1989) Histological Assessment of Ovarian Follicle Number in Mice As a Screen of Ovarian Toxicity. In: Growth Factors and the Ovary, A.N. Hirshfield (ed.), Plenum, New York, p. 421-426.

(17) Manson, J.M. and Y.J. Kang, (1989) Test Methods for Assessing Female Reproductive and Developmental Toxicology. In: Principles and Methods of Toxicology, A.W. Hayes (ed.), Raven, New York.

(18) Smith, B.J. et al., (1991) Comparison of Random and Serial Sections in Assessment of Ovarian Toxicity. Reproductive Toxicology, 5, p. 379-383.

(19) Heindel, J.J., (1999) Oocyte Quantitation and Ovarian Histology. In: An Evaluation and Interpretation of Reproductive Endpoints for Human Health Risk Assessment, G. Daston,. and C.A. Kimmel, (eds.), ILSI Press, Washington, DC.

(20) Thomas, J. A., (1991) Toxic Responses of the Reproductive System. In: Casarett and Doull's Toxicology, M.O. Amdur, J. Doull, and C.D. Klaassen (eds.), Pergamon, New York.

(21) Zenick, H. and E.D. Clegg, (1989) Assessment of Male Reproductive Toxicity: A Risk Assessment Approach. In: Principles and Methods of Toxicology, A.W. Hayes (ed.), Raven Press, New York.

(22) Palmer, A.K., (1981) In: Developmental Toxicology, Kimmel, C.A. and J. Buelke-Sam (eds.), Raven Press, New York.

(23) Palmer, A.K., (1978) In Handbook of Teratology, Vol. 4, J.G. Wilson and F.C. Fraser (eds.), Plenum Press, New York.

B.36. **TOXICOKINETICS**

1. **METHOD**

1.1. INTRODUCTION

See General introduction Part B.

1.2. DEFINITIONS

See General introduction Part B.

1.3. REFERENCE SUBSTANCES

None.

1.4. PRINCIPLE OF THE TEST METHOD

The test substance is administered by an appropriate route. Depending on the purpose of the study, the substance may be administered in single or repeated doses over defined periods to one or several groups of experimental animals. Subsequently, depending on the type of study, the substance and/or metabolites are determined in body fluids, tissues and/or excreta.

Studies may be done with 'unlabelled' or 'labelled' forms of the test substance. Where a label is used it should be positioned in the substance in such a way to provide the most information about the fate of the compound.

1.5. QUALITY CRITERIA

None.

1.6. DESCRIPTION OF THE TEST METHOD

1.6.1. **Preparations**

Healthy young adult animals are acclimatised to the laboratory conditions for at least five days prior to the test. Before the test, animals are randomised and assigned to the treatment groups. In special situations, very young, pregnant or pre-treated animals may be used.

1.6.2. **Test conditions**

1.6.2.1. *Experimental animals*

Toxicokinetic studies may be carried out in one or more appropriate animal species and should take account of the species used or intended to be used in other toxicological studies on the same test substance. Where rodents are used in a test the weight variation should not exceed ± 20 % of the mean weight.

1.6.2.2. *Number and sex*

For absorption and excretion studies, there should be four animals in each dose group initially. Sex preference is not mandatory, but under some circumstances both sexes may need to be studied. If there are sex differences in response, then four animals of each sex should be tested. In the case of studies with non-rodents fewer animals may be used. When tissue distribution is being studied, the initial group size should take into account both the number of animals to be sacrificed at each time point and the number of time points to be examined.

When metabolism is being studied, the group size is related to the needs of the study. For multiple-dose and multiple-time-point studies, the group size should take into account the number of time points and planned sacrifice(s), but may not be smaller than two animals. The group size should be sufficient to provide an acceptable characterisation of uptake, plateau and depletion (as appropriate) of the test substance and/or metabolites.

1.6.3. **Dose levels**

In the case of single-dose administration, at least two dose levels should be used. There should be a low dose at which no toxic effects are observed and a high dose at which there might be changes in toxicokinetic parameters or at which toxic effects occur.

In the case of repeated-dose administration the low dose is usually sufficient, but under certain circumstances a high dose may also be necessary.

1.6.4. **Route of administration**

Toxicokinetic studies should be performed using the same route and, where appropriate, the same vehicle as that used or intended to be used in the other toxicity studies. The test substance is usually administered orally by gavage or in the diet, applied to the skin, or administered by inhalation for defined periods to groups of experimental animals. Intravenous administration of the test substance may be useful in determining relative absorption by other routes. In addition, useful information may be provided on the pattern of distribution soon after the intravenous administration of a substance.

The possibility of interference of the vehicle with the rest substance should be taken into consideration. Attention should be given to differences in absorption between the administration of the test substance by gavage and in the diet and the need for an accurate determination of dose particularly when the test substance is given in the diet.

1.6.5. **Observation period**

All the animals should be observed daily and signs of toxicity and other relevant clinical features recorded, including time of onset, degree and duration.

1.6.6. **Procedure**

After weighing test animals, the test substance is administered by an appropriate route. If considered relevant, animals may be fasted before the test substance is administered.

Absorption

The rate and extent of absorption of the administered substance can be evaluated using various methods, with and without reference groups ([1]), for example by:

— determination of the amount of test substance and/or metabolites in excreta, such as urine, bile, faeces, exhaled air and that remaining in the carcase,

— comparison of the biological response (e.g. acute toxicity studies) between test and control and/or reference groups,

— comparison of the amount of renally excreted substance and/or metabolite in test and reference groups,

— determination of the area under the plasma-level/time curve of the lest substance and/or metabolites and comparison with data from a reference group.

Distribution

Two approaches are available at present, one or both of which may be used for analysis of distribution patterns:

— useful qualitative information is obtained using whole body autoradiographic techniques,

— quantitative information is obtained by sacrificing animals at different times after exposure and determining the concentration and amount of the test substance and/or metabolites in tissues and organs.

([1]) In this method a reference group is one in which the test substance is administered by another route that ensures complete bioavailability of the dose.

Excretion

In excretion studies, urine, faeces and expired air and, in certain circumstances, bile are collected. The amount of test substance and/or metabolites in these excreta should be measured several times after exposure, either until about 95 % of the administered dose has been excreted or for seven days, whichever comes first.

In special cases, the excretion of the test substance in the milk of lactating test animals may need to be considered.

Metabolism

To determine the extent and pattern of metabolism, biological samples should be analysed by suitable techniques. Structures of metabolites should be elucidated and appropriate metabolic pathways proposed where there is a need to answer questions arising from previous toxicological studies. It may be helpful to perform studies *in vitro* to obtain information on metabolic pathways.

Further information on the relationship of metabolism to toxicity may be obtained from biochemical studies, such as the determination of effects on metabolising enzyme systems, depletion of endogenous non-protein sulphydryl compounds and binding of the substance with macromolecules.

2. DATA

According to the type of study performed, data should be summarised in tabular form supported by graphical presentation whenever appropriate. For each test group, mean and statistical variations of measurements in relation to time, dosage, tissues and organs should be shown when appropriate. The extent of absorption and the amount and rates of excretion should be determined by appropriate methods. When metabolism studies are performed, the structure of identified metabolites should be given and possible metabolic pathways presented.

3. REPORTING

3.1. TEST REPORT

According to the type of study performed, the test report shall, if possible, contain the following information:

— species, strain, source, environmental conditions, diet,

— characterisation of labelled materials, when used,

— dosage levels and intervals used,

— route(s) of administration and any vehicles used,

— toxic and other effects observed,

— methods for determination of test substance and/or metabolites in biological samples, including expired air,

— tabulation of measurements by sex, dose, regimen, time, tissues and organs,

— presentation of the extent of absorption and excretion with time,

— methods for the characterisation and identification of metabolites in biological samples,

— methods for biochemical measurements related to metabolism,

— proposed pathways for metabolism,

— discussion of the results,

— interpretation of the results.

3.2. EVALUATION AND INTERPRETATION

See General introduction Part B.

4. **REFERENCES**

See General introduction Part B.

B.37. DELAYED NEUROTOXICITY OF ORGANOPHOSPHORUS SUBSTANCES FOLLOWING ACUTE EXPOSURE

1. METHOD

1.1. INTRODUCTION

In the assessment and evaluation of the toxic effects of substances, it is important to consider the potential of certain classes of substances to cause specific types of neurotoxicity that might not be detected in other toxicity studies. Certain organophosphorus substances have been observed to cause delayed neurotoxicity and should be considered as candidates for evaluation.

In vitro screening tests could be employed to identify those substances which may cause delayed polyneuropathy; however, negative findings from *in vitro* studies do not provide evidence that the test substance is not a neurotoxicant.

See General introduction Part B.

1.2. DEFINITIONS

Organophopsphorus substances include uncharged organophosphorus esters, thioesters or anhydrides of organophosphoric, organophosphonic or organophosphoramidic acids or of related phosphorothioic, phosphonothioic or phosphorothioamidic acids, or other substances that may cause the delayed neurotoxicity sometimes seen in this class of substances.

Delayed neurotoxicity is a syndrome associated with prolonged delayed onset of ataxia, distal axonopathies in spinal cord and peripheral nerve, and inhibition and aging of neuropathy target esterase (NTE) in neural tissue.

1.3. REFERENCE SUBSTANCES

A reference substance may be tested with a positive control group as a means of demonstrating that under the laboratory test conditions, the response of the tested species has not changed significantly.

An example of a widely used neurotoxicant is tri-o-tolyl phosphate (CAS 78-30-8, Einecs 201-103-5, CAS nomenclature: phosphoric acid, tris(2-methylphenyl)ester), also known as tris-o-cresylphosphate.

1.4. PRINCIPLE OF THE TEST METHOD

The test substance is administered orally in a single dose to domestic hens which have been protected from acute cholinergic effects, when appropriate. The animals are observed for 21 days for behavioural abnormalities, ataxia, and paralysis. Biochemical measurements, in particular neuropathy target esterase inhibition (NTE), are undertaken on hens randomly selected from each group, normally 24 and 48 hours after dosing. Twenty-one days after exposure, the remainder of the hens are killed and histopathological examination of selected neural tissues is undertaken.

1.5. DESCRIPTION OF THE TEST METHOD

1.5.1. **Preparations**

Healthy young adult hens free from interfering viral diseases and medication and without abnormalities of gait should be randomised and assigned to treatment and control groups and acclimatised to the laboratory conditions for at least five days prior to the start of the study.

Cages or enclosures which are large enough to permit free mobility of the hens, and easy observation of gait should be used.

Dosing with the test substance should normally be by the oral route using gavage, gelatine capsules, or a comparable method. Liquids may be given undiluted or dissolved in an appropriate vehicle such as corn oil; solids should be dissolved if possible since large doses of solids in gelatine capsules may not be absorbed efficiently. For non-aqueous vehicles the toxic characteristics of the vehicle should be known, and if not known should be determined before the test.

1.5.2. **Test conditions**

1.5.2.1. *Test animals*

The young adult domestic laying hen (*Gallus gallus domesticus*), aged eight to 12 months, is recommended. Standard size breeds and strains should be employed and the hens normally should have been reared under conditions which permitted free mobility.

1.5.2.2. *Number and sex*

In addition to the treatment group, both a vehicle control group and a positive control group should be used. The vehicle control group should be treated in a manner identical to the treatment group, except that administration of the test substance is omitted.

Sufficient number of hens should be utilised in each group of birds so that at least six birds can be killed for biochemical determination (three at each of two time points) and six can survive the 21-day observation period for pathology.

The positive control group may be run concurrently or be a recent historical control group. It should contain at least six hens, treated with a known delayed neurotoxicant, three hens for biochemistry and three hens for pathology. Periodic updating of historical data is recommended. New positive control data should be developed when some essential element (e.g. strain, feed, housing conditions) of the conduct of the test has been changed by the performing laboratory.

1.5.2.3. *Dose levels*

A preliminary study using an appropriate number of hens and dose levels groups should be performed to establish the level to be used in the main study. Some lethality is typically necessary in this preliminary study to define an adequate main study dose. However, to prevent death due to acute cholinergic effects, atropine or another protective agent, known to not interfere with delayed neurotoxic responses, may be used. A variety of test methods may be used to estimate the maximum non-lethal dose of test substances (See method B.1*bis*). Historical data in the hen or other toxicological information may also be helpful in dose selection.

The dose level of the test substance in the main study should be as high as possible taking into account the results of the preliminary dose selection study and the upper limit dose of 2 000 mg/kg body weight. Any mortality which might occur should not interfere with the survival of sufficient animals for biochemistry (six) and histology (six) at 21 days. Atropine or another protective agent, known to not interfere with delayed neurotoxic responses, should be used to prevent death due to acute cholinergic effects.

1.5.2.4. *Limit test*

If a test at a dose level of at least 2 000 mg/kg body weight/day, using the procedures described for this study, produces no observable toxic effects and if toxicity would not be expected based upon data from structurally related substances, then a study using a higher dose may not be considered necessary. The limit test applies except when human exposure indicates the need for a higher dose level to be used.

1.5.3. **Observation period**

Observation period should be 21 days.

1.5.4. **Procedure**

After administration of a protective agent to prevent death due to acute cholinergic effect, the test substance is administered in a single dose.

General observation

Observations should start immediately after exposure. All hens should be carefully observed several times during the first two days and thereafter at least once daily for a period of 21 days or until scheduled kill. All signs of toxicity should be recorded, including the time of onset, type, severity and duration of behavioural abnormalities. Ataxia should be measured on an ordinal grading scale consisting of at least four levels, and paralysis should be noted. At least twice a week the hens selected for pathology should be taken outside the cages and subjected to a period of forced motor activity, such as ladder climbing, in order to facilitate the observation of minimal toxic effects. Moribund animals and animals in severe distress or pain should be removed when noticed, humanely killed and necropsied.

Body weight

All hens should be weighed just prior to administration of the test substance and at least once a week thereafter.

Biochemistry

Six hens randomly selected from each of the treatment and vehicle control groups, and three hens from the positive control group (when this group is run concurrently), should be killed within a few days after dosing, and the brain and lumbar spinal cord prepared and assayed for neuropathy target esterase inhibition activity. In addition, it may also be useful to prepare and assay sciatic nerve tissue for neuropathy target esterase inhibition activity. Normally, three birds of the control and each treatment group are killed after 24 hours and three at 48 hours, whereas the three hens of the positive controls should be killed at 24 hours. If observation of clinical signs of intoxication (this can often be assessed by observation of the time of onset of cholinergic signs) indicates that the toxic agent may be disposed of very slowly then it may be preferable to sample tissue from three birds at each of two times between 24 and as late as 72 hours after dosing.

Analyses of acetylcholinesterase (AChE) may also be performed on these samples, if deemed appropriate. However, spontaneous reactivation of AChE may occur *in vivo*, and so lead to underestimation of the potency of the substance as an AChE inhibitor.

Gross necropsy

Gross necropsy of all animals (scheduled killed and killed when moribund) should include observation of the appearance of the brain and spinal cord.

Histopathological examination

Neural tissue from animals surviving the observation period and not used for biochemical studies should be subjected to microscopic examination. Tissues should be fixed *in situ*, using perfusion techniques. Sections should include cerebellum (mid-longitudinal level), medulla oblongata, spinal cord, and peripheral nerves. The spinal cord sections should be taken from the upper cervical segment, the mid-thoracic and the lumbo-sacral regions. Sections of the distal region of the tibial nerve and its branches to the gastrocnemial muscle and of the sciatic nerve should be taken. Sections should be stained with appropriate myelin and axon-specific stains.

2. **DATA**

Negative results on the endpoints selected in this method (biochemistry, histopathology and behavioural observation) would not normally require further testing for delayed neurotoxicity. Equivocal or inconclusive results for these endpoints may require further evaluation.

Individual data should be provided. Additionally, all data should be summarised in tabular form, showing for each test group the number of animals at the start of the test, the number of animals showing lesions, behavioural or biochemical effects, the types and severity of these lesions or effects, and the percentage of animals displaying each type and severity of lesion or effect.

The findings of this study should be evaluated in terms of the incidence, severity, and correlation of behavioural, biochemical and histopathological effects and any other observed effects in the treated and control groups.

Numerical results should be evaluated by appropriate and generally acceptable statistical methods. The statistical methods used should be selected during the design of the study.

3. **REPORTING**

TEST REPORT

The test report shall, if possible, include the following information:

3.1. Test animals:

— strain used,

— number and age of animals,

— source, housing conditions, etc.,

— individual weights of animals at the start of the test.

3.2. Test conditions:

— details of test substance preparation, stability and homogeneity, where appropriate,

— justification for choice of vehicle,

— details of the administration of the test substance,

— details of food and water quality,

— rationale for dose selection,

— specification of doses administered, including details of the vehicle, volume and physical form of the material administered,

— identity and details of the administration of any protective agent.

3.3. Results:

— body weight data,

— toxic response data by group, including mortality,

— nature, severity and duration of clinic observations (whether reversible or not),

— a detailed description of biochemical methods and findings,

— necropsy findings,

— a detailed description of all histopathological findings,

— statistical treatment of results, where appropriate.

Discussion of results.

Conclusions.

4. **REFERENCES**

This method is analogius to OECD TG 418.

B.38. **DELAYED NEUROTOXICITY OF ORGANOPHOSPHORUS SUBSTANCES 28-DAY REPEATED DOSE STUDY**

1. **METHOD**

1.1. INTRODUCTION

In the assessment and evaluation of the toxic effects of substances, it is important to consider the potential of certain classes of substances to cause specific types of neurotoxicity that might not be detected in other toxicity studies. Certain organophosphorus substances have been observed to cause delayed neurotoxicity and should be considered as candidates for evaluation.

In vitro screening tests could be employed to identify those substances which may cause delayed polyneuropathy; however, negative findings from *in vitro* studies do not provide evidence that the test substance is not a neurotoxicant.

This 28-day delayed neurotoxicity test provides information on possible health hazards likely to arise from repeated exposures over a limited period of time. It will provide information on dose response and can provide an estimate of a no-observed-adverse effect level, which can be of use for establishing safety criteria for exposure.

See also General introduction Part B.

1.2. DEFINITIONS

Organophosphorus substances include uncharged organophosphorus esters, thioesters or anhydrides of organophosphoric, organophosphonic or organophosphoramidic acids or of related phosphorothioic, phosphonothioic or phosphorothioamidic acids or other substances that may cause the delayed neurotoxicity sometimes seen in this class of substances.

Delayed neurotoxicity is a syndrome associated with prolonged delayed onset of ataxia, distal axonopathies in spinal cord and peripheral nerve, and inhibition and ageing of neuropathy target esterase (NTE) in neural tissue.

1.3. PRINCIPLE OF THE TEST METHOD

Daily doses of the test substance are administered orally to domestic hens for 28 days. The animals are observed at least daily for behavioural abnormalities, ataxia and paralysis until 14 days after the last dose. Biochemical measurements, in particular neuropathy target esterase inhibition (NTE), are undertaken, on hens randomly selected from each group, normally 24 and 48 hours after the last dose. Two weeks after the last dose, the remainder of the hens are killed and histopathological examination of selected neural tissues is undertaken.

1.4. DESCRIPTION OF THE TEST METHOD

1.4.1. **Preparations**

Healthy young adult hens free from interfering viral diseases and medication, and without abnormalities of gait should be randomised and assigned to treatment and control groups and acclimatised to the laboratory conditions for at least five days prior to the start of the study.

Cages or enclosures which are large enough to permit free mobility of the hens and easy observation of gait should be used.

Oral dosing each day, seven days per week, should be carried out, preferably by gavage or administration of gelatine capsules. Liquids may be given undiluted or dissolved in an appropriate vehicle such as corn oil; solids should be dissolved if possible since large doses of solids in gelatine capsules may not be absorbed efficiently. For non-aqueous vehicles the toxic characteristics of the vehicle should be known, and if not known should be determined before the test.

1.4.2. **Test conditions**

1.4.2.1. *Test animals*

The young adult domestic laying hen (*Gallus gallus domesticus*), aged eight to 12 months, is recommended. Standard size, breeds and strains should be employed and the hens normally should have been reared under conditions which permitted free mobility.

1.4.2.2. *Number and sex*

Generally at least three treatment groups and a vehicle control group should be used. The vehicle control group should be treated in a manner identical to the treatment group, except that administration of the test substance is omitted.

Sufficient number of hens should be utilised in each group of birds so that at least six birds can be killed for biochemical determinations (three at each of two timepoints) and six birds can survive the 14-day post-treatment observation period for pathology.

1.4.2.3. *Dose levels*

Dose levels should be selected taking into account the results from an acute test on delayed neurotoxicity and any other existing toxicity or kinetic data available for the test compound. The highest dose level should be chosen with the aim of inducing toxic effects, preferably delayed neurotoxicity, but not death nor obvious suffering. Thereafter, a descending sequence of dose levels should be selected with a view to demonstrate any dose-related response and no-observed-adverse effects at the lowest dose level.

1.4.2.4. *Limit test*

If a test at a dose level of at least 1 000 mg/kg body weight/day, using the procedures described for this study, produces no observable toxic effects and if toxicity would not be expected based upon data from structurally related substances, then a study using a higher dose may not be considered necessary. The limit test applies except when expected human exposure indicates the need for a higher dose level to be used.

1.4.2.5. *Observation period*

All the animals should be observed at least daily during the exposure period and 14 days after, unless scheduled necropsy.

1.4.3. **Procedure**

Animals are dosed with the test substance on seven days per week for a period of 28 days.

General observations

Observations should start immediately after treatment begins. All hens should be carefully observed at least once daily on each of the 28 days of treatment, and for 14 days after dosing or until scheduled kill. All signs of toxicity should be recorded including their time of onset, type, severity and duration. Observations should include, but not be limited to, behavioural abnormalities. Ataxia should be measured on an ordinal grading scale consisting of at least four levels, and paralysis should be noted. At least twice a week the hens should be taken outside the cages and subjected to a period of forced motor activity, such as ladder climbing, in order to facilitate the observation of minimal toxic effects. Moribund animals in severe distress or pain should be removed when noticed, humanely killed and necropsied.

Body weight

All hens should be weighed just prior to the first administration of the test substance and at least once a week thereafter.

Biochemistry

Six hens randomly selected from each of the treatment and vehicle control groups should be killed within a few days after the last dose, and the brain and lumbar spinal cord prepared and assayed for neuropathy target esterase (NTE) inhibition activity. In addition, it may also be useful to prepare and assay sciatic nerve tissue for

neuropathy target esterase (NTE) inhibition activity. Normally, three birds of the control and each treatment group are killed after 24 hours and three at 48 hours after the last dose. If data from the acute study or other studies (e.g. toxicokinetics) indicate that other times of killing after final dosing are preferable then these times should be used and the rationale documented.

Analyses of acetylcholinesterase (AChE) may also be performed on these samples, if deemed appropriate. However, spontaneous reactivation of AChE may occur *in vivo*, and so lead to underestimation of the potency of the substance as an AChE inhibitor.

Gross necropsy

Gross necropsy of all animals (scheduled killed and killed when moribund) should include observation of the appearance of the brain and spinal cord.

Histopathological examination

Neural tissue from animals surviving the observation period and not used for biochemical studies should be subjected to microscopic examination. Tissues should be fixed *in situ*, using perfusion techniques. Sections should include cerebellum (mid longitudinal level), medulla oblongata, spinal cord and peripheral nerves. The spinal cord sections should be taken from the upper cervical segment, the mid-thoracic and the lumbo-sacral regions. Sections of the distal region of the tibial nerve and its branches to the gastrocnemial muscle and of the sciatic nerve should be taken. Sections should be stained with appropriate myelin and axon-specific stains. Initially, microscopic examination should be carried out on the preserved tissues of all animals in the control and high dose group. When there is evidence of effects in the high dose group, microscopic examination should also be carried out in hens from the intermediate and low dose groups.

2. DATA

Negative results on the endpoints selected in this method (biochemistry, histopathology and behavioural observation) would not normally require further testing for delayed neurotoxicity. Equivocal or inconclusive results for these endpoints may require further evaluation.

Individual data should be provided. Additionally, all data should be summarised in tabular form, showing for each test group the number of animals at the start of the test, the number of animals showing lesions, behavioural or biochemical effects, the types and severity of these lesions or effects, and the percentage of animals displaying each type and severity of lesion or effect.

The findings of this study should be evaluated in terms of the incidence, severity, and correlation of behavioural, biochemical and histopathological effects and any other observed effects in each of the treated and control groups.

Numerical results should be evaluated by appropriate and generally acceptable statistical methods. The statistical methods should be selected during the design of the study.

3. REPORTING

TEST REPORT

The test report shall, if possible, include the following information:

3.1. Test animals:

— strain used,

— number and age of animals,

— source, housing conditions, etc.,

— individual weights of animals at the start of the test.

3.2. Test conditions:

— details of test substance preparation, stability and homogeneity, where appropriate,

— justification for choice of vehicle,

— details of the administration of the test substance,

— details of food and water quality,

— rationale for dose selection,

— specification of doses administered, including details of the vehicle, volume and physical form of the material administered,

— rationale for choosing other times for biochemical determination, if other than 24 and 48 h.

3.3. Results:

— body weight data,

— toxic response data by dose level, including mortality,

— no-observed adverse effect level,

— nature, severity and duration of clinic observations (whether reversible or not),

— a detailed description of biochemical methods and findings,

— necropsy findings,

— a detailed description of all histopathological findings,

— statistical treatment of results, where appropriate.

Discussion of results.

Conclusions.

4. **REFERENCES**

This method is analogous to OECD TG 419.

B.39. **UNSCHEDULED DNA SYNTHESIS (UDS) TEST WITH MAMMALIAN LIVER CELLS IN VIVO**

1. **METHOD**

This method is a replicate of the OECD TG 486, Unscheduled DNA Synthesis (UDS) Test with Mammalian Liver Cells *In Vivo* (1997).

1.1. INTRODUCTION

The purpose of the unscheduled DNA Synthesis (UDS) test with mammalian liver cells *in vivo* is to identify test substances that induce DNA repair in liver cells of treated animals (see 1,2,3,4).

This *in vivo* test provides a method for investigating genotoxic effects of chemicals in the liver. The end-point measured is indicative of DNA damage and subsequent repair in liver cells. The liver is usually the major site of metabolism of absorbed compounds. It is thus an appropriate site to measure DNA damage *in vivo*.

If there is evidence that the test substance will not reach the target tissue, it is not appropriate to use this test.

The end-point of unscheduled DNA synthesis (UDS) is measured by determining the uptake of labelled nucleosides in cells that are not undergoing scheduled (S-phase) DNA synthesis. The most widely used technique is the determination of the uptake of tritium-labelled thymidine (^{3}H-TdR) by autoradiography. Rat livers are preferably used for *in vivo* UDS tests. Tissues other than the livers may be used, but are not the subject of this method.

The detection of a UDS response is dependent on the number of DNA bases excised and replaced at the site of the damage. Therefore, the UDS test is particularly valuable to detect substance-induced 'longpatch repair' (20-30 bases). In contrast, 'shortpatch repair' (1-3 bases) is detected with much lower sensitivity. Furthermore, mutagenic events may result because of non-repair, misrepair or misreplication of DNA lesions. The extent of the UDS response gives no indication of the fidelity of the repair process. In addition, it is possible that a mutagen reacts with DNA but the DNA damage is not repaired via an excision repair process. The lack of specific information on mutagenic activity provided by the UDS test is compensated for by the potential sensitivity of this endpoint because it is measured in the whole genome.

See also General introduction Part B.

1.2. DEFINITIONS

Cells in repair: a net nuclear grain (NNG) higher than a preset value, to be justified at the laboratory conducting the test.

Net nuclear grains (NNG): quantitative measure for UDS activity of cells in autoradiographic UDS tests, calculated by subtracting the average number of cytoplasmic grains in nucleus-equivalent cytoplasmic areas (CG) from the number of nuclear grains (NG): NNG = NG − CG. NNG counts are calculated for individual cells and then pooled for cells in a culture, in parallel cultures, etc.

Unscheduled DNA Synthesis (UDS): DNA repair synthesis after excision and removal of a stretch of DNA containing a region of damage induced by chemical substances or physical agents.

1.3. PRINCIPLE OF THE TEST METHOD

The UDS test with mammalian liver cells *in vivo* indicates DNA repair synthesis after excision and removal of a stretch of DNA containing a region of damage induced by chemical substances or physical agents. The test is usually based on the incorporation of ^{3}H-TdR into the DNA of liver cells which have a low frequency of cells in the S-phase of the cell cycle. The uptake of ^{3}H-TdR is usually determined by autoradiography, since this technique is not as susceptible to interference from S-phrase cells as, for example, liquid scintillation counting.

1.4. DESCRIPTION OF THE METHOD

1.4.1. **Preparations**

1.4.1.1. *Selection of animal species*

Rats are commonly used, although any appropriate mammalian species may be used. Commonly used laboratory strains of young healthy adult animals should be employed. At the commencement of the study the weight variation of animals should be minimal and not exceed ± 20 % of the mean weight for each sex.

1.4.1.2. *Housing and feeding conditions*

General conditions referred in the General introduction to Part B are applied although the aim for humidity should be 50-60 %.

1.4.1.3. *Preparation of the animals*

Healthy young adult animals are randomly assigned to the control and treatment groups. Cages should be arranged in such a way that possible effects due to cage placement are minimised. The animals are identified uniquely and kept in their cages for at least five days prior to the start of the study to allow for acclimatisation to the laboratory conditions.

1.4.1.4. *Test substance/Preparation*

Solid test substances should be dissolved or suspended in appropriate solvents or vehicles and diluted, if appropriate, prior to dosing of the animals. Liquid test substances may be dosed directly or diluted prior to dosing. Fresh preparations of the test substance should be employed unless stability data demonstrate the acceptability of storage.

1.4.2. **Test conditions**

1.4.2.1. *Solvent/Vehicle*

The solvent/vehicle should not produce toxic effects at the dose levels used, and should not be suspected of chemical reaction with the test substance. If other than well-known solvents/vehicles are used, their inclusion should be supported with data indicating their compatibility. It is recommended that wherever possible, the use of an aqueous solvent/vehicle should be considered first.

1.4.2.2. *Controls*

Concurrent positive and negative controls (solvent/vehicle) should be included in each independently performed part of the experiment. Except for treatment with the test substance, animals in the control group should be handled in an identical manner to the animals in the treated groups.

Positive controls should be substances known to produce UDS when administered at exposure levels expected to give a detectable increase over background. Positive controls needing metabolic activation should be used at doses eliciting a moderate response (4). The doses may be chosen so that the effects are clear but do not immediately reveal the identity of the coded slides to the reader. Examples of positive control substances include:

Sampling Times	Substance	CAS No	EINECS No
Early sampling times (2-4 hours)	N-Nitrosodimethylamine	62-75-9	200-249-8
Late sampling times (12-16 hours)	N-2-Fluorenylacetamide (2-AAF)	53-96-3	200-188-6

Other appropriate positive control substances may be used. It is acceptable that the positive control should be administered by a route different from the test substance.

1.5. PROCEDURE

1.5.1. **Number and sex of animals**

An adequate number of animals should be used, to take account of natural biological variation in test response. The number of animals should be at least three analysable animals per group. Where a significant historical database has been accumulated, only one or two animals are required for the concurrent negative and positive control groups.

If at the time of the study there are data available from studies in the same species and using the same route of exposure that demonstrate that there are no substantial differences in toxicity between sexes, then testing in a single sex, preferably males, will be sufficient. Where human exposure to chemicals may be sex-specific, as for example with some pharmaceutical agents, the test should be performed with animals of the appropriate sex.

1.5.2. **Treatment schedule**

Test substances are generally administered as a single treatment.

1.5.3. **Dose levels**

Normally, at least two dose levels are used. The highest dose is defined as the dose producing signs of toxicity such that higher dose levels, based on the same dosing regimen, would be expected to produce lethality. In general, the lower dose should be 50 % to 25 % of the high dose.

Substances with specific biological activities at low non-toxic doses (such as hormones and mitogens) may be exceptions to the dose-setting criteria and should be evaluated on a case-by-case basis. If a range finding study is performed because there are no suitable data available, it should be performed in the same laboratory, using the same species, strain, sex, and treatment regimen to be used in the main study.

The highest dose may also be defined as a dose that produces some indication of toxicity in the liver (e.g. pyknotic nuclei).

1.5.4. **Limit test**

If a test at one dose level of at least 2 000 mg/kg body weight, applied in a single treatment, or in two treatments on the same day, produces no observable toxic effects, and if genotoxicity would not be expected, based upon data from structurally related substances, then a full study may not be necessary. Expected human exposure may indicate the need for a higher dose level to be used in the limit test.

1.5.5. **Administration of doses**

The test substance is usually administered by gavage using a stomach tube or a suitable intubation cannula. Other routes of exposure may be acceptable where they can be justified. However, the intraperitoneal route is not recommended as it could expose the liver directly to the test substance rather than via the circulatory system. The maximum volume of liquid that can be administered by gavage or injection at one time depends on the size of the test animal. The volume should not exceed 2 ml/100 g body weight. The use of volumes higher than these must be justified. Except for irritating or corrosive substances, which will normally reveal exacerbated effects with higher concentrations, variability in test volume should be minimised by adjusting the concentration to ensure a constant volume at all dose levels.

1.5.6. **Preparation of liver cells**

Liver cell are prepared from treated animals normally 12-16 hours after dosing. An additional earlier sampling time (normally two to four hours post-treatment) is generally necessary unless there is a clear positive response at 12-16 hours. However, alternative sampling times may be used when justified on the basis of toxicokinetic data.

Short-term cultures of mammalian liver cells are usually established by perfusing the liver *in situ* with collagenase and allowing freshly dissociated liver cells to attach themselves to a suitable surface. Liver cells from negative control animals should have a viability (5) of at least 50 %.

1.5.7. **Determination of UDS**

Freshly isolated mammalian liver cells are incubated usually with medium containing ^{3}H-TdR for an appropriate length of time, e.g. 3-8 hours. At the end of the incubation period, medium should be removed from the cells, which may then be incubated with medium containing excess unlabelled thymidine to diminish unincorporated radioactivity ('cold chase'). The cells are then rinsed, fixed and dried. For more prolonged incubation times, cold chase may not be necessary. Slides are dipped in autoradiographic emulsion, exposed in the dark (e.g. refrigerated for 7-14 days), developed, stained, and exposed silver grains are counted. Two to three slides are prepared from each animal.

1.5.8. **Analysis**

The slide preparations should contain sufficient cells of normal morphology to permit a meaningful assessment of UDS. Preparations are examined microscopically for signs of overt cytotoxicity (e.g. pyknosis, reduced levels of radiolabelling).

Slides should be coded before grain counting. Normally 100 cells are scored from each animal from at least two slides; the scoring of less than 100 cells/animal should be justified. Grain counts are not scored for S-phase nuclei, but the proportion of S-phase cells may be recorded.

The amount of ^{3}H-TdR incorporation in the nuclei and the cytoplasm of morphologically normal cells, as evidenced by the deposition of silver grains, should be determined by suitable methods.

Grain counts are determined over the nuclei (nuclear grains, NG) and nucleus equivalent areas over the cytoplasm (cytoplasmic grains, CG). CG counts are measured by either taking the most heavily labelled area of cytoplasm, or by taking an average of two to three random cytoplasmic grain counts adjacent to the nucleus. Other counting methods (e.g. whole cell counting) may be used if they can be justified (6).

2. **DATA**

2.1. TREATMENT OF RESULTS

Individual slide and animal data should be provided. Additionally, all data should be summarised in tabular form. Net nuclear grain (NNG) counts should be calculated for each cell, for each animal and for each dose and time by subtracting CG counts from NG counts. If 'cells in repair' are counted, the criteria for defining 'cells in repair' should be justified and based on historical or concurrent negative control data. Numerical results may be evaluated by statistical methods. If used, statistical tests should be selected and justified prior to conducting the study.

2.2. EVALUATION AND INTERPRETATION OF RESULTS

Examples of criteria for positive/negative responses include:

positive (i) NNG values above a pre-set threshold which is justified on the basis of laboratory historical data; or

(ii) NNG values significantly greater than concurrent control;

negative (i) NNG values within/below historical control threshold; or

(ii) NNG values not significantly greater than concurrent control.

The biological relevance of data should be considered: i.e. parameters such as inter-animal variation, dose-response relationship and cytotoxicity should be taken into account. Statistical methods may be used as an aid in evaluating the test results. However, statistical significance should not be the only determining factor for a positive response.

Although most experiments will give clearly positive or negative results, in rare cases the data set will preclude making a definite judgement about the activity of the test substance. Results may remain equivocal or questionable regardless of the number of times the experiment is repeated.

A positive result from the UDS test with mammalian liver cells *in vivo* indicate that a test substance induces DNA damage in mammalian liver cells *in vivo* that can be repaired by unscheduled DNA synthesis *in vitro*. A negative result indicates that, under the test conditions, the test substance does not induce DNA damage that is detectable by this test.

The likelihood that the test substance reaches the general circulation or specifically the target tissue (e.g. systemic toxicity) should be discussed.

3. **REPORTING**

TEST REPORT

The test report must include the following information:

Solvent/Vehicle:

— justification for choice of vehicle,

— solubility and stability of the test substance in solvent/vehicle, if known.

Test animals:

— species/strain used,

— number, age and sex of animals,

— source, housing conditions, diet, etc.,

— individual weight of the animals at the start of the test, including body weight range, mean and standard deviation for each group,

Test conditions:

— positive and negative vehicle/solvent controls,

— data from range-finding study, if conducted,

— rationale for dose level selection,

— details of test substance preparation,

— details of the administration of the test substance,

— rationale for route of administration,

— methods for verifying that test agent reached the general circulation or target tissue, if applicable,

— conversion from diet/drinking water test substance concentration (ppm) to the actual dose (mg/kg body weight/day), if applicable,

— details of food and water quality,

— detailed description of treatment and sampling schedules,

— methods for measurement of toxicity,

— method of liver cell preparation and culture,

— autoradiographic technique used,

— number of slides prepared and numbers of cells scored,

— evaluation criteria,

— criteria for considering studies as positive, negative or equivocal,

Results:

— individual slide, animal and group mean values for nuclear grains, cytoplasmic grains, and net nuclear grains,

— dose-response relationship, if available,

— statistical evaluation if any,

— signs of toxicity,

— concurrent negative (solvent/vehicle) and positive control data,

— historical negative (solvent/vehicle) and positive control data with range, means and standard deviations,

— number of 'cells in repair' if determined,

— number of S-phase cells if determined,

— viability of the cells.

Discussion of results.

Conclusions.

4. **REFERENCES**

(1) Ashby, J., Lefevre, P.A., Burlinson, B. and Penman, M.G., (1985) An Assessment of the *In Vivo* Rat Hepatocyte DNA Repair Assay. Mutation Res., 156, p. 1-18.

(2) Butterworth, B.E., Ashby, J., Bermudez, E., Casciano, D., Mirsalis, J., Probst, G. and Williams, G., (1987) A Protocol and Guide for the *In Vivo* Rat Hepatocyte DNA-Repair Assay. Mutation Res., 189, p. 123-133.

(3) Kennelly, J.C., Waters, R., Ashby, J., Lefevre, P.A., Burlinson, B., Benford, D.J., Dean, S.W. and Mitchell, I. de G., (1993) In Vivo Rat Liver UDS Assay. In: Kirkland D.J. and Fox M., (Eds) Supplementary Mutagenicity Tests: UKEM Recommended Procedures. UKEMS Subcommittee on Guidelines for Mutagenicity Testing. Report. Part II revised. Cambridge University Press, Cambridge, New York, Port Chester, Melbourne, Sydney, p. 52-77.

(4) Madle, S., Dean, S.W., Andrae, U., Brambilla, G., Burlinson, B., Doolittle, D.J., Furihata, C., Hertner, T., McQueen, C.A. and Mori, H., (1993) Recommendations for the Performance of UDS Tests *In Vitro* and *In Vivo*. Mutation Res., 312, p. 263-285.

(5) Fautz, R., Hussain, B., Efstathiou, E. and Hechenberger-Freudl, C., (1993) Assessment of the Relation Between the Initial Viability and the Attachment of Freshly Isolated Rat Hepatocytes Used for the *In Vivo*/*In Vitro* DNA Repair Assay (UDS). Mutation Res., 291, p. 21-27.

(6) Mirsalis, J.C., Tyson, C.K. and Butterworth, B.E., (1982) Detection of Genotoxic Carcinogens in the *In Vivo*/*In Vitro* Hepatocyte DNA Repair Assay. Environ. Mutagen, 4, p. 553-562.

B.40. ***IN VITRO* SKIN CORROSION: TRANSCUTANEOUS ELECTRICAL RESISTANCE TEST (TER)**

1. **METHOD**

This testing method is equivalent to the OECD TG 430 (2004).

1.1. INTRODUCTION

Skin corrosion refers to the production of irreversible tissue damage in the skin following the application of a test material (as defined by the Globally Harmonised System for the Classification and Labelling of Chemical Substances and Mixtures (GHS)) (1). This method provides a procedure by which the assessment of corrosivity is not carried out in live animals.

The assessment of skin corrosivity has typically involved the use of laboratory animals (2). Concern for the pain and suffering of animals involved with this procedure has been addressed in the revision of testing method B.4 that allows for the determination of skin corrosion by using alternative, *in vitro,* methods, avoiding pain and suffering.

A first step towards defining alternative tests that could be used for skin corrosivity testing for regulatory purposes was the conduct of prevalidation studies (3). Following this, a formal validation study of *in vitro* methods for assessing skin corrosion (4)(5) was conducted (6)(7)(8). The outcome of these studies and other published literature led to the recommendation that the following tests could be used for the assessment of in vivo skin corrosivity (9)(10)(11): the human skin model test (see tesing method B.40*bis*) and the transcutaneous electrical resistance test (this method).

A validation study and other published studies have reported that the rat skin transcutaneous electrical resistance (TER) assay (12)(13) is able to reliably discriminate between known skin corrosives and non-corrosives (5)(9).

The test described in this method allows the identification of corrosive chemical substances and mixtures. It further enables the identification of non-corrosive substances and mixtures when supported by a weight of evidence determination using other existing information (e.g. pH, structure-activity-relationships, human and/or animal data) (1)(2)(11)(14). It does not provide information on skin irritation, nor does it allow the sub-categorisation of corrosive substances as permitted in the Globally Harmonised Classification System (GHS) (1).

For a full evaluation of local skin effects after a single dermal exposure, it is recommended to follow the sequential testing strategy as appended to testing method B.4 (2) and provided in the Globally Harmonised System (1). This testing strategy includes the conduct of *in vitro* tests for skin corrosion (as described in this method) and skin irritation before considering testing in live animals.

1.2. DEFINITIONS

Skin corrosion *in vivo*: is the production of irreversible damage of the skin: namely, visible necrosis through the epidermis and into the dermis, following the application of a test subsance for up to four hours. Corrosive reactions are typified by ulcers, bleeding, bloody scabs, and, by the end of the observation at 14 days, by discolouration due to blanching of the skin, complete areas of alopecia, and scars. Histopathology should be considered to evaluate questionable lesions.

Transcutaneous Electrical Resistance (TER): is a measure of the electrical impendance of the skin, as a resistance value in kilo Ohms. A simple and robust method of assessing barrier function by recording the passage of ions through the skin using a Wheatstone bridge apparatus.

1.3. REFERENCE SUBSTANCES

Table 1

Reference chemicals

Name	EINECS No	CAS No	
1,2-Diaminopropane	201-155-9	78-90-0	Severely corrosive

Name	EINECS No	CAS No	
Acrylic Acid	201-177-9	79-10-7	Severely Corrosive
2-tert. Butylphenol	201-807-2	88-18-6	Corrosive
Potassium hydroxide (10 %)	215-181-3	1310-58-3	Corrosive
Sulfuric acid (10 %)	231-639-5	7664-93-9	Corrosive
Octanoic acid (caprylic acid)	204-677-5	124-07-02	Corrosive
4-Amino-1,2,4-triazole	209-533-5	584-13-4	Not corrosive
Eugenol	202-589-1	97-53-0	Not corrosive
Phenethyl bromide	203-130-8	103-63-9	Not corrosive
Tetrachloroethylene	204-825-9	27-18-4	Not corrosive
Isostearic acid	250-178-0	30399-84-9	Not corrosive
4-(Methylthio)-benzaldehyde	222-365-7	3446-89-7	Not corrosive

Most of the chemicals listed are taken from the list of chemicals selected for the ECVAM international validation study (4). Their selection is based on the following criteria:

(i) equal number of corrosive and non-corrosive substances;

(ii) commercially available substances covering most of the relevant chemical classes;

(iii) inclusion of severely corrosive as well as less corrosive substances in order to enable discrimination based on corrosive potency;

(iv) choice of chemicals that can be handled in a laboratory without posing other serious hazards than corrosivity.

1.4. PRINCIPLE OF THE TEST METHOD

The test material is applied for up to 24 hours to the epidermal surfaces of skin discs in a two-compartment test system in which the skin discs function as the separation between the compartments. The skin discs are taken from humanely killed rats aged 28-30 days. Corrosive materials are identified by their ability to produce a loss of normal stratum corneum integrity and barrier function, which is measured as a reduction in the TER below a threshold level (12). For rat TER, a cut-off value of 5 kΩ has been selected based on extensive data for a wide range of chemicals where the vast majority of values were either clearly well above (often > 10 kΩ), or well below (often < 3 kΩ) this value (12). Generally, materials which are non-corrosive in animals but are irritating or non-irritating do not reduce the TER below this cut-off value. Furthermore, use of other skin preparations or other equipment may alter the cut-off value, necessitating further validation.

A dye-binding step is incorporated into the test procedure for confirmation testing of positive results in the TER including values around 5 kΩ. The dye-binding step determines if the increase in ionic permeability is due to physical destruction of the stratum corneum. The TER method utilising rat skin has shown to be predictive of *in vivo* corrosivity in the rabbit assessed under Testing Method B.4 (2). It should be noted that the *in vivo* rabbit test is highly conservative with respect to skin corrosivity and skin irritation when compared with the human skin patch test (15).

1.5. DESCRIPTION OF THE TEST METHOD

1.5.1. **Animals**

Rats are the species of choice because the sensitivity of their skin to chemicals in this test has been previously demonstrated (10). The age (when the skin is collected) and strain of the rat is particularly important to ensure that the hair follicles are in the dormant phase before adult hair growth begins.

The dorsal and flank hair from young, approximately 22 day-old, male or female rats (Wistar-derived or a comparable strain), is carefully removed with small clippers. Then, the animals are washed by careful wiping, whilst submerging the clipped area in antibiotic solution (containing, for example, streptomycin, penicillin, chloramphenicol, and amphotericin, at concentrations effective in inhibiting bacterial growth). Animals are washed with antibiotics again on the third or fourth day after the first wash and are used within three days of the second wash, when the stratum corneum has recovered from the hair removal.

1.5.2. **Preparation of the skin discs**

Animals are humanely killed when 28-30 days old; this age is critical. The dorso-lateral skin of each animal is then removed and stripped of excess subcutaneous fat by carefully peeling it away from the skin. Skin discs, with a diameter of approximately 20 mm each, are removed. The skin may be stored before disks are used where it is shown that positive and negative control data are equivalent to that obtained with fresh skin.

Each skin disc is placed over one of the ends of a PTFE (polytetrafluoroethylene) tube, ensuring that the epidermal surface is in contact with the tube. A rubber 'O' ring is press-fitted over the end of the tube to hold the skin in place and excess tissue is trimmed away. Tube and 'O' ring dimensions are shown in Figure 2. The rubber 'O' ring is then carefully sealed to the end of the PTFE tube with petroleum jelly. The tube is supported by a spring clip inside a receptor chamber containing $MgSO_4$ solution (154 mM) (Figure 1). The skin disc should be fully submerged in the $MgSO_4$ solution. As many as 10-15 skin discs can be obtained from a single rat skin.

Before testing begins, the electrical resistance of two skin discs is measured as a quality control procedure for each animal skin. Both discs should give resistance values greater than 10 kΩ for the remainder of the discs to be used for the test. If the resistance value is less than 10 kΩ, the remaining discs from that skin should be discarded.

1.5.3. **Application of the test and control substances**

Concurrent positive and negative controls should be used for each study to ensure adequate performance of the experimental model. Skin discs from a single animal should be used. The suggested positive and negative control substances are 10 M hydrochloric acid and distilled water, respectively.

Liquid test substances (150 µL) are applied uniformly to the epidermal surface inside the tube. When testing solid materials, a sufficient amount of the solid is applied evenly to the disc to ensure that the whole surface of the epidermis is covered. Deionised water (150 µL) is added on top of the solid and the tube is gently agitated. In order to achieve maximum contact with the skin, solids may need to be warmed to 30 °C to melt or soften the test substance, or ground to produce a granular material or powder.

Three skin discs are used for each test and control substance. Test substances are applied for 24 hours at 20-23 °C. The test substance is removed by washing with a jet of tap water at up to 30 °C until no further material can be removed.

1.5.4. **TER measurements**

The skin impedance is measured as TER is measured by using a low-voltage, alternating current Wheatstone databridge (13). General specifications of the bridge are 1-3 Volt operating voltage, a sinus or rectangular shaped alternating current of 50 – 1 000 Hz, and a measuring range of at least 0,1 – 30 kΩ. The databridge used in the validation study measureds inductance, capacitance and resistance up to values of 2 000 H, 2 000 µF, and 2 MΩ, respectively at frequencies of 100 Hz or 1 kHz, using series or parallel values. For the purposes of the TER corrosivity assay measurements are recorded in resistance, at a frequency of 100 Hz and using series values. Prior to measuring the electrical resistance, the surface tension of the skin is reduced by adding a sufficient volume of 70 % ethanol to cover the epidermis. After a few seconds, the ethanol is removed from the tube and the tissue is then hydrated by the addition of 3 mL $MgSO_4$ solution (154 mM). The databridge electrodes are placed on either side of the skin disc to measure the resistance in kΩ/skin disc (Figure 1). Electrode dimensions and the length of the electrode exposed below the crocodile clips are shown in Figure 2. The clip attached to the inner electrode is rested on the top of the PTFE tube during resistance measurement to ensure that a consistent length of electrode is submerged in the $MgSO_4$ solution. The outer electrode is positioned inside the receptor chamber so that it rests on the bottom of the chamber. The distance

between the spring clip and the bottom of the PTFE tube is maintained as a constant (Figure 2), because this distance affects the resistance value obtained. Consequently, the distance between the inner electrode and the skin disc should be constant and minimal (1-2 mm).

If the measured resistance value is greater than 20 kΩ, this may be due to the remains of the test substance coating the epidermal surface of the skin disc. Further removal of this coating can be attempted, for example, by sealing the PTFE tube with a gloved thumb and shaking it for approximately 10 seconds; the $MgSO_4$ solution is discarded and the resistance measurement is repeated with fresh $MgSO_4$.

The properties and dimensions of the test apparatus and the experimental procedure used may influence the TER values obtained. The 5 kΩ corrosive threshold was developed from data obtained with the specific apparatus and procedure described in this method. Different threshold and control values may apply if the test conditions are altered or a different apparatus is used. Therefore, it is necessary to calibrate the methodology and resistance threshold values by testing a series of reference standards chosen from the chemicals used in the validation study (4)(5), or from similar chemical classes to the chemicals being investigated. A set of suitable reference chemicals is shown in Table 1.

1.5.5. **Dye binding methods**

Exposure of certain non-corrosive materials can result in a reduction of resistance below the cut-off of 5 kΩ allowing the passage of ions through the stratum corneum, thereby reducing the electrical resistance (5). For example, neutral organics and chemicals that have surface-active properties (including detergents, emulsifiers and other surfactants) can remove skin lipids making the barrier more permeable to ions. Thus, if the TER values of test substances are less than or around 5 kΩ in the absence of visual damage, an assessment of dye penetration should be carried out on the control and treated tissues to determine if the TER values obtained were the result of increased skin permeability, or skin corrosion (3)(5). In case of the latter where the stratum corneum is disrupted, the dye sulforhodamine B, when applied to the skin surface rapidly penetrates and stains the underlying tissue. This particular dye is stable to a wide range of chemicals and is not affected by the extraction procedure described below.

1.5.5.1. *Sulforhodamine B dye application and removal*

Following TER assessment, the magnesium sulfate is discarded from the tube and the skin is carefully examined for obvious damage. If there is no obvious major damage, Sulforhodamine B dye (Acid Red 52; C.I. 45100; EINECS Number 222-529-8; CAS number 3520-42-1), 150 μL of a 10 % (w/v) dilution in distilled water, is applied to the epidermal surface of each skin disc for two hours. These skin discs are then washed with tap water at up to room temperature for approximately 10 seconds to remove any excess/unbound dye. Each skin disc is carefully removed from the PTFE tube and placed in a vial (e.g. a 20 mL glass scintillation vial) containing deionised water (8 mL). The vials are agitated gently for five minutes to remove any additional unbound dye. This rinsing procedure is then repeated, after which the skin discs are removed and placed into vials containing 5 ml of 30 % (w/v) sodium dodecyl sulphate (SDS) in distilled water and are incubated overnight at 60 °C.

After incubation, each skin disc is removed and discarded and the remaining solution is centrifuged for eight minutes at 21 °C (relative centrifugal force ~175 × g). A 1 ml sample of the supernatant is diluted 1 in 5 (v/v) [i.e. 1 mL + 4 mL] with 30 % (w/v) SDS in distilled water. The optical density (OD) of the solution is measured at 565 nm.

1.5.5.2. *Calculation of dye content*

The sulforhodamine B dye content per disc is calculated from the OD values (5) (sulforhodamine B dye molar extinction coefficient at 565 nm = $8{,}7 \times 10^4$; molecular weight = 580). The dye content is determined for each skin disc by the use of an appropriate calibration curve and a mean dye content is then calculated for the replicates.

2. **DATA**

Resistance values (kΩ) and mean dye content values (μg/disc), where appropriate, for the test material, as well as for positive and negative controls should be reported in tabular form (individual trial data and means ± S.D.), including data for replicates/repeat experiments, mean and individual values.

2.1. INTERPRETATION OF RESULTS

The mean TER results are accepted if the concurrent positive and negative control values fall within the acceptable ranges for the method in the testing laboratory. The acceptable resistance ranges for the methodology and apparatus described above are given in the following table:

Control	Substance	Resistance range (kΩ)
Positive	10M Hydrochloric acid	0,5-1,0
Negative	Distilled water	10-25

The mean dye binding results are accepted on condition that concurrent control values fall within the acceptable ranges for the method. Suggested acceptable dye content ranges for the control substances for the methodology and apparatus described above are given below:

Control	Substance	Dye content range (μg/disc)
Positive	10M Hydrochloric acid	40-100
Negative	Distilled water	15-35

The test substance is considered to be non-corrosive to skin:

(i) if the mean TER value obtained for the test substance is greater than 5 kΩ; or

(ii) the mean TER value is less than or equal to 5 kΩ; and

— the skin disc is showing no obvious damage, and

— the mean disc dye content is well below the mean disc dye content of the 10M HCl positive control obtained concurrently.

The test substance is considered to be corrosive to skin:

(i) if the mean TER value is less than or equal to 5 kΩ and the skin disk is obviously damaged; or

(ii) the mean TER value is less than or equal to 5 kΩ; and

— the skin disc is showing no obvious damage, but

— the mean disc dye content is greater than or equal to the mean disc dye content of the 10M HCl positive control obtained concurrently.

3. **REPORTING**

3.1. TEST REPORT

The test report must include the following information:

Test and control substances:

— chemical name(s) such as IUPAC or CAS name and CAS number, if known,

— purity and composition of the substance or preparation (in percentage(s) by weight) and physical natur,

— physico-chemical properties such as physical state, pH, stability, water solubility, relevant to the conduct of the study,

— treatment of the test/control substances prior to testing, if applicable (e.g. warming, grinding),

— stability, if known.

Test animals:

— strain and sex used,

— age of the animals when used as donor animals,

— source, housing condition, diet, etc,

— details of the skin preparation.

Test conditions:

— calibration curves for test apparatus,

— calibration curves for dye binding test performance,

— details of the test procedure used for TER measurements,

— details of the test procedure used for the dye binding assessment; if appropriate,

— description of any modification of the test procedures,

— description of evaluation criteria used.

Results:

— tabulation of data from the TER and dye binding assay (if appropriate) for individual animals and individual skin samples;

— description of any effects observed.

Discussion of the results.

Conclusions.

4. **REFERENCES**

(1) OECD, (2001) Harmonised Integrated Classification System for Human Health and Environmental Hazards of Chemical Substances and Mixtures. OECD Series on Testing and Assessment Number 33. ENV/JM/MONO(2001)6, Paris. http://www.olis.oecd.org/olis/2001doc.nsf/LinkTo/env-jm-mono(2001)6.

(2) Testing Method B.4. Acute Toxicity: Dermal Irritation/Corrosion.

(3) Botham, P.A., Chamberlain, M., Barratt, M.D., Curren, R.D., Esdaile, D.J., Gardner, J.R., Gordon, V.C., Hildebrand, B., Lewis, R.W., Liebsch, M., Logemann, P., Osborne, R., Ponec, M., Regnier, J.F., Steiling, W., Walker, A.P., and Balls, M., (1995) A prevalidation study on *in vitro* skin corrosivity testing. The report and recommendations of ECVAM Workshop 6. ATLA 23, p. 219-255.

(4) Barratt, M.D., Brantom, P.G., Fentem, J.H., Gerner, I., Walker, A.P., and Worth, A.P. (1998). The ECVAM international validation study on *in vitro* tests for skin corrosivity. 1. Selection and distribution of the test chemicals. Toxicology. *In Vitro* 12, p. 471-482.

(5) Fentem, J.H., Archer, G.E.B., Balls, M., Botham, P.A., Curren, R.D., Earl, L.K., Esdaile, D.J., Holzhütter, H.-G., and Liebsch, M., (1998) The ECVAM international validation study on *in vitro* tests for skin corrosivity. 2. Results and evaluation by the Management Team. Toxicology. *In Vitro* 12, p. 483- 524.

(6) OECD, (1996) Final Report of the OECD Workshop on Harmonisation of Validation and Acceptance Criteria for Alternative Toxicological Test Methods, p. 62.

(7) Balls, M., Blaauboer, B.J., Fentem. J.H., Bruner. L., Combes, R.D., Ekwall, B., Fielder. R.J., Guillouzo, A., Lewis, R.W., Lovell, D.P., Reinhardt, C.A., Repetto, G., Sladowski. D., Spielmann, H., and Zucco, F., (1995) Practical aspects of the validation of toxicity test procedures. The report and recommendations of ECVAM workshops. ATLA 23, p. 129-147.

(8) ICCVAM, (Interagency Coordinating Committee on the Validation of Alternative Methods)., (1997) Validation and Regulatory Acceptance of Toxicological Test Methods. NIH Publication No 97-3981. National Institute of Environmental Health Sciences, Research Triangle Park, NC, USA. http://iccvam.niehs.nih.gov/docs/guidelines/validate.pdf.

(9) ECVAM, (1998) ECVAM News & Views. ATLA 26, 275-280.

(10) ICCVAM (Interagency Coordinating Committee on the Validation of Alternative Methods)., (2002) ICCVAM evaluation of EpiDerm™, EPISKIN™ (EPI-200), and the Rat Skin Transcutaneous Electrical Resistance (TER) assay: In Vitro test methods for assessing dermal corrosivity potential of chemicals. NIH Publication No 02-4502. National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods, National Institute of Environmental Health Sciences, Research Triangle Park, NC, USA. http://iccvam.niehs.nih.gov/methods/epiddocs/epis_brd.pdf.

(11) OECD (2002) Extended Expert Consultation Meeting on The In Vitro Skin Corrosion Test Guideline Proposal, Berlin, 1st–2nd November 2001, Secretariat's Final Summary Report, 27th March 2002, OECD ENV/EHS, available upon request from the Secretariat.

(12) Oliver, G.J.A., Pemberton, M.A., and Rhodes, C., (1986) An *in vitro* skin corrosivity test -modificationsand validation. Fd. Chem. Toxicol. 24, 507-512.

(13) Botham, P.A., Hall, T.J., Dennett, R., McCall, J.C., Basketter, D.A., Whittle, E., Cheeseman, M., Esdaile, D.J., and Gardner, J., (1992) The skin corrosivity test *in vitro:* results of an interlaboratory trial. Toxic. *In Vitro* 6, p. 191-194.

(14) Worth A.P., Fentem J.H., Balls, M., Botham, P.A., Curren, R.D., Earl, L.K., Esdaile D.J., Liebsch, M., (1998) An Evaluation of the Proposed OECD Testing Strategy for Skin Corrosion. ATLA 26, p. 709-720

(15) Basketter, D.A., Chamberlain, M., Griffiths, H.A., Rowson, M., Whittle, E., York, M., (1997) The classification of skin irritants by human patch test. Fd. Chem. Toxicol. 35, p. 845-852.

(16) Oliver G.J.A, Pemberton M.A and Rhodes C., (1988) An In Vitro model for identifying skin-corrosive chemicals. I. Initial Validation. Toxicology *In Vitro*. 2, p. 7-17.

Figure 1

Apparatus for the rat skin TER assay

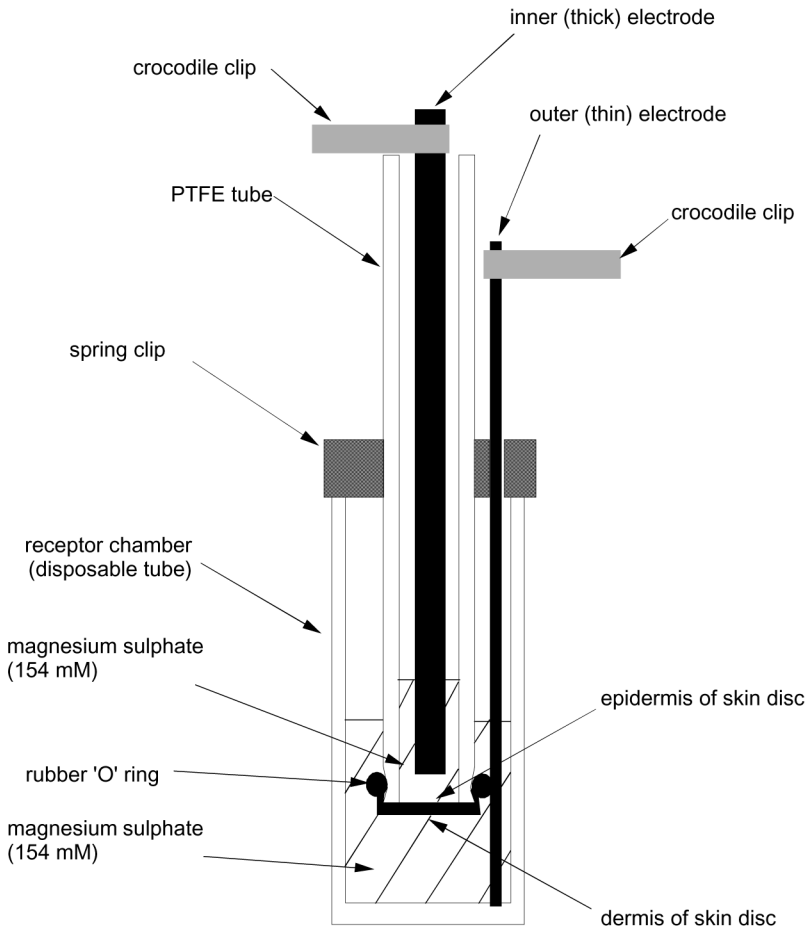
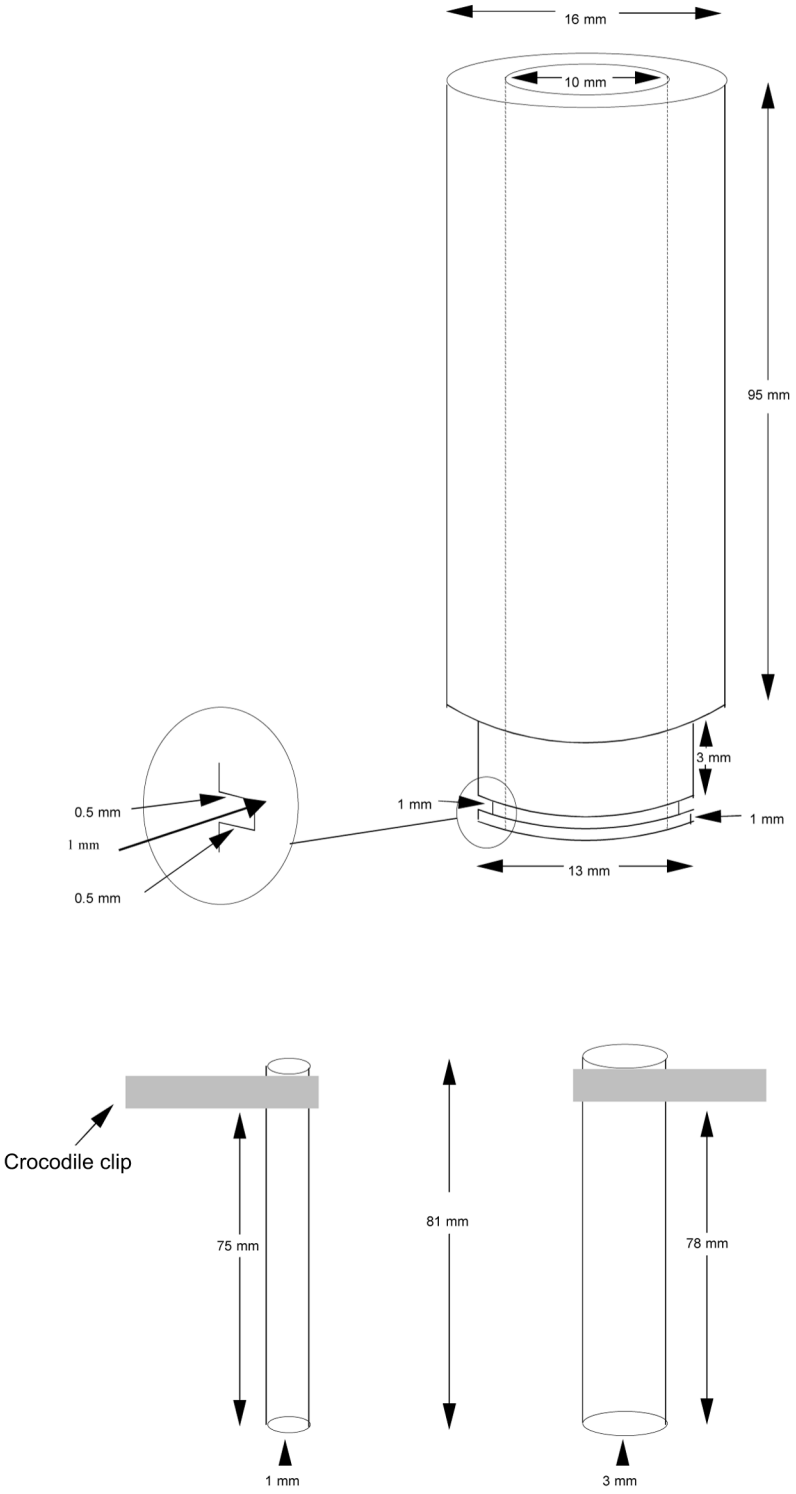


Figure 2

Dimensions of the polytetrafluoroethylene (PFTE) and receptor tubes and electrodes used



Critical factors of the apparatus shown above:

— the inner diameter of the PTFE tube,

— the length of the electrodes relative to the PTFE tube and receptor tube, such that the skin disc is not touched by the electrodes and that a standard length of electrode is in contact with the $MgSO_4$ solution,

— the amount of $MgSO_4$ solution in the receptor tube should give a depth of liquid, relative to the level in the PFTE tube, as shown in Figure 1,

— the skin disk should be fixed well enough to the PFTE tube, such that the electrical resistance is a true measure of the skin properties.

B.40 BIS. ***IN VITRO* SKIN CORROSION: HUMAN SKIN MODEL TEST**

1. **METHOD**

This testing method is equivalent to the OECD TG 431 (2004).

1.1. INTRODUCTION

Skin corrosion refers to the production of irreversible tissue damage in the skin following the application of a test material [as defined by the Globally Harmonised System for the Classification and Labelling of Chemical Substances and Mixtures (GHS)] (1). This Testing Method does not require the use of live animals or animal tissue for the assessment of skin Corrosivity.

The assessment of skin corrosivity has typically involved the use of laboratory animals (2). Concern for the pain and suffering involved with this procedure has been addressed in the revision of testing method B.4 that allows for the determination of skin corrosion by using alternative, *in vitro*, methods, avoiding pain and suffering of animals.

A first step towards defining alternative tests that could be used for skin corrosivity testing for regulatory purposes was the conduct of prevalidation studies (3). Following this, a formal, validation study of *in vitro* methods for assessing skin corrosion (4)(5) was conducted (6)(7)(8). The outcome of these studies and other published literature (9) led to the recommendation that the following tests could be used for the assessment of the *in vivo* skin corrosivity (10)(11)12)(13): the human skin model test (this method) and the transcutaneous electrical resistance test (see testing method B.40).

Validation studies have reported that tests employing human skin models (3)(4)(5)(9) are able to reliably discriminate between known skin corrosives and non-corrosives. The test protocol may also provide an indication of the distinction between severe and less severe skin corrosives.

The test described in this method allows the identification of corrosive chemical substances and mixtures. It further allows the identification of non-corrosive substances and mixtures when supported by a weight of evidence determination using other existing information (e.g. pH, structure-activity relationships, human and/or animal data) (1)(2)(13)(14). It does not normally provide adequate information on skin irritation, nor does it allow the subcategorisation of corrosive substances as permitted in the Globally Harmonised Classification System (GHS) (1).

For a full evaluation of local skin effects after single dermal exposure, it is recommended to follow the sequential testing strategy as appended to testing method B.4 (2) and provided in the Globally Harmonised System (GHS) (1). This testing strategy includes the conduct of *in vitro* tests for skin corrosion (as described in this method) and skin irritation before considering testing in live animals.

1.2. DEFINITIONS

Skin corrosion *in vivo*: is the production of irreversible damage of the skin: namely, visible necrosis through the epidermis and into the dermis, following the application of a test subsance for up to four hours. Corrosive reactions are typified by ulcers, bleeding, bloody scabs, and, by the end of the observation at 14 days, by discolouration due to blanching of the skin, complete areas of alopecia, and scars. Histopathology should be considered to evaluate questionable lesions.

Cell viability: parameter measuring total activity of a cell population (e.g. ability of cellular mitochondrial dehidrogenases to reduce the vital dye MTT), which, depending on the end point measured and the test design used, correlates with the total number and/or vitality of the cells.

1.3. REFERENCE SUBSTANCES

Table 1

Reference chemicals

Name	EINECS No	CAS No	
1,2-Diaminopropane	201-155-9	78-90-0	Severely corrosive

Name	EINECS No	CAS No	
Acrylic Acid	201-177-9	79-10-7	Severely corrosive
2-tert. Butylphenol	201-807-2	88-18-6	Corrosive
Potassium hydroxide (10 %)	215-181-3	1310-58-3	Corrosive
Sulfuric acid (10 %)	231-639-5	7664-93-9	Corrosive
Octanoic acid (caprylic acid)	204-677-5	124-07-02	Corrosive
4-Amino-1,2,4-triazole	209-533-5	584-13-4	Not corrosive
Eugenol	202-589-1	97-53-0	Not corrosive
Phenethyl bromide	203-130-8	103-63-9	Not corrosive
Tetrachloroethylene	204-825-9	27-18-4	Not Corrosive
Isostearic acid	250-178-0	30399-84-9	Not corrosive
4-(Methylthio)-benzaldehyde	222-365-7	3446-89-7	Not corrosive

Most of the chemicals listed are taken from the list of chemicals selected for the ECVAM international validation study (4). Their selection is based on the following criteria:

(i) equal number of corrosive and non-corrosive substances;

(ii) commercially available substances covering most of the relevant chemical classes;

(iii) inclusion of severely corrosive as well as less corrosive substances in order to enable discrimination based on corrosive potency;

(iv) choice of chemicals that can be handled in a laboratory without posing other serious hazards than corrosivity.

1.4. PRINCIPLE OF THE TEST METHOD

The test material is applied topically to a three-dimensional human skin model, comprising at least a reconstructed epidermis with a functional stratum corneum. Corrosive materials are identified by their ability to produce a decrease in cell viability (as determined, for example, by using the MTT reduction assay (15)) below defined threshold levels at specified exposure periods. The principle of the human skin model assay is based on the hypothesis that corrosive chemicals are able to penetrate the stratum corneum by diffusion or erosion, and are cytotoxic to the underlying cell layers.

1.4.1. **Procedure**

1.4.1.1. *Human skin models*

Human skin models can be constructed or obtained commercially (e.g. the EpiDerm™ and EPISKIN™ models) (16)(17)(18)(19) or be developed or constructed in the testing laboratory (20)(21). It is recognised that the use of human skin is subject to national and international ethical considerations and conditions. Any new model should be validated (at least to the extent described under 1.4.1.1.2). Human skin models used for this test must comply with the following:

1.4.1.1.1. General model conditions:

Human keratinocytes should be used to construct the epithelium. Multiple layers of viable epithelial cells should be present under a functional stratum corneum. The skin model may also have a stromal component layer. Stratum corneum should be multi-layered with the necessary lipid profile to produce a functional barrier with robustness to resist rapid penetration of cytotoxic markers. The containment properties of the model should prevent passage of material around the stratum corneum to the viable tissue. Passage of test chemicals around the stratum corneum will lead to poor modeling of the exposure to skin. The skin model should be free of contamination with bacteria (including mycoplasma) or fungi.

1.4.1.1.2. Functional model conditions:

The magnitude of viability is usually quantified by using MTT or the other metabolically converted vital dyes. In these cases the optical density (OD) of the extracted (solubilised) dye from the negative control tissue should be at least 20 fold greater than the OD of the extraction solvent alone (for an overview, see (22)). The negative control tissue should be stable in culture (provide similar viability measurements) for the duration of the test exposure period. The stratum corneum should be sufficiently robust to resist the rapid penetration of certain cytotoxic marker chemicals (e.g. 1 % Triton X-100). This property can be estimated by the exposure time required to reduce cell viability by 50 % (ET_{50}) (e.g. for the EpiDerm™ and EPISKIN™ models this is > 2 hours). The tissue should demonstrate reproductivity over time and preferably between laboratories. Moreover it should be capable of predicting the corrosive potential of the reference chemicals (see Table 1) when used in the testing protocol selected.

1.4.1.2. *Application of the test and control substances*

Two tissue replicates are used for each treatment (exposure time), including controls. For liquid materials, sufficient test substance must be applied to uniformly cover the skin surface: a minimum of 25 $\mu L/cm^2$ should be used. For solid materials, sufficient test substance must be applied evenly to cover the skin, and it should be moistened with deionised or distilled water to ensure good contact with the skin. Where appropriate, solids should be ground to a powder before application. The application method should be appropriate for the test substance (see e.g. reference 5). At the end of the exposure period, the test material must be carefully washed from the skin surface with an appropriate buffer, or0,9 % NaCl.

Concurrent positive and negative controls should be used for each study to ensure adequate performance of the experimental model. The suggested positive control substances are glacial acetic acid or 8N KOH. The suggested negative controls are 0,9 % NaCl or water.

1.4.1.3. *Cell viability measurements*

Only quantitative, validated, methods can be used to measure cell viability. Furthermore, the measure of viability must be compatible with use in a three-dimensional tissue construct. Non-specific dye binding must not interfere with the viability measurement. Protein binding dyes and those which do not undergo metabolic conversion (e.g. neutral red) are therefore not appropriate. The most frequently used assay is MTT (3-(4,5-Dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide, Thiazolyl blue: EINECS number 206-069-5, CAS number 298-93-1)) reduction, which has been shown to give accurate and reproducible results (5) but others may be used. The skin sample is placed in an MTT solution of appropriate concentration (e.g. 0,3-1 mg/mL) at appropriate incubation temperature for three hours. The precipitated blue formazan product is then extracted using a solvent (isopropanol), and the concentration of the formazan is measured by determining the OD at wavelength between 540 and 595 nm.

Chemical action by the test material on the vital dye may mimic that of cellular metabolism leading to a false estimate of viability. This has been shown to happen when such a test material is not completely removed from the skin by rinsing (9). If the test material directly acts on the vital dye, additional controls should be used to detect and correct for the test substances interference with the viability measurement (9)(23).

2. **DATA**

For each tissue, OD values and calculated percentage cell viability data for the test material, positive and negative controls, should be reported in tabular form, including data from replicate repeat experiments as appropriate, mean and individual values.

2.1. INTERPRETATION OF RESULTS

The OD values obtained for each test sample can be used to calculate a percentage viability relative to the negative control, which is arbitrarily set at 100 %. The cut-off percentage cell viability value distinguishing corrosive from non-corrosive test materials (or discriminating between different, corrosive classes), or the statistical procedure(s) used to evaluate the results and identify corrosive materials, must be clearly defined and documented, and be shown to be appropriate. In general, these cut-off values are established during test optimisation, tested during a prevalidation phase, and confirmed in a validation study. As an example, the prediction of Corrosivity associated with the EpiDerm™ model is (9):

The test substance is considered to be corrosive to skin:

(i) if the viability after three minutes exposure is less than 50 %; or

(ii) if the viability after three minutes exposure is greater than or equal to 50 % and the viability after 1 hour exposure is less than 15 %.

The test substance is considered to be non-corrosive to skin:

(i) if the viability after three minutes exposure is greater than or equal to 50 % and the viability after 1 hour exposure is greater than or equal to 15 %.

3. **REPORTING**

3.1. TEST REPORT

The test report must include the following information:

Test and control substance:

— chemical names(s) such as IUPAC or CAS name and CAS number, if known,

— purity and composition of the substance or preparation (in percentage(s) by weight),

— physico-chemical properties such as physical state, pH, stability, water solubility relevant to the conduct of the study,

— treatment of the test/control substances prior to testing, if applicable (e.g. warming, grinding),

— stability, if known.

Justification of the skin model and protocol used.

Test conditions:

— cell system used,

— calibration information for measuring device used for measuring cell viability (e.g. Spectrophotometer),

— complete supporting information for the specific skin model used including its validity,

— details of the test procedure used,

— test doses used,

— description of any modifications of the test procedure,

— reference to historical data of the model,

— description of evaluation criteria used.

Results:

— tabulation of data from individual test samples,

— description of other effects observed.

Discussion of the results.

Conclusion.

4. **REFERENCES**

(1) OECD (2001) Harmonised Integrated Classification System for Human Health and Environmental Hazards of Chemical Substances and Mixtures. OECD Series on Testing and Assessment Number 33. ENV/JM/MONO(2001)6, Paris. http://www.olis.oecd.org/olis/2001doc.nsf/LinkTo/env-jm-mono(2001)6.

(2) Testing Method B.4. Acute Toxicity: Dermal Irritation/Corrosion.

(3) Botham, P.A., Chamberlain, M., Barratt, M.D., Curren, R.D., Esdaile, D.J., Gardner, J.R., Gordon, V.C., Hildebrand, B., Lewis, R.W., Liebsch, M., Logemann, P., Osborne, R., Ponec, M., Regnier, J.F., Steiling, W., Walker, A.P., and Balls, M., (1995) A prevalidation study on *in vitro* skin corrosivity testing. The report recommendations of ECVAM Workshop 6 ATLA 23, p. 219-255.

(4) Barratt, M.D., Brantom, P.G., Fentem, J.H., Gerner, I., Walker, A.P., and Worth, A.P. (1998). The ECVAM international validation study on *in vitro* tests for skin corrosivity. 1. Selection and distribution of the test chemicals. Toxicology In Vitro 12, p. 471-482.

(5) Fentem, J.H., Archer, G.E.B., Balls, M., Botham, P.A., Curren, R.D., Earl, L.K., Esdaile, D.J., Holzhutter, H.G. and Liebsch, M., (1998) The ECVAM international validation study on *in vitro* tests for skin corrosivity. 2. Results and evaluation by the Management Team. Toxicology In Vitro 12, p. 483-524.

(6) OECD, (1996) Final Report of the OECD Workshop on Harmonisation of Validation and Acceptance Criteria for Alternative Toxicological Test Methods, p. 62.

(7) Balls, M., Blaauboer, B.J., Fentem, J.H., Bruner, L., Combes, R.D., Ekwall, B., Fielder, R.J., Guillouzo, A., Lewis, R.W., Lovell, D.P., Reinhardt, C.A., Repetto, G., Sladowski, D., Spielmann, H., and Zucco, F., (1995) Practical aspects of the validation of toxicity test procedures. Test report and recommendations of ECVAM workshops. ATLA 23, p. 129-147.

(8) ICCVAM (Interagency Coordinating Committee on the Validation of Alternative Methods)., (1997) Validation and Regulatory Acceptance of Toxicological Test Methods. NIH Publication No 97-3981. National Institute of Environmental Health Sciences, Research Triangle Park, NC, USA. http://iccvam.niehs.nih.gov/docs/guidelines/validate.pdf.

(9) Liebsch, M., Traue, D., Barrabas, C., Spielmann, H., Uphill, P., Wilkins, S., McPherson, J.P., Wiemann, C., Kaufmann, T., Remmele, M. and Holzhutter, H.G., (2000) The ECVAM prevalidation study on the use of EpiDerm for skin Corrosivity testing. ATLA 28, p. 371-401.

(10) ECVAM, (1998) ECVAM News & Views. ATLA 26, p. 275-280.

(11) ECVAM, (2000) ECVAM News & Views. ATLA 28, p. 365-67.

(12) ICCVAM (Interagency Coordinating Committee on the Validation of Alternative Methods)., (2002) ICCVAM evaluation of EpiDerm™, EPISKIN™ (EPI-200), and the Rat Skin Transcutaneous Electrical Resistance (TER) assay: In Vitro test methods for assessing dermal corrosivity potential of chemicals. NIH Publication No 02-4502. National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods, National Institute of Environmental Health Sciences, Research Triangle Park, NC, USA. http://iccvam.niehs.nih.gov/methods/epiddocs/epis_brd.pdf.

(13) OECD, (2002) Extended Expert Consultation Meeting on The In Vitro Skin Corrosion Test Guideline Proposal, Berlin, 1st–2nd November 2001, Secretariat's Final Summary Report, 27th March 2002, OECD ENV/EHS, available upon request from the Secretariat

(14) Worth, A.P., Fentem, J.H., Balls, M., Botham, P.A., Curren, R.D., Earl, L.K., Esdaile, D.J., Liebsch, M., (1998) An Evaluation of the Proposed OECD Testing Strategy for Skin Corrosion. ATLA 26, p. 709-720.

(15) Mosmann, T., (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity asssays. J.Immunol. Meth. 65, p. 55-63.

(16) Cannon, C.L., Neal, P.J., Southee, J.A., Kubilus, J., and Klausner, M., 1994. New epidermal model for dermal irritancy testing. Toxicology *In Vitro* 8, p. 889-891.

(17) Ponec, M., Boelsma, E., Weerheim, A., Mulder, A., Boutwstra, J., and Mommaas, M., 2000. Lipid and ultrastructural characterisation of reconstructed skin models. International Journal of Pharmaceutics. 203, p. 211-225.

(18) Tinois, E., Gaetani, Q., Gayraud, B., Dupont, D., Rougier, A., Pouradier, D.X., (1994) The Episkin model: Successful reconstruction of human epidermis in vitro. In *In vitro* Skin Toxicology. Edited by A Rougier, AM Goldberg and HI Maibach, p. 133-140

(19) Tinois E, Tiollier J, Gaucherand M, Dumas H, Tardy M, Thivolet J (1991). *In vitro* and post–transplantation differentiation of human keratinocytes grown on the human type IV collagen film of a bilayered dermal substitute. Experimental Cell Research 193, p. 310-319

(20) Parentau, N.L., Bilbo, P., Molte, C.J., Mason, V.S., and Rosenberg, H., (1992) The organotypic culture of human skin keratinocytes and fibroblasts to achieve form and function. Cytotechnology 9, p. 163-171.

(21) Wilkins, L.M., Watson, S.R., Prosky, S.J., Meunier, S.F., Parentau, N.L., (1994) Development of a bilayered living skin construct for clinical applications. Biotechnology and Bioengineering 43/8, p. 747-756.

(22) Marshall, N.J., Goodwin, C.J., Holt, S.J., (1995) A critical assessment of the use of microculture tetrazolium assays to measure cell growth and function. Growth Regulation 5, p. 69-84.

(23) Fentem, J.H., Briggs, D., Chesne', C., Elliot, G.R., Harbell, J.W., Heylings, J.R., Portes, P., Rouget, R., and van de Sandt, J.J.M., and Botham, P.A., (2001) A prevalidation study on *in vitro* tests for acute skin irritation: results and evaluation by the Management Team. Toxicology *In Vitro* 15, p. 57-93.

B.41. *IN VITRO* 3T3 NRU PHOTOTOXICITY TEST

1. METHOD

This method is equivalent to OECD TG 432 (2004).

1.1. INTRODUCTION

Phototoxicity is defined as a toxic response from a substance applied to the body which is either elicited or increased (apparent at lower dose levels) after subsequent exposure to light, or that is induced by skin irradiation after systemic administration of a substance.

The *in vitro* 3T3 NRU phototoxicity test is used to identify the phototoxic potential of a test substance induced by the excited chemical after exposure to light. The test evaluates photo-cytotoxicity by the relative reduction in viability of cells exposed to the chemical in the presence versus absence of light. Substances identified by this test are likely to be phototoxic *in vivo* following systemic application and distribution to the skin, or after topical application.

Many types of chemicals have been reported to induce phototoxic effects (1)(2)(3)(4). Their common feature is their ability to absorb light energy within the sunlight range. According to the first law of photochemistry (Grotthaus-Draper Law), photoreaction requires sufficient absorption of light quanta. Thus, before biological testing is considered, a UV/vis absorption spectrum of the test chemical must be determined according to OECD Test Guideline 101. It has been suggested that if the molar extinction/absorption coefficient is less than 10 litre × mol^{-1} × cm^{-1} the chemical is unlikely to be photoreactive. Such chemical may not need to be tested in the *in vitro* 3T3 NRU phototoxicity test or any other biological test for adverse photochemical effects (1)(5). See also Appendix 1.

The reliability and relevance of the *in vitro* 3T3 NRU phototoxicity test was recently evaluated (6)(7)(8) (9). The *in vitro* 3T3 NRU phototoxicity test was shown to be predictive of acute phototoxicity effects in animals and humans *in vivo*. The test is not designed to predict other adverse effects that may arise from combined action of a chemical and light, e.g. it does not address photogenotoxicity, photoallergy, or photocarcinogenicity, nor does it allow an assessment of phototoxic potency. In addition, the test has not been designed to address indirect mechanisms of phototoxicity, effects of metabolites of the test substance, or effects of mixtures.

Whereas the use of metabolising systems is a general requirement for all *in vitro* tests for the prediction of genotoxic and carcinogenic potential, up to now, in the case of phototoxicology, there are only rare examples where metabolic transformation is needed for the chemical to act as a phototoxin *in vivo* or *in vitro*. Thus, it is neither considered necessary nor scientifically justified for the present test to be performed with a metabolic activation system.

1.2. DEFINITIONS

Irradiance: the intensity of ultraviolet (UV) or visible light incident on a surface, measured in W/m^2 or mW/cm^2.

Dose of light: the quantity (= intensity × time) of ultraviolet (UV) or visible radiation incident on a surface, expressed in Joules (= W × s) per surface area, e.g. J/m^2 or J/cm^2.

UV light wavebands: the designations recommended by the CIE (Commission Internationale de L'Eclairage) are: UVA (315-400 nm), UVB (280-315 nm) and UVC (100-280 nm). Other designations are also used; the division between UVB and UVA is often placed at 320 nm, and the UVA may be divided into UV-A1 and UV-A2 with a division made at about 340 nm.

Cell viability: parameter measuring total activity of a cell population (e.g. uptake of the vital dye Neutral Red into cellular lysosomes), which, depending on the endpoint measured and the test design used, correlates with the total number and/or vitality of the cells.

Relative cell viability: cell viability expressed in relation of solvent (negative) controls which have been taken through the whole test procedure (either +Irr or -Irr) but not treated with test chemical.

PIF (Photo-Irritation-Factor): factor generated by comparing two equally effective cytotoxic concentrations (IC_{50}) of the test chemical obtained in the absence (-Irr) and in the presence (+Irr) of a non-cytotoxic irradiation with UVA/vis light.

IC_{50}: the concentration of the test chemical by which the cell viability is reduced by 50 %.

MPE (Mean-Photo-Effect): measurement derived from mathematical analysis of the concentration response curves obtained in the absence (-Irr) and in the presence (+Irr) of a non-cytotoxic irradiation with UVA/vis light.

Phototoxicity: acute toxic response that is elicited after the first exposure of skin to certain chemicals and subsequent exposure to light, or that is induced similarly by skin irradiation after systemic administration of a chemical.

1.3. PRINCIPLE OF THE TEST METHOD

The *in vitro* 3T3 NRU phototoxicity test is based on a comparison of the cytotoxicity of a chemical when tested in the presence and in the absence of exposure to a non-cytotoxic dose of simulated solar light. Cytotoxicity in this test is expressed as a concentration-dependent reduction of the uptake of the vital dye Neutral Red when measured 24 hours after treatment with the test chemical and irradiation (10). NR is a weak cationic dye that readily penetrates cell membranes by non-diffusion, accumulating intracellulary in lysosomes. Alterations of the surface of the sensitive lysosomal membrane lead to lysosomal fragility and other changes that gradually become irreversible. Such changes brought about by the action of xenobiotics result in a decreased uptake and binding of NR. It is thus possible to distinguish between viable, damaged or dead cells, which is the basis of this test.

Balb/c 3T3 cells are maintained in culture for 24 h for formation of monolayers. Two 96-well plates per test chemical are pre-incubated with eight different concentrations of the test substance for 1 h. Thereafter one of the two plates is exposed to the highest non-cytotoxic irradiation dose whereas the other plate is kept in the dark. In both plates the treatment medium is then replaced by culture medium and after another 24 h of incubation cell viability is determined by Neutral Red uptake. Cell viability is expressed as percentage of untreated solvent controls and is calculated for each test concentration. To predict the phototoxic potential, the concentration responses obtained in the presence and in the absence of irradiation are compared, usually at the IC_{50} level, i.e., the concentration reducing cell viability to 50 % compared to the untreated controls.

1.4. DESCRIPTION OF THE TEST METHOD

1.4.1. **Preparations**

1.4.1.1. *Cells*

A permanent mouse fibroblast cell line, Balb/c 3T3, clone 31, either from the American Type Culture Collection (ATCC), Manassas, VA, USA, or from the European Collection of Cell Cultures (ECACC), Salisbury, Wiltshire, UK, was used in the validation study, and therefore is recommended to obtain from a well qualified cell depository. Other cells or cell lines may be used with the same test procedure if culture conditions are adapted to the specific needs of the cells, but equivalency must be demonstrated.

Cells should be checked regularly for the absence of mycoplasma contamination and only used if none is found (11).

It is important that UV sensitivity of the cells is checked regularly according to the quality control procedure described in this method. Because the UVA sensitivity of cells may increase with the number of passages, Balb/c 3T3 cells of the lowest obtainable passage number, preferably less than 100, should be used. (See Section 1.4.2.2.2 and Appendix 2).

1.4.1.2. *Media and culture conditions*

Appropriate culture media and incubation conditions should be used for routine cell passage and during the test procedure, e.g. for Balb/c 3T3 cells these are DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10 % new-born calf serum, 4 mM glutamine, penicillin (100 IU), and streptomycin (100 µg/mL), and humidified incubation at 37 °C, 5-7,5 % CO_2 depending on the buffer (See Section 1.4.1.4, second paragraph.). It is particularly important that cell culture conditions assure a cell cycle time within the normal historical range of the cells or cell line used.

1.4.1.3. *Preparation of cultures*

Cells from frozen stock cultures are seeded in culture medium at an appropriate density and subcultured at least once before they are used in the *in vitro* 3T3 NRU phototoxicity test.

Cells used for the phototoxicity test are seeded in culture medium at the appropriate density so that cultures will not reach confluence by the end of the test, i.e., when cell viability is determined 48 h after seeding of the cells. For Balb/c 3T3 cells grown in 96-well plates, the recommended cell seeding density is 1×10^4 cells per well.

For each test chemical cells are seeded identically in two separate 96-well plates, which are then taken concurrently through the entire test procedure under identical culture conditions except for the time period where one of the plates is irradiated (+Irr) and the other one is kept in the dark (-Irr).

1.4.1.4. *Preparation of test substance*

Test substances must be prepared fresh, immediately prior to use unless data demonstrate their stability in storage. It is recommended that all chemical handling and the initial treatment of cells be performed under light conditions that would avoid photoactivation or degradation of the test substance prior to irradiation.

Test chemicals shall be dissolved in buffered salt solutions, e.g. Earle's Balanced Salt Solution (EBSS), or other physiologically balanced buffer solutions, which must be free from protein components, light absorbing components (e.g. pH-indicator colours and vitamins) to avoid interference during irradiation. Since during irradiation cells are kept for about 50 minutes outside of the CO_2 incubator, care has to be taken to avoid alkalisation. If weak buffers like EBSS are used this can be achieved by incubating the cells at 7,5 % CO_2. If the cells are incubated at 5 % CO_2 only, a stronger buffer should be selected.

Test chemicals of limited solubility in water should be dissolved in an appropriate solvent. If a solvent is used it must be present at a constant volume in all cultures, i.e. in the negative (solvent) controls as well as in all concentrations of the test chemical, and be noncytotoxic at that concentration. Test chemical concentrations should be selected so as to avoid precipitate or cloudy solutions.

Dimethylsulphoxide (DMSO) and ethanol (ETOH) are the recommended solvents. Other solvents of low cytotoxicity may be appropriate. Prior to use, all solvents should be assessed for specific properties, e.g. reaction with the test chemical, quenching of the phototoxic effect, radical scavenging properties and/or chemical stability in the solvent.

Vortex mixing and/or sonication and/or warming to appropriate temperatures may be used to aid solubilisation unless this would affect the stability of the test chemical.

1.4.1.5. *Irradiation conditions*

1.4.1.5.1. Light source

The choice of an appropriate light source and filters is a crucial factor in phototoxicity testing. Light of the UVA and visible regions is usually associated with phototoxic reactions *in vivo* (3)(12), whereas generally UVB is of less relevance but is highly cytotoxic; the cytotoxicity increases 1 000-fold as the wavelength goes from 313 to 280 nm (13). Criteria for the choice of an appropriate light source must include the requirement that the light source emits wavelengths absorbed by the test chemical (absorption spectrum) and that the dose of light (achievable in a reasonable exposure time) should be sufficient for the detection of known photocytotoxic chemicals. Furthermore, the wavelengths and doses employed should not be unduly deleterious to the test system, e.g. the emission of heat (infrared region).

Simulation of sunlight with solar simulators is considered the optimal artificial light source. The irradiation power distribution of the filtered solar simulator should be close to that of outdoor daylight given in (14). Both, Xenon arcs and (doped) mercury-metal halide arcs are used as solar simulators (15). The latter has the advantage of emitting less heat and being cheaper, but the match to sunlight is less perfect compared to that of xenon arcs. Because all solar simulators emit significant quantities of UVB they should be suitably filtered to attenuate the highly cytotoxic UVB wavelengths. Because cell culture plastic materials contain UV stabilisers the spectrum should be measured through the same type of 96-well plate lid as will be used in the assay. Irrespective of measures taken to attenuate parts of the spectrum by filtering or by unavoidable filter effects of the equipment the spectrum recorded below these filters should not deviate from standardised outdoor daylight (14). An example of the spectral irradiance distribution of the filtered solar simulator used in the validation study of the *in vitro* 3T3 NRU phototoxicity test is given in (8)(16). See also Appendix 2 Figure 1.

1.4.1.5.2. Dosimetry

The intensity of light (irradiance) should be regularly checked before each phototoxicity test using a suitable broadband UV-meter. The intensity should be measured through the same type of 96-well plate lid as will be used in the assay. The UV-meter must have been calibrated to the source. The performance of the UV-meter

should be checked, and for this purpose the use of a second, reference UV-meter of the same type and identical calibration is recommended. Ideally, at greater intervals, a spectroradiometer should be used to measure the spectral irradiance of the filtered light source and to check the calibration of the broadband UV-meter.

A dose of 5 J/cm^2 (as measured in the UVA range) was determined to be non-cytotoxic to Balb/c 3T3 cells and sufficiently potent to excite chemicals to elicit phototoxic reactions, (6) (17) e.g. to achieve 5 J/cm^2 within a time period of 50 min, irradiance was adjusted to 1,7 mW/cm^2. See Appendix 2 Figure 2. If another cell line or a different light source are used, the irradiation dose may have to be calibrated so that a dose regimen can be selected that is not deleterious to the cells but sufficient to excite standard phototoxins. The time of light exposure is calculated in the following way:

$$t(\text{min}) = \frac{\text{irradiation dose } (J/cm^2) \times 1000}{\text{irradiance } (mW/cm^2) \times 60} \qquad (1\ \text{J} = 1\ \text{Wsec})$$

1.4.2. **Test conditions**

1.4.2.1. *Test substance concentrations*

The ranges of concentrations of a chemical tested in the presence (+Irr) and in the absence (-Irr) of light should be adequately determined in dose range-finding experiments. It may be useful to assess solubility initially and at 60 min (or whatever treatment time is to be used), as solubility can change during time or during the course of exposure. To avoid toxicity induced by improper culture conditions or by highly acidic or alkaline chemicals, the pH of the cell cultures with added test chemical should be in the range 6,5 - 7,8.

The highest concentration of the test substance should be within physiological test conditions, e.g. osmotic and pH stress should be avoided. Depending on the test chemical, it may be necessary to consider other physico-chemical properties as factors limiting the highest test concentration. For relatively insoluble substances that are not toxic at concentrations up to the saturation point the highest achievable concentration should be tested. In general, precipitation of the test chemical at any of the test concentrations should be avoided. The maximum concentration of a test substance should not exceed 1 000 μg/mL; osmolarity should not exceed 10 mmolar. A geometric dilution series of eight test substance concentrations with a constant dilution factor should be used (See Section 2.1, second paragraph).

If there is information (from a range finding experiment) that the test chemical is not cytotoxic up to the limit concentration in the dark experiment (-Irr), but is highly cytotoxic when irradiated (+Irr), the concentration ranges to be selected for the (+Irr) experiment may differ from those selected for the (−Irr) experiment to fulfill the requirement of adequate data quality.

1.4.2.2. *Controls*

1.4.2.2.1. Radiation sensitivity of the cells, establishing of historical data:

Cells should be checked regularly (about every fifth passage) for sensitivity to the light source by assessing their viability following exposure to increasing doses of irradiation. Several doses of irradiation, including levels substantially greater than those used for the 3T3 NRU Phototoxicity test should be used in this assessment. These doses are easiest quantitated by measurements of UV parts of the light source. Cells are seeded at the density used in the *in vitro* 3T3 NRU phototoxicity test and irradiated the next day. Cell viability is then determined one day later using Neutral Red uptake. It should be demonstrated that the resulting highest non-cytotoxic dose (e.g. in the validation study: 5 J/cm^2 [UVA]) was sufficient to classify the reference chemicals (Table 1) correctly.

1.4.2.2.2. Radiation sensitivity, check of current test:

The test meets the quality criteria if the irradiated negative/solvent controls show a viability of more than 80 % when compared with non-irradiated negative/solvent.

1.4.2.2.3. Viability of solvent controls:

The absolute optical density ($OD_{540\ NRU}$) of the Neutral Red extracted from the solvent controls indicates whether the 1×10^4 cells seeded per well have grown with a normal doubling time during the two days of the assay. A test meets the acceptance criteria if the mean $OD_{540\ NRU}$ of the untreated controls is ≥ 0,4 (i.e. approximately 20 times the background solvent absorbance).

1.4.2.2.4. Positive control:

A known phototoxic chemical shall be tested concurrently with each *in vitro* 3T3 NRU phototoxicity test. Chlorpromazine (CPZ) is recommended. For CPZ tested with the standard protocol in the *in vitro* 3T3 NRU phototoxicity test, the following test acceptance criteria were defined: CPZ irradiated (+Irr): IC_{50} = 0,1 to 2,0 μg/ml, CPZ non-irradiated (-Irr): IC_{50} = 7,0 to 90,0 μg/mL. The Photo Irritation Factor (PIF), should be > 6. The historical performance of the positive control should be monitored.

Other phototoxic chemicals, suitable for the chemical class or solubility characteristics of the chemical being evaluated, may be used as the concurrent positive controls in place of chlorpromazine.

1.4.3. **Test procedure (6)(7)(8)(16)(17):**

1.4.3.1. *1st day:*

Dispense 100 μL culture medium into the peripheral wells of a 96-well tissue culture microtiter plate (= blanks). In the remaining wells, dispense 100 μL of a cell suspension of 1×10^5 cells/mL in culture medium (= 1×10^4 cells/well). Two plates should be prepared for each series of individual test substance concentrations, and for the solvent and positive controls.

Incubate cells for 24 h (See Section 1.4.1.2) until they form a half confluent monolayer. This incubation period allows for cell recovery, adherence, and exponential growth.

1.4.3.2. *2nd day:*

After incubation, decant culture medium from the cells and wash carefully with 150 μL of the buffered solution used for incubation. Add 100 μL of the buffer containing the appropriate concentration of test chemical or solvent (solvent control). Apply eight different concentrations of the test chemical. Incubate cells with the test substance in the dark for 60 minutes (See Section 1.4.1.2 and 1.4.1.4 second paragraph).

From the two plates prepared for each series of test substance concentrations and the controls, one is selected, generally at random, for the determination of cytotoxicity (-Irr) (i.e., the control plate), and one (the treatment plate) for the determination of photocytotoxicity (+Irr).

To perform the +Irr exposure, irradiate the cells at room temperature for 50 minutes through the lid of the 96-well plate with the highest dose of radiation that is non-cytotoxic (see also Appendix 2). Keep non-irradiated plates (-Irr) at room temperature in a dark box for 50 min (= light exposure time).

Decant test solution and carefully wash twice with 150 μL of the buffered solution used for incubation, but not containing the test material. Replace the buffer with culture medium and incubate (See Section 1.4.1.2.) overnight (18-22 h).

1.4.3.3. *3rd day:*

1.4.3.3.1. Microscopic evaluation

Cells should be examined for growth, morphology, and integrity of the monolayer using a phase contrast microscope. Changes in cell morphology and effects on cell growth should be recorded.

1.4.3.3.2. Neutral Red uptake test

Wash the cells with 150 μL of the pre-warmed buffer. Remove the washing solution by gentle tapping. Add 100 μL of a 50 μg/mL Neutral Red (NR) (3-amino-7-dimethylamino-2-methylphenazine hydrochloride, EINECS number 209-035-8; CAS number 553-24-2; C.I. 50040) in medium without serum (16) and incubate as described in paragraph 1.4.1.2., for 3 h. After incubation, remove the NR medium, and wash cells with 150 μL of the buffer. Decant and remove excess buffer by blotting or centrifugation.

Add exactly 150 μL NR desorb solution (freshly prepared 49 parts water + 50 parts ethanol + 1 part acetic acid).

Shake the microtiter plate gently on a microtiter plate shaker for 10 min until NR has been extracted from the cells and has formed a homogeneous solution.

Measure the optical density of the NR extract at 540 nm in a spectrophotometer, using blanks as a reference. Save data in an appropriate electronic file format for subsequent analysis.

2. DATA

2.1. QUALITY AND QUANTITY OF DATA

The test data should allow a meaningful analysis of the concentration-response obtained in the presence and in the absence of irradiation, and if possible the concentration of test chemical by which cell viability is reduced to 50 % (IC_{50}). If cytotoxicity is found, both the concentration range and the intercept of individual concentrations shall be set in a way to allow the fit of a curve to the experimental data.

For both clearly positive and clearly negative results (See Section 2.3, first paragraph), the primary experiment, supported by one or more preliminary dose range-finding experiment(s), may be sufficient.

Equivocal, borderline, or unclear results should be clarified by further testing (see also section 2.4, second paragraph). In such cases, modification of experimental conditions should be considered. Experimental conditions that might be modified include the concentration range or spacing, the pre-incubation time, and the irradiation-exposure time. A shorter exposure time may be appropriate for water-unstable chemicals.

2.2. EVALUATION OF RESULTS

To enable evaluation of the data, a Photo-Irritation-Factor (PIF) or Mean Photo Effect (MPE) may be calculated.

For the calculation of the measures of photocytotoxicity (see below) the set of discrete concentration-response values has to be approximated by an appropriate continuous concentration-response curve (model). Fitting of the curve to the data is commonly performed by a non-linear regression method (18). To assess the influence of data variability on the fitted curve a bootstrap procedure is recommended.

A Photo-Irritation-Factor (PIF) is calculated using the following formula:

$$PIF = \frac{IC_{50}(-Irr)}{IC_{50}(+Irr)}$$

If an IC_{50} in the presence or absence of light cannot be calculated, a PIF cannot be determined for the test material. The mean photo effect (MPE) is based on comparison of the complete concentration-response curves (19). It is defined as the weighted average across a representative set of photo effect values

$$MPE = \frac{\sum_{i=1}^{n} w_i PE_{c_i}}{\sum_{i=1}^{n} w_i}$$

The photo effect PE_c at any concentration C is defined as the product of the response effect RE_c and the dose effect DE_c i.e. $PE_c = RE_c \times DE_c$. The response effect RE_c is the difference between the responses observed in the absence and presence of light, i.e. $RE_c = R_c$ (-Irr) − R_c (+Irr). The dose-effect is given by

$$DE_c = \left| \frac{C/C^* - 1}{C/C^* + 1} \right|$$

where C* represents the equivalence concentration, i.e. the concentration at which the +Irr response equals the –Irr response at concentration C. If C* cannot be determined because the response values of the +Irr curve are systematically higher or lower than R_C(-Irr) the dose effect is set to 1. The weighting factors w_i are given by the highest response value, i.e. w_i = MAX {R_i (+Irr), R_i (-Irr) }. The concentration grid C_i is chosen such that the same number of points falls into each of the concentration intervals defined by the concentration values used in the experiment. The calculation of MPE is restricted to the maximum concentration value at which at least one of the two curves still exhibits a response value of at least 10 %. If this maximum concentration is higher than the highest concentration used in the +Irr experiment the residual part of the +Irr curve is set to the response value '0'. Depending on whether the MPE value is larger than a properly chosen cut-off value (MPE_c = 0,15) or not, the chemical is classified as phototoxic.

A software package for the calculation of the PIF and MPE is available from (20).

2.3. INTERPRETATION OF RESULTS

Based on the validation study (8), a test substance with a PIF < 2 or an MPE < 0,1 predicts: 'no phototoxicity'. A PIF > 2 and < 5 or an MPE > 0,1 and < 0,15 predicts: 'probable phototoxicity'; and a PIF > 5 or an MPE > 0,15 predicts: 'phototoxicity'.

For any laboratory initially establishing this assay, the reference materials listed in Table 1 should be tested prior to the testing of test substances for phototoxic assessment. PIF or MPE values should be close to the values mentioned in Table 1.

Table 1

Chemical name	EINECS No	CAS No	PIF	MPE	Absorption peak	Solvent (1)
Amiodarone HCL	243-293-2	[19774-82-4]	> 3,25	0,2-0,54	242 nm 300 nm (shoulder)	ethanol
Choloropromazine HCL	200-701-3	[69-09-0]	> 14,4	0,33-0,63	309 nm	ethanol
Norfloxacin	274-614-4	[70458-96-7]	> 71,6	0,34-0,90	316 nm	acetonitrile
Anthracene	204-371-1	[120-12-7]	> 18,5	0,19-0,81	356 nm	acetonitrile
Protoporphyrin IX, Disodium	256-815-9	[50865-01-5]	> 45,3	0,54-0,74	402 nm	ethanol
L-Histidine		[7006-35-1]	no PIF	0,05-0,10	211 nm	water
Hexacholorophene	200-733-8	[70-30-4]	1,1-1,7	0,00-0,05	299 nm 317 nm (shoulder)	ethanol
Sodium lauryl sulphate	205-788-1	[151-21-3]	1,0-1,9	0,00-0,05	no absorption	water

(1) Solvent used for measuring absorption.

2.4. INTERPRETATION OF DATA

If phototoxic effects are observed only at the highest test concentration, (especially for water soluble test chemicals) additional considerations may be necessary for assessment of hazard. These may include data on skin absorption, and accumulation of the chemical in the skin and/or data from other tests, e.g. testing of the chemical in *in vitro* animal or human skin, or skin models.

If no toxicity is demonstrated (+Irr and -Irr), and if poor solubility limited the concentrations that could be tested, then the compatibility of the test substance with the assay may be questioned and confirmatory testing should be considered using, e.g. another model.

3. **REPORTING**

TEST REPORT

The test report must include at least the following information:

Test substance:

— identification data, common generic names and IUPAC and CAS number, if known,

— physical nature and purity,

— physicochemical properties relevant to conduct of the study,

— UV/vis absorption spectrum,

— stability and photostability, if known.

Solvent:

— justification for choice of solvent,

— solubility of the test chemical in solvent,

— percentage of solvent present in treatment medium.

Cells:

— type and source of cells,

— absence of mycoplasma,

— cell passage number, if known,

— Radiation sensitivity of cells, determined with the irradiation equipment used in the *in vitro* 3T3 NRU phototoxicity test.

Test conditions (1); *incubation before and after treatment:*

— type and composition of culture medium,

— incubation conditions (CO_2 concentration; temperature; humidity),

— duration of incubation (pre-treatment; post-treatment).

Test conditions (2); *treatment with the chemical:*

— rationale for selection of concentrations of the test chemical used in the presence and in the absence of irradiation,

— in case of limited solubility of the test chemical and absence of cytotoxicity: rationale for the highest concentration tested,

— type and composition of treatment medium (buffered salt solution),

— duration of the chemical treatment.

Test conditions (3); *irradiation:*

— rationale for selection of the light source used,

— manufacturer and type of light source and radiometer,

— spectral irradiance characteristics of the light source,

— transmission and absorption characteristics of the filter(s) used,

— characteristics of the radiometer and details on its calibration,

— distance of the light source from the test system,

— UVA irradiance at this distance, expressed in mW/cm^2,

— duration of the UV/vis light exposure,

— UVA dose (irradiance × time), expressed in J/cm^2,

— temperature of cell cultures during irradiation and cell cultures concurrently kept in the dark.

Test conditions (4); *Neutral Red viability test:*

— composition of Neutral Red treatment medium,

— duration of Neutral Red incubation,

— incubation conditions (CO_2 concentration; temperature; humidity),

— Neutral Red extraction conditions (extractant; duration),

— wavelength used for spectrophotometric reading of Neutral Red optical density,

— second wavelength (reference), if used,

— content of spectrophotometer blank, if used.

Results:

— cell viability obtained at each concentration of the test chemical, expressed in percent viability of mean, concurrent solvent controls,

— concentration response curves (test chemical concentration vs. relative cell viability) obtained in concurrent +Irr and -Irr experiments,

— analysis of the concentration-response curves: if possible, computation/calculation of IC_{50} (+Irr) and IC_{50} (-Irr),

— comparison of the two concentration response curves obtained in the presence and in the absence of irradiation, either by calculation of the Photo-Irritation-Factor (PIF), or by calculation of the Mean-Photo-Effect (MPE),

— test acceptance criteria; concurrent solvent control:

— absolute viability (optical density of Neutral Red extract) of irradiated and non-irradiated cells,

— historic negative and solvent control data; means and standard deviations,

— test acceptance criteria; concurrent positive control,

— IC_{50}(+Irr) and IC_{50}(-Irr) and PIF/MPE of positive control chemical,

— historic positive control chemical data: IC_{50}(+Irr) and IC_{50}(-Irr) and PIF/MPE; means and standard deviations.

Discussion of the results.

Conclusions.

4. **REFERENCES**

(1) Lovell W.W., (1993) A scheme for *in vitro* screening of substances for photoallergenic potential. Toxicology *In Vitro* 7, p. 95-102.

(2) Santamaria, L. and Prino, G., (1972) List of the photodynamic substances. In 'Research Progress in Organic, Biological and Medicinal Chemistry' Vol. 3 part 1. North Holland Publishing Co. Amsterdam. p. XI-XXXV.

(3) Spielmann, H., Lovell, W.W., Hölzle, E., Johnson, B.E., Maurer, T., Miranda, M.A., Pape, W.J.W., Sapora, O., and Sladowski, D., (1994) *In vitro* phototoxicity testing: The report and recommendations of ECVAM Workshop 2. ATLA, 22, p. 314-348.

(4) Spikes, J.D., (1989) Photosensitisation. In 'The science of Photobiology' Edited by K.C. Smith. Plenum Press, New York. 2nd edition, p. 79-110.

(5) OECD, (1997) Environmental Health and Safety Publications, Series on Testing and Assessment No 7 'Guidance Document On Direct Phototransformation Of Chemicals In Water' Environment Directorate, OECD, Paris.

(6) Spielmann, H., Balls, M., Döring, B., Holzhütter, H.G., Kalweit, S., Klecak, G., L'Eplattenier, H., Liebsch, M., Lovell, W.W., Maurer, T., Moldenhauer. F. Moore. L., Pape, W., Pfannbecker, U., Potthast, J., De Silva, O., Steiling, W., and Willshaw, A., (1994) EEC/COLIPA project on *in vitro* phototoxicity testing: First results obtained with a Balb/c 3T3 cell phototoxicity assay. Toxic. In Vitro 8, p. 793-796.

(7) Anon, (1998) Statement on the scientific validity of the 3T3 NRU PT test (an *in vitro* test for phototoxicity), European Commission, Joint Research Centre: ECVAM and DGXI/E/2, 3 November 1997, ATLA, 26, p. 7-8.

(8) Spielmann, H., Balls, M., Dupuis, J., Pape, W.J.W., Pechovitch, G. De Silva, O., Holzhütter, H.G., Clothier, R., Desolle, P., Gerberick, F., Liebsch, M., Lovell, W.W., Maurer, T., Pfannenbecker, U., Potthast, J. M., Csato, M., Sladowski, D., Steiling, W., and Brantom, P., (1998) The international EU/COLIPA *In vitro* phototoxicity validation study: results of phase II (blind trial), part 1: the 3T3 NRU phototoxicity test. Toxicology *In Vitro* 12, p. 305-327.

(9) OECD, (2002) Extended Expert Consultation Meeting on The In Vitro 3T3 NRU Phototoxicity Test Guideline Proposal, Berlin, 30th-31th October 2001, Secretariat's Final Summary Report, 15th March 2002, OECD ENV/EHS, available upon request from the Secretariat.

(10) Borenfreund, E., and Puerner, J.A., (1985) Toxicity determination *in vitro* by morphological alterations and neutral red absorption. Toxicology Lett., 24, p. 119-124.

(11) Hay, R.J., (1988) The seed stock concept and quality control for cell lines. Analytical Biochemistry 171, p. 225-237.

(12) Lambert L.A, Warner W.G., and Kornhauser A., (1996) Animal models for phototoxicity testing. In 'Dermatotoxicology', edited by F.N. Marzulli and H.I. Maibach. Taylor & Francis, Washington DC. 5th Edition, p. 515-530.

(13) Tyrrell R.M., Pidoux M., (1987) Action spectra for human skin cells: estimates of the relative cytotoxicity of the middle ultraviolet, near ultraviolet and violet regions of sunlight on epidermal keratinocytes. Cancer Res., 47, p. 1825-1829.

(14) ISO 10977., (1993) Photography — Processed photographic colour films and paper prints — Methods for measuring image stability.

(15) Sunscreen Testing (UV.B) TECHNICALREPORT, CIE, International Commission on Illumnation, Publication No 90, Vienna, 1993, ISBN 3 900 734 275

(16) ZEBET/ECVAM/COLIPA — Standard Operating Procedure: *In Vitro* 3T3 NRU Phototoxicity Test. Final Version, 7 September, 1998. p. 18.

(17) Spielmann, H., Balls, M., Dupuis, J., Pape, W.J.W., De Silva, O., Holzhütter, H.G., Gerberick, F., Liebsch, M., Lovell, W.W., and Pfannenbecker, U., (1998) A study on UV filter chemicals from Annex VII of the European Union *Directive 76/768/EEC,* in the *in vitro* 3T3 NRU phototoxicity test. ATLA 26, p. 679-708.

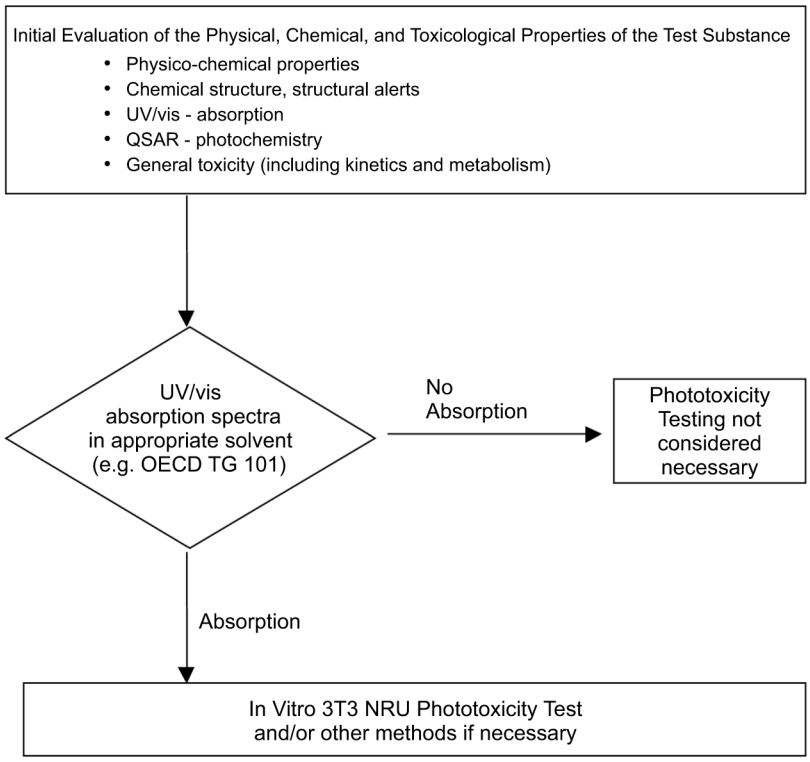
(18) Holzhütter, H.G., and Quedenau, J., (1995) Mathematical modeling of cellular responses to external signals. J. Biol. Systems 3, p. 127-138.

(19) Holzhütter, H.G., (1997) A general measure of *in vitro* phototoxicity derived from pairs of dose-response curves and its use for predicting the *in vivo* phototoxicity of chemicals. ATLA, 25, p. 445-462.

(20) http://www.oecd.org/document/55/0,2340,en_2649_34377_2349687_1_1_1_1,00.html

Appendix 1

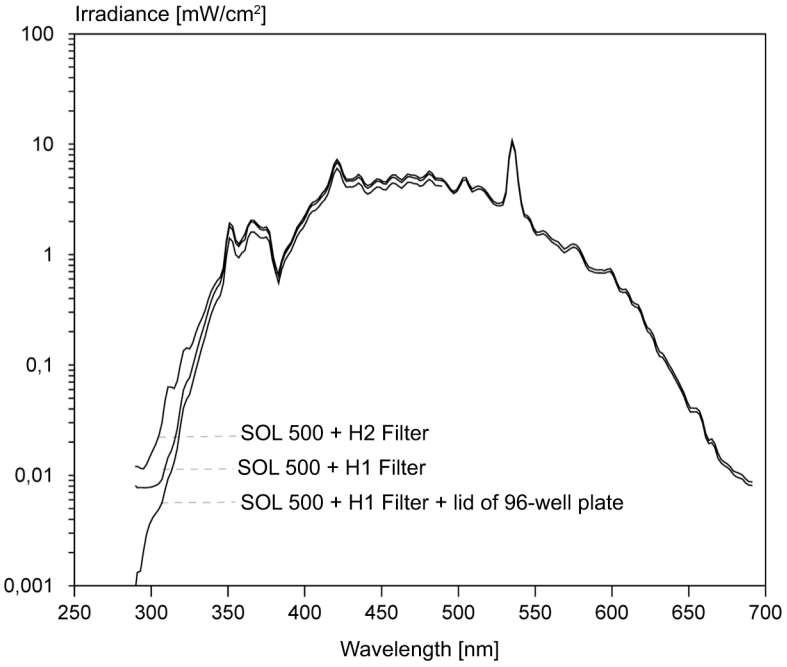
Role of the 3T3 NRU PT in a sequential approach to the phototoxicity testing of chemicals



Appendix 2

Figure 1

Spectral power distribution of a filtered solar simulator



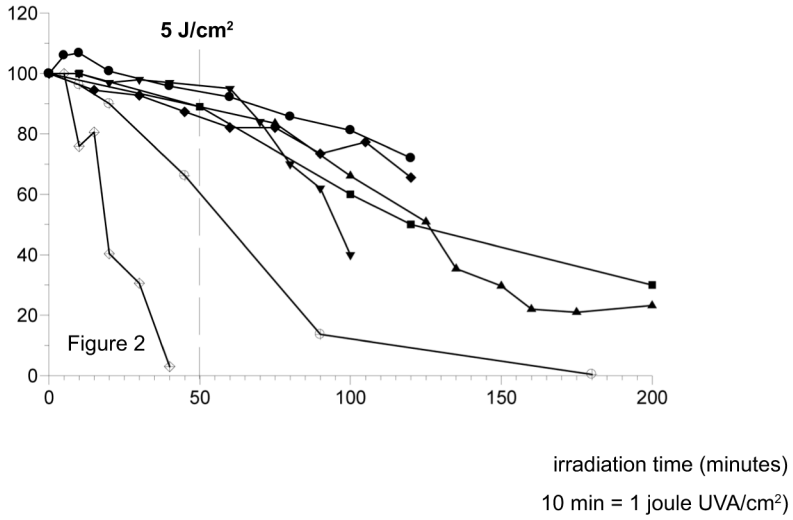
(see Section 1.4.1.5, second paragraph)

Figure 1 gives an example of an acceptable spectral irradiance distribution of a filtered solar simulator. It is from the doped metal halide source used in the validation trial of the 3T3 NRU PT (6)(8)(17). The effect of two different filters and the additional filtering effect of the lid of a 96-well cell culture plate are shown. The H2 filter was only used with test systems that can tolerate a higher amount of UVB (skin model test and red blood cell photo-haemolysis test). In the 3T3 NRU-PT the H1 filter was used. The figure shows that additional filtering effect of the plate lid is mainly observed in the UVB range, still leaving enough UVB in the irradiation spectrum to excite chemicals typically absorbing in the UVB range, like Amiodarone (see Table 1).

Figure 2

Irradiation sensivity of Balb/c 3T3 cells (as measured in the UVA range)

Cell viability (% Neutral Red uptake of dark controls)



(see Sections 1.4.1.5.2 second paragraph; 1.4.2.2.1, 1.4.2.2.2)

Sensitivity of Balb/c 3T3 cells to irradiation with the solar simulator used in the validation trial of the 3T3NRU-phototoxicity test, as measured in the UVA range. Figure shows the results obtained in seven different laboratories in the pre-validation
study (1). While the two curves with open symbols were obtained with aged cells (high number of passages), that had to be replaced by new cell stocks the curves with bold symbols show cells with acceptable irradiation tolerance.

From these data the highest non-cytotoxic irradiation dose of 5 J/cm^2 was derived (vertical dashed line). The horizontal dashed line shows in addition the maximum acceptable irradiation effect given in paragraph 1.4.2.2.

B.42. SKIN SENSITISATION: LOCAL LYMPH NODE ASSAY

1. METHOD

This test method is equivalent to the OECD TG 429 (2002)

1.1. INTRODUCTION

The Local Lymph Node Assay (LLNA) has been sufficiently validated and accepted to justify its adoption as a new Method (1)(2)(3). This is the second method for assessing skin sensitisation potential of chemicals in animals. The other method (B.6) utilises guinea pig tests, notably the guinea pig maximisation test and the Buehler test (4).

The LLNA provides an alternative method for identifying skin sensitising chemicals and for confirming that chemicals lack a significant potential to cause skin sensitisation. This does not necessarily imply that in all instances the LLNA should be used in place of guinea pig test, but rather that the assay is of equal merit and may be employed as an alternative in which positive and negative results generally no longer require further confirmation.

The LLNA provides certain advantages with regard to both scientific progress and animal welfare. It studies the induction phase of skin sensitisation and provides quantitative data suitable for dose response assessment. The details of the validation of the LLNA and a review of the associated work have been published (5)(6)(7)(8). In addition, it should be noted that the mild/moderate sensitisers, which are recommended as suitable positive control substances for guinea pig test m, are also appropriate for use with the LLNA (6)(8)(9).

The LLNA is an *in vivo* method and, as a consequence, will not eliminate the use of animals in the assessment of contact sensitising activity. It has, however, the potential to reduce the number of animals required for this purpose. Moreover, the LLNA offers a substantial refinement of the way in which animals are used for contact sensitisation testing. The LLNA is based upon consideration of immunological events stimulated by chemicals during the induction phase of sensitisation. Unlike guinea pig tests the LLNA does not require that challenged-induced dermal hypersensitivity reactions be elicited. Furthermore, the LLNA does not require the use of an adjuvant, as is the case for the guinea pig maximisation test. Thus, the LLNA reduces animal distress. Despite the advantages of the LLNA over traditional guinea pig tests, it should be recognised that there are certain limitations that may necessitate the use of traditional guinea pigs tests (e.g. false negative findings in the LLNA with certain metals, false positive findings with certain skin irritants)(10).

See also Introduction part B.

1.2. PRINCIPLE OF THE TEST METHOD

The basic principle underlying the LLNA is that sensitisers induce a primary proliferation of lymphocytes in the lymph node draining the site of chemical application. This proliferation is proportional to the dose applied (and to the potency of the allergen) and provides a simple means of obtaining an objective, quantitative measurement of sensitisation. The LLNA assesses this proliferation as a dose-response relationship in which the proliferation in test groups is compared to that in vehicle treated controls. The ratio of the proliferation in treated groups to that in vehicular controls, termed the Stimulation Index, is determined, and must be at least three before a test substance can be further evaluated as a potential skin sensitiser. The methods described here are based on the use of radioactive labelling to measure cell proliferation. However, other endpoints for assessment of proliferation may be employed provided there is justification and appropriate scientific support, including full citations and description of the methodology.

1.3. DESCRIPTION OF THE TEST METHOD

1.3.1. **Preparations**

1.3.1.1. *Housing and feeding conditions*

Animals should be individually housed. The temperature of the experimental animals room should be 22 °C (±3 °C). Although the relative humidity should be at least 30 % and preferably not exceed 70 % other than during room cleaning, the aim should be 50-60 %. Lighting should be artificial, the sequence being 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water.

1.3.1.2. *Preparation of animals*

The animals are randomly selected, marked to permit individual identification (but not by any form of ear marking), and kept in their cages for at least 5 days prior to the start of dosing to allow for acclimatisation to the laboratory conditions. Prior to the start of treatment all animals are examined to ensure that they have no observable skin lesions.

1.3.2. **Test conditions**

1.3.2.1. *Experimental animals*

The mouse is the species of choice for this test. Young adult female mice of CBA/Ca or CBA/J strain, which are nulliparous and non-pregnant are used. At the start of the study, animals should be between eight to 12 weeks old, and the weight variation of the animals should be minimal and not exceed 20 % of the mean weight. Other strains and males may be used when sufficient data are generated to demonstrate that significant strain and/or gender-specific differences in the LLNA response do not exist.

1.3.2.2. *Reliability check*

Positive controls are used to demonstrate appropriate performance of the assay and competency of the laboratory to successfully conduct the assay. The positive control should produce a positive LLNA response at an exposure level expected to give an increase in the stimulation index (SI) >3 over the negative control group. The positive control dose should be chosen such that the induction is clear but not excessive. Preferred substances are hexyl cinnamic aldehyde (CAS No 101-86-0, EINECS No 202-983-3) and mercaptobenzothiazole (CAS No 149-30-4, EINECS No 205-736-8). There may be circumstances in which, given adequate justification, other control substances, meeting the above criteria, may be used. While ordinarily a positive control group may be required in each assay, there may be situations in which test laboratories will have available historic positive control data to show consistency of a satisfactory response over a six-month or more extended period. In those situations, less frequent testing with positive controls may be appropriate at intervals no greater than six months. Although the positive control substance should be tested in the vehicle that is known to elicit a consistent response (e.g. acetone: olive oil), there may be certain regulatory situations in which testing in non-standard vehicle (clinically/chemically relevant formulation) will also be necessary. In such situation the possible interaction of a positive control with this unconventional vehicle should be tested.

1.3.2.3. *Number of animals, dose levels and vehicle selection.*

A minimum of four animals is used per dose group, with a minimum of three concentrations of the test substance, plus a negative control group treated only with the vehicle for the test substance, and, as appropriate, a positive control. In those cases in which individual animal data are to be collected, a minimum of five animals per dose group are used. Except for absence of treatment with the test substance, animals in the control groups should be handled and treated in a manner identical to that of animals in the treatment groups.

Dose and vehicle selection should be based on the recommendations given in reference (1). Doses are selected from the concentration series 100 %, 50 %, 25 %, 10 %, 5 %, 2,5 %, 1 %, 0,5 % etc. Existing acute toxicity and dermal irritation data should be considered, where available, in selecting the three consecutive concentrations so that the highest concentration maximises exposure whilst avoiding systemic toxicity and excessive local skin irritation (2)(11).

The vehicle should be selected on the basis of maximising the test concentrations and solubility whilst producing a solution/suspension suitable for application of the test substance. In order of preference, recommended vehicles are acetone/olive oil (4:1 v/v), dimethylformamide, methyl ethyl ketone, propylene glycol and dimethyl sulphoxide (2)(10), but others may be used if sufficient scientific rationale is provided. In certain situations it may be necessary to use a clinically relevant solvent or the commercial formulation in which the test substance is marketed as an additional control. Particular care should be taken to ensure that hydrophilic materials are incorporated into a vehicle system, which wets the skin and does not immediately run off. Thus, wholly aqueous vehicles are to be avoided.

1.3.3. **Test procedure**

1.3.3.1. *Experimental schedule*

The experimental schedule of the assay is as follows:

Day 1:

individually identify and record the weight of each animal. Open application of 25µl of the appropriate dilution of the test substance, the vehicle alone, or the positive control (as appropriate), to the dorsum of each ear.

Days 2 and 3:

repeat the application procedure carried out on day 1.

Days 4 and 5:

no treatment.

Day 6:

record the weight of each animal. Inject 250µl of phosphate-buffered saline (PBS) containing 20 µCi (7,4e + 8 Bq) of ^{3}H-methyl thymidine into all test and control mice via the tail vein. Alternatively inject 250 µL PBS containing 2 µCi (7,4e + 7 Bq) of ^{125}I-iododeoxyuridine and 10^{-5} M fluorodeoxyuridine into all mice via the tail vein.

Five hours later, the animals are killed. The draining auricular lymph nodes from each ear are excised and pooled in PBS for each experimental group (pooled treatment group approach); alternatively pairs of lymph nodes from individual animals may be excised and pooled in PBS for each animal (individual animal approach). Details and diagrams of the node identification and dissection can be found in Appendix I of reference 10.

1.3.3.2. *Preparation of cell suspensions*

A single cell suspension of lymph node cells (LNC) either from pooled treatment groups or bilaterally from individual animals is prepared by gentle mechanical disaggregation through 200 µm-mesh stainless steel gauze. Lymph node cells are washed twice with an excess of PBS and precipitated with 5 % trichloroacetic acid (TCA) at 4 °C for 18h (2). Pellets are either re-suspended in 1 ml TCA and transferred to scintillation vials containing 10 ml of scintillation fluid for ^{3}H-counting, or transferred directly to gamma counting tubes for ^{125}I-counting.

1.3.3.3. *Determination of cell proliferation (incorporated radioactivity)*

Incorporation of ^{3}H-methyl thymidine is measured by β-scintillation counting as disintegrations per minute (DPM). Incorporation of ^{125}I-iododeoxyuridine is measured by ^{125}I-counting and also is expressed as DPM. Depending on the approach used, the incorporation will be expressed as DPM/treatment group (pooled approach) or the DPM/animal (individual approach).

1.3.3.4. *Observations*

1.3.3.4.1. Clinical observations

Animals should be carefully observed once daily for any clinical signs, either of local irritation at the application site or of systemic toxicity. All observations are systematically recorded with individual records being maintained for each animal.

1.3.3.4.2. Body weights

As stated in section 1.3.3.1, individual animal body weights should be measured at the start of the test and at the scheduled kill of the animals.

1.3.4. **Calculation of results**

Results are expressed as the Stimulation Index (SI). When using the pooled approach, the SI is obtained by dividing the pooled radioactive incorporation for each treatment group by the incorporation of the pooled vehicle control group; this yields a mean SI. When using the individual approach, the SI is derived by dividing the mean DPM/animal within each test substance group and the positive control group by the mean DPM/animal for the solvent/vehicle control group. The average SI for vehicle treated controls is then 1.

Use of the individual approach to calculate the SI will enable the performance of a statistical analysis of the data. In choosing an appropriate method of statistical analysis the investigator should maintain an awareness of possible inequalities of variances and other related problems that may necessitate a data transformation or a non-parametric statistical analysis. An adequate approach for interpreting the data is to evaluate all individual data of treated and vehicle controls, and derive from these the best fitting dose response curve, taking confidence limits into account (8)(12)(13). However, the investigator should be alert to possible 'outlier' responses for individual animals within a group that may necessitate the use of an alternative measure of response (e.g. median rather than mean) or elimination of the outlier.

The decision process with regard to a positive response includes a stimulation index ≥ 3 together with consideration of dose-response and, where appropriate, statistical significance (3)(6)(8)(12)(14).

If it is necessary to clarify the results obtained, consideration should be given to various properties of the test substance, including whether it has a structural relationship to known skin sensitisers, whether it causes excessive skin irritation and the nature of the dose response seen. These and other considerations are discussed in detail elsewhere (7).

2. **DATA**

Data should be summarised in tabular form showing the mean and individual DPM values and stimulation indexes for each dose (including vehicle control) group.

3. **REPORTING**

3.1. TEST REPORT

The test report should contain the following information

test substance:

— identification data (e.g. CAS number, if available, source, purity, known impurities, lotnumber),

— physical nature and physicochemical properties (e.g. volatility, stability, solubility),

— if mixture, composition and relative percentages of components.

Vehicle:

— identification data (purity, concentration (where appropriate); volume used)

— justification for choice of vehicle.

Test animals:

— strain of mice used,

— microbiological status of the animals, when known,

— number, age and sex of animals,

— source of animals, housing conditions, diet, etc.

Test conditions:

— details of test substance preparation and application,

— justification for dose selection, including results from range finding study, if conducted; vehicle and test substance concentrations used and the total amount of substance applied,

— details of food and water quality (including diet type/source, water source).

Reliability check:

— a summary of the results of the latest reliability check including information on substance, concentration and vehicle used,

— concurrent and/or historical positive and negative control data for testing laboratory.

Results:

— individual weights of animals at the start of dosing and at scheduled kill,

— a table of mean (pooled approach) and individual (individual approach) DPM values as well as the range of values for both approaches and the stimulation indices for each dose (including vehicle control) group,

— statistical analysis where appropriate,

— time course of onset and signs of toxicity, including dermal irritation at site of administration, if any, for each animal.

Discussion of results:

— a brief commentary on the results, the dose-response analysis, and statistical analyses, where appropriate, with a conclusion as to whether the test substance should be considered a skin sensitiser.

4. **REFERENCES**

(1) Kimber, I. and Basketter, D.A., (1992) The murine local lymph node assay; collaborative studies and new directions: A commentary. Food and Chemical Toxicology 30, p. 165-169.

(2) Kimber, I, Derman, R.J., Scholes E.W, and Basketter, D.A. (1994). The local lymph node assay: developments and applications. Toxicology, 93, p. 13-31.

(3) Kimber, I., Hilton, J., Dearman, R.J., Gerberick, G.F., Ryan, C.A., Basketter, D.A., Lea, L., House, R.V., Ladies, G.S., Loveless, S.E., Hastings, K.L., (1998) Assessment of the skin sensitisation potential of topical medicaments using the local lymph node assay: An interlaboratory exercise. Journal of Toxicology and Environmental Health, 53, p. 563-79.

(4) Testing Method B.6.

(5) Chamberlain, M. and Basketter, D.A., (1996) The local lymph node assay: status of validation. Food and Chemical Toxicology, 34, p. 999-1002.

(6) Basketter, D.A., Gerberick, G.F., Kimber, I. and Loveless, S.E., (1996) The local lymph node assay- A viable alternative to currently accepted skin sensitisation tests. Food and Chemical Toxicology, 34, p. 985-997.

(7) Basketter, D.A., Gerberick, G.F. and Kimber, I., (1998) Strategies for identifying false positive responses in predictive sensitisation tests. Food and Chemical Toxicology. 36, p. 327-33.

(8) Van Och, F.M.M, Slob, W., De Jong, W.H., Vandebriel, R.J., Van Loveren, H., (2000) A quantitative method for assessing the sensitising potency of low molecular weight chemicals using a local lymph node assay: employement of a regression method that includes determination of uncertainty margins. Toxicology, 146, p. 49-59.

(9) Dearman, R.J., Hilton, J., Evans, P., Harvey, P., Basketter, D.A. and Kimber, I., (1998) Temporal stability of local lymph node assay responses to hexyl cinnamic aldehyde. Journal of Applied Toxicology, 18, p. 281-4.

(10) National Institute of Environmental Health Sciences, (1999) The Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals/Compounds: The Results of an Independent Peer Review Evaluation Coordinated by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and the National Toxicology Program Center for the Evaluation of Alternative Toxicological Methods (NICETAM). NIH Publication No: 99-4494, Research Triangle Park, N.C. (http://iccvam.niehs.nih.gov).

(11) Testing method B.4.

(12) Basketter, D.A., Selbie, E., Scholes, E.W. Lees, D. Kimber, I. and Botham, P.A., (1993) Results with OECD recommended positive control sensitisers in the maximisation, Buehler and local lymph node assays. Food and Chemical Toxicology, 31, p. 63-67.

(13) Basketter D.A., Lea L.J., Dickens A., Briggs D., Pate I., Dearman R.J., Kimber I., (1999) A comparison of statistical approaches to the derivation of EC_3 values from local lymph node assay dose responses. J. Appl. Toxicology, 19, p. 261-266.

(14) Basketter D.A., Blaikie L., Derman R.J., Kimber I., Ryan C.A., Gerberick G.F., Harvey P., Evans P., White I.R. and Rycroft R.T.G., (2000) Use of local lymph node assay for the estimation of relative contact allergenic potency. Contact Dermatitis 42, p. 344-48.

B.43. NEUROTOXICITY STUDY IN RODENTS

1. METHOD

This method is equivalent of OECD TG 424 (1997).

This test method has been designed to obtain the information necessary to confirm or to further characterise the potential neurotoxicity of chemicals in adult animals. It can either be combined with existing test methods for repeated dose toxicity studies or to be carried out as a separate study. It is recommended that the OECD Guidance Document on Neurotoxicity Testing Strategies and Methods (1) be consulted to assist in the design of studies based on this test method. This is particularly important when modifications of the observations and test procedures as recommended for routine use of this method are considered. The guidance document has been prepared to facilitate the selection of other test procedures for use in specific circumstances.

The assessment of developmental neurotoxicity is not the subject of this method.

1.1. INTRODUCTION

In the assessment and evaluation of the toxic characteristics of chemicals, it is important to consider the potential for neurotoxic effects. Already the test method for repeated dose systemic toxicity includes observations that screen for potential neurotoxicity. This test method can be used to design a study to obtain further information on, or to confirm, the neurotoxic effects observed in the repeated dose systemic toxicity studies. However, consideration of the potential neurotoxicity of certain classes of chemicals may suggest that they may be more appropriately evaluated using this Method without prior indications of the potential neurotoxicity from repeated dose systemic toxicity studies. Such considerations include, for example:

— observation of neurological signs or neuropathological lesions in toxicity studies other than repeated dose systemic toxicity studies, or

— structural relationship or other information linking them to known neurotoxicants.

In addition there may be other instances when use of this test method is appropriate; for further details see (1).

This method has been developed so that it can be tailored to meet particular needs to confirm the specific histopathological and behavioural neurotoxicity of a chemical as well as provide a characterisation and quantification of the neurotoxic responses.

In the past, neurotoxicity was equated with neuropathy involving neuropathological lesions or neurological dysfunctions, such as seizure, paralysis or tremor. Although neuropathy is an important manifestation of neurotoxicity, it is now clear that there are many other signs of nervous system toxicity (e.g. loss of motor co-ordination, sensory deficits, learning and memory dysfunctions) that may not be reflected in neuropathy or other types of studies.

This neurotoxicity test method is designed to detect major neurobehavioural and neuropathological effects in adult rodents. While behavioural effects, even in the absence of morphological changes, can reflect an adverse impact on the organism, not all behavioural changes are specific to the nervous system. Therefore, any changes observed should be evaluated in conjunction with correlative histopathological, haematological or biochemical data as well as data on other types of systemic toxicity. The testing called for in this method to provide a characterisation and quantification of the neurotoxic responses includes specific histopathological and behavioural procedures that may be further supported by electrophysiological and/or biochemical investigations (1)(2)(3)(4).

Neurotoxicants may act on a number of targets within the nervous system and by a variety of mechanisms. Since no single array of tests is capable of thoroughly assessing the neurotoxic potential of all substances, it may be necessary to utilise other *in vivo* or *in vitro* tests specific to the type of neurotoxicity observed or anticipated.

This test method can also be used, in conjunction with the guidance set out in the OECD Guidance Document on Neurotoxicity Testing Strategies and Methods (1) to design studies intended to further characterise or increase the sensitivity of the dose-response quantification in order or better estimate a no-observed-adverse effect level or to substantiate known or suspected hazards of the chemical. For example, studies may be

designed to identify and evaluate the neurotoxic mechanism(s) or supplement the data already available from the use of basic neurobehavioural and neuropathological observation procedures. Such studies need not replicate data that would be generated from the use of the standard procedures recommended in this Method, if such data are already available and are not considered necessary for the interpretation of the results of the study.

This neurotoxicity study, when used alone or in combination, provides information that can:

— identify whether the nervous system is permanently or reversibly affected by the chemical tested;

— contribute to the characterisation of the nervous system alterations associated with exposure to the chemical, and to understanding the underlying mechanism.

— determine dose-and time-response relationships in order to estimate a no-observed-adverse-effect level (which can be used to establish safety criteria for the chemical).

This test method uses oral administration of the test substance. Other routes of administration (e.g. dermal or inhalation) may be more appropriate, and may require modification of the procedures recommended. Considerations of the choice of the route of administration depend on the human exposure profile and available toxicological or kinetic information.

1.2. DEFINITIONS

Adverse effect: is any treatment-related alteration from baseline that diminishes an organism's ability to survive, reproduce or adapt to the environment.

Dose: is the amount of test substance administered. Dose is expressed as weight (g, mg) or as weight of test substance per unit weight of the test animal (e.g. mg/Kg), or as constant dietary concentrations (ppm).

Dosage: is a general term comprising of dose, its frequency and the duration of dosing.

Neurotoxicity: is an adverse change in the structure or function of the nervous system that results from exposure to a chemical, biological or physical agent.

Neurotoxicant: is any chemical, biological or physical agent having the potential to cause neurotoxicity.

NOAEL: is the abbreviation for no-observed-adverse effect level and is the highest dose level where no adverse treatment-related findings are observed.

1.3. PRINCIPLE OF THE TEST METHOD

The test chemical is administered by the oral route across a range of doses to several groups of laboratory rodents. Repeated doses are normally required, and the dosing regimen may be 28 days, subchronic (90 days) or chronic (1 year or longer). The procedures set out in this test method may also be used for an acute neurotoxicity study. The animals are tested to allow the detection or the characterisation of behavioural and/or neurological abnormalities. A range of behaviours that could be affected by neurotoxicants is assessed during each observation period. At the end of the test, a subset of animals of each sex from each group are perfused *in situ* and sections of the brain, spinal cord, and peripheral nerves are prepared and examined.

When the study is conducted as a stand-alone study to screen for neurotoxicity or to characterise neurotoxic effects, the animals in each group not used for perfusion and subsequent histopathology (see Table 1) can be used for specific neurobehavioural, neuropathological, neurochemical or electrophysiological procedures that may supplement the data obtained from the standard examinations required by this method (1). These supplemental procedures can be particularly useful when empirical observations or anticipated effects indicate a specific type or target of a chemical's neurotoxicity. Alternatively, the remaining animals can be used for evaluations such as those called for in test methods for repeated dose toxicity studies in rodents.

When the procedures of this test method are combined with those of other test methods, a sufficient number of animals is needed to satisfy the requirements for the observations of both studies.

1.4. DESCRIPTION OF THE TEST METHOD

1.4.1. **Selection of animal species**

The preferred rodent species is the rat, although other rodent species, with justification, may be used. Commonly used laboratory strains of young adult healthy animals should be employed. The females should be nulliparous and non-pregnant. Dosing should normally begin as soon as possible after weaning, preferably not later than when animals are six weeks, and, in any case, before the animals are nine weeks age. However, when this study is combined with other studies this age requirement may need adjustment. At the commencement of the study the weight variation of animals used should not exceed ± 20 % of the mean weight of each sex. Where a repeated dose study of short duration is conducted as a preliminary to a long term study, animals from the same strain and source should be used in both studies.

1.4.2. **Housing and feeding conditions**

The temperature in the experimental animal room should be 22 °C (± 3 °C). Although the relative humidity should be at least 30 % and preferably not exceed 70 % other than during room cleaning, the aim should be 50-60 %. Lighting should be artificial, the sequence being 12 hours light, 12 hours dark. Loud intermittent noise should be kept to a minimum. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. The choice of diet may be influenced by the need to ensure a suitable admixture of a test substance when administered by this method. Animals may be housed individually, or be caged in small groups of the same sex.

1.4.3. **Preparation of animals**

Healthy young animals are randomly assigned to the treatment and control groups. Cages should be arranged in such a way that possible effects due to cage placement are minimised. The animals are identified uniquely and kept in their cages for at least (5) five days prior the start of the study to allow for acclimatisation to the laboratory conditions.

1.4.4. **Route of administration and preparation of doses**

This test method specifically addresses the oral administration of the test substance. Oral administration may be by gavage, in the diet, in drinking water or by capsules. Other routes of administration (e.g. dermal or inhalation) can be used but may require modification of the procedures recommended. Considerations of the choice of the route of administration depend on the human exposure profile and available toxicological or kinetic information. The rationale for choosing the route of administration as well as resulting modifications to the procedures of this test method should be indicated.

Where necessary, the test substance may be dissolved or suspended in a suitable vehicle. It is recommended that the use of an aqueous solution/suspension be considered first, followed by consideration of a solution/suspension in oil (e.g. corn oil) and then by possible solution/suspension in other vehicle. The toxic characteristics of the vehicle must be known. In addition, consideration should be given to the following characteristics of the vehicle: effects of the vehicle on absorption, distribution, metabolism, or retention of the test substance which may alter its toxic characteristics; and effects on the food or water consumption or the nutritional status of the animals.

1.5. PROCEDURES

1.5.1. **Number and sex animals**

When the study is conducted as a separate study, at least 20 animals (10 females and 10 males) should be used in each dose and control group for the evaluation of detailed clinical and functional observations. At least five males and five females, selected from these 10 males and 10 females, should be perfused *in situ* and used for detailed neurohistopathology at the end of the study. In cases where only a limited number of animals in a given dose group are observed for signs of neurotoxic effects, consideration should be given to the inclusion of these animals in those selected for perfusion. When the study is conducted in combination with a repeated dose toxicity study, adequate numbers of animals should be used to meet the objectives of both studies. The minimum numbers of animals per group for various combinations of studies are given in Table 1. If interim kills or recovery groups for observation of reversibility, persistence or delayed occurrence of toxic effects post treatment are planned or when supplemental observations are considered, then the number of animals should be increased to ensure that the number of animals required for observation and histopathology are available.

1.5.2. **Treatment and control group**

At least three dose groups and a control group should generally be used, but if from the assessment of other data, no effects would be expected at a repeated dose of 1 000 mg/kg body weight/day, a limit test may be

performed. If there are no suitable data available, a range finding study may be performed to aid in the determination of the doses to be used. Except for treatment with the test substance, animals in the control group should be handled in an identical manner to the test group subjects. If a vehicle is used in administering the test substance, the control group should receive the vehicle at the highest volume used.

1.5.3. **Reliability check**

The laboratory performing the study should present data demonstrating its capability to carry out the study and the sensitivity of the procedures used. Such data should provide evidence of the ability to detect and quantify, as appropriate, changes in the different end points recommended for observation, such as autonomic signs, sensory reactivity, limb grip strength and motor activity. Information on chemicals that cause different types of neurotoxic responses and could be used as positive control substances can be found in references 2 to 9. Historical data may be used if the essential aspects of the experimental procedures remain the same. Periodic updating of historical data is recommended. New data that demonstrate the continuing sensitivity of the procedures should be developed when some essential element of the conduct of the test or procedures has been changed by the performing laboratory.

1.5.4. **Dose selection**

Dose levels should be selected by taking into account any previously observed toxicity and kinetic data available for the test compound or related materials. The highest dose level should be chosen with the aim of inducing neurotoxic effects or clear systemic toxic effects. Thereafter, a descending sequence of dose levels should be selected with a view to demonstrating any dose-related response and no-observed-adverse effect (NOAEL) at the lowest dose level. In principle, dose levels should be set so that primary toxic effects on the nervous system can be distinguished from effects related to systemic toxicity. Two to three intervals are frequently optimum and addition of a fourth test group is often preferable to using very large intervals (e.g. more than a factor of 10) between dosages. Where there is a reasonable estimation of human exposure this should also be taken into account.

1.5.5. **Limit test**

If a study at one dose level of at least 1 000 mg/kg body weight/day, using the procedures described, produces no observable neurotoxic effects and if toxicity would not be expected based upon data from structurally related compounds, then a full study using three dose levels may not be considered necessary. Expected human exposure may indicate the need for a higher oral dose level to be used in the limit test. For other types of administration, such as inhalation or dermal application, the physical chemical properties of the test substance often may dictate the maximum attainable level of exposure. For the conduct of an oral acute study, the dose for a limit test should be at least 2 000 mg/kg.

1.5.6. **Administration of doses**

The animals are dosed with the test substance daily, seven days each week, for a period at least 28 days; use of a five-day dosing regime or a shorter exposure period needs to be justified. When the test substance is administered by gavage, this should be done in a single dose using a stomach tube or a suitable intubation cannula. The maximum volume of a liquid that can be administered at one time depends on the size of the test animals. The volume should not exceed 1 ml/100 g body weight. However in the case of aqueous solutions, the use of up to 2 ml/100 g body weight can be considered. Except for irritating or corrosive substances, which will normally reveal exacerbated effects with higher concentrations, variability in test volume should be minimised by adjusting the concentration to ensure a constant volume at all dose levels.

For substances administered via the diet or drinking water, it is important to ensure that the quantities of the test substance involved do not interfere with normal nutrition or water balance. When the test substance is administered in the diet either a constant dietary concentration (ppm) or a constant dose level in terms of the animals' body weight may be used; the alternative used must be specified. For a substance administered by gavage, the dose should be given at similar times each day, and adjusted as necessary to maintain a constant dose level in terms of animal body weight. Where a repeat dose study is used as a preliminary to a long term study, a similar diet should be used in both studies. For acute studies, if a single dose is not possible, the dose may be given in smaller fractions over a period not exceeding 24 hours.

1.6. OBSERVATION

1.6.1. **Frequency of observations and tests**

In repeated dose studies, the observation period should cover the dosage period. In acute studies, 14-day post-treatment period should be observed. For animals in satellite groups which are kept without exposure during a post-treatment period, observations should cover this period as well.

Observations should be made with sufficient frequency to maximise the probability of detection of any behavioural and/or neurological abnormalities. Observations should be made preferably at the same times each day with consideration given to the peak period of anticipated effects after dosing. The frequency of clinical observations and functional tests is summarised in Table 2. If kinetic or other data generated from previous studies indicates the need to use different time points for observations, tests or post-observation periods, an alternative schedule should be adopted in order to achieve maximum information. The rationale for changes to the schedule should be provided.

1.6.1.1. *Observations of general health condition and mortality/morbidity*

All animals should be carefully observed at least once daily with respect to their health condition as well as at least twice daily for morbidity and mortality.

1.6.1.2. *Detailed clinical observations*

Detailed clinical observations should be made on all animals selected for this purpose (see Table 1) once before the first exposure (to allow for within-subject comparisons) and at different intervals thereafter, dependant on the duration of the study (see Table 2). Detailed clinical observations on satellite recovery groups should be made at the end of the recovery period. Detailed clinical observations should be made outside the home cage in a standard arena. They should be carefully recorded using scoring systems that include criteria or scoring scales for each measurement in the observations. The criteria or scales used should be explicitly defined by the testing laboratory. Effort should be made to ensure that variations in the test conditions are minimal (not systematically related to treatment) and that observations are conducted by trained observers unaware of the actual treatment.

It is recommended that the observations be carried out in a structured fashion in which well-defined criteria (including the definition of the normal 'range') are systematically applied to each animal at each observation time. The 'normal range' should be adequately documented. All observed signs should be recorded. Whenever feasible, the magnitude of the observed signs should also be recorded. Clinical observations should include, but not be limited to, changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions and autonomic activity (e.g. lacrimation, piloerection, pupil size, unusual respiratory pattern and/or mouth breathing, any unusual signs of urination or defecation, and discoloured urine).

Any unusual responses with respect to body position, activity level (e.g. decreased or increased exploration of the standard arena) and co-ordination of movement should also be noted. Changes in gait (e.g. waddling, ataxia), posture (e.g. hunched-back) and reactivity to handling, placing or other environmental stimuli, as well as the presence of clonic or tonic movements, convulsions or tremors, stereotypes (e.g. excessive grooming, unusual head movements, repetitive circling) or bizarre behaviour (e.g. biting or excessive licking, self mutilation, walking backwards, vocalisation) or aggression should be recorded.

1.6.1.3. *Functional tests*

Similar to the detailed clinical observations, functional tests should also be conducted once prior to exposure and frequently thereafter in all animals selected for this purpose (see Table 1). The frequency of functional testing is also dependent on the study duration (see Table 2). In addition to the observation periods as set out in Table 2, functional observations on satellite recovery groups should also be made as close as possible to the terminal kill. Functional tests should include sensory reactivity to stimuli of different modalities (e.g. auditory, visual and proprioceptive stimuli (5)(6)(7)), assessment of limb grip strength (8) and assessment of motor activity (9). Motor activity should be measured with an automated device capable of detecting both decreases and increases in activity. If another defined system is used it should be quantitative and its sensitivity and reliability should be demonstrated. Each device should be tested to ensure reliability across time and consistency between devices. Further details of the procedures that can be followed are given in the respective references. If there are no data (e.g. structure-activity, epidemiological data, other toxicology studies) to indicate the potential neurotoxic effects, the inclusion of more specialised tests of sensory and motor function or learning and memory to examine these possible effects in greater details should be considered. More information on more specialised tests and their use is provided in (1).

Exceptionally, animals that reveal signs of toxicity to an extent that would significantly interfere with the functional test may be omitted from that test. Justification for the elimination of animals from a functional test should be provided.

1.6.2. **Body weight and food/water consumption**

For studies up to 90 days duration, all animals should be weighed at least once a week and measurements should be made of food consumption (water consumption, when the test substance is administered by that medium) at least weekly. For long term studies, all animals should be weighed at least once at week for the first

13 weeks and at least once every four weeks thereafter. Measurements should be made of food consumption (water consumption, when the test substance is administered by that medium) at least weekly for the first 13 weeks and then at approximately three-month intervals unless the health status or body weight changes dictate otherwise.

1.6.3. **Ophthalmology**

For studies longer than 28 days duration, ophthalmologic examination, using an ophthalmoscope or an equivalent suitable instrument, should be made prior to the administration of the test substance and at the termination of the study, preferably on all animals, but at least on animals in the high dose and control groups. If changes in the eyes are detected or, if clinical signs indicate the need, all animals should be examined. For long term studies, an ophthalmologic examination should also be carried out at 13 weeks. Ophthalmologic examinations need not to be conducted if this data is already available from others studies of similar duration and at similar dose levels.

1.6.4. **Haematology and clinical biochemistry**

When the neurotoxicity study is carried out in combination with a repeated dose systemic toxicity study, haematological examinations and clinical biochemistry determinations should be carried out as set out in the respective method of the systemic toxicity study. Collection of samples should be carried out in such a way that any potential effects on neurobehaviour are minimised.

1.6.5. **Histopathology**

The neuropathological examination should be designed to complement and extend the observations made during the *in vivo* phase of the study. Tissues from at least five animals/sex/group (see Table 1 and next paragraph) should be fixed *in situ*, using generally recognised perfusion and fixation techniques (see reference 3, chapter 5 and reference 4, chapter 50). Any observable gross changes should be recorded. When the study is conducted as a stand-alone study screen for neurotoxicity or to characterise neurotoxic effects, the remainder of the animals may be used either for specific neurobehavioural (10)(11), neuropathological (10)(11)(12)(13), neurochemical (10)(11)(14)(15) or electrophysiological (10)(11)(16)(17) procedures that may supplement the procedures and examinations described here, or to increase the number of subjects examined for histophatology. These supplementary procedures are of particular use when empirical observations or anticipated effects indicate a specific type or target of neurotoxicity (2)(3). Alternatively, the remainder of the animals can also be used for routine pathological evaluations as described in Method for repeated dose studies.

A general staining procedure, such as haematoxylin and eosin (H&E), should be performed on all tissue specimens embedded in paraffin and microscopic examination should be carried out. If signs of peripheral neuropathy are observed or suspected, plastic-embedded samples of peripheral nerve tissue should be examined. Clinical signs may also suggest additional sites for examination or the use of special staining procedures. Guidance on additional sites to be examined can be found in (3)(4). Appropriate special stains to demonstrate specific types of pathological change may also be helpful (18).

Representative sections of the central and peripheral nervous system should be examined histologically (see reference 3, chapter 5 and reference 4, chapter 50). The areas examined should normally include: the forebrain, the centre of the cerebrum, including a section through the hippocampus, the midbrain, the cerebellum, the pons, the medulla oblongata, the eye with optic nerve and retina, the spinal cord at the cervical and lumbar swellings, the dorsal root ganglia, the dorsal and ventral root fibres, the proximal sciatic nerve, the proximal tibial nerve (at the knee) and the tibial nerve calf muscle branches. The spinal cord and peripheral nerve sections should include both cross or transverse and longitudinal sections. Attention should be given to the vasculature of the nervous system. A sample of skeletal muscle, particularly calf muscle, should also be examined. Special attention should be paid to sites with cellular and fibre structure and pattern in the CNS and PNS known to be particularly affected by neurotoxicants.

Guidance on neurophatological alterations that typically result from toxicant exposure can be found in the references (3)(4). A stepwise examination of tissue samples is recommended in which sections from the high dose group are first compared with those of the control group. If no neurophatological alterations are observed in the samples from these groups, subsequent analysis is not required. If neuropathological alterations are observed in the high dose group, sample from each of the potentially affected tissues from the intermediate and low dose groups should then be coded and examined sequentially.

If any evidence of neuropathological alterations is found in the qualitative examination, then a second examination should be performed on all regions of the nervous system showing these alterations. Sections from all dose groups from each of the potentially affected regions should be coded and examined at random without knowledge of the code. The frequency and severity of each lesion should be recorded. After all regions from all dose groups have been rated, the code can be broken and statistical analysis performed to evaluate dose-response relationships. Examples of different degrees of severity of each lesion should be described.

The neuropathological findings should be evaluated in the context of behavioural observations and measurements, as well as other data from preceding and concurrent systemic toxicity studies of the test substance.

2. DATA

2.1. TREATMENT OF RESULTS

Individual data should be provided. Additionally, all data should be summarised in tabular form showing for each test or control group the number of animals at the start of the test, the number of animals found dead during the test or killed for humane reasons and the time of any death or humane kill, the number showing signs of toxicity, a description of the signs of toxicity observed, including time of onset, duration, type and severity of any toxic effects, the number of animals showing lesions, including the type and severity of the lesion(s).

2.2. EVALUATION AND INTERPRETATION OF RESULTS

The findings of the study should be evaluated in terms of the incidence, severity and correlation of neurobehavioural and neuropathological effects (neurochemical or electrophysiological effects as well if supplementary examinations are included) and any other adverse effects observed. When possible, numerical results should be evaluated by an appropriate and generally acceptable statistical method. The statistical methods should be selected during the design of the study.

3. REPORTING

3.1. TEST REPORT

the test report must include the following information:

Test substance:

— physical nature (including isomerism, purity and physicochemical properties),

— identification data.

Vehicle (if appropriate):

— justification for choice of vehicle.

Test animals:

— species/strain used,

— number, age and sex of animals,

— source, housing conditions, acclimatisation, diet, etc,

— individual weights of animals at the start of the test.

Test conditions:

— details of test substance formulation/diet preparation, achieved concentration, stability and homogeneity of the preparation,

— specification of the doses administered, including details of the vehicle, volume and physical form of the material administered,

— details of the administration of the test substance,

— rationale for dose levels selected,

— rationale for the route and duration of the exposure,

— conversion from diet/drinking water test substance concentration (ppm) to the actual dose (mg/kg body weight/day), if applicable,

— details of the food and water quality.

Observation and test procedures:

— details of the assignment of animals in each group to the perfusion subgroups,

— details of scoring systems, including criteria and scoring scales for each measurement in the detailed clinical observations,

— details on the functional tests for sensory reactivity to stimuli of different modalities (e.g. auditory, visual and proprioceptive), for assessment of limb grip strength, for motor activity assessment (including details of automated devices for detecting activity), and other procedures used,

— details of ophthalmologic examinations and, if appropriate, haematological examinations and clinical biochemistry tests with relevant base-line values,

— details for specific neurobehavioural, neuropathological, neurochemical or electrophysiological procedures.

Results:

— body weight/body weight changes including body weight at kill,

— food consumption and water consumption, as appropriate,

— toxic response data by sex and dose level, including signs of toxicity or mortality,

— nature, severity and duration (time of onset and subsequent course) of the detailed clinical observations (whether reversible or not),

— a detailed description of all functional test results,

— necropsy findings,

— a detailed description of all neurobehavioural, neuropathological, and neurochemical or electrophysiological findings, if available,

— absorption and metabolism data, if available,

— statistical treatment of results, where appropriate.

Discussion of results:

— dose response information;

— relationship of any other toxic effects to a conclusion about the neurotoxic potential of the test chemical;

— no-observed-adverse effect level.

Conclusions:

— a specific statement of the overall neurotoxicity of the test chemical is encouraged.

4. **REFERENCES**

(1) OECD Giudance Document on Neurotoxicity Testing Strategies and Test Methods. OECD, Paris, In Preparation.

(2) Test Guideline for a Developmental Neurotoxicity Study, OECD Guidelines for the Testing of Chemicals. In preparation.

(3) World Health Organisation (WHO) (1986) Environmental Health Criteria document 60: Principles and Methods for the Assessment of Neurotoxicity associated with Exposure to Chemicals.

(4) Spencer, P.S. and Schaumburg, H.H. (1980) Experimental and Clinical Neurotoxicology. Eds. Spencer, P.S. and Schaumburg, H.H. eds. Williams and Wilkins, Baltimore/London.

(5) Tupper, D.E. and Wallace, R.B., (1980) Utility of the Neurological Examination in Rats. Acta Neurobiol. Exp., 40, p. 999-1003.

(6) Gad, S.C., (1982) A Neuromuscular Screen for Use in Industrial Toxicology. J. Toxicol. Environ. Health, 9, p. 691-704.

(7) Moser, V.C., McDaniel, K.M. and Phillips, P.M., (1991) Rat Strain and Stock Comparisons Using a Functional Observational Battery: Baseline Values and Effects of amitraz. Toxic. Appl. Pharmacol., 108, p. 267-283.

(8) Meyer, O.A., Tilson, H.A., Byrd, W.C. and Riley, M.T., (1979) A Method for the Routine Assessment of Fore- and Hind- limb Grip Strength of Rats and Mice. Neurobehav. Toxicol., 1, p. 233-236.

(9) Crofton, K.M., Haward, J.L., Moser, V.C., Gill, M.W., Reirer, L.W., Tilson, H.A. and MacPhail, R.C. (1991) Interlaboratory Comparison of Motor Activity Experiments: Implication for Neurotoxicological Assessments. Neurotoxicol. Teratol., 13, p. 599-609.

(10) Tilson, H.A., and Mitchell, C.L. eds., (1992) Neurotoxicology Target Organ Toxicology Series. Raven Press, New York.

(11) Chang, L.W., ed., (1995) Principles of Neurotoxicology. Marcel Dekker, New York.

(12) Broxup, B., (1991) Neuopathology as a screen for Neurotoxicity Assessment. J. Amer. Coll. Toxicol., 10, p. 689-695.

(13) Moser, V.C., Anthony, D.C., Sette, W.F. and MacPhail, R.C., (1992) Comparison of Subchronic Neurotoxicity of 2-Hydroxyethyl Acrylate and Acrylamide in Rats. Fund. Appl.Toxicol., 18, p. 343-352.

(14) O'Callaghan, J.P. (1988). Neurotypic and Gliotypic Proteins as Biochemical Markers of Neurotoxicity. Eurotoxicol. Teratol., 10, p. 445-452.

(15) O'Callaghan J.P. and Miller, D.B., (1988) Acute Exposure of the Neonatal Rat to Triethyltin Results in Persistent Changes in Neurotypic and Gliotypic Proteins. J. Pharmacol. Exp. Ther., 244, p. 368-378.

(16) Fox. D.A., Lowndes, H.E. and Birkamper, G.G., (1982) Electrophysiological Techniques in Neurotoxicology. In: Nervous System Toxicology. Mitchell, C.L. ed. Raven Press, New York, p. 299-335.

(17) Johnson, B.L., (1980) Electrophysiological Methods in neurotoxicity Testing. In: Experimental and Clinical Neurotoxicology. Spencer, P.S. and Schaumburg, H.H. eds., Williams and Wilkins Co.,. Baltimore/London, p. 726-742.

(18) Bancroft, J.D. and Steven A., (1990) Theory and Pratice of Histological Techniques. Chapter 17, Neuropathological Techniques. Lowe, James and Cox, Gordon eds. Churchill Livingstone.

Table 1

Minimum numbers of animals needed per group when the neurotoxicity study is conducted separately or in combination whit studies

	NEUROTOXICITY STUDY CONDUCTED AS:			
	Separate study	Combined study with the 28-day study	Combined study with the 90-day study	Combined study with the chronic toxicity study
Total number of animals per group	10 males and 10 females	10 males and 10 females	15 males and 15 females	25 males and 25 females
Number of animals selected for functional testing including detailed clinical observations	10 males and 10 females	10 males and 10 females	10 males and 10 females	10 males and 10 females
Number of animals selected per perfusion *in situ* and neurohistopathology	5 males and 5 females	5 males and 5 females	5 males and 5 females	5 males and 5 females
Number of animals selected for repeated dose/subchronic/chronic toxicity observations, haematology, clinical biochemistry, histopathology, etc. as indicate in the respective *Guidelines*		5 males and 5 females	10 males † and 10 females †	20 males † and 20 females †
Supplemental observations, as appropriate	5 males and 5 females			

† Includes five animals selected for functional testing and detailed clinical observations as part of the neurotoxicity study.

Table 2

Frequency of clinical observation and functional tests

Type of observations		Study duration			
		Acute	28-day	90-day	Chronic
In all animals	General health condition	daily	daily	daily	daily
	Mortality/morbidity	Twice daily	Twice daily	Twice daily	Twice daily
In animals selected for functional observations	Detailed clinical observations	— prior to first exposure — within 8 hours of dosing at estimate time of peak effect — at day 7 and 14 after dosing	— prior to first exposure — once weekly thereafter	— prior to first exposure — once during the first or second week of exposure — monthly thereafter	— prior to first exposure — once at the end of the first month of exposure — every three months thereafter
	Functional tests	— prior to first exposure — within 8 hours of dosing at estimate time of peak effect — at day 7 and 14 after dosing	— prior to first exposure — during the fourth week of treatment as close as possible to the end of the exposure period	— prior to first exposure — once during the first or second week of exposure — monthly thereafter	— prior to first exposure — once at the end of the first month of exposure — every three months thereafter

B.44. SKIN ABSORPTION: *IN VIVO* METHOD

1. METHOD

This testing method is equivalent to the OECD TG 427 (2004).

1.1. INTRODUCTION

Exposure to many chemicals occurs mainly *via* the skin whilst the majority of toxicological studies performed in laboratory animals use the oral route of administration. The *in vivo* percutaneous absorption study set out in this guideline provides the linkage necessary to extrapolate from oral studies when making safety assessments following dermal exposure.

A substance must cross a large number of cell layers of the skin before it can reach the circulation. The rate-determining layer for most substances is the *stratum corneum* consisting of dead cells. Permeability through the skin depends both on the lipophilicity of the chemical and the thickness of the outer layer of epidermis, as well on factors such as molecular weight and concentration of the substance. In general, the skin of rats and rabbits is more permeable than that of humans, whereas the skin permeability of guinea pigs and monkeys is more similar to that of humans.

The methods for measuring percutaneous absorption can be divided into two categories; *in vivo* and *in vitro*.The *in vivo* method is capable of providing good information, in various laboratory species, on skin absorption. More recently *in vitro* methods have been developed. These utilise transport across full or partial thickness animal or human skin to a fluid reservoir. The *in vitro* method is described in a separate testing method (1). It is recommended that the OECD Guidance Document for the Conduct of Skin Absorption Studies (2) be consulted to assist in the selection of the most appropriate method in the given situation, as it provides more details on the suitability of both *in vivo* and in vitro methods.

The *in vivo* method, described in this method, allows the determination of the penetration of the test substance through the skin into the systemic compartment. The technique has been widely used for many years (3)(4)(5)(6)(7). Although *in vitro* percutaneous absorption studies may in many cases be appropriate there may be situations in which only an *in vivo* study can provide the necessary data.

Advantages of the *in vivo* method are that it uses a physiologically and metabolically intact system, uses a species common to many toxicity studies and can be modified for use with other species. The disadvantages are the use of live animals, the need for radiolabelled material to facilitate reliable results, difficulties in determining the early absorption phase and the differences in permeability of the preferred species (rat) and human skin. Animal skin is generally more permeable and therefore may overestimate human percutaneous absorption (6)(8)(9). Caustic/corrosive substances should not be tested in live animals.

1.2. DEFINITIONS

Unabsorbed dose: represents that washed from the skin surface after exposure and any present on the non-occlusive cover, including any dose shown to volatilise from the skin during exposure.

Absorbed dose (*in vivo*): comprises that present in urine, cage wash, faeces, expired air (if measured), blood, tissues (if collected) and the remaining carcass, following removal of application site skin.

Absorbable dose: represents that present on or in the skin following washing.

1.3. PRINCIPLE OF THE TEST METHOD

The test substance, preferably radiolabelled, is applied to the clipped skin of animals at one or more appropriate dose levels in the form of a representative in-use preparation. The test preparation is allowed to remain in contact with the skin for a fixed period of time under a suitable cover (non-occlusive, semi-occlusive, or occlusive) to prevent ingestion of the test preparation. At the end of the exposure time the cover is removed and the skin is cleaned with an appropriate cleansing agent, the cover and the cleansing materials are retained for analysis and a fresh cover applied. The animals are housed prior to, during and after the exposure period in individual metabolism cages and the excreta and expired air over these periods are collected for analysis. The

collection of expired air can be omitted when there is sufficient information that little or no volatile radioactive metabolite is formed. Each study will normally involve several groups of animals that will be exposed to the test preparation. One group will be killed at the end of the exposure period. Other groups will be killed at scheduled time intervals thereafter (2). At the end of the sampling time the remaining animals are killed, blood is collected for analysis, the application site removed for analysis and the carcass is analysed for any unexcreted material. The samples are assayed by appropriate means and the degree of percutaneous absorption is estimated (6)(8)(9).

1.4. DESCRIPTION OF THE METHOD

1.4.1. **Selection of animal species**

The rat is the most commonly used species, but hairless strains and species having skin absorption rates more similar to those of human, can also be used (3)(6)(7)(8)(9). Young adult healthy animals of a single sex (with males as the default sex) of commonly used laboratory strains should be employed. At the commencement of the study, the weight variation of animals used should not exceed ± 20 % of the mean weight. As an example, male rats of 200 g – 250 g are suitable, particularly in the upper half of this range.

1.4.2. **Number and sex of animals**

A group of at least four animals of one sex should be used for each test preparation and each scheduled termination time. Each group of animals will be killed after different time intervals, for example at the end of the exposure period (typically 6 or 24 hours) and subsequent occasions (e.g. 48 and 72 hours). If there are data available that demonstrate substantial differences in dermal toxicity between males and females, the more sensitive sex should be chosen. If there are no such data, then either gender can be used.

1.4.3. **Housing and feeding conditions**

The temperature in the experimental animal room should be 22 °C (± 3 °C). Although the relative humidity should be at least 30 % and preferably not exceed 70 % other than during room cleaning, the aim should be 50-60 %. Lighting should be artificial, the sequence being 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used and should be freely available together with an unlimited supply of drinking water. During the study, and preferably also during the acclimatisation, the animals are individually housed in metabolism cages. Since food and water spillage would compromise the results, the probability of such events should be minimised.

1.4.4. **Preparation of animals**

The animals are marked to permit individual identification and kept in their cages for at least five days prior to the start of the study to allow for acclimatisation to the laboratory conditions.

Following the acclimatisation period, and approximately 24 hours prior to dosing, each animal will have an area of skin in the region of the shoulders and the back clipped. The permeation properties of damaged skin are different from intact skin and care should be taken to avoid abrading the skin. Following the clipping and approximately 24 hours before the test substance is applied to the skin, (See Section 1.4.7) the skin surface should be wiped with acetone to remove sebum. An additional soap and water wash is not recommended because any soap residue might promote test substance absorption. The area must be large enough to allow reliable calculation of the absorbed amount of test chemical per cm^2 skin, preferably at least 10 cm^2. This area is practicable with rats of 200-250 g bodyweight. After preparation, the animals are returned to metabolism cages.

1.4.5. **Test substance**

The test substance is the entity whose penetration characteristics are to be studied. Ideally, the test substance should be radiolabelled.

1.4.6. **Test preparation**

The test substance preparation (e.g. neat, diluted, or formulated material containing the test chemical which is applied to the skin) should be the same (or realistic surrogate) as that to which humans or other potential target species may be exposed. Any variations from the 'in-use' preparation must be justified. Where necessary, the test substance is dissolved or suspended in a suitable vehicle. For vehicles other than water the absorption characteristics and potential interaction with the test substance should be known.

1.4.7. **Application to the skin**

An application site of a specific surface area is defined on the skin surface. A known amount of the test preparation is then evenly applied to the site. This amount should normally mimic potential human exposure, typically 1-5 mg/cm^2 for a solid or up to 10 µl/cm^2 for liquids. Any other quantities should be justified by the expected use conditions, the study objectives or physical characteristics of the test preparation. Following application, the treated site must be protected from grooming. An example of a typical device is shown in Figure 1. Normally, the application site will be protected by a non-occlusive cover (e.g. a permeable nylon gauze cover). However, for infinite applications the application site should be occluded. In case of evaporation of semivolatile test substances reduces the recovery rate of the test substance to an unacceptable extend (see also section 1.4.10, first paragraph), it is necessary to trap the evaporated substance in a charcoal filter covering the application device (see Figure 1). It is important that any device does not damage the skin, nor absorb or react with the test preparation. The animals are returned to individual metabolism cages in order to collect excreta.

1.4.8. **Duration of exposure and sampling**

The duration of exposure is the time interval between application and removal of test preparation by skin washing. A relevant exposure period (typically 6 or 24 hours) should be used, based on the expected human exposure duration. Following the exposure period, the animals are maintained in the metabolism cages until the scheduled termination. The animals should be observed for signs of toxicity/abnormal reactions at regular intervals for the entire duration of the study. At the end of the exposure period the treated skin should be observed for visible signs of irritation.

The metabolism cages should permit separate collection of urine and faeces throughout the study. They should also allow collection of ^{14}C-carbon dioxide and volatile ^{14}C-carbon compounds, which should be analysed when produced in quantity (> 5 %). The urine, faeces and trap fluids (e.g. ^{14}C-carbon dioxide and volatile ^{14}C-compounds) should be individually collected from each group at each sampling time. If there is sufficient information that little or no volatile radioactive metabolite is formed, open cages can be used.

Excreta are collected during the exposure period, up to 24 hours after the initial skin contact and then daily until the end of the experiment. Whilst three excreta collection intervals will normally be sufficient, the envisaged purpose of the test preparation or existing kinetic data may suggest more appropriate or additional time points for study.

At the end of the exposure period the protective device is removed from each animal and retained separately for analysis. The treated skin of all animals should be washed at least three times with cleansing agent using suitable swabs. Care must be taken to avoid contaminating other parts of the body. The cleansing agent should be representative of normal hygiene practice, e.g. aqueous soap solution. Finally, the skin should be dried. All swabs and washings must be retained for analysis. A fresh cover should be applied to protect the treated site of those animals forming later time point groups prior to their return to individual cages.

1.4.9. **Terminal procedures**

For each group, the individual animals should be killed at the scheduled time and blood collected for analysis. The protective device or cover should be removed for analysis. The skin from the application site and a similar area of non-dosed, clipped skin should be removed from each animal for separate analysis. The application site may be fractioned to separate the *stratum corneum* from the underlying epidermis to provide more information on the test chemical disposition. The determination of this disposition over a time course after the exposure period should provide some indication of the fate of any test chemical in the *stratum corneum*. To facilitate skin fractionation (following the final skin wash and killing the animal) each protective cover is removed. The application site skin, with annular ring of surrounding skin, is excised from the rat and pinned on a board. A strip of adhesive tape is applied to the skin surface using gentle pressure and the tape removed together with part of the *stratum corneum*. Successive strips of tape are applied until the tape no longer adheres to the skin surface, when all of the *stratum corneum* has been removed. For each animal, all the tape strips may be combined in a single container to which a tissue digestant is added to solubilise the *stratum corneum*. Any potential target tissues may be removed for separate measurement before the residual carcass is analysed for absorbed carcass dose. The carcasses of the individual animals should be retained for analysis. Usually analysis of the total content will be sufficient. Target organs may be removed for separate analysis (if indicated by other studies). Urine present in the bladder at scheduled kill should be added to the previous urine collection. After collection of the excreta from metabolism cages at the time scheduled kill, the cages and their traps should be washed with an appropriate solvent. Other potentially contaminated equipment should likewise be analysed.

1.4.10. **Analysis**

In all studies adequate recovery (i.e. mean of 100 ± 10 % of the radioactivity) should be achieved. Recoveries outside this range must be justified. The amount of the administered dose in each sample should be analysed by suitably validated procedures.

Statistical considerations should include a measure of variance for the replicates for each application.

2. **DATA**

The following measurements should be made for each animal, at each sampling time for the test chemical and/or metabolites. In addition to individual data, data grouped according to sampling times should be reported as means.

— quantity associated with the protective appliances,

— quantity that can be dislodged from the skin,

— quantity in/on skin that cannot be washed from the skin,

— quantity in the sampled blood,

— quantity in the excreta and expired air (if appropriate),

— quantity remaining in the carcass and any organs removed for separate analysis.

The quantity of test substance and/or metabolites in the excreta, expired air, blood and in the carcass will allow determination of the total amount absorbed at each time point. A calculation of the amount of test chemical absorbed per cm^2 of skin exposed to the test substance over the exposure period can also be obtained.

3. **REPORTING**

3.1. TEST REPORT

The test report must include the requirements stipulated in the protocol, including a justification for the test system used and should comprise the following:

test substance:

— identification data (e.g. CAS number, if available, source, purity (radiochemical purity), known impurities, lot number),

— physical nature, physicochemical properties (e.g. pH, volatility, solubility, stability, molecular weight and log P_{ow}).

Test preparation:

— formulation and justification of use,

— details of the test preparation, amount applied, achieved concentration, vehicle, stability and homogeneity.

Test animal:

— species/strain used,

— number, age and sex of animals,

— source of animals, housing conditions, diets, etc.,

— individual animal weights at start of test.

Test conditions:

— details of the administration of the test preparation (site of application, assay methods, occlusion/non-occlusion, volume, extraction, detection),

— details of food and water quality.

Results:

— any signs of toxicity,

— tabulated absorption data (expressed as rate, amount or percentage),

— overall recoveries of the experiment,

— interpretation of the results, comparison with any available data on percutaneous absorption of the test compound.

Discussion of the results.

Conclusions.

4. **REFERENCES**

(1) Testing Method B.45. Skin Absorption: *In vitro* Method.

(2) OECD (2002). Guidance Document for the Conduct of Skin Absorption Studies. OECD, Paris.

(3) ECETOC, (1993) Percutaneous Absorption. European Centre for Ecotoxicology and Toxicology of Chemicals, Monograph No 20.

(4) Zendzian R.P. (1989) Skin Penetration Method suggested for Environmental Protection Agency Requirements. *J. Am. Coll. Toxicol.* 8(5), p. 829-835.

(5) Kemppainen, B.W., Reifenrath WG (1990) Methods for skin absorption. CRC Press Boca Raton, FL, USA.

(6) EPA, (1992) Dermal Exposure Assessment: Principles and Applications. Exposure Assessment Group, Office of Health and Environmental Assessment.

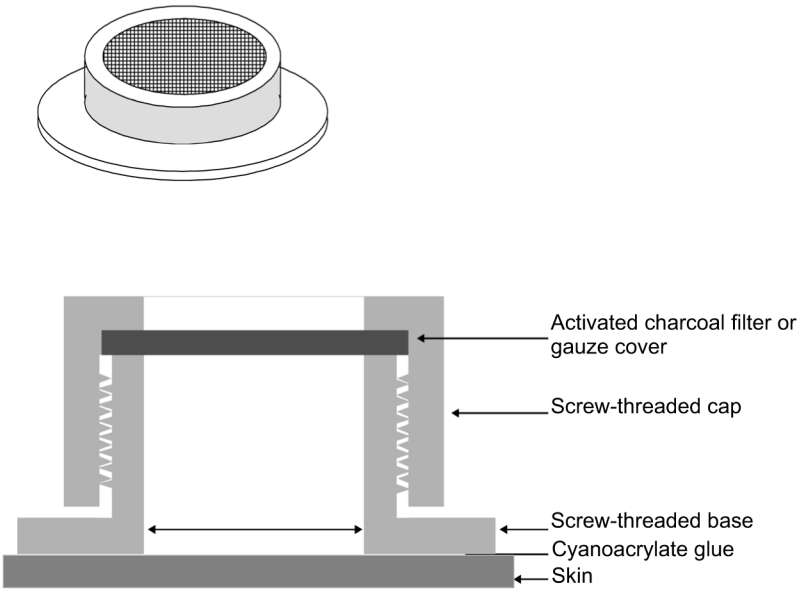
(7) EPA, (1998) Health Effects Test Guidelines, OPPTS 870-7600, Dermal Penetration. Office of Prevention, Pedsticides and Toxic Substances.

(8) Bronaugh, R.L., Wester, R.C., Bucks, D., Maibach H.I. and Sarason, R., (1990) In vivo percutaneous absorption of fragrance ingredients in reshus monkeys and humans. *Fd. Chem. Toxic.* 28, p. 369-373.

(9) Feldman, R.J. and Maibach, H.I., (1970) Absorption of some organic compounds through the skin in man. *J. Invest Dermatol.* 54, p. 399-404.

Figure 1

An example of a design of a typical device used to define and protect dermal application site during *in vivo* percutaneous absorption studies



B.45. SKIN ABSORPTION: *IN VITRO* METHOD

1. METHOD

This testing method is equivalent to the OECD TG 428 (2004).

1.1. INTRODUCTION

This method has been designed to provide information on absorption of a test substance applied to excised skin. It can either be combined with the method for skin absorption: *in vivo* method (1), or be conducted separately. It is recommended that the OECD Guidance Document for the Conduct of Skin Absorption Studies (2) be consulted to assist in the design of studies based on this method. The Guidance Document has been prepared to facilitate the selection of appropriate *in vitro* procedures for use in specific circumstances, to ensure the reliability of results obtained by this method.

The methods for measuring skin absorption and dermal delivery can be divided into two categories: *in vivo* and *in vitro*. *In vivo* methods on skin absorption are well established and provide pharmacokinetic information in a range of animal species. An *in vivo* method is separately described in another testing method (1). *In vitro* methods have also been used for many years to measure skin absorption. Although formal validation studies of the *in vitro* methods covered by this testing method have not been performed, OECD experts agreed in 1999 that there was sufficient data evaluated to support the *in vitro* method (3). Further details that substantiate this support, including a significant number of direct comparisons of *in vitro* and *in vivo* methods, are provided with the Guidance Document (2). There are a number of monographs that review this topic and provide detailed background on the use of an *in vitro* method (4)(5)(6)(7)(8)(9)(10)(11)(12). *In vitro* methods measure the diffusion of chemicals into and across skin to a fluid reservoir and can utilise non-viable skin to measure diffusion only, or fresh, metabolically active skin to simultaneously measure diffusion and skin metabolism. Such methods have found particular use as a screen for comparing delivery of chemicals into and through skin from different formulations and can also provide useful models for the assessment of percutaneous absorption in humans.

The *in vitro* method may not be applicable for all situations and classes of chemicals. It may be possible to use the *in vitro* test method for an initial qualitative evaluation of skin penetration. In certain cases, it may be necessary to follow this up with *in vivo* data. The guidance document (2) should be consulted for further elaboration of situations where the *in vitro* method would be suitable. Additional detailed information to support the decision is provided in (3).

This method presents general principles for measuring dermal absorption and delivery of a test substance using excised skin. Skin from many mammalian species, including humans, can be used. The permeability properties of skin are maintained after excision from the body because the principal diffusion barrier is the non-viable *stratum corneum*; active transport of chemicals through the skin has not been identified. The skin has been shown to have the capability to metabolise some chemicals during percutaneous absorption (6), but this process is not rate limiting in terms of actual absorbed dose, although it may affect the nature of the material entering the bloodstream.

1.2. DEFINITIONS

Unabsorbed dose: represents that washed from the skin surface after exposure and any present on the non-occlusive cover, including any dose shown to volatilise from the skin during exposure.

Absorbed dose (*in vitro*): mass of test substance reaching the receptor fluid or systemic circulation within a specified period of time.

Absorbable dose (*in vitro*): represents that present on or in the skin following washing.

1.3. PRINCIPLE OF THE TEST METHOD

The test substance, which may be radiolabelled, is applied to the surface of a skin sample separating the two chambers of a diffusion cell. The chemical remains on the skin for a specified time under specified conditions, before removal by an appropriate cleansing procedure. The receptor fluid is sampled at time points throughout the experiment and analysed for the test chemical and/or metabolites.

When metabolically active systems are used, metabolites of the test chemical may be analysed by appropriate methods. At the end of the experiment the distribution of the test chemical and its metabolites are quantified, when appropriate.

Using appropriate conditions, which are described in this method and the guidance document (2), absorption of a test substance during a given time period is measured by analysis of the receptor fluid and the treated skin. The test substance remaining in the skin should be considered as absorbed unless it can be demonstrated that absorption can be determined from receptor fluid values alone. Analysis of the other components (material washed off the skin and remaining within the skin layers) allows for further data evaluation, including total test substance disposition and percentage recovery.

To demonstrate the performance and reliability of the test system in the performing laboratory, the results for relevant reference chemicals should be available and in agreement with published literature for the method used. This requirement could be met by testing an appropriate reference substance (preferably of a lipophilicity close to the test substance) concurrently with the test substance or by providing adequate historical data for a number of reference substances of different lipophilicity (e.g. caffeine, benzoic acid, and testosterone).

1.4. DESCRIPTION OF THE TEST METHOD

1.4.1. **Diffusion cell**

A diffusion cell consists of a donor chamber and a receptor chamber between which the skin is positioned (an example of a typical design is provided in Figure 1). The cell should provide a good seal around the skin, enable easy sampling and good mixing of the receptor solution in contact with the underside of the skin, and good temperature control of the cell and its contents. Static and flow-through diffusion cells are both acceptable. Normally, donor chambers are left unoccluded during exposure to a finite dose of a test preparation. However, for infinite applications and certain scenarios for finite doses, the donor chambers may be occluded.

1.4.2. **Receptor fluid**

The use of a physiologically conducive receptor fluid is preferred although others may also be used provided that they are justified. The precise composition of the receptor fluid should be provided. Adequate solubility of the test chemical in the receptor fluid should be demonstrated so that it does not act as a barrier to absorption. In addition, the receptor fluid should not affect skin preparation integrity. In a flow-through system, the rate of flow must not hinder diffusion of a test substance into the receptor fluid. In a static cell system, the fluid should be continuously stirred and sampled regularly. If metabolism is being studied, the receptor fluid must support skin viability throughout the experiment.

1.4.3. **Skin preparations**

Skin from human or animal sources can be used. It is recognised that the use of human skin is subject to national and international ethical considerations and conditions. Although viable skin is preferred, non-viable skin can also be used provided that the integrity of the skin can be demonstrated. Either epidermal membranes (enzymically, heat or chemically separated) or split thickness skin (typically 200-400 μm thick) prepared with a dermatome, are acceptable. Full thickness skin may be used but excessive thickness (approximately > 1 mm) should be avoided unless specifically required for determination of the test chemical in layers of the skin. The selection of species, anatomical site and preparative technique must be justified. Acceptable data from a minimum of four replicates per test preparation are required.

1.4.4. **Skin preparation integrity**

It is essential that the skin is properly prepared. Inappropriate handling may result in damage to the *stratum corneu*m, hence the integrity of the prepared skin must be checked. When skin metabolism is being investigated, freshly excised skin should be used as soon as possible, and under conditions known to support metabolic activity. As a general guidance, freshly excised skin should be used within 24 hours, but the acceptable storage period may vary depending on the enzyme system involved in metabolisation and storage temperatures (13). When skin preparations have been stored prior to use, evidence should be presented to show that barrier function is maintained.

1.4.5. **Test substance**

The test substance is the entity whose penetration characteristics are to be studied. Ideally, the test substance should be radiolabelled.

1.4.6. **Test preparation**

The test substance preparation (e.g. neat, diluted or formulated material containing the test substance which is applied to the skin) should be the same (or a realistic surrogate) as that to which humans or other potential target species may be exposed. Any variation from the 'in-use' preparation must be justified.

1.4.7. **Test substances concentrations and formulations**

Normally more than one concentration of the test substance is used spanning the upper of potential human exposures. Likewise, testing a range of typical formulations should be considered.

1.4.8. **Application to the skin**

Under normal conditions of human exposure to chemicals, finite doses are usually encountered. Therefore, an application that mimics human exposure, normally 1-5 mg/cm^2 of skin for a solid and up to10 µl/cm^2 for liquids, should be used. The quantity should be justified by the expected use conditions, the study objectives or physical characteristics of the test preparation. For example, applications to the skin surface may be infinite, where large volumes per unit area are applied.

1.4.9. **Temperature**

The passive diffusion of chemicals (and therefore their skin absorption) is affected by temperature. The diffusion chamber and skin should be maintained at a constant temperature close to normal skin temperature of 32 ± 1 °C. Different cell designs will require different water bath or heated block temperatures to ensure that the receptor/skin is at its physiological norm. Humidity should preferably be between 30 and 70 %.

1.4.10. **Duration of exposure and sampling**

Skin exposure to the test preparation may be for the entire duration of the experiment or for shorter times (i.e., to mimic a specific type of human exposure). The skin should be washed of excess test preparation with a relevant cleansing agent, and the rinses collected for analysis. The removal procedure of the test preparation will depend on the expected use condition, and should be justified. A period of sampling of 24 hours is normally required to allow for adequate characterisation of the absorption profile. Since skin integrity may start to deteriorate beyond 24 hours, sampling times should not normally exceed 24 hours. For test substances that penetrate the skin rapidly this may not be necessary but, for test substances that penetrate slowly, longer times may be required. Sampling frequency of the receptor fluid should allow the absorption profile of the test substance to be presented graphically.

1.4.11. **Terminal procedures**

All components of the test system should be analysed and recovery is to be determined. This includes the donor chamber, the skin surface rinsing, the skin preparation and the receptor fluid/chamber. In some cases, the skin may be fractionated into the exposed area of skin and area of skin under the cell flange, and into *stratum corneu*m, epidermis and dermis fractions, for separate analysis.

1.4.12. **Analysis**

In all studies adequate recovery should be achieved (the aim should be a mean of 100±10 % of the radioactivity and any deviation should be justified). The amount of test substance in the receptor fluid, skin preparation, skin surface washings and apparatus rinse should be analysed, using a suitable t echnique.

2. **DATA**

The analysis of receptor fluid, the distribution of the test substance chemical in the test system and the absorption profile with time, should be presented. When finite dose conditions of exposure are used, the quantity washed from the skin, the quantity associated with the skin (and in the different skin layers if analysed) and the amount present in the receptor fluid (rate, and amount or percentage of applied dose) should be calculated. Skin absorption may sometimes be expressed using receptor fluid data alone. However, when the test substance remains in the skin at the end of the study, it may need to be included in the total amount absorbed (see paragraph 66 in reference (3)). When infinite dose conditions of exposure are used the data may permit the calculation of a permeability constant (Kp). Under the latter conditions, the percentage absorbed is not relevant.

3. **REPORTING**

3.1. TEST REPORT

The test report must include the requirements stipulated in the protocol, including a justification for the test system used and should, comprise the following:

test substance:

— physical nature, physicochemical properties (at least molecular weight and log P_{ow}), purity (radiochemical purity),

— identification information (e.g. batch number),

— solubility in receptor fluid.

Test preparation:

— formulation and justification of use,

— homogeneity.

Test conditions:

— sources and site of skin, method of preparation, storage conditions prior to use, any pre-treatment (cleaning, antibiotic treatments, etc.), skin integrity measurements, metabolic stat us, justification of use,

— cell design, receptor fluid composition, receptor fluid flow rate or sampling times and procedures,

— details of application of test preparation and quantification of dose applied,

— duration of exposure,

— details of removal of test preparation from the skin, e.g. skin rinsing,

— details of analysis of skin and any fractionation techniques employed to demonstrate skin distribution,

— cell and equipment washing procedures,

— assay methods, extraction techniques, limits of detection and analytical method validation.

Results:

— overall recoveries of the experiment (Applied dose ≡ Skin washings + Skin + Receptor fluid + Cell washings),

— tabulation of individual cell recoveries in each compartment,

— absorption profile,

— tabulated absorption data (expressed as rate, amount or percentage).

Discussion of results.

Conclusions.

4. **REFERENCES**

(1) Testing Method B.44. Skin Absorption: *In Vivo* Method.

(2) OECD, (2002) Guidance Document for the Conduct of Skin Absorption Studies. OECD, Paris.

(3) OECD, (2000) Report of the Meeting of the OECD Extended Steering Committee for Percutaneous Absorption Testing, Annex 1 to ENV/JM/TG(2000)5. OECD, Paris.

(4) Kemppainen B.W. and Reifenrath W.G., (1990) Methods for skin absorption. CRC Press, Boca Raton.

(5) Bronaugh R.L. and Collier, S.W., (1991) Protocol for In vitro Percutaneous Absorption Studies, in *In vitro Percutaneous Absorption: Principles, Fundamentals and Applications*, RL Bronaugh and HI Maibach, Eds., CRC Press, Boca Raton, p. 237-241.

(6) Bronaugh R.L. and Maibach H.I., (1991) *In vitro Percutaneous Absorption: Principles, Fundamentals and Applications*. CRC Press, Boca Raton.

(7) European Centre for Ecotoxicology and Toxicology of Chemicals, (1993) Monograph No 20, Percutaneous Absorption, ECETOC, Brussels.

(8) Diembeck W, Beck H, Benech-Kieffer F, Courtellemont P, Dupuis J, Lovell W, Paye M, Spengler J, Steiling W., (1999) Test Guidelines for *In Vitro* Assessment of Dermal Absorption and Percutaneous Penetration of Cosmetic Ingredients, Fd Chem Tox, 37, p. 191-205.

(9) Recommended Protocol for *In vitro* Percutaneous Absorption Rate Studies (1996). US Federal Register, Vol. 61, No 65.

(10) Howes, D., Guy, R., Hadgraft, J., Heylings, J.R. *et al.* (1996). Methods for assessing Percutaneous absorption. ECVAM Workshop Report ATLA 24, 81 R10.

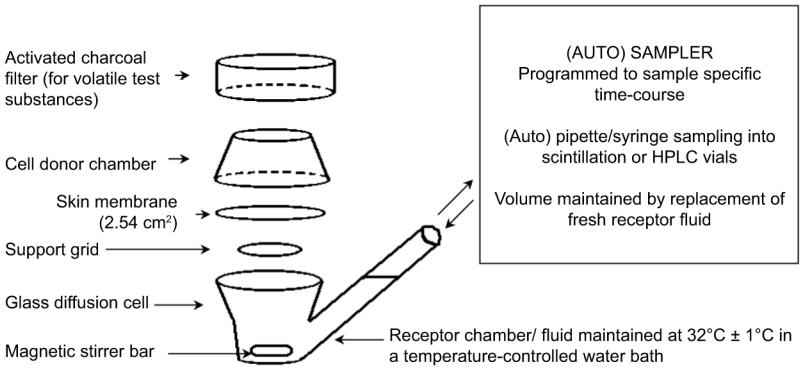
(11) Schaefer, H. and Redelmeier, T.E., (1996). Skin barrier: principles of percutaneous absorption. Karger, Basel.

(12) Roberts, M.S. and Walters, K.A., (1998). Dermal absorption and toxicity assessment. Marcel Dekker, New York.

(13) Jewell, C., Heylings, J.R., Clowes, H.M. and Williams, F.M. (2000). Percutaneous absorption and metabolism of dinitrochlorobenzene in vitro. Arch Toxicol 74, p. 356-365.

Figure 1

An example of a typical design of a static diffusion cell for *in vitro* percutaneous absorption studies



PART C: METHODS FOR THE DETERMINATION OF ECOTOXICITY

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C.1. ACUTE TOXICITY FOR FISH

1. METHOD

1.1. INTRODUCTION

The purpose of this test is to determine the acute lethal toxicity of a substance to fish in fresh water. It is desirable to have, as far as possible, information on the water solubility, vapour pressure, chemical stability, dissociation constants and biodegradability of the substance to help in the selection of the most appropriate test method (static, semi-static or flow-through) for ensuring satisfactorily constant concentrations of the test substance over the period of the test.

Additional information (for instance structural formula, degree of purity, nature and percentage of significant impurities, presence and amounts of additives, and n-octanol/water partition coefficient) should be taken into consideration in both the planning of the test and interpretation of the results.

1.2. DEFINITIONS AND UNITS

Acute toxicity is the discernible adverse effect induced in an organism within a short time (days) of exposure to a substance. In the present test, acute toxicity is expressed as the median lethal concentration (LC_{50}) that is the concentration in water which kills 50 % of a test batch of fish within a continuous period of exposure which must be stated.

All concentrations of the test substance are given in weight by volume (milligrams per litre). They may also be expressed as weight by weight (mg/kg^{-1}).

1.3. REFERENCE SUBSTANCES

A reference substance may be tested as a means of demonstrating that under the laboratory test conditions the response of tested species have not changed significantly.

No reference substances are specified for this test.

1.4. PRINCIPLE OF THE TEST METHOD

A limit test may be performed at 100 mg per litre in order to demonstrate that the LC_{50} is greater than this concentration.

The fish are exposed to the test substance added to water at a range of concentrations for a period of 96 hours. Mortalities are recorded at least at 24-hour intervals, and the concentrations killing 50 % of the fish (LCso) at each observation time are calculated where possible.

1.5. QUALITY CRITERIA

The quality criteria shall apply to the limit test as well as the full test method.

The mortality in the controls must not exceed 10 % (or one fish if less than ten are used) by the end of the test.

The dissolved oxygen concentration must have been more than 60 % of the air-saturation value throughout.

The concentrations of the test substance shall be maintained to within 80 % of the initial concentrations throughout the duration of the test.

For substances which dissolve easily in the test medium, yielding stable solutions i.e. those which will not to any significant extent volatilise, degrade, hydrolyze or adsorb, the initial concentration can be taken as being equivalent to the nominal concentration. Evidence shall be presented that the concentrations have been maintained throughout the test and that the quality criteria have been satisfied.

For substances that are:

(i) poorly soluble in the test medium, or

(ii) capable of forming stable emulsions or dispersions, or

(iii) not stable in aqueous solutions,

the initial concentration shall be taken as the concentration measured in solution (or, if technically not possible, measured in the water column) at the start of the test. The concentration shall be determined after a period of equilibration but before the introduction of the test fish.

In any of these cases, further measurements must be made during the test to confirm the actual exposure concentrations or that the quality criteria have been met.

The pH should not vary by more than 1 unit.

1.6. DESCRIPTION OF THE TEST METHOD

Three types of procedure can be used:

Static test:

Toxicity test in which no flow of test solution occurs. (Solutions remain unchanged throughout the duration of the test.)

Semi-static test:

Test without flow of test solution, but with regular batchwise renewal of test solutions after prolonged periods (e.g. 24 hours).

Flow-through test:

Toxicity test in which the water is renewed constantly in the test chambers, the chemical under test being transported with the water used to renew the test medium.

1.6.1. **Reagents**

1.6.1.1. *Solutions of test substances*

Stock solutions of the required strength are prepared by dissolving the substance in deionised water or water according to 1.6.1.2.

The chosen test concentrations are prepared by dilution of the stock solution. If high concentrations are tested, the substance may be dissolved in the dilution water directly.

The substances should normally only be tested up to the limit of solubility. For some substances (e.g. substances having low solubility in water, or high P_{ow}, or those forming stable dispersion rather than true solution in water), it is acceptable to run a test concentration above the solubility limit of the substance to ensure that the maximum soluble/stable concentration has been obtained. It is important, however, that this concentration will not otherwise disturb the test system (e.g. film of the substance on the water surface preventing the oxygenation of the water, etc.).

Ultrasonic dispersion, organic solvents, emulsifiers or dispersants may be used as an aid to prepare stock solutions of substances with low aqueous solubility or to help to disperse these substances in the test medium. When such auxiliary substances are used, all test concentrations should contain the same amount of auxiliary substance, and additional control fish should be exposed to the same concentration of the auxiliary substance as that used in the test series. The concentration of such auxiliaries should be minimised, but in no case should exceed 100 mg per litre in the test medium.

The test should be carried out without adjustment of the pH. If there is evidence of marked change in the pH, it is advised that the test should be repeated with pH adjustment and the results reported. In that case, the pH value of the stock solution should be adjusted to the pH value of the dilution water unless there are specific reasons not to do so. HCl and NaOH are preferred for this purpose. This pH adjustment should be made in such a way that the concentration of test substance in the stock solution is not changed to any significant extent. Should any chemical reaction or physical precipitation of the test compound be caused by the adjustment, this should be reported.

1.6.1.2. *Holding and dilution water*

Orinking-water supply (uncontaminated by potentially harmful concentrations of chlorine, heavy metals or other substances), good-quality natural water or reconstituted water (See Appendix 1) may be used. Waters with a total hardness of between 10 and 250 mg per litre (as $CaCO_3$) and with a pH from 6,0 to 8,5 are preferred.

1.6.2. **Apparatus**

All apparatus must be made of chemically inert material:

— automatic dilution system (for flow-through test),

— oxygen meter,

— equipment for determination of hardness of water,

— adequate apparatus for temperature control,

— pH meter.

1.6.3. **Test fish**

The fish should be in good health and free from any apparent malformation.

The species used should be selected on the basis of practical criteria, such as their ready availability throughout the year, ease of maintenance, convenience for testing, relative sensitivity to chemicals, and any economic, biological or ecological factors which have any bearing. The need for comparability of the data obtained and existing international harmonisation (reference 1) should also be borne in mind when selecting the fish species.

A list of fish species which are recommended for the performance of this test is given in Appendix 2; Zebra fish and rainbow trout are the preferred species.

1.6.3.1. *Holding*

Test fish should preferably come from a single stock of similar length and age. The fish must be held for at least 12 days, in the following conditions:

loading:

appropriate to the system (recirculation or flow-through) and the fish species,

water:

see 1.6.1.2,

light:

12 to 16 hours illumination daily,

dissolved oxygen concentration:

at least 80 % of air-saturation value,

feeding:

three times per week or daily, ceasing 24 hours before the start of the test.

1.6.3.2. *Mortality*

Following a 48-hour settling-in period, mortalities are recorded and the following criteria applied:

— greater than 10 % of population in seven days:

rejection of entire batch,

— between 5 and 10 % of population:

holding period continued for seven additional days.

If no further mortalities occur, the batch is acceptable, otherwise it must be rejected,

— less than 5 % of population:

acceptance of the batch.

1.6.4. **Adaptation**

All fish must be exposed to water of the quality and the temperature to be used in the test for at least seven days before they are used.

1.6.5. **Test procedure**

A range-finding test can precede a definitive test, in order to obtain information about the range of concentrations to be used in the main test.

One control without the test substance is run and, if relevant, one control containing the auxiliary substance is also run, in addition to the test series.

Depending on the physical and chemical properties of the test compound, a static, semi-static, or a flow-through test should be selected as appropriate, to fulfil the quality criteria.

Fish are exposed to the substance as described below:

— duration: 96 hours,

— number of animals: at least seven per concentration,

— tanks: of suitable capacity in relation to the recommended loading,

— loading: maximum loading of 1 g per litre for static and semi-static tests is recommended; for flow-through systems, higher loading is acceptable,

— test concentration: At least five concentrations differing by a constant factor not exceeding 2,2 and as far as possible spanning the range of 0 to 100 % mortality,

— water: see 1.6.1.2,

— light: 12 to 16 hours illumination daily,

— temperature: appropriate to the species (Appendix 2) but within ± 1 °C within any particular test,

— dissolved oxygen concentration: not less than 60 % of the air-saturation value at the selected temperature,

— feeding: none.

The fish are inspected after the first two to four hours and at least at 24-hour intervals. Fish are considered dead if touching of the caudal peduncle produces no reaction, and no breathing movements are visible. Dead fish are removed when observed and mortalities are recorded. Records are kept of visible abnormalities (e.g. loss of equilibrium, changes in swimming behaviour, respiratory function, pigmentation, etc.).

Measurements of pH, dissolved oxygen and temperature must be carried out daily.

Limit test

Using the procedures described in this test method, a limit test may be performed at 100 mg per litre in order to demonstrate that the LC_{50} is greater than this concentration.

If the nature of the substance is such that a concentration of 100 mg per litre in the test water cannot be attained, the limit test should be performed at a concentration equal to the solubility of the substance (or the maximum concentration forming a stable dispersion) in the medium used (see also point 1.6.1.1).

The limit test should be performed using seven to 10 fish, with the same number in the control(s). (Binomial theory dictates that when 10 fish are used with zero mortality, there is a 99,9 % confidence that the LC_{50} is greater than the concentration used in the limit test. With 7, 8 or 9 fish, the absence of mortality provides at least 99 % confidence that the LC_{50} is greater than the concentration used.)

If mortalities occur, a full study must be carried out. If sublethal effects are observed, these should be recorded.

2. DATA AND EVALUATION

For each period where observations were recorded (24, 48, 72 and 96 hours), plot percentage mortality for each recommended exposure period against concentration on logarithmic-probability paper.

When possible and for each observation time, the LC_{50} and the confidence limits ($p = 0,05$) should be estimated using standard procedures; these values should be rounded off to one, or at most two significant figures (examples of rounding off to two figures: 170 for 173,5; 0,13 for 0,127; 1,2 for 1,21).

In those cases where the slope of the concentration/percentage response curve is too steep to permit calculation of the LC_{50}, a graphical estimate of this value is sufficient.

When two consecutive concentrations, at a ratio of 2,2 give only 0 and 100 % mortality, these two values are sufficient to indicate the range within which the LC_{50} falls.

If it is observed that the stability or homogeneity of the test substance cannot be maintained, this should be reported and care should be taken in the interpretation of the results.

3. REPORTING

The test report shall, if possible, include the following information:

— information about test fish (scientific name, strain, supplier, any pretreatment, size and number used in each test concentration),

— dilution-water source and major chemical characteristics (pH, hardness, temperature),

— in the case of a substance of low aqueous solubility, the method of preparation of stock and test solutions,

— concentration of any auxiliary substances,

— list of the concentrations used and any available information on the stability at the concentrations of the tested chemical in the test solution,

— if chemical analyses are performed, methods used and results obtained,

— results of the limit test if conducted,

— reasons for the choice and details of the test procedure used (e.g. static, semi-static, dosing rate, flow-through rate, whether aerated, fish loading, etc.),

— description of test equipment,

— lighting regime,

— dissolved oxygen concentrations, pH values and temperatures of the test solutions every 24 hours,

— evidence that the quality criteria have been fulfilled,

— a table showing the cumulative mortality at each concentration and the control (and control with the auxiliary substance if required) at each of the recommended observation times,

— graph of the concentration/percentage response curve at the end of the test,

— if possible, the LC_{50} values at each of the recommended observation times (with 95 % confidence limits),

— statistical procedures used for determining the LC_{50} values,

— if a reference substance is used, the results obtained,

— highest test concentration causing no mortality within the period of the test,

— lowest test concentration causing 100 % mortality within the period of the test.

4. **REFERENCES**

(1) OECD, Paris, 1981, Test Guideline 203, Decision of the Council C(81) 30 final and updates.

(2) AFNOR — Determination of the acute toxicity of a substance to *Brachydanio rerio* — Static and Flow Through methods — NFT 90-303 June 1985.

(3) AFNOR- Determination of the acute toxicity of a substance to *Salmo gairdneri* — Static and Flow — Through methods — NFT 90-305 June 1985.

(4) ISO 7346/1,/2 and/3 — Water Quality — Determination of the acute lethal toxicity of substances to a fresh water fish (*Brachydanio rerio* Hamilton-Buchanan-Teleostei, Cyprinidae). Part 1: Static method. Part 2: Semi-static method. Part 3: Flow-through method.

(5) Eidgenössisches Department des Innern, Schweiz: Richtlinien fur Probenahme und Normung von Wasseruntersuchungsmethoden -Part II 1974.

6) DIN Testverfahren mit Wasserorganismen, 38 412 (11) und 1 (15).

(7) JIS K 0102, Acute toxicity test for fish.

(8) NEN 6506- Water -Bepaling van de akute toxiciteit met behulp van *Poecilia reticulata*, 1980.

9) Environmental Protection Agency, Methods for the acute toxicity tests with fish, macroinvertebrates and amphibians. The Committee on Methods for Toxicity Tests with Aquatic Organisms, Ecological Research Series EPA-660-75-009, 1975.

(10) Environmental Protection Agency, Environmental monitoring and support laboratory, Office of Research and Development, EPA-600/4-78-012, January 1978.

11) Environmental Protection Agency, Toxic Substance Control, Part IV, 16 March 1979.

(12) Standard methods for the examination of water and wastewater, fourteen edition, APHA-AWWA-WPCF,1975.

13) Commission of the European Communities, Inter-laboratory test programme concerning the study of the ecotoxicityof a chemical substance with respect to the fish. EEC Study D.8368, 22 March 1979.

(14) Verfahrensvorschlag des Umweltbundesamtes zum akuten Fisch-Test. Rudolph, P. und Boje, R. Okotoxikologie, Grundlagen fur die okotoxikologische Bewertung von Umweltchemikalien nach dem Chemikaliengesetz, ecomed 1986.

(15) Litchfield, J. T.and Wilcoxon, F., A simplified method for evaluating dose effects experiments, J. Pharm, tExp. Therap., 1949, vol. 96,99.

(16) Finney, D.J. Statistical Methods in Biological Assay. Griffin, Weycombe, U.K., 1978.

17) Sprague, J.B. Measurement of pollutant toxicity to fish. Bioassay methods for acute toxicity. Water Res., 1969, vol. 3,793-821.

(18) Sprague, J.B. Measurement of pollutant toxicity to fish. II Utilising and applying bioassay results. Water Res. 1970, vol. 4, 3-32.

(19) Stephan, C.E. Methods for calculating an LC_{50}. In Aquatic Toxicology and Hazard Evaluation (edited by F.I. Mayer and J.L. Hamelink). American Society for Testing and Materials, ASTM STP 634,1977, 65-84.

(20) Stephan, C.E., Busch, K.A., Smith, R., Burke, J. and Andrews, R.W. A computer program for calculating an LC_{50}. US EPA.

Appendix 1

Reconstituted water

Example of a suitable dilution water

All chemicals must be of analytical grade.

The water should be good-quality distilled water, or deionised water with a conductivity of less than 5 μScm^{-1}.

Apparatus for distillation of water must not contain any parts made of copper.

Stock solutions

$CaCl_2$. $2H_2O$ (calcium chloride dihydrate):	11,76 g
Dissolve in, and make up to 1 litre with water.	
$MgSO_4$. $7H_2O$ (magnesium sulphate heptahydrate):	4,93 g
Dissolve in, and make up to 1litre with water.	
$NaHCO_3$ (sodium hydrogen carbonate):	2,59 g
Dissolve in, and make up to 1 litre with water.	
KCI (potassium chloride):	0,23 g
Dissolve in, and make up to 11itre with water.	

Reconstituted dilution water

Mix 25 ml of each of the four stock solutions and make up to 1 litre with water.

Aerate until the dissolved oxygen concentration equals the air-saturation value.

The pH should be 7,8 ± 0,2.

If necessary adjust the pH with NaOH (sodium hydroxide) or HCI (hydrochloric acid).

The dilution water so prepared is set aside for about 12 hours and must not be further aerated.

The sum of the Ca and Mg ions in this solution is 2,5 mmol per litre. The ratio of Ca:Mg ions is 4:1 and of Na:K ions is 10:1. The total alkalinity of this solution is 0,8 mmol per litre.

Any deviation in the preparation of the dilution water must not change the composition or properties of the water.

Appendix 2

Fish species recommended for testing

Recommended species	Recommended range of test temperature (°C)	Recommended total length of test animal (cm)
Brachydanio rerio (Teleostei, Cyprinidae) (Hamilton-Buchanan) Zebrab-fish	20 to 24	3,0 ± 0,5
Pimephales promelas (Teleostei, Cyprinidae) (Rafinesque) Fat-head minnow	20 to 24	5,0 ± 2,5
Cyprinus carpio (Teleostei, Cyprinidae) (Linneaus 1758) Common carp	20 to 24	6,0 ± 2,0
Oryzias latipes (Teleostei, Poeciliidae) Cyprinodontidae (Tom-minck and Schlege 1850) Red killifish	20 to 24	3,0 ± 1,0
Poecilia reticulata (Teleostei, Poeciliidae) (Peters 1859) Guppy	20 to 24	3,0 ± 1,0
Lepomis macrochirus (Teleostei, Centrarchidae) (Rafinesque Linneaus 1758) Bluegill	20 to 24	5,0 ± 2,0
Onchorhynchus mykiss (Teleostei, Salmonidae) (Walbaum 1988) Rainbow trout	12 to 17	6,0 ± 2,0
Leuciscus idus (Teleostei, Cyprinidae) (Linneaus 1758) Golden Orfe	20 to 24	6,0 ± 2,0

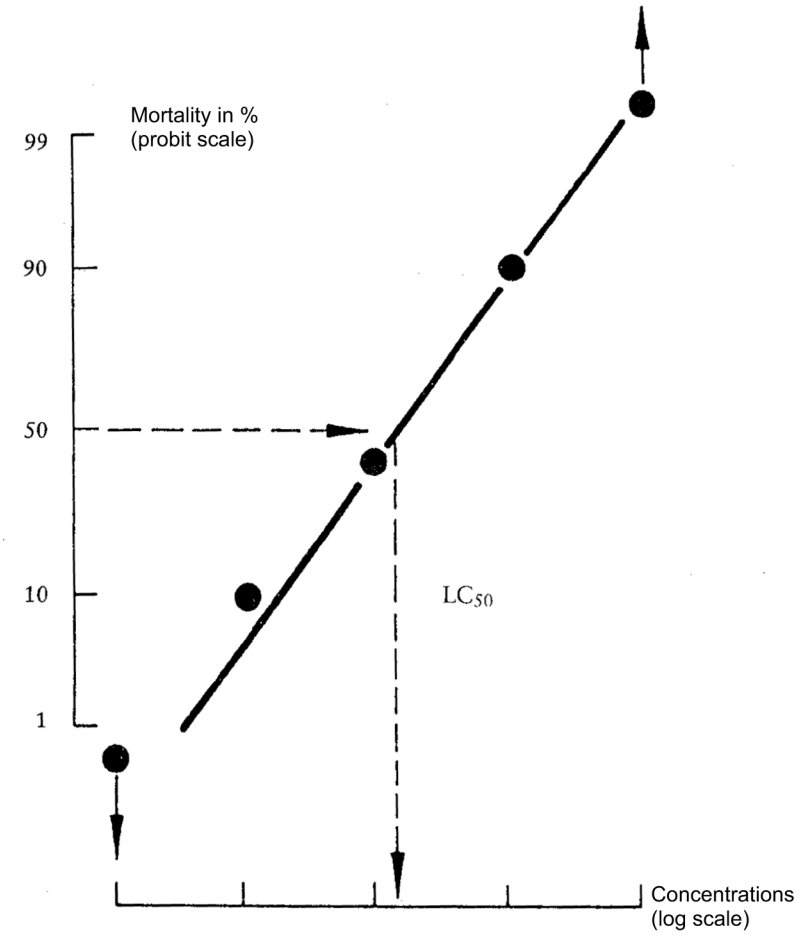
Collection

The fish listed above are easy to rear and/or are widely available throughout the year. They are capable of being bred and cultivated either in fish farms or in the laboratory, under disease — and parasite — controlled conditions, so that the test animal will be healthy and of known parentage. These fish are available in many parts of the world.

Appendix 3

Example of concentration: percentage mortality

Example of determination of LC_{50} using log-probit paper



C.2. *DAPHNIA* SP. ACUTE IMMOBILISATION TEST

1. METHOD

This acute immobilisation testing method is equivalent to the OECD TG 202 (2004).

1.1. INTRODUCTION

This method describes an acute toxicity test to assess effects of chemicals towards daphnids. Existing test methods were used to the extent possible (1)(2)(3).

1.2. DEFINITIONS

In the context of this method, the following definitions are used:

EC_{50}: is the concentration estimated to immobilise 50 % of the daphnids within a stated exposure period. If another definition is used, this must be reported, together with its reference.

Immobilisation: those animals that are not able to swim within 15 seconds, after gentle agitation of the test vessel are considered to be immobilised (even if they can still move their antennae).

1.3. PRINCIPLE OF THE TEST METHOD

Young daphnids, aged less than 24 hours at the start of the test, are exposed to the test substance at a range of concentrations for a period of 48 hours. Immobilisation is recorded at 24 hours and 48 hours and compared with control values. The results are analysed in order to calculate the EC_{50} at 48h (see Section 1.2 for definitions). Determination of the EC_{50} at 24h is optional.

1.4. INFORMATION ON THE TEST SUBSTANCE

The water solubility and the vapour pressure of the test substance should be known and a reliable analytical method for the quantification of the substance in the test solutions with reported recovery efficiency, and limit of determination should be available. Useful information includes the structural formula, purity of the substance, stability in water or light, P_{ow} and results of a test for ready biodegradability (see method C.4).

Note: guidance for testing substances with physical chemical properties that made them difficult to test is provided in (4).

1.5. REFERENCE SUBSTANCES

A reference substance may be tested for EC_{50} as a means of assuring that the test conditions are reliable. Toxicants used in international ring-tests (1)(5) are recommended for this purpose ([1]). Test(s) with a reference substance should be done preferably every month and at least twice a year.

1.6. QUALITY CRITERIA

For a test to be valid, the following performance criteria apply:

— in the controls, including the control containing the solubilising agent, not more that 10 % of the daphnids should have been immobilised;

— the dissolved oxygen concentration at the end of the test should be ≥ 3 mg/l in control and test vessels.

([1]) The results of these inter laboratory tests and a Technical Corrigendum to ISO 6341 give an EC_{50} — 24 h of the potassium dichromate ($K_2Cr_2O_7$) within the range 0,6 mg/l to 1,7 mg/l.

Note: For the first criterion, not more than 10 % of the control daphnids should show immobilisation or other signs of disease or stress, for example, discoloration, unusual behaviour such as trapping at surface of water.

1.7. DESCRIPTION OF THE TEST METHOD

1.7.1. **Apparatus**

Test vessels and other apparatus that will come into contact with the test solutions should be made entirely of glass or other chemically inert material. Test vessels will normally be glass test tubes or beakers; they should be cleaned before each use using standard laboratory procedures. Test vessels should be loosely covered to reduce the loss of water due to evaporation and to avoid the entry of dust into the solutions. Volatile substances should be tested in completely filled closed vessels, large enough to prevent oxygen becoming limiting or too low (see Section 1.6 and first paragraph of Section 1.8.3).

In addition some or all of the following equipment will be used: oxygen-meter (with microelectrode or other suitable equipment for measuring dissolved oxygen in low volumes samples); pH-meter; adequate apparatus for temperature control; equipment for the determination of total organic carbon concentration (TOC); equipment for the determination of chemical oxygen demand (COD); equipment for the determination of hardness, etc.

1.7.2. **Test organism**

Daphnia magna Straus is the preferred test species although other suitable *Daphnia* species can be used in this test (e.g. *Daphnia pulex*). At the start of the test, the animals should be less than 24 hours old and to reduce variability, it is strongly recommended they are not first brood progeny. They should be derived from a healthy stock (i.e. showing no signs of stress such as high mortality, presence of males and ephippia, delay in the production of the first brood, discoloured animals, etc.). All organisms used for a particular test should have originated from cultures established from the same stock of daphnids. The stock animals must be maintained in culture conditions (light, temperature, medium) similar to those to be used in the test. If the daphnids culture medium to be used in the test is different from that used for routine daphnids culture, it is good practice to include a pre-test acclimation period. For that, brood daphnids should be maintained in dilution water at the test temperature for at least 48 hours prior to the start of the test.

1.7.3. **Holding and dilution water**

Natural water (surface or ground water), reconstituted water or dechlorinated tap water are acceptable as holding and dilution water if daphnids will survive in it for the duration of the culturing, acclimation and testing without showing signs of stress. Any water which conforms to the chemical characteristics of an acceptable dilution water as listed in Appendix 1 is suitable as a test water. It should be of constant quality during the period of the test. Reconstituted water can be made up by adding specific amounts of reagents of recognised analytical grade to deionised or distilled water. Examples of reconstituted water are given in (1) (6) and in Appendix 2. Note that media containing known chelating agents, such as M4 and M7 media in Appendix 2, should be avoided for testing substances containing metals. The pH should be in the range of 6 to 9. Hardness between 140 and 250 mg/l (as $CaCO_3$) is recommended for *Daphnia magna*, while lower hardness may be also appropriate for other *Daphnia* species. The dilution water may be aerated prior to use for the test so that the dissolved oxygen concentration has reached saturation.

If natural water is used, the quality parameters should be measured at least twice a year or whenever it is suspected that these characteristics may have changed significantly (see previous paragraph and Appendix 1). Measurements of heavy metals (e.g. Cu, Pb, Zn, Hg, Cd, Ni) should also be made. If dechlorinated tap water is used, daily chlorine analysis is desirable. If the dilution water is from a surface or ground water source, conductivity and total organic carbon (TOC) or chemical oxygen demand (COD) should be measured.

1.7.4. **Test solutions**

Test solutions of the chosen concentrations are usually prepared by dilution of a stock solution. Stock solutions should preferably be prepared by dissolving the test substance in the dilution water. As far as possible, the use of solvents, emulsifiers or dispersants should be avoided. However, such compounds may be required in some cases in order to produce a suitably concentrated stock solution. Guidance for suitable solvents, emulsifiers and dispersants is given in (4). In any case, the test substance in the test solutions should not exceed the limit of solubility in the dilution water.

The test should be carried out without the adjustment of pH. If the pH does not remain in the range 6-9, then a second test could be carried out, adjusting the pH of the stock solution to that of the dilution water before addition of the test substance. The pH adjustment should be made in such a way that the stock solution concentration is not changed to any significant extent and that no chemical reaction or precipitation of the test substance is caused. HCl and NaOH are preferred.

1.8. PROCEDURE

1.8.1. **Conditions of exposure**

1.8.1.1. *Test groups and controls*

Test vessels are filled with appropriate volumes of dilution water and solutions of test substance. Ratio of air/water volume in the vessel should be identical for test and control group. Daphnids are then placed into test vessels. At least 20 animals, preferably divided into four groups of five animals each, should be used at each test concentration and for the controls. At least 2 ml of test solution should be provided for each animal (i.e. a volume of 10 ml for five daphnids per test vessel). The test may be carried out using semi-static renewal or flow-through system when the concentration of the test substance is not stable.

One dilution-water control series and also, if relevant, one control series containing the solubilising agent must be run in addition to the treatment series.

1.8.1.2. *Test concentrations*

A range-finding test may be conducted to determine the range of concentrations for the definitive test unless information on toxicity of the test substance is available. For this purpose, the daphnids are exposed to a series of widely spaced concentrations of the test substance. Five daphnids should be exposed to each test concentration for 48 hours or less, and no replicates are necessary. The exposure period may be shortened (e.g. 24 hours or less) if data suitable for the purpose of the range-finding test can be obtained in less time.

At least five test concentrations should be used. They should be arranged in a geometric series with a separation factor preferably not exceeding 2,2. Justification should be provided if fewer than five concentrations are used. The highest concentration tested should preferably result in 100 % immobilisation, and the lowest concentration tested should preferably give no observable effect.

1.8.1.3. *Incubation conditions*

The temperature should be within the range of 18 °C and 22 °C, and for each single test it should be constant within ± 1 °C. A 16-hour light and eight-hour dark cycle is recommended. Complete darkness is also acceptable, especially for the test substances unstable in light.

The test vessels must not be aerated during the test. The test is carried out without adjustment of pH. The daphnids should not be fed during the test.

1.8.1.4. *Duration*

The test duration is 48 hours.

1.8.2. **Observations**

Each test vessel should be checked for immobilised daphnids at 24 and 48 hours after the beginning of the test (see Section 1.2 for definitions). In addition to immobility, any abnormal behaviour or appearance should be reported.

1.8.3. **Analytical measurements**

The dissolved oxygen and pH are measured at the beginning and end of the test in the control(s) and in the highest test substance concentration. The dissolved oxygen concentration in controls should be in compliance with the validity criterion (see Section 1.6). The pH should normally not vary by more than 1,5 units in any one test. The temperature is usually measured in control vessels or in ambient air and it should be recorded preferably continuously during the test or, as a minimum, at the beginning and end of the test.

The concentration of the test substance should be measured, as a minimum, at the highest and lowest test concentration, at the beginning and end of the test (4). It is recommended that results be based on measured concentrations. However, if evidence is available to demonstrate that the concentration of the test substance has been satisfactorily maintained within ± 20 % of the nominal or measured initial concentration throughout the test, then the results can be based on nominal or measured initial values.

1.9. LIMIT TEST

Using the procedures described in this Method, a limit test may be performed at 100 mg/l of test substance or up to its limit of solubility in the test medium (whichever is the lower) in order to demonstrate that the EC_{50} is greater than this concentration. The limit test should be performed using 20 daphnids (preferably divided into four groups of five), with the same number in the control(s). If any immobilisation occurs, a full study should be conducted. Any observed abnormal behaviour should be recorded.

2. DATA

Data should be summarised in tabular form, showing for each treatment group and control, the number of daphnids used, immobilisation at each observation. The percentages immobilised at 24 hours and 48 hours are plotted against test concentrations. Data are analysed by appropriate statistical methods (e.g. probit analysis, etc.) to calculate the slopes of the curves and the EC_{50} with 95 % confidence limits ($p = 0,05$) (7) (8).

Where the standard methods of calculating the EC_{50}, are not applicable to the data obtained, the highest concentration causing no immobility and the lowest concentration producing 100 % immobility should be used as an approximation for the EC_{50} (this being considered the geometric mean of these two concentrations).

3. REPORTING

3.1. TEST REPORT

The test report must include the following:

Test substance:

— physical nature and relevant physical-chemical properties,

— chemical identification data, including purity.

Test species:

— source and species of *Daphnia*, supplier of source (if known) and the culture conditions used (including source, kind and amount of food, feeding frequency).

Test conditions:

— description of test vessels: type of vessels, volume of solution, number of daphnids per test vessel, number of test vessels (replicates) per concentration,

— methods of preparation of stock and test solutions including the use of any solvent or dispersants, concentrations used,

— details of dilution water: source and water quality characteristics (pH, hardness, Ca/Mg ratio, Na/K ratio, alkalinity, conductivity, etc.); composition of reconstituted water if used,

— incubation conditions: temperature, light intensity and periodicity, dissolved oxygen, pH, etc.

Results:

— the number and percentage of daphnids that were immobilised or showed any adverse effects (including abnormal behaviour) in the controls and in each treatment group, at each observation time and a description of the nature of the effects observed,

— results and date of test performed with reference substance, if available,

— the nominal test concentrations and the result of all analyses to determine the concentration of the test substance in the test vessels; the recovery efficiency of the method and the limit of determination should also be reported,

— all physical-chemical measurements of temperature, pH and dissolved oxygen made during the test,

— the EC_{50} at 48 h for immobilisation with confidence intervals and graphs of the fitted model used for their calculation, the slopes of the dose-response curves and their standard error; statistical procedures used for determination of EC_{50}; (these data items for immobilisation at 24 h should also be reported when they were measured),

— explanation for any deviation from the Testig Method and whether the deviation affected the test results.

4. **REFERENCES**

(1) ISO 6341. (1996). Water quality — Determination of the inhibition of the mobility of *Daphnia magna* Straus (Cladocera, Crustacea) — Acute toxicity test. Third edition, 1996.

(2) EPA OPPTS 850.1010. (1996). Ecological Effects Test Guidelines — Aquatic Invertebrate Acute Toxicity Test, Freshwater Daphnids.

(3) Environment Canada. (1996) Biological test method. Acute Lethality Test Using *Daphnia* spp. EPS 1/RM/11. Environment Canada, Ottawa, Ontario, Canada.

(4) Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures. OECD Environmental Health and Safety Publication. Series on Testing and Assessment. No 23. Paris 2000.

(5) Commission of the European Communities. Study D8369. (1979). Inter-laboratory Test Programme concerning the study of the ecotoxicity of a chemical substance with respect to *Daphnia*.

(6) OECD Guidelines for the Testing of Chemicals. Guideline 211: *Daphnia magna* Reproduction Test, adopted September 1998.

(7) Stephan C.E. (1977). Methods for calculating an LC_{50}. In Aquatic Toxicology and Hazard Evaluation (edited by F.I. Mayer and J.L. Hamelink). ASTM STP 634 — American Society for Testing and Materials. p. 65-84

(8) Finney D.J. (1978). Statistical Methods in Biological Assay. 3rd ed. London. Griffin, Weycombe, UK.

Appendix 1

SOME CHEMICAL CHARACTERISTICS OF AN ACCEPTABLE DILUTION WATER

Substance	Concentration
Particulate matter	< 20 mg/l
Total organic carbon	< 2 mg/l
Unionised ammonia	< 1 µg/l
Residual chlorine	< 10 µg/l
Total organophosphorus pesticides	< 50 ng/l
Total organochorine pesticides plus polychlorinated biphenyls	< 50 ng/l
Total organic chlorine	< 25 ng/l

Appendix 2

EXAMPLES OF SUITABLE RECONSTITUTED TEST WATER

ISO Test water (1)

Stock solutions (single substance)		To prepare the reconstituted water, add the following volumes of stock solutions to 1 litre water (*)
Substance	Amount added to 1 litre water (*)	
Calcium chloride $CaCl_2$, $2H_2O$	11,76 g	25 ml
Magnesium sulfate $MgSO_4$, $7H_2O$	4,93 g	25 ml
Sodium bicarbonate $NaHCO_3$	2,59 g	25 ml
Potassium chloride KCl	0,23 g	25 ml

(*) Water of suitable purity, e.g. deionised, distilled or reverse osmosis with conductivity preferably not exceeding 10 $\mu S.cm^{-1}$.

Elendt M7 and M4 medium

Acclimation to Elendt M4 and M7 medium

Some laboratories have experienced difficulty in directly transferring *Daphnia* to M4 and M7 media. However, some success has been achieved with gradual acclimation, i.e. moving from own medium to 30 % Elendt, then to 60 % Elendt and then to 100 % Elendt. The acclimation periods may need to be as long as one month.

Preparation

Trace element

Separate stock solutions (I) of individual trace elements are first prepared in water of suitable purity, e.g. deionised, distilled or reverse osmosis. From these different stock solutions (I) a second single stock solution (II) is prepared, which contains all trace elements (combined solution), i.e.:

Stock solution(s) I (single substance)	Amount added to water (mg/l)	Concentration (related to medium M4)	To prepare the combined stock solution II, add the following amount of stock solution I to water (ml/l)	
			M4	M7
$H_3 BO_3$	57 190	20 000-fold	1,0	0,25
$MnCl_2.4H_2O$	7 210	20 000-fold	1,0	0,25
LiCl	6 120	20 000-fold	1,0	0,25
RbCl	1 420	20 000-fold	1,0	0,25
$SrCl_2.6H_2O$	3 040	20 000-fold	1,0	0,25
NaBr	320	20 000-fold	1,0	0,25
$Na_2 MoO_4.2H_2O$	1 230	20 000-fold	1,0	0,25
$CuCl_2.2H_2O$	335	20 000-fold	1,0	0,25
$ZnCl_2$	260	20 000-fold	1,0	1,0
$CoCl_2.6H_2O$	200	20 000-fold	1,0	1,0

Stock solution(s) I (single substance)	Amount added to water (mg/l)	Concentration (related to medium M4)	To prepare the combined stock solution II, add the following amount of stock solution I to water (ml/l)	
			M4	M7
KI	65	20 000-fold	1,0	1,0
Na_2 SeO_3	43,8	20 000-fold	1,0	1,0
NH_4 VO_3	11,5	20 000-fold	1,0	1,0
Na_2 EDTA.$2H_2O$	5 000	2 000-fold	—	—
$FeSO_4.7H_2O$	1 991	2 000-fold	—	—

Both Na 2 EDTA and $FeSO_4$ solutions are prepared singly, poured together and autoclaved immediately.

This gives:

2 l Fe-EDTA solution		1 000-fold	20,0	5,0

M4 and M7 media

M4 and M7 media are prepared using stock solution II, the macro-nutrients and vitamin as follows:

	Amount added to water (mg/l)	Concentration (related to medium M4)	Amount of stock solution II added to prepare medium (ml/l)	
			M4	M7
Stock solution II (combined trace elements)		20-fold	50	50
Macro nutrient stock solutions (single substance)				
$CaCl_2 \cdot 2H_20$	293 800	1 000-fold	1,0	1,0
$MgSO_4 \cdot 7H_2O$	246 600	2 000-fold	0,5	0,5
KCl	58 000	10 000-fold	0,1	0,1
$NaHCO_3$	64 800	1 000-fold	1,0	1,0
Na_2 $SiO_3 \cdot 9H_2O$	50 000	5 000-fold	0,2	0,2
$NaNO_3$	2 740	10 000-fold	0,1	0,1
KH_2 PO_4	1 430	10 000-fold	0,1	0,1
K_2HPO_4	1 840	10 000-fold	0,1	0,1
Combined Vitamin stock	—	10 000-fold	0,1	0,1

The combined vitamin stock solution is prepared by adding the 3 vitamin to 1 litre water, as shown below:

Thiamine hydrochloride	750	10 000-fold		
Cyanocobalamine (B_{12})	10	10 000-fold		
Biotine	7,5	10 000-fold		

The combined vitamin stock is stored frozen in small aliquots. Vitamins are added to the media shortly before use.

N.B: to avoid precipitation of salts when preparing the complete media, add the aliquots of stock solutions to about 500-800 ml deionised water and then fill up to 1 litre.

N.B: the first publication of the M4 medium can be found in Elendt, B. P. (1990). Selenium deficiency in crustacea; an ultrastructual approach to antennal damage in *Daphnia magna* Straus. Protoplasma, 154, 25-33.

C.3. ALGAL INHIBITION TEST

1. METHOD

1.1. INTRODUCTION

The purpose of this test is to determine the effects of a substance on the growth of a unicellular green algal species. Relatively brief (72 hours) tests can assess effects over several generations. This method can be adapted for use with several unicellular algal species, in which case a description of the method used must be provided with the test report.

This method is most easily applied to water-soluble substances which, under the conditions of the test, are likely to remain in the water.

The method can be used for substances that do not interfere directly with the measurement of algal growth.

It is desirable to have, as far as possible, information on the water solubility, vapour pressure, chemical stability, dissociation constants and biodegradability of the substance before starting the test.

Additional information (for instance structural formula, degree of purity, nature and percentage of significant impurities, presence and amount of additives, and n-octanol/water partition coefficient) should be taken into consideration in both the planning of the test and interpretation of the results.

1.2. DEFINITIONS AND UNITS

Cell density: the number of cells per millilitre.

Growth: the increase in cell density over the test period.

Growth rate: the increase in cell density per unit time.

EC_{50}: in this method, that concentration of test substance which results in a 50 % reduction in either growth (E_bC_{50}) or growth rate (E_rC_{50}) relative to the control.

NOEC (no observed effect concentration): in this method, the highest tested concentration at which no significant inhibition of growth is observed relative to the control.

All concentrations of the test substance are given in weight by volume (milligrams per litre). They may also be expressed as weight by weight ($mg./kg^{-1}$).

1.3. REFERENCE SUBSTANCES

A reference substance may be tested as a means of demonstrating that under the laboratory test conditions the sensitivity of the test species has not changed significantly.

If a reference substance is used, the results should be given in the test report. Potassium dichromate can be used as a reference substance, but its colour may affect the light quality and intensity avai1able to the cells and also the spectrophotometric determinations if used. Potassium dichromate has been used in an international inter-laboratory test (see ref. (3) and Appendix 2).

1.4. PRINCIPLE OF THE TEST METHOD

A limit test may be performed at 100 mg per litre in order to demonstrate that the EC_{50} is greater than this concentration.

Exponentially-growing cultures of selected green algae are exposed to various concentrations of the test substance over several generations under defined conditions.

The test solutions are incubated for a period of 72 hours, during which the cell density in each solution is measured at least every 24 hours. The inhibition of growth in relation to a control culture is determined.

1.5. QUALITY CRITERIA

The quality criteria shall apply to the limit test as well as the full test method.

The cell density in the control cultures should have increased by a factor of at least 16 within three days.

The concentrations of the test substance shall be maintained to within 80 % of the initial concentrations/ throughout a time corresponding to the duration of the test.

For substances which dissolve easily in the test medium, yielding stable solutions i.e. those which will not to any significant extent volatilise, degrade, hydrolyze or adsorb, the initial concentration can be taken as being equivalent to the nominal concentration. Evidence shall be presented that the concentrations have been maintained throughout the test and that the quality criteria have been satisfied.

For substances that are:

(i) poorly soluble in the test medium, or

(ii) capable of forming stable emulsions or dispersions, or

(iii) not stable in aqueous solutions,

the initial concentration shall be taken as the concentration measured at the start of the test. The concentration shall be determined after a period of equilibration.

In any of these cases, further measurements must be made during the test to confirm the actual exposure concentrations or that the quality criteria have been met.

It is recognised that significant amounts of the test substance may be incorporated into the algal biomass during the period of the test. Therefore, for the purpose of demonstrating compliance with the above quality criteria, both the amount of the substance incorporated into the algal biomass and the substance in solution (or, if not technically possible measured in the water column) should be taken into account. However, as determination of the substance concentration in the algal biomass may pose significant technical problems, compliance with the quality criteria may be demonstrated by running a test vessel at the highest substance concentration but without algae and measuring concentrations in solution (or, if not technically possible in the water column) at the beginning and at the end of the test period.

1.6. DESCRIPTION OF THE TEST PROCEDURE

1.6.1. **Reagents**

1.6.1.1. *Solutions of test substances*

Stock solutions of the required strength are prepared by dissolving the substance in deionised water or water according to 1.6.1.2.

The chosen test concentrations are prepared by adding suitable aliquots to algal pre-cultures (see Appendix 1).

Substances should normally only be tested up to the limit of solubility. For some substances (e.g. substances having low solubility in water, or high P_{ow} or those forming stable dispersion rather than true solution in water), it is acceptable to run a test concentration above the solubility limit of the substance to ensure that the maximum soluble stable concentration has been obtained. It is important, however, that this concentration will not otherwise disturb the test system (e.g. film of the substance on the water surface preventing the oxygenation of the water, etc.).

Ultrasonic dispersion, organic solvents, emulsifiers or dispersants may be used as an aid to prepare stock solutions of substances with low aqueous solubility or to help to disperse these substances in the test medium. When such auxiliary substances are used, all test concentrations should contain the same amount of auxiliary substances, and additional controls should be exposed to the same concentration of the auxiliary substance as that used in the test series. The concentration of such auxiliaries should be minimised, but in no case should exceed 100 mg per litre in the test medium.

The test should be carried out without adjustment of the pH. If there is evidence of marked change in the pH, it is advised that the test should be repeated with pH adjustment and the results reported. In that case, the pH value of the stock solution should be adjusted to the pH value of the solution water unless there are specific reasons not to do so. HCl and NaOH are preferred for this purpose. This pH adjustment should be made in such a way that the concentration of test substance in the stock solution is not changed to any significant extent. Should any chemical reaction or physical precipitation of the test compound be caused by the adjustment, this should be reported.

1.6.1.2. *Test medium*

The water should be good-quality distilled water, or deionised water with a conductivity less than 5 $\mu S.cm^{-1}$. The apparatus for distillation of water must not contain any part made of copper.

The following medium is recommended.

Four stock solutions are prepared, according to the following table. The stock solutions are sterilised by membrane filtration or by autoclaving, and stored in the dark at 4 °C. Stock solution No 4 should be sterilised only by membrane filtration. These stock solutions are diluted to achieve the final nutrient concentrations in the test solutions.

Nutrient	Concentration in the stock solution	Final concentration in the test solution
Stock solution 1: macro-nutrients		
NH_4Cl	1,5 g/l	15 mg/l
$MgCl_2.6H_2O$	1,2 g/l	12mg/l
$CaCl_2.2H_2O$	1,8 g/l	18 mg/l
$MgSO_4.7H_2O$	1,5 g/l	15 mg/l
KH_2PO_4	0,16 g/l	1,6 mg/l
Stock solution 2: Fe-EDTA		
$FeCl_3.6H_2O$	80 mg/l	0,08 mg/l
$Na_2EDTA.2H_2O$	100 mg/l	0,1 mg/l
Stock solution 3: trace elements		
H_3BO_3	185 mg/l	0,185 mg/l
$MnCl_2.4H_2O$	415 mg/l	0,415 mg/l
$ZnCl_2$	3 mg/l	3×10^{-3} mg/l
$CoCl_2.6H_2O$	1,5 mg/l	$1,5 \times 10^{-3}$ mg/l
$CuCl_2.2H_2O$	0,01 mg/l	10^{-5} mg/l
$Na_2MoO_4.2H_2O$	7 mg/l	7×10^{-3}mg/l
Stock solution 4: $NaHCO_3$		
$NaHCO_3$	50 g/l	50 mg/l

The pH of the medium after equilibration with air is approximately 8.

1.6.2. **Apparatus**

— Normal laboratory equipment.

— test flasks of suitable volume (e.g. 250 ml conical flasks are suitable when the volume of the test solution is 100 ml). All test flasks should be identical as regards to material and dimensions,

— culturing apparatus: cabinet or chamber in which a temperature in the range 21 °C to 25 °C can be maintained at ± 2 °C, and continuous uniform illumination provided in the spectral range 400 to 700 nm. If algae in control cultures have achieved the recommended growth rates, it can be assumed that the conditions for growth, including light intensity, have been adequate.

It is recommended to use, at the average level of the test solutions, a light intensity in the range 60 to 120 $\mu E.m^{-2}.s^{-1}$ (35 to 70 × 10^{18} $photons.m^{-2}.s^{-1}$) when measured in the range 400 to 700 nm using an appropriate receptor. For light measuring instruments calibrated in lux, an equivalent range of 6000 to 10000 lx is acceptable.

The light intensity could be obtained using four to seven 30 W fluorescent lamps of the universal white type (colour temperature of approximately 4300 K), at a distance of 0,35 m from the algal culture,

— Cell density measurements should be made using a direct counting method of living cells, e.g. a microscope with counting chambers. However, other procedures (photometry, turbidimetry, ...) may be used if sufficiently sensitive and if shown to be sufficiently well correlated with cell density.

1.6.3. **Test organisms**

It is suggested that the species of green algae used be a fast-growing species that is convenient for culturing and testing. The following species are preferred:

— *Selenastrum capricornutum*, e.g. ATCC 22662 or CCAP 278/4,

— Scenedesmus subspicatus, e.g. 86.81 SAG,

Note:

ATCC = American Type Culture Collection (U.S.A.)
CCAP = Culture Centre of Algae and Protozoa (U.K.)
SAG = Collection of algal culture (Gottingen, F.R.G.)

If other species are used, the strain should be reported.

1.6.4. **Test procedure**

The concentration range in which effects are likely to occur is determined on the basis of results from range-finding tests.

The two measures of growth (biomass and growth rate) may result in widely disparate measures of growth inhibition; both should be used in the range finding test to ensure that the geometric progression of concentrations will allow estimation of both the E_bC_{50} and the E_rC_{50}.

Initial cell density

It is recommended that the initial cell density in the test cultures be approximately 10^4 cells/ml for *Selenastrum capricornutum* and *Scenedesmus subspicatus*. When other species are used the biomass should be comparable.

Concentrations of test substance

For the test, at least five concentrations are made up in a geometric series at a concentration ratio not exceeding 2,2. The lowest concentration tested should have no observed effect on the growth of the algae. The highest concentration tested should inhibit growth by at least 50 % relative to the control and, preferably, stop growth completely.

Replicates and controls

The test design should include three replicates at each test concentration. Three controls without test substance are run and, if relevant, three controls containing the auxiliary substance are also run. If justified, the test design may be altered to increase the number of concentrations and reduce the number of replicates per concentration.

Performance of the test

Test cultures containing the desired concentrations of test substance and the desired quantity of algal inoculum are prepared by adding aliquots of stock solutions of the test substance to suitable amounts of algal pre-cultures (see Appendix 1).

The culture flasks are shaken and placed in the culturing apparatus. The algal cells are kept in suspension by shaking, stirring or bubbling with air, in order to improve gas exchange and reduce pH variation in the test solutions. The cultures should be maintained at a temperature in the range of 21 to 25 °C, controlled at ± 2 °C.

The cell density in each flask is determined at least at 24, 48 and 72 hours after the start of the test. Filtered algal medium containing the appropriate concentration of the test chemical is used to determine the background when using cell density measurements other than direct counting methods.

The pH is measured at the beginning of the test and at 72 hours.

The pH of the controls should not normally deviate by more than 1,5 units during the test.

Testing volatile substances

There is to date no generally accepted way to test volatile substances. When a substance is known to have a tendency to vaporise, closed test flasks with increased head-space may be used. The possibility of shortage of CO_2 should be considered when calculating the head-space of the closed flasks. Variations to this method have been proposed (see reference (4)).

Attempts should be made to determine the amount of the substance which remains in solution, and extreme caution is advised when interpreting results of tests with volatile chemicals using closed systems.

Limit test

Using the procedures described in this method, a limit test may be performed at 100 mg per litre in order to demonstrate that the EC_{50} is greater than this concentration.

If the nature of the substance is such that a concentration of 100 mg per litre in the test water cannot be attained, the limit test should be performed at a concentration equal to the solubility of the substance (or the maximum concentration forming a stable dispersion) in the medium used (see also point 1.6.1.1).

The limit test should be performed at least in triplicate, with the same number of controls. The two measures of growth (biomass and growth rate) should be used for the limit test.

If, in a limit test, a mean decrease of 25 % or more is found in either biomass or growth rate between the limit test and the control, a full test should be carried out.

2. **DATA AND EVALUATION**

The measured cell density in the test cultures and controls are tabulated together with the concentrations of the test substance and the times of measurements. The mean value of the cell density for each test substance concentration and for the controls is plotted against time (0-72 h) to produce growth curves.

To determine the concentration/effect relationship, the two following approaches should be used. Some substances can stimulate the growth at low concentrations. Only data points indicating inhibition between 0 and 100 % should be considered.

2.1. COMPARISON OF AREAS UNDER THE GROWTH CURVES

The area between the growth curves and the horizontal line $N = N_0$ may be calculated according to the formula:

$$A = \frac{N_1 - N_0}{2} \times t_1 + \frac{N_1 + N_2 - 2N_0}{2} \times (t_2 - t_1) + \ldots + \frac{N_{n-1} + N_n - 2N_0}{2} \times (t_n - t_{n-1})$$

Where:

A = area

N_o = number of cells/ml at time t_0 (beginning of the test),$_o$,

N_1 = measured number of cells/ml at t_1

N_n = measured number of cells/ml at time

t_l = time of first measurement after beginning of test

t_n = time of n_{th} measurement after beginning of test.

n = number of measurements taken after the beginning of the test.

The percentage inhibition of the cell growth at each test substance concentration (I_A) is calculated according to the formula:

$$I_A = \frac{A_c - A_t}{A_c} \times 100$$

Where:

A_c = area between the control growth curve and the horizontal line $N = N_o$.

A_t = area between the growth curve at the concentration t and the horizontal line $N = N_o$.

I_A = values are plotted on semilogarithmic paper or on semilogarithmic probit paper against the corresponding concentrations. If plotted on probit paper, the points are fitted by a straight line, either by eye or by a computed regression.

The EC_{50} is estimated from the regression line by reading off the concentration that is equivalent to a 50 % inhibition (I_A = 50 %). To denote this value unambiguously in relation to this method of calculation, it is proposed to use the symbol E_bC_{50}. It is essential that the E_bC_{50} is quoted with the appropriate exposure period, e.g. E_bC_{50} (0-72h).

2.2. COMPARISON OF GROWTH RATES

The average specific growth rate (μ) for exponentially growing cultures can be calculated as

$$\mu = \frac{\ln N_n - \ln N_0}{t_n - t_0}$$

where t_o is the time at the beginning of the test.

Alternatively, the average specific growth rate may be derived from the slope of the regression line in a plot of ln N *versus* time.

The percentage inhibition of specific growth rate at each test substance concentration (Iμt) is calculated according to the formula:

$$I_{\mu t} = \frac{\mu_c - \mu_t}{\mu_c} \times 100$$

Where:

μ_c = mean control specific growth rate
μ_t = mean specific growth rate for the test concentration t

The percentage reduction in average specific growth rate at each test substance concentration compared to the control value is plotted against the logarithm of the concentration. The EC_{5o} may be read from the resulting graph. To denote unambiguously the EC_{50} derived by this method it is proposed to use the symbol E_rC_{50}. The times of measurement must be indicated, e.g. if the value relates to times 0 and 72 hours, the symbol becomes E_rC_{50} (0-72h).

Note: specific growth rate is a logarithmic term, and small changes in growth rate may lead to great changes in biomass. E_bC and E_rC values are therefore not numerically comparable.

2.3. CALCULATION OF THE NOEC

The No Observed Effect Concentration is determined by a suitable statistical procedure for multisample comparison (e.g. analysis of variance and Dunnett's test), using the individual replicates values of the areas under the growth curves A (see point 2.1) or the specific growth rates μ (see point 2.2).

3. **REPORTING**

The test report shall, if possible, include the following information:

— test substance: chemical identification data,

— test organisms: origin, laboratory culture, strain number, method of cultivation,

— test conditions:

  — date of the start and the end of the test and its duration,

  — temperature,

  — composition of medium,

  — culturing apparatus,

  — pH of solutions at the start and end of the test (an explanation should be provided if pH deviations of more than 1,5 unit are observed),

  — vehicle and method used for solubilising the test substance and concentration of the vehicle in the test solutions,

  — light intensity and quality,

  — concentrations tested (measured or nominal).

— results:

  — cell density for each flask at each measuring point and method for measuring cell density,

— mean values of cell density,

— growth curves,

— graphical presentation of the concentration effect relationship,

— EC values and method of calculation,

— NOEC,

— other observed effects.

4. **REFERENCES**

1) OECD, Paris, 1981, Test Guideline 201, Decision of the Council C(81) 30 Final.

2) Umweltbundesamt, Berlin, 1984, Verfahrensvorschlag 'Hemmung der Zellvermehrung bei der Grünalge Scenedesmus subspicatus', in: Rudolph/Boje: bkotoxikologie, ecomed, Landsberg, 1986.

3) ISO 8692 — Water quality — Fresh water algal growth inhibition test with *Scenedesmus subspicatus* and *Selenastrum capricornutum*.

4) S.Galassi and M.Vighi — Chemosphere, 1981, vol.10, 1123-1126.

Appendix 1

Example of a procedure for the culturing of algae

General observations

The purpose of culturing on the basis of the following procedure is to obtain algal cultures for toxicity tests.

Suitable methods should be used to ensure that the algal cultures are not infected with bacteria (ISO 4833). Axenic cultures may be desirable but unialgal cultures are essential.

All operations should be carried out under sterile conditions in order to avoid contamination with bacteria and, other algae. Contaminated cultures should be rejected.

Procedures for obtaining algal cultures

Preparation of nutrient solutions (media):

the medium can be prepared by diluting concentrated stock solutions of nutrients. For solid medium, 0,8 % of agar is added. The medium used should be sterile. Sterilisation by autoclaving may lead to a loss of NH_3.

Stock culture:

the stock cultures are small algal cultures that are regularly transferred to fresh medium to act as initial test material. If the cultures are not used regularly they are streaked out on sloped agar tubes. These are transferred to fresh medium at least once every two months.

the stock cultures are grown in conical flasks containing the appropriate medium (volume about 100 ml). When the algae are incubated at 20 °C with continuous illumination, a weekly transfer is required.

During transfer an amount of 'old' culture is transferred with sterile pipettes into a flask of fresh medium, so that with the fast-growing species the initial concentration is about 100 times smaller than in the old culture.

the growth rate of a species can be determined from the growth curve. If this is known, it is possible to estimate the density at which the culture should be transferred to new medium. This must be done before the culture reaches the death phase.

Pre-culture:

the pre-culture is intended to give an amount of algae suitable for the inoculation of test cultures. The pre-culture is incubated under the conditions of the test and used when still exponentially growing, normally after an incubation period of about three days. When the algal cultures contain deformed or abnormal cells, they must be discarded.

Appendix 2

The ISO 8692 — Water quality — Fresh water algal growth inhibition test with *Scenedesmus subspicatus* and *Selenastrum capricornutum* reports the following results for an inter-laboratory test among 16 laboratories, testing potassium dichromate:

	Means (mg/l)	Range (mg/l)
E_rC_{50} (0-72 h)	0,84	0,60 to 1,03
E_bC_{50} (0-72 h)	0,53	0,20 to 0,75

C.4. DETERMINATION OF 'READY' BIODEGRADABILITY

PART I. GENERAL CONSIDERATIONS

I.1. INTRODUCTION

Six test methods are described that permit the screening of chemicals for ready biodegradability in an aerobic aqueous medium:

(a) Dissolved Organic Carbon (DOC) Die-Away (Method C.4-A)

(b) Modified OECD Screening — DOC Die-Away (Method C.4-B)

(c) Carbon dioxide (CO_2) Evolution (Modified Sturm Test) (Method C.4-C)

(d) Manometric Respirometry (Method C.4-D)

(e) Closed Bottle (Method C.4-E)

(f) MITI (Ministry of International Trade and Industry — Japan) (Method C.4-F)

General and common considerations to all six tests are given in Part I of the method. Items specific for individual methods are given in Parts II to VII. The appendices contain definitions, formulas and guidance material.

An OECD inter-laboratory comparison exercise, done in 1988, has shown that the methods give consistent results. However, depending on the physical characteristics of the substance to be tested, one or other of the methods may be preferred.

I.2. SELECTION OF THE APPROPRIATE METHOD

In order to select the most appropriate method, information on the chemical's solubility, vapour pressure and adsorption characteristics is essential. The chemical structure or formula should be known in order to calculate theoretical values and/or check measured values of parameters, e.g. ThOD, $ThCO_2$, DOC, TOC, COD (see Appendices 1 and 2).

Test chemicals which are soluble in water to at least 100 mg/l may be assessed by all methods, provided they are non-volatile and non-adsorbing. For those chemicals which are poorly soluble in water, volatile or adsorbing, suitable methods are indicated in Table 1. The manner in which poorly water-soluble chemicals and volatile chemicals can be dealt with is described in Appendix 3. Moderately volatile chemicals may be tested by the DOC Die-Away method if there is sufficient gas space in the test vessels (which should be suitably stoppered). In this case, an abiotic control must be set up to allow for any physical loss.

Table 1:

Applicability of test methods

Test	Analytical Method	Suitability for substances which are:		
		poorly soluble	volatile	adsorbing
DOC Die-Away	Dissolved organic carbon	–	–	+/–
Mod. OECD Die-Away	Dissolved organic carbon	–	–	+/–
CO_2 Evolution	Respirometry: CO_2 evolution	+	–	+
Manometric Respirometry	Manometric respirometry: oxygen consumption	+	+/–	+

Test	Analytical Method	Suitability for substances which are:		
		poorly soluble	volatile	adsorbing
Closed Bottle	Respirometry: dissolved oxygen	+/–	+	+
MITI	Respirometry: oxygen consumption	+	+/–	+

Information on the purity or the relative proportions of major components of the test material is required to interpret the results obtained, especially when the results are low or marginal.

Information on the toxicity of the test chemical to bacteria (Appendix 4) may be very useful for selecting appropriate test concentrations and may be essential for the correct interpretation of low biodegradation values.

I.3. REFERENCE SUBSTANCES

In order to check the procedure, reference chemicals which meet the criteria for ready biodegradability are tested by setting up an appropriate flask in parallel to the normal test runs.

Suitable chemicals are aniline (freshly distilled), sodium acetate and sodium benzoate. These reference chemicals all degrade in these methods even when no inoculum is deliberately added.

It was suggested that a reference chemical should be sought which was readily biodegradable but required the addition of an inoculum. Potassium hydrogen phthalate has been proposed but more evidence needs to be obtained with this substance before it can be accepted as a reference substance.

In the respirometric tests, nitrogen-containing compounds may affect the oxygen uptake because of nitrification (see Appendices 2 and 5).

I.4. PRINCIPLE OF THE TEST METHODS

A solution, or suspension, of the test substance in a mineral medium is inoculated and incubated under aerobic conditions in the dark or in diffuse light. The amount of DOC in the test solution due to the inoculum should be kept as low as possible compared to the amount of DOC due to the test substance. Allowance is made for the endogenous activity of the inoculum by running parallel blank tests with inoculum but without test substance, although the endogenous activity of cells in the presence of the substance will not exactly match that in the endogenous control. A reference substance is run in parallel to check the operation of the procedures.

In general, degradation is followed by the determination of parameters, such as DOC, CO_2 production and oxygen uptake, and measurements are taken at sufficiently frequent intervals to allow the identification of the beginning and end of biodegradation. With automatic respirometers the measurement is continuous. DOC is sometimes measured in addition to another parameter but this is usually done only at the beginning and the end of the test. Specific chemical analysis can also be used to assess primary degradation of the test substance, and to determine the concentration of any intermediate substances formed (obligatory in the MITI test).

Normally, the test lasts for 28 days. Tests however may be ended before 28 days, i.e. as soon as the biodegradation curve has reached a plateau for at least three determinations. Tests may also be prolonged beyond 28 days when the curve shows that biodegradation has started but that the plateau has not been reached
day 28.

I.5. QUALITY CRITERIA

I.5.1. **Reproducibility**

Because of the nature of biodegradation and of the mixed bacterial populations used as inocula, determinations should be carried out at least in duplicate.

It is common experience that the larger the concentration of micro-organisms initially added to the test medium, the smaller will be the variation between replicates. Ring tests have also shown that there can be large variations between results obtained by different laboratories, but good agreement is normally obtained with easily biodegradable compounds.

I.5.2. **Validity of the test**

A test is considered valid if the difference of extremes of replicate values of the removal of test chemical at the plateau, at the end of the test or at the end of the 10-day window, as appropriate, is less than 20 % and if the percentage degradation of the reference substance has reached the level for ready biodegradability by 14 days. If either of these conditions is not met, the test should be repeated. Because of the stringency of the methods, low values do not necessarily mean that the test substance is not biodegradable under environmental conditions, but indicates that more work will be necessary to establish biodegradability.

If in a toxicity test, containing both the test substance and a reference chemical, less than 35 % degradation (based on DOC) or less than 25 % (based on ThOD or $ThCO_2$) occurred in 14 days, the test chemicals can be assumed to be inhibitory (see also Appendix 4). The test series should be repeated, if possible using a lower concentration of test chemical and/or a higher concentration of inoculum, but not greater than 30 mg solids/litre.

I.6. GENERAL PROCEDURES AND PREPARATIONS

General conditions applying to the tests are summarised in Table 2. Apparatus and other experimental conditions pertaining specifically to an individual test are described later under the heading for that test.

Table 2

Test conditions

Test	DOC Die-Away	CO_2 Evolution	Manometric Respirometry	Modified OECD Screeing	Closed Bottle	MITI (I)
Concentration of Test Substance						
as mg/l			100		2-10	100
mg DOC/1	10-40	10-20		10-40		
mg ThOD/1			50-100		5-10	
Concentration of Inoculum (in cells/l, approximatively)	≤ 30 mg/l SS or ≤ 100 ml effluent/l (10^7-10^8)			0,5 ml secondary effluent/1 (10^5)	≤ 5 ml of effluent/l (10^4-10^6)	30 mg/l SS (10^7-10^8)
Concentration of elements in mineral medium (in mg/l):						
P	116				11,6	29
N	1,3				0,13	1,3
Na	86				8,6	17,2
K	122				12,2	36,5
Mg	2,2				2,2	6,6
Ca	9,9				9,9	29,7
Fe	0,05- 0,1				0,05- 0,1	0,15
pH	7,4 ± 0,2					preferably 7,0

Test	DOC Die-Away	CO_2 Evolution	Manometric Respirometry	Modified OECD Screeing	Closed Bottle	MITI (I)
Temperature	22 ± 2 °C					25 ± 1 °C

DOC = Dissolved organic Carbon ThoD = Theoretical Oxygen Demand SS =Suspended Solids

I.6.1. **Dilution water**

Deionised or distilled water, free from inhibitory concentrations of toxic substances (e.g. Cu^{++} ions) is used. It must contain no more than 10 % of the organic carbon content introduced by the test material. The high purity of the test water is necessary to eliminate high blank values. Contamination may result from inherent impurities and also from the ion-exchange resins and lysed material from bacterial and algae. For each series of tests use only one batch of water, checked beforehand by DOC analysis. Such a check is not necessary for the closed bottle test, but the oxygen consumption of the water must be low.

I.6.2. **Stock solutions of mineral components**

To make up the test solutions, stock solutions of appropriate concentrations of mineral components are made up. The following stock solutions may be used (with different dilution factors) for the methods DOC Die-Away, Modified OECD Screening, CO_2 Evolution, Manometric Respirometry, Closed Bottle test.

The dilution factors and, for the MITI test, the specific preparation of the mineral medium are given under the headings of the specific tests.

Stock solutions:

Prepare the following stock solutions, using analytical grade reagents.

(a) Monopotassium dihydrogen orthophosphate, KH_2PO_4 8,50 g

Dipotassium monohydrogen orthophosphate, K_2HPO_4 21,75 g

Disodium monohydrogen orthophosphate dihydrate $Na_2HPO_4 . 2 H_2O$ 33,40 g

Ammonium chloride, NH_4Cl 0,50 g

Dissolve in water and make up to 1 litre The pH of the solution should be 7,4.

(b) Calcium chloride, anhydrous, $CaCl_2$ 27,50g

or Calcium chloride dihydrate, $CaCl_2, 2 H_2O$ 36,40 g

Dissolve in water and make up to 1 litre

(c) Magnesium sulphate heptahydrate, $MgSO_4 . 7 H_2O$ 22,50 g

Dissolve in water and make up to 1 litre.

(d) Iron (III) chloride hexahydrate, $FeC1_3 . 6H_2O$ 0,25 g

Dissolve in water and make up to 1 litre.

Note: in order to avoid having to prepare this solution immediately before use add one drop of conc. HCL or 0,4 g ethylenediaminetetra-acetic acid disodium salt (EDTA) par litre.

I.6.3. **Stock solutions of chemicals**

For example, dissolve 1-10 g, as appropriate, of test or reference chemical in deionised water and make up to 1 litre when the solubility exceeds 1 g/l. Otherwise, prepare stock solutions in the mineral medium or add the chemical direct to the mineral medium. For the handling of less soluble chemicals, see Appendix 3, but in the MITI test (Method C.4-F), neither solvents nor emulsifying agents are to be used.

I.6.4. **Inocula**

The inoculum may be derived from a variety of sources: activated sludge, sewage effluents (unchlorinated), surface waters and soils or from a mixture of these. For the DOC Die-Away, CO_2 Evolution and Manometric Respirometry tests, if activated sludge is used, it should be taken from a treatment plant or laboratory-scale unit receiving predominantly domestic sewage. Inocula from other sources have been found to give higher scattering of results. For the Modified OECD Screening and the Closed Bottle tests a more dilute inoculum without sludge fiocs is needed and the preferred source is a secondary effluent from a domestic waste water treatment plant or laboratory-scale unit. For the MITI test the inoculum is derived from a mixture of sources and is described under the heading of this specific test.

I.6.4.1. *Inoculum from activated sludges*

Collect a sample of activated sludge freshly from the aeration tank of a sewage treatment plant or laboratory-scale unit treating predominantly domestic sewage. Remove coarse particles if necessary by filtration through a fine sieve and keep the sludge aerobic thereafter.

Alternatively, settle or centrifuge (e.g. at 100 g for 10 min) after removal of any coarse particles. Discard the supernatant. The sludge may be washed in the mineral medium. Suspend the concentrated sludge in mineral medium to yield a concentration of 3-5 g suspended solids/l and aerate until required.

Sludge should be taken from a properly working conventional plant. If sludge has to be taken from a high rate treatment plant, or is thought to contain inhibitors, it should be washed. Settle or centrifuge the re-suspended sludge after thorough mixing, discard the supernatant and again re-suspend the washed sludge in a further volume of mineral medium. Repeat this procedure until the sludge is considered to be free from excess substrate or inhibitor.

After complete re-suspension is achieved, or with untreated sludge, withdraw a sample just before use for the determination of the dry weight of the suspended solids.

A further alternative is to homogenise activated sludge (3-5 g suspended solids/l). Treat the sludge in a mechanical blender for two min at medium speed. Settle the blended sludge for 30 min or longer if required and decant liquid for use as inoculum at the rate of 10 mill of mineral medium.

I.6.4.2. *Other sources of inoculum*

It can be derived from the secondary effluent of a treatment plant or laboratory-scale unit receiving predominantly domestic sewage. Collect a fresh sample and keep it aerobic during transport. Allow to settle for 1 h. or filter through a coarse filter paper and keep the decanted effluent or filtrate aerobic until required. Up to 100 ml of this type of inoculum may be used per litre of medium.

A further source for the inoculum is surface water. In this case, collect a sample of an appropriate surface water, e.g. river, lake, and keep aerobic until required. If necessary, concentrate the inoculum by filtration or centrifugation.

I.6.5. **Pre-conditioning of inocula**

Inocula may be pre-conditioned to the experimental conditions, but not pre-adapted to the test chemical. Pre-conditioning consists of aerating activated sludge in mineral medium or secondary effluent for five to seven days at the test temperature. Pre-conditioning sometimes improves the precision of the test methods by reducing blank values. It is considered unnecessary to pre-condition MITI inoculum.

I.6.6. **Abiotic controls**

When required, check for the possible abiotic degradation of the test substance by determining the removal of DOC, oxygen uptake or carbon dioxide evolution in sterile controls containing no inoculum. Sterilise by filtration through a membrane (0,2- 0,45 micrometre) or by the addition of a suitable toxic substance at an appropriate concentration. If membrane filtration is used, take samples aseptically to maintain sterility. Unless adsorption of the test chemical has been ruled out beforehand, tests which measure biodegradation as the removal of DOC, especially with activated sludge inocula, should include an abiotic control which is inoculated and poisoned.

I.6.7. **Number of flasks**

The number of flasks in a typical run is described under the headings of each tests.

The following type of flask may be used:

— test suspension: containing test substance and inoculum

— inoculum blank: containing only inoculum

— procedure control: containing reference substance and inoculum

— abiotic sterile control: sterile, containing test substance (see 1.6.6)

— adsorption control: containing test substance, inoculum and sterilising agent

— toxicity control: containing test substance, reference substance and inoculum

It is mandatory that determination in test suspension and inoculum blank is made in parallel. It is advisable to make the determinations in the other flasks in parallel as well.

This may, however, not always be possible. Ensure that sufficient samples or readings are taken to allow the percentage removal in the 10-day window to be assessed.

I.7. DATA AND EVALUATION

In the calculation of D_t, percentage degradation, the mean values of the duplicate measurement of the parameter in both test vessels and inoculum blank are used. The formulas are set out in the sections below on specific tests. The course of degradation is displayed graphically and the 10-day window is indicated. Calculate and report the percentage removal achieved at the end of the 10-day window and the value at the plateau or at the end of the test, whichever is appropriate.

In respirometric tests nitrogen-containing compounds may affect the oxygen uptake because of nitrification (see Appendices 2 and 5).

I.7.1. **Degradation measured by means of DOC determination**

The percentage degradation D_t at each time a sample was taken should be calculated separately for the flasks containing test substance using mean values of duplicate DOC measurements in order that the validity of the test can be assessed (see 1.5.2). It is calculated using the following equation:

$$D_t = \left(1 - \frac{C_t - C_{bt}}{C_o - C_{b0}}\right) \times 100$$

where:

D_t = % degradation at time t

C_o = mean starting concentration of DOC in the inoculated culture medium containing the test substance (mg DOC/l)

C_t = mean concentration of DOC in the inoculated culture medium containing test substance at time t (mg DOC/l)

C_{bo} = mean starting concentration of DOC in blank inoculated mineral medium (mg DOC/l)

C_{bt} = mean concentration of DOC blank inoculated mineral medium at time t (mg DOC/l).

All concentrations are measured experimentally.

I.7.2. **Degradation measured by means of specific analysis**

When specific analytical data are available, calculate primary biodegradation from:

$$D_t = \frac{S_b - S_a}{S_b} \times 100$$

where:

D_t = % degradation at time t, normally 28 days,
S_a = residual amount of test substance in inoculated medium at end of test (mg),
S_b = residual amount of test substance in the blank test with water/medium to which only the test substance was added (mg).

1.7.3. **Abiotic degradation**

When an abiotic sterile control is used, calculate the percentage abiotic degradation using:

$$\% \text{ abiotic degradation} = \frac{C_{s(o)} - C_{s(t)}}{C_{s(o)}} \times 100$$

Where:

$C_{s(o)}$ = DOC Concentration in sterile control at day 0
$C_{s(t)}$ = DOC Concentration in sterile control at day t.

I.8. REPORTING

The test report shall, if possible, contain the following:

— test and reference chemicals, and their purity,

— test conditions,

— inoculum: nature and sampling site(s), concentration and any pre-conditioning treatment,

— proportion and nature of industrial waste present in sewage if known,

— test duration and temperature,

— in the case of poorly soluble test chemicals, treatment given,

— test method applied; scientific reasons and explanation should be given for any change of procedure,

— data sheet,

— any observed inhibition phenomena,

— any observed abiotic degradation,

— specific chemical analytical data, if available,

— analytical data on intermediates, if available,

— the graph of percentage degradation against time for the test and reference substances; the lag phase, degradation phase, 10-day window and slope should be clearly indicated (Appendix 1). If the test has complied with the validity criteria, the mean of the degradation percentages of the flasks containing test substance may be used for the graph,

— percentage removal after 10-day window, and at plateau or at end of the test.

PART II. **DOC DIE-AWAY TEST** (Method C.4-A)

II.1. PRINCIPLE OF THE METHOD

A measured volume of inoculated mineral medium containing a known concentration of the test substance (10-40 mg DOC/l) as the nominal sole source of organic carbon is aerated in the dark or diffused light at 22 ± 2 °C.

Degradation is followed by DOC analysis at frequent intervals over a 28-day period. The degree of biodegradation is calculated by expressing the concentration of DOC removed (corrected for that in the blank inoculum control) as a percentage of the concentration initially present. The degree of primary biodegradation may also be calculated from supplemental chemical analysis made at the beginning and end of incubation.

II.2. DESCRIPTION OF THE METHOD

II.2.1. **Apparatus**

(a) Conical flasks, e. g. 250 ml to 2 l, depending on the volume needed for DOC analysis;

(b) shaking machine to accommodate the conical flasks, either with automatic temperature control or used in a constant temperature room; and of sufficient power to maintain aerobic conditions in all flasks;

(c) filtration apparatus, with suitable membranes;

(d) DOC analyser;

(e) apparatus for determining dissolved oxygen;

(f) centrifuge.

II.2.2. **Preparation of mineral medium**

For the preparation of the stock solutions, see I.6.2.

Mix 10 ml of solution (a) with 800 ml dilution water, add 1 ml of solutions (b) to (d) and make up to 11 with dilution water.

II.2.3. **Preparation and pre-conditioning of inoculum**

The inoculum may be derived from a variety of sources: activated sludge; sewage effluents; surface waters; soils or from a mixture of these.

See I.6.4., I.6.4.1., I.6.4.2. and I.6.5.

II.2.4. **Preparation of flasks**

As an example, introduce 800 ml portions of mineral medium into 2 l conical flasks and add sufficient volumes of stock solutions of the test and reference substances to separate flasks to give a concentration of chemical equivalent to 10-40 mg DOC/l. Check the pH values and adjust, if necessary, to 7,4. Inoculate the flasks with activated sludge or other source of inocula (see I.6.4.), to give a final concentration not greater than 30 mg suspended solids/l. Also prepare inoculum controls in the mineral medium but without test or reference chemical.

If needed, use one vessel to check the possible inhibitory effect of the test chemical by inoculating a solution containing, in the mineral medium, comparable concentrations of both the test and a reference chemical.

Also, if required, set up a further, sterile flask to check whether the test chemical is degraded abiotically by using an uninoculated solution of the chemical (see I.6.6).

Additionally, if the test chemical is suspected of being significantly adsorbed on to glass, sludge, etc., make a preliminary assessment to determine the likely extent of adsorption and thus the suitability of the test for the chemical (see Table 1). Set up a flask containing the test substance, inoculum and sterilising agent.

Make up the volumes in all flasks to 11 with mineral medium and, after mixing, take a sample from each flask to de.termine the initial concentration of DOC (see Appendix 2.4). Cover the openings of the flasks, e.g. with aluminium foil, in such a way as to allow free exchange of air between the flask and the surrounding atmosphere. Then insert the vessels into the shaking machine for starting the test.

II.2.5. **Number of flasks in typical run**

Flasks 1 and 2: Test suspension

Flasks 3 and 4: Inoculum blank

Flask 5: Procedure control

preferably and when necessary:

Flask 6: Abiotic sterile control

Flask 7: Adsorption control

Flask 8: Toxicity control

See also I.6.7.

II.2.6. **Performance of the test**

Throughout the test, determine the concentrations of DOC in each flask in duplicate at known time intervals, sufficiently frequently to be able to determine the start of the 10-day window and the percentage removal at the end of the 10-day window. Take only the minimal volume of test suspension necessary for each determination.

Before sampling make good evaporation losses from the flasks by adding dilution water (I.6.1) in the required amount if necessary. Mix the culture medium thoroughly before withdrawing a sample and ensure that material adhering to the walls of the vessels is dissolved or suspended before sampling. Membrane-filter or centrifuge (see Appendix 2..4) immediately after the sample has been taken. Analyse the filtered or centrifuged samples on the same day, otherwise store at 2-4 °C for a maximum of 48 h, or below - 18 °C for a longer period.

II.3. DATA AND REPORTING

II.3.1. **Treatment of results**

Calculate the percentage degradation at time t as given under I.7.1 (DOC determination) and, optionally, under I.7.2 (specific analysis).

Record all results on the data sheets provided.

II.3.2. **Validity of results**

See I.5.2.

II.3.3. **Reporting**

See I.8.

II.4. DATA SHEET

An example of a data sheet is given hereafter.

DOC DIE-AWAY TEST

1. **LABORATORY**

2. **DATE AT START OF TEST**

3. **TEST SUBSTANCE**

Name:

Stock solution concentration: … mg/1 as chemical

Initial concentration in medium, to: … mg/1 as chemical

4. **INOCULUM**

Source:

Treatment given:

Pre-conditioning, if any:

Concentration of suspended solids in reaction mixture: mg/1

5. **CARBON DETERMINATIONS**

Carbon analyser:

	Flask nr		DOC after n-days (mg/l)				
			0	n_1	n_2	n_3	n_x
Test chemical plus inoculum	1	a_1					
		a_2					
		a, mean $C_{a(t)}$					
	2	b1					
		b2					
		b, men $C_{b(t)}$					

	Flask nr		DOC after n-days (mg/l)				
			0	n_1	n_2	n_3	n_x
Blank inoculum without test chemical	3	C_1					
		C_2					
		C, mean $C_{c(t)}$					
	4	d_1					
		d_2					
		d, mean $C_{d(t)}$					
	$C_{bl(t)} = \frac{C_{c(t)} + C_{d(t)}}{2}$						

6. **EVALUATION OF RAW DATA**

Flask nr		% degradation afer n days				
		0	n_1	n_2	n_3	n_x
1	$D_1 = \left(1 - \frac{C_{a(t)} - C_{bl(t)}}{C_{a(o)} - C_{bl(o)}}\right) \times 100$	0				
2	$D_2 = \left(1 - \frac{C_{b(t)} - C_{bl(t)}}{C_{b(o)} - C_{bl(o)}}\right) \times 100$	0				
Mean (*)	$D = \frac{D_1 - D_2}{2}$	0				

(*) D1 and D_2 should not be averaged if there is a considerable difference.

Note: similar formats may be used for the reference chemical and toxicity controls.

7. **ABIOTIC CONTROL** (optional)

	Time (days)	
	0	t
DOC conc. (mg/l) in sterile control	$C_{s(o)}$	$C_{s(t)}$

$$\% \text{ abiotic degradation} = \frac{C_{s(o)} - C_{s(t)}}{C_{s(o)}} \times 100$$

8. **SPECIFIC CHEMICAL ANALYSIS** (optional)

	residual amount of test chemical at end of test (mg/l)	% primary degradation
Sterile control	S_b	

	residual amount of test chemical at end of test (mg/l)	% primary degradation
Inoculated test medium	S_a	$\frac{S_b - S_a}{S_b} \times 100$

PART III. **MODIFIED OECD SCREENING TEST** (Method C.4-B)

III.1. PRINCIPLE OF THE METHOD

A measured volume of mineral medium containing a known concentration of the test substance (10-40 mg DOC/litre) as the nominal sole source of organic carbon is inoculated with 0,5 ml effluent per litre of medium. The mixture is aerated in the dark or diffused light at 22 ± 2 °C.

Degradation is followed by DOC analysis at frequent intervals over a 28-day period. The degree of biodegradation is calculated by expressing the concentration of DOC removed (corrected for that in the blank inoculum control) as a percentage of the concentration initially present. The degree of primary biodegradation may also be calculated from supplemental chemical analysis made at the beginning and end of incubation.

III.2. DESCRIPTION OF THE METHOD

III.2.1. Apparatus

(a) Conical flasks, e.g. 250 ml to 2 litres, depending on the volume needed for DOC analysis;

(b) shaking machine — to accommodate the conical flasks, either with automatic temperature control or used in a constant temperature room, and of sufficient power to maintain aerobic conditions in all flasks;

(c) filtration apparatus, with suitable membranes;

(d) DOC analyser;

(e) apparatus for determining dissolved oxygen;

(f) centrifuge.

III.2.2. **Preparation of mineral medium**

For the preparation of the stock solutions, see I.6.2.

Mix 10 ml of solution (a) with 80 ml dilution water, add 1 ml of solutions (b) to (d) and make up to 1 litre with dilution water.

This method uses only 0,5 ml effluent/litre as inoculum and therefore the medium may need to be fortified with trace elements and growth factors. This is done by adding 1 ml each of the following solutions per litre of final medium:

Trace element solution:

Manganese sulfate tetrahydrate, $MnSO_4 . 4H_2O$	39,9 mg
Boric acid, H_3BO_3	57,2 mg
Zinc sulfate heptahydrate, $ZnSO_4 . 7H_2O$	42,8 mg
Ammonium heptamolybdate $(NH_4)_6\ Mo_7O_{24}$	34,7 mg
Fe-chelate ($FeCl_3$ ethylenediamine-tetra-acetic acid)	100,0 mg

Dissolve in, and make up to 1 000 ml with dilution water

Vitamin solution:

Yeast extract	15,0 mg

Dissolve the yeast extract in 100 ml water. Sterilise by passage through a 0,2 micron membrane, or make up freshly.

III.2.3. **Preparation and pre-conditioning of inoculum**

The inoculum is derived from the secondary effluent of a treatment plant or laboratory scale unit receiving predominantly domestic sewage. See I.6.4.2. and I.6.5.

0,5 ml per litre of mineral medium is used.

III.2.4. **Preparation of flasks**

As an example, introduce 800 ml portions of mineral medium into 2-litre conical flasks and add sufficient volumes of stock solutions of the test and reference substances to separate flasks to give a concentration of chemical equivalent to 10-40 mg DOC/litre. Check the pH value and adjust, if necessary, to 7,4. Inoculate the flasks with sewage effluent at 0,5 ml/litre (see I.6.4.2). Also prepare inoculum controls in the mineral medium but without test or reference chemical.

If needed, use one vessel to check the possible inhibitory effect of the test chemical by inoculating a solution containing, in the mineral medium, comparable concentrations of both the test and a reference chemical.

Also, if required, set up a further, sterile flask to check whether the test chemical is degraded abiotically by using an uninoculated solution of the chemical (see I.6.6).

Additionally, if the test chemical is suspected of being significantly adsorbed on to glass, sludge, etc., make a preliminary assessment to determine the likely extent of adsorption and thus the suitability of the test for the chemical (see Table 1). Set up a flask containing the test substance, inoculum and sterilising agent.

Make up the volumes in all flasks to 1 litre with mineral medium and, after mixing, take a sample from each flask to determine the initial concentration of DOC (see Appendix 2.4). Cover the openings of the flasks, e.g. with aluminium foil, in such a way as to allow free exchange of air between the flask and the surrounding atmosphere. Then insert the vessels into the shaking machine for starting the test.

III.2.5. **Number of flasks in typical run**

Flasks 1 and 2: test suspension

Flasks 3 and 4: inoculum blank

Flask 5: procedure control

and preferably and when necessary:

Flask 6: abiotic sterile control

Flask 7: adsorption control

Flask 8: toxicity control

See also 1.6.7.

III.2.6. **Performance of the test**

Throughout the test, determine the concentrations of DOC in each flask in duplicate at known time intervals, sufficiently frequently to be able to determine the start of the 10-day window and the percentage removal at the end of the 10-day window. Take only the minimal volume of test suspension necessary for each determination.

Before sampling make good evaporation losses from the flasks by adding dilution water (I.6.1) in the required amount if necessary. Mix the culture medium thoroughly before withdrawing a sample and ensure that material adhering to the walls of the vessels is dissolved or suspended before sampling. Membrane-filter or centrifuge (see Appendix 2.4) immediately after the sample has been taken. Analyse the filtered or centrifuged samples on the same day, otherwise store at 2-4 °C for a maximum of 48 h, or below 18 °C for a longer period.

III.3. DATA AND REPORTING

III.3.1. **Treatment of results**

Calculate the percentage degradation at time t as given under I.7.1 (DOC determination) and, optionally, under I.7.2 (specific analysis).

Record all results on the data sheets provided.

III.3.2. Validity of results

See I.5.2.

III.3.3. Reporting

See I.8.

III.4. DATA SHEET

An example of a data sheet is given hereafter

MODIFIED OECD SCREENING TEST

1. **LABORATORY**

2. **DATE AT START OF TEST**

3. **TEST SUBSTANCE**

Name:

Stock solution concentration: … mg/litre as chemical

Initial concentration in medium, to: … mg/litre as chemical

4. **INOCULUM**

Source:

Treatment given:

Pre-conditioning, if any:

Concentration of suspended solids in reaction mixture: mg/1

5. CARBON DETERMINATIONS

Carbon analyser:

	Flask nr		DOC after n-days (mg/l)				
			0	n_1	n_2	n_3	n_x
Test chemical plus inoculum	1	a_1					
		a_2					
		a, mean $C_{a(t)}$					
	2	b1					
		b2					
		b, men $C_{b(t)}$					
Blank inoculum without test chemical	3	C_1					
		C_2					
		C, mean $C_{c(t)}$					
	4	d_1					
		d_2					
		d, mean $C_{d(t)}$					
	$C_{bl(t)} = \frac{C_{c(t)} + C_{d(t)}}{2}$						

6. **EVALUATION OF RAW DATA**

Flask nr		% degradation afer n days				
		0	n_1	n_2	n_3	n_x
1	$D_1 = \left(1 - \frac{C_{a(t)} - C_{bl(t)}}{C_{a(o)} - C_{bl(o)}}\right) \times 100$	0				
2	$D_2 = \left(1 - \frac{C_{b(t)} - C_{bl(t)}}{C_{b(o)} - C_{bl(o)}}\right) \times 100$	0				
Mean (*)	$D = \frac{D_1 - D_2}{2}$	0				

(*) D_1 and D_2 should not be averaged if there is a considerable difference.

Note: similar formats may be used for the reference chemical and toxicity controls.

7. **ABIOTIC CONTROL** (optional)

	Time (days)	
	0	t
DOC conc. (mg/l) in sterile control	$C_{s(o)}$	$C_{s(t)}$

$$\% \text{ abiotic degradation} = \frac{C_{s(o)} - C_{s(t)}}{C_{s(o)}} \times 100$$

8. **SPECIFIC CHEMICAL ANALYSIS** (optional)

	residual amount of test chemical at end of test (mg/l)	% primary degradation
Sterile control	S_b	
Inoculated test medium	S_a	$\frac{S_b - S_a}{S_b} \times 100$

PART IV. CO_2 EVOLUTION TEST (Method C.4-C)

IV.1. PRINCIPLE OF THE METHOD

A measured volume of inoculated mineral medium containing a known concentration of the test chemical (10-20 mg DOC or TOC/l) as the nominal sole source of organic carbon is aerated by the passage of carbon, dioxide-free air at a controlled rate in the dark or in diffuse light. Degradation is followed over 28 days by determining the carbon dioxide produced, which is trapped in barium or sodium hydroxide and which is measured by titration of the residual hydroxide or as inorganic carbon. The amount of carbon dioxide produced from the test chemical (corrected for that derived from the blank inoculum) is expressed as a percentage of $ThCO_2$. The degree of biodegradation may also be calculated from supplemental DOC analysis made at the beginning and end of incubation.

IV.2. DESCRIPTION OF THE METHOD

IV.2.1. **Apparatus**

(a) Flasks, 2-5 litres, each fitted with an aeration tube reaching nearly the bottom of the vessel and an outlet;

(b) magnetic stirrers, when assessing poorly soluble chemicals;

(c) gas-absorption bottles;

(d) device for controlling and measuring airflow;

(e) apparatus for carbon dioxide scrubbing, for preparation of air which is free from carbon dioxide; alternatively, a mixture of CO_2-free oxygen and CO_2-free nitrogen, from gas cylinders, in the correct proportions (20 % O_2: 80 % N_2) may be used;

(f) device for determination of carbon dioxide, either titrimetrically or by some form of inorganic carbon analyser;

(g) membrane filtration device (optional);

(h) DOC analyser (optional).

IV.2.2. **Preparation of mineral medium**

For the preparation of the stock solutions, see I.6.2.

Mix 10 ml of solution (a) with 800 ml dilution water, add 1 ml of solutions (b) to (d) and make up to 11 with dilution water.

IV.2.3. **Preparation and pre-conditioning of inoculum**

The inoculum may be derived from a variety of sources: activated sludge; sewage effluents; surface waters; soils or from a mixture of these.

See I.6.4., I.6.4.1., I.6.4.2. and I.6.5.

IV.2.4. **Preparation of flasks**

As an example the following volumes and weights indicate the values for 5-litre flasks containing 3 l of suspension. If smaller volumes are used modify the values accordingly, but ensure that the carbon dioxide formed can be measured accurately.

To each 5-litre flask add 2400 ml mineral medium. Add an appropriate volume of the prepared activated sludge (see I.6.4.1 and I.6.5) to give a concentration of suspended solids of not more than 30 mg/l in the final 3 l of inoculated mixture. Alternatively first dilute the prepared sludge to give a suspension of 500-1000 mg/l in the mineral medium before adding an aliquot to the contents of the 5 litre flask to attain a concentration of 30 mg/l; this ensures greater precision. Other sources of inoculum may be used (see I.6.4.2.).

Aerate these inoculated mixtures with CO_2-free air overnight to purge the system of carbon dioxide.

Add the test material and reference substance, separately, as known volume of stock solutions, to replicate flasks to yield concentrations, contributed by the added chemicals, of 10 to 20 mg DOC or TOC/l; leave some flasks without addition of chemicals as inoculum controls. Add poorly soluble test substances directly to the flasks on a weight or volume basis or handle as described in Appendix 3.

If required, use one flask to check the possible inhibitory effect of the test chemical by adding both the test and reference chemicals at the same concentrations as present in the other flasks.

Also, if required, use a sterile flask to check whether the test chemical is degraded abiotically by using an uninoculated solution of the chemical (see I.6.6). Sterilise by the addition of a toxic substance at an appropriate concentration.

Make up the volumes of suspensions in all flasks to 3 l by the addition of mineral medium previously aerated with CO_2-free air. Optionally, samples may be withdrawn for analysis of DOC (see Appendix 2.4.) and/or specific analysis. Connect the absorption bottles to the air outlets of the flasks.

If barium hydroxide is used, connect three absorption bottles, each containing 100 ml of 0,0125 M barium hydroxide solution, in series to each 5-litre flask. The solution must be free of precipitated sulphate and carbonate and its strength must be determined immediately before use. If sodium hydroxide is used, connect two traps, the second acting as a control to demonstrate that all the carbon dioxide was absorbed in the first. Absorption bottles fitted with serum bottle closures are suitable. Add 200 ml 0,05 M sodium hydroxide to each bottle, which is sufficient to absorb the total quantity of carbon dioxide evolved when the test chemical is completely degraded. The sodium hydroxide solution, even when freshly prepared, will contain traces of carbonates; this is corrected by deduction of the carbonate in the blank.

IV.2.5. **Number of flasks in a typical run**

Flasks 1 and 2: Test suspension

Flasks 3 and 4: Inoculum blank

Flask 5: Procedure control

and, preferably and when necessary:

Flask 6: Abiotic sterile control

Flask 7: Toxicity control

See also I.6.7.

IV.2.6. **Performance of the test**

Start the test by bubbling CO_2-free air through the suspensions at a rate of 30-100 ml/min. Take samples of the carbon dioxide absorbent periodically for analysis of the CO_2-content. During the first ten days it is recommended that analyses should be made every second or third day and then every fifth day until the 28th day so that the 10-day window period can be identified.

On the 28th day, withdraw samples (optionally) for DOC and/or specific analysis, measure the pH of the suspensions and add 1 ml of concentrated hydrochloric acid to each flask; aerate the flasks overnight to drive off the carbon dioxide present in the test suspensions. On day 29 make the last analysis of evolved carbon dioxide.

On the days of measurement of CO_2, disconnect the barium hydroxide absorber closest to the flask and titrate the hydroxide solution with HCl 0,05 M using phenolphthalein as the indicator. Move the remaining absorbers one place closer to the flask and place a new absorber containing 100 ml fresh 0,0125 M barium hydroxide at the far end of the series. Make titrations as needed, for example, when substantial precipitation is seen in the first trap and before any is evident in the second, or at least weekly. Alternatively, with NaOH as absorbent, withdraw with a syringe a small sample (depending on the characteristics of the carbon analyser used) of the sodium hydroxide solution in the absorber nearer to the flask. Inject the sample into the IC part of the carbon analyser for analysis of evolved carbon dioxide directly.

Analyse the contents of the second trap only at the end of the test to correct for any carryover of carbon dioxide.

IV.3. DATA AND REPORTING

IV.3.1. **Treatment of results**

The amount of CO_2 trapped in an absorber when titrated is given by:

$$mgCO_2 = (100 \times C_B - 0,5 \times V \times C_A) \times 44$$

where:

V = volume of HCl used for titration of the 100 ml in the absorber (ml)
C_B = concentration of the barium hydroxide solution (M)
C_A = concentration of the hydrochloric acid solution (M)

if C_B is 0,0125 M and C_A is 0,05 M, the titration for 100 ml barium hydroxide is 50 ml and the weight of CO_2 is given by:

$$\frac{0,05}{2} \times 44 \times \text{ml HCI titrated} = 1,1 \times \text{ml HCI}$$

Thus, in this case, to convert volume of HCl titrated to mg CO_2 produced the factor is 1,1.

Calculate the weights of CO_2 produced from the inoculum alone and from the inoculum plus test chemical using the respective titration values and the difference is the weight of CO_2 produced from the test chemical alone.

For example, if the inoculum alone gives a titration of 48 ml and inoculum plus test chemical gives 45 ml,

CO_2 from inoculum = 1,1 × (50-48) = 2,2 mg

CO_2 from inoculum plus test chemical = 1,1 × (50-45) = 5,5 mg

and thus the weight of CO_2 produced from the test chemical is 3,3 mg.

The percentage biodegradation is calculated from:

$$\% \text{ degradation} = \frac{\text{mg } CO_2 \text{ produced} \times 100}{ThCO_2 \times \text{mg test chemical added}},$$

or,

$$\% \text{ degradation} = \frac{\text{mg } CO_2 \text{ produced} \times 100}{\text{mg TO added in test} \times 3{,}67}$$

3,67 being the conversion factor (44/12) for carbon to carbon dioxide.

Obtain the percentage degradation after any time interval by adding the percentage of $ThCO_2$ values calculated for each of the days, up to that time, on which it was measured.

For sodium hydroxide absorbers, calculate the amount of carbon dioxide prodllced, expressed as IC (mg), by multiplying the concentration of IC in the absorbent by the volume of the absorbent.

Calculate the percentage degradation from:

$$\% \text{ of } ThCO_2 = \frac{\text{mg IC flask} - \text{mg IC blank}}{\text{MG TOC added as test chemical}} \times 100$$

Calculate DOC removals (optional) as described under I.7. Record these and all other results on the data sheets provided.

IV.3.2. **Validity of results**

The IC content of the test chemical suspension in the mineral medium at the beginning of the test must be less than 5 % of the TC, and the total CO_2 evolution in the inoculum blank at the end of the test should not normally exceed 40 mg/1 medium. If values greater than 70 mg CO_2/litre are obtained, the data and experimental technique should be examined critically.

See also I.5.2.

IV.3.3. **Reporting**

See I.8.

IV.4. DATA SHEET

An example of a data sheet is given hereafter.

CARBON DIOXIDE EVOLUTION TEST

1. **LABORATORY**

2. **DATE AT START OF TEST**

3. **TEST SUBSTANCE**

Name:

Stock solution concentration: … mg/litre as chemical

Initial conc. in medium: … mg/litre as chemical

Total C added to flask: … mg C

$ThCO_2$: mg CO_2

4. **INOCULUM**

Source:

Treatment given:

Pre-conditioning if any:

Concentration of suspended solids in reaction mixture: mg/litre

Time (day)	CO_2 formed Test (mg)		CO_2 formed blank (mg)		CO_2 formed cumulative (mg) (test minus blank mean)		$ThCO_2$ cumulative $\frac{CO_2}{ThCO_2} \times 100$		
	1 / 2	mean	3 / 4	mean	1	2	1	2	mean
0									
n_1									
n_2									
n_3									
28									

Note: similar formats may be used for the reference chemical and toxicity controls.

6. **CARBON ANALYSIS** (optional)

Carbon analyser:

Time (day)	Blank mg/l	Test chemical mg/l
0	$C_{b(o)}$	C_o
28 (*)	$C_{b(t)}$	C_t

(*) Or at end of incubation

$$\% \text{ DOC removed} = \left(1 - \frac{C_t - C_{b(t)}}{C_o - C_{b(o)}}\right) \times 100$$

7. **ABIOTIC DEGRADATION** (optional)

$$\% \text{ abiotic degradation} = \frac{CO_2 \text{ formation in sterile in flask after 28 day (mg)}}{ThCO_2 \text{ (mg)}} \times 100$$

PART V. **MANOMETRIC RESPIROMETRY TEST** (Method C.4-D)

V.1. PRINCIPLE OF THE METHOD

A measured volume of inoculated mineral medium, containing a known concentration of test chemical (100 mg/litre of the test substance, to give at least 50-100 mg ThOD/litre) as the nominal sole source of organic carbon, is stirred in a closed flask at a constant temperature (± 1° C or closer) for up to 28 days. The consumption of oxygen is determined either by measuring the quantity of oxygen (produced electrolytically) required to maintain constant gas volume in the respirometer flask, or from the change in volume or pressure (or a combination of the two) in the apparatus. Evolved carbon dioxide is absorbed in a solution of potassium hydroxide or another suitable absorbent. The amount of oxygen taken up by the test chemical (corrected for uptake by blank inoculum, run in parallel) is expressed as a percentage of ThOD or COD. Optionally, primary biodegradation may also be calculated from supplemental specific analysis made at the beginning and end of incubation, and ultimate biodegradation by DOC analysis.

V.2. DESCRIPTION OF THE METHOD

V.2.1. **Apparatus**

(a) suitable respirometer;

(b) temperature control, maintaining ± 1 °C or better;

(c) membrane-filtration assembly (optional);

(d) carbon analyser (optional).

V.2.2. **Preparation of mineral medium**

For the preparation of the stock solutions, see I.6.2.

Mix 10 ml of solution (a) with 800 ml dilution water, add 1 ml of solutions (b) to (d) and make up to 1 litre with dilution water.

V.2.3. **Preparation and pre-conditioning of inoculum**

The inoculum may be derived from a variety of sources: activated sludge; sewage effluents; surface waters and soils or from a mixture of these.

See I.6.4., I.6.4.1., I.6.4.2. and I.6.5.

V.2.4. **Preparation of flasks**

Prepare solutions of the test and reference chemicals, in separate batches, in mineral medium equivalent to a concentration, normally, of 100 mg chemical/litre (giving at least 50-100 mg ThOD/litre), using stock solutions.

Calculate the ThOD on the basis of formation of ammonium salts unless nitrification is anticipated, when the calculation should be based on nitrate formation (see Appendix 2.2)

Determine the pH values and if necessary adjust to 7,4 ± 0,2.

Poorly soluble substances should be added at a later stage (see below).

If the toxicity of the test chemical is to be determined, prepare a further solution in mineral medium containing both test and reference chemicals at the same concentrations as in the individual solutions.

If measurement of the physico-chemical uptake of oxygen is required, prepare a solution of the test chemical at, normally, 100 mg ThOD/litre which has been sterilised by the addition of a suitable toxic substance (see I.6.6).

Introduce the requisite volume of solutions of test and reference chemicals, respectively, into at least duplicate flasks. Add to further flasks mineral medium only (for inoculum controls) and, if required, the mixed test/reference chemical solution and the sterile solution.

If the test chemical is poorly soluble, add it directly at this stage on a weight or volume basis or handle it as described in Appendix 3. Add potassium hydroxide, soda lime pellets or other absorbent to the CO_2-absorber compartments.

V.2.5. **Number of flasks in a typical run**

Flasks 1 and 2: test suspension

Flasks 3 and 4: inoculum blank

Flask 5: procedure control

preferably, and when necessary:

Flask 6: sterile control

Flask 7: toxicity control

See also I.6.7.

V.2.6. **Performance of the test**

Allow the vessels to reach the desired temperature and inoculate appropriate vessels with prepared activated sludge or other source of inoculum to give a concentration of suspended solids not greater than 30 mg/litre. Assemble the equipment, start the stirrer and check for air-tightness, and start the measurement of oxygen uptake. Usually no further attention is required other than taking the necessary readings and making daily checks to see that the correct temperature and adequate stirring are maintained.

Calculate the oxygen uptake from the readings taken at regular and frequent intervals, using the methods given by the manufacturer of the equipment. At the end of incubation, normally 28 days, measure the pH of the contents of the flasks, especially if oxygen uptakes are low or greater than $ThODNH_4$ (for nitrogen-containing compounds).

If required, withdraw samples from the respirometer flasks, initially and finally, for analysis of DOC or specific chemical (see Appendix 2.4). At the initial withdrawal, ensure that the volume of test suspension remaining in the flask is known. 'When oxygen is taken up by N-containing test substance, determine the increase in concentration of nitrite and nitrate over 28 days and calculate the correction for the oxygen consumed by nitrification (Appendix 5).

V.3. DATA AND REPORTING

V.3.1. **Treatment of results**

Divide the oxygen uptake (mg) of the test chemical after a given time (corrected for that by the blank inoculum control after the same time) by the weight of the test chemical used. This yields the BOD expressed as mg oxygen/mg test chemical, that is:

$$BOD = \frac{(\text{mg } O_2 \text{ uptake by test chemical} - \text{mg } O_2 \text{ uptake by blank})}{(\text{mg test chemical in flask})}$$

= mg O_2 per mg test chemical

calculate the percentage biodegradation either from:

$$\% \text{ biodegradation} = \% \text{ ThOD} = \frac{\text{BOD}(\text{mg } O_2/\text{mg chemical})}{\text{ThOD}(\text{mg } O_2 \text{ chemical})} \times 100$$

or form

$$\% \text{ COD} = \frac{\text{BOD}(\text{mg } O_2/\text{mg chemical})}{\text{COD}(\text{mg } O_2 \text{ chemical})} \times 100$$

It should be noted that these two methods do not necessarily give the same value; it is preferable to use the former method.

For test substances containing nitrogen, use the appropriate ThOD (NH_4 or NO_3) according to what is known or expected about the occurrence of nitrification (Appendix 2.2). If nitrification occurs but is not complete, calculate a correction for the oxygen consumed by nitrification from the changes in concentration of nitrite and nitrate (Appendix 5).

When optional determinations of organic carbon and/or specific chemical are made, calculate the percentage degradation, as described under I.7.

Record all results on the data sheets attached.

V.3.2. **Validity of results**

The oxygen uptake of the inoculum blank is normally 20-30 mg O_2/litre and should not be greater than 60 mg/litre in 28 days. Values higher than 60 mg/litre require critical examination of the data and experimental techniques. If the pH value is outside the range 6-8,5 and the oxygen consumption by the test chemical is less than 60% , the test should be repeated with a lower concentration of test chemical.

See also I.5.2.

V.3.3. **Reporting**

See I.8.

V.4. **DATA SHEET**

An example of a data sheet is given hereafter.

MANOMETRIC RESPIROMETRY TEST

1. **LABORATORY**

2. **DATE AT START OF TEST**

3. **TEST SUBSTANCE**

Name:

Stock solution concentration: … mg/litre

Initial concentration in medium, C_o: … mg/litre

Volume in test flask (V): … ml

ThOD or COD: … mg O_2/mg test substance (NH_4 or NO_3)

4. **INOCULUM**

Source:

Treatment given:

Pre-conditioning, if any:

Concentration of suspended solids in reaction mixture: … mg/1

5. **OXYGEN UPTAKE: BIODEGRADABILITY**

		Time (Days)											
		0		7		14			21			28	
O_2 upt. (mg) test chemical	1												
	2												
	a, mean												
O_2 upt. (mg) blank	3												
	4												
	b, mean												
Corrected BOD (mg)	$(a_1 - b_m)$												
	$(a_2 - b_m)$												
BOD per mg test chemical	$\frac{(a_1 - b)}{C_o V}$												
	$\frac{(a_2 - b)}{C_o V}$												
% degradation $\frac{BOD}{ThOD} \times 100$	D_1 (a_1)												
	D_2 (a_2)												
	Mean (*)												

V = volume of medium in test flask

(*) D_1 and D_2 should not be averaged if there is a considerable difference.

N.B.: Similar formats may be used for the reference chemical and the toxicity controls.

6. **CORRECTION FOR NITRIFICATION** (see Annex V)

Day		0	28	Difference
(i)	Concentration of nitrate (mg N/litre)			(N)
(ii)	Oxygen equivalent (4,57 × N × V) (mg)	—	—	
(iii)	Concentration of nitrite (mg N/litre)			(N)
(iv)	Oxygen equivalent (3,43 × N × V) (mg)	—	—	
(ii + iv)	Total oxygen equivalent	—	—	

7. **CARBON ANALYSIS** (optional)

Carbon analyser:

Time (day)	Blank mg/litre	Test chemical mg/litre
0	(C_{blo})	(C_o)
28 (*)	(C_{blt})	(C_t)

(*) Or at end of incubation

$$\% \text{ DOC removed} = \left(1 - \frac{C_t - C_{blt}}{C_o - C_{blo}}\right) \times 100$$

8. **SPECIFIC CHEMICAL** (optional)

S_b = concentration in physico-chemical (sterile) control at 28 days
S_a = concentration in inoculated flask at 28 days,

$$\% \text{ biodegradation} = \frac{S_b - S_a}{S_b} \times 100$$

9. **ABIOTIC DEGRADATION** (optional)

a = oxygen consumption in sterile flasks after 28 days, (mg)

$$\text{oxygen consumption per mg test chemical} = \frac{a \times 100}{C_o V}$$

(see Sections 1 and 3)

$$\% \text{ abiotic degradation} = \frac{a \times 100}{C_o V \times \text{ThOD}}$$

PART VI. **CLOSED BOTTLE TEST** (Method C.4-E)

VI.1 PRINCIPLE OF THE TEST METHOD

The solution of the test chemical in mineral medium, usually at 2-5 mg/litre, is inoculated with a relatively small number of micro-organisms from a mixed population and kept in completely full, closed bottles in the dark at constant temperature. Degradation is followed by analysis of dissolved oxygen over a 28-day period. The amount of oxygen taken up by the test chemical, corrected for uptake by the blank inoculum run in parallel, is expressed as a percentage of ThOD or COD.

VI.2 DESCRIPTION OF THE METHOD

VI.2.1. **Apparatus**

a) BOD bottles, with glass stoppers, e.g. 250-300 ml;

b) water bath or incubator, for keeping bottles at constant temperature (± 1 °C or better) with the exclusion of light;

c) large glass bottles (2-5 litres) for the preparation of media and for filling the BOD bottles;

d) oxygen electrode and meter, or equipment and reagents for Winkler titration.

VI.2.2. **Preparation of mineral medium**

For the preparation of the stock solutions, see I.6.2.

Mix 1 (one) ml of solution (a) to (d) and make up to 1 litre with dilution water.

VI.2.3. **Preparation of the inoculum**

The inoculum is normally derived from the secondary effluent of a treatment plant or laboratory-scale unit receiving predominantly domestic sewage. An alternative source for the inoculum is surface water. Normally use from one drop (0,05 ml) to 5 ml of filtrate per litre of medium; trials may be needed to discover the optimum volume for a given effluent (See I.6.4.2 and I.6.5).

VI.2.4. **Preparation of flasks**

Strongly aerate mineral medium for at least 20 min. Carry out each test series with mineral medium derived from the same batch. Generally, the medium is ready for use after standing for 20 h, at the test temperature. Determine the concentration of dissolved oxygen for control purposes; the value should be about 9 mg/litre at 20 °C. Conduct all transfer and filling operations of the air-saturated medium bubble-free, for example, by the use of siphons.

Prepare parallel groups of BOD bottles for the determination of the test and reference chemicals in simultaneous experimental series. Assemble a sufficient number of BOD bottles, including inoculum blanks, to allow at least duplicate measurements of oxygen consumption to be made at the desired test intervals, for example, after 0, 7, 14, 21 and 28 days. To ensure being able to identify the 10-day window, more bottles may be required.

Add fully aerated mineral medium to large bottles so that they are about one-third full. Then add sufficient of the stock solutions of the test chemical and reference chemical to separate large bottles so that the final concentration of the chemicals is normally not greater than 10 mg/litre. Add no chemicals to the blank control medium contained in a further large bottle.

In order to ensure that the inoculum activity is not limited, the concentration of dissolved oxygen must not fall below 0,5 mg/litre in the BOD bottles. This limits the concentration of test chemical to about 2 mg/litre. However, for poorly degradable compounds and those with a low ThOD, 5-10 mg/litre can be used. In some cases, it would be advisable to run parallel series of test chemical at two different concentrations, for example, 2 and 5 mg/litre. Normally, calculate the ThOD on the basis of formation of ammonium salts but, if nitrification is expected or known to occur, calculate on the basis of the formation of nitrate ($ThOD_{NO3}$: see Appendix 2.2). However, if nitrification is not complete but does occur, correct for the changes in concentration of nitrite and nitrate, determined by analysis, (see Appendix 5).

If the toxicity of the test chemical is to be investigated (in the case, for example, of a previous low biodegradability value having been found), another series of bottles is necessary.

Prepare another large bottle to contain aerated mineral medium (to about one-third of its volume) plus test chemical and reference chemical at final concentrations normally the same as those in the other large bottles.

Inoculate the solutions in the large bottles with secondary effluent (one drop or about 0,05 ml, to 5 ml/litre) or with another source such as river water (see I.6.4.2.). Finally, make up the solutions to volume with aerated mineral medium using a hose which reaches down to the bottom of the bottle to achieve adequate mixing.

VI.2.5. **Number of flasks in a typical run**

In a typical run the following bottles are used:

— at least 10 containing test chemical and inoculum (test suspension),

— at least 10 containing only inoculum (inoculum blank),

— at least 10 containing reference chemical and inoculum (procedure control),

— and, when necessary, six bottles containing test chemical, reference chemical and inoculum (toxicity control). However, to ensure being able to identify the 10-day window, about twice as many bottles would be necessary.

VI.2.6. Performance of the test

Dispense each prepared solution immediately into the respective group of BOD bottles by hose from the lower quarter (not the bottom) of the appropriate large bottle, so that all the BOD bottles are completely filled. Tap gently to remove any air bubbles. Analyse the zero-time bottles immediately for dissolved oxygen by the Winkler or electrode methods. The contents of the bottles can be preserved for later analysis by the Winkler method by adding manganese (II) sulfate and sodium hydroxide (the first Winkler reagent). Store the carefully stoppered bottles, containing the oxygen fixed as brown manganese (III) hydrated oxide, in the dark at 10-20 °C for no longer than 24 hours before proceeding with the remaining steps of the Winkler method. Stopper the remaining replicate bottles ensuring that no air bubbles are enclosed, and incubate at 20 °C in the dark. Each series must be accompanied by a complete parallel series for the determination of the inoculated blank medium. Withdraw at least duplicate bottles of all series for dissolved oxygen analysis at time intervals (at least weekly) over the 28 days incubation.

Weekly samples should allow the assessment of percentage removal in a 14-day window, whereas sampling every 3-4 days should allow the 10-day window to be identified, which would require about twice as many bottles.

For N-containing test substances, corrections for uptake of oxygen by any nitrification occurring should be made. To do this, use the O_2-electrode method for determining the concentration of dissolved oxygen and then withdraw a sample from the BOD bottle for analysis for nitrite and nitrate. From the increase in concentration of nitrite and nitrate, calculate the oxygen used (see Annex V).

VI.3. DATA AND REPORTING

VI.3.1. **Treatment of results**

First calculate the BOD exerted after each time period by subtracting the oxygen depletion (mg O_2/litre) of the inoculum blank from that exhibited by the test chemical. Divide this corrected depletion by the concentration (mg/litre) of the test chemical, to obtain the specific BOD as mg oxygen per mg test chemical. Calculate the percentage biodegradability by dividing the specific BOD by the specific ThOD (calculated according to Appendix 2.2) or COD (determined by analysis, see Appendix 2.3), thus:

$$\text{BOD} = \frac{(\text{mg } O_2 \text{ uptake by test chemical} - \text{mg } O_2 \text{ uptake by blank})}{(\text{mg test chemical in flask})}$$

= mg O_2 per mg test chemical

$$\% \text{ degradation} = \frac{\text{BOD } (\text{mg } O_2/\text{mg test chemical})}{\text{ThOD}(\text{mg } O_2/\text{mg test chemical})} \times 100$$

Or

$$\% \text{ degradation} = \frac{\text{BOD } (\text{mg } O_2/\text{mg test chemical})}{\text{COD}(\text{mg } O_2/\text{mg test chemical})} \times 100$$

It should be noted that these two methods do not necessarily give same value; it is preferable to use the former method.

For test substances containing nitrogen, use the appropriate ThOD (NH_4 or NO_3) according to what is known or expected about the occurrence of nitrification (Appendix 2.2). If nitrification occurs but is not complete, calculate a correction for the oxygen consumed by nitrification from the changes in concentration of nitrite and nitrate (Appendix 5).

VI.3.2. **Validity of results**

Oxygen depletion in the inoculum blank should not exceed 1,5 mg dissolved oxygen/litre after 28 days. Values higher than this require investigation of the experimental techniques. The residual concentration of oxygen in the test bottles should not fall below 0,5 mg/litre at any time. Such low oxygen levels are valid only if the method of determining dissolved oxygen used is capable of measuring such levels accurately.

See also I.5.2.

VI.3.3. **Reporting**

See I.8.

VI.4. DATA SHEET

An example of a data sheet is given hereafter.

CLOSED BOTTLE TEST

1. **LABORATORY**

2. **DATE AT START OF TEST**

3. **TEST SUBSTANCE**

Name:

Stock solution concentration: … mg/litre

Initial concentration in bottle: … mg/litre

ThOD or COD: … mg O_2/mg test substance

4. **INOCULUM**

Source:

Treatment given:

Pre-conditioning if any:

Concentration in the reaction mixture: … mg/litre

5. **DO DETERMINATION**

Method: Winkler/electrode

Flask analyses

Time of incubation (d)			DO (mg/l)			
			0	n_1	n_2	
Blank (without chemical)	1	C_1				
	2	C_2				
Mean	$m_b = \frac{C_1 + C_2}{2}$					
Test chemical	1	a_1				
	2	a_2				
Mean	$m_t = \frac{a_1 + a_2}{2}$					

Note: Similar format may be used for reference and toxicity control.

6. **CORRECTION FOR NITRIFICATION** (see Annex V)

Time of incubation (d)		0	n_1	n_2	n_3
(i)	Concentration of nitrate (mg N/litre)				
(ii)	Change in nitrate concentration (mg N/litre)	—			
(iii)	Oxygen equivalent (mg/litre)	—			
(iv)	Concentration of nitrite (mg N/litre)				
(v)	Change in nitrite concentration (mg N/litre)	—			
(vi)	Oxygen equivalent (mg/litre)	—			
(iii + vi)	Total oxygen equivalent (mg/litre)	—			

7. **DO DEPLETION: % DEGRADATION**

	Depletion after n days (mg/litre)			
	n_1	n_2	n_3	
FLASK 1: $(m_{to} - m_{tx}) - (m_{bo} - m_{bx})$				
FLASK 2: $(m_{to} - m_{tx}) - (m_{bo} - m_{bx})$				
FLASK 1: $\%D_1 = \frac{[(m_{to} - m_{tx}) - (m_{bo} - m_{bx})] \times 100}{\text{conc. of test} \times \text{ThOD chemical}}$				
FLASK 2: $\%\ D_2 = \frac{[(m_{to} - m_{tx}) - (m_{bo} - m_{bx})] \times 100}{\text{conc. of test} \times \text{ThOD chemical}}$				
$\%\ D\ \text{mean}\ (*) = \frac{D_1 - D_2}{2}$				

(*) Do not take mean if there is considerable difference between duplicates.

m_{to} = value in the flask at time 0
m_{tx} = value in the flask at time x
m_{bo} = mean blank value at time 0
m_{bx} = mean blank value at time x

Apply also correction for nitrification from iii + vi in section 6.

8. **BLANK DO DEPLETIONS**

Oxygen consumption by blank: ($m_{bo} - m_{b28}$) mg/litre. This consumption is important for the validity of the test. It should be less than 1,5 mg/litre.

PART VII. **M.I.T.I. TEST** (Method C.4-F)

VII.1. PRINCIPLE OF THE METHOD

The oxygen uptake by a stirred solution, or suspension, of the test chemical in a mineral medium, inoculated with specially grown, unadapted micro-organisms, is measured automatically over a period of 28 days in a darkened, enclosed respirometer at 25 ± 1 °C. Evolved carbon dioxide is absorbed by soda lime. Biodegradability is expressed as the percentage oxygen uptake (corrected for blank uptake) of the theoretical uptake (ThOD). The percentage of primary biodegradability is also calculated from supplemental specific chemical analysis made at the beginning and end of incubation and, optionally, by DOC analysis.

VII.2. DESCRIPTION OF THE METHOD

VII.2.1. **Apparatus**

(a) Automatic electrolytic BOD meter or respirometer normally equipped with six bottles, 300 ml each and equipped with cups to contain CO_2 absorbent;

(b) constant temperature room and/or water-bath at 25 °C ± 1 °C or better;

(c) membrane-filtration assembly (optional);

(d) carbon analyser (optional).

VII.2.2. **Preparation of mineral medium**

Prepare the following stock solutions, using analytical grade reagents and water (I.6.1.):

(a)	Monopotassium dihydrogen ortho phosphate, KH_2PO_4	8,50 g
	Dipotassium monohydrogen ortho phosphate, K_2HPO_4	21,75 g
	Disodium monohydrogen ortho phosphate dodecahydrate Na_2HPO_4 12 H_2O	44,60 g
	Ammonium chloride, NH_4Cl	1,70 g
	Dissolve in water and make up to 1 litre	
	The pH value of the solution should be 7,2	
(b)	Magnesium sulphate heptahydrate, $MgSO_4$ 7 H_2O	22,50 g
	Dissolve in water and make up to 1 litre	
(c)	Calcium chloride anhydrous, $CaCl_2$	27,50 g
	Dissolve in water and make up to 1 litre	
(d)	Iron (III) chloride hexahydrate, $FeCl_3$ 6 H_2O	0,25 g
	Dissolve in water and make up to 1 litre	

Take 3 ml of each solution (a), (b), (c) and (d) and make up to 1 litre.

VII.2.3. **Preparation of inoculum**

Collect fresh samples from no fewer than ten sites, mainly in areas where a variety of chemicals are used and discharged. From sites such as sewage treatment works, industrial waste-water treatment, rivers, lakes, seas, collect 11 samples of sludge, surface soil, water, etc. and mix thoroughly together. After removing floating matter and allowing to stand, adjust the supernatant to pH 7 ± 1 with sodium hydroxide or phosphoric acid.

Use an appropriate volume of the filtered supernatant to fill a fill-and-draw activated sludge vessel and aerate the liquid for about 23 1/2 h. 30 minutes after stopping aeration, discard about one third of the whole volume of supernatant and add an equal volume of a solution (pH 7) containing 0,1 % each of glucose, peptone and monopotassium ortho phosphate, to the settled material and recommence aeration. Repeat this procedure once per day. The sludge unit must be operated according to good practice: effluents should be clear, temperature should be kept at 25 ± 2° C, pH should be 7 ± 1, sludge should settle well, sufficient aeration to keep the mixture aerobic at all times, protozoa should be present and the activity of the sludge should be tested against a reference substance at least every three months. Do not use sludge as inoculum until after at least one month's operation, but not after more than four months. Thereafter, sample from at least 10 sites arregular intervals, once every three months.

In order to maintain fresh and old sludge at the same activity, mix the filtered supernatant of an activated sludge in use with an equal volume of the filtered supernatant of a freshly collected ten-source mixture and culture the combined liquor as above. Take sludge for use as inoculum 18-24 h after the unit has been fed.

VII.2.4. **Preparation of flasks**

Prepare the following six flasks:

No 1: test chemical in dilution water at 100 mg/1

No 2, 3 and 4: test chemical in mineral medium at 100 mg/1

No 5: reference chemical (e.g. aniline) in mineral medium at 100 mg/1

No 6: mineral medium only

Add poorly soluble test chemicals directly on a weight or volume basis or handle as described in Appendix 3, except that neither solvents nor emulsifying agents should be used. Add the CO_2 absorbent to all flasks in the special cups provided. Adjust the pH in flasks No 2, 3 and 4 to 7,0.

VII.2.5. **Performance of the test**

Inoculate flasks No 2, 3 and 4 (test suspensions), No 5 (activity control) and No 6 (inoculum blank) with a small volume of the inoculum to give a concentration of 30 mg/1 suspended solids. No inoculum is added to flask No 1 which serves as an abiotic control. Assemble the equipment, check for air-tightness, start the stirrers, and start the measurement of oxygen uptake under conditions of darkness. Daily check the temperature, stirrer and coulometric oxygen uptake recorder, and note any changes in colour of the contents of the flasks. Read the oxygen uptakes for the six flasks directly by an appropriate method, for example, from the six-point chart recorder, which produces a BOD curve. At the end of incubation, normally 28 days, measure the pH of the contents of the flasks and determine the concentration of the residual test chemical and any intermediate and, in the case of water soluble substance, the concentration o f DOC (Appendix 2.4). Take special care in the case of volatile chemicals. If nitrification is anticipated, determine nitrate and nitrite concentration, if possible.

VII.3. DATA AND REPORTING

VII.3.1. **Treatment of results**

Divide the oxygen uptake (mg) by the test chemical after a given time, corrected for that taken up by the blank inoculum conttol after the same time, by the weight of the test chemical used. This yields the BOD expressed as mg oxygen/mg test chemical, that is:

$$\text{BOD} = \frac{(\text{mg } O_2 \text{ uptake by test chemical} - \text{mg } O_2 \text{ uptake by blank})}{(\text{mg test chemical in flask})}$$

= mg O_2 per mg test chemical

The percentage biodegradation is then obtained from:

$$\% \text{ biodegradation} = \% \text{ ThOD} = \frac{\text{BOD } (\text{mg } O_2/\text{mg chemical})}{\text{ThOD } (\text{mg } O_2/\text{mg chemical})} \times 100$$

For mixtures, calculate the ThOD from the elemental analysis, as for simple compound. Use the appropriate ThOD ($ThOD_{NH4}$ or $ThOD_{NO3}$) according to whether nitrification is absent or complete (Appendix 2.2). If however, nitrification occurs but is incomplete, make a correction for the oxygen consumed by nitrification calculated from the changes in concentrations of nitrite and nitrate (Appendix 5).

Calculate the percentage primary biodegradation from loss of specific (parent) chemical (see 1.7.2).

$$D_t = \frac{S_b - S_a}{S_b} \times 100\ \%$$

If there has been a loss of test chemical in the flask No 1 measuring physico-chemical removal, report this and use the concentration of test chemical (S_b) after 28 days in this flask to calculate the percentage biodegradation.

When determinations of DOC are made (optional), calculate the percentage ultimate biodegradation from:

$$D_t = \left(1 - \frac{C_t - C_{bt}}{C_o - C_{bo}}\right) \times 100\ \%$$

as described under point I.7.1. If there has been a loss of DOC in the flask No 1, measuring physico-chemical removal, use the DOC concentration in this flask to calculate the percentage biodegradation.

Record all results on the data sheets attached.

VII.3.2. **Validity of results**

The oxygen uptake of the inoculum blank is normally 20-30 mg O_2/1 and should not be greater than 60 mgl/l in 28 days. Values higher than 60 mgl/l require critical examination of the data and experimental techniques. If the pH value is outside the range 6-8,5 and the oxygen consumption by the test chemical is less than 60 %, the test should be repeated with a lower concentration of test chemical.

See also I.5.2.

If the percentage degradation of aniline calculated from the oxygen consumption does not exceed 40 % after seven days and 65 % after 14 days, the test is regarded as invalid.

VII.3.3. **Reporting**

See I.8.

VII.4. DATA SHEET

An example of a data sheet is given below.

MITI (I) TEST

1. **LABORATORY**

2. **DATE AT START OF TEST**

3. **TEST SUBSTANCE**

Name:

Stock solution concentration: mg/1 as chemical

Initial concentration in medium, C_o: … mg/1 as chemical

Volume of reaction mixture, V: … ml

ThOD: … mg O_2/1

4. **INOCULUM**

Sludge sampling sites:

(1) … (6) …
(2) … (7) …
(3) … (8) …
(4) … (9) …
(5) … (10) …

Concentration of suspended solids in activated sludge after acclimatisation with synthetic sewage = ... mg/1

Volume of activated sludge per litre of final medium = ... ml

Concentration of sludge in final medium = ... mg/1

5. **OXYGEN UPTAKE: BIODEGRADABILITY**

Type of respirometer used:

			Time (Days)				
			0	7	14	21	28
O_2 upt. (mg) test chemical	a_1						
	a_2						
	a_3						
O_2 upt. (mg) blank	b						
Corrected BOD (mg)	$(a_1 - b_1)$ $(a_1 - b_1)$ $(a_1 - b_1)$						
BOD per mg test chemical	$\frac{(a-b)}{C_oV}$	Flask 1					
		Flask 2					
		Flask 3					

			Time (Days)				
			0	7	14	21	28
% degradation $\frac{BOD}{ThOD} \times 100$		1					
		2					
		3					
		Mean (*)					

(*) Do not take a mean if there are considerable differences between replicates.

N.B.: Similar formats may be used for the reference compound.

6. **CARBON ANALYSIS** (optional)

Carbon analyser:

Flask	DOC				% DOC removed	Mean
	Measured		Corrected			
Water + test substance	a				—	—
Sludge + test substance	b_1		$b_1 - c$			
Sludge + test substance	b_2		$b_2 - c$			
Sludge + test substance	b_3		$b_3 - c$			
Control blank	c		—		—	—

% DOC removed : $\frac{a_1 - (b - c)}{a} \times 100$

7. **SPECIFIC CHEMICAL ANALYTICAL DATA**

	Residual amount of test chemical at end of test	% degradation
blank test with water	S_b	
inoculated medium	S_{a1}	
	S_{a2}	
	S_{a3}	

% degradation = $\frac{S_b - S_a}{S_b} \times 100$

Calculate % degradation for flasks a_1, and a_3 respectively

8. **REMARKS**

BOD curve against time, if available, should be attached.

Appendix 1

ABBREVIATIONS AND DEFINITIONS

DO: Dissolved oxygen (mg/l) is the concentration of oxygen dissolved in an aqueous sample.

BOD: Biochemical oxygen demand (g) is the amount of oxygen consumed by micro-organisms when metabolising a test compound; also expressed as g oxygen uptake per g test compound. (See method C.5).

COD: Chemical oxygen demand (g) is the amount of oxygen consumed during oxidation of a test compound with hot, acidic dichromate; it provides a measure of the amount of oxidisable matter present; also expressed as 9 oxygen consumed per g test compound. (See method C.6).

DOC: Dissolved organic carbon is the organic carbon present in solution or that which passes through a 0,45 micrometre filter or remains in the supernatant after centrifuging at 40 000 $m.s^{-2}$ (± 4 000 g) for 15 min.

ThOD: Theoretical oxygen demand (mg) is the total amount of oxygen required to oxidise a chemical completely; it is calculated from the molecular formula (see Appendix 2.2) and is also expressed as mg oxygen required per mg test compound.

$ThCO_2$: Theoretical carbon dioxide (mg) is the quantity of carbon dioxide calculated to be produced from the known or measured carbon content of the test compound when fully mineralised; also expressed as mg carbon dioxide evolved per mg test compound.

TOC: Total organic carbon of a sample is the sum of the organic carbon in solution and in suspension.

IC: Inorganic carbon

TC: Total carbon, is the sum of the organic and inorganic carbon present in a sample.

Primary biodegradation:

is the alteration in the chemical structure of a substance, brought about by biological action, resulting in the loss of specific property of that substance.

Ultimate biodegradation (aerobic):

is the level of degradation achieved when the test compound is totally utilised by micro-organisms resulting in the production of carbon dioxide, water, mineral salts and new microbial cellular constituents (biomass).

Readily biodegradable:

an arbitrary classification of chemicals which have passed certain specified screening tests for ultimate biodegradability; these tests are so stringent that it is assumed that such compounds will rapidly and completely biodegrade in aquatic environments under aerobic conditions.

Inherently biodegradable:

a classification of chemicals for which there is unequivocal evidence of biodegradation (primary or ultimate) in any recognized test of biodegradability.

Treatability:

is the amenability of compounds to removal during biological wastewater treatment without adversely affecting the normal operation of the treatment processes. Generally, readily biodegradable compounds are treatable but not all inherently biodegradable compounds are. Abiotic processes may also operate.

Lag time

is the time from inoculation, in a die-away test, until the degradation percentage has increased to at least 10 %. The lag time is often highly variable and poorly reproducible.

Degradation time

is the time from the end of the lag time till the time that 90 % of maximum level of degradation has been reached.

10-day window

is the 10 days immediately following the attainment of 10 % degradation.

Appendix 2

CALCULATION AND DETERMINATION OF SUITABLE SUMMARY PARAMETERS

Depending on the method chosen, certain summary parameters will be required. The following section describes the derivation of these values. The use of these parameters is described in the individual methods.

1. **Carbon content**

The carbon content is calculated from the known elemental composition or determined by elemental analysis of the test substance.

2. **Theoretical oxygen demand (ThOD)**

The theoretical oxygen demand (ThOD) may be calculated if the elemental composition is known or derermined by elemental analysis. It is for the compound:

$C_cH_hCl_{cl}N_nNa_{na}O_oP_pS_s$

without nitrification,

$$ThOD_{NH4} = \frac{16\ [2c + 1/2\ (h - cl - 3n) + 3s + 5/2p + 1/2na - o]}{MW}\ mg/mg$$

or with nitrification

$$ThOD_{NO3} = \frac{16\ [2c + 1/2\ (h - cl) + 5/2n + 3s + 5/2p + 1/2na - o]}{MW}\ mg/mg$$

3. **Chemical Oxygen Demand (COD)**

The chemical oxygen demand (COD) is determined according to method C.6.

4. **Dissolved organic carbon (DOC)**

Dissolved organic carbon (DOC) is by definition the organic carbon of any chemical or mixture in water passing through a 0,45 micrometre filter.

Samples from the test vessels are withdrawn and filtered immediately in the filtration apparatus using an appropriate membrane filter. The first 20 ml (amount can be reduced when using small filters) of the filtrate are discarded. Volumes of 10-20 ml or lower, if injected (volume depending on the amount required for carbon analyser) are retained for carbon analysis. The DOC concentration is determined by means of an organic carbon analyser which is capable of accurately measuring a carbon concentration equivalent or lower than 10 % of the initial DOC concentration used in the test.

Filtered samples which cannot be analysed on the same working day can be preserved by storage in a refrigerator at 2-4 °C for 48 h, or below - 18 °C for longer periods.

Remarks:

Membrane filters are often impregnated with surfactants for hydrophilisation. Thus the filter may contain up to several mg of soluble organic carbon which would interfere in the biodegradability determinations. Surfactants and other soluble organic compounds are removed from the filters by boiling them in deionised water for three periods each of one hour. The filters may then be stored in water for one week. If disposable filter cartridges are used each lot must be checked to confirm that it does not release soluble organic carbon.

Depending on the type of membrane filter the test chemical may be retained by adsorption. It may therefore be advisable to ensure that the test chemical is not retained by the filter.

Centrifugation at 40 000 $m.sec^{-2}$ (4 000 g) for 15 min may be used for differentiation of TOC versus DOC instead of filtration. The method is not reliable at initial concentration of < 10 mg DOC/l since either not all bacteria are removed or carbon as part of the bacterial plasma is redissolved.

BIBLIOGRAPHY

— Standard Methods for the Examination of Water and Wastewater, 12th ed, Am. Pub. Hlth. Ass., Am. Wat. Poll. Control Fed., Oxygen Demand, 1965, P 65.

— Wagner, R., Von Wasser, 1976, vol. 46, 139.

— DIN-Entwurf 38 409 Teil 41 Deutsche Einheitsverfahren zur Wasser-, Abwasser- und Schlammuntersuchung, Summarische Wirkungs- und Stoffkenngrößen (Gruppe H). Bestimmung des Chemischen Sauerstoffbedarfs (CSB) (H 41), Normenausschuß Wasserwesen (NAW) in DIN Deutsches Institut für Normung e. V.

— Gerike, P., The biodegradability testing of poorly water soluble compounds. Chemosphere, 1984, vol 13 (1), 169.

Appendix 3

EVALUATION OF THE BIODEGRADABILITY OF POORLY SOLUBLE SUBSTANCES

In biodegradability tests with poorly soluble substances the following aspects should receive special attention.

While homogeneous liquids will seldom present sampling problems, it is recommended that solid materials be homogenised by appropriate means to avoid errors due to non-homogeneity. Special care must be taken when representative samples of a few milligrams are required from mixtures of chemicals or substances with large amounts of impurities.

Various forms of agitation during the tests may be used. Care should be taken to use only sufficient agitation to keep the chemical dispersed, and to avoid overheating, excessive foaming and excessive shear forces.

An emulsifier which gives a stable dispersion of the chemical may be used. It should not be toxic to bacteria and must not be biodegraded or cause foaming under test conditions.

The same criteria apply to solvents as to the emulsifiers.

It is not recommended that solid carriers be used for solid test substances but they may be suitable for only substances.

When auxiliary substances such as emulsifiers, solvents and carriers are used, a blank run containing the auxiliary substance should be performed.

Any of the three respirometric tests CO_2, BOD, MITI can be used to study the biodegradability of poorly soluble compounds.

BIBLIOGRAPHY

— de Morsier, A. et al., Biodegradation tests for poorly soluble compounds. Chemosphere, 1987, vol. 16, 833.

— Gerike, P, The Biodegradability testing of poorly water soluble compounds. Chemosphere, 1984, vol. 13, 169.

Appendix 4

EVALUATION OF THE BIODEGRADABILITY OF CHEMICALS SUSPECTED TO BE TOXIC TO THE INOCULUM

When a chemical is subjected to ready biodegradability testing and appears to be non-biodegradable, the following procedure is recommended if a distinction between inhibition and inertness is desired (Reynolds et al., 1987).

Similar or identical inocula should be used for the toxicity and biodegradation tests.

To assess the toxicity of chemicals studied in ready biodegradability tests, the application of one or a combination of the inhibition of Sludge Respiration rate (activated sludge respiration inhibition test — Directive 87/302/EEC), BOD and/or Growth Inhibition methods would seem appropriate.

If inhibition due to toxicity is to be avoided, it is suggested that the test substance concentrations used in ready biodegradability testing should be less than 1/10 of the EC_{50} values (or less than EC_{20} values) obtained in toxicity testing. Compounds with an EC_{50} value of greater than 300 mg/1 are not likely to have toxic effects in ready biodegradability testing.

EC_{50} values of less than 20 mg/1 are likely to pose serious problems for the subsequent testing. Low test concentrations should be employed, necessitating the use of the stringent and sensitive Closed Bottle test or the use of ^{14}C-labelled material. Alternatively, an acclimatised inoculum may permit higher test substance concentrations to be used. In the latter case, however, the specific criterion of the ready biodegradability test is lost.

BIBLIOGRAPHY

Reynolds, L. et al., Evaluation of the toxicity of substances to be assessed for biodegradability. Chemosphere, 1987, vol. 16, 2259.

Appendix 5

CORRECTION FOR OXYGEN UPTAKE FOR INTERFERENCE BY NITRIFICATION

Errors due to not considering nitrification in the assessment by oxygen uptake of the biodegradability of test substances not containing N are marginal (not greater than 5 %), even if oxidation of the ammonium-N in the medium occurs erratically as between test and blank vessels. However, for test substances containing N, serious errors can arise.

If nitrification has occurred but is not complete the observed oxygen uptake by the reaction mixture may be corrected for the amount of oxygen used in oxidising ammonium to nitrite and nitrate, if the changes in concentration during incubation of nitrite and nitrate are determined by consideration of the following equations:

$$2\ NH_4Cl + 3\ O_2 = 2\ HNO_2 + 2\ HCl + 2\ H_2O \quad (1)$$

$$2\ HNO_2 + O_2 = 2\ HNO_3 \quad (2)$$

Overall:

$$2\ NH_4Cl + 4\ O_2 = 2\ HNO_3 + 2\ HCl + 2\ H_2O \quad (3)$$

From equation (1), the oxygen uptake by 28 g of nitrogen contained in ammonium chloride (NH_4Cl) in being oxidised to nitrite is 96 g, i.e. a factor of 3,43 (96/28). In the same way, from equation (3) the oxygen uptake by 28 g of nitrogen in being oxidised to nitrate is 128 g, i.e. a factor of 4,57 (128/28).

Since the reactions are sequential, being carried out by distinct and different bacterial species, it is possible for the concentration of nitrite to increase or decrease; in the latter case an equivalent concentration of nitrate would be formed. Thus, the oxygen consumed in the formation of nitrate is 4,57 multiplied by the increase in concentration of nitrate, whereas the oxygen associated with the formation of nitrite is 3,43 multiplied by the increase in the concentration of nitrite or with the decrease in its concentration the oxygen loss is −3,43 multiplied by the decrease in concentration.

That is:

O_2 consumed in nitrate formation = 4,57 × increase in nitrate concentration (4)

and

O_2 consumed in nitrite formation = 3,43 × increase in nitrite concentration (5)

and

O_2 lost in nitrite disappearance = - 3,43 × decrease in nitrate concentration (6)

So that

O_2 uptake due to nitrification = ± 3,43 × change in nitrite conc. + 4,57 × increase in nitrate conc. (7)

and thus

O_2 uptake due to C oxidation = total observed uptake uptake due to nitrification (8).

Alternatively, if only total oxidised N is determined, the oxygen uptake due to nitrification may be taken to be, as a first approximation, 4,57 × increase in oxidised N

The corrected value for oxygen consumption due to C oxidation is then compared with ThOD NH_3, as calculated in Appendix 2.

C.5 DEGRADATION — BIOCHEMICAL OXYGEN DEMAND

1. METHOD

1.1. INTRODUCTION

The purpose of the method is the measurement of the biochemical oxygen demand (BOD) of solid or liquid organic substances.

Data elaborated with this test pertain to water-soluble compounds; however, volatile compounds and those of low water solubility may also, at least in principle, be tested.

The method is applicable only to those organic test materials which are not inhibitory to bacteria at the concentration used in the test. If the test material is not soluble at the test concentration, special measures, such as the use of ultrasonic dispersion, may have to be employed to achieve good dispersion of test material.

Information on the toxicity of the chemical may be useful to the interpretation of low results and in the selection of appropriate test concentrations.

1.2. DEFINITION AND UNITS

The BOD is defined as the mass of dissolved oxygen required by a specified volume of solution of the substance for the process of biochemical oxidation under prescribed conditions.

The results are expressed as grams of BOD per gram of tested substance.

1.3. REFERENCE SUBSTANCES

The use of a suitable reference substance to check the activity of the inoculum is desirable.

1.4. PRINCIPLE OF THE TEST METHOD

A predetermined amount of the substance, dissolved or dispersed in a well-aerated suitable medium, is inoculated with micro-organisms and incubated at a constant defined ambient temperature in the dark.

The BOD is determined by the difference in dissolved oxygen content at the beginning and at the end of the test. The duration of the test must be at least five days and not more than 28 days.

A blank must be determined in a parallel assay containing no test substance.

1.5. QUALITY CRITERIA

The BOD determination cannot be considered as a valid determination of the biodegradability of a substance. This test may only be regarded as a screening test.

1.6. DESCRIPTION OF THE TEST METHOD

A preliminary solution or dispersion of the substance is prepared to obtain a BOD concentration compatible with the method used. The BOD is then determined following any suitable national or international standardised method.

2. DATA AND EVALUATION

The BOD contained in the preliminary solution is calculated according to the selected normalised method, and converted into grams of BOD per gram of tested substance.

3. **REPORTING**

The method used shall be stated.

The biochemical oxygen demand should be a mean of at least three valid measurements.

All information and remarks relevant for the interpretation of results have to be reported, especially with regard to impurities, physical state, toxic effects and inherent composition of the substance which would affect the results.

The use of an additive to inhibit biological nitrification must be reported.

4. REFERENCES

List of standardised methods, for example:

NF T 90-103: Determination of the biochemical oxygen demand.

NBN 407: Biochemical oxygen demand.

NEN 32355.4: Bepaling van het biochemish zuurstofverbruik (BZV).

The determination of biochemical oxygen demand, Methods for the examination of water and associated materials, HMSO, London.

ISO 5815: Determination of biochemical oxygen demand after n days.

C.6. DEGRADATION — CHEMICAL OXYGEN DEMAND

1. METHOD

1.1. INTRODUCTION

The purpose of the method is the measurement of the chemical oxygen demand (COD) of solid or liquid organic substances in a standard, arbitrary manner, under fixed laboratory conditions.

Information on the formula of the substance will be useful to conduct this test and interpret the result obtained (e.g. halogen salts, ferrous salts of organic compounds, organochlorine compounds).

1.2. DEFINITIONS AND UNITS

The chemical oxygen demand is a measure of the oxidisability of a substance, expressed as the equivalent amount in oxygen of an oxidising reagent consumed by the substance under fixed laboratory conditions.

The result is expressed in grams of COD per gram of tested substance.

1.3. REFERENCE SUBSTANCES

Reference substances do not need to be employed in all cases when investigating a new substance. They should serve primarily to calibrate the method from time to time and to allow comparison of results when another method is applied.

1.4. PRINCIPLE OF THE TEST METHOD

A predetermined amount of the substance, dissolved or dispersed in water, is oxidised by potassium dichromate in a strong sulphuric acid medium with silver sulphate as a catalyst, under reflux for two hours. The residual dichromate is determined by titration with standardised ferrous ammonium sulphate.

In case of chlorine-containing substances, mercuric sulphate ([1]) is added to reduce chloride interference.

1.5. QUALITY CRITERIA

Because of the arbitrary manner of determination, COD is an 'oxidisability indicator' and as such is used as a practical method to measure organic matter.

Chloride can interfere in this test; inorganic reducing or oxidising agents may also interfere with the COD determination.

Some cyclic compounds and many volatile substances (e.g. lower fatty acids) are not fully oxidised by this test.

1.6. DESCRIPTION OF THE TEST METHOD

A preliminary solution or dispersion of the substance is prepared to obtain a COD between 250 and 600 mg per litre.

Remarks:

In the case of poorly soluble and non-dispersible substances, an amount of finely powdered substance or liquid substance corresponding to about 5 mg of COD can be weighed and put in the experimental apparatus with water.

([1]) After use, solutions containing mercury salts should be treated to avoid dissemination of mercury in the environment.

The chemical oxygen demand (COD) is often and especially in case of poorly soluble substances determined advantageously in a variant of the method, i.e., in a closed system with a pressure equaliser (H. Kelkenberg, 1975). In this modification compounds which are only with difficulty determined by the conventional method — e.g. acetic acid — may often be successfully quantified. The method also fails, however, in the case of pyridine. If the potassium dichromate concentration, as prescribed in ref.(1), is raised to 0,25 N (0,0416 M), the direct weighing-in of 5-10 mg of substance is facilitated which is essential for the COD determination of poorly water soluble substances (ref. (2)).

Otherwise, the COD is then determined following any suitable national or international standardised method.

2. DATA AND EVALUATION

The COD contained in the experimental flask is calculated following the selected normalised method, and converted to grams of COD per gram of tested substance.

3. REPORTING

The reference method used should be stated.

The chemical oxygen demand should be a mean of at least three measurements. All information and remarks relevant to the interpretation of the results have to be reported, especially with regard to impurities, physical state and inherent properties of the substance (if known) which would affect the results.

The use of mercuric sulphate to minimise the chloride interference must be reported.

4. REFERENCES

(1) Kelkenberg, H.,Z. von Wasser und Abwasserforschung, 1975, vol. 8, 146.

(2) Gerike, P. The biodegradability testing of poorly water soluble compounds. Chemosphere, 1984, vol. 13,169.

List of standardised methods, for example:

NBN T 91-201 Determination of the chemical oxygen demand.

ISBN O 11 7512494 Chemical oxygen demand (dichromate value) of polluted and waste waters.

NF T 90-101 Determination of the chemical oxygen demand.

DS 217 = water analysis Determination of the chemical oxygen demand.

DIN 38409-H-41 Determination of the chemical oxygen demand (COD) within the range above 15 mg per litre.

NEN 3235 5.3 Bepaling van het chemisch zuurstofverbruik.

ISO 6060 Water quality: chemical oxygen demand dichromate methods.

C.7. DEGRADATION — ABIOTIC DEGRADATION: HYDROLYSIS AS A FUNCTION OF PH

1. METHOD

This testing method is equivalent to the OECD TG 111 (2004).

1.1. INTRODUCTION

Chemicals can enter surface waters by such routes as direct application, spray drift, run-off, drainage, waste disposal, industrial, domestic or agricultural effluent and atmospheric deposition and may be transformed in those waters by chemical (e.g. hydrolysis, oxidation), photochemical and/or microbial processes. This Guideline describes a laboratory test method to assess abiotic hydrolytic transformations of chemicals in aquatic systems at pH values normally found in the environment (pH 4-9) and is based on existing Guidelines (1)(2)(3)(4)(5)(6)(7).

The experiments are performed to determine (i) the rate of hydrolysis of the test substance as a function of pH and (ii) the identity or nature and rates of formation and decline of hydrolysis products to which organisms may be exposed. Such studies may be required for chemicals which are directly applied to water or that are likely to reach the environment by the other routes described above.

1.2. DEFINITIONS AND UNITS

See Appendix 2

1.3. APPLICABILITY OF THE METHOD

The method is generally applicable to chemical substances (unlabelled or labelled) for which an analytical method with sufficient accuracy and sensitivity is available. It is applicable to slightly volatile and non-volatile compounds of sufficient solubility in water. The test should not be applied to chemicals that are highly volatile from water (e.g. fumigants, organic solvents) and thus cannot be kept in solution under the experimental conditions of this test. The test may be difficult to conduct with substances of minimal solubility in water (8).

1.4. PRINCIPLE OF THE TEST METHOD

Sterile aqueous buffer solutions of different pH values (pH 4, 7 and 9) are treated with the test substance and incubated in the dark under controlled laboratory conditions (at constant temperatures). After appropriate time intervals, buffer solutions are analysed for the test substance and for hydrolysis products. With labelled test substance (e.g. ^{14}C), a mass balance can be more easily established.

This testing method is designed as a tiered approach which is shown and explained in Appendix 1. Each tier is triggered by the results of the previous tier.

1.5. INFORMATION ON THE TEST SUBSTANCE

Non-labelled or labelled test substance can be used to measure the rate of hydrolysis. Labelled material is generally preferred for studying the pathway of hydrolysis and for establishing mass balance; however, in special cases, labelling may not be absolutely necessary. ^{14}C-labelling is recommended but the use of other isotopes, such as ^{13}C, ^{15}N, ^{3}H, may also be useful. As far as possible, the label should be positioned in the most stable part(s) of the molecule. For example, if the test substance contains one ring, labelling on this ring is required; if the test substance contains two or more rings, separate studies may be needed to evaluate the fate of each labelled ring and to obtain suitable information on formation of hydrolysis products. The purity of the test substance should be at least 95 %.

Before carrying out a hydrolysis test, the following information on the test substance should be available:

(a) solubility in water [Testing Method A.6];

(b) solubility in organic solvents;

(c) vapour pressure [Testing Method A.4] and/or Henry's Law constant;

(d) n-octanol/water partition coefficient [Testing Method A.8];

(e) dissociation constant (pK_a) [OECD Guideline 112] (9);

(f) direct and indirect phototransformation rate in water where appropriate.

Analytical methods for quantification of the test substance and, if it is relevant, for identification and quantification of hydrolysis products in aqueous solutions should be available (see also Section 1.7.2).

1.6. REFERENCE SUBSTANCES

Where possible, reference substances should be used for the identification and quantification of hydrolysis products by spectroscopic and chromatographic methods or other suitably sensitive methods.

1.7. QUALITY CRITERIA

1.7.1. **Recovery**

Analysis of, at least, duplicate buffer solutions or of their extracts immediately after the addition of the test substance gives a first indication of the repeatability of the analytical method and of the uniformity of the application procedure for the test substance. Recoveries for later stages of the experiments are given by the respective mass balances (when labelled material is used). Recoveries should range from 90 % to 110 % for labelled and non labelled chemicals (7). In case it is technically difficult to reach this range, a recovery of 70 % for non labelled chemicals is acceptable, but justification should be given.

1.7.2. **Repeatability and sensitivity of analytical method**

Repeatability of the analytical method(s) used to quantify the test substance and hydrolysis products at later times can be checked by duplicate analysis of the same buffer solutions (or of their extracts) after sufficient quantities of hydrolysis products have formed for quantification

The analytical method should be sufficiently sensitive to quantify test substance concentrations down to 10 % or less of the initial concentration. If relevant, analytical methods should also be sufficiently sensitive to quantify any hydrolysis product representing 10 % or more of applied (at any time during the study) down to 25 % or less of its peak concentration.

1.7.3. **Confidence intervals for hydrolysis kinetic data**

Confidence intervals should be computed and presented for all regression coefficients, rate constants, half-lives, and any other kinetic parameters (e.g. DT50).

1.8. DESCRIPTION OF THE TEST METHOD

1.8.1. **Equipment and apparatus**

The study should be performed in glass containers (e.g. test tubes, small flasks) under dark and sterile conditions, if necessary, unless preliminary information (such as the n-octanol-water partition coefficient) indicates that the test substance may adhere to glass. In such cases, alternative materials (such as Teflon) may have to be considered. It may also be possible to alleviate the problem of adhere to glass by using one or more of the following methods:

— determine the mass of test substance and hydrolysis products sorbed to the test vessel,

— use of an ultrasonic bath,

— ensure a solvent wash of all glassware at each sampling interval,

— use of formulated products,

— use an increased amount of co-solvent for addition of test substance to the system; if a co-solvent is used it should be a co-solvent that does not hydrolyse the test substance.

Temperature-controlled water bath shakers or thermostatically controlled incubators for incubation of the various test solutions are normally required.

Standard laboratory equipment is required, including, in particular, the following:

— pH meter,

— analytical instruments such as GC, HPLC, TLC equipment, including the appropriate detection systems for analysing radiolabelled and non-labelled substances or inverse isotopes dilution method,

— instruments for identification purposes (e.g. MS, GC-MS, HPLC-MS, NMR, etc.),

— liquid scintillation counter,

— separating funnels for liquid-liquid extraction,

— instrumentation for concentrating solutions and extracts (e.g. rotating evaporator),

— temperature control devise (e.g. water bath).

Chemical reagents include, for example:

— organic solvents, analytical grade, such as hexane, dichloromethane, etc.,

— scintillation liquid,

— buffer solutions (for details see Section 1.8.3).

All glassware, reagent-grade water and buffer solutions used in the hydrolysis tests should be sterilised.

1.8.2. **Test substance application**

The test substance should be applied as aqueous solution into the different buffer solutions (see Appendix 3). If it is necessary for adequate dissolution, the use of low amounts of water miscible solvents (such as acetonitrile, acetone, ethanol) is permitted for application and distribution of the test substance but this should not normally exceed 1 % v/v. In case a higher concentration of solvents is considered (e.g. in the case of poorly soluble test substances), this could only be allowed when it can be shown that the solvent has no effect on the hydrolysis of the test substances.

The use of formulated product is not routinely recommended, as it cannot be excluded that the formulation ingredients may influence the hydrolysis process. However, for poorly water-soluble test substances or for substances that adhere to glass (see Section 1.8.1), the use of formulated material may be an appropriate alternative.

One concentration of the test substance should be used; it should not exceed 0,01 M or half of the saturation concentration (see Appendix 1).

1.8.3. **Buffer solutions**

The hydrolysis test should be performed at pH values of 4, 7 and 9. For this purpose, buffer solutions should be prepared using reagent grade chemicals and water. Some useful buffer systems are presented in Appendix 3. It should be noted that the buffer system used may influence the rate of hydrolysis and where this is observed an alternate buffer system should be employed ([1]).

([1]) Mabey and Mill recommend the use of borate or acetate buffers instead of phosphate (11).

The pH of each buffer solution should be checked with a calibrated pH meter to a precision of at least 0,1 at the required temperature.

1.8.4. **Test conditions**

1.8.4.1. *Test temperature*

The hydrolysis experiments should be carried out at constant temperatures. For extrapolation purposes, it is important to maintain the temperature to at least ± 0,5 °C.

A preliminary test (Tier 1) should be conducted at a temperature of 50 °C if the hydrolytic behaviour of the test substance is unknown. Higher Tier kinetic tests should be carried out with a minimum of three temperatures (including the test at 50 °C) unless the test substance is stable to hydrolysis as determined by the Tier 1 testing. A suggested temperature range is 10-70 °C (preferably with at least one temperature below 25 °C utilised), which will encompass the reporting temperature of 25 °C and most of the temperatures encountered in the field.

1.8.4.2. *Light and oxygen*

All of the hydrolysis tests should be carried out using any suitable method to avoid photolytic effects. All suitable measures should be taken to avoid oxygen (e.g. by bubbling helium, nitrogen or argon for five minutes before preparation of the solution).

1.8.4.3. *Test duration*

The preliminary test should be carried out for 5 days whereas the higher Tier tests should be conducted until 90 % hydrolysis of the test substance or for 30 days whichever comes first.

1.8.5. **Performance of the test**

1.8.5.1. *Preliminary test (Tier 1)*

The preliminary test is performed at 50 ± 0,5 °C and pH 4,0, 7,0 and 9,0. If less than 10 % of hydrolysis is observed after 5 days ($t_{0,5\ 25\ °C}$ > 1 year), the test substance is considered hydrolytically stable and, normally, no additional testing is required. If the substance is known to be unstable at environmentally relevant temperatures ([1]), the preliminary test is not required. The analytical method must be sufficiently precise and sensitive to detect a reduction of 10 % in the initial concentration.

1.8.5.2. *Hydrolysis of unstable substances (Tier 2)*

The higher Tier (advanced) test should be performed at the pH values at which the test substance was found unstable as defined by the preliminary test above. The buffered solutions of the test substance should be thermostated at the selected temperatures. To test for first-order behaviour, each reaction solution should be analysed in time intervals which provide a minimum of six spaced data points normally between 10 % and 90 % hydrolysis of the test substance. Individual replicate test samples (a minimum of duplicate samples contained in separate reaction vessels) should be removed and the contents analysed at each of at least six sampling times (for a minimum of twelve replicate data points). The use of a single bulk sample from which individual aliquots of the test solution are removed at each sampling interval is considered to be inadequate, as it does not allow for the analysis of data variability and it may lead to problems with contamination of the test solution. Sterility confirmation tests should be conducted at the end of the higher Tier test (i.e. at 90 % hydrolysis or 30 days). However, if no degradation (i.e. transformation) is observed, sterility tests are not considered necessary.

1.8.5.3. *Identification of hydrolysis products (Tier 3)*

Any major hydrolysis products at least those representing > 10 % of the applied dose should be identified by appropriate analytical methods.

1.8.5.4. *Optional tests*

Additional tests at pH values other than 4, 7 and 9 may be required for a hydrolytically unstable test substance. For example, for physiological purposes a test under more acidic conditions (e.g. pH 1,2) may be required employing a single physiologically relevant temperature (37 °C).

([1]) Such information may come from other sources such as hydrolysis data of structurally similar compounds from the literature or from other preliminary, semi-quantitative hydrolysis tests with the test substance at an earlier development stage.

2. **DATA**

The amounts of test substance and of hydrolysis products, if relevant, should be given as % of applied initial concentration and, where appropriate, as mg/L for each sampling interval and for each pH and test temperature. In addition, a mass balance should be given in percentage of the applied initial concentration when labelled test substance has been used.

A graphical presentation of the log-transformed data of the test substance concentrations against time should be reported. Any major hydrolysis products at least those representing ≥ 10 % of the applied dose should be identified and their log-transformed concentrations should also be plotted in the same manner as the parent substance to show their rates of formation and decline.

2.1. TREATMENT OF RESULTS

More accurate determinations of half-lives or DT_{50} values should be obtained by applying appropriate kinetic model calculations. The half-life and/or DT_{50} values (including confidence limits) should be reported for each pH and temperature together with a description of the model used the order of kinetics and the coefficient of determination (r^2). If appropriate, the calculations should also be applied to the hydrolysis products.

In the case of rate studies carried out at different temperatures, the pseudo first-order hydrolysis rate constants (k_{obs}) should be described as a function of temperature. The calculation should be based on both the separation of k_{obs} into rate constants for acid catalysed, neutral, and base catalysed hydrolysis (k_H, $k_{neutral}$, and k_{OH} respectively) and the Arrhenius equation:

$$k_{obs} = k_H[H^+] + k_{neutral} + k_{OH}[OH^-] = \sum_{i = H,neutral,OH} A_i e^{-B_i/T}$$

where A_i and B_i are regression constants from the intercept and slope, respectively, of the best fit lines generated from linearly regressing ln k_i against the reciprocal of the absolute temperature in Kelvin (T). Through the use of the Arrhenius relationships for acid, neutral and base catalysed hydrolysis, pseudo first-order rate constants, and thus half-lives can be calculated for other temperatures for which the direct experimental determination of a rate constant is not practicable (10).

2.2. EVALUATION AND INTERPRETATION OF RESULTS

Most hydrolysis reactions follow apparent first order reaction rates and, therefore, half-lives are independent of the concentration (see equation 4 in Appendix 2). This usually permits the application of laboratory results determined at 10^{-2} to 10^{-3} M to environmental conditions (≤ 10^{-6} M) (10). Several examples of good agreement between rates of hydrolysis measured in both pure and natural waters for a variety of chemicals were reported by Mabey and Mill (11), provided both pH and temperature had been measured.

3. **REPORTING**

3.1. TEST REPORT

The test report must include at least the following information:

Test substance:

— common name, chemical name, CAS number, structural formula (indicating position of label when radiolabelled material is used) and relevant physical-chemical properties (see Section 1.5);

— purity (impurities) of test substance,

— label purity of labelled chemical and molar activity (where appropriate).

— Buffer solutions:

— dates and details of preparation,

— buffers and waters used,

— molarity and pH of buffer solutions.

Test conditions:

— dates of the performance of the studies,

— amount of test substance applied,

— method and solvents (type and amount) used for application of the test substance,

— volume of buffered test substance solutions incubated,

— description of the incubation system used,

— pH and temperature during the study,

— sampling times,

— method(s) of extraction,

— methods for quantification and identification of the test substance and its hydrolysis products in the buffer solutions,

— number of replicates.

Results:

— repeatability and sensitivity of the analytical methods used,

— recoveries (% values for a valid study are given in Section 1.7.1),

— replicate data and means in a tabular forms,

— mass balance during and at the end of the studies (when labelled test substance is used),

— results of preliminary test,

— discussion and interpretation of results,

— all original data and figures.

The following information is only required when hydrolysis rate is determined:

— plots of concentrations versus time for the test substances and, where appropriate, for the hydrolysis products at each pH value and temperature;

— tables of results of Arrhenius equation for the temperature 20 °C/25 °C, with pH, rate constant [h^{-1} or day^{-1}], half-life or DT_{50}, temperatures [°C] including confidence limits and the coefficients of correlation (r^2) or comparable information;

— proposed pathway of hydrolysis.

4. **REFERENCES**

(1) OECD, (1981) Hydrolysis as a Function of pH. OECD Guideline for Testing of Chemicals No 111, adopted 12 May 1981.

(2) US-Environmental Protection Agency, (1982) 40 CFR 796.3500, Hydrolysis as a Function of pH at 25 °C. Pesticide Assessment Guidelines, Subdivision N. Chemistry: Environmental Fate.

(3) Agriculture Canada, (1987) Environmental Chemistry and Fate Guidelines for registration of pesticides in Canada.

(4) European Union (EU), (1995) Commission Directive 95/36/EC amending Council Directive 91/414/EEC concerning the placing of plant protection products on the market. Annex V: Fate and Behaviour in the Environment.

(5) Dutch Commission for Registration of Pesticides, (1991) Application for registration of a pesticide. Section G: Behaviour of the product and its metabolites in soil, water and air.

(6) BBA, (1980) Merkblatt No 55, Teil I und II: Prüfung des Verhaltens von Pflanzenbehandlungsmitteln im Wasser (October 1980).

(7) SETAC, (1995) Procedures for Assessing the Environmental Fate and Ecotoxicity of Pesticides. Mark R. Lynch, Ed.

(8) OECD, (2000) Guidance document on aquatic toxicity testing of difficult substances and mixtures, OECD Environmental Health and Safety Publications Series on Testing and Assessment No 23.

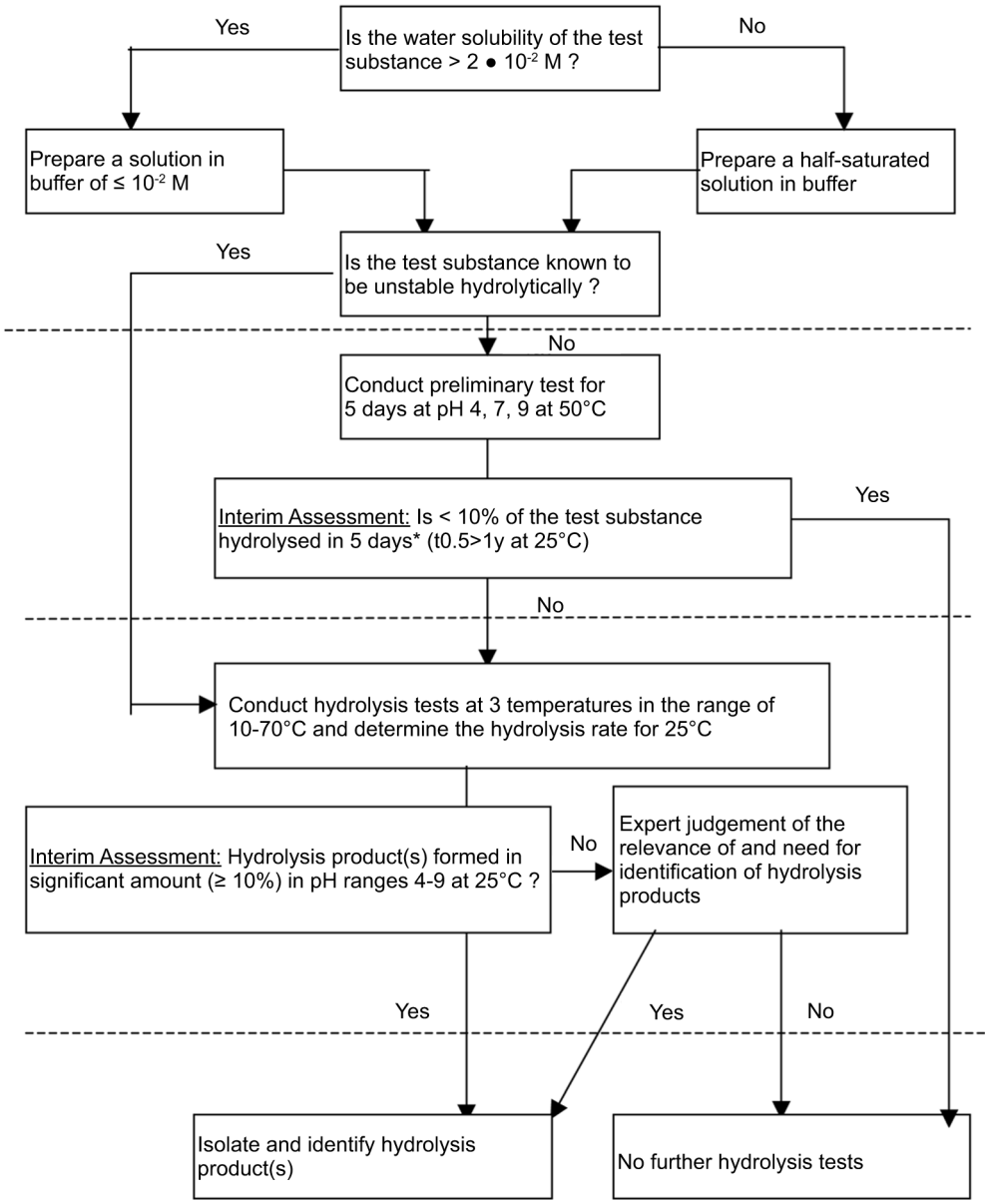
(9) OECD, (1993) Guidelines for the Testing of Chemicals. Paris. OECD (1994-2000): Addenda 6-11 to Guidelines for the Testing of Chemicals.

(10) Nelson, H, Laskowski D, Thermes S, and Hendley P., (1997) Recommended changes in pesticide fate study guidelines for improving input to computer models. (Text version of oral presentation at the 14th Annual Meeting of the Society of Environmental Toxicology and Chemistry, Dallas TX, November 1993).

(11) Mabey, W. and Mill, T., (1978) Critical review of hydrolysis of organic compounds in water under environmental conditions. J. Phys. Chem. Ref. Data 7, p. 383-415.

Appendix 1

Tiered hydrolysis test scheme



* 10% hydrolysis of a test substance at 50°C corresponds to a half-life of approx. 30 days which corresponds to a value of approx. 1 year at 20°C.

Appendix 2

Definitions and units

Standard International (SI) units should be used in any case.

Test substance: any substance, whether the parent compound or relevant transformation products.

Transformation products: all substances resulting from biotic or abiotic transformation reactions of the test substance.

Hydrolysis products: all substances resulting from hydrolytic transformation reactions of the test substance.

Hydrolysis refers to a reaction of a test substance RX with water, with the net exchange of the group X with OH at the reaction centre:

$$RX + HOH \rightarrow ROH + HX \qquad [1]$$

The rate at which the concentration of RX decreases in this simplified process is given by

$$\text{rate} = k\,[H_2O]\,[RX] \qquad \text{second order reaction}$$

or

$$\text{rate} = k\,[RX] \qquad \text{first order reaction}$$

depending on the rate determining step. Because the water is present in great excess compared to the test substance, this type of reaction is usually described as a pseudo-first order reaction in which the observed rate constant is given by the relationship

$$k_{obs} = k\,[H_2O] \qquad [2]$$

and can be determined from the expression (*)

$$k_{obs} = \frac{1}{t} \ln \frac{C_o}{C_t} \qquad [3]$$

where:

t = time

and C_o, C_t = concentrations of RX at times 0 and t.

The units of this constant have the dimensions of $(\text{time})^{-1}$ and the half-life of the reaction (time for 50 % of RX to react) is given by

$$t_{0,5} = \frac{\ln 2}{k_{obs}} \qquad [4]$$

Half-life: ($t_{0,5}$) is the time taken for 50 % hydrolysis of a test substance when the reaction can be described by first order kinetics; it is independent of the concentration.

DT_{50} (Disappearance Time 50): is the time within which the concentration of the test substance is reduced by 50 %; it is different from the half-life $t_{0,5}$ when the reaction does not follow first order kinetics.

(*) If the plot of the log-transformed data *vs.* time does not indicate a linear function (equated with a first-order reaction rate), then the use of equation [3] is not appropriate for determining the hydrolysis rate constant of the test compound.

Estimation of k at different temperature

When the rate constants are known for two temperatures, the rate constants at other temperatures can be calculated using the Arrhenius equation:

$$k = A \times e^{-\frac{E}{R \times T}} \text{ or } \ln k = \frac{-E}{R \times T} + \ln A$$

A plot of ln k versus 1/T gives a straight line with a slope of − E/R

where:

k = rate constant, measured at different temperatures
E = activation energy [kJ/mol]
T = absolute temperature [K]
R = gas constant [8,314 J/mol.K]

The activation energy was calculated by regression analysis or the following equation:

$$E = R \times \frac{\ln k_2 - \ln k_1}{\left(\frac{1}{T_1} - \frac{1}{T_2}\right)}$$

where: $T_2 > T_1$.

Appendix 3

Buffer Systems

A. **CLARK AND LUBS:**

Buffer mixtures of CLARK and LUBS (*)

Composition	pH
0,2 N HCl and 0,2 N KCl at 20 °C	
47,5 ml. HCl + 25 ml. KCl dil. to 100 ml	1,0
32,25 ml. HCl + 25 ml. KCl dil. to 100 ml	1,2
20,75 ml. HCl + 25 ml. KCl dil. to 100 ml	1,4
13,15 ml. HCl + 25 ml. KCl dil. to 100 ml	1,6
8,3 ml. HCl + 25 ml. KCl dil. to 100 ml	1,8
5,3 ml. HCl + 25 ml. KCl dil. to 100 ml	2,0
3,35 ml. HCl + 25 ml. KCl dil. to 100 ml	2,2
0,1 M potassium biphthalate + 0,1 N HCl at 20 °C	
46,70 ml. 0,1 N HCl + 50 ml. biphthalate to 100 ml	2,2
39,60 ml. 0,1 N HCl + 50 ml. biphthalate to 100 ml	2,4
32,95 ml. 0,1 N HCl + 50 ml. biphthalate to 100 ml	2,6
26,42 ml. 0,1 N HCl + 50 ml. biphthalate to 100 ml	2,8
20,32 ml. 0,1 N HCl + 50 ml. biphthalate to 100 ml	3,0
14,70 ml. 0,1 N HCl + 50 ml. biphthalate to 100 ml	3,2
9,90 ml. 0,1 N HCl + 50 ml. biphthalate to 100 ml	3,4
5,97 ml. 0,1 N HCl + 50 ml. biphthalate to 100 ml	3,6
2,63 ml. 0,1 N HCl + 50 ml. biphthalate to 100 ml	3,8
0,1 M potassium biphthalate + 0,1 N NaOH at 20 °C	
0,40 ml. 0,1 N NaOH + 50 ml. biphthalate to 100 ml	4,0
3,70 ml. 0,1 N NaOH + 50 ml. biphthalate to 100 ml	4,2
7,50 ml. 0,1 N NaOH + 50 ml. biphthalate to 100 ml	4,4
12,15 ml. 0,1 N NaOH + 50 ml. biphthalate to 100 ml	4,6
17,70 ml. 0,1 N NaOH + 50 ml. biphthalate to 100 ml	4,8

(*) The pH values reported in these tables have been calculated from the potential measurements using Sörensen's standard equations (1909). The corresponding pH values are 0,04 units higher than the tabulated values.

Composition	pH
23,85 ml. 0,1 N NaOH + 50 ml. biphthalate to 100 ml	5,0
29,95 ml. 0,1 N NaOH + 50 ml. biphthalate to 100 ml	5,2
35,45 ml. 0,1 N NaOH + 50 ml. biphthalate to 100 ml	5,4
39,85 ml. 0,1 N NaOH + 50 ml. biphthalate to 100 ml	5,6
43,00 ml. 0,1 N NaOH + 50 ml. biphthalate to 100 ml	5,8
45,45 ml. 0,1 N NaOH + 50 ml. biphthalate to 100 ml	6,0

Buffer mixtures of CLARK and LUBS (Continued)

0,1 M monopotassium phosphate + 0,1 N NaOH at 20 °C	
5,70 ml. 0,1 N NaOH + 50 ml. phosphate to 100 ml	6,0
8,60 ml. 0,1 N NaOH + 50 ml. phosphate to 100 ml	6,2
12,60 ml. 0,1 N NaOH + 50 ml. phosphate to 100 ml	6,4
17,80 ml. 0,1 N NaOH + 50 ml. phosphate to 100 ml	6,6
23,45 ml. 0,1 N NaOH + 50 ml. phosphate to 100 ml	6,8
29,63 ml. 0,1 N NaOH + 50 ml. phosphate to 100 ml	7,0
35,00 ml. 0,1 N NaOH + 50 ml. phosphate to 100 ml	7,2
39,50 ml. 0,1 N NaOH + 50 ml. phosphate to 100 ml	7,4
42,80 ml. 0,1 N NaOH + 50 ml. phosphate to 100 ml	7,6
45,20 ml. 0,1 N NaOH + 50 ml. phosphate to 100 ml	7,8
46,80 ml. 0,1 N NaOH + 50 ml. phosphate to 100 ml	8,0
0,1 M H_3B0_3 in 0,1 M KCl + 0,1 N NaOH at 20 °C	
2,61 ml. 0,1 N NaOH + 50 ml. boric acid to 100 ml	7,8
3,97 ml. 0,1 N NaOH + 50 ml. boric acid to 100 ml	8,0
5,90 ml. 0,1 N NaOH + 50 ml. boric acid to 100 ml	8,2
8,50 ml. 0,1 N NaOH + 50 ml. boric acid to 100 ml	8,4
12,00 ml. 0,1 N NaOH + 50 ml. boric acid to 100 ml	8,6
16,30 ml. 0,1 N NaOH + 50 ml. boric acid to 100 ml	8,8
21,30 ml. 0,1 N NaOH + 50 ml. boric acid to 100 ml	9,0
26,70 ml. 0,1 N NaOH + 50 ml. boric acid to 100 ml	9,2

32,00 ml. 0,1 N NaOH + 50 ml. boric acid to 100 ml	9,4
36,85 ml. 0,1 N NaOH + 50 ml. boric acid to 100 ml	9,6
40,80 ml. 0,1 N NaOH + 50 ml. boric acid to 100 ml	9,8
43,90 ml. 0,1 N NaOH + 50 ml. boric acid to 100 ml	10,0

B. **KOLTHOFF AND VLEESCHHOUWER:**

Citrate buffers of KOLTHOFF and VLEESCHHOUWER

Composition	pH
0,1 M monopotassium citrate and 0,1 N HCl at 18 ºC (*)	
49,7 ml. 0,1 N HCl + 50 ml. citrate to 100 ml	2,2
43,4 ml. 0,1 N HCl + 50 ml. citrate to 100 ml	2,4
36,8 ml. 0,1 N HCl + 50 ml. citrate to 100 ml	2,6
30,2 ml. 0,1 N HCl + 50 ml. citrate to 100 ml	2,8
23,6 ml. 0,1 N HCl + 50 ml. citrate to 100 ml	3,0
17,2 ml. 0,1 N HCl + 50 ml. citrate to 100 ml	3,2
10,7 ml. 0,1 N HCl + 50 ml. citrate to 100 ml	3,4
4,2 ml. 0,1 N HCl + 50 ml. citrate to 100 ml	3,6
0,1 M monopotassium citrate and 0,1 N NaOH at 18 ºC (*)	
2,0 ml. 0,1 N NaOH + 50 ml. citrate to 100 ml	3,8
9,0 ml. 0,1 N NaOH + 50 ml. citrate to 100 ml	4,0
16,3 ml. 0,1 N NaOH + 50 ml. citrate to 100 ml	4,2
23,7 ml. 0,1 N NaOH + 50 ml. citrate to 100 ml	4,4
31,5 ml. 0,1 N NaOH + 50 ml. citrate to 100 ml	4,6
39,2 ml. 0,1 N NaOH + 50 ml. citrate to 100 ml	4,8
46,7 ml. 0,1 N NaOH + 50 ml. citrate to 100 ml	5,0
54,2 ml. 0,1 N NaOH + 50 ml. citrate to 100 ml	5,2
61,0 ml. 0,1 N NaOH + 50 ml. citrate to 100 ml	5,4
68,0 ml. 0,1 N NaOH + 50 ml. citrate to 100 ml	5,6
74,4 ml. 0,1 N NaOH + 50 ml. citrate to 100 ml	5,8
81,2 ml. 0,1 N NaOH + 50 ml. citrate to 100 ml	6,0

(*) Add tiny crystal of thymol or a similar substance to prevent growth of molds.

C. **SÖRENSEN:**

Borate mixtures of SÖRENSEN

Composition		Sörensen 18 °C	Walbum, pH at		
ml. Borax	ml. HCl/ NaOH		10 °C	40 °C	70 °C
0,05 M borax + 0,1 N HCl					
5,25	4,75	7,62	7,64	7,55	7,47
5,50	4,50	7,94	7,98	7,86	7,76
5,75	4,25	8,14	8,17	8,06	7,95
6,00	4,00	8,29	8,32	8,19	8,08
6,50	3,50	8,51	8,54	8,40	8,28
7,00	3,00	8,08	8,72	8,56	8,40
7,50	2,50	8,80	8,84	8,67	8,50
8,00	2,00	8,91	8,96	8,77	8,59
8,50	1,50	9,01	9,06	8,86	8,67
9,00	1,00	9,09	9,14	8,94	8,74
9,50	0,50	9,17	9,22	9,01	8,80
10,00	0,00	9,24	9,30	9,08	8,86
0,05 M borax + 0,1 N NaOH					
10,0	0,0	9,24	9,30	9,08	8,86
9,0	1,0	9,36	9,42	9,18	8,94
8,0	2,0	9,50	9,57	9,30	9,02
7,0	3,0	9,68	9,76	9,44	9,12
6,0	4,0	9,97	10,06	9,67	9,28

Phosphate mixtures of SÖRENSEN

Composition	pH
0,0667 M Monopotassium phosphate + 0,0667 M Disodium phosphate at 20 °C	
99,2 ml. KH_2PO_4 + 0,8 ml Na_2HPO_4	5,0
98,4 ml. KH_2PO_4 + 1,6 ml Na_2HPO_4	5,2
97,3 ml. KH_2PO_4 + 2,7 ml Na_2HPO_4	5,4
95,5 ml. KH_2PO_4 + 4,5 ml Na_2HPO_4	5,6
92,8 ml. KH_2PO_4 + 7,2 ml Na_2HPO_4	5,8
88,9 ml. KH_2PO_4 + 11,1 ml Na_2HPO_4	6,0
83,0 ml. KH_2PO_4 + 17,0 ml Na_2HPO_4	6,2
75,4 ml. KH_2PO_4 + 24,6 ml Na_2HPO_4	6,4
65,3 ml. KH_2PO_4 + 34,7 ml Na_2HPO_4	6,6
53,4 ml. KH_2PO_4 + 46,6 ml Na_2HPO_4	6,8

41,3 ml. KH_2PO_4 + 58,7 ml Na_2HPO_4	7,0
29,6 ml. KH_2PO_4 + 70,4 ml Na_2HPO_4	7,2
19,7 ml. KH_2PO_4 + 80,3 ml Na_2HPO_4	7,4
12,8 ml. KH_2PO_4 + 87,2 ml Na_2HPO_4	7,6
7,4 ml. KH_2PO_4 + 92,6 ml Na_2HPO_4	7,8
3,7 ml. KH_2PO_4 + 96,3 ml Na_2HPO_4	8,0

C.8. TOXICITY FOR EARTHWORMS

ARTIFICIAL SOIL TEST

1. METHOD

1.1. INTRODUCTION

In this laboratory test, the test substance is added to an artificial soil in which worms are placed for 14 days. After this period (and optionally after seven days) the lethal effect of the substance on the earthworms is examined. The test provides a method for relatively short-term screening of the effect of chemicals on earthworms, by dermal and alimentary uptake.

1.2. DEFINITION AND UNIT

LC_{50}: the concentration of a substance estimated as killing 50 % of the test animals during the test period.

1.3. REFERENCE SUBSTANCE

A reference substance is used periodically as a means of demonstration that the sensitivity of the test system has not changed significantly.

Analytical grade chloroacetamide is recommended as the reference substance.

1.4. PRINCIPLE OF THE TEST

Soil is a variable medium, so for this test a carefully defined artificial loam soil is used. Adult earthworms of the species *Eisenia foetida* (see note in Appendix) are kept in a defined artificial soil treated with different concentrations of the test substance. The content of the containers is spread on a tray 14 days (and optionally seven days) after the beginning of the test, and the earthworms surviving at each concentration counted.

1.5. QUALITY CRITERIA

The test is designed to be as reproducible as possible with respect to the test substrate and organism. Mortality in the controls must not exceed 10 % at the end of the test, or the test is invalid.

1.6. DESCRIPTION OF THE TEST METHOD

1.6.1. *Materials*

1.6.1.1. Test substrate

A defined artificial soil is used as a basic test substrate.

(a) Basic substrate (percentages are in terms of dry weight)

— 10 % sphagnum peat (as close to pH 5,5 to 6,0 as possible with no visible plant remains and finely ground),

— 20 % kaolinite clay with preferably more than 50 % kaolinite,

— about 69 % industrial quartz sand (dominant fine sand with more than 50 % of particle size 0,05 to 0,2 mm). If the substance is not sufficiently dispersible in water, 10 g per test container should be kept available for mixing with the test substance later on,

— about 1 % calcium carbonate ($CaCO_3$), pulverised, chemically pure, added to bring the pH to 6,0 ± 0,5.

(b) Test substrate

The test substrate contains the basic substrate, the test substance and deionised water.

Water content is about 25 to 42 % of the dry weight of the basic substrate. The water content of the substrate is determined by drying a sample to constant weight at 105 °C. The key criterion is that the artificial soil must be wetted to a point where there is no standing water. Care should be taken in mixing to obtain an even distribution of the test substance and the substrate. The way of introducing the test substance to the substrate has to be reported.

(c) Control substrate

The control substrate contains the basic substrate and water. If an additive agent is used, an additional control should contain the same quantity of the additive agent.

1.6.1.2. Test containers

Glass containers of about one litre capacity (adequately covered with plastic lids, dishes or plastic film with ventilation holes) filled with an amount of wet test or control substrate equivalent to 500 g dry weight of substrate.

1.6.2. *Test conditions*

Containers should be kept in climatic chambers at a temperature of 20 ± 2 °C with continuous light. Light intensity should be 400 to 800 lux.

The test period is 14 days, but mortality can be assessed optionally seven days after starting the test.

1.6.3. *Test procedure*

Test concentrations

Concentrations of the test substance are expressed as weight of substance per dry weight of basic substrate (mg/kg).

Range finding test

The range of concentrations just causing mortalities of 0 to 100 % may be determined in a range-finding test to provide information on the range of concentrations to be used in the definitive test.

The substance should be tested at the following concentrations: 1 000; 100; 10; 1; 0,1 mg substance/kilogram test substrate (dry weight).

If a full definitive test is to be carried out, one test batch per concentration and one for the untreated control, each with 10 worms, could be sufficient for the range-finding test.

Definitive test

The results of the range-finding test are used to choose at least five concentrations in a geometric series just spanning the range 0 to 100 % mortality and differing by a constant factor not exceeding 1,8.

Tests using these series of concentration should allow the LC_{50} value and its confidence limits to be estimated as precisely as possible.

In the definitive test at least four test batches per concentration and four untreated controls, each with 10 worms, are used. The results of these replicate batches are given as a mean and standard deviation.

When two consecutive concentrations, at a ratio of 1,8, give only 0 % and 100 % mortality, these two values are sufficient to indicate the range within which the LC_{50} falls.

Mixture of the basic test substrate and the test substance

The test substrate should, whenever possible, be made up without any additional agents other than water. Immediately before the start of the test, an emulsion or dispersion of the test substance in deionised water or other solvent is mixed with the basic test substrate, or sprayed evenly over it with a fine chromatographic or similar spray.

If insoluble in water, the test substance can be dissolved in as small a volume as possible of suitable organic solvent (e.g. hexane, acetone or chloroform).

Only agents which volatilise readily may be used to solubilise, disperse or emulsify the test substance. The test substrate must be ventilated before use. The amount of water evaporated must be replaced. The control should contain the same quantity of any additive agent.

If the test substance is not soluble, dispersible or emulsifiable in organic solvents, 10 g of a mixture of fine ground quartz sand and a quantity of test substance necessary to treat 500 g dry weight of artificial soil are mixed with 490 g of dry weight of test substrate.

For each test batch, an amount of wet test substrate equivalent to 500 g dry weight is placed in each glass container and 10 earthworms, which have been conditioned for 24 hours in a similar wet basic substrate and then washed quickly and surplus water absorbed on filter paper before use, are placed on the test substrate surface.

The containers are covered with perforated plastic lids, dishes or film to prevent the substrate drying and they are kept under the test conditions for 14 days.

The assessments should be made 14 days (and optionally seven days) after setting up the test. The substrate is spread on a plate made of glass or stainless steel. The earthworms are examined and the numbers of surviving earthworms determined. Earthworms are considered dead if they do not respond to a gentle mechanical stimulus to the front end.

When the examination is made at seven days, the container is refilled with the substrate and the surviving earthworms are replaced on the same test substrate surface.

1.6.4. *Test organisms*

Test organisms should be adult *Eisenia foetida* (see note in Appendix) (at least two months old with clitellum) wet weight 300 to 600 mg. (For breeding method see Appendix.)

2. **DATA**

2.1. TREATMENT AND EVALUATION OF RESULTS

The concentrations of the substance tested are reported with reference to the corresponding percentages of dead earthworms.

When the data are adequate the LC_{50} value and the confidence limits ($p = 0,05$) should be determined using standard methods (Litchfield and Wilcoxon, 1949, for equivalent method). The LC_{50} should be given as mg of test substance per kilogram of the test substrate (dry weight).

In those cases where the slope of the concentration curve is too steep to permit calculation of the LC_{50}, a graphical estimate of this value is sufficient.

When two consecutive concentrations at a ratio of 1,8 give only 0 % and 100 % mortality, the two values are sufficient to indicate the range within which the LC_{50} falls.

3. **REPORTING**

3.1. TEST REPORT

The test report shall, if possible, contain the following:

— statement that the test has been carried out in accordance with the abovementioned quality criteria,

— test carried out (range finding test and/or definitive test),

— exact description of the test conditions or statement that the test has been carried out in accordance with the method; any deviations have to be reported,

— exact description of how the test substance has been mixed into the basic test substrate,

— information about test organisms (species, age, mean and range in weight, keeping and breeding conditions, supplier),

— method used for determination of LC_{50},

— test results including all data used,

— description of observed symptoms or changes in behaviour of test organisms,

— mortality in the controls,

— LC_{50} or highest tested concentration without mortality and lowest tested concentration with a mortality of 100 %, 14 days (and optionally seven days) after setting up the test,

— plotting of the concentration/response curve,

— results obtained with the reference substance, whether in association with the present test or from previous quality control exercises.

4. **REFERENCES**

1) OECD, Paris, 1981, Test Guideline 207, Decision of the Council C(81)30 final.

2) Edwards, C. A. and Lofty, J. R., 1977, Biology of Earthworms, Chapman and Hall, London, p. 331.

3) Bouche. M. B., 1972, Lombriciens de France, Ecologie et Systematique, Institut National de la Recherche Agronomique, p. 671.

4) Litchfield, J. T. andWilcoxon, F., A simplified method of evaluation dose effect experiments. I. Pharm. Exp. Therap., vol. 96, 1949, p. 99.

5) Commission of the European Communities, Development of a standardised laboratory method for assessing the toxicity of chemical substances to earthworms, Report EUR 8714 EN, 1983.

6) Umweltbundesamt/Biologische Bundesanstalt für land- und Forstwirtschaft, Berlin, 1984, Verfahrensvorschlag 'Toxizitätstest am Regenwurm *Eisenia foetida* in künstlichem Boden', in: Rudolph/Boje, Ökotoxikologie, ecomed, Landsberg, 1986.

Appendix

Breeding and keeping of the worms before testing

For breeding the animals, 30 to 50 adult worms, are put in a breeding box with fresh substrate and removed after 14 days. These animals maybe used for further breeding batches. The earthworms hatched from the cocoons are used for testing when mature (under the prescribed conditions after two to three months).

Keeping and breeding conditions

Climatic chamber:	temperature 20 ± 2 °C preferably with continuous light (intensity 400 to 800 lux).
Breeding boxes:	suitable shaI1ow containers of 10 to 20 l volume.
Substrate:	*Eisenia foetida* may be bred in various animal excrements. It is recommended to use as breeding medium a mixture of 50 % by volume peat and 50 % cow or horse dung. The medium should have a pH value of about 6 to 7 (regulated with calcium carbonate) and a low ionic conductivity (less than 6 mmhos or 0,5 % salt concentration).
	The substrate should be moist but not too wet.
	Other successful procedures may be used besides the method given above.

Note: *Eisenia foetida* exists in two races which some taxonomists have separated into species (Bouche, 1972). These are morphologically similar but one, *Eisenia foetida foetida*, has typically transverse striping or banding on the segments and the other, *Eisenia foetida andrei*, lacks this and has a variegated reddish colour. Where possible *Eisenia foetida andrei* should be used. Other species may be used if the necessary methodology is available.

C.9. **BIODEGRADATION**

ZAHN-WELLENS TEST

1. **METHOD**

1.1. INTRODUCTION

The purpose of the method is the evaluation of the potential ultimate biodegradability of water-soluble, non-volatile organic substances when exposed to relatively high concentrations of micro-organisms in a static test.

Physico-chemical adsorption on the suspended solids may take place and this must be taken into account when interpreting results (see 3.2).

The substances to be studied are used in concentrations corresponding to DOC-values in the range of 50 to 400 mg/litre or COD-values in the range of 100 to 1 000 mg/litre (DOC = dissolved organic carbon; COD = chemical oxygen demand). These relatively high concentrations have the advantage of analytical reliability. Compounds with toxic properties may delay or inhibit the degradation process.

In this method, the measure of the concentration of dissolved organic carbon or the chemical oxygen demand is used to assess the ultimate biodegradability of the test substance.

A simultaneous use of a specific analytical method may allow the assessment of the primary biodegradation of the substance (disappearance of the parent chemical structure).

The method is applicable only to those organic test substances which, at the concentration used in the test:

— are soluble in water under the test conditions,

— have negligible vapour pressure under the test conditions,

— are not inhibitory to bacteria,

— are adsorbed within the test system only to a limited extent,

— are not lost by foaming from the test solution.

Information on the relative proportions of the major components of the test material will be useful in interpreting the results obtained, particularly in those cases where the results are low or marginal.

Information on the toxicity of the substance to micro-organisms is desirable for the interpretation of low results and in the selection of appropriate test concentrations.

1.2. DEFINITIONS AND UNITS

The amount of degradation attained at the end of the test is reported as the 'Biodegradability in the Zahn-Wellens test':

$$D_T(\%) = \left[1 - \frac{(C_T - C_B)}{(C_A - C_{BA})}\right] \times 100$$

where:

DT = biodegradation (%) at time T,
CA = DOC (or COD) values in the test mixture measured three hours after the beginning of the test (mg/l) (DOC = dissolved organic carbon, COD = chemical oxygen demand),
CT = DOC or COD values in the test mixture at time of sampling (mg/l),
CB = DOC or COD value of the blank at time of sampling (mg/l),
CBA = DOC or COD value of the blank, measured three hours after the beginning of the test (mg/l).

The extent of degradation is rounded to the nearest full percent.

Percentage degradation is stated as the percentage DOC (or COD) removal of the tested substance.

The difference between the measured value after three hours and the calculated or preferably measured initial value may provide useful information on the elimination of the substance (see 3.2, Interpretation of results).

1.3. REFERENCE SUBSTANCES

In some cases when investigating new substances reference substances may be useful; however, specific reference substances cannot yet be recommended.

1.4. PRINCIPLE OF THE TEST METHOD

Activated sludge, mineral nutrients and the test material as the sole carbon source in an aqueous solution are placed together in a one to four litre glass vessel equipped with an agitator and an aerator. The mixture is agitated and aerated at 20 to 25 °C under diffuse illumination or in a dark room for up to 28 days. The degradation process is monitored by determination of the DOC (or COD) values in the filtered solution at daily or other appropriate regular time intervals. The ratio of eliminated DOC (or COD) after each interval to the value three hours after the start is expressed as percentage biodegradation and serves as the measure of the extent of degradation at this time. The result is plotted versus time to give the biodegradation curve.

When a specific analytical method is used, changes in the concentration of the parent molecule due to biodegradation can be measured (primary biodegradability).

1.5. QUALITY CRITERIA

The reproducibility of this test has been proven to be satisfactory in a ring test.

The sensitivity of the method is largely determined by the variability of the blank and, to a lesser extent, by the precision of the determination of dissolved organic carbon and the level of test compound in the liquor.

1.6. DESCRIPTION OF THE TEST PROCEDURE

1.6.1. *Preparations*

1.6.1.1. Reagents

Test water: drinking water with an organic carbon content < 5 mg/litre. The concentration of calcium and magnesium ions together must not exceed 2,7 mmole/litre; otherwise adequate dilution with deionised or distilled water is required.

Sulphuric acid, analytical reagent (A.R.):	50 g/l
Sodium hydroxide solution A.R.:	40 g/l
Mineral nutrient solution: dissolve in one litre deionised water:	
ammonium chloride, NH4Cl, A.R.:	38,5 g
sodium dihydrogenphosphate, NaH2PO4.2H2O, A.R.:	33,4 g
potassium dihydrogenphosphate, KH2PO4, A.R.:	8,5 g
di-potassium mono-hydrogenphosphate, K2HPO4, A.R.:	21,75 g

The mixture serves both as a nutrient and as buffering system.

1.6.1.2. Apparatus

Glass vessels with a volume of one to four litres (e.g. cylindrical vessels).

Agitator with a glass or metal stirrer on a suitable shaft (the stirrer should rotate about 5 to 10 cm above the bottom of the vessel). A magnetic stirrer with a 7 to 10 cm long rod can be used instead.

Glass tube of 2 to 4 mm inner diameter to introduce air. The opening of the tube should be about 1 cm above the bottom of the vessel.

Centrifuge (about 3 550 g).

pH-meter.

Dissolved-oxygen meter.

Paper filters.

Membrane filtration apparatus.

Membrane filters, pore size 0,45 μm. Membrane filters are suitable if it is assured that they neither release carbon nor absorb the substance in the filtration step.

Analytical equipment for determining organic carbon content and chemical oxygen demand.

1.6.1.3. Preparation of the inoculum

Activated sludge from a biological treatment plant is washed by (repeatedly) centrifuging or settling with test water (above).

The activated sludge must be in an appropriate condition. Such sludge is available from a properly working waste-water treatment plant. To get as many different species or strains of bacteria as possible, it may be preferred to mix inocula from different sources (e.g. different treatment plants, soil extracts, river waters, etc.). The mixture is to be treated as described above.

For checking the activity of the activated sludge see 'Functional control', below.

1.6.1.4. Preparation of the test solutions

To the test vessel add 500 ml of test water, 2,5 ml/litre mineral nutrient solution and activated sludge in an amount corresponding to 0,2 to 1,0 g/litre dry matter in the final mixture. Add sufficient stock solution of the substance to be tested so that a DOC concentration of 50 to 400 mg/litre results in the final mixture. The corresponding COD-values are 100 to 1 000 mg/litre. Make up with test water to a total volume of one to four litres. The total volume to be chosen is dependent on the number of samples to be taken for DOC or COD determinations and the volumes necessary for the analytical procedure.

Normally a volume of two litres can be regarded as satisfactory. At least one control vessel (blank) is set up to run in parallel with each test series; it contains only activated sludge and mineral nutrient solution made up with test water to the same total volume as in the test vessels.

1.6.2. *Performance of the test*

The test vessels are agitated with magnetic stirrers or screw propellers under diffuse illumination or in a dark room at 20 to 25 °C. Aeration is accomplished by compressed air cleaned by a cotton-wool strainer and a wash bottle if necessary. It must be ensured that the sludge does not settle and the oxygen concentration does not fall below 2 mg/litre.

The pH-value must be checked at regular intervals (e.g. daily) and adjusted to pH 7 to 8, if necessary.

Losses from evaporation are made up just before each sampling with deionised or distilled water in the required amounts. A good procedure is to mark the liquid level on the vessel before starting the test. New marks are made after each sampling (without aeration arid stirring). The first samples are always taken three hours after the start of the test in order to detect adsorption of test material by the activated sludge.

The elimination of the test material is followed by DOC or COD determinations made daily or at some other regular interval. The samples from the test vessel and the blank are filtered through a carefully washed paper filter. The first 5 ml of test solution filtrate are discarded. Sludges difficult to filter may be removed previously

by centrifugation for 10 minutes. DOC and COD determinations are made at least in duplicate. The test is run for up to 28 days.

Note: samples remaining turbid are filtered through membrane filters. The membrane filters must not release or adsorb any organic material.

Functional control of activated sludge

A vessel containing a known substance should be run in parallel with each test series in order to check the functional capacity of the activated sludge. Diethyleneglycol has been found useful for this purpose.

Adaptation

If analyses are carried out at relatively short intervals (e.g. daily), adaptation can be clearly recognised from the degradation curve (see Figure 2). The test should therefore not be started immediately before the weekend.

If the adaptation occurs in the end of the period, the test can be prolonged until the degradation is finished.

Note: if a broader knowledge of the behaviour of the adapted sludge is needed, the same activated sludge is exposed once again to the same test material in accordance with the following procedure:

Switch of the agitator and the aerator and allow the activated sludge to settle. Draw off the supernatant liquid, fill up to two litres with test water, stir for 15 minutes and allow to settle again. After the supernatant liquid is drawn off again, use the remaining sludge to repeat the test with the same material in accordance with 1.6.1.4 and 1.6.2, above. The activated sludge can also be isolated by centrifuging instead of settling.

The adapted sludge may be mixed with fresh sludge to a concentration of 0,2 to 1 g dry weight/litre.

Analytical means

Normally samples are filtered through a carefully washed paper filter (for washing use deionised water).

Samples which remain turbid are filtered through membrane filters (0,45 μm).

The DOC concentration is determined in duplicate in the sample filtrates (the first 5 ml are discarded) by means of the TOC instrument. If the filtrate cannot be analysed on the same day, it must be stored in the refrigerator until the next day. Longer storage cannot be recommended.

The COD concentration: is determined in the sample filtrates with a COD analytical set-up by the procedure described in reference (2) below.

2. **DATA AND EVALUATION**

DOC and/or COD concentrations are determined at least in duplicate in the samples according to 1.6.2 above. The degradation at time T is calculated according to the formula (with definitions) given unter 1.2 above.

The extent of degradation is rounded to the nearest full percent. The amount of degradation attained at the end of the test is reported as the 'Biodegradability in the Zahn-Wellens test'.

Note: if complete degradation is attained before the test time is over and this result is confirmed by a second analysis on the next day, the test can be concluded.

3. **REPORTING**

3.1. TEST REPORT

The test report shall, if possible, contain the following:

— the initial concentration of the substance,

— all other information and the experimental results concerning the tested substance, the reference substance if used, and the blank,

— the concentration after three hours,

— biodegradation: curve with description,

— date and location where test organisms were sampled, status of adaptation, concentration used, etc.,

— scientific reasons for any changes of test procedure.

3.2. INTERPRETATION OF RESULTS

Removal of DOC (COD) which takes place gradually over days or weeks indicates that the test substance is being biodegraded.

However, physico-chemical adsorption can, in some cases, play a role and this is indicated when there is complete or partial removal from the outset, within the first three hours, and the difference between control and test supernatant liquors remains at an unexpectedly low level.

Further tests are necessary if a distinction is to be drawn between biodegradation (or partial biodegradation) and adsorption.

This can be done in a number of ways, but the most convincing is to use the supernatant or sludge as inoculum in a base-set test (preferably a respirometric test).

Test substances giving high, non-adsorptive removal of DOC (COD) in this test should be regarded as potentially biodegradable. Partial, non-adsorptive removal indicates that the chemical is at least subject to some biodegradation. Low, or zero removals of DOC (COD) may be due to inhibition of microorganisms by the test substance and this may also be revealed by lysis and loss of sludge, giving turbid supernatants. The test should be repeated using a lower concentration of test substance.

The use of a compound-specific analytical method or of ^{14}C-labelled test substance may allow greater sensitivity. In the case of ^{14}C test compound, the recovery of the $^{14}CO_2$ will confirm that biodegradation has occurred.

When results are given in terms of primary biodegradation, an explanation should, if possible, be given on the chemical structure change that leads to the loss of response of the parent test substance.

The validation of the analytical method must be given together with the response found on the blank test medium.

4. **REFERENCES**

(1) OECD, Paris, 1981, Test Guideline 302 B, Decision of the Council C(81) 30 final.

(2) Annex V C.9 Degradation: Chemical Oxygen Demand, Commission Directive 84/449/EEC, (OJ L 251,19.9.1984, p. 1).

Appendix

EVALUATION EXAMPLE

Organic compound:	4-Ethoxybenzoic acid
Theoretical test concentration:	600 mg/1
Theoretical DOC:	390 mg/l
Inoculum	Sewage treatment plant of…
Concentration:	1 gram dry material/litre
Adaptation status:	not adapted
Analysis:	DOC-determination
Amount of sample:	3 ml
Control substance:	Diethyleneglycol
Toxicity of compound:	No toxic effects below 1 000 mg/l Test used: fermentation tubes test

Test time	Control substance				Test substance		
	Blank DOC (1) mg/l	DOC (1) mg/l	DOC net mg/l	Degradation %	DOC (1) mg/l	DOC net mg/l	Degradation %
0	—	—	300,0	—	—	390,0	—
3 hours	4,0	298,0	294,0	2	371,6	367,6	6
1 day	6,1	288,3	282,2	6	373,3	367,2	6
2 days	5,0	281,2	276,2	8	360,0	355,0	9
5 days	6,3	270,5	264,2	12	193,8	187,5	52
6 days	7,4	253,3	245,9	18	143,9	136,5	65
7 days	11,3	212,5	201,2	33	104,5	93,2	76
8 days	7,8	142,5	134,7	55	58,9	51,1	87
9 days	7,0	35,0	28,0	91	18,1	11,1	97
10 days	18,0	37,0	19,0	94	20,0	2,0	99

(1) Mean values of triplicate determinations.

Figure 1

Examples of biodegradation curves

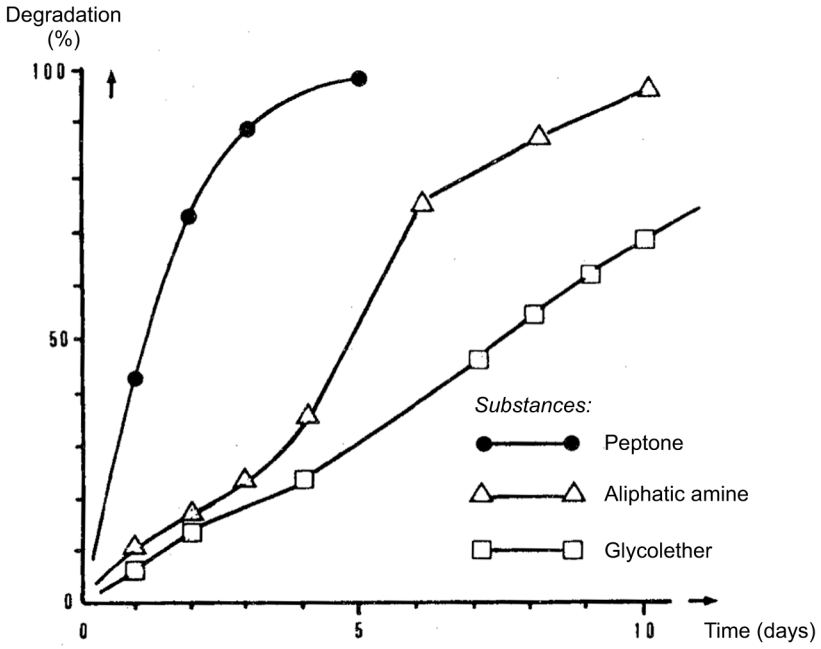
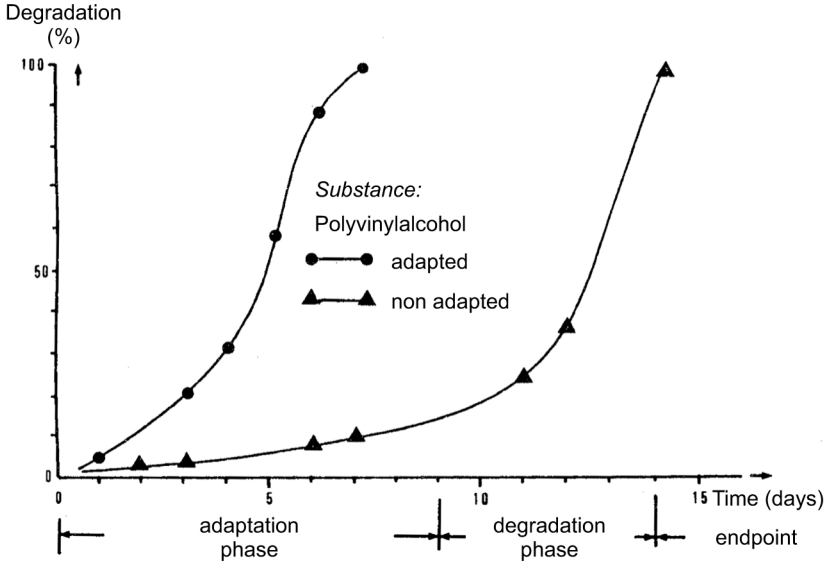


Figure 2

Examples of sludge adaptation



C.10. **BIODEGRADATION**

ACTIVATED SLUDGE SIMULATION TESTS

1. **METHOD**

1.1. INTRODUCTION

1.1.1. *General remarks*

The method is applicable only to those organic substances which, at the concentration used in the test:

— are soluble in water to the extent necessary for the preparation of the test solutions,

— have negligible vapour pressure under the test conditions,

— are not inhibitory to bacteria.

Information on the relative proportions of the major components of the test material will be useful in interpreting the results obtained, particularly in those cases where the results are low or marginal.

Information on the toxicity of the substance to micro-organisms is desirable for the interpretation of the low results and in the selection of appropriate test concentrations.

1.1.2. *Determination of ultimate biodegradability (DOC/COD analysis)*

The purpose of the method is to determine the ultimate biodegradability by the measurement of the removal of the substance and any metabolites in an activated sludge plant model at a concentration corresponding to > 12 mg DOC/litre (or approximately 40 mg COD/litre); 20 mg DOC/litre seem to be optimal. (DOC = dissolved organic carbon; COD = chemical oxygen demand).

The organic carbon content (or the chemical oxygen demand) of the test material must be established.

1.1.3. *Determination of primary biodegradability (specific analysis)*

The purpose of the method is the determination of the primary biodegradability of a substance in an activated sludge plant model, at a concentration of about 20 mg/litre, using a specific analytical method (lower or higher concentration can be used if analytical method and consideration of toxicity permits). This allows the assessment of the primary biodegradability of the substance (disappearance of the parent chemical structure).

The purpose of this method is not the determination of the mineralisation of the tested substance.

An adequate analytical method for the determination of the tested substance must be available.

1.2. DEFINITIONS AND UNITS

1.2.1. *DOC/COD analysis*

The degree of removal of the substance is given by:

$$TD = \frac{T - (E - E_o)}{T} \times 100\ \% \qquad [1(a)]$$

where:

DR = degree of removal in percent DOC (or COD) within the given mean retention time with respect to the test material

T = concentration of the test material in the influent in mg DOC/litre (or mg COD/litre)

E = DOC (or COD) concentration in the effluent of the test unit in mg DOC/litre (or mg COD/litre)

E_o = DOC (or COD) concentration in the effluent of the blank unit in mg DOC/litre (or mg COD/litre).

The degradation is stated as the percentage DOC (or COD) removal within the given retention time with respect to the test material.

1.2.2. *Specific analysis*

The percentage elimination of the tested substance from the aqueous phase (R_w) within the given mean retention time is given by:

$$R_W = \frac{C_I - C_O}{C_I} \times 100\ \% \qquad [1(b)]$$

where:

C_I = concentration of the substance in the influent of the test unit (mg substance/litre, determined by specific analysis)

C_o = concentration of the substance in the effluent of the test unit (mg substance/litre, determined by specific analysis).

1.3. REFERENCE SUBSTANCES

In some cases when investigating a new substance, reference substances may be useful; however, specific reference substances cannot yet be recommended.

1.4. PRINCIPLE OF THE TEST METHODS

For the determination of ultimate biodegradability, two activated sludge pilot units (OECD confirmatory test or porous pot units) are run in parallel. The test substance is added to the influent (synthetic or domestic sewage) of one of the units, while the other receives the sewage alone. For the determination of primary biodegradation with specific analysis in the influent and effluent, only one unit is used.

The DOC (or COD) concentrations are measured in the effluents, or the substance concentrations are determined by specific analysis.

The DOC due to test material is not measured but simply stated.

When DOC (or COD) measurements are performed, the difference in mean concentrations between the test and the control effluents is assumed to be due to undegraded test material.

When specific analyses are preformed, change in the concentration of the parent molecule can be measured (primary biodegradation).

The units may be operated following the 'coupled units mode', by a transinoculation procedure.

1.5. QUALITY CRITERIA

The starting concentration of the substance depends on the type of analysis performed and its limitation.

1.6. DESCRIPTION OF THE TEST METHOD

1.6.1. *Preparation*

1.6.1.1. Apparatus

A pair of units of the same type are needed except when specific analyses are performed. Two types of device may be used:

OECD confirmatory test

The equipment (Appendix 1) consists of a storage vessel (A) for synthetic sewage, dosing pump (B), aeration vessel (C), separator (D), air-lift pump (E), to recycle activated sludge, and vessel (F) for collecting the treated effluent.

Vessels (A) and (F) must be of glass or suitable plastic and hold at least 24 litres. Pump (B) must provide a constant flow of synthetic sewage to the aeration vessel; any suitable system may be used, providing that input flow and concentration are assured. During normal operation the height of separator (D) is so fixed that the volume contained in the aeration vessel is three litres of mixed liquor. A sintered aeration cube (G) is suspended in vessel (C) at the apex of the cone. The quantity of air blown through the aerator may be monitored by means of a flow meter.

Air-lift pump (E) is set so that the activated sludge from the separator is continually and regulariy recycled to aeration vessel (C).

'Porous pot'

The porous pot is constructed from sheets of porous polyethylene (2 mm thick, maximum pore size 95 μm), which are made into cylinders 14 cm in diameter with a conical base at 45° (Figures 1 and 2 of Appendix 2). The porous pot is contained in an impervious vessel of suitable plastic 15 cm in diameter with an outlet at a height of 17,2 cm on the cylindrical part, which determines the volume (3 litres) in the pot. There is a rigid supporting ring made of suitable plastic around the top of the inner vessel, so that there is an effluent space of 0,5 cm between the inner and outer vessels.

The porous pots may be mounted in the base of a thermostatically controlled water-bath. There is an air supply to the base of the inner vessel on which are placed suitable diffusers.

Vessels (A) and (E) must be of glass or suitable plastic and hold at least 24 litres. Pump (B) must provide a constant flow of synthetic sewage to the aeration vessel; any suitable system may be used, providing that input flow and concentration are assured.

Spare inner porous pots are required to replace any which may block in use; blocked pots are cleaned by 24-hour immersion in hypochlorite solution followed by thorough washing in tap water.

1.6.1.2. Filtration

Membrane filtration apparatus and membrane filters with a pore size of 0,45 μm. Membrane filters are suitable if it is assured that they neither release carbon nor adsorb the substance in the filtration step.

1.6.1.3. Sewage

Either suitable synthetic feed or domestic sewage may be used.

Example of synthetic feed

Dissolve in each litre of tap water:

Peptone:	160 mg,
Meat extract:	10 mg,
Urea:	30 mg,

NaCl:	7 mg,
$CaCl_2 .2H_2O$:	4 mg,
$MgSO_4 .7H_2O$:	2 mg,
K_2HPO_4:	28 mg.

Domestic sewage

This should be collected freshly each day from the overflow of the primary settlement tank of a treatment plant treating predominantly domestic sewage.

1.6.1.4. Stock solution of test material

A solution of test material, e.g. 1 %, should be prepared for addition to the test unit. The concentration of the material must be determined, so that the appropriate volume to be added to the sewage or directly to the unit via a second pump to give the required test concentration is known.

1.6.1.5. Inoculum

Remark: when domestic sewage is used, there would be no point in using an inoculum of low bacterial concentration, but activated sludge may be used.

A variety of inocula may be used.

Three examples of suitable inoculum are given:

(a) Inoculum from secondary effluent

The inoculum should be obtained from a secondary effluent of good quality collected from a treatment plant dealing with predominantly domestic sewage. The effluent must be kept under aerobic conditions in the period between sampling and use. To prepare the inoculum, the sample is filtered through a coarse filter, the first 200 ml being discarded. The filtrate is kept aerobic until used. The inoculum must be used on the day of collection. At least 3 ml are to be used for inoculation.

(b) Composite inoculum

Inoculum from secondary effluent:

See description above.

Inoculum from soil:

100 g of garden soil (fertile, not sterile) are suspended in 1 000 ml chlorine-free drinking water. (Soils with an extremely large fraction of clay, sand or humus are unsuitable). After stirring, the suspension is allowed to settle for 30 minutes. The supernatant is filtered through a coarse filter paper, the first 200 ml being discarded. The filtrate is aerated immediately and until use. The inoculum must be used on the day of collection.

Inoculum from a surface water:

A further partial inoculum is drawn from a mesosaprobic surface water. The sample is filtered through a coarse paper, the first 200 ml being discarded. The filtrate is kept aerobic until used. The inoculum must be used on the, day of collection.

Equal volumes of the three partial inoculum samples are united, mixed well, and the final inoculum drawn from this mixture. At least 3 ml are to be used for inoculation.

c) Inoculum from activated sludge

A volume (not more than 3 litres) of activated sludge (suspended solids content of up to 2,5 g/litre) taken from the aeration tank of a plant treating predominantly domestic sewage may be used as an inoculum.

1.6.2. *Procedure*

The test is performed at room temperature; this should be kept between 18 and 25 °C.

If it is appropriate, the test may be performed at a lower temperature (down to 10 °C); if the substance is degraded then no further work is normally required. If, however, the substance is not degraded, the test must be conducted at a steady temperature between 18 and 25 °C.

1.6.2.1. Running-in period: Sludge formation/stabilisation of the units

The sludge growth/stabilisation period is the period during which the concentration of the activated sludge suspended solids and the performance of the units progress to a steady state under the operating conditions used.

The running-in period is the period which lasts from the time the test substance is first added to the time when its removal reaches a plateau (relatively constant value). This period must not exceed six weeks.

The evaluation period is a three-week period, three weeks from the time that the removal of the test substance reaches a relatively constant, and usually high, value. For those substances which show little or no degradation in the first six weeks, the evaluation period is taken as the following three weeks.

Initially, fill the unit(s) needed for one test with the inoculum mixed with influent.

The aerator (and air lift (E) in the case of the OECD confirmatory test units) and dosing device (B) are then set in operation.

Influent without substance to be tested must pass through the aeration vessel (C) either at the rate of one litre per hour or a rate of one half litre per hour; this gives a mean retention time of either three or six hours.

The rate of aeration should be regulated so that the content of vessel (C) is kept constantly in suspension while the dissolved oxygen content is at least 2 mg/litre.

Foaming must be prevented by appropriate means. Anti-foaming agents which inhibit the activated sludge must not be used.

The sludge which has accumulated around the top of the aeration vessel (C) (and, in the case of the OECD confirmatory test units, in the base of the settling vessel (D), and in the circulation circuit) must be returned to the mixed liquor at least once each day by brushing or some other appropriate means.

When sludge fails to settle, its density may be increased by addition of 2 ml portions of a 5 % solution of ferric chloride, repeated as necessary.

The effluent is collected in vessel (E or F) for 20 to 24 hours, and a sample is taken after thorough mixing. Vessel (E or F) must be carefully cleaned.

In order to monitor and control the efficiency of the process, the chemical oxygen demand (COD) or the dissolved organic carbon (DOC) of the filtrate of the accumulated effluent is measured at least twice weekly, as well as that of the filtered influent (using a membrane of pore size 0,45 μm, the first 20 ml (approximately) of the filtrate are discarded).

The reduction in COD or DOC should level off when a roughly regular daily degradation is obtained.

The dry matter content of the activated sludge in the aeration tank should be determined twice a week (in g/litre). The units may be operated in one of two ways: either the content of dry matter in the activated sludge should be determined twice a week, and, if it is more than 2,5 g/litre, the excess activated sludge must be discarded, or 500 ml of mixed liquor is wasted from each pot daily to give a mean sludge retention time of six days.

When the measured and estimated parameters (efficiency of the process (in COD or DOC removal), sludge, concentration, sludge settleability, turbidity of the effluents, etc.) of the two units are sufficiently steady, the test substance may be introduced in the influent of one of the units, following 1.6.2.2.

Alternatively, the test substance may be added at the beginning of the sludge growth period (1.6.2.1), especially when sludge is added as the inoculum.

1.6.2.2. Test procedure

The operating conditions of the running-in period are maintained and sufficient stock solution (approximately 1 %) of the test material is added to the influent of the test unit so that the desired concentration of test material (approximately 10 to 20 mg DOC/litre or40 mg COD/litre) in the sewage is obtained. This can be done by mixing the stock solution to the sewage daily or by means of a separate pumping system. This concentration may be reached progressively. If there are no toxic effects of the test substance on the activated sludge, higher concentrations can also be tested.

The blank unit is fed only with influent without added substances. Adequate volumes of the effluents are taken for analysis and filtered through membrane filters (0,45 μm) the first 20 ml (approximately) of filtrate being discarded.

The filtered samples have to be analysed on the same day, otherwise they must be preserved by any suitable method, for example, by using 0,05 ml of a 1 % mercuric chloride ($HgCl_2$) solution for each 10 ml of filtrate or by storing them at 2 to 4 °C up to 24 hours, or below −18 °C for longer periods.

The running-in time, with addition of test substance, should not exceed six weeks and the evaluation period should not be shorter than three weeks, i.e. about 14 to 20 determinations should be available for calculation of the final result.

Coupled units mode

The coupling of the units is achieved by interchanging 1,5 litres of mixed liquor (including sludge) from the activated sludge aeration vessels between the two units once a day. In the case of strongly absorbing test materials, 1,5 litres of supernatant liquid only are drawn from the settling vessels and poured into the activated sludge vessel of the other unit.

1.6.2.3. Analysis

Two kinds of analyses may be performed in order to follow the behaviour of the substance:

DOC and COD

The DOC concentrations are performed in duplicate with the carbon analyser and/or the COD values according to reference (2).

Specific analysis

The concentrations of the tested substance are determined by a suitable analytical method. When possible, specific determination of the substance absorbed on sludge should be performed.

2. DATA AND EVALUATION

2.1. COUPLED UNITS MODE

When using 'coupled units mode', the daily degrees of removal, DR are calculated according to 1.2.1.

These daily degrees of removal DR are corrected to DRc for the material transfer due to the transinoculation procedure with equation [2] for a three-hour or equation [3] for a six-hour mean retention time.

$$DRc = \frac{4}{3}DR - \frac{100}{3} \quad [2]$$

$$DRc = \frac{8}{7}DR - \frac{100}{7} \quad [3]$$

The mean of the series of DRc values is calculated and in addition the standard deviation according to equation [4]

$$S_{DRc} = \sqrt{\frac{\sum_{i=1}^{n}\left(\overline{DRc} - DRc_i\right)^2}{n-1}} \quad [4]$$

where:

S_{DRc} = standard deviation of the series of DRc values

$\overline{DR}c$ = mean of DRc value

n = number of determinations.

Outliers of the DRc series are eliminated according to a suitable statistical procedure, e.g. Nalimov (6), at the 95 % probability level and the mean and the standard deviation of the outlier free DRc data set are recalculated.

The final result is then calculated with equation [5] as

$$DRc = \overline{DR}c \pm \frac{t_{n-1;\alpha}S}{\sqrt{n}}DRc \quad [5]$$

where:

$t_{n-1};\alpha$ = table value of t for n value pairs of E and E_o and statistical confidence P (P =1-α) whereby P is at 95 % (1).

The result is stated as the mean with tolerance limits at the 95 % probability level, the respective standard deviation and the number of data of the outlier-free DRc data set, and the number of outliers, e.g.

DRc = 98,6 ± 2,3 % DOC removal

s = 4,65 % DOC removal

n = 18

x = number of outliers.

2.2. NON-COUPLED UNITS MODE

The performance of the units may be checked as follows:

$$\text{percentage removal of COD or DOC} = \frac{\text{CODorDOCsewage} - \text{CODorDOCeffluent}}{\text{CODorDOCsewage}} \times 100$$

These daily removals may be plotted graphically to reveal any trends, e.g. to acclimatisation.

2.2.1. *Using COD/DOC determinations*

The daily degree of removal DR is calculated according to 1.2.1.

The mean of the series of DR values is calculated; in addition, its standard deviation is calculated according to:

$$S_{DR} = \sqrt{\frac{\sum_{i=1}^{n} \left(\overline{DR} - DR_i\right)^2}{n-1}} \qquad [6]$$

where:

s_{DR} = standard deviation of the series of DR_i values,
$\overline{DR}$ = mean of DR_i values,
n = number of determinations.

Outliers of the DR series are eliminated according to a suitable statistical procedure, e.g. Nalimov (6), at the 95 % probability level, and the mean and the standard deviation of the outliers-free DR set are recalculated.

The final result is then calculated with equation [7] as:

$$DR = \overline{DR} \pm \frac{t_{n-1;\alpha} s}{\sqrt{n}} DR \qquad [7]$$

where:

$t_{n-1;\alpha}$ = table value of t for n value pairs of E and E_o and statistical confidence P ($P = 1 - \alpha$) whereby P is set at 95 % (1).

The result is stated as the mean with tolerance limits at the 95 % probability level, the respective standard deviation and the number of data of the outlier free DR data set, and the number of outliers, e.g.

DR = (98,6 ± 2,3) % DOC removal
s = (4,65) % DOC removal
n = 18
x = number of outliers.

2.2.2. *Using specific analysis*

The percentage of elimination of the tested substance from the aqueous phase (R_w) is calculated according to 1.2.2.

3. REPORTING

3.1. TEST REPORT

The test report shall, if possible, contain the following:

— the formsheet given in Appendix 3, showing the operating conditions for the test,

— which apparatus was chosen (OECD confirmatory test or porous pot),

— which operating mode was chosen: coupled units mode or not,

— which sewage synthetic or domestic — in the case of domestic sewage, date and location of sample,

— which inoculum, with date and location of sample,

— a statement with description of the analytical method if specific analyses were performed,

— plot of COD or DOC removal versus time, including running-in and evaluation period,

— analytical recovery of the test substance as COD of DOC in the stock solution,

— if specific analyses were performed, plot of the percentage removal of the tested substance from the aqueous phase versus time (running-in and evaluation period),

— the mean removal of DOC or COD of test substance and standard deviation are calculated from the results of the evaluation period; i.e. when there is a steady removal of test material or period of steady operation,

— plot of activated sludge concentration versus time,

— any remark concerning the activated sludge (discard of excess sludge, presence of bulking, FeCl3 etc.),

— concentration of the substance used in the test,

— any results concerning analysis done on the sludge,

— all information and experimental results concerning the test substance and the reference substance if used,

— scientific reasons for any changes of the procedure.

3.2. INTERPRETATION OF RESULTS

Low removal of the tested substance from the aqueous phase may be due to inhibition of micro-organisms by the test substance. This may also be revealed by lysis and loss of sludge, giving a turbid supernatant, and by a decrease of the COD (or DOC) removal efficiency of the pilot plant.

Physico-chemical adsorption can sometimes play a role. Differences between biological action on the molecule and phyisico-chemical adsorption may be revealed by analysis performed on the sludge after an adequate desorption.

Further tests are necessary if a distinction is to be drawn between biodegradation (or partial biogradation) and adsorption.

This can be done in a number of ways, but the most convincing is to use the supernatant as inoculum in a base-set test (respirometric test preferably).

If high DOC or COP removals are observed, then this is due to biodegradation while, at low removals, biodegradation is indistinguishable from elimination. For example, if a soluble compound exhibits a high adsorption constant of 98 % and the surplus sludge wastage rate is 10 % per day, an elimination of up to 40 % is possible; at a surplus sludge wastage rate of 30 % elimination due to adsorption on and removal with surplus sludge may amount to up to 65 % (4).

When using specific analysis, attention should be paid to the relationship between the structure of the substance and the specific analysis used. In this case, the phenomenon observed cannot be interpreted as a mineralisation of the substance.

4. **REFERENCES**

(1) OECD, Paris, 1981, Test Guideline 303 A, Decision of the Council C(81) 30 final.

(2) Annex V C9 Degradation Test — Chemical Oxygen Demand, Commission Directive 84/449/EEC, (OJ L 251, 19.9.1984, p. 1).

(3) Painter, H. A., King, E. F., WRC Porous-Pot method for assessing biodegradability, Technical Report TR70, June 1978, Water Research Centre, United Kingdom.

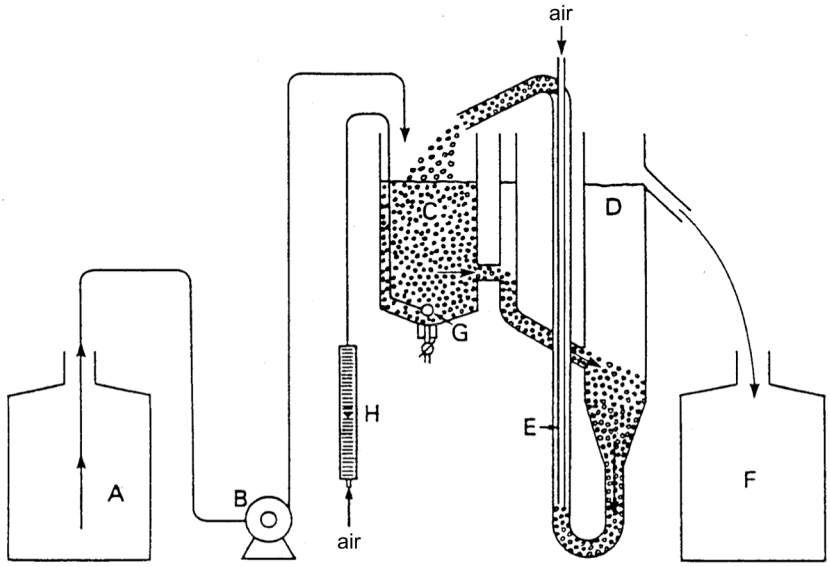
(4) Wierich, P., Gerike, P., The fate of soluble, recalcitrant, and adsorbing compounds in activated sludge plants, Ecotoxicology and Environmental Safety, vol. 5, No 2, June 1981, p. 161-171.

(5) Council Directives 82/242/EEC and 82/243/EEC, (OJ, No L 109, 22.4.1982, p. 1), amending Council Directives 73/404/EEC and 73/405/EEC on biodegradability of detergents, (OJ L 347, 17.12.1973, p. 51).

(6) Streuli, H., Fehlerhafte Interpretation und Anwendung von Ausreßertests, insbesondere bei Ringversuchen zur Oberprüfung analytisch-chemischer Untersuchungsmethoden, Fresenius-Zeitschrift für Analytische Chemie, 303 (1980), p. 406-408.

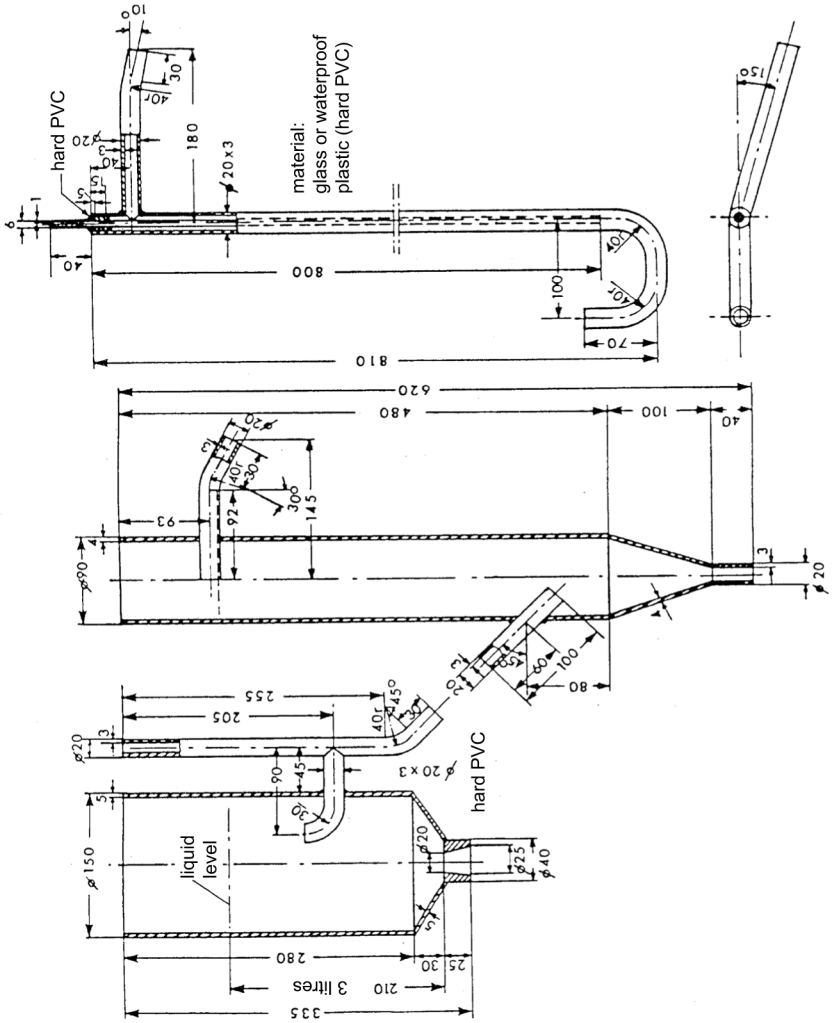
Appendix 1

Figure 1



Key: A = storage vessel;
B = dosing device;
C = aeration chamber (3 l capacity);
D = settling vessel;
E = air lift;
F = collector;
G = aerator;
H = air flow meter (optional).

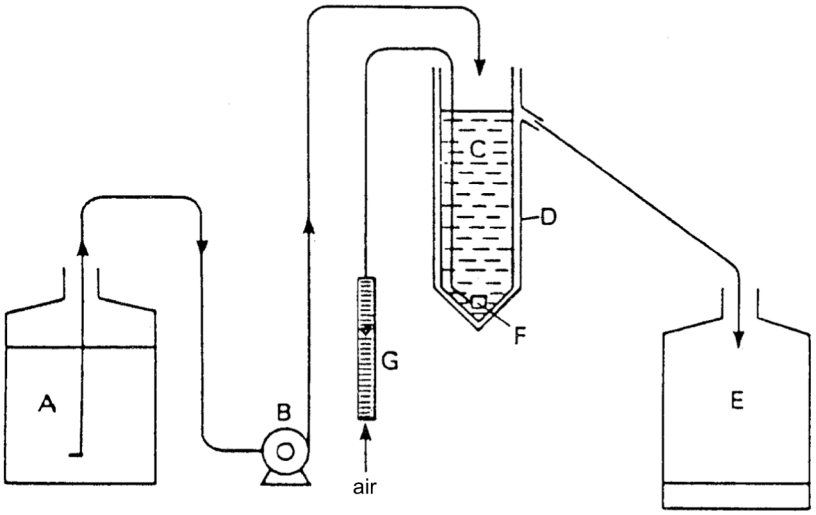
Figure 2


material:
glass or waterproof
plastic (hard PVC)
hard PVC
hard PVC
liquid
level
3 litres

Appendix 2

Figure 1

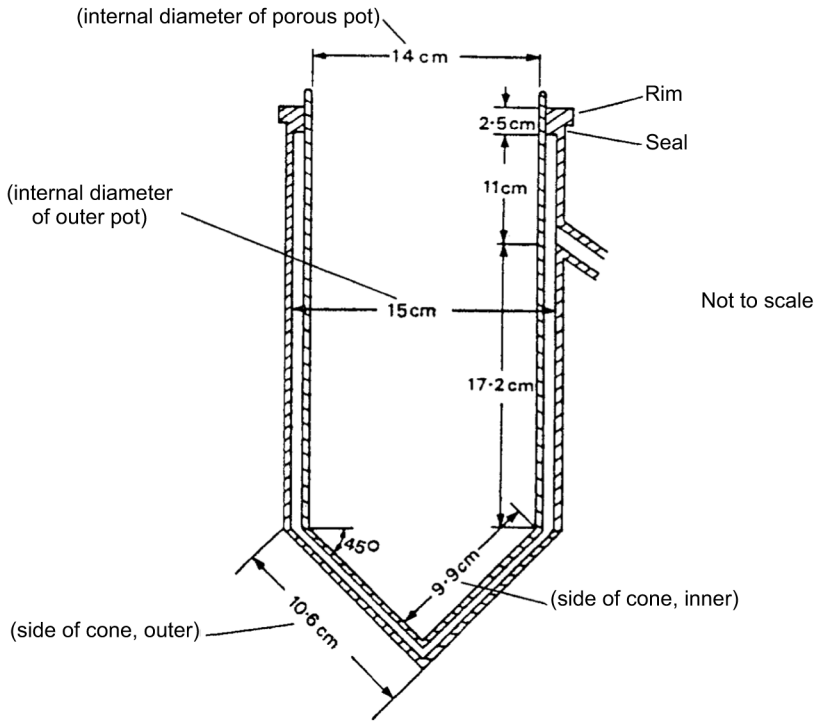
Equipment used for assessing biodegradability



Key:
A = storage vessel;
B = dosing pump;
C = porous aeration vessel;
D = outer impermeable vessel;
E = effluent collection vessel;
F = diffuser-stone aerator;
G = rotameter (optional).

Figure 2

Details of three-litre porous-pot aeration vessel



Appendix 3

Operating conditions for the Activated Sludge Simulation Test

Check in each group

Apparatus	
OECD confirmatory	☐
Porous pot	☐
Mode of operation	
Single unit	☐
Coupled units	☐
Non-coupled units	☐
Transinoculation	
None	☐
Activated sludge	☐
Supernatant	☐
Mean retention time	
Three hours	☐
Six hours	☐
Base nutrient	
Domestic sewage	☐
Synthetic sewage	☐
Inoculum	
Secondary effluent	☐
Composite	☐
Activated sludge	☐
Test material addition	
Form the start	☐
Stepwise increase	☐
After sludge has formed	☐
Analysis	
Specific	☐
COD	☐
DOC	☐

C.11. **BIODEGRADATION**

ACTIVATED SLUDGE RESPIRATION INHIBITION TEST

1. **METHOD**

1.1. INTRODUCTION

The method described assesses the effect of a test substance on micro-organisms by measuring the respiration rate under defined conditions in the presence of different concentrations of the test substance.

The purpose of this method is to provide a rapid screening method whereby substances which may adversely affect aerobic microbial treatment plants can be identified, and to indicate suitable non-inhibitory concentrations of test substances to be used in biodegradability tests.

A range-finding test may precede a definitive test. It provides information about the range of concentrations to be used in the main test.

Two controls without test substance are included in the test design, one at the start and the other at the end of the test series. Each batch of activated sludge should also be checked using a reference substance.

This method is most readily applied to substances which, due to their water solubility and low volatility, are likely to remain in water.

For substances with limited solubility in the test media, it may not be possible to determine the EC_{50}.

Results based on oxygen uptake may lead to erroneous conclusions when the test substance has the propensity to uncouple oxidative phosphorylation.

It is useful to have the following information to perform the test:

— water solubility,

— vapour pressure,

— structural formula,

— purity of the test substance.

Recommendation

Activated sludge may contain potentially pathogenic organisms and should be handled with care.

1.2. DEFINITIONS AND UNITS

The respiration rate is the oxygen consumption of waste-water micro-organisms in aerobic sludge, expressed generally as mg O_2 per mg of sludge per hour.

In order to calculate the inhibitory effect of a test substance at a particular concentration, the respiration rate is expressed as a percentage of the mean of the two control respiration rates:

$$\left(1 - \frac{2R_s}{Rc_1 + Rc_2}\right) \times 100 = \% \text{ inhibition}$$

where:

R_s = oxygen-consumption rate at tested concentration of test substance

R_{c1} = oxygen-consumption rate, control 1
R_{c2} = oxygen-consumption rate, control 2.

EC_{50} in this method is the concentration of the test substance at which the respiration rate is 50 % of that shown by the control under conditions described in this method.

1.3. REFERENCE SUBSTANCES

It is recommended that 3,5-dichlorophenol, as a known inhibitor of respiration, be used as a reference substance and tested for EC_{50} on each batch of activated sludge as a means of checking that the sensitivity of the sludge is not abnormal.

1.4. PRINCIPLE OF THE TEST METHOD

The respiration rate of an activated sludge fed with a standard amount of synthetic sewage feed is measured after a contact time of 30 minutes or three hours, or both. The respiration rate of the same activated sludge in the presence of various concentrations of the test substance under otherwise identical conditions is also measured. The inhibitory effect of the test substance at a particular concentration is expressed as a percentage of the mean respiration rates of two controls. An EC_{50} value is calculated from determinations at different concentrations.

1.5. QUALITY CRITERIA

The test results are valid if:

— the two control respiration rates are within 15 % of each other,

— the EC_{50} (30 minutes and/or three hours) of 3,5-dichlorophenol is in the accepted range 5 to 30 mg/litre.

1.6. DESCRIPTION OF THE TEST METHOD

1.6.1. *Reagents*

1.6.1.1. Solutions of the test substance

Solutions of the test substance are freshly prepared at the start of the study using a stock solution. A stock solution concentration of 0,5 g/litre is appropriate if the procedure recommended below is followed.

1.6.1.2. Solution of control substance

A solution of 5,5-dichlorophenol can for example be prepared by dissolving 0,5 g 3,5-dichlorophenol in 10 ml of 1M NaOH, diluting to approximately 30 ml with distilled water, adding under stirring 0,5M H_2SO_4 to the point of incipient precipitation — approximately 8 ml of 0,5M H_2SO_4 will be required — and finally diluting the mixture to one litre with distilled water. The pH should then be in the range 7 to 8.

1.6.1.3. Synthetic sewage

A synthetic sewage feed is made by dissolving the following amounts of substances in one litre of water:

— 16 g peptone,

— 11 g meat extract,

— 3 g urea,

— 0,7 g NaCl,

— 0,4 g $CaCl_2.2H_2O$,

— 0,2 g $MgSO_4.7H_2O$,

— 2,8 g K_2HPO_4.

Note 1: this synthetic sewage is a 100-fold concentrate of that described in the OECD Technical Report 'Proposed method for the determination of the biodegradability of surfactants used in synthetic detergents' (June 11, 1976), with the addition of dipotassium hydrogen phosphate.

Note 2: if the prepared medium is not used immediately, it shall be stored in the dark at 0 to 4 °C, for no longer than one week, in conditions which do not produce any change in its composition. The medium may also be sterilised prior to storage, or the peptone and meat extract may be added shortly before carrying out the test. Before use, it shall be mixed thoroughly and the pH adjusted.

1.6.2. *Apparatus*

Measuring apparatus: the precise design is not critical. However, there should be head space and the probe should fit tightly in the neck of the measuring flask.

Normal laboratory equipment and especially the following is necessary:

— measuring apparatus,

— aeration device,

— pH-electrode and measuring equipment,

— O_2-electrode.

1.6.3. *Preparation of the inoculum*

Activated sludge from a sewage treatment plant treating predominantly domestic sewage is used as the mircobial inoculum for the test.

If necessary, on return to the larboratory, coarse particles may be removed by settling for a short period, e.g. 15 minutes, and decanting the upper layer of finer solids for use. Alternatively, the sludge may be mixed using a blender for a few seconds.

In addition, if it is thought that inhibitory material is present, the sludge should be washed with tap water or an isotonic solution. After centrifuging, the supernatant is decanted (this procedure is repeated three times).

A small amount of the sludge is weighed and dried. From this result, the amount of wet sludge can be calculated which must be suspended in water in order to obtain an activated sludge with a mixed liquor suspended solids range between 2 and 4 g/litre. This level gives a concentration between 0,8 and 1,6 g/litre in the test medium if the procedure recommended below is followed.

If the sludge cannot be used on the day of collection, 50 ml of synthetic sewage is added to each litre of the activated sludge prepared as described above; this is then aerated overnight at 20 ± 2 °C. It is then kept aerated for use during the day. Before use the pH is checked and adjusted, if necessary, to pH 6 to 8. The mixed liquor suspended solids should be determined as described in the preceding paragraph.

If the same batch of sludge is required to be used on subsequent days (maximum four days), a further 50 ml of synthetic sewage feed is added per litre of sludge at the end of each working day.

1.6.4. *Performance of the test*

Duration/contact time:	30 minutes and/or three hours, during which aeration takes place
Water:	Drinking water (dechlorinated if necessary)
Air supply:	Clean, oil-free air. Air flow 0,5 to 1 litre/minute
Measuring apparatus:	Flat bottom flask such as a BOD-flask

Oxygen meter:	Suitable oxygen electrode, with a recorder
Nutrient solution:	Synthetic sewage (see above)
Test substance:	The test solution is freshly prepared at the start of the test
Reference substance:	e.g. 3,5-dichlorophenol (at least three concentrations)
Controls:	Inoculated sample without test substance
Temperature:	20 ± 2 °C

A suggested experimental procedure which may be followed for both the test and reference substance for the three-hour contact period is given below:

Several vessels (e.g. one-litre beakers) are used.

At least five concentrations, spaced by a constant factor preferably not exceeding 3,2, should be used.

At time '0', 16 ml of the synthetic sewage feed are made up to 300 ml with water. 200 ml of microbial inoculum are added and the total mixture (500 ml) poured into a first vessel (first control C_1).

The test vessels should be aerated continuously such as to ensure that the dissolved O_2 does not fall below 2,5 mg/litre and that, immediately before the measurement of the respiration rate, the O_2 concentration is about 6,5 mg/litre.

At time '15 minutes' (15 minutes is an arbitrary, but convenient, interval) the above is repeated, except that 100 ml of the test substance stock solution are added to the 16 ml of synthetic sewage before adding water to 300 ml and microbial inoculum to make a volume of 500 ml. This mixture is then poured into a second vessel and aerated as above. This process is repeated at 15-minute intervals with different volumes of the test substance stock solution to give a series of vessels containing different concentrations of the test substance. Finally, a second control is prepared (C_2).

After three hours the pH is recorded, and a well-mixed sample of the contents of the first vessel is poured into the measuring apparatus and the respiration rate is measured over a period of up to 10 minutes.

This determination is repeated on the contents of each vessel at 15-minute intervals, in such a way that the contact time in each vessel is three hours.

The reference substance is tested on each batch of microbial inoculum in the same way.

A different regime (e.g. more than one oxygen meter) will be necessary when measurements are to be made after 30 minutes of contact.

If measurement of the chemical oxygen consumption is required, further vessels are prepared containing test substance, synthetic sewage feed and water, but no activated sludge. Oxygen consumption is measured and recorded after an aeration time of 30 minutes and/or three hours (contact time).

2. **DATA AND EVALUATION**

The respiration rate is calculated from the recorder trace between approximately 6,5 mg O_2/litre and 2,5 mg O_2/litre, or over a 10-minute period when the respiration rate is low. The portion of the respiration curve over which the respiration rate is measured should be linear.

If the respiration rates of the two controls are not within 15 % of each other, or the EC_{50} (30 minutes and/or three hours) of the reference substance is not in the accepted range (5 to 30 mg/litre for 3,5-dichlorophenol), the test is invalid and must be repeated.

The per - cent inhibition is calculated at each test concentration (see 1.2). The per - cent inhibition is plotted against concentration on log-normal (or log-probability) paper, and an EC_{50} value derived.

95 % confidence limits for the EC_{50} values can be determined using standard procedures.

3. **REPORTING**

3.1. TEST REPORT

The test report shall, if possible, contain the following:

— test substance: chemical identification data,

— test system: source, concentration and any pre-treatment of the activated sludge,

— test conditions:

  — pH of the reaction mixture before the respiration measurement,

  — test temperature,

  — test duration,

  — reference substance and its measured EC_{50},

  — abiotic oxygen uptake (if any).

— results:

  — all measured data,

  — inhibition curve and method for calculation of EC_{50},

  — EC_{50} and, if possible, 95 % confidence limits, EC_{20} and EC_{80},

  — all observations and any deviations from this test method which could have influenced the result.

3.2. INTERPRETATION OF DATA

The EC_{50} value should be regarded merely as a guide to the likely toxicity of the test substance either to activated sludge sewage treatment or to waste-water microorganisms, since the complex interactions occurring in the environment cannot be accurately simulated in a laboratory test. In addition, test substances which may have an inhibitory effect on ammonia oxidation may also produce atypical inhibition curves. Accordingly, such curves should be interpreted with caution.

4. **REFERENCES**

(1) International Standard ISO 8192-1986.

(2) Broecker, B., Zahn, R., Water Research 11,1977, p. 165.

(3) Brown, D., Hitz, H. R., Schaefer, L., Chemosphere 10, 1981, p. 245.

(4) ETAD (Ecological and Tox.icological Association of Dyestuffs Manufacturing Industries), Recommended Method No 103, also described by:

(5) Robra, B., Wasserl Abwasser 117, 1976, p. 80.

(6) Schefer, W., Textilveredlung 6,1977, p. 247.

(7) OECD, Paris, 1981, Test Guideline 209, Decision of the Council C(84) 30 final.

C.12. **BIODEGRADATION**

MODIFIED SCAS TEST

1. **METHOD**

1.1. INTRODUCTION

The purpose of the method is the evaluation of the potential ultimate biodegradability of water-soluble, non-volatile organic substances when exposed to relatively high concentrations of micro-organisms over a long time period. The viability of the microorganisms is maintained over this period by daily addition of a settled sewage feed. (For weekend requirements, the sewage may be stored at 4 °C. Alternatively, the synthetic sewage of the OECD confirmatory test may be used.)

Physico-chemical adsorption on the suspended solids may take place and this must be taken into account when interpreting results (see 3.2).

Because of the long detention period of the liquid phase (36 hours), and the intermittent addition of nutrients, the test does not simulate those conditions experienced in a sewage treatment plant. The results obtained with various test substances indicate that the test has a high biodegradation potential.

The conditions provided by the test are highly favourable to the selection and/or adaptation of micro-organisms capable of degrading the test compound. (The procedure may also be used to produce acclimatised inocula for use in other tests.)

In this method, the measure of the concentration of dissolved organic carbon is used to assess the ultimate biodegradability of the test substances. It is preferable to determine DOC after acidification and purging rather than as the difference of C_{total}-$C_{inorganic}$.

The simultaneous use of a specific analytical method may allow the assessment of the primary degradation of the substance (disappearance of the parent chemical structure).

The method is applicable only to those organic test substances which, at the concentration used in the test:

— are soluble in water (at least 20 mg dissolved organic carbon/litre),

— have negligible vapour pressure,

— are not inhibitory to bacteria,

— do not significantly adsorb within the test system,

— are not lost by foaming from the test solution.

The organic carbon content of the test material must be established.

Information on the relative proportions of the major components of the test material will be useful in interpreting the results obtained, particularly in those cases where the results are low or marginal.

Information on the toxicity to microorganisms of the substance may be useful to the interpretation of low results and in the selection of an appropriate test concentration.

1.2. DEFINITIONS AND UNITS

C_T = concentration of test compound as organic carbon as present in or added to the settled sewage at the start of the aeration period (mg/litre),
C_t = concentration of dissolved organic carbon found in the supernatant liquor of the test at the end of the aeration period (mg/litre),
C_c = concentration of dissolved organic carbon found in the supernatant liquor of the control at the end of the aeration period (mg/litre).

The biodegradation is defined in this method as the disappearance of the organic carbon. The biodegradation can be expressed as:

1. The percentage removal D_{da} of the amount of substance added daily:

$$D_{da} = \frac{C_T - (C_T - C_c)}{C_T} \times 100 \qquad [1]$$

where

D_{da} = degradation/daily addition.

2. The percentage removal D_{ssd} of the amount of substance present at the start of each day:

$$D_{ssd} = \frac{2C_T + C_{ti} - C_{ci} - 3C_{t(i+1)} + 3C_{c(i+1)}}{2C_T + C_{ti} - C_{ci}} \times 100 \qquad [2\ (a)]$$

$$\approx \frac{2C_T - 2(C_t - C_c)}{2C_T + (C_t - C_c)} \times 100 \qquad [2\ (b)]$$

where

D_{ssd} = degradation/substance start of day;

the indices i and (i + 1) refer to the day of measurement.

Equation 2(a) is recommended if effluent DOC varies from day to day, while equation 2(b) may be used when effluent DOC remains relatively constant from day to day.

1.3. REFERENCE SUBSTANCES

In some cases, when investigating a new substance, reference substances may be useful; however, no specific reference substance is recommended here.

Data on several compounds evaluated in ring tests are provided (see Appendix 1) primarily so that calibration of the method may be performed from time to time and to permit comparison of results when another method is employed.

1.4. PRINCIPLE OF THE TEST METHOD

Activated sludge from a sewage treatment plant is placed in a semi-continuous activated sludge (SCAS) unit. The test compound and settled domestic sewage are added, and the mixture is aerated for 23 hours. The aeration is then stopped, the sludge allowed to settle and the supernatant liquor is removed.

The sludge remaining in the aeration chamber is then mixed with a further aliquot of test compound and sewage and the cycle is repeated.

Biodegradation is established by determination of the dissolved organic carbon content of the supernatant liquor. This value is compared with that found for the liquor obtained from a control tube dosed with settled sewage only.

When a specific analytical method is used, changes in the concentration of the parent molecule due to biodegradation can be measured (primary biodegradability).

1.5. QUALITY CRITERIA

The reproducibility of this method based on removal of dissolved organic carbon has not yet been established. (When primary biodegradation is considered, very precise data are obtained for materials that are extensively degraded).

The sensitivity of the method is largely determined by the variability of the blank and to a lesser extent by the precision of the determination of dissolved organic carbon and the level of test compound in the liquor at the start of each cycle.

1.6. DESCRIPTION OF THE TEST PROCEDURE

1.6.1. *Preparations*

A sufficient number of clean aeration units, alternatively, the original 1,5 litre SCAS test unit may be used, and air inlet tubes (Figure 1) for each test substance and controls are assembled. Compressed air supplied to the test units, cleaned by a cotton wool strainer, should be free of organic carbon and pre-saturated with water to reduce evaporation losses.

A sample of mixed liquor, containing 1 to 4 g suspended solids/litre, is obtained from an activated sludge plant treating predominantly domestic sewage. Approximately 150 ml of the mixed liquor are required for each aeration unit.

Stock solutions of the test substance are prepared in distilled water; the concentration normally required is 400 mg/litre as organic carbon which gives a test compound concentration of 20 mg/litre carbon at the start of each aeration cycle if no biodegradation is occurring.

Higher concentrations are allowed if the toxicity to microorganisms permits it.

The organic carbon content of the stock solutions is measured.

1.6.2. *Test conditions*

The test should be performed at 20 to 25 °C.

A high concentration of aerobic microorganisms is used (from 1 to 4 g/litre suspended solids), and the effective detention period is 36 hours. The carbonaceous material in the sewage feed is oxidised extensively, normally within eight hours after the start of each aeration cycle. Thereafter, the sludge respires endogenously for the remainder of the aeration period, during which time the only available substrate is the test compound unless this is also readily metabolised. These features, combined with daily re-inoculation of the test when domestic sewage is used as the medium, provide highly favourable conditions for both acclimatisation and high degress of biodegradation.

1.6.3. *Performance of the test*

A sample of mixed liquor from a suitable predominantly domestic activated sludge plant or laboratory unit is obtained and kept aerobic until used in the laboratory. Each aeration unit as well as the control unit are filled with 150 ml of mixed liquor (if the original SCAS test unit is used, multiply the given volumes by 10) and the aeration is started. After 23 hours, aeration is stopped and the sludge is allowed to settle for 45 minutes. The tap of each vessel is opened in turn, and 100 ml portions of the supernatant liquor are withdrawn. A sample of settled domestic sewage is obtained immediately before use, and 100 ml are added to the sludge remaining in each aeration unit. Aeration is started anew. At this stage no test materials are added, and the units are fed daily with domestic sewage only until a clear supernatant liquor is obtained on settling. This usually takes up to two weeks, by which time the dissolved organic carbon in the supernatant liquor at the end of each aeration cycle approaches a constant value.

At the end of this period, the individual settled sludges are mixed, and 50 ml of the resulting composite sludge are added to each unit.

95 ml of settled sewage and 5 ml of water are added to the control units, and 95 ml of the settled sewage plus 5 ml of the appropriate test compound stock solution (400 mg/litre) are added to the test units. Aeration is started again and continued for 23 hours. The sludge is then allowed to settle for 45 minutes and the supernatant drawn off and analysed for dissolved organic carbon content.

The above fill-and-draw procedure is repeated daily throughout the test.

Before settling, it may be necessary to clean the walls of the units to prevent the accumulation of solids above the level of the liquid. A separate scraper or brush is used for each unit to prevent cross contamination.

Ideally, the dissolved organic carbon in the supernatant liquors is determined daily, although less frequent analyses are permissible. Before analysis the liquors are filtered through washed 0,45 μm membrane filters or centrifuged. Membrane filters are suitable if it is assured that they neither release carbon nor absorb the substance in the filtration step. The temperature of the sample must not exceed 40 °C while it is in the centrifuge.

The length of the test for compounds showing little or no biodegradation is indeterminate, but experience suggests that this should be at least 12 weeks in general, but not longer than 26 weeks.

2. DATA AND EVALUATION

The dissolved organic carbon values in the supernatant liquors of the test units and the control units are plotted against time.

As biodegradation is achieved, the level found in the test will approach that found in the control. Once the difference between the two levels is found to be constant over three consecutive measurements, such number of further measurements as are sufficient to allow statistical treatment of the data are made and the percentage biodegradation of the test compound is calculated (D_{da} or D_{ssd}, see 1.2).

3. REPORTING

3.1. TEST REPORT

The test report shall, if possible, contain the following:

— all information on the kind of sewage, the type of unit used and the experimental results concerning the tested substance, the reference substance if used, and the blank,

— the temperature,

— removal curve with description, mode of calculation (see 1.2),

— date and location where the activated sludge and the sewage were sampled, status of adaptation, concentration, etc.,

— scientific reasons for any changes of test procedure,

— signature and date.

3.2. INTERPRETATION OF RESULTS

Since the substance to be tested by this method will not be readily biodegradable, any removal of DOC due solely to biodegradation will normally be gradual over days or weeks, except in such cases where acclimatisation is sudden as indicated by an abrupt disappearance occurring after some weeks.

However, physico-chemical adsorption can sometimes play an important role; this is indicated when there is complete or partial removal of the added DOC at the outset. What happens subsequently depends on factors such as the degrees of adsorption and the concentration of suspended solids in the discarded effluent. Usually the difference between the concentration of DOC in the control and test supernatant liquors gradually increases from the initial low value and this difference then remains at the new value for the remainder of the experiment, unless acclimatisation takes place.

If a distinction is to be drawn between biodegradation (or partial biodegradation) and adsorption, further tests are necessary. This can be done in a number of ways, but the most convincing is to use the supernatant liquor, or sludge, as inoculum in a base-set test (preferably a respirometric test).

Test substances giving high, non-adsorptive removal of DOC in this test should be regarded as potentially biodegradable. Partial, non-adsorptive removal indicates that the chemical is at least subject to some biodegradation.

Low, or zero removals of DOC may be due to inhibition of microorganisms by the test substance and this may also be revealed by lysis and loss of sludge, giving turbid supernatants. The test should be repeated using a lower concentration of test substance.

The use of a specific analytical method or of ^{14}C-labelled test substance may allow greater sensitivity. In the case of ^{14}C test compound, the recovery of the $^{14}CO_2$ will confirm that biodegradation has occurred.

When results are also given in terms of primary biodegradation, an explanation should, if possible, be given on the chemical structure change that leads to the loss of response of the parent test substance.

The validation of the analytical method must be given together with the response found on the blank test medium.

4. REFERENCES

(1) OECD, Paris, 1981, Test Guideline 302 A, Decision of the Council C(81) 30 final.

Appendix 1

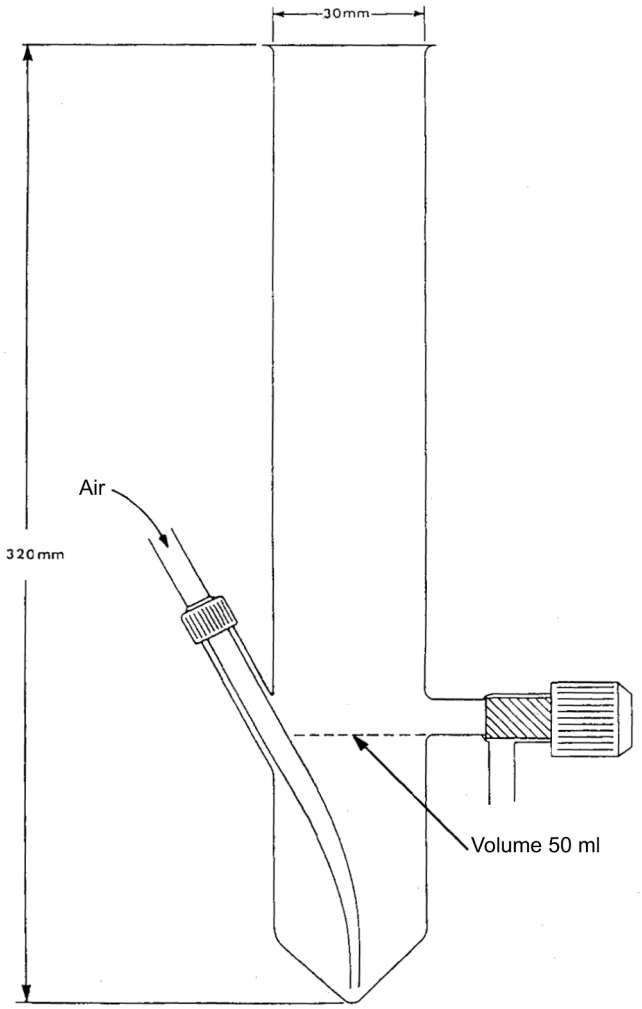
SCAS test: example of results

Substance	C_T (mg/l)	$C_t - C_c$ (mg/l)	Percentage biodegradition, D_{da}	Test duration (days)
4-acetyl aminobenzene sulphonate	17,2	2,0	85	40
Tetra propylene benzene sulphonate	17,3	8,4	51,4	40
4-nitrophenol	16,9	0,8	95,3	40
Diethylene glycol	16,5	0,2	98,8	40
Aniline	16,9	1,7	95,9	40
Cyclopentane tetra carboxylate	17,9	3,2	81,1	120

Appendix 2

Example of test apparatus

Figure 1



C.13 BIOCONCENTRATION: FLOW-THROUGH FISH TEST

1. METHOD

This bioconcentration method is a replicate of the OECD TG 305 (1996).

1.1. INTRODUCTION

This method describes a procedure for characterising the bioconcentration potential of substances in fish under flow-through conditions. Although flow-through test regimes are much to be preferred, semi-static regimes are permissible, provided that the validity criteria are satisfied.

The method gives sufficient details for performing the test while allowing adequate freedom for adapting the experimental design to the conditions in particular laboratories and for varying characteristics of test substances. It is most validly applied to stable organic chemicals with log P_{ow} values between 1,5 and 6,0 (1) but may still be applied to superlipophilic substances (having log P_{ow} > 6,0). The pre-estimate of the bioconcentration factor (BCF), sometimes denoted as K_B, for such superlipophilic substances will presumably be higher than the steady-state bioconcentration factor (BCF_{SS}) value expected to be obtained from laboratory experiments. Preestimates of the bioconcentration factor for organic chemicals with log P_{ow} values up to about 9,0 can be obtained by using the equation of Bintein at al (2). The parameters which characterise the bioconcentration potential include the uptake rate constant (k_1), the depuration rate constant (k_2) and the BCF_{ss}.

Radio-labelled test substances can facilitate the analysis of water and fish samples and may be used to determine whether degradate identification and quantification should be made. If total radioactive residues are measured (e.g. by combustion or tissue solubilisation), the BCF is based on the parent compound, any retained metabolites and also assimilated carbon. BCFs based on total radioactive residues may not, therefore, be directly comparable to a BCF derived by specific chemical analysis of the parent compound only.

Clean-up procedures may be employed in radiolabelled studies in order to determine BCF based on the parent compound, and the major metabolites may be characterised if deemed necessary. It is also possible to combine a fish metabolism study with a bioconcentration study by analysis and identification of the residues in tissues.

1.2. DEFINITIONS AND UNITS

Bioconcentration/Bioaccumulation is the increase in concentration of the test substance in or on an organism (specified tissues thereof) relative to the concentration of test substance in the surrounding medium.

The bioconcentration factor (BCF or K_B) at any time during the uptake phase of this accumulation test is the concentration of test substance in/on the fish or specified tissues thereof (C_f as µg/g (ppm)) divided by the concentration of the chemical in the surrounding medium (C_w as µg/ml (ppm)).

The steady-state bioconcentration factor (BCF_{ss} or K_B) does not change significantly over a prolonged period of time, the concentration of the test substance in the surrounding medium being constant during this period of time.

A plateau or steady-state is reached in the plot of test substance in fish (C_f) against time when the curve becomes parallel to the time axis and three successive analyses of C_f made on samples taken at intervals of at least two days are within ± 20 % of each other, and there are no significant differences among the three sampling periods. When pooled samples are analysed at least four successive analyses are required. For test substances which are taken up slowly the intervals would more appropriately be seven days.

Bioconcentration factors calculated directly from kinetic rate constants (k_1/k_2) are termed kinetic concentration factor, BCF_K.

The octanol-water partition coefficient (P_{ow}) is the ratio of a chemical's solubility in n-octanol and water at equilibrium (Method A.8) also expressed as K_{ow}. The logarithm of P_{ow} is used as an indication of a chemical's potential for bioconcentration by aquatic organisms.

The exposure or uptake phase is the time during which the fish are exposed to the test chemical.

The uptake rate constant (k_1) is the numerical value defining the rate of increase in the concentration of test substance in/on test fish (or specified tissues thereof) when the fish are exposed to that chemical (k_1 is expressed in day^{-1}).

The post-exposure or depuration (loss) phase is the time, following the transfer of the test fish from a medium containing test substance to a medium free of that substance, during which the depuration (or the net loss) of the substance from the test fish (or specified tissue thereof) is studied.

The depuration (loss) rate constant (k_2) is the numerical value defining the rate of reduction in the concentration of the test substance in the test fish (or specified tissues thereof) following the transfer of the test fish from a medium containing the test substance to a medium free of that substance (k_2 is expressed in day^{-1}).

1.3. PRINCIPLE OF THE TEST METHOD

The test consists of two phases: the exposure (uptake) and post-exposure (depuration) phases. During the uptake phase, separate groups of fish of one species are exposed to at least two concentrations of the test substance. They are then transferred to a medium free of the test substance for the depuration phase. A depuration phase is always necessary unless uptake of the substance during the uptake phase has been insignificant (e.g. the BCF is less than 10). The concentration of the test substance in/on the fish (or specified tissue thereof) is followed through both phases of the test. In addition to the two test concentrations, a control group of fish is held under identical conditions except for the absence of the test substance, to relate possible adverse effects observed in the bioconcentration test to a matching control group and to obtain background concentrations of test substance.

The uptake phase is run for 28 days unless it is demonstrated that equilibrium has been reached earlier. A prediction of the length of the uptake phase and the time to steady-state can be made from equation in Appendix 3. The depuration period is then begun by transferring the fish to the same medium but without the test substance in another clean vessel. Where possible the bioconcentration factor is calculated preferably both as the ratio (BCF_{ss}) of concentration of the fish (C_f) and in the water (C_w) at apparent steady-state and as a kinetic bioconcentration factor, BCF_K as the ratio of the rate constants of uptake (k_1) and depuration (k_2) assuming first-order kinetics. If first-order kinetics are obviously not obeyed, more complex models should be employed (Appendix 5).

If a steady-state is not achieved within 28 days, the uptake phase should be extended until steady-state is reached, or 60 days, whichever comes first; the depuration phase is then begun.

The uptake rate constant, the depuration (loss) rate constant (or constants, where more complex models are involved), the bioconcentration factor, and where possible, the confidence limits of each of these parameters are calculated from the model that best describes the measured concentrations of test substance in fish and water.

The BCF is expressed as a function of the total wet weight of the fish. However, for special purposes, specified tissues or organs (e.g. muscle, liver), may be used if the fish are sufficiently large or the fish may be divided into edible (fillet) and non-edible (viscera) fractions. Since, for many organic substances, there is a clear relationship between the potential for bioconcentration and lipophilicity, there is also a corresponding relationship between the lipid content of the test fish and the observed bioconcentration of such substances. Thus, to reduce this source of variability in test results for those substances with high lipophilicity (i.e. with log P_{ow} > 3), bioconcentration should be expressed in relation to lipid content in addition to whole body weight.

The lipid content should be determined on the same biological material as is used to determine the concentration of the test substance, when feasible.

1.4. INFORMATION ON THE TEST SUBSTANCE

Before carrying out the test for bioconcentration, the following information for the test substance should be known:

— solubility in water

— octanol-water partition coefficient P_{ow} (denoted also as K_{ow}, determined by an HPLC method in A.8)

— hydrolysis

— phototransformation in water determined under solar or simulated solar irradiation and under the irridiation conditions of the test for bioconcentration (3)

— surface tension (i.e. for substances where the log P_{ow} cannot be determined)

— vapour pressure

— ready biodegradability (where appropriate)

Other information required is the toxicity to the fish species to be used in the test, preferably the asymptotic LC_{50} (i.e. time-independent). An appropriate analytical method, of known accuracy, precision and sensitivity, for the quantification of the test substance in the test solutions and in biological material must be available, together with details of sample preparation and storage. Analytical detection limit of test substance in both water and fish tissues should also be known. When ^{14}C labelled test substance is used, the percentage of radioactivity associated with impurities should be known.

1.5. VALIDITY OF THE TEST

The following conditions should apply for a test to be valid:

— the temperature variation is less than ± 2 °C;

— the concentration of dissolved oxygen does not fall below 60 % saturation;

— the concentration of the test substance in the chambers is maintained within ± 20 % of the mean of the measured values during the uptake phase;

— the mortality or other adverse effects/disease in both control and treated fish is less than 10 % at the end of the test; where the test is extended over several weeks or months, death or other adverse effects in both sets of fish should be less than 5 % per month and not exceed 30 % in all.

1.6. REFERENCE COMPOUNDS

The use of reference compounds of known bioconcentration potential would be useful in checking the experimental procedure, when required. However, specific substances cannot yet be recommended.

1.7. DESCRIPTION OF THE TEST METHOD

1.7.1. *Apparatus*

Care should be taken to avoid the use of materials, for all parts of the equipment, that can dissolve, sorb or leach and have an adverse effect on the fish. Standard rectangular or cylindrical tanks, made of chemically inert material and of a suitable capacity in compliance with loading rate can be used. The use of soft plastic tubing should be minimised. Teflon®, stainless steel and/or glass tubing are preferably used. Experience has shown that for substances with high adsorption coefficients, such as the synthetic pyrethroids, silanised glass may be required. In these situations the equipment will have to be discarded after use.

1.7.2. *Water*

Natural water is generally used in the test and should be obtained from an uncontaminated and uniform quality source. The dilution water must be of a quality that will allow the survival of the chosen fish species for the duration of the acclimation and test periods without them showing any abnormal appearance or behaviour. Ideally, it should be demonstrated that the test species can survive, grow and reproduce in the dilution water (e.g. in laboratory culture or a life-cycle toxicity test). The water should be characterised at least by pH, hardness,

total solids, total organic carbon and, preferably also ammonium, nitrite and alkalinity and, for marine species, salinity. The parameters which are important for optimal fish well-being are fully known, but Appendix 1 gives recommended maximum concentrations of a number of parameters for fresh and marine test waters.

The water should be of constant quality during the period of a test. The pH value should be within the range 6,0 to 8,5, but during a given test it should be within a range of ± 0,5 pH units. In order to ensure that the dilution water will not unduly influence the test result (for example, by complexation of the test substance) or adversely affect the performance of the stock of fish, samples should be taken at intervals for analysis. Determination of heavy metals (e.g. Cu, Pb, Zn, Hg, Cd, Ni), major anions and cations (e.g. Ca, Mg, Na, K, Cl, SO_4), pesticides (e.g. total organophosphorous and total organochlorine pesticides), total organic carbon and suspended solids should be made, for example, every three months where a dilution water is known to be relatively constant in quality. If water quality has been demonstrated to be constant over at least one year, determinations can be less frequent and intervals extended (e.g. every six months).

The natural particle content as well as the total organic carbon (TOC) of the dilution water should be as low as possible to avoid adsorption of the test substance to organic matter which may reduce its bioavailability (4). The maximum acceptable value is 5 mg/l for particulate matter (dry matter, not passing a 0,45 μm filter) and 2 mg/l for total organic carbon (see Appendix 1). If necessary, the water should be filtered before use. The contribution to the organic carbon content in water from the test fish (excreta) and from the food residues should be as low as possible. Throughout the test, the concentration of organic carbon in the test vessel should not exceed the concentration of organic carbon originating from the test substance and, if used, the solubilising agent by more than 10 mg/l (± 20 %).

1.7.3. *Test Solutions*

A stock solution of the test substance is prepared at a suitable concentration. The stock solution should preferably be prepared by simply mixing or agitating the test substance in the dilution water. The use of solvents or dispersants (solubilising agents) is not recommended; however this may occur in some cases in order to produce a suitably concentrated stock solution. Solvents which may be used are ethanol, methanol, ethylene glycol monomethyl ether, ethylene glycol dimethyl ether, dimethylformamide and triethylene glycol. Dispersants which may be used are Cremophor RH40, Tween 80, methylcellulose 0,01 % and HCO-40. Care should be taken when using readily biodegradable agents as these can cause problems with bacterial growth in flow-through tests. The test substance may be radio-labelled and should be of the highest purity (e.g. preferably > 98 %).

For flow-through tests, a system which continuously dispenses and dilutes a stock solution of the test substance (e.g. metering pump, proportional diluter, saturator system) is required to deliver the test concentrations to the test chambers. At least five volume replacements through each test chamber per day are preferably allowed. The flow-through mode is to be preferred, but where this is not possible (e.g. when the test organisms are adversely affected) a semi-static technique may be used provided that the validity criteria are satisfied. The flow rates of stock solutions and dilution water should be checked both 48 h before and then at least daily during the test. In this check the determination of the flow-rate through each test chamber is included and ensured that it does not vary by more than 20 % either within or between chambers.

1.7.4. *Selection of species*

Important criteria in the selection of species are that they are readily available, can be obtained in convenient sizes and can be satisfactorily maintained in the laboratory. Other criteria for selecting fish species include recreational, commercial, ecological importance as well as comparable sensitivity, past successful use etc.

Recommended test species are given in Appendix 2. Other species may be used but the test procedure may have to be adapted to provide suitable test conditions. The rationale for the selection of the species and the experimental method should be reported in this case.

1.7.5. *Holding of fish*

Acclimate the stock population of fish for at least two weeks in water at the test temperature and feed throughout on a sufficient diet and of the same type to be used during the test.

Following a 48-hour settling-in period, mortalities are recorded and the following criteria applied:

— mortalities of greater than 10 % of population in seven days: reject the entire batch;

— mortalities of between 5 and 10 % of population in seven days: acclimate for seven additional days;

— mortalities of less than 5 % of population in seven days: accept the batch — if more than 5 % mortality during second seven days reject the entire batch.

Ensure that fish used in tests are free from observable diseases and abnormalities. Discard any diseased fish. Fish should not receive treatment for disease in the two weeks preceding the test, or during the test.

1.8. PERFORMANCE OF THE TEST

1.8.1. *Preliminary test*

It may be useful to conduct a preliminary experiment in order to optimise the test conditions of the definitive test, e.g. selection of test substance concentration(s), duration of the uptake and depuration phases.

1.8.2. *Conditions of exposure*

1.8.2.1. Duration of uptake phase

A prediction of the duration of the uptake phase can be obtained from practical experience (e.g. from a previous study or an accumulation related chemical) or from certain empirical relationships utilising knowledge of either the aqueous solubility or the octanol/water partition coefficient of the test substance (see Appendix 3).

The uptake phase should be run for 28 days unless it can be demonstrated that equilibrium has been reached earlier. If the steady-state has not been reached by 28 days, the uptake phase should be extended, taking further measurements, until steady-state is reached or 60 days, whichever is shorter.

1.8.2.2. Duration of the depuration phase

A period of half the duration of the uptake phase is usually sufficient for an appropriate (e.g. 95 %) reduction in the body burden of the substance to occur (see Appendix 3 for explanation of the estimation). If the time required to reach 95 % loss is impractically long, exceeding for example twice the normal duration of the uptake phase (i.e. more than 56 days) a shorter period may be used (i.e. until the concentration of test substance is less than 10 % of steady-state concentration). However, for substances having more complex patterns of uptake and depuration than are represented by a one-compartment fish model, yielding first order kinetics, allow longer depuration phases for determination of loss rate constants. The period may, however, be governed by the period over which the concentration of test substance in the fish remains above the analytical detection limit.

1.8.2.3. Numbers of test fish

Select the numbers of fish per test concentration such that minimum of four fish per sample are available at each sampling. If greater statistical power is required, more fish per sample will be necessary.

If adult fish are used, report whether male or female, or both are used in the experiment. If both sexes are used, differences in lipid content between sexes should be documented to be non-significant before the start of the exposure; pooling all male and all female fish may be necessary.

In any one test, fish of similar weight are selected, such that the smallest are no smaller than two-thirds of the weight of the largest. All should be of the same year-class and come from the same source. Since weight and age of a fish appear sometimes to have a significant effect on BCF values (1) these details are recorded accurately. It is recommended that a sub-sample of the stock of fish is weighed before the test in order to estimate the mean weight.

1.8.2.4. Loading

High water-to-fish ratios are used in order to minimise the reduction in C_W caused by the addition of the fish at the start of the test and also to avoid decreases in dissolved oxygen concentration. It is important that the loading rate is appropriate for the test species used. In any case, a loading rate of 0,1-1,0 g of fish (wet weight)

per litre of water per day is normally recommended. High loading rates can be used if it is shown that the required concentration of test substance can be maintained within ± 20 % limits, and that the concentration of dissolved oxygen does not fall below 60 % saturation.

In choosing appropriate loading regimes, account of the normal habitat of the fish species is taken. For example, bottom-living fish may demand a larger bottom area of the aquarium for the same volume of water than pelagic fish species.

1.8.2.5. Feeding

During the acclimation and test periods, fish are fed with an appropriate diet of known lipid and total protein content, in an amount sufficient to keep them in a healthy condition and to maintain body weight. Fish are fed daily throughout the acclimation and test periods at a level of approximately 1 to 2 % of body weight per day; this keeps the lipid concentration in most species of fish at a relatively constant level during the test. The amount of feed should be re-calculated, for example, once per week, in order to maintain consistent body weight and lipid content. For this calculation, the weight of the fish in each test chamber can be estimated from the weight of the fish sampled most recently in that chamber. Do not weigh the fish remaining in the chamber.

Uneaten food and faeces are siphoned daily from the test chambers shortly after feeding (30 minutes to 1 hour). Chambers are kept as clean as possible throughout the test so that the concentration of organic matter is kept as low as possible, since the presence of organic carbon may limit the bioavailability of the test substance (1).

Since many feeds are derived from fishmeal, the feed should be analysed for the test substance. It is also desirable to analyse the feed for pesticides and heavy metals.

1.8.2.6. Light and temperature

The photoperiod is usually 12 to 16 hours and the temperature (± 2 °C) should be appropriate for the test species (see Appendix 2). The type and characteristics of illumination should be known. Caution should be given to the possible phototransformation of the test substance under the irradiation conditions of the study. Appropriate illumination should be used avoiding exposure of the fish to unnatural photoproducts. In some cases it may be appropriate to use a filter to screen out UV irradiation below 290 nm.

1.8.2.7. Test concentrations

Fish are exposed under flow-through conditions to at least two concentrations of the test substance in water. Normally, the higher (or highest) concentration of the test substance are selected to be about 1 % of its acute asymptotic LC_{50}, and to be at least ten-fold higher than its detection limit in water by the analytical method used.

The highest test concentration can also be determined by dividing the acute 96 h LC_{50} by an appropriate acute/chronic ratio (appropriate ratios for some chemicals can be about 3 up to 100). If possible, choose the other concentration(s) such that it differs from the one above by a factor of ten. If this is not possible because of the 1 % of LC_{50} criterion and the analytical limit, a lower factor than ten can be used or the use of ^{14}C labelled test substance should be considered. No concentration used should be above the solubility of the test substance.

Where a solubilising agent is used its concentration should not be greater than 0,1 ml/l and should be the same in all test vessels. Its contribution, together with the test substance, to the overall content of organic carbon in the test water should be known. However, every effort should be made to avoid the use of such materials.

1.8.2.8. Controls

One dilution water control or if relevant, one control containing the solubilising agent should be run in addition to the test series, provided that it has been established that the agent has no effects on the fish. If not, both controls should be set up.

1.8.3. *Frequency of water quality measurements*

During the test, dissolved oxygen, TOC, pH and temperature should be measured in all vessels. Total hardness and salinity, if relevant, should be measured in the controls and one vessel at the higher (or highest) concentration. As a minimum, dissolved oxygen and salinity, if relevant, should be measured three times — at

the beginning, around the middle and end of the uptake period — and once a week in the depuration period. TOC should be measured at the beginning of the test (24 h and 48 h prior to test initiation of uptake phase) before addition of the fish and at least once a week, during both uptake and depuration phases. Temperature should be measured daily, pH at the beginning and end of each period and hardness once each test. Temperature should preferably be monitored continuously in at least one vessel.

1.8.4. *Sampling and analysis of fish and water*

1.8.4.1. Fish and water sampling schedule

Water from the test chambers for the determination of test substance concentration is sampled before addition of the fish and during both uptake and depuration phases. As a minimum, the water is sampled at the same time as the fish and before feeding. During the uptake phase, the concentrations of test substance are determined in order to check compliance with the validity criteria.

Fish is sampled on at least five occasions during the uptake phase and at least on four occasions during the depuration phase. Since on some occasions it will be difficult to calculate a reasonably precise estimate of the BCF value based on this number of samples, especially when other than simple first-order depuration kinetics are indicated, it may be advisable to take samples at a higher frequency in both periods (see Appendix 4). The extra samples are stored and analysed only if the results of the first round of analyses prove inadequate for the calculation of the BCF with the desired precision.

An example of an acceptable sampling schedule is given in Appendix 4. Other schedules can readily be calculated using other assumed values of P_{ow} to calculate the exposure time for 95 % uptake.

Sampling is continued during the uptake phase until a steady-state has been established or for 28 days, whichever is the shorter. If the steady-state has not been reached within 28 days sampling continues until a steady-state has been attained or for 60 days, whichever is shorter. Before beginning the depuration phase the fish are transfered to clean tanks.

1.8.4.2. Sampling and Sample Preparation

Water samples for analysis are obtained e.g. by siphoning through inert tubing from a central point in the test chamber. Since neither filtration nor centrifuging appear always to separate the non-bioavailable fraction of the test substance from that which is bioavailable (especially for super-lipophilic chemicals i.e. those chemicals with a log P_{ow} > 5) (1) (5), samples may not be subjected to those treatments.

Instead, measures should be taken to keep the tanks as clean as possible and the content of total organic carbon should be monitored during both the uptake and depuration phases.

An appropriate number of fish (normally a minimum of four) is removed from the test chambers at each sampling time. The sampled fish are rinsed quickly with water, blot 'dry', killed instantly using the most appropriate and humane method, and then weighed.

It is preferable to analyse fish and water immediately after sampling in order to prevent degradation or other losses and to calculate approximate uptake and depuration rates as the test proceeds. Immediate analysis also avoids delay in determining when a plateau has been reached.

Failing immediate analysis, samples are stored by an appropriate method. Before the beginning of the study, information on the proper method of storage for the particular test substance — for example, deep-freezing, holding at 4 ^{0}C, duration of storage, extraction, etc. are obtained.

1.8.4.3. Quality of Analytical method

Since the whole procedure is governed essentially by the accuracy, precision and sensitivity of the analytical method used for the test substance, check experimentally that the precision and reproducibility of the chemical analysis, as well as recovery of the test substance from both water and fish are satisfactory for the particular method. Also, check that the test substance is not detectable in the dilution water used.

If necessary, the values of C_w and C_f obtained from the test are corrected for the recoveries and background values of controls. Fish and water samples are handled throughout in such a manner as to minimise contamination and loss (e.g. resulting from adsorption by the sampling device).

1.8.4.4. Analysis of Fish Sample

If radiolabelled materials are used in the test, it is possible to analyse for total radio label (i.e. parent and metabolites) or, the samples may be cleaned up so that parent compound can be analysed separately. Also, the major metabolites may be characterised at steady-state or at the end of the uptake phase, whichever is the sooner. If the BCF in terms of total radiolebelled residues is ≥ 1 000 %, it may be advisable, and for certain categories of chemicals such as pesticides strongly recommended, to identify and quantify degradates representing ≥ 10 % of total residues in fish tissues at steady state. If degradates representing ≥ 10 % of total radiolabelled residues in the fish tissue are identified and quantified, then it is also recommended to identify and quantify degradates in the test water.

The concentration of the test substance should usually be determined for each weighed individual fish. If this is not possible, pooling of the samples on each sampling occasion may be done but pooling does restrict the statistical procedures which can be applied to the data. If a specific statistical procedure and power are important considerations, then an adequate number of fish to accommodate the desired pooling procedure and power should be included in the test (6) (7).

BCF should be expressed both as a function of total wet weight and, for high lipophilic substances, as a function of the lipid content. Lipid content of the fish is determined on each sampling occasion if possible. Suitable methods should be used for determination of lipid content (ref. 8 and 2 of Appendix 3). Chloroform/methanol extraction technique may be recommended as standard method (9). The various methods do not give identical values (10), so it is important to give details of the method used. When possible, the analysis for lipid should be made on the same extract as that produced for analysis for the test substance, since the lipids often have to be removed from the extract before it can be analysed chromatographically. The lipid content of the fish (as mg/kg wet weight) at the end of the experiment should not differ from that at the start by more ± 25 %. The tissue percent solids should also be reported to allow conversion of lipid concentration from a wet to a dry basis.

2. DATA

2.1. TREATMENT OF RESULTS

The uptake curve of the test substance is obtained by plotting its concentration in/on fish (or specified tissues) in the uptake phase against time on arithmetic scales. If the curve has reached a plateau, that is, become approximately asymptotic to the time axis, the steady-state BCF_{ss} is calculated from:

$$\frac{C_f \text{ as steady-state (mean)}}{C_w \text{ as steady-state (mean)}}$$

When no steady-state is reached, it may be possible to calculate a BCF_{ss} of sufficient precision for hazard assessment from a 'steady-state' at 80 % ($1{,}6/k_2$) or 95 % ($3{,}0/k_2$) of equilibrium.

Also the concentration factor (BCF_K) is determined, as the ratio k_1/k_2, the two first-order kinetic constants. The depuration rate constant (k_2) is usually determined from the depuration curve (i.e. a plot of the decrease in test substance concentration in the fish with time). The uptake rate constant (k_1) is then calculated given k_2 and a value of C_f which is derived from the uptake curve (see also Appendix 5). The preferred method for obtaining BCF_K and the rate constants, k_1 and k_2, is to use non-linear parameter estimation methods on a computer (11). Otherwise, graphical methods may be used to calculate k_1 and k_2. If the depuration curve is obviously not first-order, then more complex models should be employed (see references in Appendix 3) and advice from a biostatistician sought.

2.2. INTERPRETATION OF RESULTS

The results should be interpreted with caution where measured concentrations of test solutions occur at levels near the detection limit of the analytical method.

Clearly defined uptake and loss curves are an indication of good quality bioconcentration data. The variation in uptake/depuration constants between the two test concentrations should be less than 20 %. Observed significant differences in uptake/depuration rates between the two applied test concentrations should be recorded and possible explanations given. Generally the confidence limit of BCFs from well-designed studies approach ± 20 %.

3. **REPORTING**

The test report must include the following information:

3.1. TEST SUBSTANCE:

— physical nature and, where relevant, physicochemical properties,

— chemical identification data (including the organic carbon content, if appropriate),

— if radio labelled, the precise position of the labelled atom(s) and the percentage of radioactivity associated with impurities.

3.2. TEST SPECIES

— scientific name, strain, source, any pre-treatment, acclimation, age, size-range, etc.

3.3. TEST CONDITIONS:

— test procedure used (e.g. flow-through or semi-static),

— type and characteristics of illumination used and photoperiod(s),

— test design (e.g. number and size of test chambers, water volume replacement rate, number of replicates, number of fish per replicate, number of test concentrations, length of uptake and depuration phases, sampling frequency for fish and water samples,

— method of preparation of stock solutions and frequency of renewal (the solubilizing agent, its concentration and its contribution to the organic carbon content of test water must be given, when used),

— the nominal test concentrations, the means of the measured values and their standard deviations in the test vessels and the method by which these were attained,

— source of the dilution water, description of any pre-treatment, results of any demonstration of the ability of test fish to live in the water, and water characteristics: pH, hardness, temperature, dissolved oxygen concentration, residual chlorine levels (if measured), total organic carbon, suspended solids, salinity of the test medium (if appropriate) and any other measurements made,

— water quality within test vessels, pH, hardness, TOC, temperature and dissolved oxygen concentration,

— detailed information on feeding (e.g. type of food, source, composition — at least lipid and protein content if possible, amount given and frequency),

— information on the treatment of fish and water samples, including details of preparation, storage, extraction and analytical procedures (and precision) for the test substance and lipid content (if measured).

3.4. RESULTS:

— results from any preliminary study performed,

— mortality of the control fish and the fish in each exposure chamber and any observed abnormal behaviour,

— the lipid content of the fish (if determination on testing occasion),

— curves (including all measured data) showing the uptake and depuration of the test chemical in the fish, the time to steady-state,

— Cf and Cw (with standard deviation and range, if appropriate) for all sampling times (Cf expressed in μg/g wet weight (ppm) of whole body or specified tissues thereof e.g. lipid, and Cw in μg/ml (ppm). Cw values for the control series (background should also be reported),

— the steady-state bioconcentration factor (BCFss) and/or kinetic concentration factor (BCFK) and if applicable, 95 % confidence limits for the uptake and depuration (loss) rate constants (all expressed in relation to the whole body and the total lipid content, if measured, of the animal or specified tissues thereof), confidence limits and standard deviation (as available) and methods of computation/data analysis for each concentration of test substance used,

— where radio-labelled substances are used, and if it is required, the accumulation of any detected metabolites may be presented,

— anything unusual about the test, any deviation from these procedures, and any other relevant information,

minimise results as 'not detected at the limit of detection' by pre-test method development and experimental design, since such results cannot be used for rate constant calculations.

4. **REFERENCES**

(1) Connell D.W. (1988) Bioaccumulation behaviour of persistent chemicals with aquatic organisms. Rev. Environ. Contam. Toxicol. 102, p. 117-156.

(2) Bintein S., Devillers J. and Karcher W. (1993) Nonlinear dependence of fish bioconcentration on n-octanol/water partition coefficient. SAR and QSAR in Environmental Research, 1, p. 29-390.

(3) OECD, Paris (1996) Direct Phototransformation of chemicals in water. Environmental Health and Safety Guidance Document Series on Testing and Assessment of Chemicals. No 3.

(4) Kristensen P. (1991) Bioconcentration in fish: Comparison of bioconcentration factors derived from OECD and ASTM testing methods; influence of particulate organic matter to the bioavailability of chemicals. Water Quality Institute, Denmark.

(5) US EPA 822-R-94-002 (1994) Great Lake Water Quality Initiative Technical Support Document for the Procedure to Determine Bioaccumulation Factors. July 1994.

(6) US FDA, (Food and Drug Administration) Revision. Pesticide analytical manual, 1, 5600 Fisher's Lane, Rockville, MD 20852, July 1975.

(7) US EPA (1974). Section 5, A(1) Analysis of Human or Animal Adipose Tissue, in Analysis of Pesticide Residues in Human and Environmental Samples, Thompson J.F. (ed.) Research Triangle Park, N.C. 27711.

(8) Compaan H. (1980) in 'The determination of the possible effects of chemicals and wastes on the aquatic environment: degradation, toxicity, bioaccumulation' Ch. 2.3, Part II. Government Publishing Office, The Hague, The Netherlands.

(9) Gardner et al, (1995) Limn. & Oceanogr. 30, p. 1099-1105.

(10) Randall R.C., Lee H., Ozretich R.J., Lake J.L. and Pruell R.J. (1991). Evaluation of selected lipid methods for normalising pollutant bioaccumulation. Envir. Toxicol. Chem. 10, p. 1431-1436.

(11) CEC, Bioconcentration of chemical substances in fish: the flow-through method-Ring Test Programme, 1984 to 1985. Final report March 1987. Authors: P. Kristensen and N. Nyholm.

(12) ASTM E-1022-84 (Reapproved 1988) Standard Practice for conducting Bioconcentration Tests with Fishes and Saltwater Bivalve Molluscs.

Appendix 1

Chemical characteristics of an acceptable dilution water

	Substance	Limit concentration
1	Particulate Matter	5 mg/l
2	Total Organic Carbon	2 mg/l
3	Un-ionised ammonia	1 µg/l
4	Residual chlorine	10 µg/l
5	Total organophosphorous pesticides	50 ng/l
6	Total organochlorine pesticides plus polychlorinated biphenyls	50 ng/l
7	Total organic chlorine	25 ng/l
8	Aluminium	1µg/l
9	Arsenic	1µg/l
10	Chromium	1µg/l
11	Cobalt	1µg/l
12	Copper	1µg/l
13	Iron	1µg/l
14	Lead	1µg/l
15	Nickel	1µg/l
16	Zinc	1µg/l
17	Cadmium	100 ng/l
18	Mercury	100 ng/l
19	Silver	100 ng/l

Appendix 2

Fish species recommended for testing

	Recommended species	Recommended range of test temperature (°C)	Recommended total length of test animal (cm)
1	*Danio rerio* ([1]) (Teleostei, Cyprinidae) (Hamilton-Buchanan) Zebra — fish	20-25	3,0 ± 0,5
2	*Pimephales promelas* (Teleostei, Cyprinidae) (Rafinesque) Fathead minnow	20-25	5,0 ± 2,0
3	*Cyprinus carpio* (Teleostei, Cyprinidae) (Linnaeus) Common Carp	20-25	5,0 ± 3,0
4	*Oryzias latipes* (Teleostei, Poeciliidae) (Temminck and Schlegel) Ricefish	20-25	4,0 ± 1,0
5	*Poecilia reticulata* (Teleostei, Poeciliidae) (Peters) Guppy	20-25	3,0 ± 1,0
6	*Lepomis macrochirus* (Teleostei, Centrarchidae) (Rafinesque) Bluegill	20-25	5,0 ± 2,0
7	*Oncorhynchus mykiss* (Teleostei, Salmonidae) (Walbaum) Rainbow trout	13-17	8,0 ± 4,0
8	*Gasterosteus aculeatus* (Teleostei, Gasterosteidae) (Linnaeus) Three-spined stickleback	18-20	3,0 ± 1,0

([1]) Meyer A., Orti G. (1993) Proc. Royal Society of London, Series B., Vol. 252, p. 231.

Various estuarine and marine species have been used in different countries, for example:

Spot	*Leiostomus xanthurus*
Sheepshead minnow	*Cyprinodon variegatus*
Silverside	*Menidia beryllina*
Shiner perch	*Cymatogaster aggregata*
English sole	*Parophrys vetulus*
Staghorn sculpin	*Leptocottus armatus*
Three-spined stickleback	*Gasterosteus aculeatus*
Sea bass	*Dicentracus labrax*
Bleak	*Alburnus alburnus*

Collection

The fresh water fish listed in the table above are easy to rear and/or are widely available throughout the year, whereas the availability of marine and estuarine species is partially confined to the respective countries. They are capable of being bred and cultivated either in fish farms or in the laboratory, under disease-and parasite-controlled conditions, so that the test animal will be healthy and of known parentage. These fish are available in many parts of the world.

Appendix 3

Prediction of the duration of the uptake and depuration phases

1. *Prediction of the duration of the uptake phase*

Before performing the test, an estimate of k_2 and hence some percentage of the time needed to reach steady-state may be obtained from empirical relationships between k_2 and the n-octanol/water partition coefficient (P_{ow}) or k_2 and the aqueous solubility (s).

An estimate of k_2 (day^{-1}) may be obtained, for example from the following empirical relationship (1):

$\log_{10}k_2 = 0{,}414 \log_{10}(P_{ow}) + 1{,}47$ ($r^2 = 0{,}95$) [equation 1]

For other relationships see Ref. (2).

If the partition coefficient (P_{ow}) is not known, an estimate can be made (3) from a knowledge of the aqueous solubility (s) of the substance using:

$\log_{10}(P_{ow}) = 0{,}862 \log_{10}(s) + 0{,}710$ ($r^2 = 0{,}994$) [equation 2]

where

s = solubility (moles/l) : (n=36)

These relationships apply only to chemicals with log P_{ow} values between 2 and 6,5 (4).

The time to reach some percentage of steady-state may be obtained, by applying the k_2-estimate, from the general kinetic equation describing uptake and depuration (first-order kinetics):

$$\frac{dC_f}{dt} = k_1 \cdot C_w - k_2 \cdot C_f$$

or if C_w is constant:

$$C_f = \frac{k_1}{k_2} \cdot C_w \left(1 - e^{-k_2 t}\right) \quad \text{[equation 3]}$$

When steady-state is approached ($t \rightarrow \infty$), equation 3 may be reduced (5) (6) to:

$$C_f = \frac{k_1}{k_2} \cdot C_w \text{ or } C_f/C_w = k_1/k_2 = \text{BCF}$$

Then $k_1/k_2 \cdot C_w$ is an approach to the concentration in the fish at 'steady-state' ($C_{f,s}$).

Equation 3 may be transcribed to:

$$C_f = C_{f,s}\left(1 - e^{-k_2 t}\right) \text{ or } \frac{C_f}{C_{fs}} = 1 - e^{-k_2 t} \quad \text{[equation 4]}$$

Applying equation 4, the time to reach some percentage of steady-state may be predicted when k_2 is pre-estimated using equation 1 or 2.

As a guideline, the statistically optimal duration of the uptake phase for the production of statistically acceptable data (BCF_K) is that period which is required for the curve of the logarithm of the concentration of the test substance in fish plotted against linear time to reach its mid-point, or $1{,}6/k_2$, or 80 % of steady-state but not more than $3{,}0/k_2$ or 95 % of steady-state (7).

The time to reach 80 % of steady-state is (equation 4):

$$0{,}80 = 1 - e^{-k_2 t_{80}} \text{ or } t_{80} = \frac{1{,}6}{k_2} \quad \text{[equation 5]}$$

Similarly 95 percent of steady-state is:

$$t_{95} = \frac{3{,}0}{k_2} \quad \text{[equation 6]}$$

For example, the duration of the uptake phase (up) for a test substance with log P_{ow} = 4 would be (using equations 1,5,6):

$\log_{10} k_2 = -0{,}414.(4) + 1{,}47$ $\quad k_2 = 0{,}652$ days^{-1}

up (80 %) = 1,6/0,652, i.e. 2,45 days (59 hours)

or up (95 %) = 3,0/0,652, i.e. 4,60 days (110 hours)

Similarly, for a test substance with s = 10^{-5} mol/l (log(s) = - 5,0), the duration of up would be (using equations 1,2,5,6):

$\log_{10} (P_{ow}) = 0{,}862 (-5{,}0) + 0{,}710 = 5{,}02$

$\log_{10} K_2 = 0{,}414 (5{,}02) + 1{,}47$

$k_2 = 0{,}246$ days $^{-1}$

up (80 %) = 1,6/0,246, i.e. 6,5 days (156 hours)

or up (95 %) = 3,0/0,246, i.e. 12,2 days (293 hours)

Alternatively, the expression:

$t_{eq} = 6{,}54 \times 10^3 P_{ow} + 55{,}31$ (hours)

may be used to calculate the time for effective steady-state to be reached (4).

2. *Prediction of the duration of the depuration phase*

A prediction of the time needed to reduce the body burden to some percentage of the initial concentration may also be obtained from the general equation describing uptake and depuration (first order kinetics) (1) (8).

For the depuration phase, C_w is assumed to be zero. The equation may be reduced to:

$$\frac{dC_f}{dt} = -k_2 C_f \text{ or } C_f = C_{f,o} \cdot e^{-k_2 t}$$

where $C_{f,o}$ is the concentration at the start of the depuration period. 50 % depuration will then be reached at the time (t_{50}):

$$\frac{C_f}{C_{f,o}} = \frac{1}{2} = e^{-k_2 t_{50}} \text{ or } t_{50} = \frac{0{,}693}{k_2}$$

Similarly 95 % depuration will be reached at:

$$t_{95} = \frac{3,0}{k_2}$$

If 80 % uptake is used for the first period ($1,6/k_2$) and 95 % loss in the depuration phase ($3,0/k_2$), then the depuration phase is approximately twice the duration of the uptake phase.

It is important to note, however, that the estimations are based on the assumption that uptake and depuration patterns will follow first order kinetics. If first order kinetics are obviously not obeyed, more complex models should be employed (e.g. ref (1)).

Literature (of Appendix 3)

(1) Spacie A. and Hamelink J.L. (1982) Alternative models for describing the bioconcentration of organics in fish. Environ. Toxicol. and Chem. 1, pp 309-320.

(2) Kristensen P. (1991) Bioconcentration in fish: comparison of BCF's derived from OECD and ASTM testing methods; influence of particulate matter to the bioavailability of chemicals. Danish Water Quality Institute.

(3) Chiou C.T. and Schmedding D.W. (1982) Partitioning of organic compounds in octanol-water systems. Environ. Sci. Technol. 16 (1), pp 4-10.

(4) Hawker D.W. and Connell D.W. (1988) Influence of partition coefficient of lipophilic compounds on bioconcentration kinetics with fish. Wat. Res. 22 (6), pp 701-707.

(5) Branson D.R., Blau G.E., Alexander H.C. and Neely W.B. (1975) Transactions of the American Fisheries Society, 104 (4), pp 785-792.

(6) Ernst W. (1985) Accumulation in Aquatic organisms. In: Appraisal of tests to predict the environmental behaviour of chemicals. Ed. by Sheehman P., Korte F., Klein W. and Bourdeau P.H. Part 4.4 pp 243-255. SCOPE, 1985, John Wiley & Sons Ltd. N.Y.

(7) Reilly P.M., Bajramovic R., Blau G.E., Branson D.R. and Sauerhoff M.W. (1977) Guidelines for the optimal design of experiments to estimate parameters in first order kinetic models, Can. J. Chem. Eng. 55, pp 614-622.

(8) Könemann H. and Van Leeuwen K. (1980) Toxicokinetics in fish: Accumulation and Elimination of six Chlorobenzenes by Guppies. Chemosphere, 9, pp 3-19.

Appendix 4

Theoretical example of sampling schedule for bioconcentration tests of substances with log P_{ow} = 4

Fish sampling	Sample time schedule		No of water samples	No of fish per sample
	Minimal required frequency (days)	Additional sampling		
Uptake phase	-1 0		2 (*) 2	add 45-80 fish
1st	0,3	0,4	2 (2)	4 (4)
2nd	0,6	0,9	2 (2)	4 (4)
3rd	1,2	1,7	2 (2)	4 (4)
4th	2,4	3,3	2 (2)	4 (4)
5th	4,7		2	6
Depuration phase				Transfer fish to water free of test chemical
6th	5,0	5,3		4 (4)
7th	5,9	7,0		4 (4)
8th	9,3	11,2		4 (4)
9th	14,0	17,5		6 (4)

(*) Sample water after minimum of 3 'chamber-volume' have been delivered.

Values in brackets are numbers of samples (water, fish) to be taken if additional sampling is carried out.

Note: pre-test estimate of k_2 for log P_{ow} of 4,0 is 0,652 $days^{-1}$. The total duration of the experiment is set to 3 × up = 3 × 4,6 days, i.e. 14 days. For the estimation of 'up' refer to Appendix 3.

Appendix 5

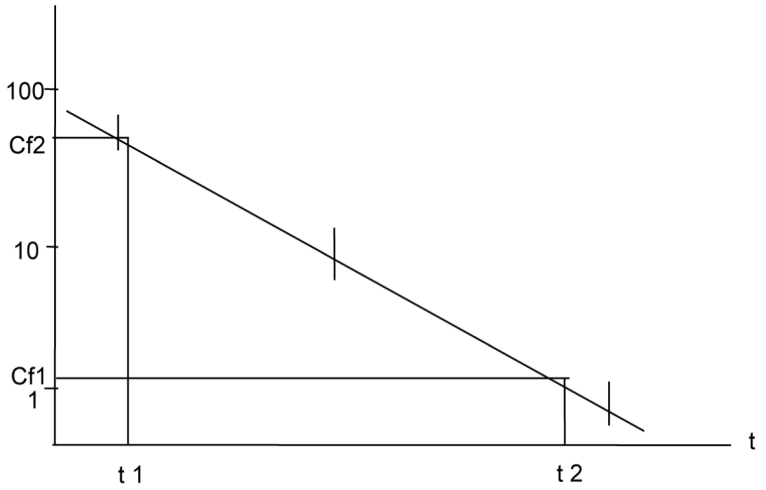
Model discrimination

Most bioconcentration data have been assumed to be 'reasonably' well described by a simple two-compartment/two-parameter model, as indicated by the rectilinear curve which approximates to the points for concentrations in fish, during the depuration phase, when these are plotted on semi-log paper. (Where these points cannot be described by a rectilinear curve then more complex models should be employed, see, for example, Spacie and Hamelink, Ref 1 in Appendix 3).

Graphical method for determination of depuration (loss) rate constant k_2

Plot the concentration of the test substance found in each sample of fish against sampling time on semi-log paper. The slope of that line is k_2.

$$k_2 = \frac{\ln\left(C_{f1}/C_{f2}\right)}{t_2 - t_1}$$



Note that deviations from straight line may indicate a more complex depuration pattern than first order kinetics. A graphical method may be applied for resolving types of depuration deviating from first order kinetics.

Graphical method for determination of uptake rate constant k_1

Given K_2, calculate k_1 as follows:

$$k_1 = \frac{C_f k_2}{C_w \times \left(1 - e^{-k_2 t}\right)} \qquad \text{[equation 1]}$$

The value of C_f is read from the midpoint of the smooth uptake curve produced by the data when log concentration is plotted versus time (on an arithmetical scale).

Computer method for calculation of uptake and depuration (loss) rate constants

The preferred means for obtaining the bioconcentration factor and k_1 and k_2 rate constants is to use non-linear parameter estimation methods on a computer. These programs find values for k_1 and k_2 given a set of sequential time concentration data and the model:

$$C_f = C_w \cdot \frac{k_1}{k_2} \times \left(1 - e^{-k_2 t}\right) \quad 0 < t < t_c \qquad \text{[equation 2]}$$

$$C_f = C_w \cdot \frac{k_1}{k_2} \times \left(e^{-k_2(t-t_c)} - e^{-k_2 t}\right) \quad t > t_c$$ [equation 3]

where t_c = time at the end of the uptake phase.

This approach provides standard deviation estimates of k_1 and k_2.

As k_2 in most cases can be estimated from the depuration curve with relatively high precision, and because a strong correlation exists between the two parameters k_1 and k_2 if estimated simultaneously, it may be advisable first to calculate k_2 from the depuration data only, and subsequently calculate k_1 from the uptake data using non-linear regression.

C.14. FISH JUVENILE GROWTH TEST

1. METHOD

This growth toxicity test method is a replicate of the OECD TG 215 (2000).

1.1. INTRODUCTION

This test is designed to assess the effects of prolonged exposure to chemicals on the growth of juvenile fish. It is based on a method, developed and ring-tested (1)(2) within the European Union, for assessing the effects of chemicals on the growth of juvenile rainbow trout (*Oncorynchus mykiss*) under flow-through conditions. Other well documented species may be used. For example, experience has been gained from growth tests with zebrafish (*Danio rerio*) ([1]) (3)(4) and ricefish (medaka, *Oryzias latipes*) (5)(6)(7).

See also General introduction Part C.

1.2. DEFINITIONS

Lowest observed effect concentration (LOEC): is the lowest tested concentration of a test substance at which the substance is observed to have a significant effect (at $p < 0,05$) when compared with the control. However, all test concentrations above the LOEC must have a harmful effect equal to or greater than those observed at the LOEC.

No observed effect concentration (NOEC): is the test concentration immediately below the LOEC.

EC_x: in this test method is the concentration of the test substance which causes a x % variation in growth rate of the fish when compared with controls.

Loading rate: is the wet weight of fish per volume of water.

Stocking density: is the number of fish per volume of water.

Individual fish specific growth rate: expresses the growth rate of one individual based on its initial weight.

Tank-average specific growth rate: expresses the mean growth rate of a tank population at one concentration.

Pseudo specific growth rate: expresses the individual growth rate compared to the mean initial weight of the tank population.

1.3. PRINCIPLE OF THE TEST METHOD

Juvenile fish in exponential growth phase are placed, after being weighted, in test chambers and are exposed to a range of sublethal concentrations of the test substance dissolved in water preferably under flow-through, or, if not possible, under appropriate semi-static (static-renewal) conditions. The test duration is 28 days. Fish are fed daily. The food ration is based on initial fish weights and may be recalculated after 14 days. At the end of the test, the fish are weighed again. Effects on growth rates are analysed using a regression model in order to estimate the concentration that would cause a x % variation in growth rate, i.e. EC_x (e.g. EC_{10}, EC_{20}, or EC_{30}). Alternatively, the data may be compared with control values in order to determine the lowest observed effect concentration (LOEC) and hence the no observed effect concentration (NOEC).

1.4. INFORMATION ON THE TEST SUBSTANCE

Results of an acute toxicity test (see Test Method C. 1.) preferably performed with the species chosen for this test, should be available. This implies that the water solubility and the vapour pressure of the test substance are known and a reliable analytical method is available for the quantification of the substance in the test solutions with known and reported accuracy and limit of detection is available.

([1]) Meyer, A., Bierman, C.H. and Orti, G. (1993). The phylogenetic position of the zebrafish (*Danio rerio*), a model system in developmental biology: an invitation to the comparative method. Proc. R. Soc. Lond. B. 252, 231-236.

Useful information includes the structural formula, purity of the substance, stability in water and light, pK_a, P_{ow} and results of a test for ready biodegradability (see Test Method C.4).

1.5. VALIDITY OF THE TEST

For the test to be valid the following conditions apply:

— the mortality in the control(s) must not exceed 10 % at the end of the test;

— the mean weight of fish in the control(s) must have increased enough to permit the detection of the minimum variation of growth rate considered as significant. A ring-test (2) has shown that for rainbow trout the mean weight of fish in the controls must have increased by at least the half (i.e. 50 %) of their mean initial weight over 28 days; e.g. initial weight: 1 g/fish (= 100 %), final weight after 28 days: ≥ 1,5 g/fish (≥ 150 %);

— the dissolved oxygen concentration must have been at least 60 % of the air saturation value (ASV) throughout the test;

— the water temperature must not differ by more than ± 1 °C between test chambers at any one time during the test and should be maintained within a range of 2 °C within the temperature ranges specified for the test species (Appendix 1).

1.6. DESCRIPTION OF THE TEST METHOD

1.6.1. **Apparatus**

Normal laboratory equipment and especially the following:

— oxygen and pH meters;

— equipment for determination of water hardness and alkalinity;

— adequate apparatus for temperature control and preferably continuous monitoring;

— tanks made of chemically inert material and of suitable capacity in relation to the recommended loading and stocking density (see Section 1.8.5 and Appendix 1);

— suitably accurate balance (i.e. accurate to ± 0,5 %).

1.6.2. **Water**

Any water in which the test species shows suitable long-term survival and growth may be used as a test water. It should be of constant quality during the period of the test. The pH of the water should be within the range 6,5 to 8,5, but during a given test it should be within a range of ± 0,5 pH units. Hardness above 140 mg/l (as $CaCO_3$) is recommended. In order to ensure that the dilution water will not unduly influence the test result (for example by complexion of test substance), samples should be taken at intervals for analysis. Measurements of heavy metals (e.g. Cu, Pb, Zn, Hg, Cd and Ni), major anions and cations (e.g. Ca, Mg, Na, K, Cl and SO_4), pesticides (e.g. total organophosphorus and total organochlorine pesticides), total organic carbon and suspended solids should be made, for example, every three months where a dilution water is known to be relatively constant in quality. If water quality has been demonstrated to be constant over at least one year, determinations can be less frequent and intervals extended (e.g. every 6 months). Some chemical characteristics of an acceptable dilution water are listed in Appendix 2.

1.6.3. **Test solutions**

Test solutions of the chosen concentrations are prepared by dilution of a stock solution.

The stock solution should preferably be prepared by simply mixing or agitating the test substance in the diluition water by using mechanical means (e.g. stirring or ultrasonication). Saturation columns (solubility columns) can be used for achieving a suitable concentrated stock solution.

The use of solvents or dispersants (solubilising agents) may be required in some cases in order to produce a suitably concentrated stock solution. Examples of suitable solvents are acetone, ethanol, methanol, dimethylsulfoxide, dimethylformamide and triethyleneglycol. Examples of suitable dispersants are Cremophor RH40, Tween 80, Methylcellulose 0,01 % and HCO-40. Care should be taken when using readily biodegradable agents (e.g. acetone) and/or highly volatile compounds as these can cause problems with bacterial built-up in flow-through tests. When a solubilising agent is used it must have no significant effects on the fish growth nor visible adverse effects on the juvenile as revealed by a solvent-only control.

For flow-through tests, a system which continually dispenses and dilutes a stock solution of the test substance (e.g. metering pump, proportional diluter, saturator system) is required to deliver a series of concentrations to the test chambers. The flow rates of stock solutions and dilution water should be checked at intervals, preferably daily, during the test and should not vary by more than 10 % throughout the test. A ring-test (2) has shown that, for rainbow trout, a frequency of water removal during the test of six litres/g of fish/day is acceptable (see Section 1.8.2.2).

For semi-static (renewal) tests, the frequency of medium renewal will depend on the stability of the test substance, but a daily water renewal is recommended. If, from preliminary stability tests (see Section 1.4), the test substance concentration is not stable (i.e. outside the range 80-120 % of nominal or falling below 80 % of the measured initial concentration) over the renewal period, consideration should be given to the use of a flow-through test.

1.6.4. **Selection of species**

Rainbow trout (*Oncorhynchus mykiss*) is the recommended species for this test since most experience has been gained from ring-test with this species (1)(2). However, other well documented species can be used but the test procedure may have to be adapted to provide suitable test conditions. For example, experience is also available with zebrafish (*Danio rerio*) (3)(4) and ricefish (medaka, *Oryzias latipes*) (5)(6)(7). The rationale for the selection of the species and the experimental method should be reported in this case.

1.6.5. **Holding of fish**

The test fish shall be selected from a population of a single stock, preferably from the same spawning, which has been held for at least two weeks prior to the test under conditions of water quality and illumination similar to those used in the test. They should be fed a minimum ration of 2 % body weight per day and preferably 4 % body weight per day throughout the holding period and during the test.

Following a 48 h setting-in period, mortalities are recorded and the following criteria applied:

— mortalities of greater than 10 % of population in seven days: reject the entire batch;

— mortalities of between 5 % and 10 % of population: acclimation for seven additional days; if more than 5 % mortality during second seven days, reject the entire batch;

— mortalities of less than 5 % of population in seven days: accept the batch.

Fish should not receive treatment for disease in the two weeks preceding the test, or during the test.

1.7. TEST DESIGN

The 'test design' relates to the selection of the number and spacing of the test concentrations, the number of tanks at each concentration level and the number of fish per tank. Ideally, the test design should be chosen with regard to:

— the objective of the study;

— the method of statistical analysis that will be used;

— the availability and cost of experimental resources.

The statement of the objective should, if possible, specify the statistical power at which a given size of difference (e.g. in growth rate) is required to be detected or, alternatively, the precision with which the EC_x (e.g. with x = 10, 20, or 30, and preferably not less than 10) is required to be estimated. Without this, a firm prescription of the size of the study cannot be given.

It is important to recognise that a design which is optimal (makes best use of resources) for use with one method of statistical analysis is not necessarily optimal for another. The recommended design for the estimation of a LOEC/NOEC would not therefore be the same as that recommended for analysis by regression.

In most of cases, regression analysis is preferable to the analysis of variance, for reasons discussed by Stephan and Rogers (8). However, when no suitable regression model is found ($r^2 < 0,9$) NOEC/LOEC should be used.

1.7.1. **Design for analysis by regression**

The important considerations in the design of a test to be analysed by regression are:

— The effect concentration (e.g. $EC_{10,20,30}$) and the concentration range over which the effect of the test substance is of interest, should necessarily be spanned by the concentrations included in the test. The precision with which estimates of effect concentrations can be made, will be best when the effect concentration is in the middle of the range of concentrations tested. A preliminary range-finding test may be helpful in selecting appropriate test concentrations.

— To enable satisfactory statistical modelling, the test should include at least one control tank and five additional tanks at different concentrations. Where appropriate, when a solubilising agent is used, one control containing the solubilising agent at the highest tested concentration should be run in addition to the test series (see Sections 1.8.3 and 1.8.4).

— An appropriate geometric series or logarithmic series (9) (see Appendix 3) may be used. Logarithmic spacing of test concentration is to be preferred.

— If more than six tanks are available, the additional tanks should either be used to provide replication or distributed across the range of concentrations in order to enable closer spacing of the levels. Either of these measures are equally desirable.

1.7.2. **Design for estimation of a NOEC/LOEC using Analysis of Variance (ANOVA)**

There should preferably be replicate tanks at each concentration, and statistical analysis should be at the tank level (10). Without replicate tanks, no allowance can be made for variability between tanks beyond that due to individual fish. However, experience has shown (11) that between-tank variability was very small compared with within-tank (i.e. between-fish) variability in the case examined. Therefore a relatively acceptable alternative is to perform statistical analysis at the level of individual fish.

Conventionally, at least five test concentrations in a geometric series with a factor preferably not exceeding 3,2 are used.

Generally, when tests are performed with replicate tanks, the number of replicate control tanks and therefore the number of fish should be the double of the number in each of the test concentrations, which should be of equal size (12)(13)(14). On the opposite, in absence of replicate tanks, the number of fish in the control group should be the same as the number in each test concentration.

If the ANOVA is to be based on tanks rather than individual fish (which would entail either individual marking of the fish or the use of 'pseudo' specific growth rates (see Section 2.1.2)), there is a need for enough replication of tanks to enable the standard deviation of 'tanks-within-concentrations' to be determined. This means that the degrees of freedom for error in the analysis of variance should be at least 5 (10). If only the controls are

replicated, there is a danger that the error variability will be biased because it may increase with the mean value of the growth rate in question. Since growth rate is likely to decrease with increasing concentration, this will tend to lead to an overestimate of the variability.

1.8. PROCEDURE

1.8.1. **Selection and weighing of test fish**

It is important to minimise variation in weight of the fish at the beginning of the test. Suitable size ranges for the different species recommended for use in this test are given in Appendix 1. For the whole batch of fish used in the test, the range in individual weights at the start of the test should ideally be kept to within ± 10 % of the arithmetic mean weight and, in any case, should not exceed 25 %. It is recommended to weight a subsample of fish before the test in order to estimate the mean weigh.

Food should be withheld from the stock population for 24 h prior to the start of the test. Fish should then be chosen at random. Using a general anaesthetic (e.g. an aqueous solution of 100 mg/l tricaine methane sulphonate (MS 222) neutralised by the addition of two parts of sodium bicarbonate per part of MS 222), fish should be weighted individually as wet weights (blotted dry) to the precision given in Appendix 1. Those fish with weights within the intended range should be retained and then should be randomly distributed between the test vessels. The total wet weight of fish in each test vessel should be recorded. The use of anaesthetics likewise handling of fish (including blotting and weighing) may cause stress and injuries to the juvenile fish, in particular for those species of small size. Therefore handling of juvenile fish must be done with the utmost care to avoid stressing and injuring test animals.

The fish are weighed again on day 28 of the test (see Section 1.8.6). However, if it is deemed necessary to recalculate the food ration, fish can be weighed again on day 14 of the test (see Section 1.8.2.3). Other method as photographic method could be used to determine changes in fish size from which food rations could be adjusted.

1.8.2. **Conditions of exposure**

1.8.2.1. *Duration*

The test duration is ≥ 28 days.

1.8.2.2. *Loading rates and stocking densities*

It is important that the loading rate and stocking density is appropriate for the test species used (see Appendix 1). If the stocking density is too high, then overcrowding stress will occur leading to reduced growth rates and possibly to disease. If it is too low, territorial behaviour may be induced which could also affect growth. In any case, the loading rate should be low enough in order that a dissolved oxygen concentration of at least 60 % ASV can be maintained without aeration. A ring-test (2) has shown that, for rainbow trout, a loading rate of 16 trouts of 3-5 g in a 40-litre volume is acceptable. Recommended frequency of water removal during the test is 6 litres/g of fish/day.

1.8.2.3. *Feeding*

The fish should be fed with an appropriate food (Appendix 1) at a sufficient rate to induce acceptable growth rate. Care should be taken to avoid microbial growth and water turbidity. For rainbow trout, a rate of 4 % of their body weight per day is likely to satisfy these conditions (2)(15)(16)(17). The daily ration may be divided into two equal portions and given to the fish in two feeds per day, separated by at least 5 h. The ration is based on the initial total fish weight for each test vessel. If the fish are weighted again on day 14, the ration is then recalculated. Food should be withheld from the fish 24 h prior to weighing.

Uneaten food and fecal material should be removed from the test vessels each day by carefully cleaning the bottom of each tank using a suction.

1.8.2.4. *Light and temperature*

The photoperiod and water temperature should be appropriate for the test species (Appendix 1).

1.8.3. **Test concentrations**

Normally five concentrations of the test substance are required, regardless of the test design (see Section 1.7.2). Prior knowledge of the toxicity of the test substance (e.g. from an acute test and/or from range-finding studies) should help in selecting appropriate test concentrations. Justification should be given if fewer than five concentrations are used. The highest tested concentration should not exceed the substance solubility limit in water.

Where a solubilising agent is used to assist in stock solution preparation, its final concentration should not be greater than 0,1 ml/l and should preferably be the same in all test vessels (see Section 1.6.3). However, every effort should be made to avoid use of such materials.

1.8.4. **Controls**

The number of dilution-water controls depends on the test design (see Sections 1.7-1.7.2). If a solubilising agent is used, then the same number of solubilising-agent controls as dilution-water controls should also be included.

1.8.5. **Frequency of analytical determinations and measurements**

During the test, the concentrations of test substance are determined at regular intervals (see below).

In flow-through tests, the flow rates of diluent and toxicant stock solution should be checked at intervals, preferably daily, and should not vary by more than 10 % throughout the test. Where the test substance concentrations are expected to be within ± 20 % of the nominal values (i.e. within the range 80-120 %; see Sections 1.6.2 and 1.6.3), it is recommended that, as a minimum, the highest and lowest test concentrations be analysed at the start of the test and at weekly intervals thereafter. For the test where the concentration of the test substance is not expected to remain within ± 20 % of nominal (on the basis of stability data of the test substance), it is necessary to analyse all test concentrations, but following the same regime.

In semi-static (renewal) tests where the concentration of the test substance is expected to remain within ± 20 % of the nominal values, it is recommended that, as a minimum, the highest and lowest test concentrations be analysed when freshly prepared and immediately prior to renewal at the start of the study and weekly thereafter. For tests where the concentration of the test substance is not expected to remain within ± 20 % of nominal, all test concentrations must be analysed following the same regime as for more stable substances.

It is recommended that results be based on measured concentrations. However, if evidence is available to demonstrate that the concentration of the test substance in solution has been satisfactorily maintained within ± 20 % of the nominal or measured initial concentration throughout the test, then the results can be based on nominal or measured values.

Samples may need to be filtered (e.g. using a 0,45 μm pore size) or centrifuged. Centrifugation is the recommended procedure. However, if the test material does not adsorb to filters, filtration may also be acceptable.

During the test, dissolved oxygen, pH and temperature should be measured in all test vessels. Total hardness, alkalinity and salinity (if relevant) should be measured in the controls and one vessel at the highest concentration. As a minimum, dissolved oxygen and salinity (if relevant) should be measured three times (at the beginning, middle and end of the test). In semi-static tests, it is recommended that dissolved oxygen be measured more frequently, preferably before and after each water renewal or at least once a week. pH should be measured at the beginning and end of each water renewal in static renewal test and at least weekly in flow-through tests. Hardness and alkalinity should be measured once each test. Temperature should preferably be monitored continuously in at least one test vessel.

1.8.6. **Observations**

Weight: at the end of the test all surviving fish must be weighed as wet weights (blotted dry) either in groups by test vessel or individually. Weighing of animals by test vessel is preferred to individual weights which require that fish be individually marked. In the case of the measurement of individual weights for determination of individual fish specific growth rate, the marking technique selected should avoid stressing the animals (alternatives to freeze marking may be appropriate, e.g. the use of coloured fine fishing line).

The fish should be examined daily during the test period and any external abnormalities (such as hemorrhage, discoloration) and abnormal behaviour noted. Any mortalities should be recorded and the dead fish removed as soon as possible. Dead fish are not replaced, the loading rate and stocking density being sufficient to avoid effects on growth through changes in number of fish per tank. However, the feeding rate will need to be adjusted.

2. DATA AND REPORTING

2.1. TREATMENT OF RESULTS

It is recommended that a statistician be involved in both the design and analysis of the test since this test method allows for considerable variation in experimental design as for example, in the number of test chambers, number of test concentrations, number of fish, etc. In view of the options available in test design, specific guidance on statistical procedure is not given here.

Growth rates should not be calculated for test vessels where the mortality exceeds 10 %. However, mortality rate should be indicated for all test concentrations.

Whichever method is used to analyse the data, the central concept is the specific growth rate r between time t_1 and time t_2. This can be defined in several ways depending on whether fish are individually marked or not or whether a tank average is required.

$$r_1 = \frac{\log_e w_2 - \log_e w_1}{t_2 - t_1} \times 100$$

$$r_2 = \frac{\log_e w_2 - \log_e w_1}{t_2 - t_1} \times 100$$

$$r_3 = \frac{\log_e w_2 - \log_e w_1}{t_2 - t_1} \times 100$$

where:

r_1 = individual fish specific growth rate
r_2 = tank-average specific growth rate
r_3 = 'pseudo' specific growth rate
w_1, w_2 = weights of a particular fish at times t_1 and t_2, respectively
$\log_e w_1$ = logarithm of the weight of an individual fish at the start of the study period
$\log_e w_2$ = logarithm of the weight of an individual fish at the end of the study period
$\log_e w_1$ = average of the logarithms of the values w_1 for the fish in the tank at the start of the study period
$\log_e w_2$ = average of the logarithms of the values w_2 for the fish in the tank at the end of the study period
t_1, t_2 = time (days) at start and end of study period

r_1, r_2, r_3 can be calculated for the 0-28 days period and, where appropriate (i.e. when measurement at day 14 has been done) for the 0-14 and 14-28 days periods.

2.1.1. Analysis of results by regression (concentration-response modelling)

This method of analysis fits a suitable mathematical relationship between the specific growth rate and concentration, and hence enables the estimation of the 'EC_x' i.e. any required EC value. Using this method the calculation of r for individual fish (r_1) is not necessary and instead, the analysis can be based on the tank-average value of r (r_2). This last method is preferred. It is also more appropriate in case of the use of smallest species.

The tank-average specific growth rates (r_2) should be plotted graphically against concentration, in order to inspect the concentration response relationship.

For expressing the relationship between r_2 and concentration, an appropriate model should be chosen and its choice must be supported by appropriate reasoning.

If the numbers of fish surviving in each tank are unequal, then the process of model fitting, whether simple or non-linear, should be weighted to allow for unequal sizes of groups.

The method of fitting the model must enable an estimate of, for example, the EC_{20} and of its dispersion (either standard error or confidence interval) to be derived. The graph of the fitted model should be shown in relation to the data so that the adequacy of the fit of the model can be seen (8)(18)(19)(20).

2.1.2. **Analysis of results for the estimation of the LOEC**

If the test has included replication of tanks at all concentration levels, the estimation of the LOEC could be based on an analysis of variance (ANOVA) of the tank-average specific growth rate (see Section 2.1), followed by a suitable method (e.g. Dunnett's or Williams' test (12)(13)(14)(21)) of comparing the average r for each concentration with the average r for the controls to identify the lowest concentration for which this difference is significant at a 0,05 probability level. If the required assumptions for parametric methods are not met — non-normal distribution (e.g. Shapiro-Wilk's test) or heterogeneous variance (Bartlett's test), consideration should be given to transforming the data to homogenise variances prior to performing the ANOVA, or to carrying out a weighted ANOVA.

If the test has not included replication of tanks at each concentration, an ANOVA based on tanks will be insensitive or impossible. In this situation, an acceptable compromise is to base the ANOVA on the 'pseudo' specific growth rate r_3 for individual fish.

The average r_3 for each test concentration may then be compared with the average r_3 for the controls. The LOEC can then be identified as before. It must be recognised that this method provides no allowance for, nor protection against, variability between tanks, beyond that which is accounted for by the variability between individual fish. However, experience has shown (8) that between-tank variability was very small compared with within-tank (i.e. between fish) variability. If individual fish are not included in the analysis, the method of outlier identification and justification for its use must be provided.

2.2. INTERPRETATION OF RESULTS

The results should be interpreted with caution where measured toxicant concentrations in test solutions occur at levels near the detection limit of the analytical method or, in semi static tests, when the concentration of the test substance decreases between freshly prepared solution and before renewal.

2.3. TEST REPORT

The test report must include the following information:

2.3.1. **Test substance:**

— physical nature and relevant physical-chemical properties;

— chemical identification data including purity and analytical method for quantification of the test substance where appropriate.

2.3.2. **Test species:**

— scientific name, possibly

— strain, size, supplier, any pre-treatment, etc.

2.3.3. **Test conditions:**

— test procedure used (e.g. semi-static/renewal, flow-through, loading, stocking density, etc.),

— test design (e.g. number of test vessels, test concentrations and replicates, number of fish per vessel),

— method of preparation of stock solutions and frequency of renewal (the solubilising agent and its concentration must be given, when used),

— the nominal test concentrations, the means of the measured values and their standard deviations in the test vessels and the method by which these were attained and evidence that the measurements refer to the concentrations of the test substance in true solution,

— dilution water characteristics: pH, hardness, alkalinity, temperature, dissolved oxygen concentration, residual chlorine levels (if measured), total organic carbon, suspended solids, salinity of the test medium (if measured) and any other measurements made,

— water quality within test vessels: pH, hardness, temperature and dissolved oxygen concentration,

— detailed information on feeding, (e.g. type of food(s), source, amount given and frequency).

2.3.4. **Results:**

— evidence that controls met the validity criterion for survival, and data on mortalities occurring in any of the test concentrations,

— statistical analytical techniques used, statistics based on replicates or fish, treatment of data and justification of techniques used,

— tabulated data on individual and mean fish weights on days 0, 14 (if measured) and 28 values of tank-average or pseudo specific growth rates (as appropriate) for the periods 0-28 days or possibly 0-14 and 14-28,

— results of the statistical analysis (i.e. regression analysis or ANOVA) preferably in tabular and graphical form and the LOEC (p = 0,05) and the NOEC or ECx with, when possible, standard errors, as appropriate,

— incidence of any unusual reactions by the fish and any visible effects produced by the test substance.

3. REFERENCES

(1) Solbe J.F. de LG (1987) Environmental Effects of Chemicals (CFM 9350 SLD). Report on a UK Ring Test of a Method for Studying the Effects of Chemicals on the Growth rate of Fish. WRc Report No PRD 1388-M/2.

(2) Ashley S., Mallett M.J. and Grandy N.J., (1990) EEC Ring Test of a Method for Determining the Effects of Chemicals on the Growth Rate of Fish. Final Report to the Commission of the European Communities. WRc Report No EEC 2600-M.

(3) Crossland N.O. (1985) A method to evaluate effects of toxic chemicals on fish growth. Chemosphere, 14, p. 1855-1870.

(4) Nagel R., Bresh H., Caspers N., Hansen P.D., Market M., Munk R., Scholz N. and Höfte B.B., (1991) Effect of 3,4-dichloroaniline on the early life stages of the Zebrafish (*Brachydanio rerio*): results of a comparative laboratory study. Ecotox. Environ. Safety, 21, p. 157-164.

(5) Yamamoto, Tokio., (1975) Series of stock cultures in biological field. Medaka (killifish) biology and strains. Keigaku Publish. Tokio, Japan.

(6) Holcombe, G.W., Benoit D.A., Hammermeister, D.E., Leonard, E.N. and Johnson, R.D., (1995) Acute and long-term effects of nine chemicals on the Japanese medaka (*Oryzias latipes*).Arch. Environ. Conta. Toxicol. 28, p. 287-297.

(7) Benoit, D.A., Holcombe, G.W. and Spehar, R.L., (1991) Guidelines for conducting early life toxicity tests with Japanese medaka (*Oryzias latipes*). Ecological Research Series EPA-600/3-91-063. U.S. Environmental Protection Agency, Duluth, Minesota.

(8) Stephan C.E. and Rogers J.W., (1985) Advantages of using regression analysis to calculate results of chronic toxicity tests. Aquatic Toxicology and Hazard Assessment: Eighth Symposium, ASTM STP 891, R C Bahner and D J Hansen, Eds., American Society for Testing and Materials, Philadelphia, p. 328-338.

(9) Environment Canada, (1992) Biological test method: toxicity tests using early life stages of salmonid fish (rainbow trout, coho salmon, or Atlantic salmon). Conservation and Protection, Ontario, Report EPS 1/RM/28, p. 81.

(10) Cox D.R., (1958) Planning of experiments. Wiley Edt.

(11) Pack S., (1991) Statistical issues concerning the design of tests for determining the effects of chemicals on the growth rate of fish. Room Document 4, OECD Ad Hoc Meeting of Experts on Aquatic Toxicology, WRc Medmenham, UK, 10-12 December 1991.

(12) Dunnett C.W., (1955) A Multiple Comparisons Procedure for Comparing Several Treatments with a Control, J. Amer. Statist. Assoc., 50, p. 1096-1121.

(13) Dunnett C.W., (1964) New tables for multiple comparisons with a control. Biometrics, 20, p. 482-491.

(14) Williams D.A., (1971) A test for differences between treatment means when several dose levels are compared with a zero dose control. Biometrics 27, p. 103-117.

(15) Johnston, W.L., Atkinson, J.L., Glanville N.T., (1994) A technique using sequential feedings of different coloured food to determine food intake by individual rainbow trout, *Oncorhynchus mykiss*: effect of feeding level. Aquaculture 120, p. 123-133.

(16) Quinton, J. C. and Blake, R.W., (1990) The effect of feed cycling and ration level on the compensatory growth response in rainbow trout, *Oncorhynchus mykiss*. Journal of Fish Biology, 37, p. 33-41.

(17) Post, G., (1987) Nutrition and Nutritional Diseases of Fish. Chapter IX in Testbook of Fish Health. T.F.H. Publications, Inc. Neptune City, New Jersey, USA. p. 288.

(18) Bruce, R.D. and Versteeg D.J., (1992) A statistical procedure for modelling continuous toxicity data. Environ. Toxicol. Chem. 11, p. 1485-1494.

(19) DeGraeve, G.M., Cooney, J.M., Pollock, T.L., Reichenbach, J.H., Dean, Marcus, M.D. and McIntyre, D.O., (1989) Precision of EPA seven-day fathead minnow larval survival and growth test; intra and interlaboratory study. report EA-6189 (American Petroleum Institute Publication, No 4468). Electric Power Research Institute, Palo alto, CA.

(20) Norbert-King T.J., (1988) An interpolation estimate for chronic toxicity: the ICp approach. US Environmental Protection Agency. Environmental Research Lab., Duluth, Minesota. Tech. Rep. No 05-88 of National Effluent Toxicity Assesment Center. Sept. 1988. p. 12.

(21) Williams D.A., (1972) The comparison of several dose levels with a zero dose control. Biometrics 28, p. 510-531.

Appendix 1

FISH SPECIES RECOMMENDED FOR TESTING AND SUITABLE TEST CONDITIONS

Species	Recommended test temperature range (°C)	Photoperiod (hours)	Recommended range for initial fish weight (g)	Required measurement precision	Loading rate (g/l)	Stocking density (per litre)	Food	Test duration (days)
Recommended species:								
Oncorhynchus mykiss rainbow trout	12,5-16,0	12-16	1-5	to nearest 100 mg	1,2-2,0	4	Dry propietary salmonid fry food	≥ 28
Other well documented species:								
Danio rerio zebrafish	21-25	12-16	0,050-0,100	to nearest 1 mg	0,2-1,0	5-10	Live food (*Brachionus Artemia*)	≥ 28
Oryzias latipes ricefish (Medaka)	21-25	12-16	0,050-0,100	to nearest 1 mg	0,2-1,0	5-20	Live food (*Brachionus Artemia*)	≥ 28

Appendix 2

SOME CHEMICAL CHARACTERISTICS OF AN ACCEPTABLE DILUTION WATER

Substance	Concentrations
Particulate matter	< 20 mg/l
Total organic carbon	< 2 mg/l
Unionised ammonia	< 1 µg/l
Residual chlorine	< 10 µg/l
Total organophosphorus pesticides	< 50 ng/l
Total organochlorine pesticides plus polychlorinated biphenyls	< 50 ng/l
Total organic chlorine	< 25 ng/l

Appendix 3

Logarithmic series of concentrations suitable for toxicity test (9)

Column (Number of concentrations between 100 and 10, or between 10 and 1) (*)						
1	2	3	4	5	6	7
100	100	100	100	100	100	100
32	46	56	63	68	72	75
10	22	32	40	46	52	56
3,2	10	18	25	32	37	42
1,0	4,6	10	16	22	27	32
	2,2	5,6	10	15	19	24
	1,0	3,2	6,3	10	14	18
		1,8	4,0	6,8	10	13
		1,0	2,5	4,6	7,2	10
			1,6	3,2	5,2	7,5
			1,0	2,2	3,7	5,6
				1,5	2,7	4,2
				1,0	1,9	3,2
					1,4	2,4
					1,0	1,8
						1,3
						1,0

(*) A series of five (or more) successive concentrations may be chosen from a column. Mid-points between concentrations in column (x) are found in column (2x + 1). The values listed can represent concentrations expressed as percentage per volume or weight (mg/l or µg/l). Values can be multiplied or divided by any power of 10 as appropriate. Column 1 might be used if there was considerable uncertainty on the toxicity level.

C.15. FISH, SHORT-TERM TOXICITY TEST ON *EMBRYO* AND SAC-FRY STAGES

1. METHOD

This short-term toxicity test method is a replicate of the OECD TG 212 (1998).

1.1. INTRODUCTION

This short-term toxicity test on Fish Embryo and Sac-Fry stages is a short-term test in which the life stages from the newly fertilised egg to the end of the sac-fry stage are exposed. No feeding is provided in the embryo and sac-fry test, and the test should thus be terminated while the sac-fry are still nourished from the yolk-sac.

The test is intended to define lethal, and to a limited extent, sub-lethal effects of chemicals on the specific stages and species tested. This test would provide useful information in that is could (a) form a bridge between lethal and sub-lethal tests, (b) be used as a screening test for either a Full Early Life Stage test or for chronic toxicity tests and (c) be used for testing species where husbandry techniques are not sufficiently advanced to cover the period of change from endogenous to exogenous feeding.

It should be borne in mind that only tests incorporating all stages of the life-cycle of fish are generally liable to give an accurate estimate of the chronic toxicity of chemicals to fish, and that any reduced exposure with respect to life stages may reduce the sensitivity and thus underestimate the chronic toxicity. It is therefore expected that the embryo and sac-fry test would be less sensitive than a Full Early Life Stage test, particularly with respect to chemicals with high lipophilicity (log P_{ow} > 4) and chemicals with a specific mode of toxic action. However smaller differences in sensitivity between the two tests would be expected for chemicals with a non-specific, narcotic mode of action (1).

Prior to the publication of this test, most experience with this embryo and sac-fry test has been with the freshwater fish *Danio rerio* Hamilton-Buchanan (Teleostei, Cyprinidae — common name zebrafish). More detailed guidance on test performance for this species is therefore given in Appendix 1. This does not preclude the use of other species for which experience is also available (Table 1).

1.2. DEFINITIONS

Lowest Observed Effect Concentration (LOEC): is the lowest tested concentration of a test substance at which the substance is observed to have a significant effect (at $p < 0,05$) when compared with the control. However, all test concentrations above the LOEC must have a harmful effect equal to or greater than those observed at the LOEC.

No Observed Effect Concentration (NOEC): is the test concentration immediately below the LOEC.

1.3. PRINCIPLE OF THE TEST

The embryo and sac-fry stages of fish are exposed to a range of concentrations of the test substance dissolved in water. Within the protocol a choice is possible between a semi-static and a flow-through procedure. The choice depends on the nature of the test substance. The test is begun by placing fertilised eggs in the test chambers and is terminated just before the yolk-sac of any larvae in any of the test chambers has been completely absorbed or before mortalities by starvation start in controls. Lethal and sub-lethal effects are assessed and compared with control values to determine the lowest observed effect concentration and hence the no observed effect concentration. Alternatively, they may be analysed using a regression model in order to estimate the concentration that would cause a given percentage effect (i.e. LC/ECx, where x is a defined % effect).

1.4. INFORMATION ON THE TEST SUBSTANCE

Results of an acute toxicity test (see Method C. 1) preferably performed with the species chosen for this test, should be available. The results may be useful in selecting an appropriate range of test concentrations in the early life stages test. Water solubility (including solubility in the test water) and the vapour pressure of the test substance should be known. A reliable analytical method for the quantification of the substance in the test solutions with known and reported accuracy and limit of detection should be available.

Information on the test substance which is useful in establishing the test conditions includes the structural formula, purity of the substance, stability in light, stability under the conditions of the test, pKa, P_{ow} and results of a test for ready biodegradability (see Method C. 4).

1.5. VALIDITY OF THE TEST

For a test to be valid, the following conditions apply:

— overall survival of fertilised eggs in the controls and where relevant, in the solvent-only vessels must be greater than or equal to the limits defined in Appendices 2 and 3

— the dissolved oxygen concentration must be between 60 and 100 % of the air saturation value (ASV) throughout the test

— the water temperature must not differ by more than ± 1,5 °C between test chambers or between successive days at any time during the test and should be within the temperature ranges specified for the test species (Appendices 2 and 3).

1.6. DESCRIPTION OF THE TEST METHOD

1.6.1. **Test chambers**

Any glass or other chemically inert vessels can be used. The dimensions of the vessels should be large enough to allow compliance with the loading rate (see Section 1.7.1.2). It is recommended that test chambers be randomly positioned in the test area. A randomised block design with each treatment being present in each block is preferable to a completely randomised design when there are systematic effects in the laboratory that can be controlled using blocking. Blocking, if used, should be taken account of in the subsequent data analysis. The test chambers should be shielded from unwanted disturbance.

1.6.2. **Selection of fish species**

Recommended fish species are given in Table 1A. This does not preclude the use of other species (examples are given in Table 1B), but the test procedure may have to be adapted to provide suitable test conditions. The rationale for the selection of the species and the experimental method should be reported in this case.

1.6.3. **Holding of the brood fish**

Details on holding the brood stock under satisfactory conditions may be found in OECD TG 210 ([1]) and in references (2)(3)(4)(5)(6).

1.6.4. **Handling of embryos and larvae**

Embryos and larvae may be exposed, within the main vessel, in smaller vessels fitted with mesh sides or ends to permit a flow of test solution through the vessel. Non-turbulent flow through these small vessels may be induced by suspending them from an arm arranged to move the vessel up and down but always keeping the organisms submerged; a siphon-flush system can also be used. Fertilised eggs of salmonid fishes can be supported on racks or meshes with apertures sufficiently large to allow larvae to drop through after hatching. The use of pasteur pipettes is appropriate to remove the embryos and larvaes in the semi-static tests with complete daily renewal (see paragraph 1.6.6)

Where egg containers, grids or meshes have been used to hold eggs within the main test vessel, these restraints should be removed after the larvae hatch ([1]), except that meshes should be retained to prevent the escape of the fish. If there is a need to transfer the larvae, they should not be exposed to the air and nets should not be used to release fish from egg containers (such a caution may not be necessary for some less fragile species, e.g. the carp). The timing of this transfer varies with the species and transfer may not always be necessary. For the semi-static technique, beakers or shallow containers may be used, and, if necessary, equipped with a mesh screen slightly elevated above the bottom of the beaker. If the volume of these containers is sufficient to comply with loading requirements, (see 1.7.1.2) no transfer of embryo or larvae may be necessary.

1.6.5. **Water**

Any water which conforms to the chemical characteristics of an acceptable dilution water as listed in Appendix 4 and in which the test species shows control survival at least as good as that described in Appendices 2 and 3 is suitable as a test water. It should be of constant quality during the period of the test. The pH should remain within a range of ± 0,5 pH units. In order to ensure that the dilution water will not unduly

([1]) OECD, Paris, 1992, Test Guideline 210, 'Fish, Early-life Stage Toxicity Test'.

influence the test result (for example by complexation of test substance), or adversely affect the performance of the brood stock, samples should be taken at intervals for analysis. Measurements of heavy metals (e.g. Cu, Pb, Zn, Hg, Cd and Ni), major anions and cations (e.g. Ca, Mg, Na, K, Cl and SO_4), pesticides (e.g. total organophosphorus and total organochlorine pesticides), total organic carbon and suspended solids should be made, for example, every three months, where a dilution water is known to be relatively constant in quality. If water quality has been demonstrated to be constant over at least one year, determinations can be less frequent and intervals extended (e.g. every six months).

1.6.6. **Test solutions**

Test solutions of the chosen concentrations are prepared by dilution of a stock solution.

The stock solution should preferably be prepared by simply mixing or agitating the test substance in the dilution water by using mechanical means (e.g. stirring and ultrasonication). Saturation columns (solubility columns) can be used for achieving a suitable concentrated stock solution. As far as possible, the use of solvents or dispersants (solubilising agents) should be avoided; however, such compounds may be required in some cases in order to produce a suitably concentrated stock solution. Examples of suitable solvents are acetone, ethanol, methanol, dimethylformamide and triethyleneglycol. Examples of suitable dispersants are Cremophor RH40, Tween 80, methylcellulose 0,01 % and HCO-40. Care should be taken when using readily biodegradable agents (e.g. acetone) and/or highly volatile as these can cause problems with bacterial built-up in flow-through tests. When a solubilising agent is used it must have no significant effect on survival nor visible adverse effect on the early-life stages as revealed by a solvent-only control. However, every effort should be made to avoid the use of such materials.

For the semi-static technique, two different renewal procedures may be followed; either (i) new test solutions are prepared in clean vessels and surviving eggs and larvae gently transferred into the new vessels in a small volume of old solution, avoiding exposure to air, or (ii) the test organisms are retained in the vessels whilst a proportion (at least three-quarters) of the test water is changed. The frequency of medium renewal will depend on the stability of the test substance, but a daily water renewal is recommended. If, from preliminary stability tests (see Section 1.4), the test substance concentration is not stable (i.e. outside the range 80-120 % of nominal or falling below 80 % of the measured initial concentration) over the renewal period, consideration should be given to the use of a flow-through test. In any case, care should be taken to avoid stressing the larvae during the water renewal operation.

For flow-through tests, a system which continually dispenses and dilutes a stock solution of the test substance (e.g. metering pump, proportional diluter, saturator system) is required to deliver a series of concentrations to the test chambers. The flow rates of stock solutions and dilution water should be checked at intervals, preferably daily, and should not vary by more than 10 % throughout the test. A flow rate equivalent to at least five test chamber volumes per 24 hours has been found suitable (2).

1.7. PROCEDURE

Useful information on the performance of fish embryo and sac-fry toxicity tests is available in the literature, some examples of which are included in the literature section of this text (7)(8)(9).

1.7.1. **Conditions of exposure**

1.7.1.1. *Duration*

The test should start preferably within 30 minutes after the eggs have been fertilised. The embryos are immersed in the test solution before, or as soon as possible after, commencement of the blastodisc cleavage stage and in any case before the onset of the gastrula stage. For eggs obtained from commercial supplier, it may not be possible to start the test immediately after fertilisation. As the sensitivity of the test may be seriously influenced by delaying the start of the test, the test should be initiated within eight hours after fertilisation. As larvae are not fed during the exposure period, the test should be terminated just before the yolk sac of any larvae in any of the test chambers has been completely absorbed or before mortalities by starvation start in controls. The duration will depend upon the species used. Some recommended durations are given in Appendices 2 and 3.

1.7.1.2. *Loading*

The number of fertilised eggs at the start of the test should be sufficient to meet statistical requirements. They should be randomly distributed among treatments, and at least 30 fertilised eggs, divided equally (or as equally as possible since it can be difficult to obtain equal batches when using some species) between at least three replicate test chambers, should be used per concentration. The loading rate (biomass per volume of test

solution) should be low enough in order that a dissolved oxygen concentration of at least 60 % ASV can be maintained without aeration. For flow-through tests, a loading rate not exceeding 0,5 g/l per 24 hours and not exceeding 5 g/l of solution at any time has been recommended (2).

1.7.1.3. *Light and temperature*

The photoperiod and test water temperature should be appropriate for the test species (Appendix 2 and 3). For the purpose of temperature monitoring, it may be appropriate to use an additional test vessel.

1.7.2. **Test concentrations**

Normally, five concentrations of the test substance spaced by a constant factor not exceeding 3,2 are required. The curve relating LC_{50} to period of exposure in the acute study should be considered when selecting the range of test concentrations. The use of fewer than five concentrations, for example in limit tests, and a narrower concentration interval may be appropriate in some circumstances. Justification should be provided if fewer than five concentrations are used. Concentrations of the substance higher than the 96 hour LC_{50} or 100 mg/l, whichever is the lower, need not be tested. Substances should not be tested above their solubility limit in the test water.

When a solubilising agent is used to aid preparation of test solutions (see Section 1.6.6), its final concentration in the test vessels should not be greater than 0,1 ml/l and should be the same in all test vessels.

1.7.3. **Controls**

One dilution-water control (replicated as appropriate) and also, if relevant, one control containing the solubilising-agent (replicated as appropriate) should be run in addition to the test series.

1.7.4. **Frequency of analytical determinations and measurements**

During the test, the concentrations of the test substance are determined at regular intervals.

In semi-static tests where the concentration of the test substance is expected to remain within ± 20 % of the nominal (i.e. within the range 80-120 %; see Section 1.4 and 1.6.6), it is recommended that, as a minimum, the highest and lowest test concentrations be analysed when freshly prepared and immediately prior to renewal on at least three occasions spaced evenly over the test (i.e. analyses should be made on a sample from the same solution — when freshly prepared and at renewal).

For tests where the concentration of the test substance is not expected to remain within ± 20 % of nominal (on the basis of stability data of the substance), it is necessary to analyse all test concentrations, when freshly prepared and at renewal, but following the same regime (i.e. on at least three occasions spaced evenly over the test). Determination of test substance concentrations prior to renewal need only be performed on one replicate vessel at each test concentration. Determinations should be made no more than seven days apart. It is recommended that results be based on measured concentrations. However, if evidence is available to demonstrate that the concentration of the test substance in solution has been satisfactorily maintained within ± 20 % of the nominal or measured initial concentration throughout the test, then results can be based on nominal or measured initial values.

For flow-through tests, a similar sampling regime to that described for semi-static tests is appropriate (but measurement of 'old' solutions is not applicable in this case). However, if the test duration is more than seven days, it may be advisable to increase the number of sampling occasions during the first week (e.g. three sets of measurements) to ensure that the test concentrations are remaining stable.

Samples may need to be centrifuged or filtered (e.g. using a 0,45 μm pore size). However, since neither centrifuging nor filtration appears always to separate the non-bioavailable fraction of the test substance from that which is bioavailable, samples may not be subjected to those treatments.

During the test, dissolved oxygen, pH and temperature should be measured in all test vessels. Total hardness and salinity (if relevant) should be measured in the controls and one vessel at the highest concentration. As a minimum, dissolved oxygen and salinity (if relevant) should be measured three times (at the beginning, middle and end of the test). In semi-static tests, it is recommended that dissolved oxygen be measured more frequently,

preferably before and after each water renewal or at least once at week. pH should be measured at the beginning and end of each water renewal in semi-static test and at least weekly in flow-through tests. Hardness should be measured once each test. Temperature should be measured daily and it should preferably be monitored continuously in at least one test vessel.

1.7.5. **Observations**

1.7.5.1. *Stage of embryonic development*

The embryonic stage (i.e. gastrula stage) at the beginning of exposure to the test substance should be verified as precisely as possible. This can be done using a representative sample of eggs suitably preserved and cleared. The literature may also be consulted for the description and illustration of embryonic stages (2)(5)(10)(11).

1.7.5.2. *Hatching and survival*

Observations on hatching and survival should be made at least once daily and numbers recorded. It may be desirable to make more frequent observations at the beginning of the test (e.g. each 30 minutes during the first three hours), since in some cases, survival times can be more relevant than only the number of deaths (e.g. when there are acute toxic effects). Dead embryos and larvae should be removed as soon as observed since they can decompose rapidly. Extreme care should be taken when removing dead individuals not to knock or physically damage adjacent eggs/larvae, these being extremely delicate and sensitive. Criteria for death vary according to life stage:

— **for eggs**: particularly in the early stages, a marked loss of translucency and change in colouration, caused by coagulation and/or precipitation of protein, leading to a white opaque appearance,

— **for embryos**: absence of body movement and/or absence of heart beat and/or opaque discoloration in species whose embryos are normally translucent,

— **for larvae**: immobility and/or absence of respiratory movement and/or absence of heart-beat and/or white opaque colouration of central nervous system and/or lack of reaction mechanical stimulus.

1.7.5.3. *Abnormal appearance*

The number of larvae showing abnormality of body form and/or pigmentation, and the stage of yolk-sac absorption, should be recorded at adequate intervals depending on the duration of the test and the nature of the abnormality described. It should be noted that abnormal embryos and larvae occur naturally and can be of the order of several per cent in the control(s) in some species. Abnormal animals should only be removed from the test vessels on death.

1.7.5.4. *Abnormal behaviour*

Abnormalities, e.g. hyperventilation, uncoordinated swimming, and atypical quiescence should be recorded at adequate intervals depending on the duration of the test. These effects, although difficult to quantify, can, when observed, aid in the interpretation of mortality data, i.e. provide information on the mode of toxic action of the substance.

1.7.5.5. *Length*

At the end of the test, measurement of individual lengths is recommended; standard, fork or total length may be used. If however, caudal fin rot or fin erosion occurs, standard lengths should be used. Generally, in a well-run test, the coefficient of variation for length among replicates in the controls should be ≤ 20 %.

1.7.5.6. *Weight*

At the end of the test, individual weights can be measured; dry weights (24 hours at 60 °C) are preferable to wet weights (blotted dry). Generally, in a well-run test, the coefficient of variation for weight among replicates in the controls should be ≤ 20 %.

These observations will result in some or all of the following data being available for statistical analysis:

— cumulative mortality,

— numbers of healthy larvae at end of test,

— time to start of hatching and end of hatching (i.e. 90 % hatching in each replicate),

— numbers of larvae hatching each day,

— length (and weight) of surviving animals at end of the test,

— numbers of larvae that are deformed or of abnormal appearance,

— numbers of larvae exhibiting abnormal behaviour.

2. DATA AND REPORTING

2.1. TREATMENT OF RESULTS

It is recommended that a statistician be involved in both the design and analysis of the test since the method allows for considerable variation in experimental design as, for example, in the number of test chambers, number of test concentrations, starting number of fertilised eggs and in the parameters measured. In view of the options available in test design, specific guidance on statistical procedures is not given here.

If LOEC/NOECs are to be estimated, it will be necessary for variations to be analysed within each set of replicates using analysis of variance (ANOVA) or contingency table procedures. In order to make a multiple comparison between the results at the individual concentrations and those for the controls, Dunnett's method may be found useful (12)(13). Other useful examples are also available (14)(15). The size of the effect detectable using ANOVA or other procedures (i.e. the power of the test) should be calculated and reported. It should be noted that not all the observations listed in Section 1.7.5.6 are suitable for statistical analysis using ANOVA. For example, cumulative mortality and numbers of healthy larvae at the end of the test could be analysed using probit methods.

If LC/EC_xs are to be estimated, (a) suitable curve(s), such as the logistic curve, should be fitted to the data of interest using a statistical method such as least squares or non-linear least squares. The curve(s) should be parameterised so that the LC/EC_x of interest and its standard error can be estimated directly. This will greatly ease the calculation of the confidence limits around the LC/EC_x. Unless there are good reasons to prefer different confidence levels, two-sided 95 % confidence should be quoted. The fitting procedure should preferably provide a means for assessing the significance of the lack of fit. Graphical methods for fitting curves can be used. Regression analysis is suitable for all observations listed in Section 1.7.5.6.

2.2. INTERPRETATION OF RESULTS

The results should be interpreted with caution where measured toxicant concentrations in test solutions occur at levels near the detection limit of the analytical method. The interpretation of results for concentrations above the water solubility of the substance should also be made with care.

2.3. THE TEST REPORT

The test report must include the following information:

2.3.1. **Test substance:**

— physical nature and relevant physical-chemical properties;

— chemical identification data, including purity and analytical method for quantification of the tests substance where appropriate.

2.3.2. **Test species:**

— scientific name, strain, numbers of parental fish (i.e. how many females were used for providing the required numbers of eggs in the test), source and method of collection of the fertilised eggs and subsequent handling.

2.3.3. **Test conditions:**

— test procedure used (e.g. semi-static or flow-through, time period from fertilisation to start the test, loading, etc),

— photoperiod(s),

— test design (e.g. number of test chambers and replicates, number of embryos per replicate),

— method of preparation of stock solutions and frequency of renewal (the solubilising agent and its concentration must be given, when used),

— the nominal test concentrations, the measured values, their means and their standard deviations in the test vessels and the method by which these were attained and, if the test substance is soluble in water at concentrations below those tested, evidence should be provided that the measurements refer to the concentrations of the test substance in solution,

— dilution water characteristics: pH, hardness, temperature, dissolved oxygen concentration, residual chlorine levels (if measured), total organic carbon, suspended solids, salinity of the test medium (if measured) and any other measurements made,

— water quality within test vessels: pH, hardness, temperature and dissolved oxygen concentration.

2.3.4. **Results:**

— results from any preliminary studies on the stability of the test substance,

— evidence that controls met the overall survival acceptability standard of the test species (Appendices 2 and 3),

— data on mortality/survival at embryo and larval stages and overall mortality/survival,

— days to hatch and numbers hatched,

— data for length (and weight),

— incidence and description of morphological abnormalities, if any,

— incidence and description of behavioural effects, if any,

— statistical analysis and treatment of data,

— for tests analysed using ANOVA, the lowest observed effect concentration (LOEC) at p=0,05 and the no observed effect concentration (NOEC) for each response assessed, including a description of the statistical procedures used and an indication of what size of effect could be detected,

— for tests analysed using regression techniques, the LC/ECx and confidence intervals and a graph of the fitted model used for its calculation,

— explanation for any deviation from this testing method.

3. **REFERENCES**

(1) Kristensen P., (1990) Evaluation of the Sensitivity of Short Term Fish Early Life Stage Tests in Relation to other FELS Test Methods. Final report to the Commission of the European Communities, p. 60. June 1990.

(2) ASTM (1988) Standard Guide for Conducting Early Life-Stage Toxicity Tests with Fishes. American Society for Testing and Materials. E 1241-88. p. 26

(3) Brauhn J.L. and Schoettger R.A., (1975) Acquisition and Culture of Research Fish: Rainbow trout, Fathead minnows, Channel Catfish and Bluegills. p. 54, Ecological Research Series, EPA-660/3-75-011, Duluth, Minnesota.

(4) Brungs W.A. and Jones B.R., (1977) Temperature Criteria for Freshwater Fish: Protocol and Procedures p. 128, Ecological Research Series EPA-600/3-77-061, Duluth, Minnesota.

(5) Laale H.W., (1977) The Biology and Use of the Zebrafish (*Brachydanio rerio*) in Fisheries Research. A Literature Review. J. Biol. 10, p. 121-173.

(6) Legault R. (1958) A Technique for Controlling the Time of Daily Spawning and Collecting Eggs of the Zebrafish, *Brachydanio rerio* (Hamilton-Buchanan) Copeia, 4, p. 328-330.

(7) Dave G., Damgaard B., Grande M., Martelin J.E., Rosander B. and Viktor T., (1987) Ring Test of an Embryo-larval Toxicity Test with Zebrafish (*Brachydanio rerio*) Using Chromium and Zinc as Toxicants. Environmental Toxicology and Chemistry, 6, p. 61-71.

(8) Birge J.W., Black J.A. and Westerman A.G., (1985) Short-term Fish and Amphibian Embryo-larval Tests for Determining the Effects of Toxicant Stress on Early Life Stages and Estimating Chronic Values for Single Compounds and Complex Effluents. Environmental Toxicology and Chemistry 4, p. 807-821.

(9) Van Leeuwen C.J., Espeldoorn A. and Mol F. (1986) Aquatic Toxicological Aspects of Dithiocarbamates and Related Compounds. III. Embryolarval Studies with Rainbow Trout (*Salmo gairdneri*). J. Aquatic Toxicology, 9, p. 129-145.

(10) Kirchen R.V. and W. R. West (1969) Teleostean Development. Carolina Tips 32(4): 1-4. Carolina Biological Supply Company.

(11) Kirchen R.V. and W. R. West (1976) The Japanese Medaka. Its care and Development. Carolina Biological Supply Company, North Carolina. p. 36.

(12) Dunnett C.W., (1955) A Multiple Comparisons Procedure for Comparing Several Treatments with Control. J. Amer. Statist. Assoc., 50, p. 1096-1121.

(13) Dunnett C.W. (1964) New Tables for Multiple Comparisons with a Control. Biometrics, 20, p. 482-491.

(14) Mc Clave J.T., Sullivan J.H. and Pearson J.G., (1980) Statistical Analysis of Fish Chronic Toxicity Test Data. Proceedings of 4th Aquatic Toxicology Symposium, ASTM, Philadelphia.

(15) Van Leeuwen C.J., Adema D.M.M. and Hermes J., (1990) Quantitative Structure-Activity Relationships for Fish Early Life Stage Toxicity. Aquatic Toxicology, 16, p. 321-334.

(16) Environment Canada., (1992) Toxicity Tests Using Early Life Stages of Salmonid Fish (Rainbow Trout, Coho Salmon or Atlantic Salmon). Biological Test Method Series. Report EPS 1/RM/28, December 1992, p. 81.

(17) Dave G. and Xiu R., (1991) Toxicity of Mercury, Nickel, Lead and Cobalt to Embryos and Larvae of Zebrafish, *Brachydanio rerio*. Arch. of Environmental Contamination and Toxicology, 21, p. 126-134.

(18) Meyer A., Bierman C.H. and Orti G., (1993) The phylogenetic position of the Zebrafish (*Danio rerio*), a model system in developmental biology — an invitation to the comperative methods. Proc. Royal Society of London, Series B, 252: 231-236.

(19) Ghillebaert F., Chaillou C., Deschamps F. and Roubaud P., (1995) Toxic Effects, at Three pH Levels, of Two Reference Molecules on Common Carp Embryo. Ecotoxicology and Environmental Safety 32, p. 19-28.

(20) US EPA, (1991) Guidelines for Culturing the Japanese Medaka, *Oryzias latipes*. EPA report EPA/600/3-91/064, Dec. 1991, EPA, Duluth.

(21) US EPA, (1991) Guidelines for Conducting Early Life Stage Toxicity Tests with Japanese Medaka, (*Oryzias latipes*). EPA report EPA/600/3-91/063, Dec. 1991, EPA, Duluth.

(22) De Graeve G.M., Cooney J.D., McIntyre D.O., Poccocic T.L., Reichenbach N.G., Dean J.H. and Marcus M.D., (1991) Validity in the performance of the seven-day Fathead minnow (*Pimephales promelas*) larval survival and growth test: an intra- and interlaboratory study. Environ. Tox. Chem. 10, p. 1189-1203.

(23) Calow P., (1993) Handbook of Ecotoxicology, Blackwells, Oxford. Vol. 1, chapter 10: Methods for spawning, culturing and conducting toxicity tests with Early Life stages of Estuarine and Marine fish.

(24) Balon E.K., (1985) Early life history of fishes: New developmental, ecological and evolutionary perspectives, Junk Publ., Dordrecht, p. 280.

(25) Blaxter J.H.S., (1988) Pattern and variety in development, In: W.S. Hoar and D.J. Randall Eds., Fish Physiology, vol. XIA, Academic press, p. 1-58.

Table 1A

Fish species recommended for testing

FRESHWATER
Oncorhynchus mykiss Rainbow trout (9)(16)
Danio rerio Zebrafish (7)(17)(18)
Cyprinus caprio Common carp (8)(19)
Oryzias latipes Japanese ricefish/Medaka (20)(21)
Pimephales promelas Fathead minnow (8)(22)

Table 1B

Examples of other well-documented species which have also been used

FRESHWATER	SALTWATER
Carassius auratus Goldfish (8)	*Menidia peninsulae* Tidewater silverside (23)(24)(25)
Lepomis macrochirus Bluegill (8)	*Clupea harengus* Herring (24)(25)
	Gadus morhua Cod (24)(25)
	Cyprinodon variegatus Sheepshead minnow (23)(24)(25)

Appendix 1

GUIDANCE ON PERFORMANCE OF A TOXICITY TEST ON EMBRYOS AND SAC-FRY OF ZEBRAFISH *(BRACHYDANIO RERIO)*

INTRODUCTION

The zebrafish originates from the Coromandel coast of India where it inhabits fast-flowing streams. It is a common aquarium fish of the carp family, and information about procedures for its care and culture can be found in standard reference books on tropical fish. Its biology and use in fishery research have been reviewed by Laale (1).

The fish rarely exceeds 45 mm in length. The body is cylindrical with 7-9 dark-blue horizontal silvery stripes. These stripes run into the caudal and anal fins. The back is olive-green. Males are slimmer than females. Females are more silvery and the abdomen is distended, particularly prior to spawning.

Adult fishes are able to tolerate large fluctuations in temperature, pH and hardness. However, in order to get healthy fish which produce eggs of good quality, optimal conditions should be provided.

During spawning the male pursues and butts the female, and as the eggs are expelled they are fertilised. The eggs, which are transparent and non-adhesive, fall to the bottom where they may be eaten by the parents. Spawning is influenced by light. If the morning light is adequate, the fish usually spawns in the early hours following daybreak.

A female can produce batches of several hundreds of eggs at weekly intervals.

CONDITIONS OF PARENTAL FISH, REPRODUCTION AND EARLY-LIFE STAGES

Select a suitable number of healthy fish and keep these in suitable water (e.g. Annex 4) for at least two weeks prior to the intended spawning. The group of fish should be allowed to breed at least once before producing the batch of eggs used in the test. The density of fish during this period should not exceed 1 gram of fish per litre. Regular changes of water or the use of purification systems will enable the density to be higher. The temperature in the holding tanks should be maintained at 25 ± 2 °C. The fish should be provided with a varied diet, which may consist of, for example, appropriate commercial dry food, live newly hatched Arthemia, chironomids, Daphnia, white worms (Enchytraeids).

Two procedures are outlined below, which in practice have led to a sufficient batch of healthy, fertilised eggs for a test to be run:

i) Eight females and 16 males are placed in a tank containing 50 litres of dilution water, shielded from direct light and left as undisturbed as possible for at least 48 hours. A spawning tray is placed at the bottom of the aquarium in the afternoon the day before start of the test. The spawning tray consists of a frame (plexi-glass or other suitable material), 5-7 cm high with a 2-5 mm coarse net attached at the top and a 10-30 µm fine net at the bottom. A number of 'spawning-trees', consisting of untwisted nylon rope, are attached to the coarse net of the frame. After the fish have been left in dark for 12 hours, a faint light is turned on which will initiate the spawning. Two to four hours after spawning, the spawning tray is removed and the eggs collected. The spawning tray will prevent the fish from eating the eggs and at the same time permit an easy collection of the eggs. The group of fish should have spawned at least once before the spawning from which eggs are used for testing.

ii) Five to 10 male and female fish are housed individually at least two weeks prior to the intended spawning. After 5-10 days, the abdomens of the females will be distended and their genital papillae visible. Male fish lack papillae. Spawning is performed in spawning tanks equipped with a false mesh bottom (as above). The tank is filled with dilution water, so that the depth of water above the mesh is 5-10 cm. One female and two males are placed in the tank the day before the intended spawning. The water temperature is gradually increased one degree higher than the acclimatisation temperature. The light is turned off and the tank is left as undisturbed as possible. In the morning a faint light is turned on which will initiate spawning. After two to four hours, the fish are removed and the eggs collected. If larger batches of eggs are needed than can be obtained from one female, a sufficient number of spawning tanks may be set-up in parallel. By recording the reproduction success of the individual females prior to the test (size of batch and quality), those females with highest reproduction success may be selected for breeding.

The eggs should be transferred to the test vessels by means of glass tubes (inner diameter not less than 4 mm) provided with a flexible suction bulb. The amount of water accompanying the eggs on their transfer should be as small as possible. The eggs are heavier than water and sink out of the tube. Care should be taken to prevent eggs (and larvae) coming into contact with the air. Microscopic examination of sample(s) of the batch(es) should be carried out to ensure that there are no irregularities in the first developmental stages. Disinfection of the eggs is not allowed.

The mortality rate of the eggs is highest within the first 24 hours after fertilisation. A mortality of 5-40 % is often seen during this period. Eggs degenerate as a result of unsuccessful fertilisation or development failures. The quality of the batch of eggs seems to depend on the female fish, as some females consistently produce good quality eggs, others never will. Also the development rate and the rate of hatching vary from one batch to another. The successfully fertilised eggs and the yolk sac larvae survive well, normally above 90 %. At 25 °C the eggs will hatch three-five days after fertilisation and the yolk sac will be absorbed approximately 13 days after fertilization.

The embryonic development has been well defined by Hisaoka and Battle (2). Due to the transparency of the eggs and post-hatch larvae, the development of the fish may be followed and the presence of malformations may be observed. Approximately four hours after spawning, the non-fertilized eggs may be distinguished from the fertilized (3). For this examination, eggs and larvae are placed in test vessels of small volume and studied under a microscope.

The test conditions, which apply to the early life stages, are listed in Appendix 2. Optimal values for pH values and hardness of the dilution water are 7,8 and 250 mg $CaCO_3$/l respectively.

CALCULATIONS AND STATISTICS

A two-stage approach is proposed. First, the data on mortality, abnormal development and hatching-time are analysed statistically. Then, for those concentrations at which no adverse effects on any of these parameters have been detected, the body length is statistically evaluated. This approach is advisable since the toxicant may selectively kill smaller fish, delay hatching-time and induce gross malformations, thus leading to biased length measurements. Furthermore, there will be roughly the same number of fish to be measured per treatment, ensuring the validity of the test statistics.

LC_{50} AND EC_{50} DETERMINATIONS

The percentage of surviving eggs and larvae is calculated and corrected for mortality in the controls in accordance with Abbott's formula (4):

$$P = 100 - \left(\frac{C - P'}{C} \times 100 \right)$$

where:

P = corrected % survival

P' = % survival observed in the test concentration

C = % survival in the control

If possible, the LC_{50} is determined by a suitable method at the end of the test.

If the inclusion of morphological abnormalities in the EC_{50} statistic is desired, guidance can be found in Stephan (5).

ESTIMATION OF LOEC AND NOEC

An objective of the egg and sac-fry test is to compare the non-zero concentrations with the control, i.e. to determine the LOEC. Therefore multiple comparison procedures should be utilised (6)(7)(8)(9)(10).

REFERENCES

(1) Laale H.W., (1977) The Biology and Use of the Zebrafish (*Brachydanio rerio*) in Fisheries Research. A Literature Review. J. Fish Biol. 10, p. 121-173.

(2) Hisaoka K.K. and Battle H.I., (1958). The Normal Development Stages of the Zebrafish *Brachydanio rerio* (Hamilton-Buchanan) J. Morph., 102, p. 311.

(3) Nagel R., (1986). Untersuchungen zur Eiproduktion beim Zebrabärbling (*Brachydanio rerio* Hamilton-Buchanan). Journal of Applied Ichthyology, 2, p. 173-181.

(4) Finney D.J., (1971). Probit Analysis, 3rd ed., Cambridge University Press, Great Britain, p. 1-333.

(5) Stephan C.E., (1982). Increasing the Usefulness of Acute Toxicity Tests. Aquatic Toxicology and Hazard Assessment: Fifth Conference, ASTM STP 766, J.G. Pearson, R.B. Foster and W.E. Bishop, Eds., American Society for Testing and Materials, p. 69-81.

(6) Dunnett C.W. (1955). A Multiple Comparisons Procedure for Comparing Several Treatments with a Control. J. Amer. Statist. Assoc., 50, p. 1096-1121.

(7) Dunnett C.W., (1964) New Tables for Multiple Comparisons with a Control. Biometrics, 20, p. 482-491.

(8) Williams D.A., (1971). A Test for Differences Between Treatment Means when Several Dose Levels are Compared with a Zero Dose Control. Biometrics, 27, p. 103-117.

(9) Williams D.A., (1972). The Comparison of Several Dose Levels with a Zero Dose Control. Biometrics 28, p. 519-531.

(10) Sokal R.R. and Rohlf F.J., (1981). Biometry, the Principles and Practice of Statistics in Biological Research, W.H. Freeman and Co., San Francisco.

Appendix 2

TEST CONDITIONS, DURATION AND SURVIVAL CRITERIA FOR RECOMMENDED SPECIES

Species	Temp (°C)	Salinity (0/00)	Photo-period (hrs)	Duration of stages (days)		Typical duration of test	Survival of control, (minimum %)	
				Embryo	Sac-fry		Hatching success	Post-hatch
FRESHWATER								
Brachydanio rerio Zebrafish	25 ± 1	—	12-16	3-5	8-10	As soon as possible after fertilisation (early gastrula stage) to 5 days post-hatch (8-10 days)	80	90
Oncorhynchus mykiss Rainbow trout	10 ± 1 (1) 12 ± 1 (2)	—	0 (a)	30-35	25-30	As soon as possible after fertilisation (early gastrula stage) to 20 days post-hatch (50-55 days)	66	70
Cyprinus carpio Common carp	21-25	—	12-16	5	> 4	As soon as possible after fertilisation (early gastrula stage) to 4 days post-hatch (8-9 days)	80	75
Oryzias latipes Japanese ricefish/Medaka	24 ± 1 (1) 23 ± 1 (2)	—	12-16	8-11	4-8	As soon as possible after fertilisation (early gastrula stage) to 5 days post-hatch (13-16 days)	80	80
Pimephales promelas Fathead minnow	25 ± 2	—	16	4-5	5	As soon as possible after fertilisation (early gastrula stage) to 4 days post-hatch (8-9 days)	60	70

(1) For embryos.
(2) For larvae.
(a) Darkness for embryo and larvae until one week after hatching except when they are being inspected. Then subdued lighting throughout the test.

Appendix 3

Test conditions, duration and survival criteria for other well documented species

Species	Temp (°C)	Salinity (0/00)	Photo-period (hrs)	Duration of stages (days)		Typical duration of embryo and sac-fry test	Survival of control (minimum %)	
				Embryo	Sac-fry test		Hatching success	Post-hatch
FRESHWATER								
Carassius auratus Goldfish	24 ± 1	—	—	3-4	> 4	As soon as possible after fertilisation (early gastrula stage) to 4 days post-hatch (7 days)	—	80
Leopomis macrochirus Blugill sunfish	21 ± 1	—	16	3	> 4	As soon as possible after fertilisation (early gastrula stage) to 4 days post-hatch (7 days)	—	75
SALTWATER								
Menidia peninsulae Tidewater silverside	22-25	15-22	12	1,5	10	As soon as possible after fertilisation (early gastrula stage) to 5 days post-hatch (6-7 days)	80	60
Clupea harengus Herring	10 ± 1	8-15	12	20-25	3-5	As soon as possible after fertilisation (early gastrula stage) to 3 days post-hatch (23-27 days)	60	80
Gadus morhua Cod	5 ± 1	5-30	12	14-16	3-5	As soon as possible after fertilisation (early gastrula stage) to 3 days post-hatch (18 days)	60	80
Cyprinodon variegatus Sheepshead minnow	25 ± 1	15-30	12	—	—	As soon as possible after fertilisation (early gastrula stage) to 4/7 days post-hatch (28 days)	> 75	80

Appendix 4

SOME CHEMICAL CHARACTERISTICS OF AN ACCEPTABLE DILUTION WATER

Substance	Concentrations
Particulate matter	< 20 mg/l
Total organic carbon	< 2 mg/l
Unionised ammonia	< 1 µg/l
Residual chlorine	< 10 µg/l
Total organophosphorus pesticides	< 50 ng/l
Total organochlorine pesticides plus polychlorinated biphenyls	< 50 ng/l
Total organic chlorine	< 25 ng/l

C.16. HONEYBEES — ACUTE ORAL TOXICITY TEST

1. METHOD

This acute toxicity test method is a replicate of the OECD TG 213 (1998).

1.1. INTRODUCTION

This toxicity test is a laboratory method, designed to assess the oral acute toxicity of plant protection products and other chemicals, to adult worker honeybees.

In the assessment and evaluation of toxic characteristics of substances, determination of acute oral toxicity in honeybees may be required, e.g. when exposure of bees to a given chemical is likely. The acute oral toxicity test is carried out to determine the inherent toxicity of pesticides and other chemicals to bees. The results of this test should be used to define the need for further evaluation. In particular, this method can be used in step-wise programmes for evaluating the hazards of pesticides to bees, based on sequential progression from laboratory toxicity tests to semi-field and field experiments (1). Pesticides can be tested as active substances (a.s.) or as formulated products.

A toxic standard should be used to verify the sensitivity of the bees and the precision of the test procedure.

1.2. DEFINITIONS

Acute oral toxicity: is the adverse effects occurring within a maximum period of 96h of an oral administration of a single dose of test substance.

Dose: is the amount of test substance consumed. Dose is expressed as mass (μg) of test substance per test animal (μg/bee). The real dose for each bee can not be calculated as the bees are fed collectively, but an average dose can be estimated (totally consumed test substance/number of test bees in one cage).

LD_{50} (Median Lethal Dose) oral: is a statistically derived single dose of a substance that can cause death in 50 % of animals when administered by the oral route. The LD_{50} value is expressed in μg of test substance per bee. For pesticides, the test substance may be either an active substance (a.s.) or a formulated product containing one or more than one active substance.

Mortality: an animal is recorded as dead when it is completely immobile.

1.3. PRINCIPLE OF THE TEST METHOD

Adult worker honeybees (*Apis mellifera*) are exposed to a range of doses of the test substance dispersed in sucrose solution. The bees are then fed the same diet, free of the test substance. Mortality is recorded daily during at least 48 h and compared with control values. If the mortality rate is increasing between 24 h and 48 h whilst control mortality remains at an accepted level, i.e. ≤ 10 %, it is appropriate to extend the duration of the test to a maximum of 96 h. The results are analysed in order to calculate the LD_{50} at 24 h and 48 h and, in case the study is prolonged, at 72 h and 96 h.

1.4. VALIDITY OF THE TEST

For a test to be valid, the following conditions apply:

— the average mortality for the total number of controls must not exceed 10 % at the end of the test,

— the LD_{50} of the toxic standard meets the specified range.

1.5. DESCRIPTION OF THE TEST METHOD

1.5.1. **Collection of bees**

Young adult worker bees of the same race should be used, i.e. bees of the same age, feeding status, etc. Bees should be obtained from adequately fed, healthy, as far as possible disease-free and queen-right colonies with known history and physiological status. They could be collected in the morning of use or in the evening before test and kept under test conditions to the next day. Bees collected from frames without brood are suitable. Collection in early spring or late autumn should be avoided as the bees have a changed physiology during this time. If tests must be conduced in early spring or late autumn, bees can be emerged in an incubator and reared for one week with 'bee bread' (pollen collected from the comb) and sucrose solution. Bees treated with chemical substances, such as antibiotics, anti-varroa products, etc., should not be used for toxicity test for four weeks from the time of the end of the last treatment.

1.5.2. **Housing and feeding conditions**

Easy to clean and well-ventilated cages are used. Any appropriate material can be used, e.g. stainless steel, wire mesh, plastic or disposable wooden cages, etc. Groups of 10 bees per cage are preferred. The size of test cages should be appropriate to the number of bees, i.e. providing adequate space.

The bees should be held in the dark in an experimental room at a temperature of 25 ± 2 °C. The relative humidity, normally around 50-70 %, should be recorded throughout the test. Handling procedures, including treatment and observations may be conducted under (day) light. Sucrose solution in water with a final concentration of 500 g/l (50 % w/v) is used as food. After given test doses, food should be provided *ad libitum*. The feeding system should allow recording food intake for each cage (see Section 1.6.3.1). A glass tube (approximately 50 mm long and 10 mm wide with the open end narrowed to about 2 mm diameter) can be used.

1.5.3. **Preparation of bees**

The collected bees are randomly allocated to test cages, which are randomly placed in the experimental room.

The bees may be starved for up to 2 h before the initiation of the test. It is recommended that the bees are deprived of food prior to treatment so that all bees are equal in terms of their gut contents at the start of the test. Moribund bees should be rejected and replaced by healthy bees before starting the test.

1.5.4. **Preparation of doses**

Where the test substance is a water miscible compound this may be dispersed directly in 50 % sucrose solution. For technical products and substances of low water solubility, vehicles such as organic solvent, emulsifiers or dispersants of low toxicity to bees may be used (e.g. acetone, dimethylformamide, dimethylsulfoxide). The concentration of the vehicle depends on the solubility of the test substance and it should be the same for all concentrations tested. However, a concentration of the vehicle of 1 % is generally appropriate and should not be exceeded.

Appropriate control solutions should be prepared, i.e. where a solvent or a dispersant is used to solubilise the test substance, two separate control groups should be used: a solution in water, and a sucrose solution with the solvent/carrier at the concentration used in dosing solutions.

1.6. PROCEDURE

1.6.1. **Test and control groups**

The number of doses and replicates tested should meet the statistical requirements for determination of LD_{50} with 95 % confidence limits. Normally, five doses in a geometric series, with a factor not exceeding 2,2, and covering the range for LD_{50}, are required for the test. However, the dilution factor and the number of concentrations for dosage have to be determined in relation to the slope of the toxicity curve (dose versus mortality) and with consideration taken to the statistical method which is chosen for analysis of the results. A range-finding test enables the choice of the appropriate concentrations for dosage.

A minimum of three replicate test groups, each of 10 bees, should be dosed with each test concentration. A minimum of three control batches, each of 10 bees, should be run in addition to the test series. Control batches should also be included for the solvents/carriers used (see Section 1.5.4).

1.6.2. **Toxic standard**

A toxic standard should be included in the test series. At least three doses should be selected to cover the expected LD_{50} value. A minimum of three replicate cages, each containing 10 bees, should be used with each test dose. The preferred toxic standard is dimethoate, for which the reported oral LD_{50}-24 h is in the range 0,10-0,35 µg a.s./bee (2). However, other toxic standards would be acceptable where sufficient data can be provided to verify the expected dose response (e.g. parathion).

1.6.3. **Exposure**

1.6.3.1. *Administration of doses*

Each test group of bees must be provided with 100-200 µl of 50 % sucrose solution in water, containing the test substance at the appropriate concentration. A larger volume is required for products of low solubility, low toxicity or low concentration in the formulation, as higher proportions in the sucrose solution have to be used. The amount of treated diet consumed per group should be monitored. Once consumed (usually within 3-4 h), the feeder should be removed from the cage and replaced with one containing sucrose solution alone. The sucrose solutions are then provided *ad libitum*. For some compounds, at higher concentrations rejection of test dose may result in little or no food being consumed. After a maximum of 6 h, unconsumed treated diet should be replaced with the sucrose solution alone. The amount of treated diet consumed should be assessed (e.g. measurement of volume/weight of treated diet remaining).

1.6.3.2. *Duration*

The duration of the test is preferably 48 h after the test solution has been replaced with sucrose solution alone. If mortality continues to rise by more than 10 % after the first 24 h, the test duration should be extended to a maximum of 96 h provided that control mortality does not exceed 10 %.

1.6.4. **Observations**

Mortality is recorded at 4 h after starting the test and thereafter at 24 h and 48 h (i.e. after giving dose). If a prolonged observation period is required, further assessments should be made at 24 h intervals, up to a maximum of 96 h, provided that the control mortality does not exceed 10 %.

The amount of diet consumed per group should be estimated. Comparison of the rates of consumption of treated and untreated diet within the given 6 h can provide information about palatability of the treated diet.

All abnormal behavioural effects observed during the testing period should be recorded.

1.6.5. **Limit test**

In some cases (e.g. when a test substance is expected to be of low toxicity) a limit test may be performed, using 100 µg a.s./bee in order to demonstrate that the LD_{50} is greater than this value. The same procedure should be used, including three replicate test groups for the test dose, the relevant controls, the assessment of the amount of treated diet consumed, and the use of the toxic standard. If mortalities occur, a full study should be conducted. If sublethal effects are observed (see Section 1.6.4), these should be recorded.

2. **DATA AND REPORTING**

2.1. DATA

Data should be summarised in tabular form, showing for each treatment group, as well as control and toxic standard groups, the number of bees used, mortality at each observation time and number of bees with adverse behaviour. Analyse the mortality data by appropriate statistical methods (e.g. probit analysis, moving-average, binomial probability) (3)(4). Plot dose-response curves at each recommended observation time and calculate the slopes of the curves and the median lethal doses (LD_{50}) with 95 % confidence limits. Corrections for control mortality could be made using Abbott's correction (4)(5). Where treated diet is not completely consumed, the dose of test substance consumed per group should be determined. LD_{50} should be expressed in µg of test substance per bee.

2.2. TEST REPORT

The test report must include the following information:

2.2.1. **Test substance:**

— physical nature and relevant physical-chemical properties (e.g. stability in water, vapour pressure),

— chemical identification data, including structural formula, purity (i.e. for pesticides, the identity and concentration of active substance(s)).

2.2.2. **Test species:**

— scientific name, race, approximate age (in weeks), collection method, date of collection,

— information on colonies used for collection of test bees including health, any adult disease, any pre-treatment, etc.

2.2.3. **Test conditions:**

— temperature and relative humidity of experimental room,

— housing conditions including type, size and material of cages,

— methods of preparation of stock and test solutions (the solvent and its concentration must be given, when used),

— test design, e.g. number and test concentrations used, number of controls; for each test concentration and control, number of replicate cages and number of bees per cage,

— date of test.

2.2.4. **Results:**

— results of preliminary range-finding study if performed,

— raw data: mortality at each dose tested at each observation time,

— graph of the dose-response curves at the end of the test,

— LD_{50} values with 95 % confidence limits, at each of the recommended observation times, for test substance and toxic standard;

— statistical procedures used for determining the LD_{50},

— mortality in controls,

— other biological effects observed or measured e.g. abnormal behaviour of the bees (including rejection of the test dose), rate of consumption of diet in treated and untreated groups,

— any deviation from the test procedures described here and any other relevant information.

3. **REFERENCES**

(1) EPPO/Council of Europe (1993) Decision-Making Scheme for the Environmental Risk Assessment of Plant Protection Products — Honeybees. EPPO bulletin, vol. 23, N.1, 151-165. March 1993.

(2) Gough, H. J., McIndoe, E.C., Lewis, G.B., (1994) The use of dimethoate as a reference compound in laboratory acute toxicity tests on honeybees (*Apis mellifera* L.) 1981-1992. Journal of Apicultural Research, 22, p. 119-125.

(3) Litchfield, J.T. and Wilcoxon, F., (1949) A simplified method of evaluating dose-effect experiments. Jour. Pharmacol. and Exper. Ther., 96, p. 99-113.

(4) Finney, D.J., (1971) Probit Analysis. 3rd ed., Cambridge, London and New-York.

(5) Abbott, W.S., (1925) A method for computing the effectiveness of an insecticide. Jour. Econ. Entomol., 18, p. 265-267.

C.17. HONEYBEES — ACUTE CONTACT TOXICITY TEST

1. METHOD

This acute toxicity test method is a replicate of the OECD TG 214 (1998).

1.1. INTRODUCTION

This toxicity test is a laboratory method, designed to assess the acute contact toxicity of plant protection products and other chemicals to adult worker honeybees.

In the assessment and evaluation of toxic characteristics of substances, determination of acute contact toxicity in honeybees may be required, e.g. when exposure of bees to a given chemical is likely. The acute contact toxicity test is carried out to determine the inherent toxicity of pesticides and other chemicals to bees. The results of this test should be used to define the need for further evaluation. In particular, this method can be used in step-wise programmes for evaluating the hazards of pesticides to bees, based on sequential progression from laboratory toxicity tests to semi-field and field experiments (1). Pesticides can be tested as active substances (a.s.) or as formulated products.

A toxic standard should be used to verify the sensitivity of the bees and the precision of the test procedure.

1.2. DEFINITIONS

Acute contact toxicity: is the adverse effects occurring within a maximum period of 96 h of a topical application of a single dose of a substance.

Dose: is the amount of test substance applied. Dose is expressed as mass (μg) of test substance per test animal (μg/bee).

LD_{50} (Median Lethal Dose) contact: is a statistically derived single dose of a substance that can cause death in 50 % of animals when administered by the contact. The LD_{50} value is given in μg of test substance per bee. For pesticides, the test substance may be either an active substance (a.s.) or a formulated product containing one or more than one active substance.

Mortality: an animal is recorded as dead when it is completely immobile.

1.3. PRINCIPLE OF THE TEST METHOD

Adult worker honeybees (*Apis mellifera*) are exposed to a range of doses of the test substance dissolved in appropriate carrier, by direct application to the thorax (droplets). The test duration is 48 h. If the mortality rate is increasing between 24 h and 48 h whilst control mortality remains at an accepted level, i.e. < 10 %, it is appropriate to extend the duration of the test to a maximum of 96 h. Mortality is recorded daily and compared with control values. The results are analysed in order to calculate the LD_{50} at 24 h and 48 h, and in case the study is prolonged at 72 h and 96 h.

1.4. VALIDITY OF THE TEST

For a test to be valid, the following conditions apply:

— the average mortality for the total numbers of controls must not exceed 10 % at the end of the test,

— the LD_{50} of the toxic standard meets the specified range.

1.5. DESCRIPTION OF THE TEST METHOD

1.5.1. **Collection of bees**

Young adult worker bees should be used, i.e. bees of the same age, feeding status, race etc. Bees should be obtained from adequately fed, healthy, as far as possible disease-free and queen-right colonies with known history and physiological status. They could be collected in the morning of use or in the evening before test and kept under test conditions to the next day. Bees collected from frames without brood are suitable. Collection in early spring or late autumn should be avoided, as the bees have a changed physiology during the time. If tests have to be conduced in early spring or late autumn, bees can be emerged in an incubator and reared for one week with 'bee bread' (pollen collected from the comb) and sucrose solution. Bees treated with chemical substances, such as antibiotics, anti-varroa products, etc., should not be used for toxicity test for four weeks from the time of the end of the last treatment.

1.5.2. **Housing and feeding conditions**

Easy to clean and well-ventilated cages are used. Any appropriate material can be used, e.g. stainless steel, wire mesh, plastic, disposable wooden cages, etc. The size of test cages should be appropriate to the number of bees, i.e. providing adequate space. Groups of 10 bees per cage are preferred.

The bees should be held in the dark in an experimental room at a temperature of 25 ± 2 °C. The relative humidity, normally around 50-70 %, should be recorded throughout the test. Handling procedures, including treatment and observations may be conducted under (day) light. Sucrose solution in water with a final concentration of 500 g/l (50 % w/v) should be used as food and provided *ad libitum* during the test time, using a bee feeder. This can be a glass tube (approximately 50 mm long and 10 mm wide with the open end narrowed to about 2 mm diameter).

1.5.3. **Preparation of bees**

The collected bees may be anaesthetised with carbon dioxide or nitrogen for application of the test substance. The amount of anaesthetic used and time of exposure should be minimised. Moribund bees should be rejected and replaced by healthy bees before starting the test.

1.5.4. **Preparation of doses**

The test substance is to be applied as solution in a carrier, i.e. an organic solvent or a water solution with a wetting agent. As organic solvent, acetone is preferred but other organic solvents of low toxicity to bees may be used (e.g. dimethylformamide, dimethylsulfoxide). For water dispersed formulated products and highly polar organic substances not soluble in organic carrier solvents, solutions may be easier to apply if prepared in a weak solution of a commercial wetting agent (e.g. Agral, Cittowett, Lubrol, Triton, Tween).

Appropriate control solutions should be prepared, i.e. where a solvent or a dispersant is used to solubilise the test substance, two separate control groups should be used, one treated with water, and one treated with the solvent/dispersant.

1.6. PROCEDURE

1.6.1. **Test and control groups**

The number of doses and replicates tested should meet the statistical requirements for determination LD_{50} with 95 % confidence limits. Normally five doses in a geometric series, with a factor not exceeding 2,2, and covering the range for LD_{50}, are required for the test. However, the number of doses has to be determined in relation to the slope of the toxicity curve (dose versus mortality) and with consideration taken to the statistical method which is chosen for analysis of the results. A range-finding test enables the choice of the appropriate doses.

A minimum of three replicate test groups, each of 10 bees, should be dosed with each test concentration.

A minimum of three control batches, each of 10 bees, should be run in addition to the test series. If an organic solvent or a wetting agent is used three additional control batches of each 10 bees for the solvent or the wetting agent have to be included.

1.6.2. **Toxic standard**

A toxic standard must be included in the test series. At least three doses should be selected to cover the expected LD_{50} value. A minimum of three replicate cages, each containing 10 bees, should be used with each test dose. The preferred toxic standard is dimethoate, for which the reported contact LD_{50}-24 h is in the range 0,10-0,30 μg a.s./bee (2). However, other toxic standards would be acceptable where sufficient data can be provided to verify the expected dose response (e.g. parathion).

1.6.3. **Exposure**

1.6.3.1. *Administration of doses*

Anaesthetised bees are individually treated by topical application. The bees are randomly assigned to the different test doses and controls. A volume of 1 μl of solution containing the test substance at the suitable concentration should be applied with a microapplicator to the dorsal side of the thorax of each bee. Other volumes may be used, if justified. After application, the bees are allocated to test cages and supplied with sucrose solutions.

1.6.3.2. *Duration*

The duration of the test is preferably 48 hours. If mortality increases by more than 10 % between 24 h and 48 h, the test duration should be extended up to a maximum of 96 h provided that control mortality does not exceed 10 %.

1.6.4. **Observations**

Mortality is recorded at 4 h after dosing and thereafter at 24 h and 48 h. If a prolonged observation period is required, further assessments should be made, at 24 h intervals, to a maximum of 96 h, provided that the control mortality does not exceeding 10 %.

All abnormal behavioural effects observed during the testing period should be recorded.

1.6.5. **Limit test**

In some cases (e.g. when a test substance is expected to be of low toxicity) limit test may be performed, using 100 μg a.s./bee in order to demonstrate that the LD_{50} is greater than this value. The same procedure should be used, including three replicate test groups for the test dose, the relevant controls, and the use of the toxic standard. If mortalities occur, a full study should be conducted. If sublethal effects are observed (see Section 1.6.4) these should be recorded.

2. **DATA AND REPORTING**

2.1. DATA

Data should be summarised in tabular form, showing for each treatment group, as well as, control and toxic standard groups, the number of bees used, mortality at each observation time and number of bees with adverse behaviour. Analyse the mortality data by appropriate statistical methods (e.g. probit analysis, moving-average, binomial probability) (3)(4). Plot dose-response curves at each recommended observation time (i.e. 24 h, 48 h and, if relevant, 72 h, 96 h) and calculate the slopes of the curves and the median lethal doses (LD_{50}) with 95 % confidence limits. Corrections for control mortality could be made using Abbott's correction (4)(5). LD_{50} should be expressed in μg of test substance per bee.

2.2. TEST REPORT

The test report must include the following information:

2.2.1. **Test substance:**

— physical nature and physical-chemical properties (e.g. stability in water, vapour pressure),

— chemical identification data, including structural formula, purity (i.e. for pesticides, the identity and concentration of active substance(s)).

2.2.2. **Test species:**

— scientific name, race, approximate age (in weeks), collection method, date of collection,

— information on colonies used for collection of test bees including health, any adult disease, any pre-treatment, etc.

2.2.3. **Test conditions:**

— temperature and relative humidity of experimental room,

— housing conditions including type, size and material of cages,

— methods of administration of test substance, e.g. carrier solvent used, volume of test solution applied anaesthetics used,

— test design, e.g. number and test doses used, number of controls; for each test dose and control, number of replicate cages and number of bees per cage,

— date of test.

2.2.4. **Results:**

— results of preliminary range-finding study if performed,

— raw data: mortality at each concentration tested at each observation time,

— graph of the dose-response curves at the end of the test,

— LD_{50} values, with 95 % confidence limits, at each of the recommended observation times, for test substance and toxic standard,

— statistical procedures used for determining the LD_{50},

— mortality in controls,

— other biological effects observed or measured and any abnormal responses of the bees,

— any deviation from the test method procedures described here and any other relevant information.

3. **REFERENCES**

(1) EPPO/Council of Europe (1993) Decision-Making Scheme for the Environmental Risk Assessment of Plant Protection Products — Honeybees. EPPO bulletin, vol. 23, N.1, p. 151-165. March, 1993.

(2) Gough, H. J., McIndoe, E.C., Lewis, G.B., (1994) The use of dimethoate as a reference compound in laboratory acute toxicity tests on honeybees (*Apis mellifera* L.), 1981-1992. Journal of Apicultural Research 22, p. 119-125.

(3) Litchfield, J.T. and Wilcoxon, F., (1949) A simplified method of evaluating dose-effect experiments. Jour. Pharmacol. and Exper. Ther., 96, p. 99-113.

(4) Finney, D.J., (1971) Probit Analysis. 3rd ed., Cambridge, London and New-York.

(5) Abbott, W.S., (1925) A method for computing the effectiveness of an insecticide. Jour. Econ. Entomol. 18, p. 265-267.

C.18. ADSORPTION/DESORPTION USING A BATCH EQUILIBRIUM METHOD

1. METHOD

This method is a replicate of the OECD TG 106, for the Determination of Soil Adsorption/Desorption, using a Batch Equilibrium Method (2000).

1.1. INTRODUCTION

The method takes into account a ring test and a workshop for soil selection for the development of an adsorption test (1)(2)(3)(4) and also existing guidelines at national level (5)(6)(7)(8)(9)(10)(11).

Adsorption/desorption studies are useful for generating essential information on the mobility of chemicals and their distribution in the soil, water and air compartments of the biosphere (12)(13)(14)(15)(16)(17)(18)(19)(20)(21). The information can be used in the prediction or estimation, for example, of the availability of a chemical for degradation (22)(23), transformation and uptake by organisms (24); leaching through the soil profile (16)(18)(19)(21)(25)(26)(27)(28); volatility from soil (21)(29)(30); run-off from land surfaces into natural waters (18)(31)(32). Adsorption data can be used for comparative and modelling purposes (19)(33)(34)(35).

The distribution of a chemical between soil and aqueous phases is a complex process depending on a number of different factors: the chemical nature of the substance (12)(36)(37)(38)(39)(40), the characteristics of the soil (4)(12)(13)(14)(41)(42)(43)(44)(45)(46)(47)(48)(49), and climatic factors such as rainfall, temperature, sunlight and wind. Thus, the numerous phenomena and mechanisms involved in the process of adsorption of a chemical by soil cannot be completely defined by a simplified laboratory model such as the present method. However, even if this attempt cannot cover all the environmentally possible cases, it provides valuable information on the environmental relevance of the adsorption of a chemical.

See also General Introduction.

1.2. SCOPE

The method is aimed at estimating the adsorption/desorption behaviour of a substance on soils. The goal is to obtain a sorption value which can be used to predict partitioning under a variety of environmental conditions; to this end, equilibrium adsorption coefficients for a chemical on various soils are determined as a function of soil characteristics (e.g. organic carbon content, clay content and soil texture and pH). Different soil types have to be used in order to cover as widely as possible the interactions of a given substance with naturally occurring soils.

In this method, adsorption represents the process of the binding of a chemical to surfaces of soils; it does not distinguish between different adsorption processes (physical and chemical adsorption) and such processes as surface catalysed degradation, bulk adsorption or chemical reaction. Adsorption that will occur on colloids particles (diameter < 0,2 μm) generated by the soils is not taken into account.

The soil parameters that are believed most important for adsorption are: organic carbon content (3)(4)(12)(13)(14)(41)(43)(44)(45)(46)(47)(48); clay content and soil texture (3)(4)(41)(42)(43)(44)(45)(46) (47)(48) and pH for ionisable compounds (3)(4)(42). Other soil parameters which may have an impact on the adsorption/desorption of a particular substance are the effective cation exchange capacity (ECEC), the content of amorphous iron and aluminium oxides, particularly for volcanic and tropical soils (4), as well as the specific surface (49).

The test is designed to evaluate the adsorption of a chemical on different soil types with a varying range of organic carbon content, clay content and soil texture, and pH. It comprises three tiers:

Tier 1: preliminary study in order to determine:

— the soil/solution ratio,

— the equilibrium time for adsorption and the amount of test substance adsorbed at equilibrium,

— the adsorption of the test substance on the surfaces of the test vessels and the stability of the test substance during the test period.

Tier 2: screening test: the adsorption is studied in five different soil types by means of adsorption kinetics at a single concentration and determination of distribution coefficient K_d and K_{oc}.

Tier 3: determination of Freundlich adsorption isotherms to determine the influence of concentration on the extent of adsorption on soils.

Study of desorption by means of desorption kinetics/Freundlich desorption isotherms (Appendix 1).

1.3. DEFINITIONS AND UNITS

Symbol	Definition	Units
A_{t_i}	adsorption percentage at the time t_i	%
A_{eq}	adsorption percentage at adsorption equilibrium	%
$m_s^{ads}(t_i)$	mass of the test substance adsorbed on the soil at the time t_i	µg
$m_s^{ads}(\Delta t_i)$	mass of the test substance adsorbed on the soil during the time interval Δt_i	µg
$m_s^{ads}(eq)$	mass of the test substance adsorbed on the soil at adsorption equilibrium	µg
m_0	mass of the test substance in the test tube, at the beginning of the adsorption test	µg
$m_m^{ads}(t_i)$	mass of the test substance measured in an aliquot (v_a^A) at the time point t_i	µg
$m_{aq}^{ads}(eq)$	mass of the substance in the solution at adsorption equilibrium	µg
m_{soil}	quantity of the soil phase, expressed in dry mass of soil	g
C_{st}	mass concentration of the stock solution of the substance	µg cm^{-3}
C_0	initial mass concentration of the test solution in contact with the soil	µg cm^{-3}
$C_{aq}^{ads}(t_i)$	mass concentration of the substance in the aqueous phase at the time t_i that the analysis is performed	µg cm^{-3}
$C_s^{ads}(eq)$	content of the substance adsorbed on soil at adsorption equilibrium an equilibrium	µg g^{-1}
$C_{aq}^{ads}(eq)$	mass concentration of the substance in the aqueous phase at adsorption equilibrium	µg cm^{-3}
V_0	initial volume of the aqueous phase in contact with the soil during the adsorption test	cm^3
v_a^A	volume of the aliquot in which the test substance is measured	cm^3
K_d	distribution coefficient for adsorption	cm^3 g^{-1}
K_{oc}	organic carbon normalised adsorption coefficient	cm^3 g^{-1}
K_{om}	organic matter normalised distribution coefficient	cm^3 g^{-1}
K_F^{ads}	Freundlich adsorption coefficient	µg $^{1-1/n}$ (cm^3) $^{1/n}$ g^{-1}
1/n	Freundlich exponent	
D_{t_i}	desorption percentage at a point time t_i	%
$D_{\Delta t_i}$	desorption percentage corresponding to a time interval Δt_i	%
K_{des}	apparent desorption coefficient	cm^3 g^{-1}
K_F^{des}	Freundlich desorption coefficient	µg $^{1-1/n}$ (cm^3) $^{1/n}$ g^{-1}
$m_{aq}^{des}(t_i)$	mass of the test substance desorbed from soil at the time t_i	µg
$m_m^{des}(\Delta t_i)$	mass of the test substance desorbed from soil during the time Δt_i	µg
$m_m^{des}(eq)$	mass of the substance determined analytically in the aqueous phase at desorption equilibrium	µg
$m_{aq}^{des}(eq)$	total mass of the test substance desorbed at desorption equilibrium	µg

Symbol	Definition	Units
$m_s^{des}(\Delta t_i)$	mass of the substance remaining adsorbed on the soil after the time interval Δt_i	µg
m_{aq}^{A}	mass of the substance left over from the adsorption equilibrium due to incomplete volume replacement	µg
$C_s^{des}(eq)$	content of the test substance remaining adsorbed on the soil at desorption equilibrium	µg g^{-1}
$C_{aq}^{des}(eq)$	mass concentration of the test substance in the aqueous phase at desorption equilibrium	µg cm^{-3}
V_T	total volume of the aqueous phase in contact with the soil during the desorption kinetics experiment performed with the serial method	cm^3
V_R	volume of the supernatant removed from the tube after the attainment of adsorption equilibrium and replaced by the same volume of a 0,01 M $CaCl_2$ solution	cm^3
v_a^D	volume of the aliquot sampled for analytical purpose from the time (i), during the desorption kinetics experiment performed with the serial method	cm^3
V_r^r	volume of the solution taken from the tube (i) for the measurement of the test substance, in desorption kinetics experiment (parallel method)	cm^3
V_r^F	volume of the solution taken from the tube for the measurement of the test substance, at desorption equilibrium	cm^3
MB	mass balance	%
m_E	total mass of the test substance extracted from soil and walls of the test vessel in two steps	µg
V_{rec}	volume of the supernatant recovered after the adsorption equilibrium	cm^3
P_{ow}	octanol/water partition coefficient	
pKa	dissociation constant	
S_w	water solubility	g l^{-1}

1.4. PRINCIPLE OF THE TEST METHOD

Known volumes of solutions of the test substance, non-labelled or radiolabelled, at known concentrations in 0,01 M $CaCl_2$ are added to soil samples of known dry weight which have been pre-equilibrated in 0,01 M $CaCl_2$. The mixture is agitated for an appropriate time. The soil suspensions are then separated by centrifugation and, if so wished, filtration and the aqueous phase is analysed. The amount of test substance adsorbed on the soil sample is calculated as the difference between the amount of test substance initially present in solution and the amount remaining at the end of the experiment (indirect method).

As an option, the amount of the test substance adsorbed can also be directly determined by analysis of soil (direct method). This procedure which involves stepwise soil extraction with appropriate solvent, is recommended in cases where the difference in the solution concentration of the substance cannot be accurately determined. Examples of such cases are: adsorption of the test substance on surface of the test vessels, instability of the test substance in the time scale of the experiment, weak adsorption giving only small concentration change in the solution; and strong adsorption yielding low concentration which cannot be accurately determined. If radiolabelled substance is used, the soil extraction may be avoided by analysis of the soil phase by combustion and liquid scintillation counting. However, liquid scintillation counting is an unspecific technique which cannot differentiate between parental and transformation products; therefore it should be used only if the test chemical is stable for the duration of the study.

1.5. INFORMATION ON THE TEST SUBSTANCE

Chemical reagents should be of analytical grade. The use of non-labelled test substances with known composition and preferably at least 95 % purity or of radiolabelled test substances with known composition and radio-purity, is recommended. In the case of short half-life tracers, decay corrections should be applied.

Before carrying out a test for adsorption-desorption, the following information about the test substance should be available:

(a) water solubility (A.6);

(b) vapour pressure (A.4) and/or Henry's Law Constant;

(c) abiotic degradation: hydrolysis as a function of pH (C.7);

(d) partition coefficient (A.8);

(e) ready biodegradability (C.4) or aerobic and anaerobic transformation in soil;

(f) pKa of ionisable substances;

(g) direct photolysis in water (i.e. UV-vis absorption spectrum in water, quantum yield) and photodegradation on soil.

1.6. APPLICABILITY OF THE TEST

The test is applicable to chemical substances for which an analytical method with sufficient accuracy is available. An important parameter that can influence the reliability of the results, especially when the indirect method is followed, is the stability of the test substance in the time scale of the test. Thus, it is a prerequisite to check the stability in a preliminary study; if a transformation in the time scale of the test is observed, it is recommended that the main study be performed by analysing both soil and aqueous phases.

Difficulties may arise in conducting this test for test substances with low water solubility ($S_w < 10^{-4}$ g l^{-1}), as well as for highly charged substances, due to the fact that the concentration in the aqueous phase cannot be measured analytically with sufficient accuracy. In these cases, additional steps have to be taken. Guidance on how to deal with these problems is given in the relevant sections of this method.

When testing volatile substances, care should be taken to avoid losses during the study.

1.7. DESCRIPTION OF THE METHOD

1.7.1. **Apparatus and chemical reagents**

Standard laboratory equipment, especially the following:

(a) tubes or vessels to conduct the experiments. It is important that these tubes or vessels,

— fit directly in the centrifuge apparatus in order to minimise handling and transfer errors,

— be made of an inert material, which minimises adsorption of the test substance on its surface,

(b) agitation device: overhead shaker or equivalent equipment; the agitation device should keep the soil in suspension during shaking,

(c) centrifuge: preferably high-speed, e.g. centrifugation forces > 3 000 g, temperature controlled, capable of removing particles with a diameter greater than 0,2 μm from aqueous solution. The containers should be capped during agitation and centrifugation to avoid volatility and water losses; to minimise adsorption on them, deactivated caps such as teflon lined screw caps should be used,

(d) optional: filtration device; filters of 0,2 μm porosity, sterile, single use. Special care should be taken in the choice of the filter material, to avoid any losses of the test substance on it; for poorly soluble test substances, organic filter material is not recommended,

(e) analytical instrumentation, suitable for measuring the concentration of the test chemical,

(f) laboratory oven, capable of maintaining a temperature of 103 °C to 110 °C,

1.7.2. **Characterisation and selection of soils**

The soils should be characterised by three parameters considered to be largely responsible for the adsorptive capacity: organic carbon, clay content and soil texture, and pH. As already mentioned (see Scope) other physico-chemical properties of the soil may have an impact on the adsorption/desorption of a particular substance and should be considered in such cases.

The methods used for soil characterisation are very important and can have a significant influence on the results. Therefore, it is recommended that soil pH should be measured in a solution of 0,01 M $CaCl_2$ (that is the solution used in adsorption/desorption testing) according to the corresponding ISO method (ISO-10390-1). It is also recommended that the other relevant soil properties be determined according to standard methods (for example ISO 'Handbook of Soil Analysis'); this permits the analysis of sorption data to be based on globally standardised soil parameters. Some guidance for existing standard methods of soil analysis and characterisation is given in references (50-52). For calibration of soil test methods, the use of reference soils is recommended.

Guidance for selection of soils for adsorption/desorption experiments is given in Table 1. The seven selected soils cover soil types encountered in temperate geographical zones. For ionisable test substances, the selected soils should cover a wide range of pH, in order to be able to evaluate the adsorption of the substance in its ionised and unionised forms. Guidance on how many different soils to use at the various stages of the test is given under 'Performance of the test' 1.9.

If other soil types are preferred, they should be characterised by the same parameters and should have similar variation in properties to those described in Table 1, even if they do not match the criteria exactly.

Table 1:

Guidance for selection of soil samples for adsorption-desorption

Soil Type	pH range (in 0,01 M $CaCl_2$)	Organic carbon content (%)	Clay content (%)	Soil texture [1]
1	4,5 - 5,5	1,0 - 2,0	65-80	clay
2	> 7,5	3,5 - 5,0	20-40	clay loam
3	5,5 - 7,0	1,5 - 3,0	15-25	silt loam
4	4,0 - 5,5	3,0 - 4,0	15-30	loam
5	< 4,0 - 6,0 [2]	< 0,5 - 1,5 [2] [3]	< 10-15 [2]	loamy sand
6	> 7,0	< 0,5 - 1,0 [2] [3]	40-65	clay loam/clay
7	< 4,5	> 10	< 10	sand/loamy sand

[1] According to FAO and the US system (85).
[2] The respective variables should preferably show values within the range given. If, however, difficulties in finding appropriate soil material occur, values below the indicated minimum are accepted.
[3] Soils with less than 0,3 % organic carbon may disturb correlation between organic content and adsorption. Thus, it is recommended the use of soils with a minimum organic carbon content of 0,3 %.

1.7.3. **Collection and storage of soil samples**

1.7.3.1. *Collection*

No specific sampling techniques or tools are recommended; the sampling technique depends on the purpose of the study (53)(54)(55)(56)(57)(58).

The following should be considered:

a) detailed information on the history of the field site is necessary; this includes location, vegetation cover, treatments with pesticides and/or fertilisers, biological additions or accidental contamination. Recommendations of the ISO standard on soil sampling (ISO 10381-6) should be followed with respect to the description of the sampling site;

b) the sampling site has to be defined by UTM (Universal Transversal Mercator-Projection/European Horizontal Datum) or geographical co-ordinates; this could allow recollection of a particular soil in the future or could help in defining soil under various classification systems used in different countries. Also, only A horizon up to a maximum depth of 20 cm should be collected. Especially for the soil type No 7 if a O_h horizon is present as part of the soil, it should be included in the sampling.

The soil samples should be transported using containers and under temperature conditions which guarantee that the initial soil properties are not significantly altered.

1.7.3.2. *Storage*

The use of soils freshly taken from the field is preferred. Only if this is not possible soil can be stored at ambient temperature and should be kept air-dried. No limit on the storage time is recommended, but soils stored for more than three years should be re-analysed prior to the use with respect to their organic carbon content, pH and CEC.

1.7.3.3. *Handling and preparation of soil samples for the test*

The soils are air-dried at ambient temperature (preferably between 20-25 °C). Disaggregation should be performed with minimal force, so that the original texture of the soil will be changed as little as possible. The soils are sieved to a particle size ≤ 2 mm; recommendations of the ISO standard on soil sampling (ISO 10381-6) should be followed with respect to the sieving process. Careful homogenisation is recommended, as this enhances the reproducibility of the results. The moisture content of each soil is determined on three aliquots with heating at 105 °C until there is no significant change in weight (approximately 12 h). For all calculations the mass of soil refers to oven dry mass, i.e. the weight of soil corrected for moisture content.

1.7.4. **Preparation of the test substance for application to soil**

The test substance is dissolved in a solution of 0,01 M $CaCl_2$ in distilled or de-ionised water; the $CaCl_2$ solution is used as the aqueous solvent phase to improve centrifugation and minimise cation exchange. The concentration of the stock solution should preferably be three orders of magnitude higher than the detection limit of the analytical method used. This threshold safeguards accurate measurements with respect to the methodology followed in this method; additionally, the stock solution concentration should be below water solubility of the test substance.

The stock solution should preferably be prepared just before application to soil samples and should be kept closed in the dark at 4 °C. The storage time depends on the stability of the test substance and its concentration in the solution.

Only for poorly soluble substances ($S_w < 10^{-4}$ g l^{-1}), an appropriate solubilising agent may be needed when it is difficult to dissolve the test substance. This solubilising agent: (a) should be miscible with water such as methanol or acetonitrile; (b) its concentration should not exceed 1 % of the total volume of the stock solution and should constitute less than that in the solution of the test substance which will come in contact with the soil (preferably less than 0,1 %); and (c) should not be a surfactant or undergo solvolytic reactions with the test chemical. The use of a solubilising agent should be stipulated and justified in the reporting of the data.

Another alternative for poorly soluble substances is to add the test substance to the test system by spiking: the test substance is dissolved in an organic solvent, an aliquot of which is added to the system of soil and 0,01 M solution of $CaCl_2$ in distilled or de-ionised water. The content of organic solvent in the aqueous phase should be kept as low as possible, normally not exceeding 0,1 %. Spiking from an organic solution may suffer from volume unreproducibility. Thus, an additional error may be introduced as the test substance and co-solvent concentration would not be the same in all tests.

1.8. PREREQUISITES FOR PERFORMING THE ADSORPTION/DESORPTION TEST

1.8.1. **The analytical method**

The key parameters that can influence the accuracy of sorption measurements include the accuracy of the analytical method in analysis of both the solution and adsorbed phases, the stability and purity of the test substance, the attainment of sorption equilibrium, the magnitude of the solution concentration change, the soil/solution ratio and changes in the soil structure during the equilibration process (35)(59-62). Some examples bearing upon the accuracy issues are given in Appendix 2.

The reliability of the analytical method used must be checked at the concentration range which is likely to occur during the test. The experimenter should feel free to develop an appropriate method with appropriate accuracy, precision, reproducibility, detection limits and recovery. Guidance on how to perform such a test is given by the experiment below.

An appropriate volume of 0,01 M $CaCl_2$, e.g. 100 cm^3, is agitated during 4 h with a weight of soil, e.g. 20 g, of high adsorbability, i.e. with high organic carbon and clay content; these weights and volumes may vary depending on analytical needs, but a soil/solution ratio of 1:5 is a convenient starting point. The mixture is centrifuged and the aqueous phase may be filtrated. A certain volume of the test substance stock solution is added to the latter to reach a nominal concentration within the concentration range which is likely to occur during the test. This volume should not exceed 10 % of the final volume of the aqueous phase, in order to change as little as possible the nature of the pre-equilibration solution. The solution is analysed.

One blank run consisting of the system soil + $CaCl_2$ solution (without test substance) must be included, in order to check for artefacts in the analytical method and for matrix effects caused by the soil.

The analytical methods which can be used for sorption measurements include gas-liquid chromatography (GLC), high-performance liquid chromatography (HPLC), spectrometry (e.g. GC/mass spectrometry, HPLC/mass spectrometry) and liquid scintillation counting (for radiolabelled substances). Independent of the analytical method used, it is considered suitable if the recoveries are between 90 % and 110 % of the nominal value. In order to allow for detection and evaluation after partitioning has taken place, the detection limits of the analytical method should be at least two orders of magnitude below the nominal concentration.

The characteristics and detection limits of the analytical method available for carrying out adsorption studies play an important role in defining the test conditions and the whole experimental performance of the test. This method follows a general experimental path and provides recommendations and guidance for alternative solutions where the analytical method and laboratory facilities may impose limitations.

1.8.2. **The selection of optimal soil/solution ratios**

Selection of appropriate soil to solution ratios for sorption studies depends on the distribution coefficient K_d and the relative degree of adsorption desired. The change of the substance concentration in the solution determines the statistical accuracy of the measurement based on the form of adsorption equation and the limit of the analytical methodology, in detecting the concentration of the chemical in solution. Therefore, in general practice it is useful to settle on a few fixed ratios, for which the percentage adsorbed is above 20 %, and preferably >50 % (62), while care should be taken to keep the test substance concentration in the aqueous phase high enough to be measured accurately. This is particularly important in the case of high adsorption percentages.

A convenient approach to selecting the appropriate soil/water ratios, is based on an estimate of the K_d value either by preliminary studies or by established estimation techniques (Appendix 3). Selection of an appropriate ratio can then be made based on a plot of soil/solution ratio versus K_d for fixed percentages of adsorption (Fig.1). In this plot it is assumed that the adsorption equation is linear [1]. The applicable relationship is obtained by rearranging equation (4) of the K_d in the form of equation (1):

$$\frac{V_0}{m_{soil}} = \left(\frac{m_0}{m_s^{ads}(eq)} - 1 \right) K_d \qquad (1)$$

[1] $C_s^{ads}(eq) = K_d \cdot C_{aq}^{ads}(eq)$.

or in its logarithmic form assuming that $R = m_{soil}/V_0$ and $A_{eq}\,\%/100 = \frac{m_s^{ads}(eq)}{m_0}$:

$$\log R = -\log K_d + \log\left[\frac{(A_{eq}\%/100)}{(1 - A_{eq}\%/100)}\right] \quad (2)$$

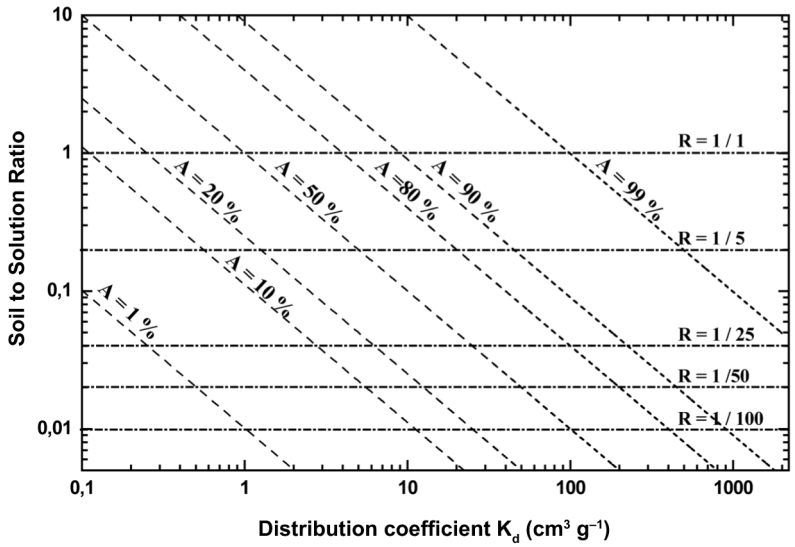


Fig. 1 Relationship between soil to solution ratios and K_d at various percentages of adsorbed test substance

Fig. 1 shows soil/solution ratios required as a function of K_d for different levels of adsorption. For example, with a soil/solution ratio of 1:5 and a K_d of 20, approximately 80 % adsorption would occur. To obtain 50 % adsorption for the same K_d, a 1:25 ratio must be used. This approach to selecting the appropriate soil/solution ratios gives the investigator the flexibility to meet experimental needs.

Areas which are more difficult to deal with are those where the chemical is highly or very slightly adsorbed. Where low adsorption occurs, a 1:1 soil/solution ratio is recommended, although for some very organic soil types smaller ratios may be necessary to obtain a slurry. Care must be taken with the analytical methodology to measure small changes in solution concentration; otherwise the adsorption measurement will be inaccurate. On the other hand, at very high distribution coefficients K_d, one can go up to a 1:100 soil/solution ratio in order to leave a significant amount of chemical in solution. However, care must be taken to ensure good mixing, and adequate time must be allowed for the system to equilibrate. An alternative approach to deal with these extreme cases when adequate analytical methodology is missing, is to predict the K_d value applying estimation techniques based, for example, on P_{ow} values (Appendix 3). This could be useful especially for low adsorbed/polar chemicals with $P_{ow} < 20$ and for lipophilic/highly sorptive chemicals with $P_{ow} > 10^4$.

1.9. PERFORMANCE OF THE TEST

1.9.1. **Test conditions**

All experiments are done at ambient temperature and, if possible, at a constant temperature between 20 °C and 25 °C.

Centrifugation conditions should allow the removal of particles larger than 0,2 μm from the solution. This value triggers the smallest sized particle that is considered as a solid particle, and is the limit between solid and colloid particles. Guidance on how to determine the centrifugation conditions is given in Appendix 4.

If the centrifugation facilities cannot guarantee the removal of particles larger than 0,2 μm, a combination of centrifugation and filtration with 0,2 μm filters could be used. These filters should be made of a suitable inert material to avoid any losses of the test substance on them. In any case, it should be proven that no losses of the test substance occur during filtration.

1.9.2. **Tier 1 — Preliminary study**

The purpose of conducting a preliminary study has already been given in the Scope section. Guidance for setting up such a test is given with the experiment suggested below.

1.9.2.1. *Selection of optimal soil/solution ratios*

Two soil types and three soil/solution ratios (six experiments) are used. One soil type has high organic carbon and low clay content, and the other low organic carbon and high clay content. The following soil to solution ratios are suggested:

— 50 g soil and 50 cm^3 aqueous solution of the test substance (ratio 1/1),

— 10 g soil and 50 cm^3 aqueous solution of the test substance (ratio 1/5),

— 2 g soil and 50 cm^3 aqueous solution of the test substance (ratio 1/25).

The minimum amount of soil on which the experiment can be carried out depends on the laboratory facilities and the performance of analytical methods used. However, it is recommended to use at least 1 g, and preferably 2 g, in order to obtain reliable results from the test.

One control sample with only the test substance in 0,01 M $CaCl_2$ solution (no soil) is subjected to precisely the same steps as the test systems, in order to check the stability of the test substance in $CaCl_2$ solution and its possible adsorption on the surfaces of the test vessels.

A blank run per soil with the same amount of soil and total volume of 50 cm^3 0,01 M $CaCl_2$ solution (without test substance) is subjected to the same test procedure. This serves as a background control during the analysis to detect interfering substances or contaminated soils.

All the experiments, included controls and blanks, should be performed at least in duplicate. The total number of the samples which should be prepared for the study can be calculated with respect to the methodology which will be followed.

Methods for the preliminary study and the main study are generally the same, exceptions are mentioned where relevant.

The air-dried soil samples are equilibrated by shaking with a minimum volume of 45 cm^3 of 0,01 M $CaCl_2$ overnight (12 h) before the day of the experiment. Afterwards, a certain volume of the stock solution of the test substance is added in order to adjust the final volume to 50 cm^3. This volume of the stock solution added: (a) should not exceed 10 % of the final 50 cm^3 volume of the aqueous phase in order to change as little as possible the nature of the pre-equilibration solution; and (b) should preferably result in an initial concentration of the test substance being in contact with the soil (C_0) at least two orders of magnitude higher than the detection limit of the analytical method; this threshold safeguards the ability to perform accurate measurements even when strong adsorption occurs (> 90 %) and to determine later the adsorption isotherms. It is also recommended, if possible, that the initial substance concentration (C_0) not exceed half of its solubility limit.

An example of how to calculate the concentration of the stock solution (C_{st}) is given below. A detection limit of 0,01 μg cm^{-3} and 90 % adsorption are assumed; thus, the initial concentration of the test substance in contact with the soil should preferably be 1 μg cm^{-3} (two orders of magnitude higher than the detection limit). Supposing that the maximum recommended volume of the stock solution is added, i.e. 5 to 45 cm^3 0,01 M $CaCl_2$ equilibration solution (= 10 % of the stock solution to 50 cm^3 total volume of aqueous phase), the concentration of the stock solution should be 10 μg cm^{-3}; this is three orders of magnitude higher than the detection limit of the analytical method.

The pH of the aqueous phase should be measured before and after contact with the soil since it plays an important role in the whole adsorption process, especially for ionisable substances.

The mixture is shaken until adsorption equilibrium is reached. The equilibrium time in soils is highly variable, depending on the chemical and the soil; a period of 24 h is generally sufficient (77). In the preliminary study, samples may be collected sequentially over a 48 h period of mixing (for example at 4, 8, 24, 48 h). However, times of analysis should be considered with flexibility with respect to the work schedule of the laboratory.

There are two options for the analysis of the test substance in the aqueous solution: (a) the parallel method and (b) the serial method. It should be stressed that, although the parallel method is experimentally more tedious, the mathematical treatment of the results is simpler (Appendix 5). However, the choice of the methodology to be followed, is left to the experimenter who will need to consider the available laboratory facilities and resources.

(a) parallel method: samples with the same soil/solution ratio are prepared, as many as the time intervals at which it is desired to study the adsorption kinetics. After centrifugation and if so wished filtration, the aqueous phase of the first tube is recovered as completely as possible and is measured after, for example, 4 h, that of the second tube after 8 h, that of the third after 24, etc.

(b) serial method: only a duplicate sample is prepared for each soil/solution ratio. At defined time intervals the mixture is centrifuged to separate the phases. A small aliquot of the aqueous phase is immediately analysed for the test substance; then the experiment continues with the original mixture. If filtration is applied after centrifugation, the laboratory should have facilities to handle filtration of small aqueous aliquots. It is recommended that the total volume of the aliquots taken not exceed 1 % of the total volume of the solution, in order not to change significantly the soil/solution ratio and to decrease the mass of solute available for adsorption during the test.

The percentage adsorption A_{t_i} is calculated at each time point (t_i) on the basis of the nominal initial concentration and the measured concentration at the sampling time (t_i), corrected for the value of the blank. Plots of the A_{t_i} versus time (Fig. 1 Appendix 5) are generated in order to estimate the achievement of equilibrium plateau ([1]). The K_d value at equilibrium is also calculated. Based on this K_d value, appropriate soil/solution ratios are selected from Fig.1, so that the percentage adsorption reaches above 20 % and preferably >50 % (61). All the applicable equations and principles of plotting are given in section on Data and Reporting and in Appendix 5.

1.9.2.2. *Determination of adsorption equilibration time and of the amount of test substance adsorbed at equilibrium*

As already mentioned, plots of A_{t_i} or C_{aq}^{ads} versus time permit estimation of the achievement of the adsorption equilibrium and the amount of test substance adsorbed at equilibrium. Figs. 1 and 2 in the Appendix 5 show examples of such plots. Equilibration time is the system needs to reach a plateau.

If, with a particular soil, no plateau but a steady increase is found, this may be due to complicating factors such as biodegradation or slow diffusion. Biodegradation can be shown by repeating the experiment with a sterilised sample of the soil. If no plateau is achieved even in this case, the experimenter should search for other phenomena that could be involved in his specific studies; this could be done with appropriate modifications of the experiment conditions (temperature, shaking times, soil/solution ratios). It is left to the experimenter to decide whether to continue the test procedure in spite of a possible failure to achieve an equilibrium.

1.9.2.3. *Adsorption on the surface of the test vessel and stability of the test substance*

Some information on the adsorption of the test substance on the surface of test vessels, as well as its stability, can be derived by analysing the control samples. If a depletion more than the standard error of the analytical method is observed, abiotic degradation and/or adsorption on the surface of the test vessel could be involved. Distinction between these two phenomena could be achieved by thoroughly washing the walls of the vessel with a known volume of an appropriate solvent and subjecting the wash solution to analysis for the test substance. If no adsorption on the surface of the test vessels is observed, the depletion demonstrates abiotic unstability of the test substance. If adsorption is found, changing the material of the test vessels is necessary. However, data on the adsorption on the surface of the test vessels gained from this experiment cannot be directly extrapolated to soil/solution experiment. The presence of soil will affect this adsorption.

([1]) Plots of the concentration of the test substance in the aqueous phase $\left(C_{aq}^{ads}\right)$ versus time could also be used to estimate the achievement of the equilibrium plateau (see Fig. 2 in Appendix 5).

Additional information on the stability of the test substance can be derived by determination of the parental mass balance over time. This means that the aqueous phase, extracts of soil and test vessel walls are analysed for the test substance. The difference between the mass of the test chemical added and the sum of the test chemical masses in the aqueous phase, extracts of the soil and test vessel walls is equal to the mass degraded and/or volatilised and/or not extracted. In order to perform a mass balance determination, the adsorbion equilibrium should have been reached within the period of the experiment.

The mass balance is performed on both soils and for one soil/solution ratio per soil that gives a depletion above 20 % and preferably > 50 % at equilibrium. When the ratio-finding experiment is completed with the analysis of the last sample of the aqueous phase after 48 h, the phases are separated by centrifugation and, if so wished, filtration. The aqueous phase is recovered as much as possible, and a suitable extraction solvent (extraction coefficient of at least 95 %) is added to the soil to extract the test substance. At least two successive extractions are recommended. The amount of test substance in the soil and test vessel extracts is determined and the mass balance is calculated (equation 10, Data and Reporting). If it is less than 90 %, the test substance is considered to be unstable in the time scale of the test. However, studies could still be continued, taking into account the unstability of the test substance; in this case it is recommended to analyse both phases in the main study.

1.9.3. *Tier 2 — Adsorption kinetics at one concentration of the test substance*

Five soils are used, selected from Table 1. There is an advantage to including some or all of the soils used in the preliminary study, if appropriate, among these five soils. In this case, Tier 2 has not to be repeated for the soils used in preliminary study.

The equilibration time, the soil/solution ratio, the weight of the soil sample, the volume of the aqueous phase in contact with the soil and concentration of the test substance in the solution are chosen based on the preliminary study results. Analysis should preferably be done approximately after 2, 4, 6, 8 (possibly also 10) and 24 h contact time; the agitation time may be extended to a maximum of 48 h in case a chemical requires longer equilibration time with respect to ratio-finding results. However, times of analysis could be considered with flexibility.

Each experiment (one soil and one solution) is done at least in duplicate to allow estimation of the variance of the results. In every experiment one blank is run. It consists of the soil and 0,01 M $CaCl_2$ solution, without test substance, and of weight and volume, respectively, identical to those of the experiment. A control sample with only the test substance in 0,01 M $CaCl_2$ solution (without soil) is subjected to the same test procedure, serving to safeguard against the unexpected.

The percentage adsorption is calculated at each time point A_{t_i} and/or time interval $A_{\Delta t_i}$ (according to the need) and is plotted versus time. The distribution coefficient K_d at equilibrium, as well as the organic carbon normalised adsorption coefficient K_{oc} (for non-polar organic chemicals), are also calculated.

Results of the adsorption kinetics test

The linear K_d value is generally accurate to describe sorptive behaviour in soil (35)(78) and represents an expression of inherent mobility of chemicals in soil. For example, in general chemicals with $K_d \leq 1$ cm^3 g^{-1} are considered to be qualitatively mobile. Similarly, a mobility classification scheme based on K_{oc} values has been developed by MacCall *et al.* (16). Additionally, leaching classification schemes exist based on a relationship between K_{oc} and DT-50 ([1]) (32)(79).

Also, according to error analysis studies (61), K_d values below 0,3 cm^3 g^{-1} cannot be estimated accurately from a decrease in concentration in the aqueous phase, even when the most favourable (from point of view of accuracy) soil/solution ratio is applied, i.e. 1:1. In this case analysis of both phases, soil and solution, is recommended.

With respect to the above remarks, it is recommended that the study of the adsorptive behaviour of a chemical in soil and its potential mobility be continued by determining Freundlich adsorption isotherms for these systems, for which an accurate determination of K_d is possible with the experimental protocol followed in this test method. Accurate determination is possible if the value which results by multiplying the K_d with the soil/solution ratio is > 0,3, when measurements are based on concentration decrease in the aqueous phase (indirect method), or > 0,1, when both phases are analysed (direct method) (61).

([1]) DT-50: degradation time for 50 % of the test substance.

1.9.4. *Tier 3 — Adsorption isotherms and desorption kinetics/desorption isotherms*

1.9.4.1. Adsorption isotherms

Five test substance concentrations are used, covering preferably two orders of magnitude; in the choice of these concentrations the water solubility and the resulting aqueous equilibrium concentrations should be taken into account. The same soil/solution ratio per soil should be kept along the study. The adsorption test is performed as described above, with the only difference that the aqueous phase is analysed only once at the time necessary to reach equilibrium as determined before in Tier 2. The equilibrium concentrations in the solution are determined and the amount adsorbed is calculated from the depletion of the test substance in the solution or with the direct method. The adsorbed mass per unit mass of soil is plotted as a function of the equilibrium concentration of the test substance (see Data and Reporting).

Results from the adsorption isotherms experiment

Among the mathematical adsorption models proposed so far, the Freundlich isotherm is the one most frequently used to describe adsorption processes. More detailed information on the interpretation and importance of adsorption models is provided in the references (41)(45)(80)(81)(82).

Note: it should be mentioned that a comparison of K_F (Freundlich adsorption coefficient) values for different substances is only possible if these K_F values are expressed in the same units (83).

1.9.4.2. Desorption kinetics

The purpose of this experiment is to investigate whether a chemical is reversibly or irreversibly adsorbed on a soil. This information is important, since the desorption process also plays an important role in the behaviour of a chemical in field soil. Moreover, desorption data are useful inputs in the computer modelling of leaching and dissolved run-off simulation. If a desorption study is desired, it is recommended that the study described below be carried out on each system for which an accurate determination of K_d in the preceding adsorption kinetics experiment was possible.

Likewise with the adsorption kinetics study, there are two options to proceed with the desorption kinetics experiment: (a) the parallel method and (b) serial method. The choice of methodology to be followed, is left to the experimenter who will need to consider the available laboratory facilities and resources.

(a) parallel method: for each soil which is chosen to proceed with the desorption study, samples with the same soil/solution ratio are prepared, as many as the time intervals at which it is desired to study the desorption kinetics. Preferably, the same time intervals as in the adsorption kinetics experiment should be used; however, the total time may be extended as appropriate in order the system to reach desorption equilibrium. In every experiment (one soil, one solution) one blank is run. It consists of the soil and 0,01 M $CaCl_2$ solution, without test substance, and of weight and volume, respectively, identical to those of the experiment. As a control sample the test substance in 0,01 M $CaCl_2$ solution (without soil) is subjected to the same test procedure. All the mixtures of the soil with the solution is agitating until to reach adsorption equilibrium (as determined before in Tier 2). Then, the phases are separated by centrifugation and the aqueous phases are removed as much as possible. The volume of solution removed is replaced by an equal volume of 0,01 M $CaCl_2$ without test substance and the new mixtures are agitated again. The aqueous phase of the first tube is recovered as completely as possible and is measured after, for example, 2 h, that of the second tube after 4 h, that of the third after 6 h, etc. until the desorption equilibrium is reached.

(b) serial method: after the adsorption kinetics experiment, the mixture is centrifuged and the aqueous phase is removed as much as possible. The volume of solution removed is replaced by an equal volume of 0,01 M $CaCl_2$ without test substance. The new mixture is agitated until the desorption equilibrium is reached. During this time period, at defined time intervals, the mixture is centrifuged to separate the phases. A small aliquot of the aqueous phase is immediately analysed for the test substance; then, the experiment continues with the original mixture. The volume of each individual aliquot should be less than 1 % of the total volume. The same quantity of fresh 0,01 M $CaCl_2$ solution is added to the mixture to maintain the soil to solution ratio, and the agitation continues until the next time interval.

The percentage desorption is calculated at each time point (D_{t_i}) and/or time interval ($D_{\Delta t_i}$) (according to the needs of the study) and is plotted versus time. The desorption coefficient of K_{des} at equilibrium is also calculated. All applicable equations are given in Data and Reporting and Appendix 5.

Results from desorption kinetics experiment

Common plots of the percentage desorption D_{t_i} and adsorption A_{t_i} versus time, allow estimation of the reversibility of the adsorption process. If the desorption equilibrium is attained even within twice the time of the adsorption equilibrium, and the total desorption is more than 75 % of the amount adsorbed, the adsorption is considered to be reversible.

1.9.4.3. Desorption isotherms

Freundlich desorption isotherms are determined on the soils used in the adsorption isotherms experiment. The desorption test is performed as described in the section 'Desorption kinetics', with the only difference that the aqueous phase is analysed only once, at desorption equilibrium. The amount of the test substance desorbed is calculated. The content of test substance remaining adsorbed on soil at desorption equilibrium is plotted as a function of the equilibrium concentration of the test substance in solution (see Data and Reporting and Appendix 5).

2. DATA AND REPORTING

The analytical data are presented in tabular form (see Appendix 6). Individual measurements and averages calculated are given. Graphical representations of adsorption isotherms are provided. The calculations are made as described below.

For the purpose of the test, it is considered that the weight of 1 cm^3 of aqueous solution is 1g. The soil/solution ratio may be expressed in units of w/w or w/vol with the same figure.

2.1. ADSORPTION

The adsorption A_{t_i} is defined as the percentage of substance adsorbed on the soil related to the quantity present at the beginning of the test, under the test conditions. If the test substance is stable and does not adsorb significantly to the container wall, A_{t_i} is calculated at each time point t_i, according to the equation:

$$A_{t_i} = \frac{m_s^{ads}(t_i) \cdot 100}{m_0} (\%) \quad (3)$$

where:

A_{t_i} = adsorption percentage at the time point t_i (%);
$m_s^{ads}(t_i)$ = mass of the test substance adsorbed on the soil at the time t_i (μg);
m_0 = mass of the test substance in the test tube, at the beginning of the test (μg).

Detailed information on how to calculate the percentage of adsorption A_{t_i} for the parallel and serial methods is given in Appendix 5.

The distribution coefficient K_d is the ratio between the content of the substance in the soil phase and the mass concentration of the substance in the aqueous solution, under the test conditions, when adsorption equilibrium is reached.

$$K_d = \frac{C_s^{ads}(eq)}{C_{aq}^{ads}(eq)} = \frac{m_s^{ads}(eq)}{m_{aq}^{ads}(eq)} \cdot \frac{V_0}{m_{soil}} (cm^3\ g^{-1}) \quad (4)$$

where:

$C_s^{ads}(eq)$ = content of the substance adsorbed on the soil at adsorption equilibrium (μg g^{-1});

$C_{aq}^{ads}(eq)$ = mass concentration of the substance in the aqueous phase at adsorption equilibrium (μg cm^{-3}). This concentration is analytically determined taking into account the values given by the blanks;

$m_s^{ads}(eq)$ = mass of the substance adsorbed on the soil at adsorption equilibrium (μg);
$m_{aq}^{ads}(eq)$ = mass of the substance in the solution at adsorption equilibrium (μg);
m_{soil} = quantity of the soil phase, expressed in dry mass of soil (g);
V_0 = initial volume of the aqueous phase in contact with the soil (cm^3).

The relation between A_{eq} and K_d is given by:

$$K_d = \frac{A_{eq}}{100 - A_{eq}} \cdot \frac{V_0}{m_{soil}} (cm^3\ g^{-1}) \quad (5)$$

where:

A_{eq} = percentage of adsorption at adsorption equilibrium, %.

The organic carbon normalised adsorption coefficient K_{oc} relates the distribution coefficient K_d to the content of organic carbon of the soil sample:

$$K_{oc} = K_d \cdot \frac{100}{\%OC} (cm^3\ g^{-1}) \quad (6)$$

where:

%OC = percentage of organic carbon in the soil sample (g g^{-1}).

K_{oc} coefficient represents a single value which characterises the partitioning mainly of non-polar organic chemicals between organic carbon in the soil or sediment and water. The adsorption of these chemicals is correlated with the organic content of the sorbing solid (7); thus, K_{oc} values depend on the specific characteristics of the humic fractions which differ considerably in sorption capacity, due to differences in origin, genesis, etc.

2.1.1. **Adsorption isotherms**

The Freundlich adsorption isotherms equation relates the amount of the test substance adsorbed to the concentration of the test substance in solution at equilibrium (equation 8).

The data are treated as under 'Adsorption' and, for each test tube, the content of the test substance adsorbed on the soil after the adsorption test ($C_s^{ads}(eq)$, elsewhere denoted as x/m) is calculated. It is assumed that equilibrium has been attained and that $C_s^{ads}(eq)$ represents the equilibrium value:

$$C_s^{ads}(eq) = \frac{m_s^{ads}(eq)}{m_{soil}} = \frac{\left[C_0 - C_{aq}^{ads}(eq)\right] \cdot V_0}{m_{soil}} (\mu g\ g^{-1}) \quad (7)$$

The Freundlich adsorption equation is shown in (8):

$$C_s^{ads}(eq) = K_F^{ads} \cdot C_{aq}^{ads}(eq)^{1/n} (\mu g\ g^{-1}) \quad (8)$$

or in the linear form:

$$\log C_s^{ads}(eq) = \log K_F^{ads} + 1/n \cdot \log C_{aq}^{ads}(eq) \quad (9)$$

where:

K_F^{ads} = Freundlich adsorption coefficient; its dimension is $cm^3\ g^{-1}$ only if 1/n = 1; in all other cases, the slope 1/n is introduced in the dimension of K_F^{ads} ($\mu g^{1-1/n}$ $(cm^3)^{1/n}$ g^{-1});

n = regression constant; 1/n generally ranges between 0,7-1,0 indicating that sorption data is frequently slightly non-linear.

Equations (8) and (9) are plotted and the values of K_F^{ads} and 1/n are calculated by regression analysis using the equation 9. The correlation coefficient r^2 of the log equation is also calculated. An example of such plots is given in Fig. 2.

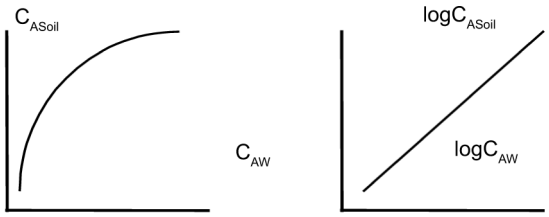


Fig. 2 Freundlich Adsorption Plot, normal and linearised

2.1.2. **Mass balance**

The mass balance (MB) is defined as the percentage of substance which can be analytically recovered after an adsorption test versus the nominal amount of substance at the beginning of the test.

The treatment of data will differ if the solvent is completely miscible with water. In the case of water-miscible solvent, the treatment of data described under 'Desorption' may be applied to determine the amount of substance recovered by solvent extraction. If the solvent is less miscible with water, the determination of the amount recovered has to be made.

The mass balance MB for the adsorption is calculated as follows; it is assumed that the term (m_E) corresponds to the sum of the test chemical masses extracted from the soil and surface of the test vessel with an organic solvent:

$$MB = \frac{\left(V_{rec} \cdot C_{aq}^{ads}(eq) + m_E\right) \cdot 100}{V_0 \cdot C_0} (\%) \qquad (10)$$

where:

MB = mass balance (%);
m_E = total mass of test substance extracted from the soil and walls of the test vessel in two steps (μg);
C_0 = initial mass concentration of the test solution in contact with the soil (μg cm^{-3});
V_{rec} = volume of the supernatant recovered after the adsorption equilibrium (cm^{-3}).

2.2. DESORPTION

The desorption (D) is defined as the percentage of the test substance which is desorbed, related to the quantity of substance previously adsorbed, under the test conditions:

$$D_{t_i} = \frac{m_{aq}^{des}(t_i)}{m_s^{ads}(eq)} \cdot 100(\%) \qquad (11)$$

where:

D_{t_i} = desorption percentage at a time point t_i(%);

$m_{aq}^{des}(t_i)$ = mass of the test substance desorbed from soil at a time point t_i (μg);

$m_s^{ads}(eq)$ = mass of the test substance adsorbed on soil at adsorption equilibrium (μg).

Detailed information on how to calculate the percentage of desorption D_{t^i} for the parallel and serial methods is given in Appendix 5.

The apparent desorption coefficient (K_{des}) is, under the test conditions, the ratio between the content of the substance remaining in the soil phase and the mass concentration of the desorbed substance in the aqueous solution, when desorption equilibrium is reached:

$$K_{des} = \frac{m_s^{ads}(eq) - m_{aq}^{des}(eq)}{m_{aq}^{des}(eq)} \cdot \frac{V_T}{m_{soil}} (cm^3\ g^{-1}) \quad (12)$$

where:

K_{des} = desorption coefficient ($cm^3\ g^{-1}$);
$m_{aq}^{des}(eq)$ = total mass of the test substance desorbed from soil at desorption equilibrium (μg);
V_T = total volume of the aqueous phase in contact with the soil during the desorption kinetics test (cm^3).

Guidance for calculating the $m_{aq}^{des}(eq)$ is given in Appendix 5 under the heading 'Desorption'.

Remark

If the adsorption test which was preceded, was performed with the parallel method the volume V_T in the equation (12) is considered to be equal to V_0.

2.2.1. **Desorption isotherms**

The Freundlich desorption isotherms equation relates the content of the test substance remaining adsorbed on the soil to the concentration of the test substance in solution at desorption equilibrium (equation 16).

For each test tube, the content of the substance remaining adsorbed on soil at desorption equilibrium is calculated as follows:

$$C_s^{des}(eq) = \frac{m_s^{ads}(eq) - m_{aq}^{des}(eq)}{m_{soil}} (\mu g\ g^{-1}) \quad (13)$$

$m_{aq}^{des}(eq)$ is defined as:

$$m_{aq}^{des}(eq) = m_m^{des}(eq) \cdot \frac{V_0}{V_r^F} - m_{aq}^A (\mu g) \quad (14)$$

where:

$C_s^{des}(eq)$ = content of the test substance remaining adsorbed on the soil at desorption equilibrium (μg g^{-1});
$m_m^{des}(eq)$ = mass of substance determined analytically in the aqueous phase at desorption equilibrium (μg);
m_{aq}^A = mass of the test substance left over from the adsorption equilibrium due to incomplete volume replacement (μg);
$m_{aq}^{des}(eq)$ = mass of the substance in the solution at adsorption equilibrium (μg);

$$m_{aq}^A = m_{aq}^{ads}(eq) \cdot \left(\frac{V_0 - V_R}{V_0}\right) \quad (15)$$

V_r^F = volume of the solution taken from the tube for the measurement of the test substance, at desorption equilibrium (cm^3);
V_R = volume of the supernatant removed from the tube after the attainment of adsorption equilibrium and replaced by the same volume of a 0,01 M $CaCl_2$ solution (cm^3);

The Freundlich desorption equation is shown in (16):

$$C_s^{des}(eq) = K_F^{des} \cdot C_{aq}^{des}(eq)^{1/n} (\mu g\ g^{-1}) \quad (16)$$

or in the linear form:

$$\log C_s^{des}(eq) = \log K_F^{des} + 1/n \cdot \log C_{aq}^{des}(eq) \quad (17)$$

where:

K_F^{des} = Freundlich desorption coefficient;
n = regression constant;
$C_{aq}^{des}(eq)$ = mass concentration of the substance in the aqueous phase at desorption equilibrium (μg cm^{-3}).

The equations (16) and (17) can be plotted and the value of K_F^{des} and 1/n are calculated by regression analysis using the equation 17.

Remark:

if the Freundlich adsorption or desorption exponent 1/n is equal to 1, the Freundlich adsorption or desorption binding constant (K_F^{ads} and K_F^{des}) will be equal to the adsorption or desorption equilibrium constants (K_d and K_{des}) respectively, and plots of C_s vs C_{aq} will be linear. If the exponents are not equal to 1, plots of C_s vs C_{aq} will be non-linear and the adsorption and desorption constants will vary along the isotherms.

2.2.2. **Test report**

The test report should include the following information:

— complete identification of the soil samples used including,

— geographical reference of the site (latitude, longitude),

— date of sampling,

— use pattern (e.g. agricultural soil, forest, etc.),

— depth of sampling,

— sand/silt/clay content;

— pH values (in 0,01 M CaCl2),

— organic carbon content,

— organic matter content,

— nitrogen content,

— C/N ratio,

— cation Exchange Capacity (mmol/kg),

— all information relating to the collection and storage of soil samples,

— where appropriate, all relevant information for the interpretation of the adsorption — desorption of the test substance,

— reference of the methods used for the determination of each parameter,

— information on the test substance as appropriate,

— temperature of the experiments,

— centrifugation conditions,

— analytical procedure used to analyse the test substance,

— justification for any use of solubilising agent for the preparation of the stock solution of the test substance,

— explanations of corrections made in the calculations, if relevant,

— data according to the form sheet (Appendix 6) and graphical presentations,

— all information and observations helpful for the interpretation of the test results.

3. **REFERENCES**

(1) Kukowski H. and Brümmer G., (1987) Investigations on the Adsorption and Desorption of Selected Chemicals in Soils. UBA Report 106 02 045, Part II.

(2) Fränzle O., Kuhnt G. and Vetter L., (1987) Selection of Representative Soils in the EC-Territory. UBA Report 106 02 045, Part I.

(3) Kuhnt G. and Muntau H. (Eds.) EURO-Soils: Identification, Collection, Treatment, Characterisation. Special Publication No 1.94.60, Joint Research Centre. European Commission, ISPRA, December 1994.

(4) OECD Test Guidelines Programme, Final Report of the OECD Workshop on Selection of Soils/Sediments, Belgirate, Italy, 18-20 January 1995 (June 1995).

(5) US-Environment Protection Agency: Pesticide Assessment Guidelines, Subdivision N, Chemistry: Environmental Fate, Series 163-1, Leaching and Adsorption/Desorption Studies, Addendum 6 on Data Reporting, 540/09-88-096, Date: 1/1988.

(6) US-Environment Protection Agency: Prevention, Pesticides and Toxic Substances, OPPTS Harmonized Test Guidelines, Series 835-Fate, Transport and Transformation Test Guidelines, 0PPTS No: 835.1220 Sediment and Soil Adsorption/Desorption Isotherm. EPA No: 712-C-96-048, April 1996.

(7) ASTM Standards, E 1195-85, Standard Test Method for Determining a Sorption Constant (K_{oc}) for an Organic Chemical in Soil and Sediments.

(8) Agriculture Canada: Environmental Chemistry and Fate. Guidelines for registration of pesticides in Canada, 15 July 1987.

(9) Netherlands Commission Registration Pesticides (1995): Application for registration of a pesticide. Section G. Behaviour of the product and its metabolites in soil, water and air.

(10) Danish National Agency of Environmental Protection (October 1988): Criteria for registration of pesticides as especially dangerous to health or especially harmful to the environment.

(11) BBA (1990) Guidelines for the Official Testing of Plant Protection Products, Biological Research Centre for Agriculture and Forestry, Braunschweig, Germany.

(12) Calvet R., (1989) 'Evaluation of adsorption coefficients and the prediction of the mobilities of pesticides in soils', in Methodological Aspects of the Study of Pesticide Behaviour in Soil (ed. P. Jamet), INRA, Paris, (Review).

(13) Calvet R., (1980) 'Adsorption-Desorption Phenomena' in Interactions between herbicides and the soil. (R.J. Hance ed.), Academic Press, London, p. 83-122.

(14) Hasset J.J., and Banwart W.L., (1989), 'The sorption of nonpolar organics by soils and sediments' in Reactions and Movement of Organic Chemicals in Soils. Soil Science Society of America (S.S.S.A), Special Publication No 22, p. 31-44.

(15) van Genuchten M. Th., Davidson J.M., and Wierenga P.J., (1974) 'An evaluation of kinetic and equilibrium equations for the prediction of pesticide movement through porous media'. Soil Sci. Soc. Am. Proc., Vol. 38(1), p. 29-35.

(16) McCall P.J., Laskowski D.A., Swann R.L., and Dishburger H.J., (1981) 'Measurement of sorption coefficients of organic chemicals and their use, in environmental fate analysis', in Test Protocols for Environmental Fate and Movement of Toxicants. Proceedings of AOAC Symposium, AOAC, Washington DC.

(17) Lambert S.M., Porter P.E., and Schieferrstein R.H., (1965) 'Movement and sorption of chemicals applied to the soil'. Weeds, 13, p. 185-190.

(18) Rhodes R.C., Belasco I.J., and Pease H.L., (1970) 'Determination of mobility and adsorption of agrochemicals in soils'. J.Agric.Food Chem., 18, p. 524-528.

(19) Russell M.H., (1995), 'Recommended approaches to assess pesticide mobility in soil' in Environmental Behavior of Agrochemicals (ed. T.R. Roberts and P.C. Kearney). John Wiley & Sons Ltd.

(20) Esser H.O., Hemingway R.J., Klein W., Sharp D.B., Vonk J.W. and Holland P.T., (1988) 'Recommended approach to the evaluation of the environmental behavior of pesticides', IUPAC Reports on Pesticides (24). Pure Appl. Chem., 60, p. 901-932.

(21) Guth J.A., Burkhard N., and D.O. Eberle, (1976) 'Experimental models for studying the persistence of pesticides in soils'. Proc. BCPC Symposium: Persistence of Insecticides and Herbicides, p. 137-157, BCPC, Surrey, UK.

(22) Furminge C.G.L., and Osgerby J.M., (1967) 'Persistence of herbicides in soil'. J. Sci. Food Agric., 18, p. 269-273.

(23) Burkhard N., and Guth J.A., (1981) 'Chemical hydrolysis of 2-Chloro-4,6-bis(alkylamino)-1,3,5-triazine herbicides and their breakdown in soil under the influence of adsorption'. Pestic. Sci. 12, p. 45-52.

(24) Guth J.A., Gerber H.R., and Schlaepfer T., (1977) 'Effect of adsorption, movement and persistence on the biological availability of soil-applied pesticides'. Proc. Br. Crop Prot. Conf., 3, p. 961-971.

(25) Osgerby J.M., (1973) 'Process affecting herbicide action in soil'. Pestic. Sci., 4, p. 247-258.

(26) Guth J.A., (1972) 'Adsorptions- und Einwascheverhalten von Pflanzenschutzmitteln in Böden'. Schr. Reihe Ver. Wass. -Boden-Lufthyg. Berlin-Dahlem, Heft 37, p. 143-154.

(27) Hamaker J.W., (1975) 'The interpretation of soil leaching experiments', in Environmental Dynamics of Pesticides (eds R. Haque and V.H. freed), p. 135-172, Plenum Press, NY.

(28) Helling C.S., (1971) 'Pesticide mobility in soils'. Soil Sci. Soc. Amer. Proc., 35, p. 732-210.

(29) Hamaker J.W., (1972) 'Diffusion and volatilization' in Organic chemicals in the soil environment (C.A.I. Goring and J.W. Hamaker eds), Vol. I, p. 49-143.

(30) Burkhard N. and Guth J.A., (1981) 'Rate of volatilisation of pesticides from soil surfaces; Comparison of calculated results with those determined in a laboratory model system'. Pestic. Sci. 12, p. 37-44.

(31) Cohen S.Z., Creeger S.M., Carsel R.F., and Enfield C.G., (1984) 'Potential pesticide contamination of groundwater from agricultural uses', in Treatment and Disposal of Pesticide Wastes, p. 297-325, Acs Symp. Ser. 259, American Chemical Society, Washington, DC.

(32) Gustafson D.I., (1989) 'Groundwater ubiquity score: a simple method for assessing pesticide leachability'. J. Environ. Toxic. Chem., 8(4), p. 339-357.

(33) Leistra M., and Dekkers W.A., (1976) 'Computed effects of adsorption kinetics on pesticide movement in soils'. J. of Soil Sci., 28, p. 340-350.

(34) Bromilov R.H., and Leistra M., (1980) 'Measured and simulated behavior of aldicarb and its oxydation products in fallow soils'. Pest. Sci., 11, p. 389-395.

(35) Green R.E., and Karickoff S.W., (1990) 'Sorption estimates for modeling', in Pesticides in the Soil Environment: Process, Impacts and Modeling (ed. H.H. Cheng). Soil Sci. Soc. Am., Book Series No 2, p. 80-101,

(36) Lambert S.M., (1967) 'Functional relationship between sorption in soil and chemical structure'. J. Agri. Food Chem., 15, p. 572-576.

(37) Hance R.J., (1969) 'An empirical relationship between chemical structure and the sorption of some herbicides by soils'. J. Agri. Food Chem., 17, p. 667-668.

(38) Briggs G.G. (1969) 'Molecular structure of herbicides and their sorption by soils'. Nature, 223, p. 1288.

(39) Briggs G.G. (1981) 'Theoretical and experimental relationships between soil adsorption, octanol-water partition coefficients, water solubilities, bioconcentration factors, and the parachor'. J. Agric. Food Chem., 29, p. 1050-1059.

(40) Sabljic A., (1984) 'Predictions of the nature and strength of soil sorption of organic polutance by molecular topology'. J. Agric. Food Chem., 32, p. 243-246.

(41) Bailey G.W., and White J.L., (1970) 'Factors influencing the adsorption, desorption, and movement of pesticides in soil'. Residue Rev., 32, p. 29-92.

(42) Bailey G.W., J.L. White and Y. Rothberg., (1968) 'Adsorption of organic herbicides by montomorillonite: Role of pH and chemical character of adsorbate'. Soil Sci. Soc. Amer. Proc. 32, p. 222-234.

(43) Karickhoff S.W., (1981) 'Semi-empirical estimation of sorption of hydrophobic pollutants on natural sediments and soils'. Chemosphere 10, p. 833-846.

(44) Paya-Perez A., Riaz M. and Larsen B., (1989) 'Soil Sorption of 6 Chlorobenzenes and 20 PCB Congeners'. Environ. Toxicol. Safety 21, p. 1-17.

(45) Hamaker J.W., and Thompson J.M., (1972) 'Adsorption in organic chemicals' in Organic Chemicals in the Soil Environment (Goring C.A.I. and Hamaker J.W., eds), Vol I and II, Marcel Dekker, Inc., New York, NY, 1972, p. 49-143.

(46) Deli J., and Warren G.F., 1971 'Adsorption, desorption and leaching of diphenamid in soils'. Weed Sci. 19, p. 67-69.

(47) Chu-Huang Wu, Buehring N., Davinson J.M. and Santelmann, (1975) 'Napropamide Adsorption, desorption and Movement in soils'. Weed Science, Vol. 23, p. 454-457.

(48) Haues M.H.B., Stacey M., and Thompson J.M., (1968) 'Adsorption of s-triazine herbicides by soil organic preparations' in Isotopes and Radiation in Soil Organic Studies, p. 75, International. Atomic Energy Agency, Vienna.

(49) Pionke H.B., and Deangelis R.J., (1980) 'Methods for distributing pesticide loss in field run-off between the solution and adsorbed phase', CREAMS, in A Field Scale Model for Chemicals, Run-off and Erosion from Agricultural Management Systems, Chapter 19, Vol. III: Supporting Documentation, USDA Conservation Research report.

(50) ISO Standard Compendium Environment: Soil Quality — General aspects; chemical and physical methods of analysis; biological methods of analysis. First Edition (1994).

(51) Scheffer F., and Schachtschabel, Lehrbuch der Bodenkunde, F. Enke Verlag, Stuttgart (1982) 11th edition.

(52) Black, Evans D.D., White J.L., Ensminger L.E., and Clark F.E., eds. 'Methods of Soil Analysis', Vol 1 and 2, American Society of Agronomy, Madison, WI, 1982.

(53) ISO/DIS 10381-1 Soil Quality — Sampling — Part 1: Guidance on the design of sampling programmes.

(54) ISO/DIS 10381-2 Soil Quality — Sampling — Part 2: Guidance on sampling techniques.

(55) ISO/DIS 10381-3 Soil Quality — Sampling — Part 3: Guidance on safety of sampling.

(56) ISO/DIS 10381-4 Soil Quality — Sampling — Part 4: Guidance on the investigation of natural and cultivated soils.

(57) ISO/DIS 10381-5 Soil Quality — Sampling — Part 5: Guidance on the investigation of soil contamination of urban and industrial sites.

(58) ISO 10381-6, 1993: Soil Quality — Sampling — Part 6: Guidance on the collection, handling and storage of soil for the assessment of aerobic microbial processes in the laboratory.

(59) Green R.E., and Yamane V.K., (1970) 'Precision in pesticide adsorption measurements'. Soil Sci. Am. Proc., 34, 353-354.

(60) Grover R., and Hance R.J. (1970) 'Effect of ratio of soil to water on adsorption of linuron and atrazine'. Soil Sci., p. 109-138.

(61) Boesten, J.J.T.I, 'Influence of soil/liquid ratio on the experimental error of sorption coefficients in pesticide/soil system'. Pest. Sci. 1990, 30, p. 31-41.

(62) Boesten, J.J.T.I. 'Influence of soil/liquid ratio on the experimental error of sorption coefficients in relation to OECD guideline 106' Proceedings of 5th international workshop on environmental behaviour of pesticides and regulatory aspects, Brussels, 26-29 April 1994.

(63) Bastide J., Cantier J.M., et Coste C., (1980) 'Comportement de substances herbicides dans le sol en fonction de leur structure chimique'. Weed Res. 21, p. 227-231.

(64) Brown D.S., and Flagg E.W., (1981) 'Empirical prediction of organic pollutants sorption in natural sediments'. J. Environ.Qual., 10(3), p. 382-386.

(65) Chiou C.T., Porter P.E., and Schmedding D.W., (1983) 'Partition equilibria of non-ionic organic compounds between soil organic matter and water'. Environ. Sci. Technol., 17(4), p. 227-231.

(66) Gerstl Z., and Mingelgrin U., (1984) 'Sorption of organic substances by soils and sediments'. J. Environm. Sci. Health, B19 (3), p. 297-312.

(67) Vowles P.D., and Mantoura R.F.C., (1987), 'Sediment-water partition coefficient and HPLC retention factors of aromatic hydrocarbons'. Chemosphere, 16(1), p. 109-116.

(68) Lyman W.J. , Reehl W.F.and Rosenblatt D.H. (1990) Handbook of Chemical Property Estimation Methods. Environmental Behaviour of Organic Compounds. American Chemical Society, Washington DC.

(69) Keniga E.E., and Goring, C.A.I. (1980) 'Relationship between water solubility, soil sorption, octanol-water partitioning and concentration of chemicals in the biota' in Aquatic Toxicology (eds J.G. Eaton, et al.), p. 78-115, ASTM STP 707, Philadelphia.

(70) Chiou C.T., Peters L.J., and Freed V.H., (1979) 'A physical concept of soil-water equilibria for non-ionic organic compounds'. Science, Vol. 206, p. 831-832.

(71) Hassett J.J., Banwart W.I., Wood S.G., and Means J.C., (1981) 'Sorption of/-Naphtol: implications concerning the limits of hydrophobic sorption'. Soil Sci. Soc. Am. J. 45, p. 38-42.

(72) Karickhoff S.W., (1981), 'Semi-empirical estimation of sorption of hydrophobic pollutants on natural sediments and soils'. Chemosphere, Vol. 10(8), p. 833-846.

(73) Moreale A., van Bladel R., (1981) 'Adsorption de 13 herbicides et insecticides par le sol. Relation solubilité — reactivité. Revue de l'Agric., 34 (4), p. 319-322.

(74) Müller M., Kördel W. (1996) 'Comparison of screening methods for the determination/estimation of adsorption coefficients on soil'. Chemosphere, 32(12), p. 2493-2504.

(75) Kördel W., Kotthoff G., Müller M. (1995) 'HPLC — screening method for the determination of the adsorption coefficient on soil — results of a ring test'. Chemosphere 30 (7), p. 1373-1384.

(76) Kördel W., Stutte J., Kotthoff G. (1993), 'HPLC — screening method for the determination of the adsorption coefficient on soil — comparison of different stationary phases. Chemosphere 27 (12), p. 2341-2352.

(77) Hance, R.J., (1967), 'The speed of Attainment of Sorption Equilibria in Some Systems Involving Herbicides'. Weed Research, Vol. 7, p. 29-36.

(78) Koskinen W.C., and Harper S.S., (1990), 'The retention processes: mechanisms' in Pesticides in the Soil Environment: Processes, Impacts and Modelling (ed. H.H. Cheng). Soil Sci. Soc. Am. Book Series, No 2, Madison, Wisconsin.

(79) Cohen S.Z., Creeger S.M., Carsel R.F., and Enfield C.G. (1984), 'Potential pesticide contamination of groundwater from agricultural uses', in Treatment and Disposal of Pesticide Wastes, p. 297-325, ACS Symp. Ser. 259, American Chemical Society, Washington, DC.

(80) Giles C.H., (1970) 'Interpretation and use of sorption isotherms' in Sorption and Transport Processes in Soils. S.C.I. Monograph No. 37, p. 14-32.

(81) Giles, C.H.; McEwan J.H.; Nakhwa, S.N. and Smith, D, (1960) 'Studies in adsorption: XI. A system of classification of solution adsorption isotherms and its use in the diagnosis of adsorption mechanisms and in measurements of pesticides surface areas of soils'. J. Chem. Soc., p. 3973-93.

(82) Calvet R., Tercé M., and Arvien J.C., (1980) 'Adsorption des pesticides par les sols et leurs constituants: 3. Caractéristiques générales de l'adsorption'. Ann. Agron. 31, p. 239-251.

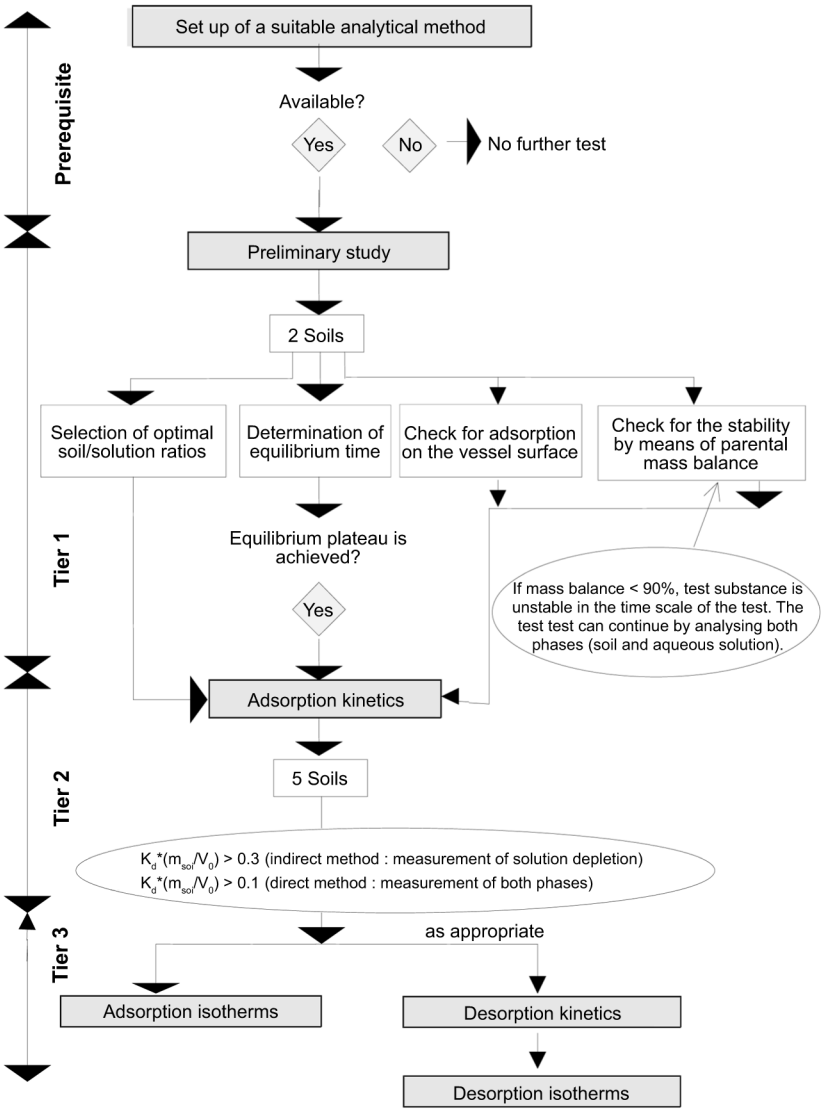
(83) Bedbur E., (1996) 'Anomalies in the Freundlich equation', Proc. COST 66 Workshop, Pesticides in soil and the environment, 13-15 May 1996, Stratford-upon-Avon, U.K.

(84) Guth, J.A., (1985) 'Adsorption/desorption', in Joint International Symposium, Physicochemical Properties and their Role in Environmental Hazard Assessment, July 1-3, Canterbury, UK.

(85) Soil Texture Classification (US and FAO systems): Weed Science, 33, Suppl. 1 (1985) and Soil Sci. Soc. Amer. Proc. 26, p. 305 (1962).

Appendix 1

Testing scheme



Appendix 2

INFLUENCE OF ACCURACY OF ANALYTICAL METHOD AND CONCENTRATION CHANGE ON ACCURACY OF ADSORPTION RESULTS

From the following table (84) it becomes obvious that when the difference between the initial mass (m_0 = 110 µg) and equilibrium mass ($m_{aq}^{ads}(eq)$ = 100 µg) of the test substance in the solution is very small, an error of 5 % in the measurement of equilibrium concentration results in an error of 50 % in the calculation of the mass of the substance adsorbed in soil ($m_s^{ads}(eq)$) and of 52,4 % in the calculation of the K_d.

Amount of soil m_{soil} = 10 g

Volume of solution V_0 = 100 cm^3

	$m_{aq}^{ads}(eq)$ (µg)	$C_{aq}^{ads}(eq)$ (µg cm^{-3})	R	$m_s^{ads}(eq)$* (µg)	$C_s^{ads}(eq)$* (µg g^{-1})	R‡	K_d*	R‡
	FOR A = 9 %							
m_0 = 110 µg or C_0 = 1,100 µg/cm^3	100	1,000	true value	10	1,00	true value	1	
	101	1,010	1 %	9	0,90	10 %	0,891	10,9 %
	105	1,050	5 %	5	0,50	50 %	0,476	52,4 %
	109	1,090	9 %	1	0,10	90 %	0,092	90,8 %
	FOR A = 55 %							
m_0= 110 µg or C_0 = 1,100 µg/cm^3	50,0	0,500	true value	60,0	6,00	true value	12,00	
	50,5	0,505	1 %	59,5	5,95	0,8 %	11,78	1,8 %
	52,5	0,525	5 %	57,5	5,75	4,0 %	10,95	8,8 %
	55,0	0,550	10 %	55,0	5,50	8,3 %	10,00	16,7 %
	FOR A = 99 %							
m_0 = 110 µg or C_0 = 1,100 µg/cm^3	1,100	0,011	true value	108,9	10,89	true value	990	
	1,111	0,01111	1 %	108,889	10,8889	0,01 %	980	1,0 %
	1,155	0,01155	5 %	108,845	10,8845	0,05 %	942	4,8 %
	1,21	0,0121	10 %	108,790	10,8790	0,10 %	899	9,2 %

$$*m_s^{ads}(eq) = m_0 - m_{aq}^{ads}(eq),\ C_s^{ads}(eq) = \frac{\left[C_0 - C_{aq}^{ads}(eq)\right]V_0}{m_{soil}} .\ Kd = \frac{m_s^{ads}(eq)}{m_{aq}^{ads}(eq)}\frac{V_0}{m_{soil}}$$

$m_s^{ads}(eq)$ = mass of the test substance in the soil phase at equilibrium, µg;

$m_{aq}^{ads}(eq)$ = mass of the test substance in the aqueous phase at equilibrium, µg;

$C_s^{ads}(eq)$ = content of the test substance in the soil phase at equilibrium, µg g^{-1};

$C_{aq}^{ads}(eq)$ = mass concentration of the test substance in the aqueous phase at equilibrium, µg cm^{-3};

R = analytical error in the determination of the $m_{aq}^{ads}(eq)$;

R‡ = calculated error due to the analytical error R.

Appendix 3

ESTIMATION TECHNIQUES FOR K_D

1. Estimation techniques permit prediction of K_d based on correlations with, for example, P_{ow} values (12)(39)(63-68), water solubility data (12)(19)(21)(39)(68-73), or polarity data derived by application of HPLC on reversed phase (74-76). As shown in Tables 1 and 2, is the K_{oc} or K_{om} that are calculated from these equations and then, indirectly, the K_d from the equations:

$$K_{oc} = K_d \cdot \frac{100}{\%oc} \ (cm^3\ g^{-1}) \qquad K_{om} = \frac{K_d}{1{,}724} \cdot \frac{100}{\%oc} \ (cm^3\ g^{-1})$$

2. The concept of these correlations is based on two assumptions: (1) it is the organic matter of the soil that mainly influences the adsorption of a substance; and (2) the interactions involved are mainly non-polar. As a result, these correlations: (1) are not, or are only to some extent, applicable to polar substances, and (2) are not applicable in cases where the organic matter content of the soil is very small (12). In addition, although satisfactory correlations have been found between P_{ow} and adsorption (19), the same cannot be said for the relationship between water solubility and extent of adsorption (19)(21); so far the studies are very contradictory.

3. Some examples of correlations between the adsorption coefficient and the octanol-water partition coefficient, as well as water solubility are given in Tables 1 and 2, respectively.

Table 1

Examples of correlations between the adsorption distribution coefficient and the octanol-water partition coefficient; for further examples (12) (68).

Substances	Correlations	Authors
Substituted ureas	$\log K_{om} = 0{,}69 + 0{,}52 \log P_{ow}$	Briggs (1981) (39)
Aromatic chlorinated	$\log K_{oc} = - 0{,}779 + 0{,}904 \log P_{ow}$	Chiou *et al.* (1983) (65)
Various pesticides	$\log K_{om} = 4{,}4 + 0{,}72 \log P_{ow}$	Gerstl and Mingelgrin (1984) (66)
Aromatic hydrocarbons	$\log K_{oc} = - 2{,}53 + 1{,}15 \log P_{ow}$	Vowles and Mantoura (1987) (67)

Table 2

Examples of correlations between the adsorption distribution coefficient and water solubility; for further examples see (68) (69).

Compounds	Correlations	Authors
Various pesticides	$\log K_{om} = 3{,}8 - 0{,}561 \log S_w$	Gerstl and Mingelgrin (1984) (66)
Aliphatic, aromatic chlorinated substances	$\log K_{om} = (4{,}040 +/- 0{,}038) - (0{,}557 +/- 0{,}012) \log S_w$	Chiou *et al.* (1979) (70)
α-naphtol	$\log K_{oc} = 4{,}273 - 0{,}686 \log S_w$	Hasset et al. (1981) (71)
Cyclic, aliphatic aromatic substances	$\log K_{oc} = - 1{,}405 - 0{,}921 \log S_w - 0{,}00953$ (mp-25)	Karickhoff (1981) (72)
Various compounds	$\log K_{om} = 2{,}75 - 0{,}45 \log S_w$	Moreale van Blade (1982) (73)

Appendix 4

CALCULATIONS FOR DEFINING THE CENTRIFUGATION CONDITIONS

1. The centrifugation time is given by the following formula, assuming spherical particles:

$$t = \frac{9}{2}\left[\frac{\eta}{\omega^2 rp^2\left(\rho_s - \rho_{aq}\right)}\right] \ln\left(R_b/R_t\right) \qquad (1)$$

For simplification purposes, all parameters are described in non-SI units (g, cm).

where:

ω = rotational speed (=2 π rpm/60), rad s^{-1}

rpm = revolutions per minute

η = viscosity of solution, g s^{-1} cm^{-1}

r_p = particle radius, cm

ρ_s = soil density, g cm^{-3}

ρ_{aq} = solution density, g cm^{-3}

R_t = distance from the centre of centrifuge rotor to top of solution in centrifuge tube, cm

R_b = distance from the centre of centrifuge rotor to bottom in centrifuge tube, cm

R_b-R_t = length of the soil/solution mixture in the centrifuge tube, cm.

In general practice, double the calculated times is used to ensure complete separation.

2. The equation (1) can be simplified further if we consider the viscosity (η) and the density (ρ_{aq}) of the solution as equal to the viscosity and density of water at 25 °C; thus, η = 8,95 × 10^{-3} g s^{-1} cm^{-1} and ρ_{aq} = 1,0 g. cm^{-3}.

Then, the centrifugation time is given by the equation (2):

$$t = \frac{3.7}{(rpm)^2 \cdot r_p^2\left(\rho_s - 1\right)} \ln\frac{Rb}{Rt} \qquad (2)$$

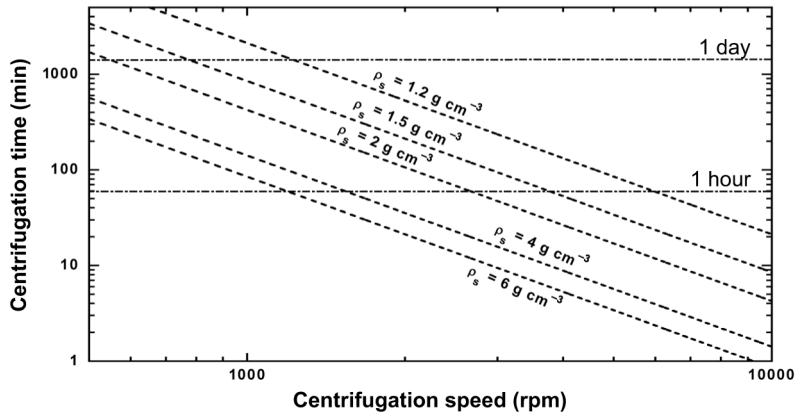
3. From the equation (2) it becomes apparent that two parameters are important in defining the centrifugation condition, i.e. time (t) and speed (rpm), in order to achieve separation of particles with a specific size (in our case 0,1 µm radius): (1) the density of the soil and (2) the length of the mixture in the centrifuge tube (R_b-R_t), i.e. the distance which a soil particle covers from the top of the solution to the bottom of the tube; obviously, for a fixed volume the length of the mixture in the tube will depend on the square of the radius of the tube.

4. Fig. 1 presents variations in the centrifugation time (t) versus centrifugation speed (rpm) for different soil densities (ρ_s) (Fig.1a) and different lengths of the mixture in the centrifuge tubes (Fig.2a). From Fig.1a the influence of the soil density appears obvious; for example, for a classical centrifugation of 3 000 rpm the centrifugation time is approximately 240 min for 1,2 g cm^3 soil density, while it is only 50 min for 2,0 g cm^3. Similarly, from Fig 1b, for a classical centrifugation of 3 000 rpm the centrifugation time is approximately 50 min for a length of the mixture of 10 cm and only seven min for a length of 1 cm. However, it is important to find an optimal relation between centrifugation which requires the less length possible and easy handling for the experimenter in separating the phases after centrifugation.

5. Moreover, when defining the experimental conditions for the separation of soil/solution phases, it is important to consider the possible existence of a third 'pseudo-phase', the colloids. These particles, with a size less than 0,2 µm, can have an important impact on the whole adsorption mechanism of a substance in a soil suspension. When centrifugation is performed as described above, colloids remain in the aqueous phase and are subjected to analysis together with the aqueous phase. Thus, the information about their impact is lost.

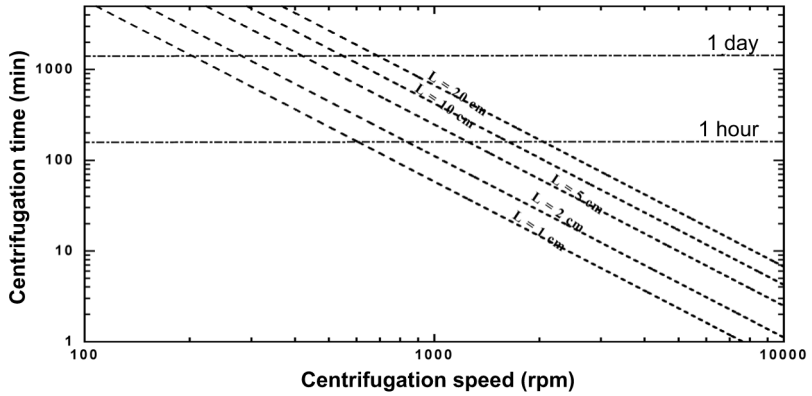
If the conducting laboratory has ultracentrifugation or ultrafiltration facilities, the adsorption/desorption of a substance in soil could be studied more in depth, including information on the adsorption of the substance on the colloids. In this case, an ultracentrifugation at 60 000 rpm/min or an ultrafiltration with filter porosity of 100 000 Daltons should be applied in order to separate the three phases soil, colloids, solution. The test protocol should also be modified accordingly, in order all three phases to be subjected to substance analysis.

Fig. 1a.



Variations of centrifugation time (t) versus centrifugation speed (rpm) for different soil densities (ρ_s). R_t = 10 cm, R_b - R_t = 10 cm, η = 8,95 × 10^{-3} g s^{-1} cm^{-1} and ρ_{aq} = 1,0 g. cm^{-3} at 25 °C.

Fig. 1b.

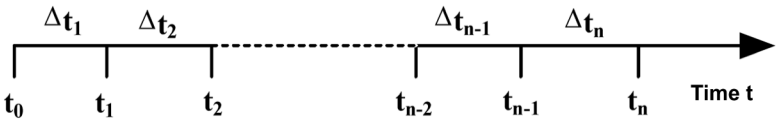


Variations of centrifugation time (t) versus centrifugation speed (rpm) for different lengths of the mixture in the centrifuge tube (R_b - R_t) = L; R_t = 10 cm, η = 8,95 × 10^{-3} g s^{-1} cm^{-1}, ρ_{aq} = 1,0 g. cm^{-3} at 25 °C and ρ_s = 2,0 g cm^{-3}.

Appendix 5

CALCULATION OF ADSORPTION A (%) AND DESORPTION D (%)

The time scheme of the procedure is:



For all the calculations it is assumed that the test substance is stable and does not adsorb significantly to the container walls.

ADSORPTION A (A%)

a) *Parallel method*

The percentage adsorption is calculated for each test tube (i) at each time point (t_i), according to the equation:

$$A_{t_i} = \frac{m_s^{ads}(t_i) \cdot 100}{m_0} \ (\%) \qquad (1)$$

The terms of this equation may be calculated as follows:

$$m_0 = C_0 \cdot V_0 \ (\mu g) \qquad (2)$$

$$m_s^{ads}(t_i) = m_0 - C_{aq}^{ads}(t_i) \cdot V_0 \ (\mu g) \qquad (3)$$

where:

A_{t_i} = adsorption percentage (%) at the time point t_i

$m_s^{ads}(t_i)$ = mass of the test substance on soil at the time t_i that the analysis is performed (μg)

m_0 = mass of test substance in the test tube, at the beginning of the test (μg)

C_0 = initial mass concentration of the test solution in contact with the soil (μg cm^{-3})

$C_{aq}^{ads}(t_i)$ = mass concentration of the substance in the aqueous phase at the time t_i that the analysis is performed (μg cm^{-3}); this concentration is analytically determined taking into account the values given by the blanks

V_0 = initial volume of the test solution in contact with the soil (cm^3).

The values of the adsorption percentage A_{t_i} or $C_{aq}^{ads}(t_i)$ are plotted versus time and the time after which the sorption equilibrium is attained is determined. Examples of such plots are given in Fig. 1 and Fig. 2 respectively.

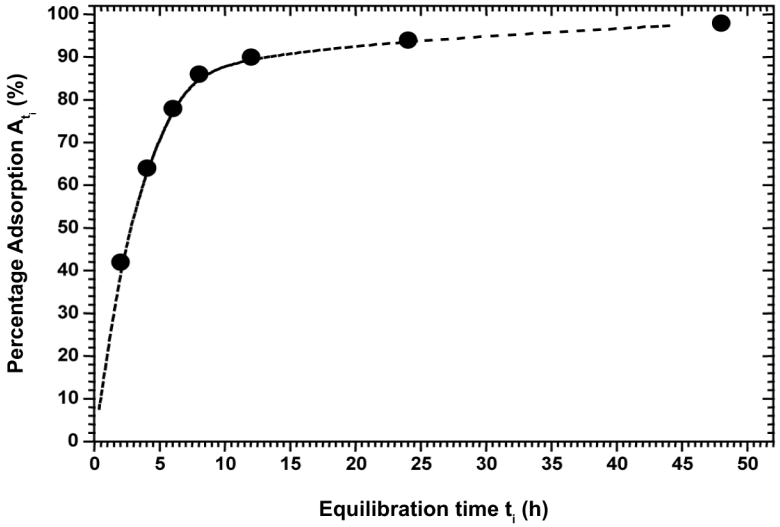


Fig. 1.

Adsorption equilibrium plot

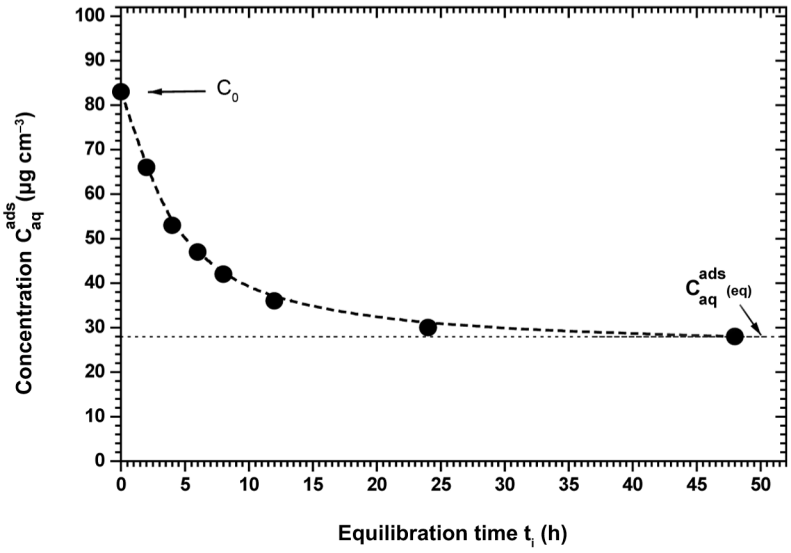


Fig. 2.

Mass concentration of the test substance in the aqueous phase (C_{aq}) versus time

b) *Serial method*

The following equations take into account that the adsorption procedure is carried out by measurements of the test substance in small aliquots of the aqueous phase at specific time intervals.

During each time interval the amount of the substance adsorbed on the soil is calculated as follows:

— for the first time interval $\Delta t_1 = t_1 - t_0$

$$m_s^{ads}(\Delta t_1) = m_0 - m_m^{ads}(t_1) \cdot \left(\frac{V_0}{V_a^A}\right) \quad (4)$$

— for the second time interval $\Delta t_2 = t_2 - t_1$

$$m_s^{ads}(\Delta t_2) = m_m^{ads}(t_1) \cdot \left(\frac{V_0}{v_a^A}\right) - m_m^{ads}(t_2) \cdot \left(\frac{V_0 - v_a^A}{v_a^A}\right) \quad (5)$$

— for the third time interval $\Delta t_3 = t_3 - t_2$

$$m_s^{ads}(\Delta t_3) = m_m^{ads}(t_2) \cdot \left(\frac{V_0 - v_a^A}{v_a^A}\right) - m_m^{ads}(t_3) \cdot \left(\frac{V_0 - 2 \cdot v_a^A}{v_a^A}\right) \quad (6)$$

— for the nth time interval $\Delta t_n = t_n - t_n - 1$

$$m_s^{ads}(\Delta t_n) = m_m^{ads}(t_{n-1}) \cdot \left(\frac{V_0 - (n-2) \cdot v_a^A}{v_a^A}\right) - m_m^{ads}(t_n) \cdot \left(\frac{\left(V_0 - (n-1) \cdot v_a^A\right)}{v_a^A}\right) \quad (7)$$

The percentage of adsorption at each time interval, $A_{\Delta t_i}$, is calculated using the following equation:

$$A_{\Delta t_i} = \frac{m_s^{ads}(\Delta t_i)}{m_0} \cdot 100\ (\%) \quad (8)$$

while the percentage of adsorption (A_{t_i}) at a time point t_i is given by the equation:

$$A_{t_i} = \frac{\sum_{j=\Delta t_1}^{\Delta t^i} m_s^{ads}(j)}{m_0} \cdot 100\ (\%) \quad (9)$$

The values of the adsorption A_{t_i} or $A_{\Delta t_i}$ (with respect to the needs of the study) are plotted versus time and the time after which the sorption equilibrium is attained is determined.

At the equilibration time t_{eq}:

— the mass of the test substance adsorbed on the soil is:

$$m_s^{ads}(eq) = \sum_{\Delta t_i = 1}^{n} m_s^{ads}(\Delta t_i) \quad (10)$$

— the mass of the test substance in the solution is:

$$m_{aq}^{ads}(eq) = m_0 - \sum_{\Delta t_i = 1}^{n} m_s^{ads}(\Delta t_i) \quad (11)$$

— and the percentage of adsorption at equilibrium is:

$$A_{eq} = \frac{m_s^{ads}(eq)}{m_0} \cdot 100\ (\%) \quad (12)$$

The parameters used above are defined as:

$m_s^{ads}(\Delta t_1)$, $m_s^{ads}(\Delta t_2)$, ..., $m_s^{ads}(\Delta t_n)$ = mass of the substance adsorbed on the soil during the time intervals Δ_{t1}, Δ_{t2},..., Δ_{tn} respectively (μg);

$m_m^{ads}(t_1)$, $m_m^{ads}(t_2)$, ..., $m_n^{ads}(t_n)$ = mass of the substance measured in an aliquot v_a^A at the time points t_1, t_2,t_n respectively (μg);

$m_s^{ads}(eq)$ = mass of the substance adsorbed on the soil at adsorption equilibrium (μg);

$m_{aq}^{ads}(eq)$ = mass of the substance in the solution at adsorption equilibrium (μg);

v_a^A = volume of the aliquot in which the test substance is measured (cm^3);

$A_{\Delta t_i}$ = percentage of adsorption corresponding at a time interval Δt_i (%);

A_{eq} = percentage of adsorption at adsorption equilibrium (%).

DESORPTION D (A %)

The time t_0 that the desorption kinetics experiment begins, is considered as the moment that the maximal recovered volume of the test substance solution (after that the adsorption equilibrium is attained) is replaced by an equal volume of 0,01 M $CaCl_2$ solution.

(a) *Parallel method*

At a time point t_i, the mass of the test substance is measured in the aqueous phase taken from the tube i (V_r^i), and the mass desorbed is calculated according to the equation:

$$m_{aq}^{des}(t_i) = m_m^{des}(t_i) \cdot \left(\frac{V_0}{v_r^i}\right) - m_{aq}^A \quad (13)$$

At desorption equilibrium $t_i = t_{eq}$ and therefore $m_{aq}^{des}(t_i) = m_{aq}^{des}(eq)$

The mass of the test substance desorbed during a time interval (Δt_i) is given by the equation:

$$m_{aq}^{des}(\Delta t_i) = m_{aq}^{des}(t_i) - \sum_{j=1}^{i=1} m_{aq}^{des}(j) \quad (14)$$

The percentage of desorption is calculated:

at a time point t_i from the equation:

$$D_{t_i} = \frac{m_{aq}^{des}(t_i)}{m_s^{ads}(eq)} \cdot 100\ (\%) \quad (15)$$

and during a time interval (Δt_i) from the equation:

$$D_{\Delta t_i} = \frac{m_{aq}^{des}(\Delta t_i)}{m_s^{ads}(eq)} \cdot 100\ (\%) \quad (16)$$

where:

D_{t_i} = desorption percentage at a time point t_i (%)

$D_{\Delta t_i}$ = desorption percentage corresponding to a time interval Δt_i (%)

$m_{aq}^{des}(t_1)$ = mass of the test substance desorbed at a time point t_i, (μg)

$m_{aq}^{des}(\Delta t_1)$ = mass of the test substance desorbed during a time interval Δt_i (μg)

$m_m^{des}(t_i)$ = mass of the test substance analytically measured at a time t_i in a solution volume V_r^i,which is taken for the analysis (μg)

m_{aq}^A = mass of the test substance left over from the adsorption equilibrium due to incomplete volume replacement (μg)

$$m_{aq}^A = m_{aq}^{ads}(eq) \cdot \left(\frac{V_0 - V_R}{V_0}\right) \quad (17)$$

$m_{aq}^{ads}(eq)$ = mass of the test substance in the solution at adsorption equilibrium (μg)

V_R = volume of the supernatant removed from the tube after the attainment of adsorption equilibrium and replaced by the same volume of a 0,01 M $CaCl_2$ solution (cm^3)

V_r^i = volume of the solution taken from the tube (i) for the measurement of the test substance, in desorption kinetics experiment (cm^3).

The values of desorption D_{t_i} or $D_{\Delta t_i}$ (according to the needs of the study) are plotted versus time and the time after which the desorption equilibrium is attained is determined.

(b) *Serial method*

The following equations take into account that the adsorption procedure, which was preceded, was carried out by measurement of test substance in small aliquots $\left(v_a^A\right)$ of the aqueous phase (serial method in 'Performance of the test' 1,9). It is assumed that: (a) the volume of the supernatant removed from the tube after the adsorption kinetics experiment was replaced by the same volume of 0,01 M $CaCl_2$ solution (V_R) and (b) and the total volume of the aqueous phase in contact with the soil (V_T) during the desorption kinetics experiment remains constant and is given by the equation:

$$V_T = V_0 - \sum_{i=1}^{n} v_a^A(i) \tag{18}$$

At a time point t_i:

— The mass of the test substance is measured in a small aliquot $\left(v_a^D\right)$ and the mass desorbed is calculated, according to the equation:

$$m_{aq}^{des}(t_i) = m_m^{des}(t_i) \cdot \left(\frac{V_T}{v_a^D}\right) - m_{aq}^A \cdot \left(\frac{\left(V_T - (i-1) \cdot v_a^D\right)}{V_T}\right) \tag{19}$$

— At desorption equilibrium $t_i = t_{eq}$ and therefore $m_{aq}^{des}(t_i) = m_{aq}^{des}(eq)$.

— The percentage of desorption D_{t_i} is calculated, from the following equation:

$$D_{t_i} = \frac{m_{aq}^{des}(t_i)}{m_s^{ads}(eq)} \cdot 100\ (\%) \tag{20}$$

At a time interval (Δt_i):

During each time interval the amount of the substance desorbed is calculated as follows:

— for the first time interval $\Delta t_1 = t_1 - t_0$

$$m_{aq}^{des}(\Delta t_1) = m_m^{des}(t_1) \cdot \left(\frac{V_T}{v_a^D}\right) - m_{aq}^A \text{ and } m_s^{des}(t_1) = m_s^{aq}(eq) - m_{aq}^{des}(\Delta t_1) \tag{21}$$

— for the second time interval $\Delta t_2 = t_2 - t_1$

$$m_{aq}^{des}(\Delta t_2) = m_m^{des}(t_2) \cdot \left(\frac{V_T}{v_a^D}\right) - m_{aq}^{des}(\Delta t_1) \cdot \left(\frac{\left(V_T - v_a^D\right)}{V_T}\right) - m_{aq}^A \cdot \left(\frac{\left(V_T - v_a^D\right)}{V_T}\right) \text{ and}$$
$$m_s^{des}(t_2) = m_s^{ads}(eq) - \left[m_{aq}^{des}(\Delta t_1) + m_{aq}^{des}(\Delta t_2)\right] \tag{22}$$

— for the nth interval $\Delta t_n = t_n - t_{n-1}$

$$m_{aq}^{des}(\Delta t_n) = \left[m_m^{des}(t_n) \cdot \left(\frac{V_T}{v_a^D}\right) - m_{aq}^A \cdot \left(\frac{\left(V_T - (n-1) \cdot v_a^D\right)}{V_T}\right) - \sum_{i=1,n\neq 1}^{n-1} \left(\frac{\left(V_T - (n-i) \cdot v_a^D\right)}{V_T} \cdot m_{aq}^{des}(\Delta t_i)\right)\right]$$
and
$$m_s^{des}(t_n) = m_s^{ads}(eq) - \sum_{i=1,n\neq 1}^{n} m_{aq}^{des}(\Delta t_i) \tag{23}$$

Finally, the percentage of desorption at each time interval, $D_{\Delta t_i}$, is calculated using the following equation:

$$D_{\Delta t_i} = \frac{m_{aq}^{des}(\Delta t_i)}{m_s^{ads}(eq)} \cdot 100(\%) \tag{24}$$

while the percentage of desorption D_{t_i} at a time point t_i is given by the equation:

$$D_{t_i} = \frac{\sum_{j=\Delta t_1}^{\Delta t_i} m_{aq}^{des}(j)}{m_s^{ads}(eq)} \cdot 100 = \frac{m_{aq}^{des}(t_i)}{m_s^{ads}(eq)} \cdot 100(\%) \quad (25)$$

where the above used parameters are defined as:

$m_s^{des}(\Delta t_1)$, $m_s^{des}(\Delta t_2)$, ..., $m_s^{des}(\Delta t_n)$ = mass of the substance remaining adsorbed on the soil after the time intervals Δt_1, Δt_2, ..., Δt_n respectively (μg)

$m_{aq}^{des}(\Delta t_1)$, $m_{aq}^{des}(\Delta t_2)$, ..., $m_{aq}^{des}(\Delta t_n)$ = mass of the test substance desorbed during the time intervals Δt_1, Δt_2, ..., Δt_n respectively (μg)

$m_m^{des}(t_1)$, $m_m^{des}(t_2)$, ..., $m_m^{des}(t_n)$ = mass of the substance measured in an aliquot $\left(v_a^D\right)$ at time points t_1,t_2, ..., t_n, respectively (μg)

V_T = total volume of the aqueous phase in contact with the soil during the desorption kinetics experiment performed with the serial method (cm^3)

m_{aq}^A = mass of the test substance left over from the adsorption equilibrium due to incomplete volume replacement (μg)

$$m_{aq}^A = \left(\frac{\left(V_0 - \sum_{i=1}^{n} v_a^A(i) \right) - V_R}{\left(V_0 - \sum_{i=1}^{n} v_a^A(i) \right)} \right) \cdot m_{aq}^{ads}(eq) \quad (26)$$

V_R = volume of the supernatant removed from the tube after the attainment of adsorption equilibrium and replaced by the same volume of a 0,01 M $CaCl_2$ solution (cm^3)

v_a^D = volume of the aliquot sampled for analytical purpose from the tube (i), during the desorption kinetics experiment performed with the serialmethod (cm^3)

$$v_a^D \leq 0{,}02 \cdot V_T \quad (27)$$

Appendix 6

ADSORPTION-DESORPTION IN SOILS: DATA REPORTING SHEETS

Substance tested:

Soil tested:

Dry mass content of the soil (105 °C, 12h): ..... %

Temperature: ..... °C

Suitability of the analytical method

Weighed soil	g	
Soil: dry mass	g	
Volume $CaCl_2$ sol.	cm^3	
Nominal conc. final sol.	$\mu g\ cm^{-3}$	
Analytical conc. final sol.	$\mu g\ cm^{-3}$	

Principle of the analytical method used:

Calibration of the analytical method:

Substance tested:

Soil tested

Dry mass content of the soil (105 °C, 12 h): ..... %

Temperature: ..... °C

Analytical methodology followed: Indirect □ Parallel □ Serial □
Direct □

Adsorption test: test samples

	Symbol	Units	Equilibration time		Equilibration time		Equilibration time		Equilibration time	
Tube No										
Weighed soil	—	g								
Soil: dry mass	m_{soil}	g								
Water volume in weighed soil (calculated)	V_{WS}	cm^3								

	Symbol	Units	Equilibration time		Equilibration time		Equilibration time		Equilibration time	
Volume 0,01 M $CaCl_2$ sol. to equilibrate the soil		cm^3								
Volume of stock solution		cm^3								
Total volume of aq. phase in contact with soil	V_0	cm^3								
Initial concentration Test solution	C_0	µg cm^{-3}								
Mass test subst. at the beginning of the test	m_0	µg								
After agitation and centrifugation										
INDIRECT METHOD										
Parallel method										
Concentration test subst. aq. phase Blank correction included	$C_{aq}^{ads}(t_i)$	µg cm^{-3}								
Serial method										
Measured mass test subst. in the aliquot V_a [A]	$m_m^{ads}(t_i)$	µg								
DIRECT METHOD										
Mass test substance adsorbed on soil	$m_s^{ads}(t_i)$	µg								
Calculation of adsorption										
Adsorption	A_{t_i}	%								
	$A_{\Delta t_i}$	%								
Means										
Adsorption coefficient	K_d	$cm^3\ g^{-1}$								
Means										
Adsorption coefficient	K_{oc}	$cm^3\ g^{-1}$								
Means										

Substance tested:

Soil tested:

Dry mass content of the soil (105 °C, 12 h): ..... %

Temperature: ..... °C

Adsorption test: blanks and control

	Sym-bol	Units	Blank		Blank		Control	
Tube No								
Weighed soils		g					0	0
Water amount in weighed soil (calculated)		cm^3					—	—
Volume of 0,01 M $CaCl_2$ solution added		cm^3						
Volume of the stock solution of the test substance added		cm^3	0	0				
Total volume of aq. phase (calculated)		cm^3					—	—
Initial concentration of the test substance in aqueous phase		$\mu g\ cm^{-3}$						
After agitation and centrifugation								
Concentration in aqueous phase		$\mu g\ cm^{-3}$						

Remark: add columns if necessary

Substance tested:

Soil tested:

Dry mass content of the soil (105 °C 12 h): ..... %

Temperature: ..... °C

Mass balance

	Symbol	Units				
Tube No						
Weighed soil	—	g				
Soil: dry mass	ms_{oil}	g				
Water volume in weighed soil (calculated)	V_{WS}	ml				
Volume 0,01 M $CaCl_2$ sol. to equilibrate the soil		ml				
Volume of stock solution		cm^3				
Total volume of aq. phase in contact with soil	V_0	cm^3				
Initial concentration test solution	C_0	$\mu g\ cm^{-3}$				
Equilibration time	—	h				

After agitation and centrifugation						
Concentr. test subst. aq. phase at adsorption equilibrium blank correction included	$C_{aq}^{ads}(eq)$	µg cm^{-3}				
Equalibration time	t_{eq}	h				
1st dilution with solvent						
Removed volume aq. phase	V_{rec}	cm^3				
Added volume of solvent	ΔV	cm^3				
1st extraction with solvent						
Signal analysed in solvent	S_{E1}	var.				
Conc. test subst. in solvent	C_{E1}	µg cm^{-3}				
Mass of substance extracted from soil and vessel walls	m_{E1}	µg				
2nd dilution with solvent						
Removed volume of solvent	ΔV_s	cm^3				
Added volume of solvent	$\Delta V'$	cm^3				
2nd extraction with solvent						
Signal analysed in solvent phase	S_{E2}	var.				
Conc. test subst. in solvent	C_{E2}	µg cm^{-3}				
Mass of substance extracted from soil and vessel walls	m_{E2}	µg				
Total mass test subst. extracted in two steps	m_E	µg				
Mass balance	MB	%				

Substance tested:

Soil tested:

Dry mass content of the soil (105 °C, 12 h): ..... %

Temperature: ..... °C

Adsorption isotherms

	Symbol	Units								
Tube No										
Weighed soil	—	g								
Soil: dry mass	E	g								

	Symbol	Units								
Water volume in weighed soil (calculated)	V_{WS}	cm^3								
Volume 0,01 M $CaCl_2$ sol. to equilibrate the soil		cm^3								
Volume of stock solution added		cm^3								
Total volume of aq. phase in contact with soil (calculated)	V_0	cm^3								
Concentration solution	C_0	$\mu g\ cm^{-3}$								
Equilibration time	—	h								

After agitation and centrifugation

Concentration subst. aq. phase, blank correction included	$C_{aq}^{ads}(eq)$	$\mu g\ cm^{-3}$								
Temperature		°C								
Adsorb. mass per unit soil	$C_s^{ads}(eq)$	$\mu g\ g^{-1}$								

Regression analysis:

value of K_F^{ads}:

value of 1/n:

regression coefficient r^2:

Substance tested:

Soil tested:

Dry mass content of the soil (105 °C, 12 h): ..... %

Temperature: ..... °C

Analytical methodology followed: Indirect ☐ Parallel ☐ Serial ☐

Desorption test

		Symbol	Units	Time interval	Time interval	Time interval	Time interval
Tube No coming from adsorption step							
Mass of substance adsorbed on soil at adsorption equilibrium		$m_s^{ads}(eq)$	µg				
Removed volume aq. phase, replaced by 0,01 M $CaCl_2$		V_R	cm^3				
Total volume of aq. phase in contact with soil	PM	V_0	cm^3				
	SM	V_T	cm^3				

		Symbol	Units	Time interval	Time interval	Time interval	Time interval
Mass test subst. left over the adsorption equilibrium due to incomplete volume replacement		m_{aq}^{A}	µg				
Desorption kinetics							
Measured mass of substance desorbed from soil at time t_i		$m_{m}^{des}(t_i)$	µg				
Volume of the solution taken from the tube (i) for the measurement of the test substance	PM	V_r^i	cm^3				
	SM	v_a^D	cm^3				
Mass of substance desorbed from soil at time t_i (calculated)		$m_{aq}^{des}(t_i)$	µg				
Mass of substance desorbed from soil during time interval Δt_i (calculated)		$m_{aq}^{des}(\Delta t_i)$	µg				
Desorption percentage							
Desorption at time t_i		D_{t_i}	%				
Desorption at time interval Δt_i		$D_{\Delta t_i}$	%				
Apparent desorption coefficient		K_{des}					

PM: parallel method

SM: serial method

C.19. ESTIMATION OF THE ADSORPTION COEFFICIENT (K_{OC}) ON SOIL AND ON SEWAGE SLUDGE USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

1. METHOD

This method is a replicate of OECD TG121 (2001).

1.1. INTRODUCTION

The sorption behaviour of substances in soils or sewage sludges can be described through parameters experimentally determined by means of the test method C.18. An important parameter is the adsorption coefficient which is defined as the ratio between the concentration of the substance in the soil/sludge and the concentration of the substance in the aqueous phase at adsorption equilibrium. The adsorption coefficient normalised to the organic carbon content of the soil K_{oc} is a useful indicator of the binding capacity of a chemical on organic matter of soil and sewage sludge and allows comparisons to be made between different chemicals. This parameter can be estimated through correlations with the water solubility and the n-octanol/water partition coefficient (1)(2)(3)(4)(5)(6)(7).

The experimental method described in this test uses HPLC for the estimation of the adsorption coefficient K_{oc} in soil and in sewage sludge (8). The estimates are of higher reliability than those from QSAR calculations (9). As an estimation method it cannot fully replace batch equilibrium experiments used in the test method C18. However, the estimated K_{oc} may be useful for choosing appropriate test parameters for adsorption/desorption studies according to the test method C.18 by calculating K_d (distribution coefficient) or K_f (Freundlich adsorption coefficient) according to the equation 3 (see Section 1.2).

1.2. DEFINITIONS

K_d: distribution coefficient is defined as the ratio of equilibrium concentrations C of a dissolved test substance in a two phase system consisting of a sorbent (soil or sewage sludge) and an aqueous phase; it is a dimensionless value when concentrations in both phases are expressed on a weight/weight base. In case the concentration in the aqueous phase is given on a weight/volume base then the units are ml· g^{-1}. K_d can vary with sorbent properties and can be concentration dependent.

$$K_d = \frac{C_{soil}}{C_{aq}} or \frac{C_{sludge}}{C_{aq}} \quad (1)$$

where:

C_{soil} = concentration of test substance in soil at equilibrium (µg· g^{-1})
C_{sludge} = concentration of test substance in sludge at equilibrium (µg· g^{-1})
C_{aq} = concentration of test substance in aqueous phase at equilibrium (µg· g^{-1}, µg· ml^{-1}).

K_f: Freundlich adsorption coefficient is defined as the concentration of the test substance in soil or sewage sludge (x/m) when the equilibrium concentration C_{aq} in the aqueous phase is equal to one; units are µg.g^{-1} sorbent. The value can vary with sorbent properties.

$$\log\frac{x}{m} = \log K_f + \frac{1}{n}\cdot\log C_{aq} \quad (2)$$

where:

x/m = amount of test substance x (µg) adsorbed on amount of sorbent m (g) at equilibrium
1/n = slope of Freundlich adsorption isotherm
C_{aq} = concentration of test substance in aqueous phase at equilibrium (µg· ml- [1])

$$At C_{aq} = 1;\ \log K_f = \log\frac{x}{m}$$

K_{oc}: distribution coefficient (K_d) or Freundlich adsorption coefficient (K_f) normalised to the organic carbon content (f_{oc}) of a sorbent; particularly for non-ionised chemicals, it is an approximate indicator for the extent of adsorption between a substance and the sorbent and allows comparisons to be made between different chemicals. Depending on the dimensions of K_d and K_f, K_{oc} can be dimensionless or have the units ml· g^{-1} or µg· g^{-1} organic matter.

$$Koc = \frac{Kd}{foc}\left(\text{dimensionless or } ml \cdot g^{-1}\right) or \frac{Kf}{foc}(\mu g \cdot g^{-1}) \quad (3)$$

The relationship between K_{oc} and K_d is not always linear and thus K_{oc} values can vary from soil to soil but their variability is greatly reduced compared to K_d or K_f values.

The adsorption coefficient (K_{oc}) is deduced from the capacity factor (k') using a calibration plot of log k' versus log K_{oc} of the selected reference compounds.

$$k' = \frac{t_R - t_0}{t_0} \quad (4)$$

where:

t_R = HPLC retention time of test and reference substance (minutes);

t_0 = HPLC dead time (minutes) (see Section 1.8.2).

P_{OW}: The octanol-water partition coefficient is defined as the ratio of the concentrations of dissolved substance in n-octanol and water; it is a dimensionless value.

$$Pow = \frac{Coctanol}{Caq} (= Kow) \quad (5)$$

1.3. REFERENCE SUBSTANCES

The structural formula, the purity and the dissociation constant (if appropriate) should be known before using the method. Information on solubility in water and organic solvents, on octanol-water partition coefficient and on hydrolysis characteristics is useful.

To correlate the measured HPLC-retention data of a test substance with its adsorption coefficient K_{oc}, a calibration graph of log K_{oc} versus log k' has to be established. A minimum of six reference points, at least one above and one below the expected value of the test substance should be used. The accuracy of the method will be significantly improved if reference substances that are structurally related to the test substance are used. If such data are not available, it is up to the user to select the appropriate calibration substances. A more general set of structurally heterogeneous substances should be chosen in this case. Substances and K_{oc}-values which may be used are listed in the Appendix in Table 1 for sewage sludge and in Table 3 for soil. The selection of other calibration substances should be justified.

1.4. PRINCIPLE OF THE TEST METHOD

HPLC is performed on analytical columns packed with a commercially available cyanopropyl solid phase containing lipophilic and polar moieties. A moderately polar stationary phase based on a silica matrix is used:

— O — Si	— CH_2 — CH_2 — CH_2	— CN
silica	non-polar spacer	polar moiety

The principle of the test method is similar to testing method A.8 (Partition coefficient, HPLC method). While passing through the column along with the mobile phase the test substance interacts with the stationary phase. As a result of partitioning between mobile and stationary phases the test substance is retarded. The dual composition of the stationary phase having polar and non-polar sites allows for interaction of polar and non-polar groups of a molecule in a similar way as is the case for organic matter in soil or sewage sludge matrices. This enables the relationship between the retention time on the column and the adsorption coefficient on organic matter to be established.

pH has a significant influence on sorption behaviour in particular for polar substances. For agricultural soils or tanks of sewage treatment plants pH normally varies between pH 5,5 and 7,5. For ionisable substances, two tests should be performed with both ionised and non-ionised forms in appropriate buffer solutions but only in cases where at least 10 % of the test compound will be dissociated within pH 5,5 to 7,5.

Since only the relationship between the retention on the HPLC column and the adsorption coefficient is employed for the evaluation, no quantitative analytical method is required and only the determination of the retention time is necessary. If a suitable set of reference substances is available and standard experimental conditions can be used, the method provides a fast and efficient way to estimate the adsorption coefficient K_{oc}.

1.5. APPLICABILITY OF THE TEST

The HPLC method is applicable to chemical substances (unlabelled or labelled) for which an appropriate detection system (e.g. spectrophotometer, radioactivity detector) is available and which are sufficiently stable during the duration of the experiment. It may be particularly useful for chemicals which are difficult to study in other experimental systems (i.e. volatile substances; substances which are not soluble in water at a concentration which can be measured analytically; substances with a high affinity to the surface of incubation systems). The method can be used for mixtures which give unresolved elution bands. In such a case, upper and lower limits of the log K_{oc} values of the compounds of the test mixture should be stated.

Impurities may sometimes cause problems for interpretation of HPLC results, but they are of minor importance as long as the test substance can analytically be clearly identified and separated from the impurities.

The method is validated for the substances listed in Table 1 in the Appendix and was also applied to a variety of other chemicals belonging to the following chemical classes:

— aromatic amines (e.g. trifluralin, 4-chloroaniline, 3,5-dinitroaniline, 4-methylaniline, N-methylaniline, 1-naphthylamine),

— aromatic carboxilic acid esters (e.g. benzoic acid methylester, 3,5-dinitrobenzoic acid ethylester),

— aromatic hydrocarbons (e.g. toluene, xylene, ethylbenzene, nitrobenzene),

— aryloxyphenoxypropionic acid esters (e.g. diclofop-methyl, fenoxaprop-ethyl, fenoxaprop-P-ethyl),

— benzimidazole and imidazole fungicides (e.g. carbendazim, fuberidazole, triazoxide),

— carboxilic acid amides (e.g. 2-chlorobenzamide, N,N-dimethylbenzamide, 3,5-dinitrobenzamide, N-methylbenzamide, 2-nitrobenzamide, 3-nitrobenzamide),

— chlorinated hydrocarbons (e.g. endosulfan, DDT, hexachlorobenzene, quintozene, 1,2,3-trichlorobenzene),

— organophosphorus insecticides (e.g. azinphos-methyl, disulfoton, fenamiphos, isofenphos, pyrazophos, sulprofos, triazophos),

— phenols (e.g. phenol, 2-nitrophenol, 4-nitrophenol, pentachlorophenol, 2,4,6-trichlorophenol, 1-naphthol),

— phenylurea derivatives (e.g. isoproturon, monolinuron, pencycuron),

— pigment dyestuffs (e.g. Acid Yellow 219, Basic Blue 41, Direct Red 81),

— polyaromatic hydrocarbons (e.g. acenaphthene, naphthalene),

— 1,3,5-triazine herbicides (e.g. prometryn, propazine, simazine, terbutryn),

— triazole derivatives (e.g. tebuconazole, triadimefon, tradimenol, triapenthenol).

The method is not applicable for substances which react either with the eluent or the stationary phase. It is also not applicable for substances that interact in a specific way with inorganic components (e.g. formation of cluster complexes with clay minerals). The method may not work for surface active substances, inorganic

compounds and moderate or strong organic acids and bases. Log K_{oc} values ranging from 1,5 to 5,0 can be determined. Ionisable substances must be measured using a buffered mobile phase, but care has to be taken to avoid precipitation of buffer components or test substance.

1.6. QUALITY CRITERIA

1.6.1. **Accuracy**

Normally, the adsorption coefficient of a test substance can be estimated to within +/- 0,5 log unit of the value determined by the batch equilibrium method (see Table 1 in the Appendix). Higher accuracy may be achieved if the reference substances used are structurally related to the test substance.

1.6.2. **Repeatability**

Determinations should be run at least in duplicate. The values of log K_{oc} derived from individual measurements should be within a range of 0,25 log unit.

1.6.3. **Reproducibility**

Experience gained so far in the application of the method is supportive of its validity. An investigation of the HPLC method, using 48 substances (mostly pesticides) for which reliable data on K_{oc} on soils were available gave a correlation coefficient of R = 0,95 (10) (11).

An inter-laboratory comparison test with 11 participating laboratories was performed to improve and validate the method (12). Results are given in Table 2 of the Appendix.

1.7. DESCRIPTION OF THE TEST METHOD

1.7.1. **Preliminary Estimation of the Adsorption Coefficient**

The octanol-water partition coefficient P_{ow} (= K_{ow}) and, to some extent, the water solubility can be used as indicators for the extent of adsorption, particularly for non-ionised substances, and thus may be used for preliminary range finding. A variety of useful correlations have been published for several groups of chemicals (1)(2)(3)(4)(5)(6)(7).

1.7.2. **Apparatus**

A liquid chromatograph, fitted with a pulse-free pump and a suitable detection device is required. The use of an injection valve with an injection loop is recommended. Commercial cyanopropyl chemically bound resins on a silica base shall be used (e.g. Hypersil and Zorbax CN). A guard column of the same material may be positioned between the injection system and the analytical column. Columns from different suppliers may vary considerably in their separation efficiency. As a guidance, the following capacity factors k' should be reached: log k'> 0,0 for log K_{oc} = 3,0 and log k' > — 0,4 for log K_{oc} = 2,0 when using methanol/water 55/45 % as mobile phase.

1.7.3. **Mobile phases**

Several mobile phases have been tested and the following two are recommended:

— methanol/water (55/45 % v/v)

— methanol/0,01M citrate-buffer pH 6,0 (55/45 % v/v)

HPLC grade methanol and distilled water or citrate-buffer are used to prepare the eluting solvent. The mixture is degassed before use. Isocratic elution should be employed. If methanol/water mixtures are not appropriate, other organic solvent/water mixtures may be tried, e.g. ethanol/water or acetonitrile/water mixtures. For ionisable compounds the use of buffer solution is recommended to stabilise pH. Care must be taken to avoid salt precipitation and column deterioration, which may occur with some organic phase/buffer mixtures.

No additives such as ion pair reagents may be used because they can affect the sorption properties of the stationary phase. Such changes of the stationary phase may be irreversible. For this reason, it is mandatory that experiments using additives are carried out on separate columns.

1.7.4. **Solutes**

Test and reference substances should be dissolved in the mobile phase.

1.8. PERFORMANCE OF THE TEST

1.8.1. **Test condition**

The temperature during the measurements should be recorded. The use of a temperature controlled column compartment is highly recommended to guarantee constant conditions during calibration and estimation runs and measurement of the test substance.

1.8.2. **Determination of dead time to**

For the determination of the dead time t_o two different methods may be used (see also Section 1.2).

1.8.2.1. *Determination of the dead time to by means of a homologous series*

This procedure has proven to yield reliable and standardised t_o values. For details see Testing Method A.8: Partition coefficient (n-octanol/water), HPLC method.

1.8.2.2. *Determination of the dead time to by inert substances which are not retained by the column*

This technique is based on the injection of solutions of formamide, urea or sodium nitrate. Measurements should be performed at least in duplicate.

1.8.3. **Determination of the retention times tR**

Reference substances should be selected as described in Section 1.3. They may be injected as a mixed standard to determine their retention times, provided it has been confirmed that the retention time of each reference standard is unaffected by the presence of the other reference standards. The calibration should be performed at regular intervals at least twice daily in order to account for unexpected changes in column performance. For best practice the calibration injections should be carried out before and after injections of the test substance to confirm retention times have not drifted. The test substances are injected separately in quantities as small as possible (to avoid column overload) and their retention times are determined.

In order to increase the confidence in the measurement, at least duplicate determinations should be made. The values of log K_{oc} derived from individual measurements should fall within a range of 0,25 log unit.

1.8.4. **Evaluation**

The capacity factors k' are calculated from the dead time t_o and retention times t_R of the selected reference substances according to equation 4 (see Section 1.2). The log k' data of the reference substances are then plotted against their log K_{oc} values from batch equilibrium experiments given in Tables 1 and 3 of the Appendix. Using this plot, the log k' value of a test substance is then used to determine its log K_{oc} value. If the actual results show that the log K_{oc} of the test substance is outside the calibration range the test should be repeated using different, more appropriate reference substances.

2. **DATA AND REPORTING**

The report must include the following information:

— identity of test and reference substances and their purity, and pK_a values if relevant,

— description of equipment and operating conditions, e.g. type and dimension of analytical (and guard) column, means of detection, mobile phase (ratio of components and pH), temperature range during measurements,

— dead time and the method used for its determination,

— quantities of test and reference substances introduced in the column,

— retention times of reference compounds used for calibration,

— details of fitted regression line (log k' vs log K_{oc}) and a graph of the regression line,

— average retention data and estimated d log K_{oc} value for the test compound,

— chromatograms.

3. **REFERENCES**

(1) W.J. Lyman, W.F. Reehl, D.H. Rosenblatt (ed), (1990) Handbook of chemical property estimation methods, chapt. 4, McGraw-Hill, New York.

(2) J. Hodson, N.A. Williams, (1988) The estimation of the adsorption coefficient (K_{oc}) for soils by HPLC. Chemosphere, 17, p. 167.

(3) G.G. Briggs, (1981) Theoretical and experimental relationships between soil adsorption, octanol-water partition coefficients, water solubilities, bioconcentration factors, and the parachor. J. Agric. Food Chem., 29, p. 1050-1059.

(4) C.T. Chiou, P.E. Porter, D.W. Schmedding, (1983) Partition equilibria of nonionic organic compounds between soil organic matter and water. Environ. Sci. Technol., 17, p. 227-231.

(5) Z. Gerstl, U. Mingelgrin (1984) Sorption of organic substances by soils and sediment. J. Environm. Sci. Health, B19, p. 297-312.

(6) C.T. Chiou, L.J. Peters, V. H. Freed, (1979) A physical concept of soil water equilibria for nonionic organic compounds, Science, 106, p. 831-832.

(7) S.W. Karickhoff, (1981) Semi-empirical estimation of sorption of hydrophobic pollutants on natural sediments and soils. Chemosphere, 10, p. 833-846.

(8) W. Kördel, D. Hennecke, M. Herrmann, (1997) Application of the HPLC-screening method for the determination of the adsorption coefficient on sewage sludges. Chemosphere, 35(1/2), p. 121-128.

(9) M. Mueller, W. Kördel (1996) Comparison of screening methods for the estimation of adsorption coefficients on soil. Chemosphere, 32(12), p. 2493-2504.

(10) W. Kördel, J. Stutte, G. Kotthoff (1993) HPLC-screening method for the determination of the adsorption coefficient in soil-comparison of different stationary phases, Chemosphere, 27(12), p. 2341-2352.

(11) B. von Oepen, W. Kördel, W. Klein (1991) Sorption of nonpolar and polar compounds to soils: Processes, measurements and experience with the applicability of the modified OECD Guideline 106, Chemosphere, 22, p. 285-304.

(12) W. Kördel, G. Kotthoff, J. Müller (1995) HPLC-screening method for the determination of the adsorption coefficient on soil-results of a ring test. Chemosphere, 30(7), p. 1373-1384.

Appendix

Table 1

Comparison of K_{oc} values for soils and sewage sludges, and calculated values by the HPLC screening method ([1]), ([2])

substance	CAS-No	log K_{oc} sewage sludges	log K_{oc} HPLC	Δ	log K_{oc} soils	log K_{oc} HPLC	Δ
Atrazine	1912-24-9	1,66	2,14	0,48	1,81	2,20	0,39
Linuron	330-55-2	2,43	2,96	0,53	2,59	2,89	0,30
Fenthion	55-38-9	3,75	3,58	0,17	3,31	3,40	0,09
Monuron	150-68-5	1,46	2,21	0,75	1,99	2,26	0,27
Phenanthrene	85-01-8	4,35	3,72	0,63	4,09	3,52	0,57
Benzoic acid phenylester	93-99-2	3,26	3,03	0,23	2,87	2,94	0,07
Benzamide	55-21-0	1,60	1,00	0,60	1,26	1,25	0,01
4-Nitrobenzamide	619-80-7	1,52	1,49	0,03	1,93	1,66	0,27
Acetanilide	103-84-4	1,52	1,53	0,01	1,26	1,69	0,08
Aniline	62-53-3	1,74	1,47	0,27	2,07	1,64	0,43
2,5-Dichloroaniline	95-82-9	2,45	2,59	0,14	2,55	2,58	0,03

([1]) W. Kördel, D. Hennecke, M. Herrmann, (1997) Application of the HPLC-screening method for the determination of the adsorption coefficient on sewage sludges. Chemosphere, 35(1/2), p. 121-128.
([2]) W. Kördel, D. Hennecke, C. Franke, (1997) Determination of the adsorption-coefficients of organic substances on sewage sludges. Chemosphere, 35 (1/2), p. 107-119.

Table 2

Results of a laboratory inter-comparison test (11 participating laboratories) performed to improve and validate the HPLC-method ([1])

substance	CAS-No	log K_{oc}	K_{oc}	log K_{oc}
		[OECD 106]	[HPLC-method]	
Atrazine	1912-24-9	1,81	78 ± 16	1,89
Monuron	150-68-5	1,99	100 ± 8	2,00
Triapenthenol	77608-88-3	2,37	292 ± 58	2,47
Linuron	330-55-2	2,59	465 ± 62	2,67
Fenthion	55-38-9	3,31	2062 ± 648	3,31

([1]) W. Kördel, G. Kotthoff, J. Müller (1995). HPLC-screening method for the determination of the adsorption coefficient on soil-results of a ring test. Chemosphere, 30(7), 1373-1384.

Table 3

Recommended reference substances for the HPLC screening method based on soil adsorption data.

Reference substance	CAS-No	log K_{oc} mean values from batch equilibrium	number of K_{oc} data	log S.D.	source
Acetanilide	103-84-4	1,25	4	0,48	[a]
Phenol	108-95-2	1,32	4	0,70	[a]
2-Nitrobenzamide	610-15-1	1,45	3	0,90	[b]
N.N-dimethylbenzamide	611-74-5	1,52	2	0,45	[a]
4-Methylbenzamide	619-55-6	1,78	3	1,76	[a]
Methylbenzoate	93-58-3	1,80	4	1,08	[a]
Atrazine	1912-24-9	1,81	3	1,08	[c]
Isoproturon	34123-59-6	1,86	5	1,53	[c]
3-Nitrobenzamide	645-09-0	1,95	3	1,31	[b]
Aniline	62-53-3	2,07	4	1,73	[a]
3,5-Dinitrobenzamide	121-81-3	2,31	3	1,27	[b]
Carbendazim	10605-21-7	2,35	3	1,37	[c]
Triadimenol	55219-65-3	2,40	3	1,85	[c]
Triazoxide	72459-58-6	2,44	3	1,66	[c]
Triazophos	24017-47-8	2,55	3	1,78	[c]
Linuron	330-55-2	2,59	3	1,97	[c]
Naphthalene	91-20-3	2,75	4	2,20	[a]
Endosulfan-diol	2157-19-9	3,02	5	2,29	[c]
Methiocarb	2032-65-7	3,10	4	2,39	[c]
Acid Yellow 219	63405-85-6	3,16	4	2,83	[a]
1,2,3-Trichlorobenzene	87-61-6	3,16	4	1,40	[a]
γ-HCH	58-89-9	3,23	5	2,94	[a]
Fenthion	55-38-9	3,31	3	2,49	[c]
Direct Red 81	2610-11-9	3,43	4	2,68	[a]
Pyrazophos	13457-18-6	3,65	3	2,70	[c]
α-Endosulfan	959-98-8	4,09	5	3,74	[c]
Diclofop-methyl	51338-27-3	4,20	3	3,77	[c]
Phenanthrene	85-01-8	4,09	4	3,83	[a]
Basic Blue 41 (mix)	26850-47-5 12270-13-2	4,89	4	4,46	[a]
DDT	50-29-3	5,63	1	—	[b]

[a] W. Kördel, J. Müller (1994). Bestimmung des Adsorptionskoeffizienten organischer Chemikalien mit der HPLC. UBA R & D Report No. 106 01 044 (1994).
[b] B.V. Oepen, W. Kördel, W. Klein., (1991) Chemosphere, 22, p. 285-304.
[c] Data provided by industry.

C.20 *DAPHNIA MAGNA* REPRODUCTION TEST

1. METHOD

This Reproduction toxicity test method is a replicate of the OECD TG 211 (1998).

1.1. INTRODUCTION

The primary objective of the test is to assess the effect of chemicals on the reproductive output of *Daphnia magna*.

1.2. DEFINITIONS AND UNITS

Parent Animals: are those female *Daphnia* present at the start of the test and of which the reproductive output is the object of the study.

Offspring: are the young *Daphnia* produced by the parent animals in the course of the test.

Lowest Observed Effect Concentration (LOEC): is the lowest tested concentration at which the substance is observed to have a statistically significant effect on reproduction and parent mortality (at $p < 0,05$) when compared with the control, within a stated exposure period. However, all test concentrations above the LOEC must have a harmful effect equal to or greater than those observed at the LOEC. When these two conditions cannot be satisfied, a full explanation must be given for how the LOEC (and hence the NOEC) has been selected.

No Observed Effect Concentration (NOEC): is the test concentration immediately below the LOEC, which when compared with the control, has no statistically significant effect ($p < 0,05$), within a stated exposure period.

EC_x: is the concentration of the test substance dissolved in water that results in a x % reduction in reproduction of *Daphnia magna* within a stated exposure period.

Intrinsic rate of increase: is a measure of population growth which integrates reproductive output and age-specific mortality (20) (21) (22). In steady state populations it will be zero. For growing populations it will be positive and for shrinking populations it will be negative. Clearly, the latter is not sustainable and ultimately will lead to extinction.

Limit of Detection: is the lowest concentration that can be detected but not quantified.

Limit of Determination: is the lowest concentration that can be measured quantitatively.

Mortality: an animal is recorded as dead when it is immobile, i.e. when it is not able to swim, or if there is no observed movement of appendages or postabdomen, within 15 seconds after gentle agitation of the test container. (If another definition is used, this must be reported together with its reference).

1.3. PRINCIPLE OF THE TEST METHOD

Young female *Daphnia* (the parent animals), aged less than 24 hours at the start of the test, are exposed to the test substance added to water at a range of concentrations. The test duration is 21 days. At the end of the test, the total number of living offspring produced per parent animal alive at the end of the test is assessed. This means that juveniles produced by adults that die during the test are excluded from the calculations. Reproductive output of parent animals can be expressed in other ways (e.g. number of living offspring produced per animal per day from the first day offspring were observed) but these should be reported in addition to the total number of juveniles produced per parent alive at the end of the test. The reproductive output of the animals exposed to the test substance is compared to that of the control(s) in order to determine the lowest observed effect concentration (LOEC) and hence the no observed effect concentration (NOEC). In addition, and as far as possible, the data are analysed using a regression model in order to estimate the concentration that would cause a x % reduction in reproductive output (i.e. the EC_{50}, EC_{20}, or EC_{10}).

The survival of the parent animals and time to production of first brood must also be reported. Other substance-related effects on parameters such as growth (e.g. length) and possibly intrinsic rate of increase, may also be examined.

1.4. INFORMATION ON THE TEST SUBSTANCE

Results of an acute toxicity test (see Method C.2, Part I) performed with *Daphnia magna* should be available. The result may be useful in selecting an appropriate range of test concentrations in the reproduction tests. The water solubility and the vapour pressure of the test substance should be known and a reliable analytical method for the quantification of the substance in the test solutions with reported recovery efficiency and limit of determination should be available.

Information on the test substance which may be useful in establishing the test conditions includes the structural formula, purity of the substance, stability in light, stability under the conditions of the test, pKa, P_{ow} and results of the test for ready biodegradability (see Method C.4).

1.5. VALIDITY OF THE TEST

For a test to be valid, the following performance criteria should be met in the control(s):

— the mortality of the parent animals (female *Daphnia*) does not exceed 20 % at the end of the test,

— the mean number of live offspring produced per parent animal surviving at the end of the test is ≥ 60.

1.6. DESCRIPTION OF THE TEST METHOD

1.6.1. **Apparatus**

Test vessels and other apparatus which will come into contact with the test solutions should be made entirely of glass or other chemically inert material. The test vessels will normally be glass beakers.

In addition, some or all of the following equipment will be required:

— oxygen meter (with microelectrode or other suitable equipment for measuring dissolved oxygen in low volume samples),

— adequate apparatus for temperature control,

— pH meter,

— equipment for the determination of the hardness of water,

— equipment for the determination of the total organic carbon concentration (TOC) of water or equipment for the determination of the chemical oxygen demand (COD),

— adequate apparatus for the control of the lighting regime and the measurement of light intensity.

1.6.2. **Test organism**

The species to be used in the test is *Daphnia magna* Straus. Other *Daphnia* species may be used providing they meet the validity criteria as appropriate (the validity criterion relating to the reproductive output in the controls should be relevant for the *Daphnia* species). If other species of *Daphnia* are used they must be clearly identified and their use justified.

Preferably, the clone should have been identified by genotyping. Research (1) has shown that the reproductive performance of Clone A (which originated from IRCHA in France) (3) consistently meets the validity criterion of a mean of ≥ 60 offspring per parent animal surviving when cultured under the conditions described in this method. However, other clones are acceptable provided that the *Daphnia* culture is shown to meet the validity criteria for a test.

At the start of the test, the animals should be less than 24 hours old and must not be first brood progeny. They should be derived from a healthy stock (i.e. showing no signs of stress such as high mortality, presence of males and ephippia, delay in the production of the first brood, discoloured animals etc.). The stock animals must be maintained in culture conditions (light, temperature, medium, feeding and animals per unit volume) similar to those to be used in the test. If the *Daphnia* culture medium to be used in the test is different from that used for routine *Daphnia* culture, it is good practice to include a pre-test acclimation period of normally about 3 weeks (i.e. one generation) to avoid stressing the parent animals.

1.6.3. **Test medium**

It is recommended that a fully defined medium be used in this test. This can avoid the use of additives (e.g. seaweed, soil extract etc.), which are difficult to characterise, and therefore improves the opportunities for standardisation between laboratories. Elendt M4 (4) and M7 media (see Appendix 1) have been found to be suitable for this purpose. However, other media (e.g. (5) (6)) are acceptable providing the performance of the *Daphnia* culture is shown to meet the validity criteria for the test.

If media are used which include undefined additives, these additives should be specified clearly and information should be provided in the test report on composition, particularly with regard to carbon content as this may contribute to the diet provided. It is recommended that the total organic carbon (TOC) and/or chemical oxygen demand (COD) of the stock preparation of the organic additive is determined and an estimate of the resulting contribution to the TOC/COD in the test medium made. It is recommended that TOC levels in the medium (i.e. before addition of the algae) be below 2 mg/l (7).

When testing substances containing metals, it is important to recognise that the properties of the test medium (e.g. hardness, chelating capacity) may have a bearing on the toxicity of the test substance. For this reason, a fully defined medium is desirable. However, at present, the only fully defined media which are known to be suitable for long-term culture of *Daphnia magna* are Elendt M4 and M7. Both media contain the chelating agent EDTA. Work has shown (2) that the 'apparent toxicity' of cadmium is generally lower when the reproduction test is performed in M4 and M7 media than in media containing no EDTA. M4 and M7 are not, therefore, recommended for testing substances containing metals, and other media containing known chelating agents should also be avoided. For metal-containing substances it may be advisable to use an alternative medium such as, for example, ASTM reconstituted hard fresh water (7), which contains no EDTA, with added seaweed extract (8). This combination of ASTM reconstituted hard fresh water and seaweed extract is also suitable for long-term culture and testing of *Daphnia magna* (2), although it still exerts a mild chelating action due to the organic component in the added seaweed extract.

At the beginning and during the test, the dissolved oxygen concentration should be above 3 mg/l. The pH should be within the range 6-9, and normally it should not vary by more than 1,5 units in any one test. Hardness above 140 mg/l (as $CaCO_3$) is recommended. Tests at this level and above have demonstrated reproductive performance in compliance with the validity criteria (9) (10).

1.6.4. **Test solutions**

Test solutions of the chosen concentrations are usually prepared by dilution of a stock solution. Stock solutions should preferably be prepared by dissolving the substance in test medium.

The use of organic solvents or dispersants may be required in some cases in order to produce a suitably concentrated stock solution, but every effort should be made to avoid the use of such materials. Examples of suitable solvents are acetone, ethanol, methanol, dimethylformamide and triethylene glycol. Examples of suitable dispersants are Cremophor RH40, methylcellulose 0,01 % and HCO-40. In any case, the test substance in the test solutions should not exceed the limit of solubility in the test medium.

Solvents are used to produce a stock solution which can be dosed accurately into water. At the recommended solvent concentration in the final test medium (i.e. ≤ 0,1 ml/l), the solvents listed above will not be toxic and will not increase the water solubility of a substance.

Dispersants may assist in accurate dosing and dispersion. At the recommended concentration in the final test medium (≤ 0,1 ml/l), the dispersants listed above will not be toxic and will not increase the water solubility of a substance.

1.7. TEST DESIGN

Treatments should be allocated to the test vessels and all subsequent handling of the test vessels should be done in a random fashion. Failure to do this may result in bias that could be construed as being a concentration effect. In particular, if experimental units are handled in treatment or concentration order, then some time-related effect, such as operator fatigue or other error, could lead to greater effects at the higher concentrations. Furthermore, if the test results are likely to be affected by an initial or environmental condition of the test, such as position in the laboratory, then consideration should be given to blocking the test.

1.8. PROCEDURE

1.8.1. **Conditions of exposure**

1.8.1.1. *Duration*

The test duration is 21 days.

1.8.1.2. *Loading*

Parent animals are maintained individually, one per test vessel, with 50-100 ml of medium in each vessel.

Larger volumes may sometimes be necessary to meet requirements of the analytical procedure used for determination of the test substance concentration, although pooling of replicates for chemical analysis is also allowable. If volumes greater than 100 ml are used, the ration given to the *Daphnia* may need to be increased to ensure adequate food availability and compliance with the validity criteria. For flow-through tests, alternative designs may, for technical reasons, be considered (e.g. four groups of 10 animals in a larger test volume), but any changes to the test design should be reported.

1.8.1.3. *Number of animals*

For semi-static tests, at least 10 animals individually held at each test concentration and at least 10 animals individually held in the control series.

For flow-through tests, 40 animals divided into four groups of 10 animals at each test concentration has been shown to be suitable (1). A smaller number of test organisms may be used and a minimum of 20 animals per concentration divided into two or more replicates with an equal number of animals (e.g. four replicates each with five daphnids) is recommended. Note that for tests where animals are held in groups, it will not be possible to express the reproductive output as the total number of living offspring produced per parent animal alive at the end of the test, if parent animals die. In these cases reproductive output should be expressed as 'total number of living offspring produced per parent present at the beginning of the test'.

1.8.1.4. *Feeding*

For semi-static tests, feeding should preferably be done daily, but at least three times per week (i.e. corresponding to media changes). Deviations from this (e.g. for flow-through tests) should be reported.

During the test the diet of the parent animals should preferably be living algal cells of one or more of the following: *Chlorella* sp, *Selenastrum capricornutum* (now *Pseudokirchneriella subcapitata* (11)) and *Scenedesmus subspicatus*. The supplied diet should be based on the amount of organic carbon (C) provided to each parent animal. Research (12) has shown that, for *Daphnia magna*, ration levels of between 0,1 and 0,2 mg C/*Daphnia*/day are sufficient for achieving the required number of offspring to meet the test validity criteria. The ration can be supplied either at a consistent rate throughout the period of the test, or, if desired, a lower rate can be used at the beginning and then increased during the test to take account of growth of the parent animals. In this case, the ration should still remain within the recommended range of 0,1-0,2 mg C/*Daphnia*/day at all times.

If surrogate measures, such as algal cell number or light absorbance, are to be used to feed the required ration level (i.e. for convenience since measurement of carbon content is time consuming), each laboratory must produce its own nomograph relating the surrogate measure to carbon content of the algal culture (see Appendix 2 for advice on nomograph production). Nomographs should be checked at least annually and more frequently if algal culture conditions have changed. Light absorbance has been found to be a better surrogate for carbon content than cell number (13).

A concentrated algal suspension should be fed to the *Daphnia* to minimise the volume of algal culture medium transferred to the test vessels. Concentration of the algae can be achieved by centrifugation followed by resuspension in distilled water, deionised water or *Daphnia* culture medium.

1.8.1.5. *Light*

16 hours light at an intensity not exceeding 15-20 μE· m^{-2}·s^{-1}.

1.8.1.6. *Temperature*

The temperature of the test media should be within the range 18-22 °C. However, for any one test, the temperature should not, if possible, vary by more than 2 °C within these limits (e.g. 18-20, 19-21 or 20-22 °C). It may be appropriate to use an additional test vessel for the purposes of temperature monitoring.

1.8.1.7. *Aeration*

The test vessels must not be aerated during the test.

1.8.2. **Test concentration**

Normally, there should be at least five test concentrations arranged in a geometric series with a separation factor preferably not exceeding 3.2, and the appropriate number of replicates for each test concentration should be used (see Section 1.8.1.3). Justification should be provided if fewer than five concentrations are used. Substances should not be tested above their solubility limit in the test medium.

In setting the range of concentrations, the following should be borne in mind:

If the aim is to obtain the LOEC/NOEC, the lowest test concentration must be low enough so that the fecundity at that concentration is not significantly lower than that in the control. If this is not the case, the test will have to be repeated with a reduced lowest concentration.

If the aim is to obtain the LOEC/NOEC, the highest test concentration must be high enough so that the fecundity at that concentration is significantly lower than that in the control. If this is not the case, the test will have to be repeated with an increased highest concentration.

If the EC_X for effects on reproduction is estimated, it is advisable that sufficient concentrations are used to define the EC_X with an appropriate level of confidence. If the EC_{50} for effects on reproduction is estimated, it is advisable that the highest test concentration is greater than this EC_{50}. Otherwise, although it will still be possible to estimate the EC_{50}, the confidence interval for the EC_{50} will be very wide and it may not be possible to satisfactorily assess the adequacy of the fitted model.

The range of test concentration should preferably not include any concentrations that have a statistically significant effect on adult survival since this would change the nature of the test from simply a reproduction test to a combined reproduction and mortality test requiring much more complex statistical analysis.

Prior knowledge of the toxicity of the test substance (e.g. from an acute test and/or from range-finding studies) should help in selecting appropriate test concentrations.

Where a solvent or dispersant is used to aid preparation of test solutions (see Section 1.6.4), its final concentration in the test vessels should not be greater than 0,1 ml/l and should be the same in all test vessels.

1.8.3. **Controls**

One test-medium control series and also, if relevant, one control series containing the solvent or dispersant should be run in addition to the test series. When used, the solvent or dispersant concentration should be the same as that used in the vessels containing the test substance. The appropriate number of replicates should be used (see Section 1.8.1.3).

Generally, in a well-run test, the coefficient of variation around the mean number of living offspring produced per parent animal in the control(s) should be ≤ 25 %, and this should be reported for test designs using individually held animals.

1.8.4. **Test medium renewal**

The frequency of medium renewal will depend on the stability of the test substance, but should be at least three times per week. If, from preliminary stability tests (see Section 1.4) the test substance concentration is not stable (i.e. outside the range 80-120 % of nominal or falling below 80 % of the measured initial concentration) over the maximum renewal period (i.e. 3 days), consideration should be given to more frequent medium renewal, or to the use of a flow-through test.

When the medium is renewed in semi-static tests, a second series of test vessels are prepared and the parent animals transferred to them by, for example, a glass pipette of suitable diameter. The volume of medium transferred with the *Daphnia* should be minimised.

1.8.5. **Observations**

The results of the observations made during the test should be recorded on data sheets (see examples in Appendices 3 and 4). If other measurements are required (see 1.3 and 1.8.8) additional observations may be required.

1.8.6. **Offspring**

The offspring produced by each parent animal should preferably be removed and counted daily from the appearance of the first brood, to prevent them consuming food intended for the adult. For the purpose of this method it is only the number of living offspring that needs to be counted, but the presence of aborted eggs or dead offspring should be recorded.

1.8.7. **Mortality**

Mortality among the parent animals should be recorded preferably daily, at least at the same times as offspring are counted.

1.8.8. **Other parameters**

Although this method is designed principally to assess effects on reproduction, it is possible that other effects may also be sufficiently quantified to allow statistical analysis. Growth measurements are highly desirable since they provide information on possible sublethal effects, which may be more useful than reproduction measurement alone; the measurement of the length of the parent animals (i.e. body length excluding the anal spine) at the end of the test is recommended. Other parameters that can be measured or calculated include time to production of first brood (and subsequent broods), number and size of broods per animal, number of aborted broods, presence of males or ephippia and the intrinsic rate of population increase.

1.8.9. **Frequency of analytical determinations and measurements**

Oxygen concentration, temperature, hardness and pH values should be measured at least once a week, in fresh and old media, in the control(s) and in the highest test substance concentration.

During the test, the concentrations of test substance are determined at regular intervals.

In semi-static tests where the concentration of the test substance is expected to remain within ± 20 % of the nominal (i.e. within the range 80-120 % — see 1.4 and 1.8.4), it is recommended that, as a minimum, the highest and lowest test concentrations be analysed when freshly prepared and at the time of renewal on one occasion during the first week of the test (i.e. analyses should be made on a sample from the same solution — when freshly prepared and at renewal). These determinations should be repeated at least at weekly intervals thereafter.

For tests where the concentration of the test substance is not expected to remain within ± 20 % of the nominal, it is necessary to analyse all test concentrations, when freshly prepared and at renewal. However, for those tests where the measured initial concentration of the test substance is not within ± 20 % of nominal but where sufficient evidence can be provided to show that the initial concentrations are repeatable and stable (i.e. within the range 80-120 % of initial concentrations), chemical determinations could be reduced in weeks 2 and 3 of the test to the highest and lowest test concentrations. In all cases, determination of test substance concentrations prior to renewal need only be performed on one replicate vessel at each test concentration.

If a flow-through test is used, a similar sampling regime to that described for semi-static tests is appropriate (but measurement of 'old' solutions is not applicable in this case). However, it may be advisable to increase the number of sampling occasions during the first week (e.g. three sets of measurements) to ensure that the test concentrations are remaining stable. In these types of test, the flow-rate of diluent and test substance should be checked daily.

If there is evidence that the concentration of the substance being tested has been satisfactorily maintained within ± 20 % of the nominal or measured initial concentration throughout the test, then results can be based on nominal or measured initial values. If the deviation from the nominal or measured initial concentration is greater than ± 20 %, results should be expressed in terms of the time-weighed mean (see Appendix 5).

2. DATA AND REPORTING

2.1. TREATMENT OF RESULTS

The purpose of this test is to determine the effect of the test substance on the total number of living offspring produced per parent animal alive at the end of the test. The total number of offspring per parent animal should be calculated for each test vessel (i.e. replicate). If, in any replicate the parent animal dies during the test or turns out to be male, then the replicate is excluded from the analysis. The analysis will then be based on a reduced number of replicates.

For the estimation of the LOEC, and hence the NOEC, for effects of the chemical on reproductive output, it is necessary to calculate the mean reproductive output across replicates for each concentration and the pooled residual standard deviation, and this can be done using analysis of variance (ANOVA). The mean for each concentration must then be compared with the control mean using an appropriate multiple comparison method. Dunnett's or Williams' tests may be useful (14)(15)(16)(17). It is necessary to check whether the ANOVA assumption of homogeneity of variance holds. It is recommended that this be done graphically rather than via a formal significance test (18); a suitable alternative is to run a Bartlett's test. If this assumption does not hold, then consideration should be given to transforming the data to homogenise variances prior to performing the ANOVA, or to carrying out a weighted ANOVA. The size of the effect detectable using ANOVA (i.e. the least significant difference) should be calculated and reported.

For the estimation of the concentration which would cause a 50 % reduction in reproductive output (i.e. the EC_{50}), a suitable curve, such as the logistic curve, should be fitted to the data using a statistical method such as least squares. The curve could be parameterised so that the EC_{50} and its standard error can be estimated directly. This would greatly ease the calculation of the confidence limits about the EC_{50}. Unless there are good reasons to prefer different confidence levels, two-sided 95 % confidence limits should be quoted. The fitting procedure should preferably provide a means for assessing the significance of the lack of fit. This can be done graphically or by dividing the residual sum of squares into 'lack of fit' and 'pure error components' and performing a significance test for lack of fit. Since treatments giving high fecundity are likely to have greater variance in the number of juveniles produced than treatments giving low fecundity, consideration to weighting the observed values to reflect the different variances in the different treatment groups should be given (see for background information ref. 18).

In the analysis of the data from the final ring test (2), a logistic curve was fitted using the following model, although other suitable models can be used:

$$Y = \frac{c}{1 + \left(\frac{x}{x_0}\right)^b}$$

where:

Y = the total number of juveniles per parent animal alive at the end of the test (calculated for each vessel)

x = the substance concentration

c = the expected number of juveniles when x = 0

x_0 = the EC_{50} in the population

b = the slope parameter

This model is likely to be adequate in a large number of situations, but there will be tests for which it is not appropriate. A check should be made on the validity of the model as suggested above. In some cases, a hormesis model in which low concentrations give enhanced effects may be appropriate (19).

Other Effect Concentrations, such as the EC_{10} or EC_{20} can also be estimated, although it may be preferable to use a different parameterisation of the model from that used to estimate the EC_{50}.

2.2. TEST REPORT

The test report must include the following:

2.2.1. **Test substance:**

— physical nature and relevant physicochemical properties,

— chemical identification data, including purity.

2.2.2. **Test species:**

— the clone (whether it has been genetically typed), supplier or source (if known) and the culture conditions used. If a different species to *Daphnia magna* is used, this should be reported and justified.

2.2.3. **Test conditions:**

— test procedure used (e.g. semi-static or flow-through, volume, loading in number of *Daphnia* per litre),

— photoperiod and light intensity,

— test design (e.g. number of replicates, number of parents per replicate),

— details of culture medium used,

— if used, additions of organic material including the composition, source, method of preparation, TOC/COD of stock preparations, estimation of resulting TOC/COD in test medium,

— detailed information on feeding, including amount (in mg C/*Daphnia*/day) and schedule (e.g. type of food(s), including for algae the specific name(species) and, if known, the strain, the culture conditions),

— method of preparation of stock solutions and frequency of renewal (the solvent or dispersant and its concentration must be given, when used).

2.2.4. **Results:**

— results from any preliminary studies on the stability of the test substance,

— the nominal test concentrations and the results of all analyses to determine the concentration of the test substance in the test vessels (see example data sheets in Appendix 4); the recovery efficiency of the method and the limit of determination should also be reported,

— water quality within the test vessels (i.e. pH, temperature and dissolved oxygen concentration, and TOC and/or COD and hardness where applicable) (see example data sheet in Appendix 3),

— the full record of living offspring by each parent animal (see example data sheet in Appendix 3),

— the number of deaths among the parent animals and the day on which they occurred (see example data sheet in Appendix 3),

— the coefficient of variation for control fecundity (based on total number of living offspring per parent animal alive at the end of the test),

— plot of total number of living offspring per parent animal (for each replicate) alive at the end of the test versus concentration of the test substance,

— the Lowest Observed Effect Concentration (LOEC) for reproduction, including a description of the statistical procedures used and an indication of what size of effect could be detected and the No Observed Effect Concentration (NOEC) for reproduction; where appropriate, the LOEC/NOEC for mortality of the parent animals should also be reported,

— where appropriate, the EC_x for reproduction and confidence intervals and a graph of the fitted model used for its calculation, the slope of the dose-response curve and its standard error,

— other observed biological effects or measurements: report any other biological effects which were observed or measured (e.g. growth of parent animals) including any appropriate justification,

— an explanation for any deviation from the Test Method.

3. REFERENCES

(1) OECD Test Guideline Programme, Report of the Workshop on the *Daphnia magna* Pilot Ring Test, Sheffield University, UK, 20-21 March 1993.

(2) OECD Environmental Health and Safety Publications. Series on Testing and Assessment No6. Report of the Final Ring Test of the *Daphnia magna* Reproduction Test Paris. 1997.

(3) Baird D.J., Barber J., Bradley M.C., Soares A.M.V.M. and Calow P. (1991) A comparative study of genotype sensitivity to acute toxic stress using clones of *Daphnia magna* Strauss. Ecotoxicology and Environmental Safety, 21, p. 257-265.

(4) Elendt B.P., (1990) Selenium deficiency in Crustacea; An ultrastructural approach to antennal damage in *Daphnia magna* Straus. Protoplasma, 154, p. 25-33.

(5) EPA (1993) Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms. (Fourth ed.). EPA/600/4-90/027F. C. I. Weber (ed), USEPA, Cincinnati, Ohio.

(6) Vigano L., (1991) Suitability of commercially available spring waters as standard medium for culturing *Daphnia magna*. Bull. Environ. Contam. Toxicol., 47, p. 775-782.

(7) ASTM (1988) Standard Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates and Amphibians. E729-88a. American Society for Testing and Materials, Philadelphia P.A. p. 20.

(8) Baird D.J., Soares A.M.V.M., Girling A., Barber J., Bradley M.C. and Calow P., (1989) The long term maintenance of *Daphnia magna* Straus for use in ecotoxicological tests; problems and prospects. In: Proceedings of the 1st European Conference on Ecotoxicology. Copenhagen 1988 (H.Løkke, H. Tyle & F. Bro-Rasmussen. Eds.) p. 144-148.

(9) Parkhurst B.R., Forte J.L. and Wright G.P., (1981) Reproducibility of a life-cycle toxicity test with *Daphnia magna*. Bull. Environ. Contam. and Toxicol., 26, p. 1-8.

(10) Cowgill U.M. and Milazzo D.P., (1990) The sensitivity of two cladocerans to water quality variables: salinity and hardness. Arch. Hydrobiol., 120(2), p. 185-196.

(11) Korshikov (1990) *Pseudokirchneriella subcapitata* Hindak, F-1990. Biologice Prace, 36, p. 209.

(12) Sims I.R., Watson S. and Holmes D., (1993) Toward a standard *Daphnia* juvenile production test. Environmental Toxicology and Chemistry, 12, p. 2053-2058.

(13) Sims I., (1993) Measuring the growth of phytoplankton: the relationship between total organic carbon with three commonly used parameters of algal growth. Arch. Hydrobiol., 128, 459-466.

(14) Dunnett C.W., (1955) A multiple comparisons procedure for comparing several treatments with a control. J. Amer. Statist. Assoc., 50, p. 1096-1121.

(15) Dunnett C.W., (1964) New tables for multiple comparisons with a control. Biometrics, 20, p. 482-491.

(16) Williams D.A., (1971) A test for differences between treatment means when several dose levels are compared with a zero dose control. Biometrics 27, p. 103-117.

(17) Williams D.A., (1972) The comparison of several dose levels with a zero dose control. Biometrics, 28, p. 510-531.

(18) Draper N.R. and Smith H., (1981) Applied Regression Analysis, second edition, Wiley, N.Y.

(19) Brain P. and Cousens R., (1989) An equation to describe dose responses where there is stimulation of growth at low doses. Weed Research, 29, p. 93-96.

(20) Wilson E.O. and Bossert, W.H., (1971) A Primer of Population Biology. Sinauer Associates Inc. Publishers.

(21) Poole R.W., (1974) An Introduction to quantitative Ecology. Mc Graw Hill Series in Population Biology, New York, p. 532.

(22) Meyer J.S., Ingersoll C.G., McDonald L.L. and Boyce M.S., (1986) Estimating uncertainty in population growth rates: Jackknife vs bootstrap techniques. Ecology, 67, p. 1156-1166.

Appendix 1

PREPARATION OF FULLY DEFINED ELENDT M7 AND M4 MEDIA

Acclimation to Elendt M7 and M4 media

Some laboratories have experienced difficulty in directly transferring *Daphnia* to M4 (1) and M7 media. However, some success has been achieved with gradual acclimation, i.e. moving from own medium to 30 % Elendt, then to 60 % Elendt and then to 100 % Elendt. The acclimation periods may need to be as long as one month.

PREPARATION

Trace elements

Separate stock solutions (I) of individual trace elements are first prepared in water of suitable purity, e.g. deionised, distilled or reverse osmosis. From these different stock solutions (I) a second single stock solution (II) is prepared, which contains all trace elements (combined solution), i.e.:

Stock solutions I (single substance)	Amount added to water mg/l	Concentration (in relation to medium M4) fold	To prepare the combined stock-solution II add the following amount of stock solution I to water ml/l	
			M 4	M 7
H_3BO_3	57 190	20 000	1,0	0,25
$MnCl_2 * 4 H_2O$	7 210	20 000	1,0	0,25
LiCl	6 120	20 000	1,0	0,25
RbCl	1 420	20 000	1,0	0,25
$SrCl_2 * 6 H_2O$	3 040	20 000	1,0	0,25
NaBr	320	20 000	1,0	0,25
$Na_2MoO_4 * 2 H_2O$	1 260	20 000	1,0	0,25
$CuCl_2 * 2 H_2O$	335	20 000	1,0	0,25
$ZnCl_2$	260	20 000	1,0	1,0
$CoCl_2 * 6 H_2O$	200	20 000	1,0	1,0
KI	65	20 000	1,0	1,0
Na_2SeO_3	43,8	20 000	1,0	1,0
NH_4VO_3	11,5	20 000	1,0	1,0
$Na_2EDTA * 2 H_2O$	5 000	2 000	—	—
$FeSO_4 * 7 H_2O$	1 991	2 000	—	—

Both Na_2EDTA and $FeSO_4$ solutions are prepared singly, poured together and autoclaved immediately. This gives:

21 Fe-EDTA solution		1 000-fold	20,0	5,0

M4 and M7 media

M4 and M7 media are prepared using stock solution II, the macro-nutrients and vitamins as follows:

	Amount added to water mg/l	Concentration (related to medium M4) fold	Amount of stock solution added to prepare medium ml/l	
			M 4	M 7
Stock solution II combined trace elements		20	50	50
Macro-nutrient stock solutions (single substance)				
$CaCl_2$ * 2 H_2O	293 800	1 000	1,0	1,0
$MgSO_4$ * 7 H_2O	246 600	2 000	0,5	0,5
KCl	58 000	10 000	0,1	0,1
$NaHCO_3$	64 800	1 000	1,0	1,0
Na_2SiO_3 * 9 H_2O	50 000	5 000	0,2	0,2
$NaNO_3$	2 740	10 000	0,1	0,1
KH_2PO_4	1 430	10 000	0,1	0,1
K_2HPO_4	1 840	10 000	0,1	0,1
Combined Vitamin stock	—	10 000	0,1	0,1
The combined vitamin stock solution is prepared by adding the 3 vitamins to 1 litre water as show below:				
Thiamine hydrochloride	750	10 000	—	—
Cyanocobalamine (B_{12})	10	10 000	—	—
Biotine	7,5	10 000	—	—

The combined vitamin stock is stored frozen in small aliquots. Vitamins are added to the media shortly before use.

N.B. To avoid precipitation of salts when preparing the complete media, add the aliquots of stock solutions to about 500-800 ml deionised water and then fill up to 1 litre.

N.N.B. The first publication of the M4 medium can be found in Elendt, B.P. (1990). Selenium deficiency in crustacea; an ultrastructural approach to antennal damage in *Daphnia magna* Straus. Protoplasma, 154, p. 25-33.

Appendix 2

TOTAL ORGANIC CARBON (TOC) ANALYSIS AND PRODUCTION OF A NOMOGRAPH FOR TOC CONTENT OF ALGAL FEED

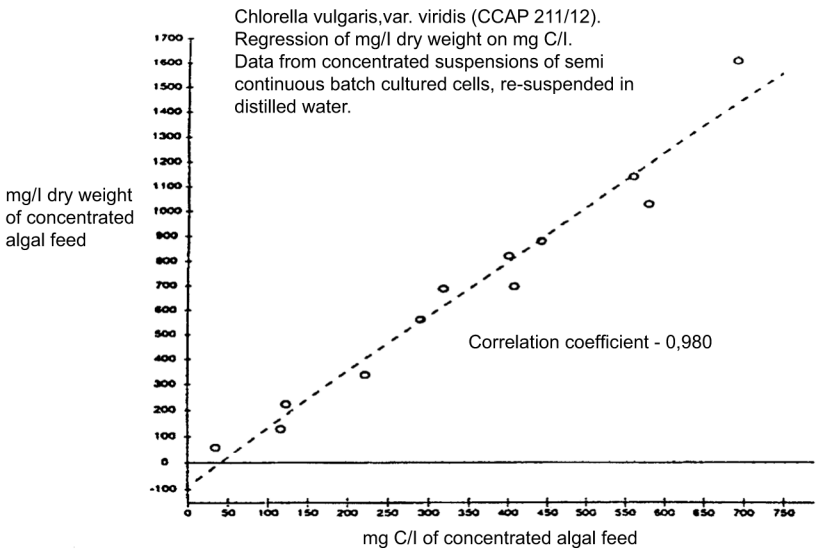
It is recognised that the carbon content of the algal feed will not normally be measured directly but from correlations (i.e. nomographs) with surrogate measures such as algal cell number or light absorbance).

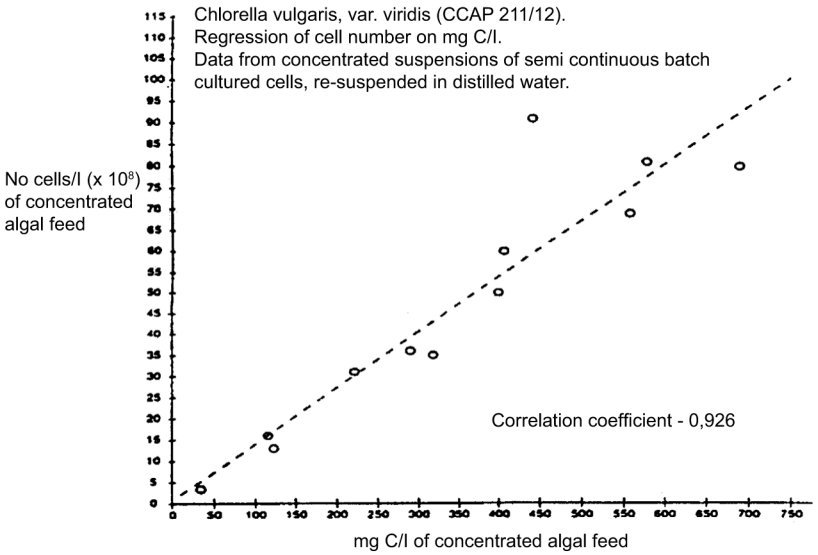
TOC should be measured by high temperature oxidation rather than by UV or persulphate methods. (See: The Instrumental Determination of Total Organic Carbon, Total Oxygen Demand and Related Determinands 1979, HMSO 1980; 49 High Holborn, London WC1V 6HB).

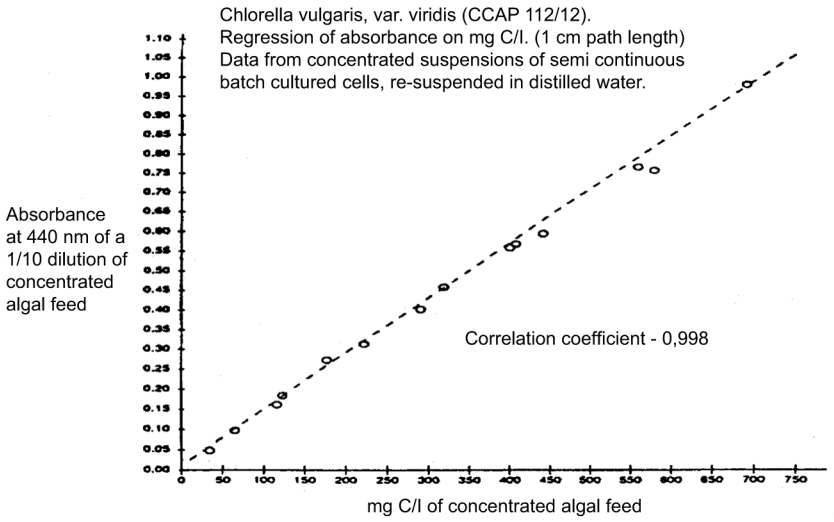
For nomograph production, algae should be separated from the growth medium by centrifugation followed by resuspension in distilled water. Measure the surrogate parameter and TOC concentration in each sample in triplicate. Distilled water blanks should be analysed and the TOC concentration deducted from that of the algal sample TOC concentration.

Nomograph should be linear over the required range of carbon concentrations. Examples are shown below.

N.B.: These should not be used for conversions; it is essential that laboratories prepare their own nomographs.






Chlorella vulgaris, var. viridis (CCAP 112/12).
Regression of absorbance on mg C/l. (1 cm path length)
Data from concentrated suspensions of semi continuous batch cultured cells, re-suspended in distilled water.
Absorbance at 440 nm of a 1/10 dilution of concentrated algal feed
Correlation coefficient - 0,998
mg C/l of concentrated algal feed

Appendix 3

EXAMPLE DATA SHEET RECORDING MEDIUM RENEWAL, PHISICAL/CHEMICAL MONITORING DATA, FEEDING, *DAPHNIA* REPRODUCTION AND ADULT MORTALITY

Experiment No: Data starded: Clone: Medium: Type of food: Test Substance: Nominal conc:

Day	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21		
Medium renewal (tick)																								
PH (1)																							new	
																							old	
O_2 mg/l (1)																							new	
																							old	
Temp (°C) (1)																							new	
																							old	
Food provided (tick)																								
No live off-spring (2)																								Total
Vessel 1																								
2																								
3																								
4																								
5																								
6																								
7																								
8																								

Experiment No: Data starded: Clone: Medium: Type of food: Test Substance: Nominal conc:

Day	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21		
9																								
10																								
																							Total	
Cumulative adult mortality ([3])																								

([1]) Indicate which vessel was used for the experiment.
([2]) Record mortality of any adult animals as 'M' in relevant box.
([3]) Record aborted broods as 'AB' in relevant box.

Appendix 4

EXAMPLE DATA SHEET FOR RECORDING RESULTS OF CHEMICAL ANALYSIS

(a) **Measured concentrations**

Nominal conc.	Week 1 sample		Week 2 sample		Week 3 sample	
	Fresh	Old	Fresh	Old	Fresh	Old

(b) **Measured concentrations as a percentage of nominal**

Nominal conc.	Week 1 sample		Week 2 sample		Week 3 sample	
	Fresh	Old	Fresh	Old	Fresh	Old

Appendix 5

CALCULATION OF A TIME-WEIGHTED MEAN

Time-weighted mean

Given that the concentration of the test substance can decline over the period between medium renewals, it is necessary to consider what concentration should be chosen as representative of the range of concentrations experienced by the parent *Daphnia*. The selection should be based on biological considerations as well as statistical ones. For example, if reproduction is thought to be affected mostly by the peak concentration experienced, then the maximum concentration should be used. However, if the accumulated or longer term effect of the toxic substance is considered to be more important, then an average concentration is more relevant. In this case, an appropriate average to use is the time-weighted mean concentration, since this takes account of the variation in instantaneous concentration over time.

Figure 1: Example of time-weighted mean

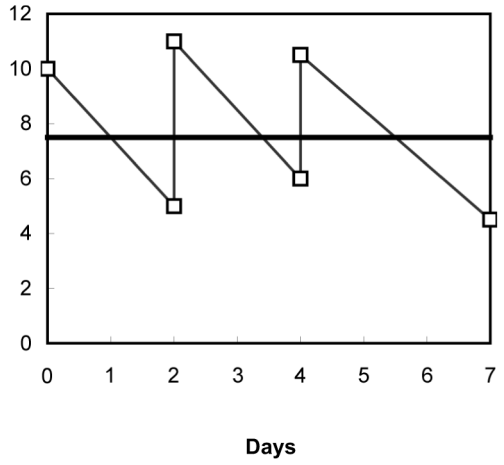


Figure 1 shows an example of a (simplified) test lasting seven days with medium renewal at Days 0, 2 and 4.

— The thin zig-zag line represents the concentration at any point in time. The fall in concentration is assumed to follow an exponential decay process.

— The six plotted points represent the observed concentrations measured at the start and end of each renewal period.

— The thick solid line indicates the position of the time-weighted mean.

The time-weighted mean is calculated so that the area under the time-weighted mean is equal to the area under the concentration curve. The calculation for the above example is illustrated in Table 1.

Table 1:

calculation of Time-weighted mean

Renewal No	Days	Conc0	Conc1	Ln(Conc0)	Ln(Conc1)	Area
1	2	10,000	4,493	2,303	1,503	13,767
2	2	11,000	6,037	2,398	1,798	16,544
3	3	10,000	4,066	2,303	1,403	19,781

Renewal No	Days	Conc0	Conc1	Ln(Conc0)	Ln(Conc1)	Area
Total Days: 7					Total Area	50,091
					TW Mean	7,156

Days is the number of days in the renewal period

Conc0 is the measured concentration at the start of each renewal period

Conc1 is the measured concentration at the end of each renewal period

Ln(Conc0) is the natural logarithm of Conc0

Ln(Conc1) is the natural logarithm of Conc1

Area is the area under the exponential curve for each renewal period. It is calculated by:

$$Area = \frac{Conc0 - Conc1}{Ln(Conc0) - Ln(Conc1)} \times Days$$

The time-weighted mean (TW *Mean*) is the *total area* divided by the *total days*.

Of course, for the *Daphnia* reproduction test the table would have to be extended to cover 21 days.

It is clear that when observation are taken only at the start and end of each renewal period, it is not possible to confirm that the decay process is, in fact, exponential. A different curve would result in a different calculation for *Area*. However, an exponential decay process is not implausible and is probably the best curve to use in the absence of other information.

However, a work of caution is required if the chemical analysis fails to find any substance at the end of the renewal period. Unless it is possible to estimate how quickly the substance disappeared from the solution, it is impossible to obtain a realistic area under the curve, and hence it is impossible to obtain a reasonable time-weighted mean.

C.21. SOIL MICROORGANISMS: NITROGEN TRANSFORMATION TEST

1. METHOD

This test method is a replicate of OECD TG 216 (2000).

1.1. INTRODUCTION

This testing method describes a laboratory method designed to investigate the long-term effects of chemicals, after a single exposure, on nitrogen transformation activity of soil microorganisms. The test is principally based on the recommendations of the European and Mediterranean Plant Protection Organization (1). However, other guideline, including those of the German Biologische Bundesanstalt (2), the US Environmental Protection Agency (3) SETAC (4) and the International Organization for Standardization (5), were also taken into account. An OECD workshop on soil/sediment selection held at Belgirate, Italy, in 1995 (6) agreed on the number and type of soils for use in this test. Recommendations for collection, handling and storage of soil sample are based on an ISO Guidance Document (7) and recommendations from the Belgirate workshop. In the assessment and evaluation of toxic characteristics of test substances, determination of effects on soil microbial activity may be required, e.g. when data on the potential side effects of crop protection products on soil microflora are required or when exposure of soil microorganisms to chemicals other than crop protection products is expected. The nitrogen transformation test is carried out to determine the effects of such chemicals on soil microflora. If agrochemicals (e.g. crop protection products, fertilisers, forestry chemicals) are tested, both nitrogen transformation and carbon transformation tests are conducted. If non-agrochemicals are tested, the nitrogen transformation test is sufficient. However, if EC_{50} values of the nitrogen transformation test for such chemicals fall within the range found for commercially available nitrification inhibitors (e.g. nitrapyrin), a carbon transformation test can be conducted to gain further information.

Soils consist of living and non-living components which exist in complex and heterogeneous mixtures. Microorganisms play an important role in break-down and transformation of organic matter in fertile soils with many species contributing to different aspects of soil fertility. Any long-term interference with these biochemical processes could potentially interfere with nutrient cycling and this could alter soil fertility. Transformation of carbon and nitrogen occurs in all fertile soils. Although the microbial communities responsible for these processes differ from soil to soil, the pathways of transformation are essentially the same.

This testing method described is designed to detect long-term adverse effects of a substance on the process of nitrogen transformation in aerobic surface soils. The test method also allows estimation of the effects of substances on carbon transformation by the soil microflora. Nitrate formation takes place subsequent to the degradation of carbon-nitrogen bonds. Therefore, if equal rates of nitrate production are found in treated and control soils, it is highly probable that the major carbon degradation pathways are intact and functional. The substrate chosen for the test (powdered lucerne meal) has a favourable carbon to nitrogen ratio (usually between 12/1 and 16/1). Because of this, carbon starvation is reduced during the test and if microbial communities are damaged by a chemical, they might recover within 100 days.

The tests from which this testing method was developed were primarily designed for substances for which the amount reaching the soil can be anticipated. This is the case, for example, for crop protection products for which the application rate in the field is known. For agrochemicals, testing of two doses relevant to the anticipated or predicted application rate is sufficient. Agrochemicals can be tested as active ingredients (a.i.) or as formulated products. However, the test is not limited to agrochemicals. By changing both the amounts of test substance applied to the soil, and the way in which the data are evaluated, the test can also be used for chemicals for which the amount expected to reach the soil is not known. Thus, with chemicals other than agrochemicals, the effects of a series of concentrations on nitrogen transformation are determined. The data from these tests are used to prepare a dose-response curve and calculate EC_x values, where x is defined % effect.

1.2. DEFINITIONS

Nitrogen transformation: is the ultimate degradation by microorganisms of nitrogen-containing organic matter, via the process of ammonification and nitrification, to the respective inorganic end-product nitrate.

EC_x (effective concentration): is the concentration of the test substance in soil that results in a x percent inhibition of nitrogen transformation to nitrate.

EC_{50} (median effective concentration): is the concentration of the test substance in soil that results in a 50 percent (50 %) inhibition of nitrogen transformation to nitrate.

1.3. REFERENCE SUBSTANCES

None.

1.4. PRINCIPLE OF THE TEST METHOD

Sieved soil is amended with powdered plant meal and either treated with the test substance or left untreated (control). If agrochemicals are tested, a minimum of two test concentrations are recommended and these should be chosen in relation to the highest concentration anticipated in the field. After 0, 7, 14 days and 28 days of incubation, samples of treated and control soils are extracted with an appropriate solvent, and the quantities of nitrate in the extracts are determined. The rate of nitrate formation in treated samples is compared with the rate in the controls, and the percent deviation of the treated from the control is calculated. All tests run for at least 28 days. If, on the 28th day, differences between treated and untreated soils are equal to or greater than 25 %, measurements are continued to a maximum of 100 days. If non-agrochemicals are tested, a series of concentrations of the test substance are added to samples of the soil, and the quantities of nitrate formed in treated and control samples are measured after 28 days of incubation. Results from tests with multiple concentrations are analysed using a regression model, and the EC_x values are calculated (i.e. EC_{50}, EC_{25} and/or EC_{10}). See definitions.

1.5. VALIDITY OF THE TEST

Evaluations of test results with agrochemicals are based on relatively small differences (i.e. average value ±25 %) between nitrate concentrations in control and treated soil samples, so large variations in the controls can lead to false results. Therefore, the variation between replicate control samples should be less than ±15 %.

1.6. DESCRIPTION OF THE TEST METHOD

1.6.1. **Apparatus**

Test containers made of chemically inert material are used. They should be of a suitable capacity in compliance with the procedure used for incubation of soils, i.e. incubation in bulk or as a series of individual soil samples (see Section 1.7.1.2). Care should be taken both to minimise water loss and to allow gas exchange during the test (e.g. the test containers may be covered with perforated polyethylene foil). When volatile substances are tested, sealable and gas-tight containers should be used. These should be of a size such that approximately one quarter of their volume is filled with the soil sample.

Standard laboratory equipment including the following is used:

— agitation device: mechanical shaker or equivalent equipment;

— centrifuge (3 000 g) or filtration device (using nitrate-free filter paper);

— instrument of adequate sensitivity and reproducibility for nitrate analysis.

1.6.2. **Selection and number of soils**

One single soil is used. The recommended soil characteristics are as follows:

— sand content: not less than 50 % and not greater than 75 %,

— pH: 5,5-7,5,

— organic carbon content: 0,5-1,5 %,

— the microbial biomass should be measured (8)(9) and its carbon content should be at least 1 % of the total soil organic carbon.

In most cases, a soil with these characteristics represents a worst case situation, since adsorption of the test chemical is minimum and its availability to the microflora is maximum. Consequently, tests with other soils are generally unnecessary. However, in certain circumstances, e.g. where the anticipated major use of the test substance is in particular soils such as acidic forest soils, or for electrostatically charged chemicals, it may be necessary to use an additional soil.

1.6.3. **Collection and storage of soil samples**

1.6.3.1. *Collection*

Detailed information on the history of the field site from where the test soil is collected should be available. Details include exact location, vegetation cover, dates of treatments with crop protection products, treatments with organic and inorganic fertilisers, additions of biological materials or accidental contaminations. The site chosen for soil collection should be one which allows long-term use. Permanent pastures, fields with annual cereal crops (except maize) or densely sown green manures are suitable. The selected sampling site should not have been treated with crop protection products for a minimum of one year before sampling. Also, no organic fertiliser should have been applied for at least six months. The use of mineral fertiliser is only acceptable when in accordance with the requirements of the crop and soil samples should not be taken until at least three months after fertiliser application. The use of soil treated with fertilisers with known biocidal effects (e.g. calcium cyanamide) should be avoided.

Sampling should be avoided during or immediately following long periods (greater than 30 days) of drought or water logging. For ploughed soils, samples should be taken from a depth of 0 down to 20 cm. For grassland (pasture) or other soils where ploughing does not occur over longer periods (at least one growing season), the maximum depth of sampling may be slightly more than 20 cm (e.g. to 25 cm).

Soil samples should be transported using containers and under temperature conditions which guarantee that the initial soil properties are not significantly altered.

1.6.3.2. *Storage*

The use of soils freshly collected from the field is preferred. If storage in the laboratory cannot be avoided, soils may be stored in the dark at 4 ± 2 °C for a maximum of three months. During the storage of soils, aerobic conditions must be ensured. If soils are collected from areas where they are frozen for at least three months per year, storage for six months at minus 18 °C to minus 22 °C can be considered. The microbial biomass of stored soils is measured prior to each experiment and the carbon in the biomass should be at least 1 % of the total soil organic carbon content (see Section 1.6.2).

1.6.4. **Handling and preparation of soil for the test**

1.6.4.1. *Pre-incubation*

If the soil was stored (see Section 1.6.3.2), pre-incubation is recommended for a period between two and 28 days. The temperature and moisture content of the soil during pre-incubation should be similar to that used in the test (see Sections 1.6.4.2 and 1.7.1.3).

1.6.4.2. *Physical-chemical characteristics*

The soil is manually cleared of large objects (e.g. stones, parts of plants, etc.) and then moist sieved without excess drying to a particle size less than or equal to 2 mm. The moisture content of the soil sample should be adjusted with distilled or deionised water to a value between 40 % and 60 % of the maximum water holding capacity.

1.6.4.3. *Amendment with organic substrate*

The soil should be amended with a suitable organic substrate, e.g. powdered lucerne-grass-green meal (main component: *Medicago sativa)* with a C/N ratio between 12/1 and 16/1. The recommended lucerne-soil ratio is 5 g of lucerne per kilogram of soil (dry weight).

1.6.5. **Preparation of the test substance for the application to soil**

The test substance is normally applied using a carrier. The carrier can be water (for water soluble substances) or an inert solid such as fine quartz sand (particle size: 0,1-0,5mm). Liquid carriers other than water (e.g. organic solvents such as acetone, chloroform) should be avoided since they can damage the microflora. If sand is used as a carrier, it can be coated with the test substance dissolved or suspended in an appropriate solvent. In such cases, the solvent should be removed by evaporation before mixing with the soil. For an optimum distribution of the test substance in soil, a ratio of 10 g of sand per kilogram of soil (dry weight) is recommended. Control samples are treated with an equivalent amount of water and/or quartz sand only.

When testing volatile chemicals, losses during treatment should be avoided as far as possible and an attempt should be made to ensure homogeneous distribution in the soil (e.g. the test substance should be injected into the soil at several places).

1.6.6. **Test concentrations**

If agrochemicals are tested, at least two concentrations should be used. The lower concentration should reflect at least the maximum amount expected to reach the soil under practical conditions whereas the higher concentration should be a multiple of the lower concentration. The concentrations of test substance added to soil are calculated assuming uniform incorporation to a depth of 5 cm and a soil bulk density of 1,5. For agrochemicals that are applied directly to soil, or for chemicals for which the quantity reaching the soil can be predicted, the test concentrations recommended are the maximum Predicted Environmental Concentration (PEC) and five times that concentration. Substances that are expected to be applied to soils several times in one season should be tested at concentrations derived from multiplying the PEC by the maximum anticipated number of applications. The upper concentration tested, however, should not exceed 10 times the maximum single application rate. If non-agrochemicals are tested, a geometric series of at least five concentrations is used. The concentrations tested should cover the range needed to determine the EC_x values.

1.7. PERFORMANCE OF THE TEST

1.7.1. **Conditions of exposure**

1.7.1.1. *Treatment and control*

If agrochemicals are tested, the soil is divided into three portions of equal weight. Two portions are mixed with the carrier containing the product, and the other is mixed with the carrier without the product (control). A minimum of three replicates for both treated and untreated soils is recommended. If non-agrochemicals are tested, the soil is divided into six portions of equal weight. Five of the samples are mixed with the carrier containing the test substance, and the sixth sample is mixed with the carrier without the chemical. Three replicates for both treatments and control are recommended. Care should be taken to ensure homogeneous distribution of the test substance in the treated soil samples. During mixing, compacting or balling of the soil should be avoided.

1.7.1.2. *Incubation of soil samples*

Incubation of soil samples can be performed in two ways: as bulk samples of each treated and untreated soil or as a series of individual and equally sized subsamples of each treated and untreated soil. However, when volatile substances are tested, the test should only be performed with a series of individual subsamples. When soils are incubated in bulk, large quantities of each treated and untreated soils are prepared and subsamples to be analysed are taken as needed during the test. The amount initially prepared for each treatment and control depends on the size of the subsamples, the number of replicates used for analysis and the anticipated maximum number of sampling times. Soils incubated in bulk should be thoroughly mixed before subsampling. When soils are incubated as a series of individual soil samples, each treated and untreated bulk soil is divided into the required number of subsamples, and these are utilised as needed. In the experiments where more than two sampling times can be anticipated, enough subsamples should be prepared to account for all replicates and all sampling times. At least three replicate samples of the test soil should be incubated under aerobic conditions (see Section 1.7.1.1). During all tests, appropriate containers with sufficient headspace should be used to avoid development of anaerobic conditions. When volatile substances are tested, the test should only be performed with a series of individual subsamples.

1.7.1.3. *Test conditions and duration*

The test is carried out in the dark at room temperature of 20 ± 2 °C. The moisture content of soil samples should be maintained during the test between 40 % and 60 % of the maximum water holding capacity of the soil (see Section 1.6.4.2) with a range of ±5 %. Distilled, deionised water can be added as needed.

The minimum duration of tests is 28 days. If agrochemicals are tested, the rates of nitrate formation in treated and control samples are compared. If these differ by more than 25 % on day 28, the test is continued until a difference equal to or less than 25 % is obtained, or for a maximum of 100 days, whichever is shorter. For non-agrochemicals, the test is terminated after 28 days. On day 28, the quantities of nitrate in treated and control soil samples are determined and the EC_x values are calculated.

1.7.2. **Sampling and analysis of soils**

1.7.2.1. *Soil sampling schedule*

If agrochemicals are tested, soil samples are analysed for nitrate on days 0, 7, 14 and 28. If a prolonged test is required, further measurements should be made at 14 days intervals after day 28.

If non-agrochemicals are tested, at least five test concentrations are used and soil samples are analysed for nitrate at the beginning (day 0) and at the end of the exposure period (28 days). An intermediate measurement, e.g. at day 7, may be added if deemed necessary. The data obtained on day 28 are used to determine EC_x value for the chemical. If desired, data from day 0 control samples can be used to report the initial quantity of nitrate in the soil.

1.7.2.2. *Analysis of soil samples*

The amount of nitrate formed in each treated and control replicate is determined at each sampling time. Nitrate is extracted from soil by shaking samples with a suitable extraction solvent, e.g. a 0,1 M potassium chloride solution. A ratio of 5 ml of KCl solution per gram dry weight equivalent of soil is recommended. To optimise extraction, containers holding soil and extraction solution should not be more than half full. The mixtures are shaken at 150 rpm for 60 minutes. The mixtures are centrifuged or filtered and the liquid phases are analysed for nitrate. Particle-free liquid extracts can be stored prior to analysis at minus 20 ± 5 °C for up to six months.

2. **DATA**

2.1. TREATMENT OF RESULTS

If tests are conducted with agrochemicals, the quantity of nitrate formed in each replicate soil sample should be recorded, and the mean values of all replicates should be provided in tabular form. Nitrogen transformation rates should be evaluated by appropriate and generally acceptable statistical methods (e.g. F-test, 5 % significance level). The quantities of nitrate formed are expressed in mg nitrate/kg dry weight soil/day. The nitrate formation rate in each treatment is compared with that in the control, and the percent deviation from the control is calculated.

If tests are conducted with non-agrochemicals, the quantity of nitrate formed in each replicate is determined, and a dose-response curve is prepared for estimation of the EC_x values. The quantities of nitrate (i.e. mg nitrate/kg dry weight soil) found in the treated samples after 28 days are compared to that found in the control. From these data, the % inhibition values for each test concentration are calculated. These percentages are plotted against concentration, and statistical procedures are then used to calculate the EC_x values. Confidence limits ($p = 0,95$) for the calculated EC_x are also determined using standard procedures (10)(11)(12).

Test substances that contain high quantities of nitrogen may contribute to the quantities of nitrate formed during the test. If these substances are tested at a high concentration (e.g. chemicals which are expected to be used in repeated applications) appropriate controls must be included in the test (i.e. soil plus test substance but without plant meal). Data from these controls must be accounted for in the EC_x calculations.

2.2. INTERPRETATION OF RESULTS

When results from tests with agrochemicals are evaluated, and the difference in the rates of nitrate formation between the lower treatment (i.e. the maximum predicted concentration) and control is equal to or less than 25 % at any sampling time after day 28, the product can be evaluated as having no long-term influence on nitrogen transformation in soils. When results from tests with chemicals other than agrochemicals are evaluated, the EC_{50}, EC_{25} and/or EC_{10} values are used.

3. **REPORTING**

The test report must include the following information:

Complete identification of the soil used including:

— geographical reference of the site (latitude, longitude),

— information on the history of the site (i.e. vegetation cover, treatments with crop protection products, treatments with fertilisers, accidental contamination, etc.),

— use pattern (e.g. agricultural soil, forest, etc.),

— depth of sampling (cm),

— sand/silt/clay content (% dry weight),

— pH (in water),

— organic carbon content (% dry weight),

— nitrogen content (% dry weight),

— initial nitrate concentration (mg nitrate/kg dry weight),

— cation exchange capacity (mmol/kg),

— microbial biomass in terms of percentage of the total organic carbon,

— reference of the methods used for the determination of each parameter,

— all information relating to the collection and storage of soil samples,

— details of pre-incubation of soil if any.

Test substance:

— physical nature and, where relevant, physical-chemical properties,

— chemical identification data, where relevant, including structural formula, purity (i.e. for crop protection products the percentage of active ingredient), nitrogen content.

Substrate:

— source of substrate,

— composition (i.e. lucerne meal, lucerne-grass-green meal),

— carbon, nitrogen content (% dry weight),

— sieve size (mm).

Test conditions:

— details of the amendment of soil with organic substrate,

— number of concentrations of test chemical used and, where appropriate, justification of the selected concentrations,

— details of the application of test substance to soil,

— incubation temperature,

— soil moisture content at the beginning and during the test,

— method of soil incubation used (i.e. as bulk or as a series of individual subsamples),

— number of replicates,

— sampling times,

— method used for extraction of nitrate from soil,

Results:

— analytical procedure and equipment used to analyse nitrate,

— tabulated data including individual and mean values for nitrate measurements,

— variation between the replicates in treated and control samples,

— explanations of corrections made in the calculations, if relevant,

— the percent variation in nitrate formation rates at each sampling time or, if appropriate, the EC_{50} value with 95 % confidence limit, other EC_x (i.e. EC_{25} or EC_{10}) with confidence intervals, and a graph of the dose-response curve,

— statistical treatment of results,

— all information and observations helpful for the interpretation of the results.

4. REFERENCES

(1) EPPO, (1994) Decision-Making Scheme for the Environmental Risk Assessment of Plant Protection Chemicals. Chapter 7: Soil Microflora. EPPO Bulletin 24: 1-16, 1994.

(2) BBA, (1990) Effects on the Activity of the Soil Microflora. BBA Guidelines for the Official Testing of Plant Protection Products, VI, 1-1 (2nd eds., 1990).

(3) EPA (1987) Soil Microbial Community Toxicity Test. EPA 40 CFR Part 797.3700. Toxic Substances Control Act Test Guidelines; Proposed rule. September 28, 1987.

(4) SETAC-Europe, (1995) Procedures for assessing the environmental fate and ecotoxicity of pesticides, Ed. M.R. Lynch, Pub. SETAC-Europe, Brussels.

(5) ISO/DIS 14238 (1995) Soil Quality — Determination of Nitrogen Mineralisation and Nitrification in Soils and the Influence of Chemicals on these Processes. Technical Committee ISO/TC 190/SC 4: *Soil Quality — Biological Methods.*

(6) OECD, (1995) Final Report of the OECD Workshop on Selection of Soils/Sediments, Belgirate, Italy, 18-20 January 1995.

(7) ISO 10381-6 (1993) Soil quality — Sampling. Guidance on the collection, handling and storage of soil for the assessment of aerobic microbial processes in the laboratory.

(8) ISO 14240-1, (1997) Soil quality — Determination of soil microbial biomass — Part 1: Substrate-induced respiration method.

(9) ISO 14240-2, (1997) Soil quality — Determination of soil microbial biomass — Part 2: Fumigation-extraction method.

(10) Litchfield, J.T. and Wilcoxon F., (1949) A simplified method of evaluating dose-effect experiments. Jour. Pharmacol. and Exper. Ther., 96, p. 99-113.

(11) Finney, D.J., (1971) Probit Analysis. 3rd ed., Cambridge, London and New-York.

(12) Finney, D.J., (1978) Statistical Methods in biological Assay. Griffin, Weycombe, UK.

C.22. SOIL MICROORGANISMS: CARBON TRANSFORMATION TEST

1. METHOD

This method is a replicate of OECD TG 217 (2000).

1.1. INTRODUCTION

This testing method describes a laboratory method designed to investigate long term potential effects of a single exposure of crop protection products and possibly other chemicals on carbon transformation activity of soil microorganisms. The test is principally based on the recommendations of the European and Mediterranean Plant Protection Organization (1). However, other guideline, including those of the German Biologische Bundesanstalt (2), the US Environmental Protection Agency (3) and SETAC (4), were also taken into account. An OECD Workshop on Soil/Sediment Selection held at Belgirate, Italy, in 1995 (5) agreed on the number and type of soils for use in this test. Recommendations for collection, handling and storage of soil sample are based on an ISO Guidance Document (6) and recommendations from the Belgirate Workshop.

In the assessment and evaluation of toxic characteristics of test substances, determination of effects on soil microbial activity may be required, e.g. when data on the potential side effects of crop protection products on soil microflora are required or when exposure of soil microorganisms to chemicals other than crop protection products is expected. The carbon transformation test is carried out to determine the effects of such chemicals on soil microflora. If agrochemicals (e.g. crop protection products, fertilisers, forestry chemicals) are tested, both carbon transformation and nitrogen transformation tests are conducted. If non-agrochemicals are tested, the nitrogen transformation test is sufficient. However, if EC_{50} values of the nitrogen transformation test for such chemicals fall within the range found for commercially available nitrification inhibitors (e.g. nitrapyrin), a carbon transformation test can be conducted to gain further information.

Soils consist of living and non-living components which exist in complex and heterogeneous mixtures. Microorganisms play an important role in breakdown and transformation of organic matter in fertile soils with many species contributing to different aspects of soil fertility. Any long-term interference with these biochemical processes could potentially interfere with nutrient cycling and this could alter the soil fertility. Transformation of carbon and nitrogen occurs in all fertile soils. Although the microbial communities responsible for these processes differ from soil to soil, the pathways of transformation are essentially the same.

This testing method is designed to detect long-term adverse effects of a substance on the process of carbon transformation in aerobic surface soils. The test is sensitive to changes in size and activity of microbial communities responsible for carbon transformation since it subjects these communities to both chemical stress and carbon starvation. A sandy soil low in organic matter is used. This soil is treated with the test substance and incubated under conditions that allow rapid microbial metabolism. Under these conditions, sources of readily available carbon in the soil are rapidly depleted. This causes carbon starvation which both kills microbial cells and induces dormancy and/or sporulation. If the test runs for more than 28 days, the sum of these reactions can be measured in (untreated soil) controls as a progressive loss of metabolically active microbial biomass (7). If the biomass in carbon-stressed soil, under the conditions of the test, is affected by the presence of a chemical, it may not return to the same level as the control. Hence, disturbances caused by the test substance at any time during the test will often last until the end of the test.

The tests from which this testing method was developed were primarily designed for substances for which the amount reaching the soil can be anticipated. This is the case, for example, for crop protection products for which the application rate in the field is known. For agrochemicals, testing of two doses relevant to the anticipated or predicted application rate is sufficient. Agrochemicals can be tested as active ingredients (a.i.) or as formulated products. However, the test is not limited to chemicals with predictable environmental concentrations. By changing both the amounts of test substance applied to the soil, and the way in which the data are evaluated, the test can also be used for chemicals for which the amount expected to reach the soil is not known. Thus, with non-agrochemicals, the effects of a series of concentrations on carbon transformation are determined. The data from these tests are used to prepare a dose-response curve and calculate EC_x values, where x is defined % effect.

1.2. DEFINITIONS

Carbon transformation: is the degradation by microorganisms of organic matter to form inorganic end-product carbon dioxide.

EC_x (Effective Concentration): is the concentration of the test substance in soil that results in a x % inhibition of carbon transformation in carbon dioxide.

EC_{50} (Median Effective Concentration): is the concentration of test substance in soil that results in a 50 % inhibition of carbon transformation in carbon dioxide.

1.3. REFERENCE SUBSTANCES

None.

1.4. PRINCIPLE OF THE TEST METHOD

Sieved soil is either treated with the test substance or left untreated (control). If agrochemicals are tested, a minimum of two test concentrations are recommended and these should be chosen in relation to the highest concentration anticipated in the field. After 0, 7, 14 and 28 days incubation, samples of treated and control soils are mixed with glucose, and glucose-induced respiration rates are measured for 12 consecutive hours. Respiration rates are expressed as carbon dioxide released (mg carbon dioxide/kg dry soil/h) or oxygen consumed (mg oxygen/kg soil/h). The mean respiration rate in the treated soil samples is compared with that in control and the percent deviation of the treated from the control is calculated. All tests run for at least 28 days. If, on the 28th day, differences between treated and untreated soils are equal to or greater than 25 % measurements are continued in 14 day intervals for a maximum of 100 days. If chemicals other than agrochemicals are tested, a series of concentrations of the test substance are added to samples of the soil, and glucose induced respiration rates (i.e. the mean of the quantities of carbon dioxide formed or oxygen consumed) are measured after 28 days. Results from tests with a series of concentrations are analysed using a regression model, and the EC_x values are calculated (i.e. EC_{50}, EC_{25} and/or EC_{10}). See definitions.

1.5. VALIDITY OF THE TEST

Evaluations of test results with agrochemicals are based on relatively small differences (i.e. average value ±25 %) between the carbon dioxide released or the oxygen consumed in (or by) control and treated soil samples, so large variations in the controls can lead to false results. Therefore, the variation between replicate control samples should be less than ±15 %.

1.6. DESCRIPTION OF THE TEST METHOD

1.6.1. **Apparatus**

Test containers made of chemically inert material are used. They should be of a suitable capacity in compliance with the procedure used for incubation of soils, i.e. incubation in bulk or as a series of individual soil samples (see Section 1.7.1.2). Care should be taken both to minimise water loss and to allow gas exchange during the test (e.g. the test containers may be covered with perforated polyethylene foil). When volatile substances are tested, sealable and gas-tight containers should be used. These should be of a size such that approximately one quarter of their volume is filled with the soil sample.

For determination of glucose-induced respiration, incubation systems and instruments for measurement of carbon dioxide production or oxygen consumption are required. Examples of such systems and instruments are found in the literature (8) (9) (10) (11).

1.6.2. **Selection and number of soils**

One single soil is used. The recommended soil characteristics are as follows:

— sand content: not less than 50 % and not greater than 75 %,

— pH: 5,5-7,5,

— organic carbon content: 0,5-1,5 %,

— the microbial biomass should be measured (12)(13) and its carbon content should be at least 1 % of the total soil organic carbon.

In most cases, a soil with these characteristics represents a worst case situation, since adsorption of the test chemical is minimised and its availability to the microflora is maximum. Consequently, tests with other soils are generally unnecessary. However, in certain circumstances, e.g. where the anticipated major use of the test substance is in particular soils such as acidic forest soils, or for electrostatically charged chemicals, it may be necessary to substitute an additional soil.

1.6.3. **Collection and storage of soil samples**

1.6.3.1. *Collection*

Detailed information on the history of the field site from where the test soil is collected should be available. Details include exact location, vegetation cover, dates of treatments with crop protection products, treatments with organic and inorganic fertilisers, additions of biological materials or accidental contaminations. The site chosen for soil collection should be one which allows long-term use. Permanent pastures, fields with annual cereal crops (except maize) or densely sown green manures are suitable. The selected sampling site should not have been treated with crop protection products for a minimum of one year before sampling. Also, no organic fertiliser should have been applied for at least six months. The use of mineral fertiliser is only acceptable when in accordance with the requirements of the crop and soil samples should not be taken until at least three months after fertiliser application. The use of soil treated with fertilisers with known biocidal effects (e.g. calcium cyanamide) should be avoided.

Sampling should be avoided during or immediately following long periods (greater than 30 days) of drought or water logging. For ploughed soils, samples should be taken from a depth of 0 down to 20 cm. For grassland (pasture) or other soils where ploughing does not occur over longer periods (at least one growing season), the maximum depth of sampling may be slightly more than 20 cm (e.g. to 25 cm). Soil samples should be transported using containers and under temperature conditions which guarantee that the initial soil properties are not significantly altered.

1.6.3.2. *Storage*

The use of soils freshly collected from the field is preferred. If storage in the laboratory cannot be avoided, soils may be stored in the dark at 4 ± 2 °C for a maximum of three months. During the storage of soils, aerobic conditions must be ensured. If soils are collected from areas where they are frozen for at least three months per year, storage for six months at minus 18 °C can be considered. The microbial biomass of stored soils is measured prior to each experiment and the carbon in the biomass should be at least 1 % of the total soil organic carbon content (see Section 1.6.2).

1.6.4. **Handling and preparation of soil for the test**

1.6.4.1. *Pre-incubation*

If the soil was stored (see Sections 1.6.4.2 and 1.7.1.3), pre-incubation is recommended for a period between two and 28 days. The temperature and moisture content of the soil during pre-incubation should be similar to that used in the test (see Sections 1.6.4.2 and 1.7.1.3).

1.6.4.2. *Physical-chemical characteristics*

The soil is manually cleared of large objects (e.g. stones, parts of plants, etc.) and then moist sieved without excess drying to a particle size less than or equal to 2 mm. The moisture content of the soil sample should be adjusted with distilled or deionised water to a value between 40 % and 60 % of the maximum water holding capacity.

1.6.5. **Preparation of the test substance for the application to soil**

The test substance is normally applied using a carrier. The carrier can be water (for water soluble substances) or an inert solid such as fine quartz sand (particle size: 0,1-0,5 mm). Liquid carriers other than water (e.g. organic solvents such as acetone, chloroform) should be avoided since they can damage the microflora. If sand is used as a carrier, it can be coated with the test substance dissolved or suspended in an appropriate solvent. In such cases, the solvent should be removed by evaporation before mixing with the soil. For an optimum distribution of the test substance in soil, a ratio of 10 g of sand per kilogram of soil (dry weight) is recommended. Control samples are treated with the equivalent amount of water and/or quartz sand only.

When testing volatile chemicals, losses during treatment should be avoided and an attempt should be made to ensure homogeneous distribution in the soil (e.g. the test substance should be injected into the soil at several places).

1.6.6. **Test concentrations**

If crop protection products or other chemicals with predictable environmental concentrations are tested, at least two concentrations should be used. The lower concentration should reflect at least the maximum amount expected to reach the soil under practical conditions whereas the higher concentration should be a multiple of the lower concentration. The concentrations of test substance added to soil are calculated assuming uniform incorporation to a depth of 5 cm and a soil bulk density of 1,5. For agrochemicals that are applied directly to soil, or for chemicals for which the quantity reaching the soil can be predicted, the test concentrations recommended are the Predictable Environmental Concentration (PEC) and five times that concentration. Substances that are expected to be applied to soils several times in one season should be tested at concentrations derived from multiplying the PEC by the maximum anticipated number of applications. The upper concentration tested, however, should not exceed 10 times the maximum single application rate.

If non-agrochemicals are tested, a geometric series of at least five concentrations is used. The concentrations tested should cover the range needed to determine the EC_x values.

1.7. PERFORMANCE OF THE TEST

1.7.1. **Conditions of exposure**

1.7.1.1. *Treatment and control*

If agrochemicals are tested, the soil is divided into three portions of equal weight. Two portions are mixed with the carrier containing the product, and the other is mixed with the carrier without the product (control). A minimum of three replicates for both treated and untreated soils is recommended. If non-agrochemicals are tested, the soil is divided into six portions of equal weight. Five of the samples are mixed with the carrier containing the test substance, and the sixth sample is mixed with the carrier without the chemical. Three replicates for both treatments and control are recommended. Care should be taken to ensure homogeneous distribution of the test substance in the treated soil samples. During mixing, compacting or balling of the soil should be avoided.

1.7.1.2. *Incubation of soil samples*

Incubation of soil samples can be performed in two ways: as bulk samples of each treated and untreated soil or as a series of individual and equally sized subsamples of each treated and untreated soil. However, when volatile substances are tested, the test should only be performed with a series of individual subsamples. When soils are incubated in bulk, large quantities of each treated and untreated soils are prepared and subsamples to be analysed are taken as needed during the test. The amount initially prepared for each treatment and control depends on the size of the subsamples, the number of replicates used for analysis and the anticipated maximum number of sampling times. Soils incubated in bulk should be thoroughly mixed before subsampling. When soils are incubated as a series of individual soil samples, each treated and untreated bulk soil is divided into the required number of subsamples, and these are utilised as needed. In the experiments where more than two sampling times can be anticipated, enough subsamples should be prepared to account for all replicates and all sampling times. At least three replicate samples of the test soil should be incubated under aerobic conditions (see Section 1.7.1.1). During all tests, appropriate containers with sufficient headspace should be used to avoid development of anaerobic conditions. When volatile substances are tested, the test should only be performed with a series of individual subsamples.

1.7.1.3. *Test conditions and duration*

The test is carried out in the dark at room temperature of 20 ± 2 °C. The moisture content of soil samples should be maintained during the test between 40 % and 60 % of the maximum water holding capacity of the soil (see Section 1.6.4.2) with a range of ±5 %. Distilled, deionised water can be added as needed.

The minimum duration of tests is 28 days. If agrochemicals are tested, the quantities of carbon dioxide released or oxygen consumed in treated and control samples are compared. If these differ by more than 25 % on day 28, the test is continued until a difference equal to or less than 25 % is obtained, or for a maximum of 100 days, whichever is shorter. If non-agrochemicals are tested, the test is terminated after 28 days. On day 28, the quantities of carbon dioxide released or oxygen consumed in treated and control soil samples are determined and the EC_x values are calculated.

1.7.2. **Sampling and analysis of soils**

1.7.2.1. *Soil sampling schedule*

If agrochemicals are tested, soil samples are analysed for glucose-induced respiration rates on days 0, 7, 14 and 28. If a prolonged test is required, further measurements should be made at 14 days intervals after day 28.

If non-agrochemicals are tested, at least five test concentrations are used and soil samples are analysed for glucose-induced respiration at the beginning (day 0) and at the end of the exposure period (28 days). An intermediate measurement, e.g. at day 7, may be added if deemed necessary. The data obtained on day 28 are used to determine EC_x value for the chemical. If desired, data from day 0 control samples can be used to estimate the initial quantities of metabolically active microbial biomass in the soil (12).

1.7.2.2. *Measurement of glucose-induced respiration rates*

The glucose-induced respiration rate in each treated and control replicate is determined at each sampling time. The soil samples are mixed with a sufficient amount of glucose to elicit an immediate maximum respiratory response. The amount of glucose needed to elicit a maximum respiratory response from a given soil can be determined in a preliminary test using a series of concentrations of glucose (14). However, for sandy soils with 0,5-1,5 % organic carbon, 2 000 mg to 4 000 mg glucose per kg dry weight soil is usually sufficient. The glucose can be ground to a powder with clean quartz sand (10 g sand/kg dry weight soil) and homogeneously mixed with the soil.

The glucose amended soil samples are incubated in a suitable apparatus for measurement of respiration rates either continuously, every hour, or every two hours (see Section 1.6.1) at 20 ± 2 °C. The carbon dioxide released or the oxygen consumed is measured for 12 consecutive hours and measurements should start as soon as possible, i.e. within one to two hours after glucose supplement. The total quantities of carbon dioxide released or oxygen consumed during the 12 hours are measured and mean respiration rates are determined.

2. **DATA**

2.1. TREATMENT OF RESULTS

If agrochemicals are tested, the carbon dioxide released from, or oxygen consumed by each replicate soil sample should be recorded, and the mean values of all replicates should be provided in tabular form. Results should be evaluated by appropriate and generally acceptable statistical methods (e.g. F-test, 5 % significance level). Glucose-induced respiration rates are expressed in mg carbon dioxide/kg dry weight soil/h or mg oxygen/dry weight soil/h. The mean carbon dioxide formation rate or mean oxygen consumption rate in each treatment is compared with that in control, and the percent deviation from the control is calculated.

If tests are conducted with non-agrochemicals, the quantities of carbon dioxide released or oxygen consumed by each replicate is determined, and a dose-response curve is prepared for estimation of the EC_x values. The glucose-induced respiration rates (i.e. mg carbon dioxide/kg dry weight soil/h or mg oxygen/dry weight soil/h) found in the treated samples after 28 days are compared to that found in control. From these data, the % inhibition values for each test concentration are calculated. These percentages are plotted against concentration, and statistical procedures are used to calculate the EC_x values. Confidence limits ($p = 0,95$) for the calculated EC_x are also determined using standard procedures (15)(16)(17).

2.2. INTERPRETATION OF RESULTS

When results from tests with agrochemicals are evaluated, and the difference in respiration rates between the lower treatment (i.e. the maximum predicted concentration) and control is equal to or less than 25 % at any sampling time after day 28, the product can be evaluated as having no long-term influence on carbon transformation in soils. When results from tests with chemicals other than agrochemicals are evaluated, the EC_{50}, EC_{25} and/or EC_{10} values are used.

3. **REPORTING**

TEST REPORT

The test report must include the following information:

Complete identification of the soil used including:

— geographical reference of the site (latitude, longitude),

— information on the history of the site (i.e. vegetation cover, treatments with crop protection products, treatments with fertilisers, accidental contamination, etc.),

— use pattern (e.g. agricultural soil, forest, etc.),

— depth of sampling (cm),

— sand/silt/clay content (% dry weight),

— pH (in water),

— organic carbon content (% dry weight),

— nitrogen content (% dry weight);

— cation exchange capacity (mmol/kg),

— initial microbial biomass in terms of percentage of the total organic carbon,

— reference of the methods used for the determination of each parameter,

— all information relating to the collection and storage of soil samples,

— details of pre-incubation of soil if any.

Test substance:

— physical nature and, where relevant, physical-chemical properties,

— chemical identification data, where relevant, including structural formula, purity (i.e. for crop protection products the percentage of active ingredient), nitrogen content.

Test conditions:

— details of the amendment of soil with organic substrate,

— number of concentrations of test chemical used and, where appropriate, justification of the selected concentrations,

— details of the application of test substance to soil,

— incubation temperature,

— soil moisture content at the beginning and during the test,

— method of soil incubation used (i.e. as bulk or as a series of individual subsamples),

— number of replicates,

— sampling times.

Results:

— method and equipment used for measurement of respiration rates,

— tabulated data including individual and mean values for quantities of carbon dioxide or oxygen,

— variation between the replicates in treated and control samples,

— explanations of corrections made in the calculations, if relevant,

— the percent variation of glucose-induced respiration rates at each sampling time or, if appropriate, the EC_{50} with 95 % confidence limit, other EC_x (i.e. EC_{25} or EC_{10}) with confidence intervals, and a graph of the dose-response curve,

— statistical treatment of results, where appropriate,

— all information and observations helpful for the interpretation of the results.

4. REFERENCES

(1) EPPO, (1994) Decision-Making Scheme for the Environmental Risk Assessment of Plant Protection Chemicals. Chapter 7: Soil Microflora. EPPO Bulletin 24: 1-16, 1994.

(2) BBA, (1990) Effects on the Activity of the Soil Microflora. BBA Guidelines for the Official Testing of Plant Protection Products, VI, 1-1 (2nd eds., 1990).

(3) EPA, (1987) Soil Microbial Community Toxicity Test. EPA 40 CFR Part 797.3700. Toxic Substances Control Act Test Guidelines; Proposed rule. September 28, 1987.

(4) SETAC-Europe, (1995) Procedures for assessing the environmental fate and ecotoxicity of pesticides, Ed. M.R. Lynch, Pub. SETAC-Europe, Brussels.

(5) OECD, (1995) Final Report of the OECD Workshop on Selection of Soils/Sediments, Belgirate, Italy, 18-20 January 1995.

(6) ISO 10381-6, (1993) Soil quality — Sampling. Guidance on the collection, handling and storage of soil for the assessment of aerobic microbial processes in the laboratory.

(7) Anderson, J.P.E., (1987) Handling and Storage of Soils for Pesticide Experiments, in 'Pesticide Effects on Soil Microflora'. Eds. L. Somerville and M.P. Greaves, Chap. 3: 45-60.

(8) Anderson, J.P.E., (1982) Soil Respiration, in 'Methods of Soil Analysis — Part 2: Chemical and Microbiological Properties'. Agronomy Monograph No 9. Eds. A.L. Page, R.H. Miller and D.R. Keeney. 41: 831- 871.

(9) ISO 11266-1, (1993) Soil Quality — Guidance on Laboratory Tests for Biodegradation in Soil: Part 1. Aerobic Conditions.

(10) ISO 14239, (1997E) Soil Quality — Laboratory incubation systems for measuring the mineralisation of organic chemicals in soil under aerobic conditions.

(11) Heinemeye r O., Insam, H., Kaiser, E.A, and Walenzik, G., (1989) Soil microbial biomass and respiration measurements; an automated technique based on infrared gas analyses. Plant and Soil, 116: 77-81.

(12) ISO 14240-1, (1997) Soil quality — Determination of soil microbial biomass — Part 1: Substrate-induced respiration method.

(13) ISO 14240-2, (1997) Soil quality — Determination of soil microbial biomass — Part 2: Fumigation-extraction method.

(14) Malkomes, H.-P., (1986) Einfluß von Glukosemenge auf die Reaktion der Kurzzeit-Atmung im Boden Gegenüber Pflanzenschutzmitteln, Dargestellt am Beispiel eines Herbizide. (Influence of the Amount of Glucose Added to the Soil on the Effect of Pesticides in Short-Term Respiration, using a Herbicide as an Example). Nachrichtenbl. Deut. Pflanzenschutzd., Braunschweig, 38: 113-120.

(15) Litchfield, J.T. and Wilcoxon, F., (1949) A simplified method of evaluating dose-effect experiments. Jour. Pharmacol. and Exper. Ther., 96, 99-113.

(16) Finney, D.J., (1971) Probit Analysis. 3rd ed., Cambridge, London and New-York.

(17) Finney D.J., (1978) Statistical Methods in biological Assay. Griffin, Weycombe, UK.

C.23. AEROBIC AND ANAEROBIC TRANSFORMATION IN SOIL

1. METHOD

This test method is a replicate of the OECD TG 307 (2002)

1.1. INTRODUCTION

This test method is based on existing guidelines (1)(2)(3)(4)(5)(6)(7)(8)(9). The method described in this test Method is designed for evaluating aerobic and anaerobic transformation of chemicals in soil. The experiments are performed to determine (i) the rate of transformation of the test substance, and (ii) the nature and rates of formation and decline of transformation products to which plants and soil organisms may be exposed. Such studies are required for chemicals which are directly applied to soil or which are likely to reach the soil environment. The results of such laboratory studies can also be used to develop sampling and analysis protocols for related field studies.

Aerobic and anaerobic studies with one soil type are generally sufficient for the evaluation of transformation pathways (8)(10)(11). Rates of transformation should be determined in at least three additional soils (8)(10).

An OECD Workshop on soil and sediment selection, held at Belgirate, Italy in 1995 (10) agreed, in particular, on the number and types of soils for use in this test. The types of soils tested should be representative of the environmental conditions where use or release will occur. For example, chemicals that may be released in subtropical to tropical climates should be tested with Ferrasols or Nitosols (FAO system). The Workshop also made recommendations relating to collection, handling and storage of soil samples, based on the ISO Guidance (15). The use of paddy (rice) soils is also considered in this method.

1.2. DEFINITIONS

Test substance: any substance, whether the parent compound or relevant transformation products.

Transformation products: all substances resulting from biotic or abiotic transformation reactions of the test substance including CO_2 and products that are in bound residues.

Bound residues: 'Bound residues' represent compounds in soil, plant or animal, which persist in the matrix in the form of the parent substance or its metabolite(s)/transformation products after extraction. The extraction method must not substantially change the compounds themselves or the structure of the matrix. The nature of the bond can be clarified in part by matrix-altering extraction methods and sophisticated analytical techniques. To date, for example, covalent ionic and sorptive bonds, as well as entrapments, have been identified in this way. In general, the formation of bound residues reduces the bioaccessibility and the bioavailability significantly (12) [modified from IUPAC 1984 (13)].

Aerobic transformation: reactions occurring in the presence of molecular oxygen (14).

Anaerobic transformation: reactions occurring under exclusion of molecular oxygen (14).

Soil: is a mixture of mineral and organic chemical constituents, the latter containing compounds of high carbon and nitrogen content and of high molecular weights, animated by small (mostly micro-) organisms. Soil may be handled in two states:

(a) undisturbed, as it has developed with time, in characteristic layers of a variety of soil types;

(b) disturbed, as it is usually found in arable fields or as occurs when samples are taken by digging and used in this test method (14).

Mineralisation: is the complete degradation of an organic compound to CO_2 and H_2O under aerobic conditions, and CH_4, CO_2 and H_2O under anaerobic conditions. In the context of this test method, when ^{14}C-labelled compound is used, mineralisation means extensive degradation during which a labelled carbon atom is oxidised with release of the appropriate amount of $^{14}CO_2$ (14).

Half-life: $t_{0,5,}$ is the time taken for 50 % transformation of a test substance when the transformation can be described by first-order kinetics; it is independent of the concentration.

DT_{50} (Disappearance Time 50): is the time within which the concentration of the test substance is reduced by 50 %; it is different from the half-life $t_{0,5}$ when transformation does not follow first order kinetics.

DT_{75} (Disappearance Time 75): is the time within which the concentration of the test substance is reduced by 75 %.

DT_{90} (Disappearance Time 90): is the time within which the concentration of the test substance is reduced by 90 %.

1.3. REFERENCE SUBSTANCES

Reference substances should be used for the characterisation and/or identification of transformation products by spectroscopic and chromatographic methods.

1.4. APPLICABILITY OF THE TEST

The method is applicable to all chemical substances (non-labelled or radiolabelled) for which an analytical method with sufficient accuracy and sensitivity is available. It is applicable to slightly volatile, non-volatile, water-soluble or water-insoluble compounds. The test should not be applied to chemicals which are highly volatile from soil (e.g. fumigants, organic solvents) and thus cannot be kept in soil under the experimental conditions of this test.

1.5. INFORMATION ON THE SUBSTANCE

Non-labelled or labelled test substance can be used to measure the rate of transformation. Labelled material is required for studying the pathway of transformation and for establishing a mass balance. ^{14}C-labelling is recommended but the use of other isotopes, such as ^{13}C, ^{15}N, ^{3}H, ^{32}P, may also be useful. As far as possible, the label should be positioned in the most stable part(s) of the molecule ([1]). The purity of the test substance should be at least 95 %.

Before carrying out a test on aerobic and anaerobic transformation in soil, the following information on the test substance should be available:

(a) solubility in water (Method A.6)

(b) solubility in organic solvents;

(c) vapour pressure (Method A.4) and Henry's law constant;

(d) n-octanol/water partition coefficient (Method A.8);

(e) chemical stability in dark (hydrolysis) (Method C.7);

(f) pK_a if a molecule is liable to protonation or deprotonation [OECD Guideline 112] (16).

Other useful information may include data on toxicity of the test substance to soil micro-organisms [testing methods C.21 and C.22] (16).

Analytical methods (including extraction and clean-up methods) for quantification and identification of the test substance and its transformation products should be available.

([1]) For example, if the test substance contains one ring, labelling on this ring is required; if the test substance contains two or more rings, separate studies may be needed to evaluate the fate of each labelled ring and to obtain suitable information on formation of transformation products.

1.6. PRINCIPLE OF THE TEST METHOD

Soil samples are treated with the test substance and incubated in the dark in biometer-type flasks or in flow-through systems under controlled laboratory conditions (at constant temperature and soil moisture). After appropriate time intervals, soil samples are extracted and analysed for the parent substance and for transformation products. Volatile products are also collected for analysis using appropriate absorption devices. Using ^{14}C-labelled material, the various mineralisation rates of the test substance can be measured by trapping evolved $^{14}CO_2$ and a mass balance, including the formation of soil bound residues, can be established.

1.7. QUALITY CRITERIA

1.7.1. **Recovery**

Extraction and analysis of, at least, duplicate soil samples immediately after the addition of the test substance gives a first indication of the repeatability of the analytical method and of the uniformity of the application procedure for the test substance. Recoveries for later stages of the experiments are given by the respective mass balances. Recoveries should range from 90 % to 110 % for labelled chemicals (8) and from 70 % to 110 % for non-labelled chemicals (3).

1.7.2. **Repeatability and sensitivity of analytical method**

Repeatability of the analytical method (excluding the initial extraction efficiency) to quantify test substance and transformation products can be checked by duplicate analysis of the same extract of the soil, incubated long enough for formation of transformation products.

The limit of detection (LOD) of the analytical method for the test substance and for the transformation products should be at least 0,01 mg· kg^{-1} soil (as test substance) or 1 % of applied dose whichever is lower. The limit of quantification (LOQ) should also be specified.

1.7.3. **Accuracy of transformation data**

Regression analysis of the concentrations of the test substance as a function of time gives the appropriate information on the reliability of the transformation curve and allows the calculation of the confidence limits for half-lives (in the case of pseudo first order kinetics) or DT_{50} values and, if appropriate, DT_{75} and DT_{90} values.

1.8. DESCRIPTION OF THE METHOD

1.8.1. **Equipment and chemical reagents**

Incubation systems consist of static closed systems or suitable flow-through systems (7)(17). Examples of suitable flow-through soil incubation apparatus and biometer-type flask are shown in Figures 1 and 2, respectively. Both types of incubation systems have advantages and limitations (7)(17).

Standard laboratory equipment is required and especially the following:

— analytical instruments such as GLC, HPLC, TLC-equipment, including the appropriate detection systems for analysing radiolabelled or non-labelled substances or inverse isotopes dilution method,

— instruments for identification purposes (e.g. MS, GC-MS, HPLC-MS, NMR, etc.),

— liquid scintillation counter,

— oxidiser for combustion of radioactive material,

— centrifuge,

— extraction apparatus (for example, centrifuge tubes for cold extraction and Soxhlet apparatus for continuous extraction under reflux),

— instrumentation for concentrating solutions and extracts (e.g. rotating evaporator),

— water bath,

— mechanical mixing device (e.g. kneading machine, rotating mixer).

Chemical reagents used include, for example:

— NaOH, analytical grade, 2 mol· dm^{-3}, or other appropriate base (e.g. KOH, ethanolamine),

— H_2SO_4, analytical grade, 0,05 mol· dm^{-3},

— ethylene glycol, analytical grade,

— solid absorption materials such as soda lime and polyurethane plugs,

— organic solvents, analytical grade, such as acetone, methanol, etc.,

— scintillation liquid.

1.8.2. **Test substance application**

For addition to and distribution in soil, the test substance can be dissolved in water (deionised or distilled) or, when necessary, in minimum amounts of acetone or other organic solvents (6) in which the test substance is sufficiently soluble and stable. However, the amount of solvent selected should not have a significant influence on soil microbial activity (see Sections 1.5 and 1.9.2-1.9.3). The use of solvents which inhibit microbial activity, such as chloroform, dichloromethane and other halogenated solvents, should be avoided.

The test substance can also be added as a solid, e.g. mixed in quartz sand (6) or in a small sub-sample of the test soil which has been air-dried and sterilised. If the test substance is added using a solvent the solvent should be allowed to evaporate before the spiked sub-sample is added to the original non-sterile soil sample.

For general chemicals, whose major route of entry into soil is through sewage sludge/farming application, the test substance should be first added to sludge which is then introduced into the soil sample. (see Sections 1.9.2 and 1.9.3)

The use of formulated products is not routinely recommended. However, e.g. for poorly soluble test substances, the use of formulated material may be an appropriate alternative.

1.8.3. **Soils**

1.8.3.1. *Soil selection*

To determine the transformation pathway, a representative soil can be used; a sandy loam or silty loam or loam or loamy sand (according to FAO and USDA classification (18)), with a pH of 5,5-8,0, an organic carbon content of 0,5-2,5 % and a microbial biomass of at least 1 % of total organic carbon is recommended (10).

For transformation rate studies at least three additional soils should be used representing a range of relevant soils. The soils should vary in their organic carbon content, pH, clay content and microbial biomass (10).

All soils should be characterised, at least, for texture (% sand, % silt, % clay) [according to FAO and USDA classification (18)], pH, cation exchange capacity, organic carbon, bulk density, water retention characteristic ([1]) and microbial biomass (for aerobic studies only). Additional information on soil properties may be useful in interpreting the results. For determination of the soil characteristics the methods recommended in references (19)(20)(21)(22)(23) can be used. Microbial biomass should be determined by using the substrate-induced respiration (SIR) method (25)(26) or alternative methods (20).

([1]) Water retention characteristic of a soil can be measured as field capacity, as water holding capacity or as water suction tension (pF). For explanations see Appendix 1. It should be reported in the test report whether water retention characteristics and bulk density of soils were determined in undisturbed field samples or in disturbed (processed) samples.

1.8.3.2. *Collection, handling, and storage of soils*

Detailed information on the history of the field site from where the test soil is collected should be available. Details include exact location, vegetation cover, treatments with chemicals, treatments with organic and inorganic fertilisers, additions of biological materials or other contamination. If soils have been treated with the test substance or its structural analogues within the previous four years, these should not be used for transformation studies (10)(15).

The soil should be freshly collected from the field (from the A horizon or top 20 cm layer) with a soil water content which facilitates sieving. For soils other than those from paddy fields, sampling should be avoided during or immediately following long periods (> 30 days) of drought, freezing or flooding (14). Samples should be transported in a manner which minimises changes in soil water content and should be kept in the dark with free access of air, as much as possible. A loosely-tied polyethylene bag is generally adequate for this purpose.

The soil should be processed as soon as possible after sampling. Vegetation, larger soil fauna and stones should be removed prior to passing the soil through a 2 mm sieve which removes small stones, fauna and plant debris. Extensive drying and crushing of the soil before sieving should be avoided (15).

When sampling in the field is difficult in winter (soil frozen or covered by layers of snow), it may be taken from a batch of soil stored in the greenhouse under plant cover (e.g. grass or grass-clover mixtures). Studies with soils freshly collected from the field are strongly preferred, but if the collected and processed soil has to be stored prior to the start of the study storage conditions must be adequate and for a limited time only (4 ± 2 °C for a maximum of three months) to maintain microbial activity ([1]). Detailed instructions on collection, handling and storage of soils to be used for biotransformation experiments can be found in (8)(10)(15)(26)(27).

Before the processed soil is used for this test, it should be pre-incubated to allow germination and removal of seeds, and to re-establish equilibrium of microbial metabolism following the change from sampling or storage conditions to incubation conditions. A pre-incubation period between two and 28 days approximating the temperature and moisture conditions of the actual test is generally adequate (15). Storage and pre-incubation time together should not exceed three months.

1.9. PERFORMANCE OF THE TEST

1.9.1. **Test conditions**

1.9.1.1. *Test temperature*

During the whole test period, the soils should be incubated in the dark at a constant temperature representative of the climatic conditions where use or release will occur. A temperature of 20 ± 2 °C is recommended for all test substances which may reach the soil in temperate climates. The temperature should be monitored.

For chemicals applied or released in colder climates (e.g. in northern countries, during autumn/winter periods), additional soil samples should be incubated but at a lower temperature (e.g. 10 ± 2 °C).

1.9.1.2. *Moisture content*

For transformation tests under aerobic conditions, the soil moisture content ([2]) should be adjusted to and maintained at a pF between 2,0 and 2,5 (3). The soil moisture content is expressed as mass of water per mass of dry soil and should be regularly controlled (e.g. in 2 week intervals) by weighing of the incubation flasks and water losses compensated by adding water (preferably sterile-filtered tap water). Care should be given to prevent or minimise losses of test substance and/or transformation products by volatilisation and/or photodegradation (if any) during moisture addition.

For transformation tests under anaerobic and paddy conditions, the soil is water-saturated by flooding.

([1]) Recent research results indicate that soils from temperate zones can also be stored at – 20 °C for more than three months (28)(29) without significant losses of microbial activity.

([2]) The soil should neither be too wet nor too dry to maintain adequate aeration and nutrition of soil microflora. Moisture contents recommended for optimal microbial growth range from 40-60 % water holding capacity (WHC) and from 0,1-0,33 bar (6). The latter range is equivalent to a pF-range of 2,0-2,5. Typical moisture contents of various soil types are given in Appendix 2.

1.9.1.3. *Aerobic incubation conditions*

In the flow-through systems, aerobic conditions will be maintained by intermittent flushing or by continuously ventilating with humidified air. In the biometer flasks, exchange of air is maintained by diffusion.

1.9.1.4. *Sterile aerobic conditions*

To obtain information on the relevance of abiotic transformation of a test substance, soil samples may be sterilised (for sterilisation methods see references 16 and 29), treated with sterile test substance (e.g. addition of solution through a sterile filter) and aerated with humidified sterile air as described in Section 1.9.1.3. For paddy soils, soil and water should be sterilised and the incubation should be carried out as described in Section 1.9.1.6.

1.9.1.5. *Anaerobic incubation conditions*

To establish and maintain anaerobic conditions, the soil treated with the test substance and incubated under aerobic conditions for 30 days or one half-life or DT_{50} (whichever is shorter) is then water-logged (1-3 cm water layer) and the incubation system flushed with an inert gas (e.g. nitrogen or argon) ([1]). The test system must allow for measurements such as pH, oxygen concentration and redox potential and include trapping devices for volatile products. The biometer-type system must be closed to avoid entrance of air by diffusion.

1.9.1.6. *Paddy incubation conditions*

To study transformation in paddy rice soils, the soil is flooded with a water layer of about 1-5 cm and the test substance applied to the water phase (9). A soil depth of at least 5 cm is recommended. The system is ventilated with air as under aerobic conditions. pH, oxygen concentration and redox potential of the aqueous layer should be monitored and reported. A pre-incubation period of at least two weeks is necessary before commencing transformation studies (see Section 1.8.3.2).

1.9.1.7. *Test duration*

The rate and pathway studies should normally not exceed 120 days ([2]) (3)(6)(8), because thereafter a decrease of the soil microbial activity with time would be expected in an artificial laboratory system isolated from natural replenishment. Where necessary to characterise the decline of the test substance and the formation and decline of major transformation products, studies can be continued for longer periods (e.g. 6 or 12 months) (8). Longer incubation periods should be justified in the test report and accompanied by biomass measurements during and at the end of these periods.

1.9.2. **Performance of the test**

About 50 to 200 g of soil (dry weight basis) are placed into each incubation flask (see Figures 1 and 2 in Appendix 3) and the soil treated with the test substance by one of the methods described in Section 1.8.2. When organic solvents are used for the application of the test substance, they should be removed from soil by evaporation. Then the soil is thoroughly mixed with a spatula and/or by shaking of the flask. If the study is conducted under paddy field conditions, soil and water should be thoroughly mixed after application of the test substance. Small aliquots (e.g. 1 g) of the treated soils should be analysed for the test substance to check for uniform distribution. For alternative method, see below.

The treatment rate should correspond to the highest application rate of a crop protection product recommended in the use instructions and uniform incorporation to an appropriate depth in the field (e.g. top 10 cm layer ([3]) of soil). For example, for chemicals foliarly or soil applied without incorporation, the appropriate depth for computing how much chemical should be added to each flask is 2,5 cm. For soil incorporated chemicals, the appropriate depth is the incorporation depth specified in the use instructions. For general chemicals, the application rate should be estimated based on the most relevant route of entry; for

([1]) Aerobic conditions are dominant in surface soils and even in sub-surface soils as shown in an EU sponsored research project [K. Takagi et al. (1992). Microbial diversity and activity in subsoils: Methods, field site, seasonal variation in subsoil temperatures and oxygen contents. Proc. Internat. Symp. Environm. Aspects Pesticides Microbiol., 270-277, 17-21 August 1992, Sigtuna, Sweden]. Anaerobic conditions may only occur occasionally during flooding of soils after heavy rainfalls or when paddy conditions are established in rice fields.

([2]) Aerobic studies might be terminated much before 120 days provided that ultimate transformation pathway and ultimate mineralisation are clearly reached at that time. Termination of the test is possible after 120 days, or when at least 90 % of the test substance is transformed, but only if at least 5 % CO_2 is formed.

([3]) Calculation of the initial concentration on an area basis using the following equation:

$$C_{soil}\left[mg/kg_{soil}\right] = \frac{A[kg/ha]\cdot 10^6[mg/kg]}{1[m]\cdot 10^4[m^2/ha]\cdot d\left[kg_{soil}/m^3\right]}$$

C_{soil} = Initial concentration in soil [mg·kg^{-1}]

A = Application rate [kg· ha^{-1}]; 1 = thickness of field soil layer [m]; d = dry bulk density of soil [kg· m^{-3}].
As a rule of thumb, an application rate of 1 kg· ha^{-1} results in a soil concentration of approximately 1 mg· kg^{-1} in a 10 cm layer (assuming a bulk density of 1 g· cm^{-3}).

example, when the major route of entry in soil is through sewage sludge, the chemical should be dosed into the sludge at a concentration that reflects the expected sludge concentration and the amount of sludge added to the soil should reflect normal sludge loading to agricultural soils. If this concentration is not high enough to identify major transformation products, incubation of separate soil samples containing higher rates may be helpful, but excessive rates influencing soil microbial functions should be avoided (see Sections 1.5 and 1.8.2).

Alternatively, a larger batch (i.e. 1 to 2 kg) of soil can be treated with the test substance, carefully mixed in an appropriate mixing machine and then transferred in small portions of 50 to 200 g into the incubation flasks (for example with the use of sample splitters). Small aliquots (e.g. 1 g) of the treated soil batch should be analysed for the test substance to check for uniform distribution. Such a procedure is preferred since it allows for more uniform distribution of the test substance into the soil.

Also untreated soil samples are incubated under the same conditions (aerobic) as the samples treated with the test substance. These samples are used for biomass measurements during and at the end of the studies.

When the test substance is applied to the soil dissolved in organic solvent(s), soil samples treated with the same amount of solvent(s) are incubated under the same conditions (aerobic) as the samples treated with the test substance. These samples are used for biomass measurements initially, during and at the end of the studies to check for effects of the solvent(s) on microbial biomass.

The flasks containing the treated soil are either attached to the flow-through system described in Figure 1 or closed with the absorption column shown in Figure 2 (see Appendix 3).

1.9.3. **Sampling and measurement**

Duplicate incubation flasks are removed at appropriate time intervals and the soil samples extracted with appropriate solvents of different polarity and analysed for the test substance and/or transformation products. A well-designed study includes sufficient flasks so that two flasks are sacrificed at each sampling event. Also, absorption solutions or solid absorption materials are removed at various time intervals (7-day intervals during the first month and after one month in 17-day intervals) during and at the end of incubation of each soil sample and analysed for volatile products. Besides a soil sample taken directly after application (0-day sample) at least five additional sampling points should be included. Time intervals should be chosen in such a way that pattern of decline of the test substance and patterns of formation and decline of transformation products can be established (e.g. 0, 1, 3, 7 days; 2, 3 weeks; 1, 2, 3 months, etc.).

When using ^{14}C-labelled test substance, non-extractable radioactivity will be quantified by combustion and a mass balance will be calculated for each sampling interval.

In the case of anaerobic and paddy incubation, the soil and water phases are analysed together for test substance and transformation products or separated by filtration or centrifugation before extraction and analysis.

1.9.4. **Optional tests**

Aerobic, non-sterile studies at additional temperatures and soil moistures may be useful for the estimation of the influence of temperature and soil moisture on the rates of transformation of a test substance and/or its transformation products in soil.

A further characterisation of non-extractable radioactivity can be attempted using, for example, supercritical fluid extraction.

2. **DATA**

2.1. TREATMENT OF RESULTS

The amounts of test substance, transformation products, volatile substances (in % only), and non-extractable should be given as % of applied initial concentration and, where appropriate, as $mg \cdot kg^{-1}$ soil (based on soil dry weight) for each sampling interval. A mass balance should be given in percentage of the applied initial concentration for each sampling interval. A graphical presentation of the test substance concentrations against

time will allow an estimation of its transformation half-life or DT_{50}. Major transformation products should be identified and their concentrations should also be plotted against time to show their rates of formation and decline. A major transformation product is any product representing ≥ 10 % of applied dose at any time during the study.

The volatile products trapped give some indication of the volatility potential of a test substance and its transformation products from soil.

More accurate determinations of half-lives or DT_{50} values and, if appropriate, DT_{75} and DT_{90} values should be obtained by applying appropriate kinetic model calculations. The half-life and DT_{50} values should be reported together with the description of the model used, the order of kinetics and the determination coefficient (r^2). First order kinetics is favoured unless $r^2 < 0,7$. If appropriate, the calculations should also be applied to the major transformation products. Examples of appropriate models are described in references 31 to 35.

In the case of rate studies carried out at various temperatures, the transformation rates should be described as a function of temperature within the experimental temperature range using the Arrhenius relationship of the form:

$$k = A \cdot e^{-B/T} \text{ or } \ln k = 1nA - \frac{B}{T},$$

where ln A and B are regression constants from the intercept and slope, respectively, of a best fit line generated from linearly regressing ln k against 1/T, k is the rate constant at temperature T and T is the temperature in Kelvin. Care should be given to the limited temperature range in which the Arrehenius relationship will be valid in case transformation is governed by microbial action.

2.2. EVALUATION AND INTERPRETATION OF RESULTS

Although the studies are carried out in an artificial laboratory system, the results will allow estimation of the rate of transformation of the test substance and also of rate of formation and decline of transformation products under field conditions (36)(37).

A study of the transformation pathway of a test substance provides information on the way in which the applied substance is structurally changed in the soil by chemical and microbial reactions.

3. REPORTING

TEST REPORT

The test report must include:

Test substance:

— common name, chemical name, CAS number, structural formula (indicating position of label(s) when radiolabelled material is used) and relevant physical-chemical properties (see Section 1.5),

— purity (impurities) of test substance,

— radiochemical purity of labelled chemical and specific activity (where appropriate),

Reference substances:

— chemical name and structure of reference substances used for the characterisation and/or identification of transformation product,

Test soils:

— details of collection site,

— date and procedure of soil sampling,

— properties of soils, such as pH, organic carbon content, texture (% sand, % silt, % clay), cation exchange capacity, bulk density, water retention characteristic, and microbial biomass,

— length of soil storage and storage conditions (if stored),

Test conditions:

— dates of the performance of the studies,

— amount of test substance applied,

— solvents used and method of application for the test substance,

— weight of soil treated initially and sampled at each interval for analysis,

— description of the incubation system used,

— air flow rates (for flow-through systems only),

— temperature of experimental set-up,

— soil moisture content during incubation,

— microbial biomass initially, during and at the end of the aerobic studies,

— pH, oxygen concentration and redox potential initially, during and at the end of the anaerobic and paddy studies,

— method(s) of extraction,

— methods for quantification and identification of the test substance and major transformation products in soil and absorption materials,

— number of replicates and number of controls.

Results:

— result of microbial activity determination,

— repeatability and sensitivity of the analytical methods used,

— rates of recovery (% values for a valid study are given in Section 1.7.1),

— tables of results expressed as % of applied initial dose and, where appropriate, as $mg \cdot kg^{-1}$ soil (on a dry weight basis),

— mass balance during and at the end of the studies,

— characterisation of non-extractable (bound) radioactivity or residues in soil,

— quantification of released CO_2 and other volatile compounds,

— plots of soil concentrations versus time for the test substance and, where appropriate, for major transformation products,

— half-life or DT_{50}, DT_{75} and DT_{90} for the test substance and, where appropriate, for major transformation products including confidence limits,

— estimation of abiotic degradation rate under sterile conditions,

— an assessment of transformation kinetics for the test substance and, where appropriate, for major transformation products,

— proposed pathways of transformation, where appropriate,

— discussion and interpretation of results,

— raw data (i.e. sample chromatograms, sample calculations of transformation rates and means used to identify transformation products).

4. **REFERENCES**

(1) US — Environmental Protection Agency, (1982) Pesticide Assessment Guidelines, Subdivision N. Chemistry: Environmental Fate.

(2) Agriculture Canada, (1987) Environmental Chemistry and Fate. Guidelines for registration of pesticides in Canada.

(3) European Union (EU), (1995) Commission Directive 95/36/EC of 14 July 1995 amending Council Directive 91/414/EEC concerning the placing of plant protection products on the market. Annex II, Part A and Annex III, Part A: Fate and Behaviour in the Environment.

(4) Dutch Commission for Registration of Pesticides, (1995) Application for registration of a pesticide. Section G: Behaviour of the product and its metabolites in soil, water and air.

(5) BBA, (1986) Richtlinie für die amtliche Prüfung von Pflanzenschutzmitteln, Teil IV, 4-1. Verbleib von Pflanzenschutzmitteln im Boden — Abbau, Umwandlung und Metabolismus.

(6) ISO/DIS 11266-1, (1994) Soil Quality -Guidance on laboratory tests for biodegradation of organic chemicals in soil — Part 1: Aerobic conditions.

(7) ISO 14239, (1997) Soil Quality — Laboratory incubation systems for measuring the mineralisation of organic chemicals in soil under aerobic conditions.

(8) SETAC, (1995) Procedures for Assessing the Environmental Fate and Ecotoxicity of Pesticides. Mark R. Lynch, Ed.

(9) MAFF — Japan 2000 — Draft Guidelines for transformation studies of pesticides in soil — Aerobic metabolism study in soil under paddy field conditions (flooded).

(10) OECD, (1995) Final Report of the OECD Workshop on Selection of Soils/Sediments. Belgirate, Italy, 18-20 January 1995.

(11) Guth, J.A., (1980) The study of transformations. In Interactions between Herbicides and the Soil (R.J. Hance, Ed.), Academic Press, p. 123-157.

(12) DFG: Pesticide Bound Residues in Soil. Wiley — VCH (1998).

(13) T.R. Roberts: Non-extractable pesticide residue in soils and plants. Pure Appl. Chem. 56, 945-956 (IUPAC 1984).

(14) OECD Test Guideline 304 A: Inherent Biodegradability in Soil (adopted 12 May 1981).

(15) ISO 10381-6 (1993) Soil Quality — Sampling — Part 6: Guidance on the collection, handling and storage of soil for the assessment of aerobic microbial processes in the laboratory.

(16) Appendix V to Directive 67/548/EEC.

(17) Guth, J.A., (1981) Experimental approaches to studying the fate of pesticides in soil. In Progress in Pesticide Biochemistry. D.H. Hutson, T.R. Roberts, Eds. J. Wiley & Sons. Vol 1, 85-114.

(18) Soil Texture Classification (US and FAO systems): Weed Science, 33, Suppl. 1 (1985) and Soil Sci. Soc. Amer. Proc. 26:305 (1962).

(19) Methods of Soil Analysis (1986) Part 1, Physical and Mineralogical Methods. A. Klute, Ed.) Agronomy Series No 9, 2nd Edition.

(20) Methods of Soil Analysis (1982) Part 2, Chemical and Microbiological Properties. A.L. Page, R.H. Miller and D.R. Kelney, Eds. Agronomy Series No 9, 2nd Edition.

(21) ISO Standard Compendium Environment (1994) Soil Quality — General aspects; chemical and physical methods of analysis; biological methods of analysis. First Edition.

(22) Mückenhausen, E., (1975) Die Bodenkunde und ihre geologischen, geomorphologischen, mineralogischen und petrologischen Grundlagen. DLG-Verlag, Frankfurt, Main.

(23) Scheffer, F., Schachtschabel, P., (1975) Lehrbuch der Bodenkunde. F. Enke Verlag, Stuttgart.

(24) Anderson, J.P.E., Domsch, K.H., (1978) A physiological method for the quantitative measurement of microbial biomass in soils. Soil Biol. Biochem. 10, p. 215-221.

(25) ISO 14240-1 and 2 (1997) Soil Quality — Determination of soil microbial biomass — Part 1: Substrate-induced respiration method. Part 2: fumigation-extraction method.

(26) Anderson, J.P.E., (1987) Handling and storage of soils for pesticide experiments. In Pesticide Effects on Soil Microflora. L. Somerville, M.P. Greaves, Eds. Taylor & Francis, 45-60.

(27) Kato, Yasuhiro. (1998) Mechanism of pesticide transformation in the environment: Aerobic and bio-transformation of pesticides in aqueous environment. Proceedings of the 16th Symposium on Environmental Science of Pesticide, p. 105-120.

(28) Keuken O., Anderson J.P.E., (1996) Influence of storage on biochemical processes in soil. In Pesticides, Soil Microbiology and Soil Quality, 59-63 (SETAC-Europe).

(29) Stenberg B., Johansson M., Pell M., Sjödahl-Svensson K., Stenström J., Torstensson L. (1996) Effect of freeze and cold storage of soil on microbial activities and biomass. In Pesticides, Soil Microbiology and Soil Quality, 68-69 (SETAC-Europe).

(30) Gennari, M., Negre, M., Ambrosoli, R., (1987) Effects of ethylene oxide on soil microbial content and some chemical characteristics. Plant and Soil 102, p. 197-200.

(31) Anderson, J.P.E. (1975) Einfluss von Temperatur und Feuchte auf Verdampfung, Abbau und Festlegung von Diallat im Boden. Z. PflKrankh Pflschutz, Sonderheft VII, p. 141-146.

(32) Hamaker, J.W., (1976) The application of mathematical modelling to the soil persistence and accumulation of pesticides. Proc. BCPC Symposium: Persistence of Insecticides and Herbicides, p. 181-199.

(33) Goring, C.A.I., Laskowski, D.A., Hamaker, J.W., Meikle, R.W., (1975) Principles of pesticide degradation in soil. In 'Environmental Dynamics of Pesticides'. R. Haque and V.H. Freed, Eds., p. 135-172.

(34) Timme, G., Frehse, H., Laska, V., (1986) Statistical interpretation and graphic representation of the degradational behaviour of pesticide residues. II. Pflanzenschutz — Nachrichten Bayer 39, p. 188-204.

(35) Timme, G., Frehse, H., (1980) Statistical interpretation and graphic representation of the degradational behaviour of pesticide residues. I. Pflanzenschutz — Nachrichten Bayer 33, p. 47-60.

(36) Gustafson D.I., Holden L.R., (1990) Non-linear pesticide dissipation in soil; a new model based on spatial variability. Environm. Sci. Technol. 24, p. 1032-1041.

(37) Hurle K., Walker A., (1980) Persistence and its prediction. In Interactions between Herbicides and the Soil (R.J. Hance, Ed.), Academic Press, p. 83-122.

Appendix 1

WATER TENSION, FIELD CAPACITY (FC) AND WATER HOLDING CAPACITY (WHC) [1]

Height of Water Column [cm]	pF [a]	bar [b]	Remarks
10^7	7	10^4	Dry Soil
$1{,}6 \cdot 10^4$	4,2	16	Wilting point
10^4	4	10	
10^3	3	1	
$6 \cdot 10^2$	2,8	0,6	
$3{,}3 \cdot 10^2$	2,5	0,33 [c]	
10^2	2	0,1	Range of
60	1,8	0,06	Field capacity [d]
33	1,5	0,033	
10	1	0,01	WHC (approximation)
1	0	0,001	Water saturated soil

[a] pF = log of cm water column.
[b] 1 bar = 10^5 Pa.
[c] Corresponds to an approximate water content of 10 % in sand, 35 % in loam and 45 % in clay.
[d] Field capacity is not constant but varies with soil type between pF 1,5 and 2,5.

Water tension is measured in cm water column or in bar. Due to the large range of suction tension it is expressed simply as pF value which is equivalent to the logarithm of cm water column.

Field capacity is defined as the amount of water which can be stored against gravity by a natural soil two days after a longer raining period or after sufficient irrigation. It is determined in undisturbed soil in situ in the field. The measurement is thus not applicable to disturbed laboratory soil samples. FC values determined in disturbed soils may show great systematic variances.

Water holding capacity (WHC) is determined in the laboratory with undisturbed and disturbed soil by saturating a soil column with water by capillary transport. It is particularly useful for disturbed soils and can be up to 30 % greater than field capacity (1). It is also experimentally easier to determine than reliable FC-values.

Notes

[1] Mückenhausen, E., (1975) Die Bodenkunde und ihre geologischen, geomorphologischen, mineralogischen und petrologischen Grundlagen. DLG-Verlag, Frankfurt, Main.

Appendix 2

SOIL MOISTURE CONTENTS (g water per 100 g dry soil) OF VARIOUS SOIL TYPES FROM VARIOUS COUNTRIES

Soil type	Country	Soil moisture content at		
		WHC [1]	pF = 1,8	pF = 2,5
Sand	Germany	28,7	8,8	3,9
Loamy sand	Germany	50,4	17,9	12,1
Loamy sand	Switzerland	44,0	35,3	9,2
Silt loam	Switzerland	72,8	56,6	28,4
Clay loam	Brazil	69,7	38,4	27,3
Clay loam	Japan	74,4	57,8	31,4
Sandy loam	Japan	82,4	59,2	36,0
Silt loam	USA	47,2	33,2	18,8
Sandy loam	USA	40,4	25,2	13,3

[1] Water holding capacity.

Appendix 3

Figure 1

Example of a flow-through apparatus to study transformation of chemicals in soil [1] [2]

1: needle valve

2: gas washing bottle containing water

3: ultramembrane (sterile conditions only), pore size 0.2 µm

4: soil metabolism flask (water-logged only for anaerobic and paddy conditions)

5: ethylene glycol trap for organic volatile compounds

6: sulphuric acid trap for alkaline volatile compounds

7, 8: sodium hydroxide trap for CO_2 & other acidic volatiles

9: flow meter.

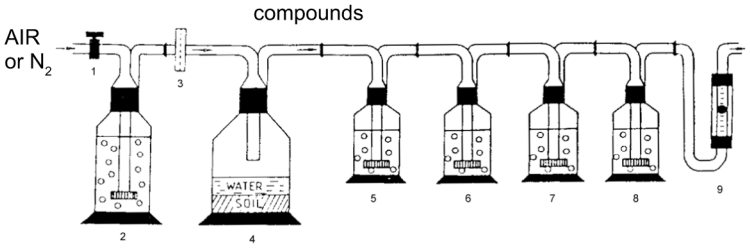
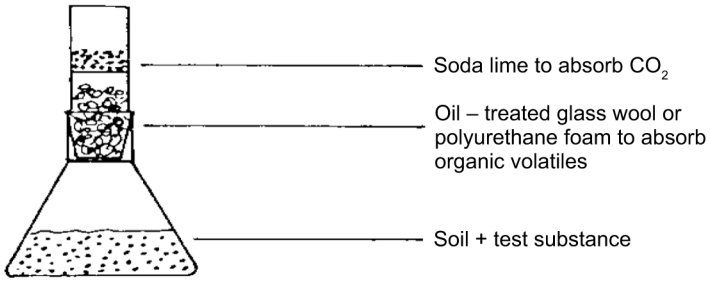


Figure 2

Example of a biometer-type flask for studying the transformation of chemicals in soil [3]



[1] Guth, J.A., (1980) The study of transformations. In Interactions between Herbicides and the Soil (R.J. Hance, Ed.), Academic Press, p. 123-157.

[2] Guth, J.A., (1981) Experimental approaches to studying the fate of pesticides in soil. In Progress in Pesticide Biochemistry. D.H. Hutson, T.R. Roberts, Eds. J. Wiley & Sons. Vol 1, p. 85-114.

[3] Anderson, J.P.E., (1975) Einfluss von Temperatur und Feuchte auf Verdampfung, Abbau und Festlegung von Diallat im Boden. Z. PflKrankh Pflschutz, Sonderheft VII, p. 141-146.

C.24. AEROBIC AND ANAEROBIC TRANSFORMATION IN AQUATIC SEDIMENT SYSTEMS

1. METHOD

This test method is a replicate of the OECD TG 308 (2002).

1.1. INTRODUCTION

Chemicals can enter shallow or deep surface waters by such routes as direct application, spray drift, run-off, drainage, waste disposal, industrial, domestic or agricultural effluent and atmospheric deposition. This testing method describes a laboratory method to assess aerobic and anaerobic transformation of organic chemicals in aquatic sediment systems. It is based on existing Guidelines (1)(2)(3)(4)(5)(6). An OECD Workshop on Soil/Sediment Selection, held in Belgirate, Italy in 1995 (7) agreed, in particular, on the number and type of sediments for use in this test. It also made recommendations relating to collection, handling and storage of sediment samples, based on the ISO Guidance (8). Such studies are required for chemicals which are directly applied to water or which are likely to reach the aqueous environment by the routes described above.

The conditions in natural aquatic sediment systems are often aerobic in the upper water phase. The surface layer of sediment can be either aerobic or anaerobic, whereas the deeper sediment is usually anaerobic. To encompass all of these possibilities both aerobic and anaerobic tests are described in this document. The aerobic test simulates an aerobic water column over an aerobic sediment layer that is underlain with an anaerobic gradient. The anaerobic test simulates a completely anaerobic water-sediment system. If circumstances indicate that it is necessary to deviate significantly from these recommendations, for example by using intact sediment cores or sediments that may have been exposed to the test substance, other methods are available for this purpose (9).

1.2. DEFINITIONS

Standard International (SI) units should be used in any case.

Test substance: any substance, whether the parent or relevant transformation products.

Transformation products: all substances resulting from biotic and abiotic transformation reactions of the test substance including CO_2 and bound residues.

Bound residues: 'bound residues' represent compounds in soil, plant or animal that persist in the matrix in the form of the parent substance or its metabolite(s) after extractions. The extraction method must not substantially change the compounds themselves or the structure of the matrix. The nature of the bond can be clarified in part by matrix-altering extraction methods and sophisticated analytical techniques. To date, for example, covalent ionic and sorptive bonds, as well as entrapments, have been identified in this way. In general, the formation of bound residues reduces the bioaccessibility and the bioavailability significantly (10) (modified from IUPAC 1984 (11)).

Aerobic transformation: (oxidising): reactions occurring in the presence of molecular oxygen (12).

Anaerobic transformation: (reducing): reactions occurring under exclusion of molecular oxygen (12).

Natural waters: are surface waters obtained from ponds, rivers, streams, etc.

Sediment: is a mixture of mineral and organic chemical constituents, the latter containing compounds of high carbon and nitrogen content and of high molecular masses. It is deposited by natural water and forms an interface with that water.

Mineralisation: is the complete degradation of an organic compound to CO_2, H_2O under aerobic conditions, and CH_4, CO_2 and H_2O under anaerobic conditions. In the context of this test method, when radiolabelled compound is used, mineralisation means extensive degradation of a molecule during which a labelled carbon atom is oxidised or reduced quantitatively with release of the appropriate amount of $^{14}CO_2$ or $^{14}CH_4$, respectively.

Half-life, $t_{0,5}$, is the time taken for 50 % transformation of a test substance when the transformation can be described by first-order kinetics; it is independent of the initial concentration.

DT_{50} (Disappearance Time 50): is the time within which the initial concentration of the test substance is reduced by 50 %.

DT_{75} (DISAPPEARANCE TIME 75): IS THE TIME WITHIN WHICH THE INITIAL CONCENTRATION OF THE TEST SUBSTANCE IS REDUCED BY 75 %.

DT_{90} (Disappearance Time 90): is the time within which the initial concentration of the test substance is reduced by 90 %.

1.3. REFERENCE SUBSTANCES

Reference substances should be used for the identification and quantification of transformation products by spectroscopic and chromatographic methods.

1.4. INFORMATION ON THE TEST SUBSTANCE

Non-labelled or isotope-labelled test substance can be used to measure the rate of transformation although labelled material is preferred. Labelled material is required for studying the pathway of transformation and for establishing a mass balance. ^{14}C-labelling is recommended, but the use of other isotopes, such as ^{13}C, ^{15}N, ^{3}H, ^{32}P, may also be useful. As far as possible, the label should be positioned in the most stable part(s) of the molecule ([1]). The chemical and/or radiochemical purity of the test substance should be at least 95 %.

Before carrying out a test, the following information about the test substance should be available:

(a) solubility in water (Method A.6);

(b) solubility in organic solvents;

(c) vapour pressure (Method A.4) and Henry's Law constant;

(d) n-octanol/water partition coefficient (Method A.8);

(e) adsorption coefficient (K_d, K_f or K_{oc}, where appropriate) (Method C.18);

(f) hydrolysis (Method C.7);

(g) dissociation constant (pK_a) (OECD Guideline 112) (13);

(h) chemical structure of the test substance and position of the isotope-label(s), if applicable.

Note: the temperature at which these measurements were made should be reported.

Other useful information may include data on toxicity of the test substance to microorganisms, data on ready and/or inherent biodegradability, and data on aerobic and anaerobic transformation in soil.

Analytical methods (including extraction and clean-up methods) for identification and quantification of the test substance and its transformation products in water and in sediment should be available (see Section 1.7.2).

([1]) For example, if the substance contains one ring, labelling on this ring is required; if the test substance contains two or more rings, separate studies may be needed to evaluate the fate of each labelled ring and to obtain suitable information on formation of transformation products.

1.5. PRINCIPLE OF THE TEST METHOD

The method described in this test employs an aerobic and an anaerobic aquatic sediment (see Appendix 1) system which allows:

the measurement of the transformation rate of the test substance in a water-sediment system,

the measurement of the transformation rate of the test substance in the sediment,

the measurement of the mineralisation rate of the test substance and/or its transformation products (when ^{14}C-labelled test substance is used),

the identification and quantification of transformation products in water and sediment phases including mass balance (when labelled test substance is used),

the measurement of the distribution of the test substance and its transformation products between the two phases during a period of incubation in the dark (to avoid, for example, algal blooms) at constant temperature. Half-lives, DT_{50}, DT_{75} and DT_{90} values are determined where the data warrant, but should not be extrapolated far past the experimental period (see Section 1.2).

At least two sediments and their associated waters are required for both the aerobic and the anaerobic studies respectively (7). However, there may be cases where more than two aquatic sediments should be used, for example, for a chemical that may be present in freshwater and/or marine environments.

1.6. APPLICABILITY OF THE TEST

The method is generally applicable to chemical substances (unlabelled or labelled) for which an analytical method with sufficient accuracy and sensitivity is available. It is applicable to slightly volatile, non-volatile, water-soluble or poorly water-soluble compounds. The test should not be applied to chemicals which are highly volatile from water (e.g. fumigants, organic solvents) and thus cannot be kept in water and/or sediment under the experimental conditions of this test.

The method has been applied so far to study the transformation of chemicals in fresh waters and sediments, but in principle can also be applied to estuarine/marine systems. It is not suitable to simulate conditions in flowing water (e.g. rivers) or the open sea.

1.7. QUALITY CRITERIA

1.7.1. **Recovery**

Extraction and analysis of, at least, duplicate water and sediment samples immediately after the addition of the test substance gives a first indication of the repeatability of the analytical method and of the uniformity of the application procedure for the test substance. Recoveries for later stages of the experiments are given by the respective mass balances (when labelled material is used). Recoveries should range from 90 % to 110 % for labelled chemicals (6) and from 70 % to 110 % for non-labelled chemicals.

1.7.2. **Repeatability and sensitivity of analytical method**

Repeatability of the analytical method (excluding the initial extraction efficiency) to quantify test substance and transformation products can be checked by duplicate analysis of the same extract of the water or the sediment samples which were incubated sufficiently long enough for formation of transformation products.

The limit of detection (LOD) of the analytical method for the test substance and for the transformation products should be at least 0,01 mg· kg^{-1} in water or sediment (as test substance) or 1 % of the initial amount applied to a test system whichever is lower. The limit of quantification (LOQ) should also be specified.

1.7.3. **Accuracy of transformation data**

Regression analysis of the concentrations of the test substance as a function of time gives the appropriate information on the accuracy of the transformation curve and allows the calculation of the confidence limits for half-lives (if pseudo first-order kinetics apply) or DT_{50} values and, if appropriate, DT_{75} and DT_{90} values.

1.8. DESCRIPTION OF THE METHOD

1.8.1. **Test system and apparatus**

The study should be performed in glass containers (e.g. bottles, centrifuge tubes), unless preliminary information (such as n-octanol-water partition coefficient, sorption data, etc.) indicates that the test substance may adhere to glass, in which case an alternative material (such as Teflon) may have to be considered. Where the test substance is known to adhere to glass, it may be possible to alleviate this problem using one or more of the following methods:

— determine the mass of test substance and transformation products sorbed to glass,

— ensure a solvent wash of all glassware at the end of the test,

— use of formulated products (see also Section 1.9.2),

— use an increased amount of co-solvent for addition of test substance to the system; if a co-solvent is used it should be a co-solvent that does not solvolyse the test substance.

Examples of typical test apparatus, i.e. gas flow-through and biometer-type systems, are shown in Appendices 2 and 3, respectively (14). Other useful incubation systems are described in reference 15. The design of the experimental apparatus should permit the exchange of air or nitrogen and the trapping of volatile products. The dimensions of the apparatus must be such that the requirements of the test are complied with (see Section 1.9.1). Ventilation may be provided by either gentle bubbling or by passing air or nitrogen over the water surface. In the latter case gentle stirring of the water from above may be advisable for better distribution of the oxygen or nitrogen in the water. CO_2-free air should not be used as this can result in increases in the pH of the water. In either case, disturbance of the sediment is undesirable and should be avoided as far as possible. Slightly volatile chemicals should be tested in a biometer-type system with gentle stirring of the water surface. Closed vessels with a headspace of either atmospheric air or nitrogen and internal vials for the trapping of volatile products can also be used (16). Regular exchange of the headspace gas is required in the aerobic test in order to compensate for the oxygen consumption by the biomass.

Suitable traps for collecting volatile transformation products include but are not restricted to 1 mol· dm^{-3} solutions of potassium hydroxide or sodium hydroxide for carbon dioxide ([1]) and ethylene glycol, ethanolamine or 2 % paraffin in xylene for organic compounds. Volatiles formed under anaerobic conditions, such as methane, can be collected, for example, by molecular sieves. Such volatiles can be combusted, for example, to CO_2 by passing the gas through a quartz tube filled with CuO at a temperature of 900 °C and trapping the CO_2 formed in an absorber with alkali (17).

Laboratory instrumentation for chemical analysis of test substance and transformation products is required (e.g. gas liquid chromatography (GLC), high performance liquid chromatography (HPLC), thin-layer chromatography (TLC), mass spectroscopy (MS), gas chromatography-mass spectroscopy (GC-MS), liquid chromatography-mass spectrometry (LC-MS), nuclear magnetic resonance (NMR), etc.), including detection systems for radiolabelled or non-labelled chemicals as appropriate. When radiolabelled material is used a liquid scintillation counter and combustion oxidiser (for the combustion of sediment samples prior to analysis of radioactivity) will also be required.

Other standard laboratory equipment for physical-chemical and biological determinations (see Section Table 1, Section 1.8.2.2), glassware, chemicals and reagents are required as appropriate.

1.8.2. **Selection and number of aquatic sediments**

The sampling sites should be selected in accordance with the purpose of the test in any given situation. In selecting sampling sites, the history of possible agricultural, industrial or domestic inputs to the catchment and the waters upstream must be considered. Sediments should not be used if they have been contaminated with the test substance or its structural analogues within the previous four years.

([1]) As these alkaline absorption solutions also absorb the carbon dioxide from the ventilation air and that formed by respiration in aerobic experiments, they have to be exchanged in regular intervals to avoid their saturation and thus loss of their absorption capacity.

1.8.2.1. *Sediment selection*

Two sediments are normally used for the aerobic studies (7). The two sediments selected should differ with respect to organic carbon content and texture. One sediment should have a high organic carbon content (2,5-7,5 %) and a fine texture, the other sediment should have a low organic carbon content (0,5-2,5 %) and a coarse texture. The difference between the organic carbon contents should normally be at least 2 %. 'Fine texture' is defined as a [clay + silt] ([1]) content of > 50 % and 'coarse texture' is defined as a [clay + silt] content of < 50 %. The difference in [clay + silt] content for the two sediments should normally be at least 20 %. In cases, where a chemical may also reach marine waters, at least one of the water-sediment systems should be of marine origin.

For the strictly anaerobic study, two sediments (including their associated waters) should be sampled from the anaerobic zones of surface water bodies (7). Both the sediment and the water phases should be handled and transported carefully under exclusion of oxygen.

Other parameters may be important in the selection of sediments and should be considered on a case-by-case basis. For example, the pH range of sediments would be important for testing chemicals for which transformation and/or sorption may be pH-dependent. pH-dependency of sorption might be reflected by the pK_a of the test substance.

1.8.2.2. *Characterisation of water-sediment samples*

Key parameters that must be measured and reported (with reference to the method used) for both water and sediment, and the stage of the test at which those parameters are to be determined are summarised in the Table hereafter. For information, methods for determination of these parameters are given in references (18)(19)(20)(21).

In addition, other parameters may need to be measured and reported on a case by case basis (e.g. for freshwater: particles, alkalinity, hardness, conductivity, NO_3/PO_4 (ratio and individual values); for sediments: cation exchange capacity, water holding capacity, carbonate, total nitrogen and phosphorus; and for marine systems: salinity). Analysis of sediments and water for nitrate, sulfate, bioavailable iron, and possibly other electron acceptors may be also useful in assessing redox conditions, especially in relation to anaerobic transformation.

Measurement of parameters for characterisation of water-sediment samples (7)(22)(23)

Parameter	Stage of test procedure					
	field sampling	post-handling	start of acclimation	start of test	during test	end of test
Water						
Origin/source	x					
Temperature	x					
pH	x		x	x	x	x
TOC			x	x		x
O_2 concentration*	x		x	x	x	x
Redox Potential*			x	x	x	x

([1]) (Clay + silt) is the mineral fraction of the sediment with particle size of < 50 μm.

Parameter	Stage of test procedure					
	field sampling	post-handling	start of acclimation	start of test	during test	end of test
Sediment						
Origin/source	x					
Depth of layer	x					
pH		x	x	x	x	x
Particle size distribution		x				
TOC		x	x	x		x
Microbial biomass (*)		x		x		x
Redox potential (**)	Observation (colour/smell)		x	x	x	x

(*) Microbial respiration rate method (26), fumigation method (27) or plate count measurements (e.g. bacteria, actinomycetes, fungi and total colonies) for aerobic studies; methanogenesis rate for anaerobic studies.

(**) Recent research results have shown that measurements of water oxygen concentrations and of redox potentials have neither a mechanistic nor a predictive value as far as growth and development of microbial populations in surface waters are concerned (24)(25). Determination of the biochemical oxygen demand (BOD, at field sampling, start and end of test) and of concentrations of micro/macro nutrients Ca, Mg and Mn (at start and end of test) in water and the measurement of total N and total P in sediments (at field sampling and end of test) may be better tools to interpret and evaluate aerobic biotransformation rates and routes.

1.8.3. **Collection, handling and storage**

1.8.3.1. *Collection*

The draft ISO guidance on sampling of bottom sediment (8) should be used for sampling of sediment. Sediment samples should be taken from the entire 5 to 10 cm upper layer of the sediment. Associated water should be collected from the same site or location and at the same time as the sediment. For the anaerobic study, sediment and associated water should be sampled and transported under exclusion of oxygen (28)(see Section 1.8.2.1). Some sampling devices are described in the literature (8)(23).

1.8.3.2. *Handling*

The sediment is separated from the water by filtration and the sediment wet-sieved to a 2 mm-sieve using excess location water that is then discarded. Then known amounts of sediments and water are mixed at the desired ratio (see Section 1.9.1) in incubation flasks and prepared for the acclimation period (see Section 1.8.4). For the anaerobic study, all handling steps have to be done under exclusion of oxygen (29)(30)(31)(32)(33).

1.8.3.3. *Storage*

Use of freshly sampled sediment and water is strongly recommended, but if storage is necessary, sediment and water should be sieved as described above and stored together, water-logged (6-10 cm water layer), in the dark, at 4 ± 2°C[4] for a maximum of four weeks (7)(8)(23). Samples to be used for aerobic studies should be stored with free access of air (e.g. in open containers), whereas those for anaerobic studies under exclusion of oxygen. Freezing of sediment and water and drying-out of the sediment must not occur during transportation and storage.

1.8.4. **Preparation of the sediment/water samples for the test**

A period of acclimation should take place prior to adding the test substance, with each sediment/water sample being placed in the incubation vessel to be used in the main test, and the acclimation to be carried out under exactly the same conditions as the test incubation (see Section 1.9.1). The acclimation period is the time needed to reach reasonable stability of the system, as reflected by pH, oxygen concentration in water, redox potential of the sediment and water, and macroscopic separation of phases. The period of acclimation should normally last

between one week and two weeks and should not exceed four weeks. Results of determinations performed during this period should be reported.

1.9. PERFORMANCE OF THE TEST

1.9.1. **Test conditions**

The test should be performed in the incubation apparatus (see Section 1.8.1) with a water sediment volume ratio between 3:1 and 4:1, and a sediment layer of 2,5 cm (± 0,5 cm). ([1]) A minimum amount of 50 g of sediment (dry weight basis) per incubation vessel is recommended.

The test should be performed in the dark at a constant temperature in the range of 10 to 30 °C. A temperature of (20 ± 2)°C is appropriate. Where appropriate, an additional lower temperature (e.g. 10 °C) may be considered on a case-by-case basis, depending on the information required from the test. Incubation temperature should be monitored and reported.

1.9.2. **Treatment and application of test substance**

One test concentration of chemical is used ([2]). For crop protection chemicals applied directly to water bodies, the maximum dosage on the label should be taken as, the maximum application rate calculated on the basis of the surface area of the water in the test vessel. In all other cases, the concentration to be used should be based on predictions from environmental emissions. Care must be taken to ensure that an adequate concentration of test substance is applied in order to characterise the route of transformation and the formation and decline of transformation products. It may be necessary to apply higher doses (e.g. 10 times) in situations where test substance concentrations are close to limits of detection at the start of the study and/or where major transformation products could not readily be detected when present at 10 % of the test substance application rate. However, if higher test concentrations are used they should not have a significant adverse effect on the microbial activity of the water-sediment system. In order to achieve a constant concentration of test substance in vessels of differing dimensions an adjustment to the quantity of the material applied may be considered appropriate, based on the depth of the water column in the vessel in relation to the depth of water in the field (which is assumed to be 100 cm, but other depths can be used). See Appendix 4 for an example calculation.

Ideally the test substance should be applied as an aqueous solution into the water phase of the test system. If unavoidable, the use of low amounts of water miscible solvents (such as acetone, ethanol) is permitted for application and distribution of the test substance, but this should not exceed 1 % v/v and should not have adverse effects on microbial activity of the test system. Care should be exercised in generating the aqueous solution of the test substance — use of generator columns and pre-mixing may be appropriate to ensure complete homogeneity. Following addition of the aqueous solution to the test system, gentle mixing of the water phase is recommended, disturbing the sediment as little as possible.

The use of formulated products is not routinely recommended as the formulation ingredients may affect the distribution of the test substance and/or transformation products between water and sediment phases. However, for poorly water-soluble test substances, the use of formulated material may be an appropriate alternative.

The number of incubation vessels depends on the number of sampling times (see Section 1.9.3). A sufficient number of test systems should be included so that two systems may be sacrificed at each sampling time. Where control units of each aquatic sediment system are employed, they should not be treated with the test substance. The control units can be used to determine the microbial biomass of the sediment and the total organic carbon of the water and sediment at the termination of the study. Two of the control units (i.e. one control unit for each aquatic sediment) can be used to monitor the required parameters in the sediment and water during the

([1]) Recent studies have shown that storage at 4 °C can lead to a decrease of the organic carbon content of the sediment which may possibly result in a decrease of microbial activity (34).

([2]) Test with a second concentration can be useful for chemicals that reach surface waters by different entry routes resulting in significantly different concentrations, as long as the lower concentration can be analysed with sufficient accuracy.

acclimation period (see Table in Section 1.8.2.2). Two additional control units have to be included in case the test substance is applied by means of a solvent to measure adverse effects on the microbial activity of the test system.

1.9.3. **Test duration and sampling**

The duration of the experiment should normally not exceed 100 days (6), and should continue until the degradation pathway and water/sediment distribution pattern are established or when 90 % of the test substance has dissipated by transformation and/or volatilisation. The number of sampling times should be at least six (including zero time), with an optional preliminary study (see Section 1.9.4) being used to establish an appropriate sampling regime and the duration of the test, unless sufficient data is available on the test substance from previous studies. For hydrophobic test substances, additional sampling points during the initial period of the study may be necessary in order to determine the rate of distribution between water and sediment phases.

At appropriate sampling times, whole incubation vessels (in replicate) are removed for analysis. Sediment and overlying water are analysed separately ([1]). The surface water should be carefully removed with minimum disturbance of the sediment. The extraction and characterisation of the test substance and transformation products should follow appropriate analytical procedures. Care should be taken to remove material that may have adsorbed to the incubation vessel or to interconnecting tubing used to trap volatiles.

1.9.4. **Optional preliminary test**

If duration and sampling regime cannot be estimated from other relevant studies on the test substance, an optional preliminary test may be considered appropriate, which should be performed using the same test conditions proposed for the definitive study. Relevant experimental conditions and results from the preliminary test, if performed, should be briefly reported.

1.9.5. **Measurements and analysis**

Concentration of the test substance and the transformation products at every sampling time in water and sediment should be measured and reported (as a concentration and as percentage of applied). In general, transformation products detected at ≥ 10 % of the applied radioactivity in the total water-sediment system at any sampling time should be identified unless reasonably justified otherwise. Transformation products for which concentrations are continuously increasing during the study should also be considered for identification, even if their concentrations do not exceed the limits given above, as this may indicate persistence. The latter should be considered on a case by case basis, with justifications being provided in the report.

Results from gases/volatiles trapping systems (CO_2 and others, i.e. volatile organic compounds) should be reported at each sampling time. Mineralisation rates should be reported. Non-extractable (bound) residues in sediment are to be reported at each sampling point.

2. **DATA**

2.1. TREATMENT OF RESULTS

Total mass balance or recovery (see Section 1.7.1) of added radioactivity is to be calculated at every sampling time. Results should be reported as a percentage of added radioactivity. Distribution of radioactivity between water and sediment should be reported as concentrations and percentages, at every sampling time.

Half-life, DT_{50} and, if appropriate, DT_{75} and DT_{90} of the test substance should be calculated along with their confidence limits (see Section 1.7.3). Information on the rate of dissipation of the test substance in the water and sediment can be obtained through the use of appropriate evaluation tools. These can range from application of pseudo-first order kinetics, empirical curve-fitting techniques which apply graphical or numerical solutions and more complex assessments using, for example, single- or multi-compartment models. Further details can be obtained from the relevant published literature (35)(36)(37).

All approaches have their strengths and weaknesses and vary considerably in complexity. An assumption of first-order kinetics may be an oversimplification of the degradation and distribution processes, but when possible gives a term (the rate constant or half-life) which is easily understood and of value in simulation modelling and calculations of predicted environmental concentrations. Empirical approaches or linear

([1]) In cases where rapid re-oxidation of anaerobic transformation products may readily occur, anaerobic conditions should be maintained during sampling and analysis.

transformations can result in better fits of curves to data and therefore allow better estimation of half-lives, DT_{50} and, if appropriate, DT_{75} and DT_{90} values., The use of the derived constants, however, is limited. Compartment models can generate a number of useful constants of value in risk assessment that describe the rate of degradation in different compartments and the distribution of the chemical. They should also be used for estimation of rate constants for the formation and degradation of major transformation products. In all cases, the method chosen must be justified and the experimenter should demonstrate graphically and/or statistically the goodness of fit.

3. REPORTING

3.1. TEST REPORT

The report must include the following information:

Test substance:

— common name, chemical name, CAS number, structural formula (indicating position of the label(s) when radiolabelled material is used) and relevant physical-chemical properties,

— purity (impurities) of test substance,

— radiochemical purity of labelled chemical and molar activity (where appropriate).

Reference substances:

— chemical name and structure of reference substances used for the characterisation and/or identification of transformation products.

Test sediments and waters:

— location and description of aquatic sediment sampling site(s) including, if possible, contamination history,

— all information relating to the collection, storage (if any) and acclimation of water-sediment systems,

— characteristics of the water-sediment samples as listed in Table in section 1.8.2.2.

Test conditions:

— test system used (e.g. flow-through, biometer, way of ventilation, method of stirring, water volume, mass of sediment, thickness of both water and sediment layer, dimension of test vessels, etc.),

— application of test substance to test system: test concentration used, number of replicates and controls mode of application of test substance (e.g. use of solvent if any), etc.,

— incubation temperature,

— sampling times,

— extraction methods and efficiencies as well as analytical methods and detection limits,

— methods for characterisation/identification of transformation products,

— deviations from the test protocol or test conditions during the study.

Results:

— raw data figures of representative analyses (all raw data have to be stored in the GLP-archive),

— repeatability and sensitivity of the analytical methods used,

— rates of recovery (% values for a valid study are given in section 1.7.1),

— tables of results expressed as % of the applied dose and in mg· kg^{-1} in water, sediment and total system (% only) for the test substance and, if appropriate, for transformation products and non-extractable radioactivity,

— mass balance during and at the end of the studies,

— a graphical representation of the transformation in the water and sediment fractions and in total system (including mineralisation),

— mineralisation rates,

— half-life, DT_{50} and, if appropriate, DT_{75} and DT_{90} values for the test substance and, where appropriate, for major transformation products including confidence limits in water, sediment and in total system,

— an assessment of the transformation kinetics of the test substance and, where appropriate, the major transformation products,

— a proposed pathway of transformation, where appropriate,

— discussion of results.

4. **REFERENCES**

(1) BBA-Guidelines for the examination of plant protectors in the registration process., (1990) Part IV, Section 5-1: Degradability and fate of plant protectors in the water/sediment system. Germany.

(2) Commission for registration of pesticides: Application for registration of a pesticide., (1991) Part G. Behaviour of the product and its metabolites in soil, water and air, Section G.2.1 (a). The Netherlands.

(3) MAFF Pesticides Safety Directorate., (1992) Preliminary guideline for the conduct of biodegradability tests on pesticides in natural sediment/water systems. Ref No SC 9046. United-Kingdom.

(4) Agriculture Canada: Environmental chemistry and fate., (1987) Guidelines for registration of pesticides in Canada. Aquatic (Laboratory) — Anaerobic and aerobic. Canada. p. 35-37.

(5) US-EPA: Pesticide assessment guidelines, Subdivision N. Chemistry: Environmental fate (1982) Section 162-3, Anaerobic aquatic metabolism.

(6) SETAC-Europe publication., (1995) Procedures for assessing the environmental fate and ecotoxicity of pesticides. Ed. Dr Mark R. Lynch. SETAC-Europe, Brussels.

(7) OECD Test Guidelines Programme., (1995) Final Report of the OECD Workshop on Selection of Soils/sediments, Belgirate, Italy, 18-20 January 1995.

(8) ISO/DIS 5667-12., (1994) Water quality — Sampling — Part 12: Guidance on sampling of bottom sediments.

(9) US-EPA (1998a) Sediment/water microcosm biodegradation test. Harmonised Test Guidelines (OPPTS 835.3180). EPA 712-C-98-080.

(10) DFG: Pesticide Bound Residues in Soil. Wiley-VCH (1998).

(11) T.R. Roberts: Non-extractable pesticide residues in soils and plants. Pure Appl. Chem. 56, 945-956 (IUPAC 1984).

(12) OECD Test Guideline 304A: Inherent Biodegradability in Soil (adopted 12 May 1981).

(13) OECD (1993): Guidelines for Testing of Chemicals. Paris. OECD (1994-2000): Addenda 6-11 to Guidelines for the Testing of Chemicals.

(14) Scholz, K., Fritz R., Anderson C. and Spiteller M. (1988) Degradation of pesticides in an aquatic model ecosystem. BCPC — Pests and Diseases, 3B-4, p. 149-158.

(15) Guth, J.A., (1981) Experimental approaches to studying the fate of pesticides in soil. In Progress in Pesticide Biochemistry (D.H. Hutson, T.R. Roberts, Eds.), Vol. 1, 85-114. J. Wiley & Sons.

(16) Madsen, T., Kristensen, P. (1997) Effects of bacterial inoculation and non-ionic surfactants on degradation of polycyclic aromatic hydrocarbons in soil. Environ. Toxicol. Chem. 16, p. 631-637.

(17) Steber, J., Wierich, P. (1987) The anaerobic degradation of detergent range fatty alcohol ethoxylates. Studies with ^{14}C-labelled model surfactants. Water Research 21, p. 661-667.

(18) Black, C.A. (1965). Methods of Soil Analysis. Agronomy Monograph No 9. American Society of Agronomy, Madison.

(19) APHA (1989) Standard Methods for Examination of Water and Wastewater (17th edition). American Public Health Association, American Water Works Association and Water Pollution Control Federation, Washington D.C.

(20) Rowell, D.L. (1994) Soil Science Methods and Applications. Longman.

(21) Light, T.S., (1972). Standard solution for redox potential measurements. Anal. Chemistry 44, p. 1038-1039.

(22) SETAC-Europe publication (1991) Guidance document on testing procedures for pesticides in freshwater mesocosms. From the Workshop 'A Meeting of Experts on Guidelines for Static Field Mesocosms Tests', 3-4 July 1991.

(23) SETAC-Europe publication. (1993) Guidance document on sediment toxicity tests and bioassays for freshwater and marine environments. From the Workshop on Sediment Toxicity Assessment (WOSTA), 8-10 November 1993. Eds.: I.R. Hill, P. Matthiessen and F. Heimbach.

(24) Vink, J.P.M., van der Zee, S.E.A.T.M. (1997) Pesticide biotransformation in surface waters: multivariate analyses of environmental factors at field sites. Water Research 31, p. 2858-2868.

(25) Vink, J.P.M., Schraa, G., van der Zee, S.E.A.T.M. (1999) Nutrient effects on microbial transformation of pesticides in nitrifying waters. Environ. Toxicol, p. 329-338.

(26) Anderson, T.H., Domsch, K.H. (1985) Maintenance carbon requirements of actively-metabolising microbial populations under *in-situ* conditions. Soil Biol. Biochem. 17, p. 197-203.

(27) ISO-14240-2., (1997) Soil quality — Determination of soil microbial biomass — Part 2: Fumigation-extraction method.

(28) Beelen, P. Van and F. Van Keulen., (1990), The Kinetics of the Degradation of Chloroform and Benzene in Anaerobic Sediment from the River Rhine. Hydrobiol. Bull. 24 (1), p. 13-21.

(29) Shelton, D.R. and Tiedje, J.M. (1984) General method for determining anaerobic biodegradation potential. App. Environ. Microbiol. 47, p. 850-857.

(30) Birch, R.R., Biver, C., Campagna, R., Gledhill, W.E., Pagga, U., Steber, J., Reust, H. and Bontinck, W.J. (1989) Screening of chemicals for anaerobic biodegradation. Chemosphere 19, p. 1527-1550.

(31) Pagga, U. and Beimborn, D.B. (1993) Anaerobic biodegradation tests for organic compounds. Chemoshpere 27, p. 1499-1509.

(32) Nuck, B.A. and Federle, T.W., (1986) A batch test for assessing the mineralisation of ^{14}C-radiolabelled compounds under realistic anaerobic conditions. Environ. Sci. Technol. 30, p. 3597-3603.

(33) US-EPA (1998b). Anaerobic biodegradability of organic chemicals. Harmonised Test Guidelines (OPPTS 835.3400). EPA 712-C-98-090.

(34) Sijm, Haller and Schrap (1997) Influence of storage on sediment characteristics and drying sediment on sorption coefficients of organic contaminants. Bulletin Environ. Contam. Toxicol. 58, p. 961-968.

(35) Timme, G., Frehse H. and Laska V. (1986) Statistical interpretation and graphic representation of the degradational behaviour of pesticide residues II. Pflanzenschutz — Nachrichten Bayer, 39, p. 187-203.

(36) Timme, G., Frehse, H. (1980) Statistical interpretation and graphic representation of the degradational behaviour of pesticide residues I. Pflanzenschutz — Nachrichten Bayer, 33, p. 47-60.

(37) Carlton, R.R., and Allen, R., (1994) The use of a compartment model for evaluating the fate of pesticides in sediment/water systems. Brighton Crop Protection Conference — Pest and Diseases, p. 1349-1354.

Appendix 1

GUIDANCE ON THE AEROBIC AND THE ANAEROBIC TEST SYSTEMS

Aerobic test system

The aerobic test system described in this test method consists of an aerobic water layer (typical oxygen concentrations range from 7 to 10 $mg \cdot l^{-1}$) and a sediment layer, aerobic at the surface and anaerobic below the surface (typical average redox potentials (E_h) in the anaerobic zone of the sediment range from — 80 to — 190 mV). Moistened air is passed over the surface of the water in each incubation unit to maintain sufficient oxygen in the head space.

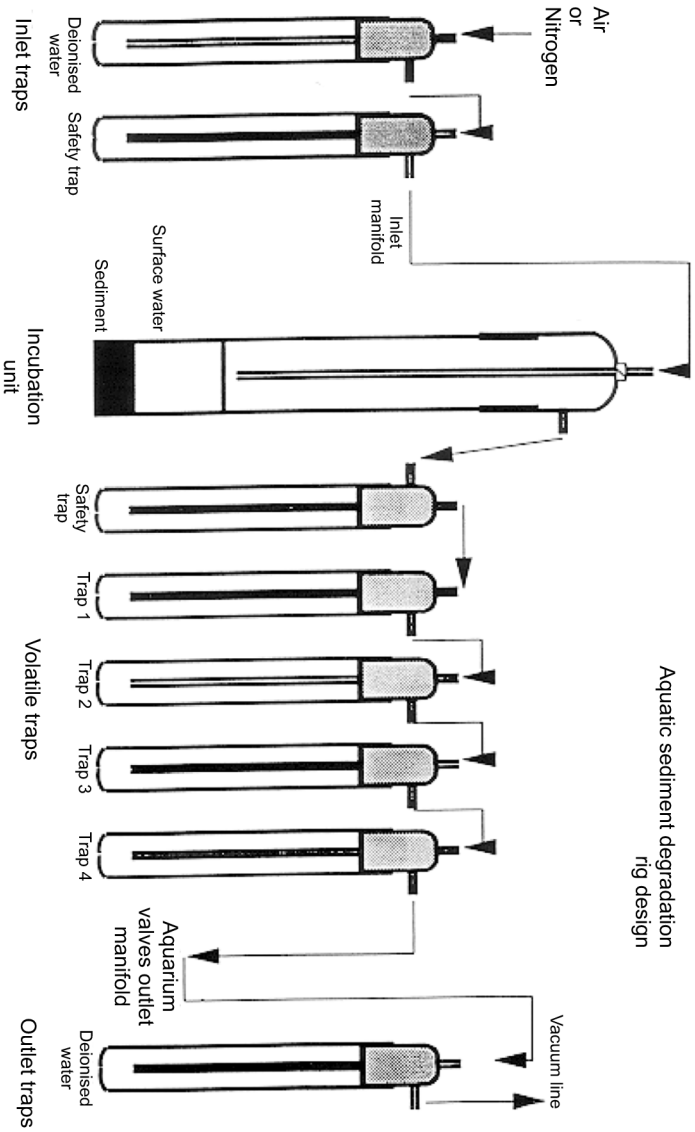
Anaerobic test system

For the anaerobic test system, the test procedure is essentially the same as that outlined for the aerobic system with the exception that moistened nitrogen is passed above the surface of the water in each incubation unit to maintain a head space of nitrogen. The sediment and water are regarded as anaerobic once the redox potential (E_h) is lower than — 100 mV.

In the anaerobic test, assessment of mineralisation includes measurement of evolved carbon dioxide and methane.

Appendix 2

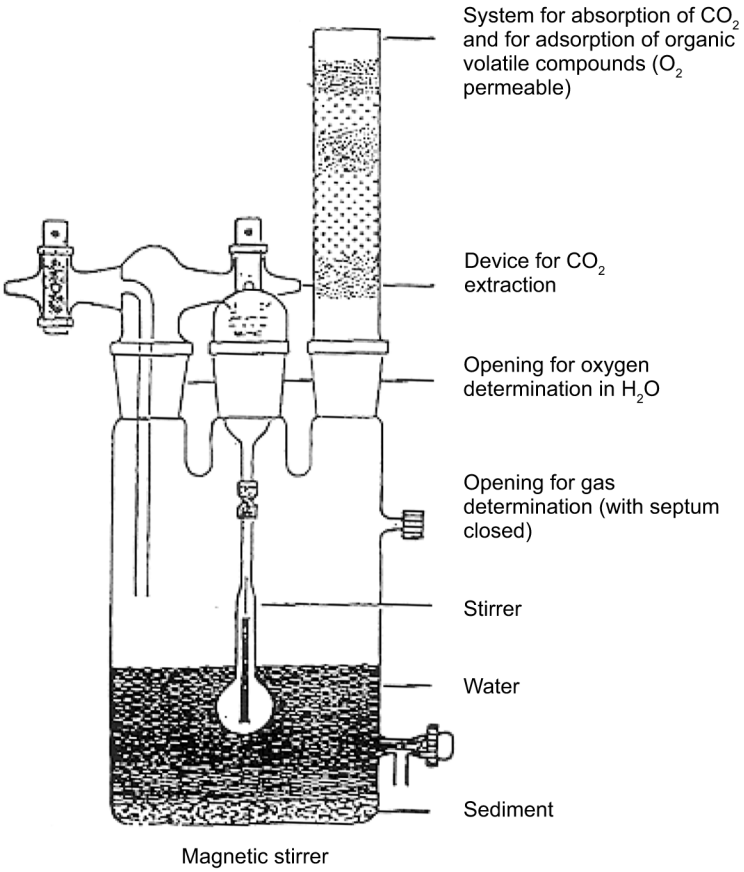
EXAMPLE OF A GAS FLOW-THROUGH APPARATUS



Safety trap, empty
Trap 1:
ethyleneglycol to trap organic volatiles
Trap 2:
sulphuric acid 0.1 M to trap alkaline volatiles

Appendix 3

EXAMPLE OF A BIOMETER APPARATUS



Appendix 4

EXAMPLE CALCULATION FOR APPLICATION DOSE TO TEST VESSELS

Cylinder internal diameter:	= 8 cm
Water column depth not including sediment:	= 12 cm
Surface area: $3{,}142 \times 4^2$	= 50,3 cm^2
Application rate: 500 g test substance/ha corresponds to 5 µg/cm^2	
Total µg: 5 × 50,3	= 251,5 µg
Adjust quantity in relation to a depth of 100 cm:	
12 × 251,5 ÷ 100	= 30,18 µg
Volume of water column: 50,3 × 12	= 603 ml
Concentration in water: 30,18 ÷ 603	= 0,050 µg/ml or 50 µg/l