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Legislation

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Price: EUR 24,50

⁽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.

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I

(Acts whose publication is obligatory)

COMMISSION DIRECTIVE 2000/32/EC**of 19 May 2000**

adapting to technical progress for the 26th time Council Directive 67/548/EEC on the approximation of the laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances^(*)

(Text with EEA relevance)

THE COMMISSION OF THE EUROPEAN COMMUNITIES,

Having regard to the Treaty establishing the European Community,

Having regard to 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⁽¹⁾, as last amended by European Parliament and Council Directive 1999/33/EC⁽²⁾, and in particular Article 28 thereof,

Whereas:

(1) Annex I to Directive 67/548/EEC contains a list of dangerous substances, together with particulars of the classification and labelling of each substance. Present scientific and technical knowledge has shown that the list of dangerous substances in that Annex should be adapted. Certain language versions of the Directive require corrections in specific sections of the foreword and of Table A to Annex I.

(2) Annex III to Directive 67/548/EEC contains a list of phrases indicating the nature of special risks attributed to dangerous substances and preparations. Annex IV to Directive 67/548/EEC contains a list of the phrases indicating the safety advice concerning dangerous substances and preparations. Annex VI to Directive 67/548/EEC contains a guide to the classification and labelling of dangerous substances and preparations. Certain language versions of the Directive require corrections in specific sections of Annexes III, IV and VI.

(3) Annex V to Directive 67/548/EEC lays down the methods for the determination of the physico-chemical properties, toxicity and ecotoxicity of substances and preparations. It is necessary to adapt that Annex to technical progress.

(4) Annex IX to Directive 67/548/EEC contains the provisions relating to child-proof fastenings. Those provisions should be adapted and updated. It is necessary to extend the scope of the use of child-proof fastenings.

(5) The measures provided for in this Directive are in accordance with the opinion of the Committee on the Adaptation to Technical Progress of the Directives for the Elimination of Technical Barriers to Trade in Dangerous Substances and Preparations,

HAS ADOPTED THIS DIRECTIVE:

Article 1

Directive 67/548/EEC is hereby amended as follows:

1. Annex I is amended as follows:

- (a) Note Q in Annex 1A to this Directive replaces the corresponding note in the Foreword.
- (b) The rows in Annex 1B to this Directive replace the corresponding rows in Table A.
- (c) The entries in Annex 1C to this Directive replace the corresponding entries.
- (d) The entries in Annex 1D to this Directive are inserted.

2. The risk phrase in Annex 2 to this Directive replaces the corresponding phrase in Annex III.

3. Annex IV is amended as follows:

- (a) The safety phrases in Annex 3A to this Directive replace the corresponding phrases in Annex IV.

(*) Adopted after the 27th adaptation.

⁽¹⁾ OJ 196, 16.8.1967, p. 1.

⁽²⁾ OJ L 199, 30.7.1999, p. 57.

- (b) The combined safety phrases in Annex 3B to this Directive replace the corresponding phrases in Annex IV.

4. Part B of Annex V is amended as follows:

- (a) The text in Annex 4A to this Directive replaces Chapter B.10.
- (b) The text in Annex 4B to this Directive replaces Chapter B.11.
- (c) The text in Annex 4C to this Directive replaces Chapter B.12.
- (d) The text in Annex 4D to this Directive replaces Chapters B.13 and B.14.
- (e) The text in Annex 4E to this Directive replaces Chapter B.17.
- (f) The text in Annex 4F to this Directive replaces Chapter B.23. The title of Chapter B.23 in the explanatory note is changed accordingly.
- (g) The text in Annex 4G to this Directive is added.

5. The fourth indent of the general introduction to Part C of Annex V is deleted.

6. The texts in Annex 5 to this Directive replace the corresponding texts in Annex VI.

7. Annex IX is amended as set out in Annex 6 to this Directive.

Article 2

1. Member States shall bring into force the laws, regulations and administrative provisions necessary to comply with this Directive by 1 June 2001 at the latest. They shall forthwith inform the Commission thereof.

When Member States adopt those provisions, they shall contain a reference to this Directive or be accompanied by such a reference on the occasion of their official publication. Member States shall determine how such reference is to be made.

2. Member States shall communicate to the Commission the main provisions of national law which they adopt in the field covered by this Directive and a correlation table between this Directive and the national provisions adopted.

Article 3

This Directive shall enter into force on the third day following its publication in the *Official Journal of the European Communities*.

Article 4

This Directive is addressed to the Member States.

Done at Brussels, 19 May 2000.

For the Commission

Margot WALLSTRÖM

Member of the Commission

ANNEX 1A

FOREWORD TO ANNEX I

Explanation of the notes relating to the identification, classification and labelling of substances

DA:

Note Q:

Klassificeringen som kræftfremkaldende kan udelades for fibre, som opfylder en af følgende betingelser:

- en kortvarig biopersistensprøve ved inhalation har vist, at fibre, der er længere end 20 µm, har en vægtet halveringstid på mindre end 10 dage
- en kortvarig biopersistensprøve ved intratrakeal instillation har vist, at fibre, der er længere end 20 µm, har en vægtet halveringstid på mindre end 40 dage
- en egnet intra-peritoneal prøve ikke har vist kræftfremkaldende virkning, eller
- en egnet langvarig inhalationsprøve ikke har vist relevante sygdomsfremkaldende virkninger eller neoplastiske forandringer.

SV:

Note Q:

Ämnet behöver inte klassificeras som cancerframkallande om det kan visas att det uppfyller ett av följande villkor:

- ett korttidstest för att bestämma den biologiska beständigheten vid inhalation har visat att fibrer längre än 20 µm har en viktad halveringstid på mindre än 10 dagar
- ett korttidstest för att bestämma den biologiska beständigheten vid intratrakeal instillation har visat att fibrer längre än 20 µm har en viktad halveringstid på mindre än 40 dagar
- ett lämpligt intraperitonealt test har inte givit belägg för förhöjd cancerogenitet
- frånvaro av relevant patogenitet eller neoplastiska förändringar i ett lämpligt långtids inhalationstest.

(Does not concern the ES version)

(Does not concern the DE version)

(Does not concern the EL version)

(Does not concern the EN version)

(Does not concern the FR version)

(Does not concern the IT version)

(Does not concern the NL version)

(Does not concern the PT version)

(Does not concern the FI version)

ANNEX 1B

TABLE A

Z	Symb.	ES	DA	DE	EL	EN	FI	FR	IT	NL	PT	SV
'18	Ar	Argón	Argon	Argon	Αργό	Argon	Argon	Argon	Argon	Argon	Árgon	Argon'
'64	Gd	Gadolinio	Gadolinium	Gadolinium	Γαδολίνιο	Gadolinium	Gadolinium	Gadolinium	Gadolinio	Gadolinium	Gadólínio	Gadolinium'

ANNEX IC

Index No	Chemical name	Notes related to substances	EC No	CAS No	Classification	Labelling	Concentration limits	Notes related to preparations
006-011-00-7	carbaryl (ISO) 1-naphthyl methylcarbamate		200-555-0	63-25-2	Carc. Cat. 3; R40 Xn; R22 N; R50	Xn; N R: 22-40-50 S: (2-)22-24-36/37-46-61		
006-013-00-8	metam-sodium (ISO) sodium methylthiocarbamate		205-293-0	137-42-8	Xn; R22 R31 C; R34 R43 N; R50-53	C; N R: 22-31-34-43-50/53 S: (1/2-)26-36/37/39-45-60-61		
006-015-00-9	diuron (ISO) 3-(3,4-dichlorophenyl)-1,1-dimethylurea		206-354-4	330-54-1	Carc. Cat. 3; R40 Muta. Cat. 3; R40 Xn; R22-48/22 N; R50-53	Xn; N R: 22-40-48/22-50/53 S: (2-)13-22-23-37-46-60-61		
006-016-00-4	propoxur (ISO) 2-isopropoxyphenyl N-methylcarbamate 2-isopropoxyphenyl methylcarbamate		204-043-8	114-26-1	T; R25 N; R50-53	T; N R: 25-50/53 S: (1/2-)37-45-60-61		
006-017-00-X	aldicarb (ISO) 2-methyl-2-(methylthio)propanal-O-(N-methylcarbamoyl)oxime		204-123-2	116-06-3	T+; R26/28 T; R24 N; R50-53	T+; N R: 24-26/28-50/53 S: (1/2-)22-36/37-45-60-61		
006-018-00-5	aminocarb (ISO) 4-dimethylamino-3-tolyl methylcarbamate		217-990-7	2032-59-9	T; R24/25 N; R50-53	T; N R: 24/25-50/53 S: (1/2-)28-36/37-45-60-61		
006-019-00-0	di-allate (ISO) S-(2,3-dichloroallyl)-N,N-disopropylthiocarbamate		218-961-1	2303-16-4	Carc. Cat. 3; R40 Xn; R22 N; R50-53	Xn; N R: 22-40-50/53 S: (2-)25-36/37-60-61		
006-020-00-6	barban (ISO) 4-chlorobut-2-ynyl N-(3-chlorophenyl)carbamate		202-930-4	101-27-9	Xn; R22 R43 N; R50-53	Xn; N R: 22-43-50/53 S: (2-)24-36/37-60-61		
006-023-00-2	mercaptodimethur (ISO) methiocarb 3,5-dimethyl-4-methylthiophenyl N-methylcarbamate		217-991-2	2032-65-7	T; R25 N; R50-53	T; N R: 25-50/53 S: (1/2-)22-37-45-60-61		
006-024-00-8	proxan-sodium (ISO) sodium O-isopropylthiocarbamate		205-443-5	140-93-2	Xn; R22 Xi; R38 N; R51-53	Xn; N R: 22-38-51/53 S: (2-)13-61		

Index No	Chemical name	Notes related to substances	EC No	CAS No	Classification	Labelling	Concentration limits	Notes related to preparations
006-026-00-9	carbofuran (ISO) 2,3-dihydro-2,2-dimethylbenzofuran-7-yl N-methylcarbamate		216-353-0	1563-66-2	T+; R26/28 N; R50-53	T+; N R: 26/28-50/53 S: (1/2-)/36/37-45-60-61		
006-028-00-X	dinobuton (ISO) 2-(1-methylpropyl)-4,6-dinitrophenyl isopropyl carbonate		213-546-1	973-21-7	T; R25 N; R50-53	T; N R: 25-50/53 S: (1/2-)/37-45-60-61		
006-029-00-5	dioxacarb (ISO) 2-(1,3-dioxolan-2-yl)phenyl N-methylcarbamate		230-253-4	6988-21-2	T; R25 N; R51-53	T; N R: 25-51/53 S: (1/2-)/37-45-61		
006-033-00-7	metoxuron (ISO) 3-(3-chloro-4-methoxyphenyl)-1,1- dimethylurea		243-433-2	19937-59-8	N; R50-53	N R: 50/53 S: 60-61		
006-034-00-2	pebulate (ISO) N-butyl-N-ethyl-S-propylthiocarbamate		214-215-4	1114-71-2	Xn; R22 N; R51-53	Xn; N R: 22-51/53 S: (2-)/23-61		
006-035-00-8	pirimicarb (ISO) 5,6-dimethyl-2-dimethylamino-pyrimidin- 4-yl-N,N-dimethylcarbamate		245-430-1	23103-98-2	T; R25 N; R50-53	T; N R: 25-50/53 S: (1/2)22-37-45-60-61		
006-037-00-9	promecarb (ISO) 3-isopropyl-5-methylphenyl N-methylcarbamate		220-113-0	2631-37-0	T; R25 N; R50-53	T; N R: 25-50/53 S: (1/2-)/24-37-45-60-61		
006-038-00-4	sulfallate (ISO) 2-chlorallyl N,N-dimethyldithiocarbamate	E	202-388-9	95-06-7	Carc. Cat. 2; R45 Xn; R22 N; R50-53	T; N R: 45-22-50/53 S: 53-45-60-61		
006-039-00-X	tri-allate (ISO) S-2,3,3-trichlorallyl diisopropylthiocarbamate		218-962-7	2303-17-5	Xn; R22-48/22 R43 N; R50-53	Xn; N R: 22-43-48/22-50/53 S: (2-)/24-37-60-61		
006-042-00-6	monuron (ISO) 3-(4-chlorophenyl)-1,1-dimethylurea		205-766-1	150-68-5	Carc. Cat. 3; R40 Xn; R22 N; R50-53	Xn; N R: 22-40-50/53 S: (2-)/36/37-60-61		
006-043-00-1	monuron-TCA 3-(4-chlorophenyl)-1,1-dimethyluronium trichloroacetate		—	140-41-0	Xi; R36/38 Carc. Cat. 3; R40 N; R50-53	Xn; N R: 36/38-40-50/53 S: (2-)/36/37-60-61		

Index No	Chemical name	Notes related to substances	EC No	CAS No	Classification	Labelling	Concentration limits	Notes related to preparations
006-045-00-2	methomyl (ISO) 1-(methylthio)ethyldeneamino N-methylcarbamate		240-815-0	16752-77-5	T+; R28 N; R50-53	T+; N R: 28-50/53 S: (1/2-)22-36/37-45-60-61		
006-046-00-8	bendiocarb (ISO) 2,2-dimethyl-1,3-benzodioxol-4-yl N-methylcarbamate		245-216-8	22781-23-3	T; R23/25 Xn; R21 N; R50-53	T; N R: 21-23/25-50/53 S: (1/2-)22-36/37-45-60-61		
006-047-00-3	bufencarb (ISO) A mixture of 3-(1-methylbutyl)phenyl N-methylcarbamate and 3-(1-ethylpropyl)phenyl N-methylcarbamate		—	8065-36-9	T; R24/25 N; R50-53	T; N R: 24/25-50/53 S: (1/2-)28-36/37-45-60-61		
006-048-00-9	ethiofencarb (ISO) 2-(ethylthiomethyl)phenyl N-methylcarbamate		249-981-9	29973-13-5	Xn; R22 N; R50-53	Xn; N R: 22-50/53 S: (2-)60-61		
006-050-00-X	fenuron-TCA 1,1-dimethyl-3-phenylurinium trichloroacetate		—	4482-55-7	Xi; R38 N; R50-53	Xi; N R: 38-50/53 S: (2-)60-61		
006-053-00-6	isoprocab (ISO) 2-isopropylphenyl N-methylcarbamate		220-114-6	2631-40-5	Xn; R22 N; R50-53	Xn; N R: 22-50/53 S: (2-)60-61		
006-054-00-1	mexacarb (ISO) 3,5-dimethyl-4-dimethylaminophenyl N-methylcarbamate		206-249-3	315-18-4	T+; R28 Xn; R21 N; R50-53	T+; N R: 21-28-50/53 S: (1/2-)36/37-45-60-61		
006-057-00-8	nitapyrin (ISO) 2-chloro-6-trichloromethylpyridine		217-682-2	1929-82-4	Xn; R22 N; R51-53	Xn; N R: 22-51/53 S: (2-)24-61		
006-060-00-4	oxycarboxin (ISO) 2,3-dihydro-6-methyl-5-(N-phenylcarbamoyl)- 1,4-oxothine 4,4-dioxide		226-066-2	5259-88-1	Xn; R22 R52-53	Xn R: 22-52/53 S: (2-)61		
006-069-00-3	thiophanate-methyl (ISO) 1,2-di-(3-methoxycarbonyl- 2-thioureido)benzene		245-740-7	23564-05-8	Muta. Cat. 3; R40 N; R50-53	Xn; N R: 40-50/53 S: (2-)36/37-60-61		
006-070-00-9	furmecyclox N-cyclohexyl-N-methoxy-2,5-dimethyl- 3-furamide		262-302-0	60568-05-0	Carc. Cat. 3; R40 N; R50-53	Xn; N R: 40-50/53 S: (2-)36/37-60-61		

Index No	Chemical name	Notes related to substances	EC No	CAS No	Classification	Labelling	Concentration limits	Notes related to preparations
006-088-00-7	berfurcarb (ISO) ethyl N-[2,3-dihydro-2,2-dimethylbenzofuran-7-yloxy]carbonyl(methylaminothio)-N-isopropyl-β-alaninate		—	82560-54-1	T; R23/25 N; R50-53	T; N R: 23/25-50/53 S: (1/2-36/37-45-60-61		
007-012-00-5	N,N-dimethylhydrazine 1,1-dimethylhydrazine	E	200-316-0	57-14-7	F; R11 Carc. Cat. 2; R45 T; R23/25 C; R34 N; R51-53	F; T; N R: 45-11-23/25-34-51/53 S: 53-45-61		
007-013-00-0	N,N-dimethylhydrazine 1,2-dimethylhydrazine	E	—	540-73-8	Carc. Cat. 2; R45 T; R23/24/25 N; R51-53	T; N R: 45-23/24/25-51/53 S: 53-45-61	C ≥ 25 %; T; R45-23/24/25 3 % ≤ C < 25 %; T; R45-20/21/22 0,01 % ≤ C < 3 %; T; R45	
009-003-00-1	hydrofluoric acid ... %	B	231-634-8	7664-39-3	T+; R26/27/28 C; R35	T+; C R: 26/27/28-35 S: (1/2-7/9-26-36/37-45	C ≥ 7 %; T+; C; R26/27/28-35 1 % ≤ C < 7 %; T; R23/24/25-34 0,1 % ≤ C < 1 %; Xn; R20/21/22-36/37/38	
015-039-00-9	azinphos-methyl (ISO) O,O-dimethyl 4-oxobenzotriazin-3-ylmethyl phosphorodithioate		201-676-1	86-50-0	T+; R26/28 T; R24 R43 N; R50-53	T+; N R: 24-26/28-43-50/53 S: (1/2-28-36/37-45-60-61		
015-048-00-8	fenthion (ISO) O,O-dimethyl-O-(4-methylthio-m-tolyl)phosphorothioate		200-231-9	55-38-9	Muta. Cat. 3; R40 T; R23-48/25 Xn; R21/22 N; R50-53	T; N R: 21/22-23-40-48/25-50/53 S: (1/2-36/37-45-60-61		
015-056-00-1	azinphos-ethyl (ISO) O,O-diethyl 4-oxobenzotriazin-3-ylmethylphosphorodithioate		220-147-6	2642-71-9	T+; R28 T; R24 N; R50-53	T+; N R: 24-28-50/53 S: (1/2-28-36/37-45-60-61		
015-140-00-8	triazophos (ISO) O,O-diethyl-O-1-phenyl-1H,2,4-triazol-3-ylphosphorothioate		245-986-5	24017-47-8	T; R23/25 Xn; R21 N; R50-53	T; N R: 21-23/25-50/53 S: (1/2-36/37-45-60-61		
016-013-00-X	sulphur dichloride		234-129-0	10545-99-0	R14 C; R34 N; R50	C; N R: 14-34-50 S: (1/2-26-36/37/39-45-61	C ≥ 10 %; C; R34 5 % ≤ C < 10 %; Xi; R36/37/38	

Index No	Chemical name	Notes related to substances	EC No	CAS No	Classification	Labelling	Concentration limits	Notes related to preparations
016-014-00-5	sulphur tetrachloride		—	13451-08-6	R14 C; R34 N; R50	C; N R: 14-34-50 S: (1/2-)/26-36/37/39-45-61	C ≥ 10 %; C; R34 5 % ≤ C < 10 %; Xi; R36/37/38	
016-023-00-4	dimethyl sulphate	E	201-058-1	77-78-1	Carc. Cat. 2; R45 Muta. Cat. 3; R40 T+; R26 T; R25 C; R34 R43	T+ R: 45-25-26-34-43 S: 53-45	C ≥ 25 %; T+; R45-25-26-34-43 10 % ≤ C < 25 %; T+; R45-22-26-34-43 7 % ≤ C < 10 %; T+; R45-22-26-36/37/38-43 5 % ≤ C < 7 %; T; R45-22-23-36/37/38-43 3 % ≤ C < 5 %; T; R45-22-23-43 1 % ≤ C < 3 %; T; R45-23-43 0,1 % ≤ C < 1 %; T; R45-20 0,01 % ≤ C < 0,1 %; T; R45	
016-024-00-X	dimexano (ISO) bis(methoxythiocarbonyl) disulphide		215-993-8	1468-37-7	Xn; R22 N; R50-53	Xn; N R: 22-50/53 S: (2-)/60-61		
016-071-00-6	trisodium-3-amino-6,1,3-dichloro-10-((3-((4-chloro-6-(2-sulphophenylamino)-1,3,5-triazin-2-yl)amino)propyl)amino)-4,1,1-triphenoxydioxazinedisulfonate		410-130-3	136248-03-8	R43	Xi R: 43 S: (2-)/22-24-37		
022-001-00-5	titanium tetrachloride		231-441-9	7550-45-0	R14 C; R34	C R: 14-34 S: (1/2-)/8-26-36/37/39-45	C ≥ 10 %; C; R34 5 % ≤ C < 10 %; Xi; R36/37/38	
030-004-00-8	dimethylzinc [1] diethylzinc [2]		208-884-1 [1] 209-161-3 [2]	544-97-8 [1] 557-20-0 [2]	R14 F; R17 C; R34 N; R50-53	F; C; N R: 14-17-34-50/53 S: (1/2-)/16-43-45-60-61		
050-002-00-0	cynhexatin (ISO) hydroxytricyclohexylstannane tri(cyclohexyl)tin hydroxide		236-049-1	13121-70-5	Xn; R20/21/22 N; R50-53	Xn; N R: 20/21/22-50/53 S: (2-)/13-60-61		

Index No	Chemical name	Notes related to substances	EC No	CAS No	Classification	Labelling	Concentration limits	Notes related to preparations
050-012-00-5	tetracyclohexylstannane [1] chlorotricyclohexylstannane [2] butyltricyclohexylstannane [3]		215-910-5 [1] 221-437-5 [2] 230-358-5 [3]	1449-55-4 [1] 3091-32-5 [2] 7067-44-9 [3]	Xn; R20/21/22 N; R50-53	Xn; N R: 20/21/22-50/53 S: (2-)26-28-60-61	C ≥ 1 %; Xn; R20/21/22	1
050-017-00-2	fenbutatin oxide (ISO) bis(tris(2-methyl-2-phenylpropyl)tin)oxide		236-407-7	13356-08-6	T+; R26 Xi; R36/38 N; R50/53	T+; N R: 26-36/38-50/53 S: (1/2-)28-36/37-45-60-61		
082-009-00-X	lead sulfochromate yellow C.I. Pigment Yellow 34 [This substance is identified in the Colour Index by Colour Index Constitution Number, C.I. 77603.]		215-693-7	1344-37-2	Carc. Cat. 3; R40 Repr. Cat. 1; R61 Repr. Cat. 3; R62 R33 N; R50-53	T; N R: 61-33-40-50/53-62 S: 53-45-60-61		1
082-010-00-5	lead chromate molybdate sulfate red C.I. Pigment red 104. [This substance is identified in the Colour Index by Colour Index Constitution Number, C.I. 77605.]		235-759-9	12656-85-8	Carc. Cat. 3; R40 Repr. Cat. 1; R61 Repr. Cat. 3; R62 R33 N; R50-53	T; N R: 61-33-40-50/53-62 S: 53-45-60-61		1
601-024-00-X	cumene [1] propylbenzene [2]		202-704-5 [1] 203-132-9 [2]	98-82-8 [1] 103-65-1 [2]	R10 Xn; R65 Xi; R37 N; R51-53	Xn; N R: 10-37-51/53-65 S: (2-)24-37-61-62		4
601-032-00-3	benzo[a]pyrene benzo[<i>def</i>]chrysene		200-028-5	50-32-8	Carc. Cat.2; R45 Muta. Cat. 2; R46 Repr. Cat. 2; R60-61 N; R50-53	T; N R: 45-46-60-61-50/53 S: 53-45-60-61		
601-034-00-4	benz[e]acephenanthrylene		205-911-9	205-99-2	Carc. Cat.2; R45 N; R50-53	T; N R: 45-50/53 S: 53-45-60-61		
602-035-00-2	1,4-dichlorobenzene <i>p</i> -dichlorobenzene		203-400-5	106-46-7	Xi; R36 N; R50-53	Xi; N R: 36-50/53 S: (2-)24/25-46-60-61		
602-054-00-6	3-iodopropene allyl iodide		209-130-4	556-56-9	R10 C; R34	C R: 10-34 S: (1/2-)7-26-45		

Index No	Chemical name	Notes related to substances	EC No	CAS No	Classification	Labelling	Concentration limits	Notes related to preparations
603-076-00-9	but-2-yne-1,4-diol 2-butyne-1,4-diol		203-788-6	110-65-6	T; R23/25 Xn; R21-48/22 C; R34	T R: 21-23/25-34-48/22 S: (1/2)-26-36/37/39-45	C ≥ 50 %; T; R21-23/25-34-48/22 25 % ≤ C < 50 %; T; R21-23/25-36/38-48/22 10 % ≤ C < 25 %; Xn; R20/22-48/22 3 % ≤ C < 10 %; Xn; R20/22	
603-091-00-0	exo-1-methyl-4-(1-methylethyl)-7-oxabicyclo[2.2.1]heptan-2-ol		402-470-6	87172-89-2	O; R8 Xn; R22 Xi; R36	O; Xn R: 8-22-36 S: (2-)26		
603-093-00-1	exo-(+)-1-methyl-4-(1-methylethyl)-2-[(2-methylphenyl)methoxy]-7-oxabicyclo[2.2.1]heptane		402-410-9	87818-31-3	Xn; R20 N; R51-53	Xn; N R: 20-51/53 S: (2-)23-61		
603-097-00-3	1,1',1''-nitrotripropan-2-ol triisopropanolamine		204-528-4	122-20-3	Xi; R36 R52-53	Xi R: 36-52/53 S: (2-)26-61		
603-117-00-0	propan-2-ol isopropyl alcohol isopropanol		200-661-7	67-63-0	F; R11 Xi; R36 R67	F; Xi R: 11-36-67 S: (2-)7-16-24/25-26		6
604-020-00-6	biphenyl-2-ol 2-hydroxybiphenyl 2-phenylphenol (ISO)		201-993-5	90-43-7	Xi; R36/37/38 N; R50	Xi; N R: 36/37/38-50 S: (2-)22-61		
604-021-00-1	2-phenylphenol, sodium salt sodium 2-biphenylate		205-055-6	132-27-4	Xn; R22 Xi; R37/38-41 N; R50	Xn; N R: 37/38-41-50 S: (2-)22-26-61		
604-024-00-8	4,4'-isobutylethylenediphenol (alt.); 2,2-bis(4'-hydroxyphenyl)-4-methylpentane		401-720-1	6807-17-6	Repr. Cat. 2; R60 Xi; R36 N; R50-53	T; N R: 60-36-50/53 S: 53-45-60-61		
604-041-00-0	acifluorfen [1] acifluorfen-sodium [2] 5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-nitrobenzoic acid [1] sodium-5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-nitrobenzoate [2]		256-634-5 [1] 263-560-7 [2]	50594-66-6 [1] 62476-59-9 [2]	Xn; R22 Xi; R38-41 N; R50-53	Xn; N R: 22-38-41-50/53 S: (2-)24-39-60-61		

Index No	Chemical name	Notes related to substances	EC No	CAS No	Classification	Labelling	Concentration limits	Notes related to preparations
604-043-00-1	monobenzene 4-hydroxyphenyl benzyl ether hydroquinone monobenzyl ether		203-083-3	103-16-2	Xi; R36 R43	Xi R: 36-43 S: (2-)24/25-26-37		
604-044-00-7	mequinol 4-methoxyphenol hydroquinone monomethyl ether		205-769-8	150-76-5	Xn; R22 Xi; R36 R43	Xn R: 22-36-43 S: (2-)24/25-26-37/39-46		
605-016-00-7	glyoxal ... % ethandial ... %	B	203-474-9	107-22-2	Muta. Cat. 3; R40 Xn; R20 Xi; R36/38 R43	Xn R: 20-36/38-40-43 S: (2-)36/37	C $\geq$ 10%; Xn; R20-36/38-40-43 1 % $\leq$ C < 10%; Xn; R40-43	
606-016-00-X	pindone (ISO) 2-pivaloylindan-1,3-dione		201-462-8	83-26-1	T; R25-48/25 N; R50-53	T; N R: 25-48/25-50/53 S: (1/2-)37-45-60-61		
606-018-00-0	dichlone (ISO) 2,3-dichloro-1,4-naphthoquinone		204-210-5	117-80-6	Xn; R22 Xi; R36/38 N; R50-53	Xn; N R: 22-36/38-50/53 S: (2-)26-60-61		
606-019-00-6	chlordecone (ISO) perchloropentacyclo[5,3,0,0 ^{2,6} ,0 ^{3,9} ,0 ^{4,8}] decan-5-one decachloropentacyclo[5,2,1,0 ^{2,6} ,0 ^{3,9} ,0 ^{5,8}] decan-4-one		205-601-3	143-50-0	Carc. Cat. 3; R40 T; R24/25 N; R50-53	T; N R: 24/25-40-50/53 S: (1/2-)22-36/37-45-60-61		
606-034-00-8	metribuzin (ISO) 4-amino-6-tert-butyl-3-methylthio-1,2,4-triazin-5(4H)-one 4-amino-4,5-dihydro-6-(1,1-dimethylethyl)-3-methylthio-1,2,4-triazin-5-one		244-209-7	21087-64-9	Xn; R22 N; R50-53	Xn; N R: 22-50/53 S: (2-)60-61		
606-035-00-3	chloridazon (ISO) 5-amino-4-chloro-2-phenylpyridazine-3-(2H)-one pyrazon		216-920-2	1698-60-8	R43 N; R50-53	Xi; N R: 43-50/53 S: (2-)24-37-60-61		
606-036-00-9	quinomethionate chinomethionat (ISO) 6-methyl-1,3-dithiolo(4,5-b)quinoxalin-2-one		219-455-3	2439-01-2	Repr. Cat. 3; R62 Xn; R20/21/22-48/22 Xi; R36 R43 N; R50-53	Xn; N R: 20/21/22-36-43-48/22-50/53-62 S: (2-)24-37-60-61		
606-037-00-4	triadimefon (ISO) 1-(4-chlorophenoxy)-3,3-dimethyl-1-(1,2,4-triazol-1-yl)butanone		256-103-8	43121-43-3	Xn; R22 N; R51-53	Xn; N R: 22-51/53 S: (2-)61		

Index No	Chemical name	Notes related to substances	EC No	CAS No	Classification	Labelling	Concentration limits	Notes related to preparations
606-044-00-2	2,4,6-trimethylbenzophenone		403-150-9	954-16-5	Xn; R22 Xi; R36 N; R50-53	Xn; N R: 22-36-50/53 S: (2-)26-60-61		
607-043-00-X	dicamba (ISO) 2,5-dichloro-6-methoxybenzoic acid 3,6-dichloro-2-methoxybenzoic acid		217-635-6	1918-00-9	Xn; R22 Xi; R41 R52-53	Xn; N R: 22-41-52/53 S: (2-)26-61		
607-057-00-6	coumachlor (ISO) 3-[1-(4-chlorophenyl)-3-oxobutyl]-4-hydroxy-coumarin		201-378-1	81-82-3	Xn; R48/22 R52-53	Xn R: 48/22-52/53 S: (2-)37-61		
607-058-00-1	cuomafuryl (ISO) fumarin (RS)-3-(1-(2-furyl)-3-oxobutyl)-4-hydroxycoumarin 4-hydroxy-3-[3-oxo-1-(2-furyl)butyl]coumarin		204-195-5	117-52-2	T; R25-48/25 R52-53	T R: 25-48/25-52/53 S: (1/2-)37-45-61		
607-079-00-6	kelevan (ISO) ethyl-5-(perchloro-5-hydroxypentacyclo(5,3,0,0 ^{2,6} ,0 ^{3,9} ,0 ^{4,8})decan-5-yl)-4-oxopentanoate ethyl-5-(1,2,3,5,6,7,8,9,10,10-decachloro-4-hydroxypentacyclo(5,2,1,0 ^{2,6} ,0 ^{3,9} ,0 ^{5,8})dec-4-yl)-4-oxovalerate		—	4234-79-1	T; R24 Xn; R22 N; R51-53	T; N R: 22-24-51/53 S: (1/2-)36/37-45-61		
607-097-00-4	benzene-1,2,4-tricarboxylic acid 1,2-anhydride trimellitic anhydride		209-008-0	552-30-7	Xi; R37-41 R42/43	Xn R: 37-41-42/43 S: (2-)22-26-36/37/39		
607-143-00-3	valeric acid		203-677-2	109-52-4	C; R34 R52-53	C R: 34-52/53 S: (1/2-)26-36-45-61		
607-152-00-2	2,3,6-TBA (ISO) 2,3,6-trichlorobenzoic acid		200-026-4	50-31-7	Xn; R22 N; R51-53	Xn; N R: 22-51/53 S: (2-)61		
607-153-00-8	benazolin (ISO) 4-chloro-2,3-dihydro-2-oxo-1,3-benzothiazol-3-ylacetic acid		223-297-0	3813-05-6	Xi; R36/38 R52-53	Xi R: 36/38-52/53 S: (2-)22-61		
607-156-00-4	chlorfenson (ISO) 4-chlorophenyl-4-chlorobenzenesulfonate		201-270-4	80-33-1	Xn; R22 Xi; R38 N; R50-53	Xn; N R: 22-38-50/53 S: (2-)37-60-61		
607-158-00-5	sodium salt of chloroacetic acid sodium chloroacetate		223-498-3	3926-62-3	T; R25 Xi; R38 N; R50	T; N R: 25-38-50 S: (1/2-)22-37-45-61		

Index No	Chemical name	Notes related to substances	EC No	CAS No	Classification	Labelling	Concentration limits	Notes related to preparations
607-159-00-0	chlorobenzilate (ISO) ethyl 2,2-di(4-chlorophenyl)-2-hydroxyacetate ethyl-4,4'-dichlorobenzilate		208-110-2	510-15-6	Xn; R22 N; R50-53	Xn; N R: 22-50/53 S: (2-)60-61		
607-176-00-3	A mixture of: α -3-(3-(2H-benzotriazol-2-yl)-5-tert-butyl-4-hydroxyphenyl)propionyl- ω -hydroxypoly(oxyethylene); α -3-(3-(2H-benzotriazol-2-yl)-5-tert-butyl-4-hydroxyphenyl)propionyl- ω -3-(3-(2H-benzotriazol-2-yl)-5-tert-butyl-4-hydroxyphenyl)propionyloxypoly(oxyethylene)		400-830-7	—	R43 N; R51-53	Xi; N R: 43-51/53 S: (2-)36/37-61		
607-188-00-9	hydrogen sodium N-carboxylatoethyl-N-octadec-9-enylmaleamate		402-970-4	—	R43 N; R51-53	Xi; N R: 43-51/53 S: (2-)24/37-61		
607-209-00-1	A mixture of: O,O-di(1-methylethyl)trithio-bis-thioformate; O,O-di(1-methylethyl)tetrathio-bis-thioformate; O,O-di(1-methylethyl)pentathio-bis-thioformate		403-030-6	—	Xn; R22 R43 N; R50-53	Xn; N R: 22-43-50/53 S: (2-)36/37-60-61		
607-213-00-3	ethyl-3,3-bis[(1,1-dimethylpropyl)peroxy]butyrate		403-320-2	67567-23-1	E; R2 O; R7 R10 N; R51-53	E; N R: 2-7-10-51/53 S: (2-)3/7-14-33-36/37/39-61		
607-217-00-5	2-ethoxyethyl-2-[4-(2,6-dihydro-2,6-dioxo-7-phenyl-1,5-dioxaindacen-3-yl)phenoxy]acetate		403-960-2	—	R43 R53	Xi R: 43-53 S: (2-)24-37-61		
607-243-00-7	sodium 3,6-dichloro-o-anisate [1] 3,6-dichloro-o-anisic acid, compound with 2,2'-iminodietanol (1:1) [2] 3,6-dichloro-o-anisic acid, compound with 2-aminoethanol (1:1) [3]	217-846-3 [1] 246-590-5 [2] 258-527-9 [3]	217-846-3 [1] 246-590-5 [2] 258-527-9 [3]	1982-69-0 [1] 25059-78-3 [2] 53404-28-7 [3]	R52-53	R: 52/53 S: 61		
607-248-00-4	naptalam-sodium sodium N-naphth-1-ylphthalamate		205-073-4	132-67-2	Xn; R22	Xn R: 22 S: (2)		

Index No	Chemical name	Notes related to substances	EC No	CAS No	Classification	Labelling	Concentration limits	Notes related to preparations
607-249-00-X	(1-methyl-1,2-ethanediyl)bis[oxymethyl-2,1-ethanediyl]diacrylate tripropylene glycol diacrylate TPGDA		256-032-2	42978-66-5	Xi; R36/37/38 R43 N; R51-53	Xi; N R: 36/37/38-43-51/53 S: (2-)24-37-61	C ≥ 10 %; Xi; R36/37/38-43 1 % ≤ C < 10 %; Xi; R43	
607-252-00-6	lambda-cyhalothrin (ISO) a 1:1 mixture of (S)-α-cyano-3-phenoxybenzyl (Z)-(1R)-cis-3-(2-chloro-3,3,3-trifluoropropenyl)- 2,2-dimethylcyclopropanecarboxylate and (R)-α-cyano-3-phenoxybenzyl (Z)-(1S)-cis- 3-(2-chloro-3,3,3-trifluoropropenyl)- 2,2-dimethylcyclopropanecarboxylate		415-130-7	91465-08-6	T+; R26 T; R25 Xn; R21 N; R50-53	T+; N R: 21-25-26-50/53 S: (1/2-)28-36/37/39-38-45-60-61		
607-255-00-2	fluroxypyr (ISO) 4-amino-3,5-dichloro-6-fluoro- 2-pyridyloxyacetic acid		—	69377-81-7	R52-53	R: 52/53 S: 61		
608-003-00-4	acrylonitrile	D E	203-466-5	107-13-1	F; R11 Carc. Cat. 2; R45 T; R23/24/25 Xi; R37/38-41 R43 N; R51-53	F; T; N R: 45-11-23-/24/25-37/38-41- 43-51/53 S: 9-16-53-45-61	C ≥ 20 %; T; R45-23/24/25-37/38- 41-43 10 % ≤ C < 20 %; T; R45-23/24/25-41-43 5 % ≤ C < 10 %; T; R45-23/24/25-36-43 1 % ≤ C < 5 %; T; R45-23/24/25-43 0,2 % ≤ C < 1 %; T; R45-20/21/22 0,1 % ≤ C < 0,2 %; T; R45	
608-016-00-5	1,4-Dicyano-2,3,5,6-tetra-chloro-benzene		401-550-8	1897-41-2	R43 N; R50-53	Xi; N R: 43-50/53 S: (2-)24-37-60-61		
609-030-00-4	dinoterb (ISO) 2-tert-butyl-4,6-dinitrophenol	E	215-813-8	1420-07-1	Repr. Cat. 2; R61 T+; R28 T; R24 R44 N; R50-53	T+; N R: 61-24-28-44-50/53 S: 53-45-60-61		
609-040-00-9	nitrofen (ISO) 2,4-dichlorophenyl 4-nitrophenyl ether	E	217-406-0	1836-75-5	Carc. Cat. 2; R45 Repr. Cat. 2; R61 Xn; R22 N; R50-53	T; N R: 45-61-22-50/53 S: 53-45-60-61		
609-044-00-0	tecnazene (ISO) 1,2,4,5-tetrachloro-3-nitrobenzene		204-178-2	117-18-0	Xn; R22 R43 N; R50-53	Xn; N R: 22-43-50/53 S: (2-)24-37-60-61		

Index No	Chemical name	Notes related to substances	EC No	CAS No	Classification	Labelling	Concentration limits	Notes related to preparations
611-008-00-4	4-aminoazobenzene 4-phenylazoaniline		200-453-6	60-09-3	Carc. Cat. 2; R45 N; R50-53	T; N R: 45-50/53 S: 53-45-60-61		
611-013-00-1	trilithium 1-hydroxy-7-(3-sulfonatoanilino)- 2-(3-methyl-4-(2-methoxy-4- (3-sulfonatophenylazo)phenylazo)phenylazo) naphthalene-3-sulfonate		403-650-7	117409-78-6	E; R2 N; R51-53	E; N R: 2-51/53 S: (2-)35-61		
611-031-00-X	4,4'-(4-iminocyclohexa-2,5-dienyldienemethyl- ene)dianiline hydrochloride C.I. Basic Red 9		209-321-2	569-61-9	Carc. Cat. 2; R45	T R: 45 S: 53-45		
612-035-00-4	2-methoxyaniline o-anisidine	E	201-963-1	90-04-0	Carc. Cat. 2; R45 Muta. Cat. 3; R40 T; R23/24/25	T R: 45-23/24/25 S: 53-45		
612-042-00-2	benzidine 1,1'-biphenyl-4,4'-diamine 4,4'-diaminobiphenyl biphenyl-4,4'-ylenediamine	E	202-199-1	92-87-5	Carc. Cat. 1; R45 Xn; R22 N; R50-53	T; N R: 45-22-50/53 S: 53-45-60-61	C ≥ 25 %; T; R45-22 0,01 % ≤ C < 25 %; T; R45	
612-051-00-1	4,4'-diaminodiphenylmethane 4,4'-methylenedianiline	E	202-974-4	101-77-9	Carc. Cat. 2; R45 Muta. Cat. 3; R40 T; R39/23/24/25Xn; R48/20/21/22 R43 N; R51-53	T; N R: 45-39/23/24/25-43-48/20/ 21/22-51/53 S: 53-45-61		
612-081-00-5	salts of 4,4'-bi-o-tolidine salts of 3,3'-dimethylbenzidine salts of o-tolidine	A E	210-322-5 265-294-7 277-985-0	612-82-8 64969-36-4 74753-18-7	Carc. Cat. 2; R45 Xn; R22 N; R51-53	T; N R: 45-22-51/53 S: 53-45-61		
612-099-00-3	4-methyl-m-phenylenediamine 2,4-toluenediamine	E	202-453-1	95-80-7	Carc. Cat. 2; R45 T; R25 Xn; R21 Xi; R36 R43 N; R51-53	T; N R: 45-21-25-36-43-51/53 S: 53-45-61		
612-105-00-4	2-piperazin-1-ylethylamine		205-411-0	140-31-8	Xn, R21/22 C; R34 R43 R52-53	C R: 21/22-34-43-52/53 S: (1/2)-26-36/37/39-45-61		

Index No	Chemical name	Notes related to substances	EC No	CAS No	Classification	Labelling	Concentration limits	Notes related to preparations
612-111-00-7	2-methyl- <i>m</i> -phenylenediamine 2,6-toluenediamine		212-513-9	823-40-5	Muta. Cat. 3; R40 Xn; R21/22 R43 N; R51-53	Xn; N R: 21/22-40-43-51/53 S: (2-)24-36/37-61		
612-125-00-3	2-methyl- <i>p</i> -phenylenediamine 2,5-toluenediamine		202-442-1	95-70-5	T; R25 Xn; R20/21 R43 N; R51-53	T; N R: 20/21-25-43-51/53 S: (1/2-)24-37-45-61		
612-144-00-7	flumetralin (ISO) N-(2-chloro-6-fluorobenzyl)-N-ethyl- α,α -trifluoro-2,6-dinitro- <i>p</i> -toluidine		—	62924-70-3	Xi; R36/38 R43 N; R50-53	Xi; N R: 36/38-43-50/53 S: (2-)36/37-60-61		
612-151-00-5	diaminotoluene	E	246-910-3	25376-45-8	Carc. Cat. 2; R45 T; R25 Xn; R20/21 Xi; R36 R43 N; R51-53	T; N R: 45-20/21-25-36-43-51/53 S: 53-45-61		
613-018-00-4	morfamquat (ISO) 1,1'-bis(3,5-dimethylmorpholinocarbonylmethyl)-4,4'-bipyridilium ion		—	7411-47-4	Xn; R22 Xi; R36/37/38 R52-53	Xn R: 22-36/37/38-52/53 S: (2-)22-36-61		
613-031-00-5	synclosene trichloroisocyanuric acid trichloro-1,3,5-triazinetron		201-782-8	87-90-1	O; R8 Xn; R22 R31 Xi; R36/37 N; R50-53	O; Xn; N R: 8-22-31-36/37-50/53 S: (2-)8-26-41-60-61		
613-038-00-3	6-phenyl-1,3,5-triazine-2,4-diyldiamine 6-phenyl-1,3,5-triazine-2,4-diamine benzoguanamine		202-095-6	91-76-9	Xn; R22 R52-53	Xn R: 22-52/53 S: (2-)61		
613-042-00-5	imazalil (ISO) 1-[2-(allyloxy)-2-(2,4-dichlorophenyl)ethyl]-1H-imidazole		252-615-0	35554-44-0	Xn; R20/22 N; R41 N; R50-53	Xn; N R: 20/22-41-50/53 S: (2-)26-39-60-61		
613-043-00-0	imazalil sulphate (ISO) 1-[2-(allyloxy)ethyl-2-(2,4-dichlorophenyl)]-1H-imidazolium hydrogen sulphate [1] (±)-1-[2-(allyloxy)ethyl-2-(2,4-dichlorophenyl)]-1H-imidazolium hydrogen sulphate [2]		261-351-5 [1] 281-291-3 [2]	58594-72-2 [1] 83918-57-4 [2]	Xn; R20/22 Xi; R41 N; R50-53	Xn; N R: 20/22-41-50/53 S: (2-)26-39-60-61		

Index No	Chemical name	Notes related to substances	EC No	CAS No	Classification	Labelling	Concentration limits	Notes related to preparations
613-066-00-6	terbutometon (ISO) 2- <i>tert</i> -butylamino-4-ethylamino-6-methoxy-1,3,5-triazin		251-637-8	33693-04-8	Xn; R22 N; R50-53	Xn; N R: 22-50/53 S: (2-)60-61		
613-091-00-2	morfamquat dichloride [1] morfamquat sulfate [2]		225-062-8 [1]	4636-83-3 [1] 29873-36-7 [2]	Xn; R22 Xi; R36/37/38 R52-53	Xn; R: 22-36/37/38-52/53 S: (2-)22-36-61		
613-098-00-0	N-(n-octyl)-2-pyrrolidinone		403-700-8	2687-94-7	C; R34 N; R51-53	C; N R: 34-51/53 S: (1/2-)23-26-36/37/39-45-61		
613-130-00-3	hexazonazole (ISO) (RS)-2-(2,4-dichlorophenyl)-1-(1H-1,2,4-triazol-1-yl)hexan-2-ol		—	79983-71-4	R43 N; R51-53	Xi; N R: 43-51/53 S: (2-)24-37-61		
613-131-00-9	pyroquilon (ISO) 1,2,5,6-tetrahydropyrrolo[3,2,1- <i>ij</i>]quinolin-4-one		—	57369-32-1	Xn; R22 R52-53	Xn R: 22-52/53 S: (2-)61		
613-134-00-5	myclobutanil (ISO) 2-(4-chlorophenyl)-2-(1H-1,2,4-triazol-1-ylmethyl)hexanenitrile		—	88671-89-0	Repr. Cat. 3; R63 Xn; R22 Xi; R36 N; R51-53	Xn; N R: 22-36-51/53-63 S: (2-)36/37-46-61		
613-137-00-1	methabenzthiazuron (ISO) 1-(1,3-benzothiazol-2-yl)-1,3-dimethylurea		242-505-0	18691-97-9	N; R50-53	N R: 50/53 S: 60-61		
613-139-00-2	met sulfuron-methyl methyl 2-(4-methoxy-6-methyl-1,3,5-triazin-2-yl)carbamoylsulfamoyl benzoate		—	74223-64-6	N; R50-53	N R: 50/53 S: 60-61		
614-001-00-4	nicotine (ISO) 3-(N-methyl-2-pyrrolidinyl)pyridine		200-193-3	54-11-5	T+; R27 T; R25 N; R51-53	T+; N R: 25-27-51/53 S: (1/2-)36/37-45-61		
614-006-00-1	brucine 2,3-dimethoxystrychnine		206-614-7	357-57-3	T+; R26/28 R52-53	T+ R: 26/28-52/53 S: (1/2-)13-45-61		

Index No	Chemical name	Notes related to substances	EC No	CAS No	Classification	Labelling	Concentration limits	Notes related to preparations
614-007-00-7	brucine sulphate [1] brucine nitrate [2] Strychnidin-10-one, 2,3-dimethoxy-, mono[(R)-1-methylheptyl 1,2-benzenedicarboxylate] [3] Strychnidin-10-one, 2,3-dimethoxy-, compd. with (S)-mono(1-methylheptyl-1,2-benzenedicarboxylate (1:1) [4]		225-432-9 [1] 227-317-9 [2] 269-439-5 [3] 269-710-8 [4]	4845-99-2 [1] 5786-97-0 [2] 68239-26-9 [3] 68310-42-9 [4]	T+; R26/28 R52-53	T+ R: 26/28-52/53 S: (1/2)-13-45-61		
615-006-00-4	2-methyl-m-phenylene diisocyanate [1] 4-methyl-m-phenylene diisocyanate [2] m-tolylidene diisocyanate [3] toluene 2,6-di-isocyanate [1] toluene 2,4-di-isocyanate [2] toluene-diisocyanate [3]	C	202-039-0 [1] 209-544-5 [2] 247-722-4 [3]	91-08-7 [1] 584-84-9 [2] 26471-62-5 [3]	Carc. Cat. 3; R40 T+; R26 Xi; R36/37/38 R42/43 R52-53	T+ R: 26-36/37/38-40-42/43-52/53 S: (1/2)-23-36/37-45-61	C ≥ 20%; T+; R26-36/37/38-40-42/43 7% ≤ C < 20%; T+; R26-40-42/43 1% ≤ C < 7%; T; R23-40-42/43 0.1% ≤ C < 1%; Xn; R20-42	2
616-010-00-9	tosylchloramide sodium		204-854-7	127-65-1	Xn; R22 R31 C; R34 R42	C R: 22-31-34-42 S: (1/2)-7-22-26-36/37/39-45		
616-034-00-X	pyracarbolid (ISO) 3,4-dihydro-6-methyl-2H-pyran-5-carboxanilide		246-419-4	24691-76-7	R52-53	R: 52/53 S: 61		
616-035-00-5	cymoxanil 2-cyano-N-[(ethylamino)carbonyl]-2-(methoxymino)acetamide		261-043-0	57966-95-7	Xn; R22 R43 N; R50-53	Xn; N R: 22-43-50/53 S: (2)-36/37-60-61		
617-004-00-9	1,2,3,4-tetrahydro-1-naphthyl hydroperoxide		212-230-0	771-29-9	O; R7 Xn; R22 C; R34 N; R50-53	O; C; N R: 7-22-34-50/53 S: (1/2)-3/7-14-26 -36/37/39-45-60-61	C ≥ 25%; C; R22-34 10% ≤ C < 25%; C; R34 5% ≤ C < 10%; Xi; R36/37/38	
617-006-00-X	bis(α,α-dimethylbenzyl) peroxide		201-279-3	80-43-3	O; R7 Xi; R36/38 N; R51-53	O; Xi; N R: 7-36/38-51/53 S: (2)-3/7-14-36/37/39-61		
617-008-00-0	dibenzoyl peroxide benzoyl peroxyde		202-327-6	94-36-0	E; R2 Xi; R36 R43	E; Xi; R: 2-36-43 S: (2)-3/7-14-36/37/39		
650-007-00-3	chlordimeform (ISO) N ² -(4-Chlor-o-tolyl)-N ¹ ,N ¹ -dimethylformamidine		228-200-5	6164-98-3	Carc. Cat. 3; R40 Xn; R21/22 N; R50-53	Xn; N R: 21/22-40-50/53 S: (2)-22-36/37-60-61		

Index No	Chemical name	Notes related to substances	EC No	CAS No	Classification	Labelling	Concentration limits	Notes related to preparations
650-008-00-9	drazoxolon (ISO) 4-(2-chlorophenylhydrazono)-3-methyl-5-isoxazalone		227-197-8	5707-69-7	T; R25 N; R50-53	T; N R: 25-50/53 S: (1/2-)22-24-36/37-45-60-61		
650-009-00-4	chlordimeform hydrochloride N'-(4-chloro-o-tolyl)-N,N'-dimethylformamidinemonohydrochloride N ² -(4-chloro-o-tolyl)-N ¹ ,N ¹ -dimethylformamidinehydrochloride		243-269-1	19750-95-9	Carc. Cat. 3; R40 Xn; R22 N; R50-53	Xn; N R: 22-40-50/53 S: (2-)22-36/37-60-61		
650-033-00-5	esfenvalerate (ISO) (S)- α -cyano-3-phenoxybenzyl-(S)-2-(4-chlorophenyl)-3-methylbutyrate		—	66230-04-4	T; R23/25 R43 N; R50-53	T; N R: 23/25-43-50/53 S: (1/2-)24-36/37/39-45-60-61		
650-041-00-9	triasulfuron (ISO) 1-[2-(2-chloroethoxy)phenylsulfonyl]-3-(4-methoxy-6-methyl-1,3,5-triazin-2-yl)urea		—	82097-50-5	N; R50-53	N R: 50/53 S: 60-61		

ANNEX 1D

Index No	Chemical name	Notes related to substances	EC No	CAS No	Classification	Labelling	Concentration limits	Notes related to preparations
006-090-00-8	2-(3-iodoprop-2-yn-1-yloxy)ethyl phenylcarbamate		408-010-0	88558-41-2	Xn; R20 Xi; R41 R52-53	Xn R: 20-41-52/53 S: (2-)22-26-39-61		
014-016-00-0	A mixture of: 1,3-dihex-5-en-1-yl-1,1,3,3-tetramethyldisiloxane; 1,3-dihex-n-en-1-yl-1,1,3,3-tetramethyldisiloxane		406-490-6	—	N; R51-53	N R: 51/53 S: 61		
015-164-00-9	calcium-P,P'-(1-hydroxyethylene)bis(hydrogenphosphonate)dihydrate		400-480-5	36669-85-9	R52-53	R: 52/53 S: 61		
015-165-00-4	A mixture of: thiobis(4,1-phenylene)-S,S',S',S'-tetraphenyldisulfoniumbis(hexafluorophosphate)diphenyl (4-phenylthiophenyl)sulfonium hexafluorophosphate		404-986-7	—	Xi; R41 N; R50-53	Xi; N R: 41-50/53 S: (2-)15-26-39-60-61		
015-166-00-X	3,9-bis(2,6-di-tert-butyl-4-methylphenoxy)-2,4,8,10-tetraoxa-3,9-diphosphaspiro[5,5]undecane		410-290-4	80693-00-1	R53	R: 53 S: 61		
015-167-00-5	3-(hydroxyphenylphosphinyl)propanoic acid		411-200-6	14657-64-8	Xi; R41	Xi R: 41 S: (2-)26-39		
601-050-00-1	benzene, C ₁₀₋₁₃ -alkyl derivatives		267-051-0	67774-74-7	N; R50	N R: 50 S: 61		
601-051-00-7	4-phenylbut-1-ene		405-980-7	768-56-9	Xi; R38 N; R51-53	Xi; N R: 38-51/53 S: (2-)37-61		
602-083-00-4	diphenyl ether, pentabromo derivative pentabromodiphenyl ether		251-084-2	32534-81-9	Xn; R48/21/22 R64 N; R50-53	Xn; N R: 48/21/22-50/53-64 S: (1/2-)36/37-45-60-61		
602-084-00-X	1,1-dichloro-1-fluoroethane		404-080-1	1717-00-6	N; R52-53-59	N R: 52/53-59 S: 59-61		
603-128-00-0	2-(phenylmethoxy)naphthalene		405-490-3	613-62-7	R53	R: 53 S: 61		

Index No	Chemical name	Notes related to substances	EC No	CAS No	Classification	Labelling	Concentration limits	Notes related to preparations
603-129-00-6	1-tert-butoxypropan-2-ol		406-180-0	57018-52-7	R10 Xi; R41	Xi R: 10-41 S: (2)-26-39		
603-130-00-1	A mixture of isomers of: α -(dimethylbiphenyl)- ω -hydroxypoly (oxyethylene)		406-325-8	—	Xn; R22 R52-53	Xn R: 22-52/53 S: (2)-39-61		
603-131-00-7	A 3:1 mixture of: 1-deoxy-1-[methyl-(1-oxododecyl)amino]-D-glucitol; 1-deoxy-1-[methyl-(1-oxotetradecyl)amino]-D-glucitol		407-290-1	—	Xi; R41	Xi R: 41 S: (2)-26-39		
603-132-00-2	2-hydroxymethyl-9-methyl-6-(1-methylethyl)-1,4-dioxaspiro[4.5]decane		408-200-3	63187-91-7	Xi; R38-41 R52-53	Xi R: 38-41-52/53 S: (2)-26-37/39-61		
603-133-00-8	A mixture of: 3-[(4-amino-2-chloro-5-nitrophenyl)amino]-propane-1,2-diol; 3'-(2-chloro-5-nitro-1,4-phenyleneimino)bis(propan-1,2-diol)		408-240-1	—	Xn; R22 R52-53	Xn R: 22-52/53 S: (2)-22-36-61		
603-134-00-3	A mixture of substituted dodecyl and/or tetradecyl, diphenyl ethers. The substance is produced by the Friedel Crafts reaction. The catalyst is removed from the reaction product. Diphenyl ether is substituted by C1-C10 alkyl groups. The alkyl groups are bonded randomly between C1 and C6. Linear C12 and C14, 50/50 used.		410-450-3	—	R53	R: 53 S: 61		
603-135-00-9	bis[[2,2',2''-nitrilotris(ethanolato)]-1-N,O]bis [2-(2-methoxyethoxy)ethoxy]-titanium		410-500-4	—	Xi; R41 N; R51-53	Xi; N R: 41-51/53 S: (2)-26-39-61		
603-136-00-4	3-((4-(bis(2-hydroxyethyl)amino)-2-nitrophenyl)amino)-1-propanol		410-910-3	104226-19-9	R43 R52-53	Xi R: 43-52/53 S: (2)-24-37-61		
603-137-00-X	A mixture of: 1-deoxy-1-[methyl-(1-oxohexadecyl)amino]-D-glucitol; 1-deoxy-1-[methyl-(1-oxooctadecyl)amino]-D-glucitol		411-130-6	—	Xi; R41	Xi R: 41 S: (2)-26-39		
603-138-00-5	3-(2,2-dimethyl-3-hydroxypropyl)toluene alt.: 2,2-dimethyl-3-(3-methylphenyl)propanol		403-140-4	103694-68-4	R52-53	R: 52/53 S: 61		

Index No	Chemical name	Notes related to substances	EC No	CAS No	Classification	Labelling	Concentration limits	Notes related to preparations
604-050-00-X	4-chloro-o-cresol 4-chloro-2-methyl phenol		216-381-3	1570-64-5	T: R23 C: R35 N: R50	T: C; N R: 23-35-50 S: (1/2)-26-36/37/39-45-61	C ≥ 25%: T; C; R23-35 10% ≤ C < 25%: C; R20-35 5% ≤ C < 10%: C; R20-34 3% ≤ C < 5%: Xn; R20-36/37/38 1% ≤ C < 3%: Xi; R36/37/38	
604-051-00-5	3,5-bis((3,5-di-tert-butyl-4-hydroxybenzyl)-2,4,6-trimethylphenol		401-110-5	87113-78-8	R52-53	R: 52/53 S: 61		
604-052-00-0	2,2'-methylenebis(6-(2H-benzotriazol-2-yl)-4-(1,1,3,3-tetramethylbutyl)phenol)		403-800-1	103597-45-1	R53	R: 53 S: 61		
604-053-00-6	2-methyl-4-(1,1-dimethylethyl)-6-(1-methyl-pentadecyl)-phenol		410-760-9	157661-93-3	Xi: R38 R43 N: R50-53	Xi: N R: 38-43-50/53 S: (2)-24-37-60-61		
604-054-00-1	A mixture of: 2-methoxy-4-(tetrahydro-4-methylene-2H-pyran-2-yl)-phenol; 4-(3,6-dihydro-4-methyl-2H-pyran-2-yl)-2-methoxyphenol		412-020-0	—	R43 R52-53	Xi R: 43-52/53 S: (2)-24-37-61		
604-055-00-7	2,2'-((3,5',5,5'-tetramethyl-(1,1'-biphenyl)-4,4'-diyl)-bis(oxymethylene))-bis-oxirane		413-900-7	85954-11-6	Muta. Cat. 3; R40	Xn R: 40 S: (2)-22-36-37		
605-027-00-7	A mixture of: 3a,4,5,6,7,7a-hexahydro-4,7-methano-1H-indene-6-carboxaldehyde; 3a,4,5,6,7,7a-hexahydro-4,7-methano-1H-indene-5-carboxaldehyde		410-480-7	—	R43 N: R51-53	Xi: N R: 43-51/53 S: (2)-24-37-61		
606-051-00-0	4-pentylcyclohexanone		406-670-4	61203-83-6	N: R51-53	N R: 51/53 S: 61		
606-052-00-6	4-(N,N-dibutylamino)-2-hydroxy-2'-carboxybenzophenone		410-410-5	54574-82-2	R52-53	R: 52/53 S: 61		
607-272-00-5	fluoroxypyr-meptyl (ISO) [1] fluoroxypyr-butometyl (ISO) [2] methylheptyl, O-((4-amino-3,5-dichloro-6-fluoro-2-pyridyloxy) acetate [1] 2-butoxy-1-methylethyl, O-(4-amino-3,5-dichloro-6-fluoro-2-pyridyloxy) acetate [2]		279-752-9 [1] —	81406-37-3 [1] 154486-27-8 [2]	N: R50-53	N R: 50/53 S: 60-61		

Index No	Chemical name	Notes related to substances	EC No	CAS No	Classification	Labelling	Concentration limits	Notes related to preparations
607-273-00-0	ammonium-7-(2,6-dimethyl-8-(2,2-dimethylbutyryloxy)-1,2,6,7,8a-hexahydro-1-naphthyl)-3,5-dihydroxyheptanoate		404-520-2	—	R52-53	R: 52/53 S: 61		
607-274-00-6	2-(N-benzyl-N-methylamino)ethyl-3-amino-2-butenate		405-350-1	54527-73-0	R43 N: R51-53	Xi: N R: 43-51/53 S: (2)-24-37-61		
607-275-00-1	sodium benzoyloxybenzene-4-sulfonate		405-450-5	66531-87-1	R43	Xi R: 43 S: (2)-24-37		
607-276-00-7	bis[(1-methylimidazol)-(2-ethyl-hexanoate)], zinc complex		405-635-0	—	Xi: R38-41 N: R50-53	Xi: N R: 38-41-50/53 S: (2)-26-37/39-60-61		
607-277-00-2	A mixture of: 2-(hexylthio)ethylamine hydrochloride; sodium propionate		405-720-2	—	Xn: R22 Xi: R41 R43 N: R51-53	Xn: N R: 22-41-43-51/53 S: (2)-24-26-37/39-61		
607-278-00-8	A mixture of isomers of sodium phenethylphthalenesulfonate; sodium naphthylethylbenzenesulfonate		405-760-0	—	Xi: R41 R43 R52-53	Xi R: 41-43-52/53 S: (2)-24-26-37/39-61		
607-279-00-3	A mixture of n-octadecylaminodiethyl bis(hydrogenmaleat); n-octadecylaminodiethyl hydrogen maleate hydrogenphthalate		405-960-8	—	R43 N: R51-53	Xi: N R: 43-51/53 S: (2)-24-37-61		
607-280-00-9	sodium 4-chloro-1-hydroxybutane-1-sulfonate		406-190-5	54322-20-2	Xn: R22 Xi: R36 R43	Xn R: 22-36-43 S: (2)-22-26-36/37		
607-281-00-4	A mixture of branched and linear C7-C9 alkyl 3-[3-(2H-benzotriazol-2-yl)-5-(1,1-dimethylethyl)-4-hydroxyphenyl]propionates		407-000-3	127519-17-9	N: R51-53	N R: 51/53 S: 61		
607-282-00-X	2-acetoxymethyl-4-benzoyloxybut-1-yl acetate		407-140-5	131266-10-9	R52-53	R: 52/53 S: 61		

Index No	Chemical name	Notes related to substances	EC No	CAS No	Classification	Labelling	Concentration limits	Notes related to preparations
607-283-00-5	E-ethyl-4-oxo-4-phenylcrotonate		408-040-4	15121-89-8	Xn; R21/22 Xi; R38-41 R43 N; R50-53	Xn; N R: 21/22-38-41-43-50/53 S: (2)-26-36/37/39-60-61		
607-284-00-0	A 9:1 mixture of: sodium 3,3'-(1,4-phenylenebis(carbonylimino-3,1-propanediylimino))bis(10-amino-6,13-dichloro-4,1,1-triphenodioxazinedisulfonate);lithium 3,3'-(1,4-phenylenebis-(carbonylimino-3,1-propanediyl-imino))bis(10-amino-6,13-dichloro)-4,1,1-triphenodioxazinedisulfonate		410-040-4	136213-76-8	N; R51-53	N R: 51/53 S: 61		
607-285-00-6	A mixture of: 7-(((3-aminophenyl)sulfonyl)amino)-naphthalene-1,3-disulfonic acid;sodium 7-(((3-aminophenyl)sulfonyl)amino)-naphthalene-1,3-disulfonate; potassium 7-(((3-aminophenyl)sulfonyl)amino)-naphthalene-1,3-disulfonate		410-065-0	—	R43	Xi R: 43 S: (2)-22-24-37		
607-286-00-1	A mixture of: sodium/potassium 7-[[[3-[[4-(2-hydroxy-naphthyl)azo]phenyl]azo]phenyl]sulfonyl]amino]-naphthalene-1,3-disulfonate		410-070-8	141880-36-6	R43 R52-53	Xi R: 43-52/53 S: (2)-22-24-37-61		
607-287-00-7	O'-methyl O-(1-methyl-2-methacryloyloxyethyl)-1,2,3,6-tetrahydrophthalate		410-140-8	—	R52-53	R: 52/53 S: 61		
607-288-00-2	Tetrasodium (3-(3-(1-(3-(6-dichloro-5-cyanopyrimidin-5-yl(methylamino)propyl)-1,6-dihydro-2-hydroxy-4-methyl-6-oxo-3-pyridylazo)-4-sulfonatophenyl)sulfamoyl)phthalocyanine-a,b,d-trisulfonato(6-))nickelato (II), where a is 1 or 2 or 3 or 4, b is 8 or 9 or 10 or 11, c is 15 or 16 or 17 or 18, d is 22 or 23 or 24 or 25 and where e and f together are 2 and 4 or 4 and 2 respectively.		410-160-7	148732-74-5	Xi; R36 R43 R52-53	Xi R: 36-43-52/53 S: (2)-22-26-36/37-61		
607-288-00-8	3-(3-(4-(2,4-bis(1,1-dimethylpropyl)phenoxyl)butylaminocarbonyl-4-hydroxy-1-naphthalenyl)thio)propanoic acid		410-370-9	105488-33-3	R53	R: 53 S: 61		
607-290-00-3	A mixture (ratio not known) of: ammonium 1-C14-C18-alkyloxycarbonyl-2-(3-allyloxy-2-hydroxypropoxycarbonyl)ethane-1-sulfonate; ammonium 2-C14-C18-alkyloxycarbonyl-1-(3-allyloxy-2-hydroxypropoxycarbonyl)ethane-1-sulfonate		410-540-2	—	Xi; R38 R43 N; R50-53	Xi; N R: 38-43-50/53 S: (2)-24-37-60-61		
607-291-00-9	dodecyl-ω-(C5/C6-cycloalkyl)alkyl carboxylate		410-630-1	104051-92-5	R53	R: 53 S: 61		

Index No	Chemical name	Notes related to substances	EC No	CAS No	Classification	Labelling	Concentration limits	Notes related to preparations
607-292-00-4	A mixture of: [1-(methoxymethyl)-2-(C12-alkoxy)-ethoxy]acetic acid; [1-(methoxymethyl)-2-(C14-alkoxy)-ethoxy]acetic acid		410-640-6	—	Xi; R38-41 N; R50-53	Xi; N R: 38-41-50/53 S: (2)-26-37/39-60-61		
607-293-00-X	A mixture of: N-aminoethylpiperazonium mono-2,4,6-trimethylnonyldiphenyl ether di-sulfonate; N-aminoethylpiperazonium di-2,4,6-trimethylnonyldiphenyl ether di-sulfonate		410-650-0	—	Xi; R41 R43 N; R51-53	Xi; N R: 41-43-51/53 S: (2)-26-36/37/39-61		
607-294-00-5	sodium 2-benzoyloxy-1-hydroxyethane-sulfonate		410-680-4	—	R43	Xi R: 43 S: (2)-24-37		
607-295-00-0	A mixture of: tetrasodium phosphonoethane-1,2-dicarboxylate; hexasodium phosphonobutane-1,2,3,4-tetracarboxylate		410-800-5	—	R43 N; R51-53	Xi; N R: 43-51/53 S: (2)-24-37-61		
607-296-00-6	A mixture of: pentaerythritol tetraesters with heptanoic acid and 2-ethylhexanoic acid		410-830-9	—	R53	R: 53 S: 61		
607-297-00-1	(E-E)-3,3'-(1,4-phenylenedimethyl- idene)bis(2-oxobornane-10-sulfonic acid)		410-960-6	92761-26-7	Xi; R41	Xi R: 41 S: (2)-26-39		
607-298-00-7	2-(trimethylammonium)ethoxycarboxybenzene-4-sulfonate		411-010-3	—	R43	Xi R: 43 S: (2)-22-36/37		
607-299-00-2	methyl 3-(acetylthio)-2-methyl-propanoate		411-040-7	97101-46-7	Xn; R22 R43 N; R50-53	Xn; N R: 22-43-50/53 S: (2)-24-37-60-61		
607-300-00-6	trisodium [2-(5-chloro-2,6-difluoropyrimidin-4-ylamino)-5-(β-sulfamoyl-c-d-sulfonatophthalocyanin-a-yl)-K4,N29,N30,N31,N32-sulfonylamino]benzoato(5-)]cuprate(II) where a = 1, 2, 3, 4 b = 8, 9, 10, 11 c = 15, 16, 17, 18d = 22, 23, 24, 25		411-430-7	—	R43	Xi R: 43 S: (2)-22-24-37		
607-301-00-1	A mixture of: dodecanoic acid; poly(1-7)lactate esters of dodecanoic acid		411-860-5	—	Xi; R38-41 R43 N; R51-53	Xi; N R: 38-41-43-51/53 S: (2)-24-26-37/39-61		

Index No	Chemical name	Notes related to substances	EC No	CAS No	Classification	Labelling	Concentration limits	Notes related to preparations
607-302-00-7	A mixture of: tetradecanoic acid; poly(1-7)lactate esters of tetradecanoic acid		411-910-6	—	Xi; R38-41 R43 N; R51-53	Xi; N R: 38-41+43-51/53 S: (2-)24-26-37/39-61		
607-303-00-2	1-cyclopropyl-6,7-difluoro-1,4-dihydro-4-oxo-quinoline-3-carboxylic acid		413-760-7	93107-30-3	Repr. Cat.3; R62 R52-53	Xn R: 62-52/53 S: (2-)22-36/37-61		
608-023-00-3	4-(4-chlorophenyl)-2-phenyl-2-[(1H-1,2,4-triazol-1-yl)methyl]butanenitrile		406-140-2	114369-43-6	N; R50-53	N R: 50/53 S: 60-61		
608-024-00-9	2-(4-(N-butyl-N-phenethylamino)phenyl)ethy-lene-1,1,2-tricarbonitrile		407-650-8	97460-76-9	R53	R: 53 S: 61		
608-025-00-4	2-nitro-4,5-bis(benzyloxy)phenylacetanitrile		410-970-0	117568-27-1	R53	R: 53 S: 61		
609-053-00-X	hydrazine-tri-nitromethane		414-850-9	—	E; R3 O; R8 Carc. Cat. 2; R45 T; R23/25 R43	E; T R: 45-3-8-23/25-43 S: 53-45		
610-010-00-2	2-bromo-1-(2-furyl)-2-nitroethylene		406-110-9	35950-52-8	Xn; R22-48/22 C; R34 R43 N; R50-53	C; N R: 22-34-43-48/22-50/53 S: (1/2-)22-26-36/37/39-45-60-61		
611-043-00-5	A 2:1:1 mixture of: trisodium N(1')-N(2):N(1'')-N(2'')-η-6-[2-amino-4-(or 6)-hydroxy-(or 4-amino-2-hydroxy)phenylazo]-6''-(1-carbaniloyl-2-hydroxyprop-1-enylazo)-5',5'''-disulfamoyl-3,3''-disulfonatobis(naphthalene-2,1'-azobenzene-1,2'-diolato-O(1),O(2'))-chromate; trisodium N(1')-N(2):N(1'')-N(2'')-η-6,6''-bis(1-carbaniloyl-2-hydroxyprop-1-enylazo)-5',5'''-disulfamoyl-3,3''-disulfonatobis(naphthalene-2,1'-azobenzene-1,2'-diolato-O(1),O(2'))-chromate; trisodium N(1')-N(2):N(1'')-N(2'')-η-6,6''-bis(1-carbaniloyl-2-hydroxyprop-1-enylazo)-5',5'''-disulfamoyl-3,3''-disulfonatobis(naphthalene-2,1'-azobenzene-1,2'-diolato-O(1),O(2'))-chromate		402-850-1		Xi; R41 52-53	Xi R: 41-52/53 S: (2-)26-39-61		

Index No	Chemical name	Notes related to substances	EC No	CAS No	Classification	Labelling	Concentration limits	Notes related to preparations
611-044-00-0	A mixture of: tert-alkyl(C12-C14)ammonium bis[1-[(2-hydroxy-5-nitrophenyl)azo]-2-naphthalenolato(2-)]-chromate(1-); tert-alkyl(C12-C14)ammonium bis[1-[(2-hydroxy-4-nitrophenyl)azo]-2-naphthalenolato(2-)]-chromate(1-); tert-alkyl(C12-C14)ammonium bis[1-[[5-(1,1-dimethylpropyl)-2-hydroxy-3-nitrophenyl]azo]-2-naphthalenolato(2-)]-chromate(1-); tert-alkyl(C12-C14)ammonium-[[1-[(2-hydroxy-5-nitrophenyl)azo]-2-naphthalenolato(2-)]-1-[(2-hydroxy-5-nitrophenyl)azo]-2-naphthalenolato(2-)]-chromate(1-); tert-alkyl(C12-C14)ammonium-[[1-[[5-(1,1-dimethylpropyl)-2-hydroxy-3-nitrophenyl]azo]-2-naphthalenolato(2-)]-1-[(2-hydroxy-5-nitrophenyl)azo]-2-naphthalenolato(2-)]-chromate(1-); tert-alkyl(C12-C14)ammonium((1-(4(or 5)-nitro-2-oxidophenylazo)-2-naphtholato(1-3-nitro-2-oxido-5-penylphenylazo)-2-naphtholato)chromate(1-))		403-720-7	117527-94-3	N; R51-53	N R: 51/53 S: 61		
611-045-00-6	2-[4-[N-(4-acetoxybutyl)-N-ethyl]amino-2-methylphenylazo]-3-acetyl-5-nitrothiophene		404-830-8	—	R53	R: 53 S: 61		
611-046-00-1	4,4'-diamino-2-methylazobenzene		407-590-2	43151-99-1	T: R25 Xn: R48/22 R43 N; R50-53	T; N R: 25-43-48/22-50/53 S: (1/2)22-28-36/37-45-60-61		
611-047-00-7	A 1:1 mixture of: 2-[[4-[N-ethyl-N-(2-acetoxyethyl)amino]phenyl]azo]-5,6-dichlorobenzothiazole; 2-[[4-[N-ethyl-N-(2-acetoxyethyl)amino]phenyl]azo]-6,7-dichlorobenzothiazole		407-890-3	111381-11-4	R53	R: 53 S: 61		
611-048-00-2	A (1:1) mixture of: 2-[[4-[bis(2-acetoxyethyl)amino]phenyl]azo]-5,6-dichlorobenzothiazole; 2-[[4-[bis(2-acetoxyethyl)amino]phenyl]azo]-6,7-dichlorobenzothiazole		407-900-6	111381-12-5	R53	R: 53 S: 61		

Index No	Chemical name	Notes related to substances	EC No	CAS No	Classification	Labelling	Concentration limits	Notes related to preparations
611-049-00-8	7-[4-(3-diethylaminopropylamino)-6-(3-diethylammoniopropylamino)-1,3,5-triazin-2-ylamino]-4-hydroxy-3-(4-phenylazophenylazo)-naphthalene-2-sulfonate, acetic acid, lactic acid (2:1:1)		408-000-6	118658-98-3	Xn; R48/22 R43 R52-53	Xn R: 43-48/22-52/53 S: (2-(22-36)/37-61		
611-051-00-9	2-(4-(N-ethyl-N-(2-hydroxyethyl)amino-2-methylphenyl)azo-6-methoxy-3-methylbenzothiazolium chloride		411-110-7	136213-74-6	N; R50-53	N R: 50/53 S: 60-61		
611-052-00-4	monosodium aqua-[5-[[2,4-dihydroxy-5-[(2-hydroxy-3,5-dinitrophenyl)azo]phenyl]azo]-2-naphthalensulfonate], iron complex		400-720-9	—	R52-53	R: 52/53 S: 61		
612-156-00-2	A mixture of: trihexadecylmethylammonium chloride; dihexadecyldimethylammoniumchloride		405-620-9	—	Xi; R41 N; R50-53	Xi; N R: 41-50/53 S: (2-26-39-60-61		
612-157-00-8	(Z)-1-benzo[b]thien-2-ylethanone oxime hydrochloride		410-780-8	—	Xn; R22-48/22 Xi; R41 R43 N; R51-53	Xn; N R: 22-41-43-48/22-51/53 S: (2-22-26-36/37/39-61		
612-158-00-3	A mixture of: bis(5-dodecyl-2-hydroxybenzaldoximate) copper (II) C12-alkyl group is branched; 4-dodecylsalicylaldoxime		410-820-4	—	R53	R: 53 S: 61		
612-159-00-9	Reaction products of: trimethylhexamethylene diamine (a mixture of 2,2,4-trimethyl-1,6-hexanediamine and 2,4,4-trimethyl-1,6-hexanediamine, EINECS listed), Epoxide 8 (mono[(C10-C16-alkyloxy)methyl]oxirane derivatives) and p-toluene-sulfonic acid		410-880-1	—	Xn; R22 C; R34 N; R50-53	C; N R: 22-34-50/53 S: (1/2-23-26-36/37/39-45-60-61		
613-149-00-7	2-tert-butyl-5-(4-tert-butylbenzylthio)-4-chloropyridazin-3(2H)-one		405-700-3	96489-71-3	T; R23/25 N; R50-53	T; N R: 23/25-50/53 S: (1/2-36/37-45-60-61		
613-150-00-2	2,2'-[3,3'-(piperazine-1,4-diyl)dipropyl]bis(1H-benzimidazo[2,1-b]benzo[l,m,n][3,8]phenanthroline-1,3,6-trione		406-295-6	—	R53	R: 53 S: 61		

Index No	Chemical name	Notes related to substances	EC No	CAS No	Classification	Labelling	Concentration limits	Notes related to preparations
613-151-00-8	1-(3-mesyloxy-5-trityloxymethyl-2-D-threofuryl)thymine		406-360-9	104218-44-2	R53	R: 53 S: 61		
613-152-00-3	phenyl N-(4,6-dimethoxypyrimidin-2-yl)carbamate		406-600-2	89392-03-0	R43 N: R51-53	Xi: N R: 43-51/53 S: (2)-24-37-61		
613-153-00-9	2,3,5-trichloropyridine		407-270-2	16063-70-0	R52-53	R: 52/53 S: 61		
613-154-00-4	2-amino-4-chloro-6-methoxypyrimidine		410-050-9	5734-64-5	Xn; R22	Xn R: 22 S: (2)-22		
613-155-00-X	5-chloro-2,3-difluoropyridine		410-090-7	89402-43-7	R10 Xn; R22 R52-53	Xn R: 10-22-52/53 S: (2)-23-36-61		
613-156-00-5	2-butyl-4-chloro-5-formylimidazole		410-260-0	83857-96-9	R43 N: R51-53	Xi: N R: 43-51/53 S: (2)-24-37-61		
613-157-00-0	2,4-diamino-5-methoxymethylpyrimidine		410-330-0	54236-98-5	Xn; R22-48/22 Xi; R36	Xn R: 22-36-48/22 S: (2)-22-26-36		
613-158-00-6	2,3-dichloro-5-trifluoromethyl-pyridine		410-340-5	69045-84-7	Xn; R20/22 Xi; R41 R43 N: R51-53	Xn; N R: 20/22-41-43-51/53 S: (2)-24-26-37/39-61		
613-159-00-1	4-[2-[4-(1,1-dimethylethyl)phenyl]-ethoxy]quinazoline		410-580-0	120928-09-8	T; R25 Xn; R20 N: R50-53	T; N R: 20-25-50/53 S: (1/2)37-45-60-61		
613-160-00-7	(1S)-2-methyl-2,5-diazobicyclo[2.2.1]heptane dihydrobromide		411-000-9	125224-62-6	R43	Xi R: 43 S: (2)-24-37		
615-022-00-1	methyl 3-isocyanatosulfonyl-2-thiophene-carboxylate		410-550-7	79277-18-2	E; R2 R14 Xn; R48/22 R42/43	E; Xn R: 2-14-42/43-48/22 S: (2)-22-30-35-36/37		

Index No	Chemical name	Notes related to substances	EC No	CAS No	Classification	Labelling	Concentration limits	Notes related to preparations
615-023-00-7	2-(isocyanatosulfonylmethyl)benzoic acid methyl ester; (alt.): methyl 2-(isocyanatosulfonylmethyl)benzoate		410-900-9	83056-32-0	R10 R14 Muta. Cat.3; R40 Xn; R20-48/22 Xi; R41 R42	Xn R: 10-14-20-40-41-42-48/22 S: (2)-23-26-36/37/39		
616-044-00-4	N-(3,5-dichloro-4-ethyl-2-hydroxyphenyl)-2-(3-pentadecylphenoxy)-butanamide		402-510-2	—	N; R51-53	N R: 51/53 S: 61		
616-045-00-X	2'-(4-chloro-3-cyano-5-formyl-2-thienylazo)-5'-diethylamino-2-methoxyacetanilide		405-190-2	122371-93-1	R43 R53	Xi R: 43-53 S: (2)-22-24-37-61		
616-046-00-5	N-(2-(6-chloro-7-methylpyrazolo(1,5-b)-1,2,4-triazol-4-yl)propyl)-2-(2,4-di-tert-pentylphenoxy)octanamide		406-390-2	—	N; R50-53	N R: 50/53 S: 60-61		
616-047-00-0	A mixture of: 2,2',2'',2'''-(ethylenedinitrilotetrakis-N,N-di(C16)alkyl)acetamide; 2,2',2'',2'''-ethylenedinitrilotetrakis-N,N-di(C18)alkylacetamide		406-640-0	—	R43	Xi R: 43 S: (2)-24-37		
616-048-00-6	3'-trifluoromethylisobutyranilide		406-740-4	1939-27-1	Xn; R48/22 N; R51-53	Xn; N R: 48/22-51/53 S: (2)-22-36-61		
616-049-00-1	2-(2,4-bis(1,1-dimethylethyl)phenoxy)-N-(3,5-dichloro-4-ethyl-2-hydroxyphenyl)-hexanamide		408-150-2	99141-89-6	R53	R: 53 S: 61		
616-050-00-7	N-[2,5-dichloro-4-(1,1,2,3,3,3-hexafluoropropoxy)-phenyl-aminocarbonyl]-2,6-difluorobenzamide		410-690-9	103055-07-8	R43 N; R50-53	Xi; N R: 43-50/53 S: (2)-24-37-60-61		
616-051-00-2	A mixture of: 2,4-bis(N'-(4-methylphenyl)-ureido)-toluene; 2,6-bis(N'-(4-methylphenyl)-ureido)-toluene		411-070-0	—	R53	R: 53 S: 61		
617-015-00-9	bis(4-methylbenzoyl)peroxide		407-950-9	895-85-2	E; R2 O; R7 N; R50-53	E; N R: 2-7-50/53 S: (2)-7-14-36/37/39-47-60-61		
650-032-00-X	cyproconazole (ISO) (2RS,3RS;2RS,3SR)-2-(4-chlorophenyl)-3-cyclopropyl-1-[(1H-1,2,4-triazol-1-yl)butan-2-ol		—	94361-06-5	Repr. Cat. 3; R63 Xn; R22 N; R50-53	Xn; N R: 22-50/53-63 S: (2)-36/37-60-61		

ANNEX 2

R 66

IT: L'esposizione ripetuta può provocare secchezza e screpolature della pelle.

(Does not concern the ES version)

(Does not concern the DA version)

(Does not concern the DE version)

(Does not concern the EL version)

(Does not concern the EN version)

(Does not concern the FR version)

(Does not concern the NL version)

(Does not concern the PT version)

(Does not concern the FI version)

(Does not concern the SV version)

ANNEX 3A

S 23

FR: Ne pas respirer les gaz/fumées/vapeurs/aérosols [terme(s) approprié(s) à indiquer par le fabricant].

(Does not concern the ES version)

(Does not concern the DA version)

(Does not concern the DE version)

(Does not concern the EL version)

(Does not concern the EN version)

(Does not concern the IT version)

(Does not concern the NL version)

(Does not concern the PT version)

(Does not concern the FI version)

(Does not concern the SV version)

S 26

DE: Bei Berührung mit den Augen sofort gründlich mit Wasser abspülen und Arzt konsultieren.

(Does not concern the ES version)

(Does not concern the DA version)

(Does not concern the EL version)

(Does not concern the EN version)

(Does not concern the FR version)

(Does not concern the IT version)

(Does not concern the NL version)

(Does not concern the PT version)

(Does not concern the FI version)

(Does not concern the SV version)

S 56

DE: Diesen Stoff und seinen Behälter der Problemabfallentsorgung zuführen.

EN: Dispose of this material and its container to hazardous or special waste collection point.

IT: Smaltire questo materiale e i relativi contenitori in un punto di raccolta di rifiuti pericolosi o speciali.

(Does not concern the ES version)

(Does not concern the DA version)

(Does not concern the EL version)

(Does not concern the FR version)

(Does not concern the NL version)

(Does not concern the PT version)

(Does not concern the FI version)

(Does not concern the SV version)

ANNEX 3B

S 27/28

DE: Bei Berührung mit der Haut beschmutzte, getränkte Kleidung sofort ausziehen und Haut sofort mit viel ... abwaschen (vom Hersteller anzugeben).

(Does not concern the ES version)

(Does not concern the DA version)

(Does not concern the EL version)

(Does not concern the EN version)

(Does not concern the FR version)

(Does not concern the IT version)

(Does not concern the NL version)

(Does not concern the PT version)

(Does not concern the FI version)

(Does not concern the SV version)

S 29/56

ES: No tirar los residuos por el desagüe; elimínese esta sustancia y su recipiente en un punto de recogida pública de residuos especiales o peligrosos.

DE: Nicht in die Kanalisation gelangen lassen; diesen Stoff und seinen Behälter der Problemabfallentsorgung zuführen.

EN: Do not empty into drains, dispose of this material and its container to hazardous or special waste collection point.

IT: Non gettare i residui nelle fognature; smaltire questo materiale e i relativi contenitori in un punto di raccolta di rifiuti pericolosi o speciali.

NL: Afval niet in de gootsteen werpen; deze stof en de verpakking naar een inzamelpunt voor gevaarlijk of bijzonder afval brengen.

SV: Töm ej i avloppet, lämna detta material och dess behållare till insamlingsställe för farligt avfall.

(Does not concern the DA version)

(Does not concern the EL version)

(Does not concern the FR version)

(Does not concern the PT version)

(Does not concern the FI version)

ANNEX 4A

'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 cells 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.

Chromatid-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 4, 8, 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. $3n$, $4n$, 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, CO₂ 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 β -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 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 elastogen 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	50-18-0	200-015-4
	cyclophosphamide monohydrate	6055-19-2	

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 3—6 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 conformed 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 1—3 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 a 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, CO₂ 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.

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ANNEX 4B

'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 human genetic diseases and there is substantial evidence that chromosome mutations and related events causing alterations in oncogenes and tumour suppresser 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 an *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 4, 8, 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. $3n$, $4n$ 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. colchicine or Colcemid®). 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 animals 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 $\pm 20\%$ 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 methansulphonate	62-50-0	200-536-7
ethyl nitrosourea	759-73-9	212-072-2
mitomycin C	50-07-7	200-008-6
cyclophosphamide	50-18-0	200-015-4
cyclophosphamide monohydrate	6055-19-2	
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 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 hours) 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 hours 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 3–5 hours; for Chinese hamsters this interval is approximately 4–5 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 fully 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/100g 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 $2n \pm 2$.

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

TEST REPORT

Test 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, means and standard deviation for each group.

Test conditions:

- positive and negative (vehicle/solvent) controls,
- data from range-finding study, if conducted,
- rationale for close 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.

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ANNEX 4C

'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 end point. 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 for four weeks or more.

This mammalian *in vivo* micronucleus test 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 $\pm 20\%$ 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 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 methansulphonate	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	50-18-0	200-015-4
cyclophosphamide monohydrate	6055-19-2	
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-hour 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 not 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 fully 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/100g body weight. The use of volumes higher can 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 supravivally (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 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 flow cytometric analysis of cell suspensions) 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 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 $\pm$ standard deviation of micronucleated immature erythrocytes per group,
- dose-response relationship, where possible,
- statistical analyses and methods applied,
- concurrent and historical negative data,
- concurrent positive control data.

Discussion of the results.

Conclusions.

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ANNEX 4D

'B.13/14. MUTAGENICITY — REVERSE MUTATION TEST 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 data base 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, pyrrolizidine 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 they 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 TA 102); 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 ultra-violet 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 β -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. $\sqrt{10}$) 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:

Substance	CAS No	Einecs No
9,10-dimethylantracene	781-43-1	212-308-4
7,12-dimethylbenz[a]anthracene	57-97-6	200-359-5
benzo[a]pyrene	50-32-8	200-028-5
2-aminoanthracene	613-13-8	210-330-9
cyclophosphamide	50-18-0	200-015-4
cyclophosphamide monohydrate	6055-19-2	

The following substance is a suitable positive control for the reductive metabolic activation method:

Substance	CAS No	Einecs No
Congo Red	573-58-0	209-358-4

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:

Substance	CAS No	Einecs No	Strain
sodium azide	26628-22-8	247-852-1	TA 1535 and TA 100
2-nitrofluorene	607-57-8	210-138-5	TA 98
9-aminoacridine	90-45-9	201-995-6	TA 1537, TA 97 and TA 97a
ICR 191	17070-45-0	241-129-4	TA 1537, TA 97 and TA 97a
cumene hydroperoxide	80-15-9	201-254-7	TA 102
mitomycin C	50-07-7	200-008-6	WP2 uvrA and TA 102
N-ethyl-N-nitro-N-nitrosoguanidine	70-25-7	200-730-1	WP2, WP2uvrA and WP2uvrA(pKM101)
4-nitroquinoline-1-oxide	56-57-5	200-281-1	WP2, WP2uvrA and WP2uvrA(pKM101)
furylfuramide (AF2)	3688-53-7		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 minutes 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.

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ANNEX 4E

'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 of 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 $TK^{+/-} \rightarrow TK^{-/-}$ 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 L5171Y, 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*

Cells 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 β -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 last 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 $\sqrt{10}$. 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 μ l/ml, or 1,01 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 methansulphonate	62-50-0	200-536-7
		Ethyl nitrosourea	759-73-9	212-072-2
	TK (small and large colonies)	Methyl methansulphonate	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
		Cyclophosphamidmonohydrate	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 No 59-14-3, Einecs No 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 3—6 hours is effective). Exposure time may be extended over once 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 6-8 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 TK6TK⁺, 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, CO₂ 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

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ANNEX 4F

'B.23. MAMMALIAN SPERMATOGENIAL 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. $3n$, $4n$ 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. colchicine or Colcemid®). 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 $\pm 20\%$ 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 substance 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	50-18-0	200-015-4
cyclophosphamide monohydrate	6055-19-2	
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 3—5 hours, for Chinese hamsters this interval is approximately 4—5 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 2 000 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/100g 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 concluded,
- 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

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ANNEX 4G

'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 (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 (³H-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 to 30 bases). In contrast "shortpatch repair" (one to three 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 end point 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 ³H-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 ³H-TdR is usually determined by autoradiography, since this technique is not as susceptible to interference from S-phase cells as, for example, liquid scintillation counting.

1.4. DESCRIPTION OF THE METHOD

1.4.1. Preparations

1.4.1.1. Selections 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 $\pm 20\%$ 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 to 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 to 4 hours)	N-nitrosodimethylamine	62-75-9	200-249-8
Late sampling times (12 to 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 animal 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/100g 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 cells are prepared from treated animals normally 12 to 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 to 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 ^3H -TdR for an appropriate length of time, e.g. three to eight 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 to 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/animals 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 ^3H -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.

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 preset 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* indicates 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, pp. 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, pp. 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, pp. 52—77.
- (4) Madle, S., Dean, S. W., Andrac, 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, pp. 263—285.
- (5) Fautz, R., Hussain, B., Efsthathiou, 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, pp. 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, pp. 553—562.

ANNEX 5

SV:

3.2.3. *Farligt*

R65 Farligt: kan ge lungskador vid förtäring.

Flytande ämnen och beredningar som på grund av sin låga viskositet utgör en fara för människa vid aspiration

- a) För ämnen och beredningar som innehåller alifatiska, alicykliska och aromatiska kolväten i en total koncentration av 10 % eller mer och
- har en flödestid mindre än 30 sekunder, uppmätt med en 3 mm utloppsbägare enligt ISO 2431, eller
 - har en kinematisk viskositet lägre än 7×10^{-6} m²/s vid 40 °C, uppmätt med en kalibrerad kapillärviskosimeter av glas, enligt ISO 3104 och ISO 3105, eller
 - har en kinematisk viskositet lägre än 7×10^{-6} m²/s vid 40 °C, bestämd från rotationsviskosimetri enligt ISO 3219.

Ämnen och beredningar, som uppfyller dessa kriterier, behöver dock inte klassificeras om de har en genomsnittlig ytspänning högre än 33 mN/m vid 25 °C, uppmätt med en Noüy tensiometer eller enligt de testmetoder som finns beskrivna i bilaga V del A.5.

- b) För ämnen och beredningar, baserat på praktiska erfarenheter från människa.

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FI:

3.2.6.1 *Ihon tulehtuminen*

Seuraava vaaraa osoittava lauseka määräytyy alla esiteltävien perusteitten mukaan:

R38 Ärsyttää ihoa

- Aineet ja valmisteet aiheuttavat ihon merkittävän tulehtumisen enintään neljän tunnin altistuksessa määritettynä kanilla liitteessä V mainitulla ihoärsytystestillä. Tulehdus kestää vähintään 24 tuntia.

Ihon tulehdus on merkittävää, jos:

- a) punoituksen ja ruvenmuodostuksen tai turvotuksen voimakkuutta kuvaavien lukuarvojen keskiarvo laskettuna kaikista koe-eläimistä on vähintään 2;
- b) tai kun liitteessä V tarkoitettua testiä on täydennetty käyttämällä kolmea koe-eläintä, vähintään kahden koe-eläimen ihon punoituksen ja ruvenmuodostuksen tai turvotuksen voimakkuutta kuvaavien lukuarvojen keskiarvo on, jokaiselle koe-eläimelle laskettuna erikseen, vähintään 2.

Kummassakin tapauksessa keskiarvojen lasekmiseen on käytettävä kaikkia niitä lukuarvoja, jotka saadaan arvioitaessa vaikutusta 24 tunnin, 48 tunnin ja 72 tunnin välein.

Tulehdusta pidetään myös merkittävänä, jos ihon tulehtuminen jatkuu ainakin kahdella koe-eläimellä havainnointiajan päättymiseen asti. Erityiset vaikutukset kuten esimerkiksi hyperplasia, hilseileminen, värin muutokset, halkeamat, ruvet ja karvojenlähtö on otettava huomioon.

Tähän liittyviä tietoja voidaan saada myös eläimillä tehtävistä ei-akuuttisista altistuskokeista (katso lauseketta R48 koskevat huomautukset jaksossa 2.d). Vaikutuksia pidetään merkittävänä, jos ne vastaavat edellä kuvattuja vaikutuksia.

- Aineet ja valmisteet, jotka aiheuttavat ihmisillä merkittävää ihotulehdusta, kun kosketus on ollut välitön, jatkuva tai toistuva.
- Orgaaniset peroksidit, paitsi jos on olemassa näyttöä siitä, että tällaista vaikutusta ei ole.

Tuntoharha ('paresthesia'):

Pyretroiditorjunta-aineen ihokosketuksen aiheuttamaa tuntoharhaa ihmisessä ei pidetä ärsytysvaikutuksena, joka oikeuttaisi luokituksen Xi; R38. S-lauseketta S24 on kuitenkin sovellettava aineisiin, joilla on tällainen vaikutus.

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In point 6.2 (Safety phrases for substances and preparations):

DE:

S 28 Bei Berührung mit der Haut sofort mit viel ... abwaschen (vom Hersteller anzugeben)

— Anwendungsbereich:

- sehr giftige, giftige oder ätzende Stoffe und Zubereitungen;

— Verwendung:

- *obligatorisch* für sehr giftige Stoffe und Zubereitungen;
- empfohlen für sonstige obengenannte Stoffe und Zubereitungen, insbesondere, wenn Wasser nicht die geeignete Spülflüssigkeit ist;
- empfohlen für ätzende Stoffe und Zubereitungen, die an die allgemeine Öffentlichkeit abgegeben werden.

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FI:

S 29 Ei saa tyhjentää viemäriin

— Soveltamisala:

- erittäin helposti syttyvät tai helposti syttyvät veteen sekoittumattomat nesteet,
- erittäin myrkylliset tai myrkylliset aineet ja valmisteet,
- ympäristölle vaaralliset aineet.

— Käytön perusteet:

- *pakollinen* yleisessä kulutuksessa todennäköisesti käytettäville ympäristölle vaarallisille ja tunnuksella N luokitelluille aineille, jollei kyseessä ole aineen tarkoitettu käyttö,
- suositeltava yleisessä kulutuksessa todennäköisesti käytettäville muille edellä mainituille aineille tai valmisteille, jollei kyseessä ole kemikaalin tarkoitettu käyttö.

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ANNEX 6

ANNEX IX

PART A

Provisions relating to child-proof fastenings

In addition to the provisions in Article 22(1)(e) of this Directive, containers of whatever capacity containing substances presenting an aspiration hazard (Xn; R65) and classified and labelled according to paragraph 3.2.3 of Annex VI to this Directive, with the exception of substances placed on the market in the form of aerosols or in a container fitted with a sealed spray attachment, shall be fitted with child-proof fastenings.

1. *Reclosable packages*

Child-proof fastenings used on reclosable packages shall comply with ISO standard 8317 (1 July 1989 edition) relating to "Child-resistant packages — Requirements and methods of testing for reclosable packages" adopted by the International Standard Organisation (ISO).

2. *Non-reclosable packages*

Child-proof fastenings used on non-reclosable packages shall comply with CEN standard EN 862 (March 1997 edition) relating to "Packaging — Child-resistant packaging — Requirements and testing procedures for non-reclosable packages for non-pharmaceutical products" adopted by the European Committee for Standardisation (CEN).

3. *Notes*

1. Evidence of conformity with the above standards may be certified only by laboratories which conform with European Standards Series EN 45 000.

2. *Specific cases*

If it seems obvious that packaging is sufficiently safe for children because they cannot get access to the contents without the help of a tool, the test does not need to be performed.

In all other cases and when there are sufficient grounds for doubting the security of the closure for a child, the national authority may ask the person responsible for putting the product on the market to give it a certificate from a laboratory, described in 3.1, stating that either:

- the type of closure is such that it is not necessary to test to the ISO and CEN standards referred to above,
- or
- the closure has been tested and has been found to conform with the standards referred to above.

PART B

Provisions relating to tactile warning devices

The technical specifications for tactile warning devices shall conform with EN ISO standard 11683 (1997 edition) relating to "Packaging — Tactile warnings of danger — Requirements".

COMMISSION DIRECTIVE 2000/33/EC**of 25 April 2000****adapting to technical progress for the 27th time Council Directive 67/548/EEC on the approximation of laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances^(*)****(Text with EEA relevance)**

THE COMMISSION OF THE EUROPEAN COMMUNITIES,

HAS ADOPTED THIS DIRECTIVE:

Having regard to the Treaty establishing the European Community,

Article 1

The texts in Annexes I and II to this Directive are added to Part B of Annex V to Directive 67/548/EEC.

Having regard to Council Directive 67/548/EEC of 27 June 1967 on the approximation of laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances⁽¹⁾, as last amended by European Parliament and Council Directive 1999/33/EC⁽²⁾, and in particular Article 28 thereof,*Article 2*

1. Member States shall bring into force the laws, regulations and administrative provisions necessary to comply with this Directive by 1 October 2001 at the latest. They shall forthwith inform the Commission thereof.

Whereas:

When Member States adopt those provisions, they shall contain a reference to this Directive or be accompanied by such a reference on the occasion of their official publication. Member States shall determine how such reference is to be made.

(1) Annex V to Directive 67/548/EEC lays down the methods for the determination of the physico-chemical properties, toxicity and ecotoxicity of substances and preparations. It is necessary to adapt that Annex to technical progress.

2. Member States shall communicate to the Commission the main provisions of national law which they adopt in the field covered by this Directive and a correlation table between this Directive and the national provisions adopted.

(2) According to Article 7(2) of Council Directive 86/609/EEC of 24 November 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes⁽³⁾, an experiment entailing the use of animals shall not be performed if another scientifically satisfactory method to obtain the result sought is reasonably and practicably available.

*Article 3*This Directive shall enter into force on the third day following its publication in the *Official Journal of the European Communities*.

(3) The Commission intends to introduce into Annex V to Directive 67/548/EEC certain alternative testing methods not entailing the use of animals, in order to make them available for the testing of chemicals according to Article 3(1) of Directive 67/548/EEC.

Article 4

This Directive is addressed to the Member States.

(4) The measures provided for in this Directive are in accordance with the opinion of the Committee on the Adaptation to Technical Progress of the Directives for the Elimination of Technical Barriers to Trade in Dangerous Substances and Preparations,

Done at Brussels, 25 April 2000.

For the Commission
Margot WALLSTRÖM
Member of the Commission

(*) Adopted before the 26th adaptation.

⁽¹⁾ OJ 196, 16.8.1967, p. 1.

⁽²⁾ OJ L 199, 30.7.1999, p. 57.

⁽³⁾ OJ L 358, 18.12.1986, p. 1.

ANNEX I

B.40. SKIN CORROSION

1. METHOD

1.1. Introduction

Two *in vitro* test for skin corrosivity, the rat skin transcutaneous electrical resistance (TER) assay and a test employing a human skin model, have been endorsed as scientifically valid by the European Centre for the Validation of Alternative Methods (ECVAM, Joint Research Centre, European Commission) (1) (2) (3). The ECVAM validation study demonstrated that both tests were able to reliably discriminate between known skin corrosives and non-corrosives. Furthermore, the test protocol based on a human skin model enabled correct distinction between degrees of corrosive effects (known severe skin corrosives, R35, and other skin corrosives, R34) (2). Description and procedures for both tests are given; the choice of which test to use depends on the specific requirements and preferences of the user.

See also General Introduction, Part B.

1.2. Definitions

Skin corrosion: the production of irreversible tissue damage in the skin following the application of a test material.

1.3. Reference substances

None specified, but see points 1.5.3.4 and 1.7.2.3.

1.4. Principle of the test method — rat skin TER assay

The test material is applied for up to 24 hours to the epidermal surfaces of skin discs taken from the pelts of humanely killed young rats. 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 inherent TER below a threshold level (5 k Ω) (4) (5). Irritant and non-irritant materials do not reduce the TER below the threshold level. A dye-binding step can be incorporated into the test procedure for surfactants and neutral organics (for definition see reference (6)) to reduce the number of false positive results obtained specifically with these chemical types (2) (7).

1.5. Description of the test method — rat skin TER assay

1.5.1. Animals

Young (20-23 days) rats (Wistar or a comparable strain) are required for the preparation of skin discs. The dorsal and flank hair is carefully removed with small animal clippers. The animals are then washed by careful wiping, whilst submerging the 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 3 days (animals must not be older than 31 days for pelt preparation).

1.5.2. Preparation of the skin discs

Animals are humanely killed. The dorsal skin of each animal is then removed and stripped of excess fat by carefully peeling it away from the skin. The pelt is placed over the end of a polytetrafluoroethylene (PTFE) 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 1. 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 magnesium sulphate solution (154 mM) (Figure 2).

1.5.3. Test procedure

1.5.3.1. Application of the test material

Liquid test substances (150 μ l) are applied to the epidermal surface inside the tube (Figure 2). When testing solid materials, a sufficient amount of the solid is applied to the disc to ensure that the whole surface of the epidermis is covered. Deionised water (150 μ l) is then added on top of the solid and the tubes are gently agitated. Test substances should have maximum contact with the skin. For some solids this may be achieved by warming up to 30°C to melt the test substance, or by grinding to produce a granular material or powder.

Three skin discs are used for each test substance. Test substances are applied for 24 hours (see also 1.5.3.4). 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. The removal of test substances which have solidified in the tube can be facilitated by jet washing with warm water at approximately 30°C.

1.5.3.2. TER measurements

The TER is measured by using a low-voltage, alternating current databridge (e.g. AIM 401 or 6401, or equivalent). 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 by inverting the tube, and the tissue is then hydrated by the addition of 3 ml magnesium sulphate solution (154 mM). The databridge electrodes are placed on either side of the skin disc to take the resistance measurement in k Ω /skin disc (Figure 2). Electrode dimensions and the length of the electrode exposed below the crocodile clips are shown in Figure 1. The inner (thick) electrode clip is rested on the top of the PTFE tube during resistance measurement, to ensure that a consistent length of electrode is submerged in the magnesium sulphate solution. The outer (thin) electrode is positioned inside the receptor chamber so that it rests on the bottom of the chamber. The distance between the bottom of the spring clip and the bottom of the PTFE tube is maintained as a constant (Figure 1), since this distance affects the resistance value obtained.

Note that if the measured resistance value is greater than 20 k Ω , this may be due to the test substance coating the epidermal surface of the skin disc. 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 magnesium sulphate solution is discarded and the resistance measurement is repeated with fresh magnesium sulphate.

The mean TER results are accepted on condition that concurrent positive and negative control values fall within the acceptable ranges for the method. The suggested control substances and their associated acceptable resistance ranges for the methodology and apparatus described are:

Control	Substance	Resistance range (k Ω)
Positive	10 M hydrochloric acid (36%)	0,5-1,0
Negative	Distilled water	10-25

1.5.3.3. Modified procedure for surfactants and neutral organics

If the TER values of test substances which are either surfactants or neutral organics are less than or equal to 5 k Ω , an assessment of dye penetration can be carried out on the tissues. This procedure will determine whether the results are false positives (2).

1.5.3.3.1. Sulforhodamine B dye application and removal

Following initial treatment with the test substance, 150 μ l of a 10% (w/v) dilution of sulforhodamine B dye in distilled water is applied to the epidermal surface of each skin disc for 2 hours. The skin discs are then jet 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 5 minutes to remove any further excess/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 8 minutes at 21°C (relative centrifugal force $\sim$ 175). A 1 ml sample of the supernatant is then 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 approximately 565 nm.

1.5.3.3.2. Calculation of dye content

The sulforhodamine B dye content per disc is calculated from the OD values (sulforhodamine B dye molar extinction coefficient at 565 nm = $8,7 \times 10^4$; molecular weight = 580). The sulforhodamine B dye content is determined for each skin disc and a mean dye content is then calculated for the replicates. 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 are:

Control	Substance	Dye content range (µg/disc)
Positive	10 M hydrochloric acid (36 %)	40-100
Negative	Distilled water	15-35

1.5.3.4. Additional information

Test substances can also be applied to the skin discs for shorter periods (e.g. 2 hours) to identify those materials which are severely corrosive. However, in the validation study, the TER assay was found to overestimate the corrosive potential of several test chemicals following their application to the skin discs for 2 hours (2), although it enabled the correct identification of corrosives and non-corrosives after a 24-hour application.

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 significantly. Therefore, it is recommended that the methodology and resistance threshold value are calibrated by testing a series of reference standards chosen from the chemicals used in the validation study (3).

1.6. Principle of the test method — human skin model assay

The test material is applied topically for up to 4 hours to a three-dimensional human skin model, comprising 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) below defined threshold levels at specified exposure periods. The principle of the assay is in accordance with the hypothesis that chemicals which are corrosive are those which are able to penetrate the stratum corneum (by diffusion or erosion) and are sufficiently cytotoxic to cause cell death in the underlying cell layers.

1.7. Description of the test method — human skin model assay

1.7.1. Human skin models

Human skin models can come from various sources, but they must meet certain criteria. The model must have a functional stratum corneum with an underlying layer of living cells. The barrier function of the stratum corneum must be adequate. This can be shown by demonstrating the model's resistance to cytotoxicity following the application of substances which are known to be cytotoxic to cells, but which do not normally pass through the stratum corneum. The model must be shown to give reproducible results under defined experimental conditions.

The viability of the living cells in the model must be sufficiently high to discriminate well between the positive and negative control substances. Cell viability (for example, as measured by the amount of MTT reduction, i.e. an OD value) following exposure to the negative control substance must fall within acceptable limits for the particular model. Similarly, cell viability values with the positive control substance (relative to those for the negative control) must fall within specified limits. Most importantly, the prediction model used must have been shown to meet the international validation standard (2).

1.7.2. Test procedure

1.7.2.1. Application of the test material

For liquid materials, sufficient test substance must be applied to cover the skin surface (a minimum of 25 µl/cm²). For solid materials, sufficient test substance must be applied to cover the skin, and it should then be moistened to ensure good contact with the skin; where appropriate, solids should be ground to a powder before application. The application method must be shown to be adequate for a wide range of chemical types (2). At the end of the exposure period, the test material must be carefully washed from the skin surface with saline solution.

1.7.2.2. Cell viability measurements

Any quantitative, validated, method can be used to measure cell viability. The most frequently used assay is MTT reduction, which has been shown to give accurate and reproducible results in various laboratories (2). The skin disc is placed in an MTT solution of 0,3 mg/ml at 20-28°C for 3 hours. The precipitated blue formazan product is then extracted (solvent extraction) and the concentration of the formazan is measured by determining the OD at a wavelength between 545 and 595 nm.

1.7.2.3. Additional information

The skin model used, and the exact protocol of exposure time and washing procedures, etc. will have a major impact on the cell viability results. It is recommended that the methodology and prediction model are calibrated by testing a series of reference standards chosen from the chemicals used in the ECVAM validation study (3). It is critical that the method used has been shown to be reproducible within and between laboratories for a wide range of chemicals, in accordance with international standards. As a minimum, the method should meet the criteria for scientific validity defined previously (2), and the results of such a validation study must be published in a peer-reviewed scientific journal.

2. DATA

2.1. Treatment of results

2.1.1. Rat skin TER assay

Resistance values ($k\Omega$) for the test material, positive and negative controls, and any standard reference chemicals should be reported in tabular form, including data for replicates/repeat experiments, mean values and the classification derived.

2.1.2. Human skin model assay

OD values and calculated percentage cell viability data for the test material, positive and negative controls, and any standard reference chemicals should be reported in tabular form, including data for replicates/repeat experiments, mean values and the classification derived.

2.2. Evaluation and interpretation of results

2.2.1. Rat skin TER assay

If the mean TER value obtained for the test substance is greater than 5 $k\Omega$, then it is non-corrosive. If the TER value is less than or equal to 5 $k\Omega$, and the test substance is not a surfactant or neutral organic, then it is corrosive.

For surfactants or neutral organics which give TER values less than or equal to 5 $k\Omega$, dye penetration can be carried out. If the mean disc dye content is greater than or equal to the mean disc dye content of the 36 % HCl positive control obtained concurrently, then the test substance is a true positive and is therefore corrosive. If the mean disc dye content is less than the mean disc content of the 36 % HCl positive control obtained concurrently, then the test substance is a false positive and is therefore non-corrosive.

2.2.2. Human skin model assay

The negative control OD value represents 100% cell viability; hence, the OD values obtained for each test sample can be used to calculate a percentage viability relative to the negative control. The cut-off percentage cell viability value distinguishing corrosive from non-corrosive test materials (or discriminating between different corrosive classes) must be clearly defined in the prediction model before the method is validated, and the subsequent validation study must show that the cut-off value is appropriate (2).

3. REPORTING

Test report

The test report must include at least the following information:

Test substance:

- identification data, physical nature and, where relevant, physicochemical properties. Similar information should be provided for reference substances, if used.

Test conditions:

- details of test procedure used,
- description and justification of any modifications.

Results:

- tabulation of resistance values (TER assay) or percentage cell viability values (human skin model assay) for the test material, positive and negative controls and any standard reference chemicals, including data for replicates/repeat experiments and mean values,
- description of any other effects observed.

*Discussion of the results.**Conclusions.***4. REFERENCES**

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Figure 1

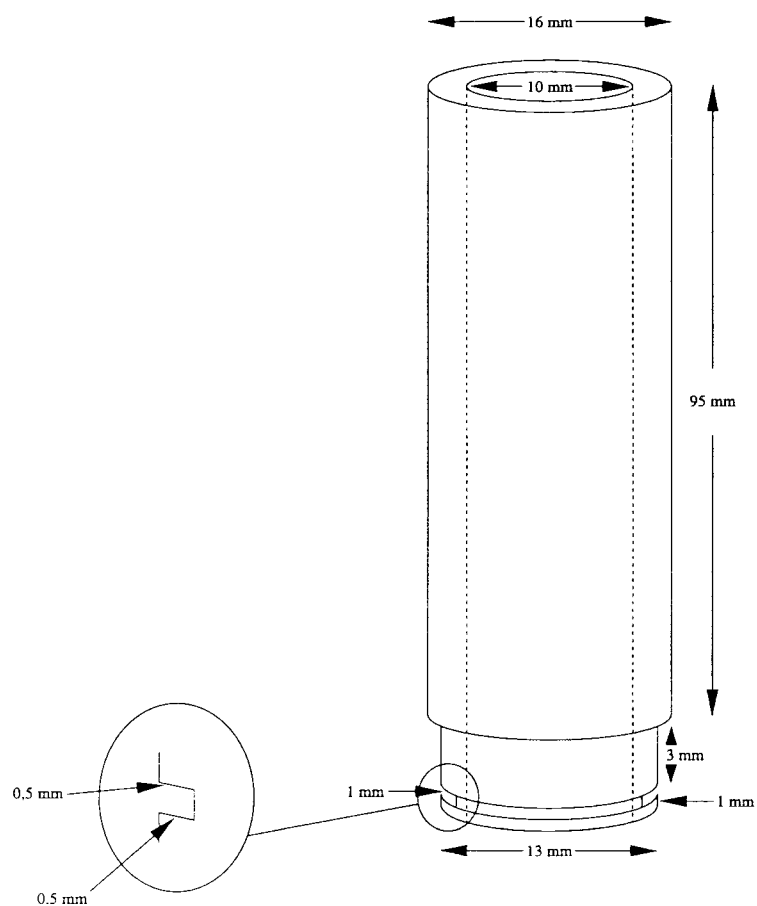
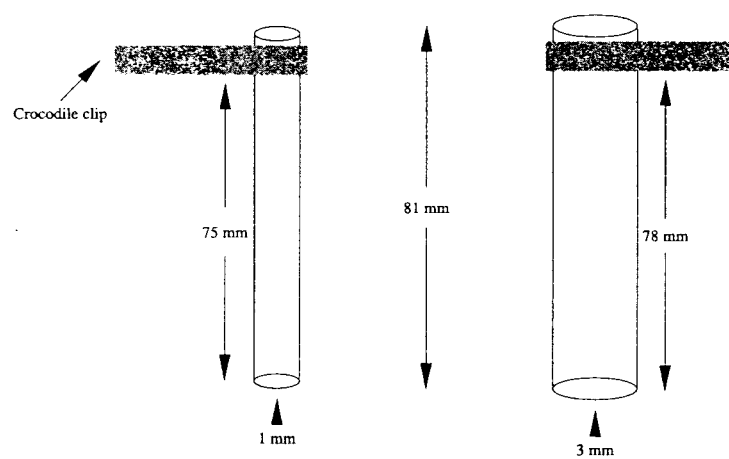
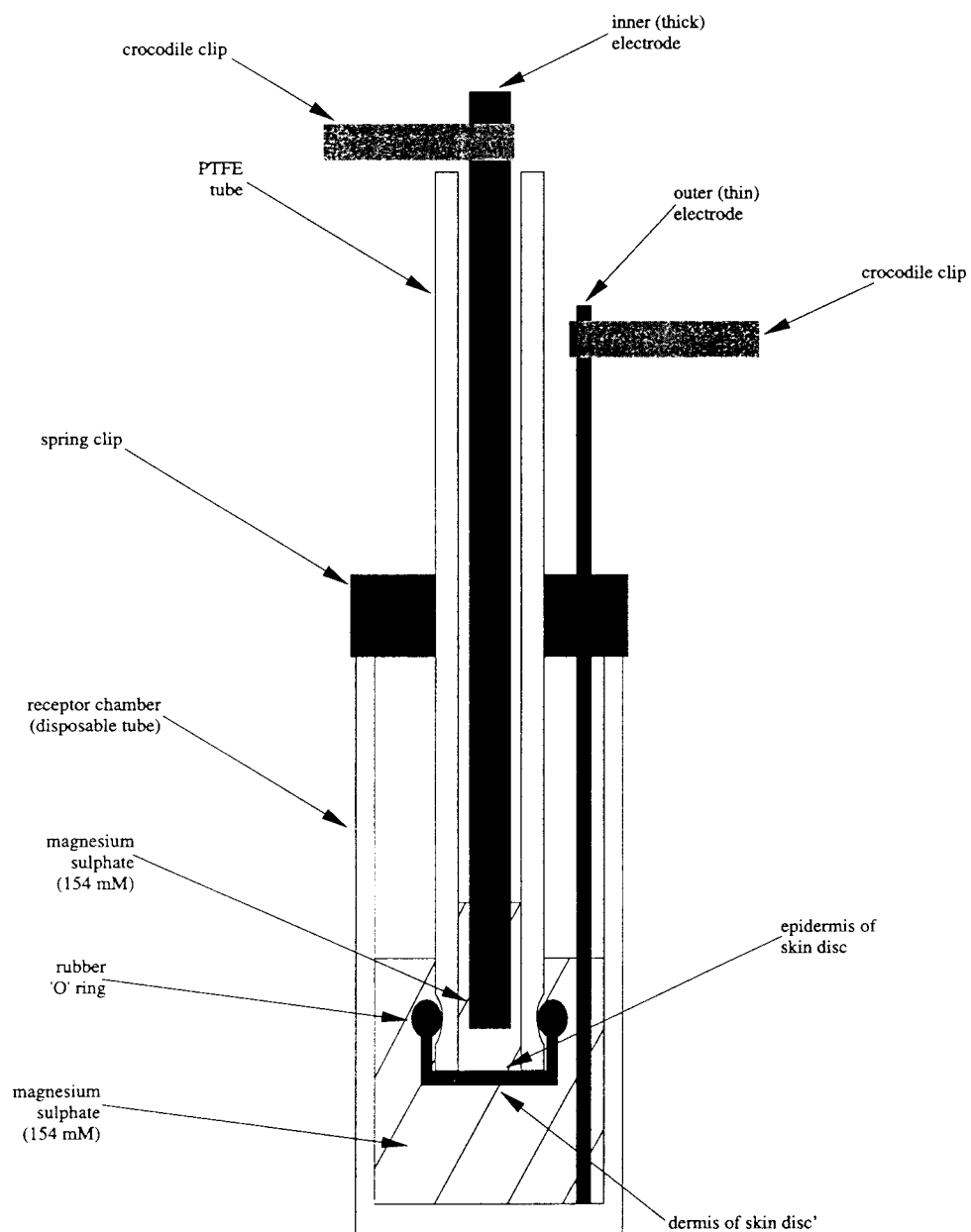
PTFE tube dimensions**Electrode dimensions**

Figure 2

Apparatus for the rat skin TER assay

ANNEX II

B.41. PHOTOTOXICITY — *IN VITRO* 3T3 NRU PHOTOTOXICITY TEST1. **METHOD**1.1. **Introduction**

Phototoxicity is defined as a 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.

Information derived from the *in vitro* 3T3 NRU phototoxicity test serves to identify the phototoxic potential of a test substance, i.e. the existence or absence of possible hazards likely to arise from a test substance in association with exposure to UV and visible light.

Since the toxicological endpoint of the *in vitro* test is determination of photocytotoxicity, induced by the combined action of a chemical and light, compounds that are phototoxic *in vivo* after systemic application and distribution to the skin, as well as compounds that act as photoirritants after topical application to the skin, can be identified by the test.

The *in vitro* 3T3 NRU phototoxicity test was developed and validated in a joint EU/COLIPA project from 1992-1997 (1) (2) (3), to establish a valid *in vitro* alternative to the various *in vivo* tests in use. In 1996 an OECD workshop recommended an *in vitro* tier testing approach for phototoxicity assessment (4).

Results from the *in vitro* 3T3 NRU phototoxicity test were compared with acute phototoxicity/photoirritation effects *in vivo* in animals and humans, and the test has been shown to give excellent predictivity for these effects. The test is not designed to predict other adverse effects that may arise from the combined action of a chemical and light, e.g. photogenotoxicity, photoallergy, and photocarcinogenicity, although many chemicals which show these specific properties will react positive in the *in vitro* 3T3 NRU phototoxicity test. In addition, the test is not designed to permit an assessment of phototoxic potency.

A sequential approach to phototoxicity testing of chemicals is set out in the Appendix.

1.2. **Definitions**

Irradiance: the intensity of ultraviolet (UV) or visible light incident on a surface, measured in W/m² or mW/cm².

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/m² or J/cm².

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 to negative (solvent) controls which have been taken through the whole test procedure (either +UV or -UV), but not treated with a test chemical.

Prediction model: an algorithm used to transform the results of a toxicity test into a prediction of toxic potential. In the present test guideline, PIF and MPE can be used for transformation of the results of the *in vitro* 3T3 NRU phototoxicity test into a prediction of phototoxic potential.

PIF (photo irritation factor): a factor generated by comparing two equally effective cytotoxic concentrations (EC₅₀) of the test chemical obtained in the absence (-UV) and in the presence (+UV) of a non-cytotoxic irradiation with UVA/vis light.

MPE (mean photo effect): a novel measure derived from mathematical analysis of the complete shape of two concentration response curves obtained in the absence (-UV) and in the presence (+UV) of a noncytotoxic irradiation with UVA/vis light.

Phototoxicity: an 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 the systemic administration of a chemical.

Photoirritation: a sub-species of the term "phototoxicity", which is used to describe only those phototoxic reactions which are produced at the skin after exposure to chemicals (topically or orally). These phototoxic reactions lead always to non-specific cell damage (sunburn like reactions).

Photoallergy: an acquired immunological reactivity, which does not occur on first treatment with chemical and light, and needs an induction period of one or two weeks before skin reactivity can be demonstrated.

Photogenotoxicity: a genotoxic response observed with a genetic endpoint, which is elicited after the exposure of cells to a non-genotoxic dose of UV/visible light and a non-genotoxic chemical.

Photocarcinogenicity: carcinogenicity induced by repeated application of light and a chemical. The term "photo co-carcinogenesis", is used if UV induced tumorigenesis is enhanced by a chemical.

1.3. Reference substances

Besides the positive control chemical chlorpromazine which should be concurrently tested in each assay, for newly establishing the 3T3 NRU phototoxicity test it is recommended to use as reference chemicals a subset from the chemicals used in interlaboratory trials with the present test (1) (3) (13).

1.4. Initial considerations

Many types of chemicals have been reported to induce phototoxic effects (5) (6) (7) (8). The only common feature is their ability to absorb light energy within the sunlight region. According to the first law of photochemistry (Grotthaus-Draper's Law) photoreaction requires sufficient absorption of light quanta. Thus, before biological testing according to the present test guideline is considered, a UV/vis absorption spectrum of the test chemical should be determined (e.g. according to OECD Test Guideline 101). If the molar extinction /absorption coefficient is less than $10 \text{ litre} \times \text{mol}^{-1} \times \text{cm}^{-1}$, the chemical has no photoreactive potential and does not need to be tested in the *in vitro* 3T3 NRU phototoxicity test or any other biological test for adverse photochemical effects (Appendix).

1.5. Principle of the test method

Four mechanisms have been identified by which absorption of light by a (chemical) chromophore can result in a phototoxic response (7). All of them result in cell damage. Therefore, 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 UVA/vis light. Cytotoxicity in this test is expressed as a concentration dependent reduction of the uptake of the vital dye neutral red (NR) (9) 24 hours after treatment with the test chemical and irradiation.

Balb/c 3T3 cells are maintained in culture for 24 h for the formation of monolayers. Two 96-well plates per test chemical are then preincubated with eight different concentrations of the chemical for 1 h. Thereafter one of the two plates is exposed to a non-cytotoxic UVA/vis light dose of 5 J/cm^2 UVA (+UV experiment), whereas the other plate is kept in the dark (-UV experiment). 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 (NRU) for 3 h. Relative cell viability, expressed as percentage of untreated negative controls, is calculated for each of the eight test concentrations. To predict the phototoxic potential, the concentration responses obtained in the presence (+UV) and in the absence (-UV) of irradiation are compared, usually at the EC_{50} level, i.e. at the concentration inhibiting cell viability by 50% cf. untreated controls.

1.6. Quality criteria

UVA sensitivity of the cells, historical data: cells should be regularly checked for sensitivity to UVA. Cells are seeded at the density used in the *in vitro* 3T3 NRU phototoxicity test, irradiated the next day with UVA doses from $1\text{-}9 \text{ J/cm}^2$, and cell viability is determined one day later using the NRU assay. Cells meet the quality criteria, if their viability after irradiation with 5 J/cm^2 UVA is not less than 80% of the viability of dark controls. At the highest UVA dose of 9 J/cm^2 , viability should not be less than 50% of that of dark controls. This check should be repeated about every 10th passage of the cells.

UVA sensitivity of the negative control cells, current test: the test meets the quality criteria if negative controls (cells in Earl's balanced salt solution (EBSS) with or without 1% dimethylsulfoxide (DMSO) or 1% ethanol (EtOH)) in the +UVA experiment show a viability of not less than 80% of that of non-irradiated cells in the same solvent of the concurrent dark experiment (-UVA).

Viability of negative controls: the absolute optical density ($OD_{540 \text{ NRU}}$) measured in the NR extract of the negative controls indicates whether the 1×10^4 cells seeded per well have grown with normal doubling time during the two days of the assay. A test meets the acceptance criteria if the mean $OD_{540 \text{ NRU}}$ of untreated controls is $\geq 0,2$.

Positive control: a known phototoxic chemical shall be tested concurrently with each *in vitro* 3T3 NRU phototoxicity test. Chlorpromazine (CPZ) was used as positive control in the EU/COLIPA validation study and is therefore 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 (+UVA): $EC_{50} = 0,1$ to $2,0 \mu\text{g/ml}$, CPZ non-irradiated (-UVA): $EC_{50} = 7,0$ to $90,0 \mu\text{g/ml}$. The photo irritation factor (PIF), i.e. the shift of EC_{50} should be at least 6.

Other known phototoxic chemicals, suitable for the chemical class or solubility characteristics of the test chemical being evaluated, may be used as the concurrent positive controls, in place of CPZ. In this case, based on historical data, the ranges of EC_{50} values and PIF or MPE (mean photo effect) should be adequately defined as acceptance criteria for the test.

1.7. Description of the test method

1.7.1. Preparations

1.7.1.1. Cells

A permanent mouse fibroblast cell line — Balb/c 3T3, clone 31 — either from ATCC or from ECACC was used in the validation study, and is therefore recommended. Other cells or cell lines may be successfully used with the same test protocol, if the 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 should only be used if the results of such checking was satisfactory.

Since the UVA sensitivity of cells may increase with the number of passages, Balb/c 3T3 cells of the lowest obtainable passage number should be used, preferably less than 100. It is important that UVA sensitivity of the Balb/c 3T3 cells is regularly checked according to the quality control procedure described in this guideline.

1.7.1.2. Media and culture conditions

Appropriate culture media and incubation conditions should be used for routine cell passage and during the test procedure. For Balb/c 3T3 cells, these are DMEM supplemented with 10% new-born calf serum, 4 mM glutamine, penicillin and streptomycin; and humidified incubation at 37°C / 7,5% CO_2 . It is particularly important that cell culture conditions ensure a cell cycle time within the normal historical range of the cells or cell line used.

1.7.1.3. 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.

For the phototoxicity test cells are seeded in culture medium at a density such that cultures will not reach confluence by the end of the test, i.e. when cell viability is determined 48 h after the seeding of the cells. For Balb/c 3T3 cells grown in 96-well plates, 1×10^4 cells per well is the recommended cell density.

For each test chemical, cells are seeded identically in two separate 96-well plates, which are then taken concurrently through the whole test procedure under identical culture conditions, except for the time period where one of the plates is irradiated (+UVA/vis) and the other one is kept in the dark (-UVA/vis).

1.7.1.4. Metabolic activation

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, no chemical is known for which 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.

1.7.1.5. Test chemical/preparation

Test chemicals must be freshly prepared immediately prior to use, unless stability data demonstrate the acceptability of storage. Preparation under red light may be required when rapid photodegradation is likely to occur.

Test chemicals should be dissolved in buffered salt solutions, e.g. Earl's balanced salt solution, (EBSS) or phosphate buffered saline (PBS), which, to avoid interference during irradiation, must be free from protein components and light absorbing pH indicator colours.

Test chemicals of limited solubility in water should be dissolved in appropriate solvents at 100-fold the desired final concentration and then diluted 1:100 with the buffered salt solution. If a solvent is used it must be present at a constant volume of 1% (v/v) in all cultures, i.e. in the negative controls as well as in all concentrations of the test chemical.

Dimethylsulphoxide (DMSO) and ethanol (EtOH) are the recommended solvents. Other solvents of low cytotoxicity (e.g. acetone) may be appropriate, but they should carefully be assessed for specific properties, e.g. reaction with the test chemical, quenching of the phototoxic effect, radical catching properties.

Vortex mixing and/or sonication and/or warming to 37°C may be used, if necessary, to aid solubilisation.

1.7.1.6. UV irradiation/preparation

Light source: the choice of an appropriate light source and appropriate filtering is the most crucial factor in phototoxicity testing. UVA and visible regions are usually associated with photosensitisation (7) (10), whereas UVB is of less relevance and is directly highly cytotoxic, increasing its cytotoxicity through 1000 fold from 313 to 280 nm (11). Criteria for the choice of an appropriate light source should include the essential requirement that the light source emits wavelengths absorbed by the test chemical and that the dose of light (achievable in a reasonable time) should be sufficient for the detection of known photosensitisers. Furthermore, the wavelengths and doses employed should not be unduly deleterious to the test system, which includes the emission of heat (infra-red region).

The simulation of sunlight with solar simulators is considered the optimal light source. Both, Xenon arcs and (doped) mercury-metal halide arcs are used in solar simulators. The latter have the advantage of emitting less heat and of being cheaper, but the match to sunlight is not perfect. Since all solar simulators emit significant quantities of UVB, they should be suitably filtered to attenuate the highly cytotoxic UVB wavelengths.

For the *in vitro* 3T3 NRU phototoxicity test an irradiance spectrum practically devoid of UVB should be used (UVA:UVB ~ 1:20). 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 has been published (3).

Dosimetry: the intensity of light (irradiance) should be regularly checked before each phototoxicity test, by using a suitable broadband UV-meter. 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, but such instruments require skilled operation by appropriately trained persons.

A dose of 5 J/cm² (UVA) was determined in the validation study to be non-cytotoxic to Balb/c 3T3 cells and sufficiently potent to excite even weak phototoxic chemicals. To achieve 5 J/cm² within a time period of 50 min, irradiance has to be adjusted to 1,666 mW/cm². If another cell line or a different light source are used, the UVA dose may have to be slightly adapted, by using the criteria of being non-deleterious to the cells and sufficient to detect standard phototoxins. The time of light exposure is calculated in the following way:

$$t(\text{min}) = \frac{\text{Strahlungsdosis (J/cm}^2\text{)} \times 1000}{\text{Strahlung (mW/cm}^2\text{)} \times 60} \quad (1 \text{ J} = 1 \text{ W sec})$$

1.7.2. Test conditions

The maximum concentration of a test chemical should not exceed 100 µg/ml, since all phototoxic chemicals were detected at lower concentrations, whereas at higher concentrations the incidence of false positives (overpredictions) increases (13). The pH of the highest concentration of the test chemical should be satisfactory (pH range: 6,5 - 7,8).

The ranges of concentrations of a chemical tested in the presence (+UVA) and in the absence (–UVA) of light should be adequately determined in preceding range-finder experiments. Range and intercept of a concentration series shall be adjusted in such a way that concentration-response curves are sufficiently supported by experimental data. Geometric concentration series (with a constant dilution factor) should be used.

1.7.3. Test procedure⁽¹⁾

1.7.3.1. First day

Prepare a cell suspension of 1×10^5 cells/ml in culture medium and dispense 100 µl culture medium only 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 (= 1×10^4 cells/well). For each test chemical, prepare two plates: one for determination of cytotoxicity (–UVA), and the other for determination of photocytotoxicity (+UVA).

Incubate the cells for 24 h (7,5% CO₂, 37°C) until they form a half-confluent monolayer. This incubation period allows for cell recovery and adherence, and for exponential growth.

1.7.3.2. Second day

After incubation, decant the culture medium from the cells and wash twice with 150 µl EBSS/PBS per well. Add 100 µl of EBSS/PBS containing the appropriate concentration of test chemical or just solvent (negative control). Apply 8 different concentrations of the test chemical. Incubate cells with the test chemical in the dark for 60 minutes (7,5% CO₂, 37°C).

To perform the (+UVA) part of the assay, irradiate the cells at room temperature for 50 minutes through the lid of the 96-well plate with 1,7 mW/cm² UVA (= 5 J/cm²). Ventilate with a fan to prevent H₂O condensation under the lid. Keep duplicate plates (–UVA) at room temperature in a dark box for 50 min (= UVA exposure time).

Decant test solution and wash twice with 150 µl EBSS/PBS. Replace EBSS/PBS with culture medium and incubate (7,5% CO₂, 37°C) overnight (18–22 h).

1.7.3.3. Third day

Microscopic evaluation

Examine the cells under a phase-contrast microscope. Record changes in morphology of the cells due to cytotoxic effects of the test chemical. This check is recommended, to exclude experimental errors, but these records are not used for evaluation of cytotoxicity or phototoxicity

Neutral red uptake test

Wash the cells with 150 µl prewarmed EBSS/PBS. Remove the washing solution by gentle tapping. Add 100 µl NR medium and incubate at 37°C, in a humidified atmosphere of 7,5% CO₂, for 3 h.

After incubation, remove the NR medium, and wash the cells with 150 µl EBSS/PBS. Decant and blot EBSS/PBS totally. (Optionally: centrifuge reversed plate.)

Add exactly 150 µl NR desorb solution (freshly prepared ethanol/acetic acid)

Shake microtiter plate rapidly on a microtiter plate shaker for 10 min, until the NR has been extracted from the cells and has formed a homogeneous solution.

Measure the optical density of NR extract at 540 nm in a spectrophotometer, using blanks as a reference. Save the data in appropriate file format (e.g. ASCII) for subsequent analysis.

⁽¹⁾ Additional details can be found in reference 12.

2. DATA

2.1. Quality and quantity of data

The data should permit a meaningful analysis of the concentration-response obtained in the presence and in the absence of UVA/vis irradiation. If cytotoxicity is found, both the concentration range and the intercept of individual concentrations should be set in such a way as to allow the fit of a curve to experimental data. Due to the fact that a test chemical might not be cytotoxic up to the defined limit concentration of 100 µg/ml in the dark experiment (–UVA), but highly cytotoxic when irradiated (+UVA), the concentration ranges to be tested in both parts of the experiment may need to differ by orders of magnitude to fulfil the requirement of adequate data quality. If no cytotoxicity is found in both parts of the experiment (–UVA and +UVA), testing with a great intercept between single doses up to the highest concentration is sufficient.

There is no requirement for verification of a clear positive result by performing a repeat experiment. In addition, clear negative results need not to be verified, provided the test chemical was tested at sufficiently high concentrations. In such cases, one main experiment, supported by one or more range-finding preliminary experiments, is sufficient.

Tests with borderline results near to the cut-off line of the prediction model should be repeated for verification.

If repeat testing is considered necessary, then variation of the experimental conditions may be important to achieve a clear result. A key variable in this test is preparation of solutions of the test chemical. Hence, variation of these conditions (co-solvent, trituration, sonication) may be most relevant in the repetition of a test. Alternatively, variation of the pre-irradiation incubation time may be considered. A shorter time can be relevant for water-unstable chemicals.

2.2. Treatment of results

Where possible, the concentration of a test chemical reflecting a 50% inhibition of the cellular NRU (EC₅₀) is determined. This can be done by applying any appropriate non-linear regression procedure (preferably a Hill function or logistic regression) to the concentration-response data, or by using other fitting procedures (14). Before using an EC₅₀ for further calculations, the quality of the fit should be appropriately checked. Alternatively, graphical fitting methods can be used to calculate the EC₅₀. In this case, the use of probability paper is recommended (x-scale: log, y-scale: probit), as in many cases the concentration response function will become almost linear after this transformation.

2.3. Evaluation of results (prediction models)

2.3.1. Prediction model version 1: photo-irritation-factor (PIF)

If both, in the presence (+UVA) and in the absence (–UVA) of light, complete concentration response curves are obtained, a photo-irritation-factor (PIF) is calculated by means of the following formula:

$$(a) \quad PIF = \frac{EC_{50}(-UV)}{EC_{50}(+UV)}$$

A PIF < 5, predicts no phototoxic potential, whereas a PIF ≥ 5 predicts phototoxic potential.

If a chemical is only cytotoxic +UVA and is not cytotoxic when tested –UVA, the PIF cannot be calculated, although this is a result that indicates phototoxic potential. In such cases, a “> PIF” can be calculated if the (–UV) cytotoxicity test is performed up to the highest test concentration (C_{max}) and this value is used for calculation of the “> PIF”;

$$(b) \quad > PIF = \frac{C_{max}(-UV)}{EC_{50}(+UV)}$$

If only a “> PIF” can be obtained, then any value > 1 predicts phototoxic potential.

If both EC₅₀ (–UV) and EC₅₀ (+UV) cannot be calculated due to the fact that a chemical does not show any cytotoxicity up to the highest test concentration, this indicates no phototoxic potential. In such cases, a formal “PIF = *1” is used to characterise the result;

$$(c) \quad PIF = *1 = \frac{C_{max}(-UV)}{C_{max}(+UV)}$$

If only a "PIF = *1" can be obtained, this predicts no phototoxic potential.

In cases (b) and (c), concentrations achieved in the *in vitro* 3T3 NRU phototoxicity test should be carefully taken into consideration when predicting phototoxic potential.

2.3.2. Prediction model version 2: mean-photo effect (MPE)

Alternatively, a novel version of the model for predicting phototoxic potential can be applied, which has been developed by using data of the EU/COLIPA validation study (15) and tested under blind conditions in a subsequent study on the *in vitro* phototoxicity of UV filter chemicals (13). This model overcomes the limitation of the PIF model in cases where an EC_{50} cannot be obtained. The model uses the "mean photo effect" (MPE), a measure which is based on comparison of the complete concentration response curves. For application of the MPE model, a special computer software was developed at the Humboldt University (Berlin), which can be obtained free of charge.

2.4. Interpretation of results

A positive result in the *in vitro* 3T3 NRU phototoxicity test ($PIF \geq 5$ or $MPE \geq 0,1$) indicates that the test substance has phototoxic potential. If this result is obtained at concentrations below 10 µg/ml, the test chemical is also likely to act as phototoxin also under various exposure conditions *in vivo*. If a positive result is obtained only at the highest test concentration of 100 µg/ml, further considerations may be necessary for the assessment of hazard or phototoxic potency. These may include data on penetration, absorption and possible accumulation of the chemical in the skin, or testing of the chemical in a confirmatory alternative test, e.g. using a human *in vitro* skin model.

A negative result from the *in vitro* 3T3 NRU phototoxicity test ($PIF < 5$ or $MPE < 0,1$) indicates that the test substance was not phototoxic to the cultured mammalian cells under the conditions used. In cases where the chemical could be tested up to the highest concentration of 100 µg/ml, a negative result indicates that the chemical has no phototoxic potential, and phototoxicity *in vivo* may be considered unlikely. In cases where identical concentration-toxicity responses ($EC_{50} + UV$ and $EC_{50} - UV$) were obtained at lower concentrations, the interpretation of data would be the same. In contrast, if no toxicity was demonstrated (+UV and -UV) and if aqueous solubility limited concentrations to values less than 100 µg/ml, then compatibility of the test substance with the assay may be questioned and confirmatory testing should be considered (e.g. using an *in vitro* skin model, or an *ex vivo* skin model or an *in vivo* test).

3. REPORTING

Test report

The test report must include the following information:

Test chemical:

- identification data and CAS No, if known,
- physical nature and purity,
- physicochemical properties relevant to conduct of the study,
- stability and photostability, if known.

Solvent:

- justification for choice of solvent,
- solubility of the test chemical in this solvent,
- percentage of solvent present in treatment medium (EBSS or PBS).

Cells:

- type and source of cells,
- absence of mycoplasma,
- number of cell passages, if known,
- UVA sensitivity of cells, determined with the irradiation equipment, used in the *in vitro* 3T3 NRU phototoxicity test.

Test conditions (a) — 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 (b) — treatment with the chemical:

- rationale for selection of concentrations of the test chemical used both in the presence and in the absence of UV/vis 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 (c) — irradiation:

- rationale for selection of the light source used,
- spectral irradiance characteristics of the light source,
- transmission/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 $\times$ time), expressed in J/cm^2 ,
- temperature employed to cell cultures during irradiation, and for cell cultures concurrently kept in the dark.

Test conditions (d) — NRU test:

- composition of NR medium,
- duration of NR incubation,
- incubation conditions (CO_2 concentration, temperature, humidity),
- NR extraction conditions (extractant, duration),
- wavelength used for spectrophotometric reading of NR optical density,
- second wavelength (reference), if used,
- content spectrophotometer blank, if used.

Results:

- cell viability obtained at each concentration of the test chemical, expressed in percent mean viability of controls,
- concentration-response curves, (test chemical concentration v. relative cell viability), obtained in concurrent +UVA and -UVA experiments,
- data analysis of the concentration response curves: if possible, computation/calculation of EC_{50} (+UVA) and EC_{50} (-UVA),
- comparison of the two concentration response curves, obtained in the presence and in the absence of UVA/vis irradiation, either by calculation of the photo irritation factor (PIF), or by calculation of the mean photo effect (MPE),
- classification of phototoxic potential,
- test acceptance criteria (a) — concurrent negative control:
 - absolute viability (optical density of NR extract) of irradiated and non irradiated cells,
 - historical data of negative control, mean and standard deviation.
- test acceptance criteria (b) — concurrent positive control:
 - EC_{50} (+UVA) and EC_{50} (-UVA) and PIF of positive control chemical,
 - historical data of positive control chemical: EC_{50} (+UVA) and EC_{50} (-UVA) and PIF, mean and standard deviation.

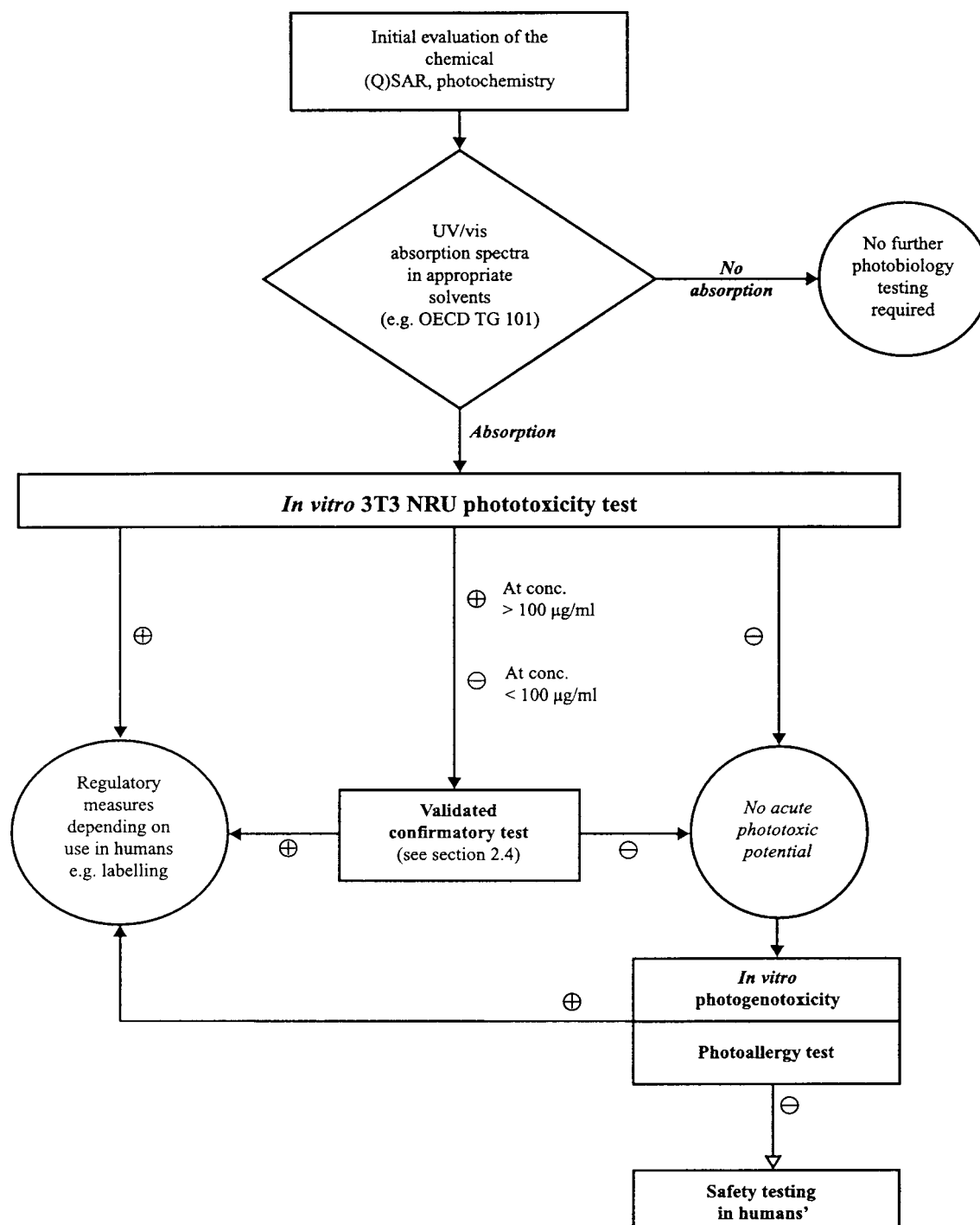
*Discussion of the results.**Conclusions.*

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Appendix

Role of the 3T3 NRU PT in a sequential approach to the phototoxicity testing of chemicals



II

(Acts whose publication is not obligatory)

COMMISSION

COMMISSION DECISION

of 19 May 2000

correcting Directive 98/98/EC adapting to technical progress for the 25th time Council Directive 67/548/EEC on the approximation of the laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances

(notified under document number C(2000) 1333)

(Text with EEA relevance)

(2000/368/EC)

THE COMMISSION OF THE EUROPEAN COMMUNITIES,

Having regard to the Treaty establishing the European Community,

Having regard to 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⁽¹⁾, as last amended by Directive 1999/33/EC of the European Parliament and of the Council⁽²⁾, and in particular Article 28 thereof,

Whereas:

(1) Annex VI to Directive 67/548/EEC contains a guide to the classification and labelling of dangerous substances and preparations. Annex VI was last amended by Annex 4 to Directive 98/98/EC⁽³⁾. Sections 3.2.3, 3.2.8, 6.2 and 8 of Annex 4 to Directive 98/98/EC are incomplete. In consequence it is necessary to correct Annex 4 to Directive 98/98/EC.

(2) The measures provided for in this Decision are in accordance with the opinion of the Committee on the

adaptation to technical progress of the Directives for the elimination of technical barriers to trade in dangerous substances and preparations,

HAS ADOPTED THIS DECISION:

Article 1

Annex 4 to Directive 98/98/EC is replaced by the Annex to this Decision.

Article 2

This Decision is addressed to the Member States.

Done at Brussels, 19 May 2000.

For the Commission
Margot WALLSTRÖM
Member of the
Commission

⁽¹⁾ OJ 196, 16.8.1967, p. 1.

⁽²⁾ OJ L 199, 30.7.1999, p. 57.

⁽³⁾ OJ L 355, 30.12.1998, p. 1.

ANNEX

'ANNEX 4

1.6. For substances, the data required for classification and labelling may be obtained:

- (a) as regards substances for which the information specified in Annex VII is required, most of the necessary data for classification and labelling appear in the base set. This classification and labelling must be reviewed, if necessary, when further information is available (Annex VIII);
- (b) as regards other substances (e.g. those referred to in section 1.5), the data required for classification and labelling may, if necessary, be obtained from a number of different sources, for example the results of previous tests, information required by international rules on the transport of dangerous substances, information taken from reference works and the literature or information derived from practical experience. The results of validated structure-activity relationships and expert judgement may also be taken into account where appropriate.

For preparations, the data required for classification and labelling may be obtained:

- (a) if it concerns physicochemical data, by the application of the methods specified in Annex V. For gaseous preparations, a calculation method may be used for flammable and oxidising properties (see Chapter 9);
- (b) if it concerns data on health effects:
 - by the application of the methods specified in Annex V and/or by the application of the conventional method referred to in Article 3(5)(a) to (i) of Directive 88/379/EEC, or, in the case of R 65, by the application of the rules under 3.2.3,
 - however, if it concerns the evaluation of the carcinogenic, mutagenic and reproductive properties, by the application of the conventional method referred to in Article 3(5)(j) to (q) of Directive 88/379/EEC.

Note concerning the performance of animal tests

The performance of animal tests to establish experimental data is subject to the provisions of Directive 86/609/EEC regarding the protection of animals used for experimental purposes.

1.7.2. *Application of the guide criteria for substances*

The guidance criteria set out in this Annex are directly applicable when the data in question have been obtained from test methods comparable with those described in Annex V. In other cases, the available data must be evaluated by comparing the test methods employed with those indicated in Annex V and the rules specified in this Annex for determining the appropriate classification and labelling.

In some cases there may be doubt over the application of the relevant criteria, especially where these require the use of expert judgement. In such cases the manufacturer, distributor or importer should provisionally classify and label the substance on the basis of an assessment of the evidence by a competent person.

Without prejudice to Article 6, where the above procedure has been followed and there is concern over possible inconsistencies then a proposal may be submitted for the entry of the provisional classification into Annex I. The proposal should be made to one of the Member States and should be accompanied by appropriate scientific data (see also section 4.1).

A similar procedure may be followed when information is identified which gives cause for concern over the accuracy of an existing entry in Annex I.

2.2.2.1. *Remarks concerning peroxides*

For the explosive properties, an organic peroxide or preparation thereof in the form in which it is placed on the market is classified according to the criteria in section 2.2.1 on the basis of tests carried out in accordance with the methods given in Annex V.

For the oxidising properties the existing methods in Annex V cannot be applied to organic peroxides.

For substances, organic peroxides not already classified as explosive are classified as dangerous on the basis of their structure (e.g. R-O-O-H; R₁-O-O-R₂).

Preparations not already classified as explosive shall be classified using the calculation method based on the percentage of active oxygen shown in section 9.5.

Any organic peroxide or preparation thereof not already classified as explosive is classified as oxidising, if the peroxide or its formulation contains:

- more than 5 % of organic peroxides or,
- more than 0,5 % available oxygen from the organic peroxides, and more than 5 % hydrogen peroxide.

3.2.3. Harmful

Substances and preparations shall be classified as harmful and assigned the symbol "Xn" and the indication of danger "harmful" in accordance with the criteria specified below. Risk phrases shall be assigned in accordance with the following criteria:

R 22 Harmful if swallowed

Acute toxicity results:

- LD₅₀ per oral, rat: 200 < LD₅₀ ≤ 2 000 mg/kg,
- discriminating dose, oral, rat 50, mg/kg: 100 % survival but evident toxicity,
- less than 100 % survival at 500 mg/kg, rat oral by the fixed dose procedure. Refer to the evaluation table in the test method Bl(a) of Annex V.

R 21 Harmful in contact with skin

Acute toxicity results:

- LD₅₀ dermal, rat or rabbit: 400 < LD₅₀ ≤ 2 000 mg/kg.

R 20 Harmful by inhalation

Acute toxicity results:

- LC₅₀ inhalation, rat, for aerosols or particulates: 1 < LC₅₀ ≤ 5 mg/litre/4 hr,
- LC₅₀ inhalation, rat, for gases or vapours: 2 < LC₅₀ ≤ 20 mg/litre/4 hr.

R 65 Harmful: may cause lung damage if swallowed

Liquid substances and preparations presenting an aspiration hazard in humans because of their low viscosity

(a) For substances and preparations containing aliphatic, alicyclic and aromatic hydrocarbons in a total concentration equal to or greater than 10 % and having either:

- a flow time of less than 30 seconds in a 3 mm ISO cup according to ISO 2431, or
- a kinematic viscosity measured by a calibrated glass capillary viscometer in accordance with ISO 3104/3105 of less than 7×10^{-6} m²/sec at 40 °C, or
- a kinematic viscosity derived from measurements of rotational viscometry in accordance with ISO 3129 of less than 7×10^{-6} m²/sec at 40 °C.

Note that substances and preparations meeting these criteria need not be classified if they have a mean surface tension greater than 33 mN/m at 25 °C as measured by the du Nouy tensiometer or by the test methods shown in Annex V Part A(5);

(b) For substances and preparations, based on practical experience in humans.

R 40 Possible risk of irreversible effects

- strong evidence that irreversible damage other than the effects referred to in Chapter 4 is likely to be caused by a single exposure by an appropriate route, generally in the abovementioned dose range.

In order to indicate route of administration/exposure one of the following combinations shall be used: R 40/20, R 40/21, R 40/22, R 40/20/21, R 40/20/22, R 40/21/22, R 40/20/21/22.

R 48 Danger of serious damage to health by prolonged exposure

- serious damage (clear functional disturbance or morphological change which has toxicological significance) is likely to be caused by repeated or prolonged exposure by an appropriate route.

Substances and preparations are classified at least as harmful when these effects are observed at levels of the order of:

- oral, rat ≤ 50 mg/kg (body weight)/day,
- dermal, rat or rabbit ≤ 100 mg/kg (body weight)/day,
- inhalation, rat $\leq 0,25$ mg/l, 6 h/day.

These guide values can apply directly when severe lesions have been observed in a subchronic (90 days) toxicity test. When interpreting the results of a subacute (28 days) toxicity test these figures should be increased approximately three fold. If a chronic (two years) toxicity test is available it should be evaluated on a case-by-case basis. If results of studies of more than one duration are available, then those from the study of the longest duration should normally be used.

In order to indicate route of administration/exposure one of the following combinations shall be used: R 48/20, R 48/21, R 48/22, R 48/20/21, R 48/20/22, R 48/21/22, R 48/20/21/22.

3.2.3.1. Comments regarding volatile substances

For certain substances with a high saturated vapour concentration, evidence may be available to indicate effects that give cause for concern. Such substances may not be classified under the criteria for health effects in this guide (3.2.3) or not covered by Section 3.2.8. However, where there is appropriate evidence that such substances may present a risk in normal handling and use, then classification on a case-by-case basis in Annex I may be necessary.

3.2.6.1. Inflammation of the skin

The following risk phrase shall be assigned in accordance with the criteria given:

R 38 Irritating to skin

- substances and preparations which cause significant inflammation of the skin which persists for at least 24 hours after an exposure period of up to four hours determined on the rabbit according to the cutaneous irritation test method cited in Annex V.

Inflammation of the skin is significant if:

- (a) the mean value of the scores for either erythema and eschar formation or oedema formation, calculated over all the animals tested, is two or more;
- (b) or, in the case where the Annex V test has been completed using three animals, either erythema and eschar formation or oedema formation equivalent to a mean value of two or more calculated for each animal separately has been observed in two or more animals.

In both cases all scores at each of the reading times (24, 48 and 72 hours) for an effect should be used in calculating respective mean values.

Inflammation of the skin is also significant if it persists in at least two animals at the end of the observation time. Particular effects, for example, hyperplasia, scaling, discoloration, fissures, scabs and alopecia should be taken into account.

Relevant data may also be available from non-acute animal studies (see comments on R 48, section 2(d)). These are considered significant if the effects seen are comparable to those described above;

- substances and preparations which cause significant inflammation of the skin, based on practical observations in humans on immediate, prolonged or repeated contact;
- organic peroxides, except where evidence to the contrary is available.

Paresthesia: Paresthesia caused in humans by skin contact with pyrethroid pesticides is not regarded as an irritant effect justifying classification as Xi; R 38. The S-phrase S 24 should however be applied for substances seen to cause this effect.

3.2.8. Other toxicological properties

Additional risk phrases shall be assigned in accordance with the following criteria (based on experience obtained during compilation of Annex I) to substances and preparations classified by virtue of 2.2.1 to 3.2.7 above and/or Chapters 4 and 5:

R 29 Contact with water liberates toxic gas

For substances and preparations which in contact with water or damp air, evolve very toxic or toxic gases in potentially dangerous amounts, for example, aluminium phosphide, phosphorus pentasulphide.

R 31 Contact with acids liberates toxic gas

For substances and preparations which react with acids to evolve toxic gases in dangerous amounts, for example, sodium hypochlorite, barium polysulphide. For substances used by members of the general public, the use of S 50 (do not mix with ... (to be specified by the manufacturer)) would be more suitable.

R 32 Contact with acids liberates very toxic gas

For substances and preparations which react with acids to evolve very toxic gases in dangerous amounts; e.g. salts of hydrogen cyanide, sodium azide. For substances used by members of the general public, the use of S 50 (do not mix with ... (to be specified by the manufacturer)) would be more suitable.

R 33 Danger of cumulative effects

For substances and preparations when accumulation in the human body is likely and may cause some concern which, however, is not sufficient to justify the use of R 48.

R 64 May cause harm to breastfed babies

For substances and preparations which are absorbed by women and may interfere with lactation or which may be present (including metabolites) in breast milk in amounts sufficient to cause concern for the health of a breastfed child.

For comments on the use and this R-phrase (and in some cases R 33) see section 4.2.3.3.

R 66 Repeated exposure may cause skin dryness or cracking

For substances and preparations which may cause concern as a result of skin dryness, flaking or cracking but which do not meet the criteria for R 38:

based on either:

- practical observation after normal handling and use, or
- relevant evidence concerning their predicted effects on the skin.

See also sections 1.6 and 1.7.

R 67 Vapours may cause drowsiness and dizziness

For volatile substances and preparations containing such substances which cause clear symptoms of central nervous system depression by inhalation and which are not already classified with respect to acute inhalation toxicity (R 20, R 23, R 26, R 40/20, R 39/23 or R 39/26).

The following evidence may be used:

- (a) data from animal studies showing clear signs of CNS depression such as narcotic effects, lethargy, lack of coordination (including loss of righting reflex) and ataxia either:
 - at concentrations/exposure times not exceeding 20 mg/l/4 h or,
 - for which the ratio of the effect concentration at ≤ 4 h to the saturated vapour concentration (SVC) at 20 °C is $\leq 1/10$;
- b) practical experience in humans (e.g. narcosis, drowsiness, reduced alertness, loss of reflexes, lack of coordination, vertigo) from well documented reports under comparable exposure conditions to the effects specified above for animals.

See also sections 1.6 and 1.7.

For other supplementary risk phrases see section 2.2.6.

- 4.1.2. If a manufacturer, distributor or importer has information available which indicates that a substance should be classified and labelled in accordance with the criteria given in section 4.2.1, 4.2.2 or 4.2.3, he shall provisionally label the substance in accordance with these criteria, on the basis of the assessment of the evidence by a competent person.
- 4.1.3. The manufacturer, distributor or importer shall submit as soon as possible a document summarising all relevant information to one Member State in which the substance is placed on the market. This summary document should include a bibliography containing all relevant references, including any relevant unpublished data.
- 4.1.4. Furthermore, a manufacturer, distributor or importer who has new data which are relevant to the classification and labelling of a substance in accordance with the criteria given in section 4.2.1, 4.2.2 or 4.2.3, shall submit this data as soon as possible to one Member State in which the substance is placed on the market.

5.2.2. *Non-aquatic environment*

- 5.2.2.1. Substances shall be classified as dangerous for the environment and assigned the symbol "N" and the appropriate indication of danger, and assigned risk phrases in accordance with the following criteria:

R 54: Toxic to flora

R 55: Toxic to fauna

R 56: Toxic to soil organisms

R 57: Toxic to bees

R 58: May cause long term adverse effects on the environment

Substances which on the basis of the available evidence concerning their properties, persistence, potential to accumulate and predicted or observed environmental fate and behaviour may present a danger, immediate or long-term and/or delayed, to the structure and/or functioning of natural ecosystems other than those covered under 5.2.1. Detailed criteria will be elaborated later.

- 5.2.2.2. Substances shall be classified as dangerous for the environment and assigned the symbol "N" and the appropriate indication of danger, and assigned risk phrases in accordance with the following criteria:

R 59: Dangerous for the ozone layer

Substances which on the basis of the available evidence concerning their properties and their predicted or observed environmental fate and behaviour may present a danger to the structure and/or functioning of the stratospheric ozone layer. This includes the substances which are listed in Annex I to Council Regulation (EC) No 3093/94 on substances that deplete the ozone layer (OJ L 333, 22.12.1994, p. 1) and its subsequent amendments.

6.2. **Safety phrases for substances and preparations.**

S 1 Keep locked up

- Applicability:
 - very toxic, toxic and corrosive substances and preparations,
- Criteria for use:
 - obligatory for those substances and preparations mentioned above if sold to the general public.

S 2 Keep out of the reach of children

- Applicability:
 - all dangerous substances and preparations,
- Criteria for use:
 - obligatory for all dangerous substances and preparations sold to the general public, except for those only classified as dangerous for the environment.

S 3 Keep in a cool place

- Applicability:
 - organic peroxides,
 - other dangerous substances and preparations having a boiling point $\leq 40^{\circ}\text{C}$,
- Criteria for use:
 - obligatory for organic peroxides unless S 47 is used,
 - recommended for other dangerous substances and preparations having a boiling point $\leq 40^{\circ}\text{C}$.

S 4 Keep away from living quarters

- Applicability:
 - very toxic and toxic substances and preparations,
- Criteria for use:
 - normally limited to very toxic and toxic substances and preparations when desirable to supplement S 13; for example when there is an inhalation risk and the substance or preparation should be stored away from living quarters. The advice is not intended to preclude proper use of the substance or preparation in living quarters.

S 5 Keep contents under ... (appropriate liquid to be specified by the manufacturer)

- Applicability:
 - spontaneously flammable solid substances and preparations,
- Criteria for use:
 - normally limited to special cases, for example, sodium, potassium or white phosphorous.

S 6 Keep under ... (inert gas to be specified by the manufacturer)

- Applicability:
 - dangerous substances and preparations which must be kept under an inert atmosphere,
- Criteria for use:
 - normally limited to special cases, for example, certain organometallic compounds.

S 7 Keep container tightly closed

- Applicability:
 - organic peroxides,
 - substances and preparations which can give off very toxic, toxic, harmful or extremely flammable gases,
 - substances and preparations which in contact with moisture give off extremely flammable gases,
 - highly flammable solids,
- Criteria for use:
 - obligatory for organic peroxides,
 - recommended for the other fields of application mentioned above.

S 8 Keep container dry

- Applicability:
 - substances and preparations which may react violently with water,
 - substances and preparations which on contact with water liberate extremely flammable gases,
 - substances and preparations which on contact with water liberate very toxic or toxic gases,
- Criteria for use:
 - normally limited to the fields of application mentioned above when necessary to reinforce warnings given by R 14, R 15 in particular, and R 29.

S 9 Keep container in a well-ventilated place

- Applicability:
 - volatile substances and preparations which may give off very toxic, toxic or harmful vapours,
 - extremely flammable or highly flammable liquids and extremely flammable gases,
- Criteria for use:
 - recommended for volatile substances and preparations which may give off very toxic, toxic or harmful vapours,
 - recommended for extremely flammable or highly flammable liquids or extremely flammable gases.

S 12 Do not keep the container sealed

- Applicability:
 - substances and preparations which will by giving off gases or vapours be liable to burst the container,
- Criteria for use:
 - normally limited to the special cases mentioned above.

S 13 Keep away from food, drink and animal feedingstuffs

- Applicability:
 - very toxic, toxic and harmful substances and preparations,
- Criteria for use:
 - recommended when such substances and preparations are likely to be used by the general public.

S 14 Keep away from ... (incompatible materials to be indicated by the manufacturer)

- Applicability:
 - organic peroxides,
- Criteria for use:
 - obligatory for and normally limited to organic peroxides. However, may be useful in exceptional cases when incompatibility is likely to produce a particular risk.

S 15 Keep away from heat

- Applicability:
 - substances and preparations which may decompose or which may react spontaneously under the effect of heat,
- Criteria for use:
 - normally limited to special cases, for example monomers, but not assigned if risk phrases R 2, R 3 and/or R 5 have already been applied.

S 16 Keep away from sources of ignition — no smoking

- Applicability:
 - extremely flammable or highly flammable liquids and extremely flammable gases,
- Criteria for use:
 - recommended for the substances and preparations mentioned above but not assigned if risk phrases R 2, R 3 and/or R 5 have already been applied.

S 17 Keep away from combustible material

- Applicability:
 - substances and preparations which may form explosive or spontaneously flammable mixtures with combustible material,
- Criteria for use:
 - available for use in special cases, for example to emphasise R 8 and R 9.

S 18 Handle and open container with care

- Applicability:
 - substances and preparations liable to produce an overpressure in the container,
 - substances and preparations which may form explosive peroxides,
- Criteria for use:
 - normally limited to the abovementioned cases when there is risk of damage to the eyes and/or when the substances and preparations are likely to be used by the general public.

S 20 When using do not eat or drink

- Applicability:
 - very toxic, toxic and corrosive substances and preparations,
- Criteria for use:
 - normally limited to special cases (for example arsenic and arsenic compounds; (fluoracetates) in particular when any of these are likely to be used by the general public.

S 21 When using do not smoke

- Applicability:
 - substances and preparations which produce toxic products on combustion.
- Criteria for use:
 - normally limited to special cases (e.g. halogenated compounds).

S 22 Do not breathe dust

- Applicability:
 - all solid substances and preparations dangerous for health,
- Criteria for use:
 - obligatory for those substances and preparations mentioned above to which R 42 is assigned,
 - recommended for those substances and preparations mentioned above which are supplied in the form of an inhalable dust and for which the health hazards following inhalation are not known.

S 23 Do not breathe gas/fumes/vapour/spray (appropriate wording to be specified by the manufacturer)

- Applicability:
 - all liquid or gaseous substances and preparations dangerous to health,
- Criteria for use:
 - obligatory for those substances and preparations mentioned above to which R 42 is assigned,
 - obligatory for substances and preparations intended for use by spraying. Either S 38 or S 51 must be ascribed in addition,
 - recommended when it is necessary to draw the attention of the user to inhalation risks not mentioned in the risk phrases which have to be ascribed.

S 24 Avoid contact with skin

- Applicability:
 - all substances and preparations dangerous for health,
- Criteria for use:
 - obligatory for those substances and preparations to which R 43 has been ascribed, unless S 36 has also been ascribed,
 - recommended when it is necessary to draw the attention of the user to skin contact risks not mentioned in the risk phrases (e.g. paresthesia) which have to be ascribed. However, may be used to emphasise such risk phrases.

S 25 Avoid contact with eyes

- Applicability:
 - all substances and preparations dangerous to health,
- Criteria for use:
 - recommended when it is necessary to draw the attention of the user to eye contact risks not mentioned in the risk phrases which have to be applied. However, may be used to emphasise such risk phrases,
 - recommended for substances ascribed R 34, R 35, R 36 or R 41 which are likely to be used by the general public.

S 26 In case of contact with eyes, rinse immediately with plenty of water and seek medical advice

- Applicability:
 - corrosive or irritant substances and preparations,
- Criteria for use:
 - obligatory for corrosive substances and preparations and those to which R 41 has already been ascribed,
 - recommended for irritant substances and preparations to which the risk phrase R 36 has already been ascribed.

S 27 Take off all contaminated clothing immediately

- Applicability:
 - very toxic, toxic or corrosive substances and preparations,
- Criteria for use:
 - obligatory for very toxic substances and preparations to which R 27 has been ascribed and which are likely to be used by the general public.
 - recommended for very toxic substances and preparations to which R 27 has been ascribed used in industry. However, this safety phrase should not be used if S 36 has been ascribed.
 - recommended for toxic substances and preparations to which R 24 has been ascribed as well as corrosive substances and preparations which are likely to be used by the general public.

S 28 After contact with skin, wash immediately with plenty of ... (to be specified by the manufacturer).

- Applicability:
 - very toxic, toxic or corrosive substances and preparations,
- Criteria for use:
 - obligatory for very toxic substances and preparations,
 - recommended for the other substances and preparations mentioned above, in particular when water is not the most appropriate rinsing fluid,
 - recommended for corrosive substances and preparations which are likely to be used by the general public.

S 29 Do not empty into drains

- Applicability:
 - extremely or highly flammable liquids immiscible with water,
 - very toxic and toxic substances and preparations,
 - substances dangerous for the environment,
- Criteria for use:
 - obligatory for substances dangerous for the environment and assigned the symbol "N", which are likely to be used by the general public, unless this is the intended use.
 - recommended for other substances and preparations mentioned above which are likely to be used by the general public, unless this is the intended use.

S 30 Never add water to this product

- Applicability:
 - substances and preparations which react violently with water,
- Criteria for use:
 - normally limited to special cases (e.g. sulphuric acid) and may be used, as appropriate, to give the clearest possible information, either to emphasise R 14 or as an alternative to R 14.

S 33 Take precautionary measures against static discharges

- Applicability:
 - extremely or highly flammable substances and preparations,
- Criteria for use:
 - recommended for substances and preparations used in industry which do not absorb moisture. Virtually never used for substances and preparations as placed on the market for use by the general public.

S 35 This material and its container must be disposed of in a safe way.

- Applicability:
 - all dangerous substances and preparations,
- Criteria for use:
 - recommended for substances and preparations where special guidance is needed to ensure proper disposal.

S 36 Wear suitable protective clothing

- Applicability:
 - organic peroxides,
 - very toxic, toxic or harmful substances and preparations,
 - corrosive substances and preparations,
- Criteria for use:
 - obligatory for very toxic and corrosive substances and preparations,
 - obligatory for those substances and preparations to which either R 21 or R 24 has been ascribed,
 - obligatory for category 3 carcinogens, mutagens and substances toxic to reproduction unless the effects are produced solely by inhalation of the substance or preparation,
 - obligatory for organic peroxides,
 - recommended for toxic substances and preparations if the LD₅₀ dermal value is unknown but the substance or preparation is likely to be toxic through skin contact,
 - recommended for substances and preparations used in industry which are liable to damage health by prolonged exposure.

S 37 Wear suitable gloves

- Applicability:
 - very toxic, toxic, harmful or corrosive substances and preparations,
 - organic peroxides,
 - substances and preparations irritating to the skin or causing sensitisation by skin contact,

- Criteria for use:
 - obligatory for very toxic and corrosive substances and preparations,
 - obligatory for those substances and preparations to which either R 21, R 24 or R 43 has been ascribed,
 - obligatory for category 3 carcinogens, mutagens and substances toxic to reproduction unless the effects are produced solely by inhalation of the substances and preparations,
 - obligatory for organic peroxides,
 - recommended for toxic substances and preparations if the LD₅₀ dermal value is unknown but the substance or preparation is likely to be harmful by skin contact,
 - recommended for substances and preparations irritating to the skin.

S 38 In case of insufficient ventilation, wear suitable respiratory equipment

- Applicability:
 - very toxic or toxic substances and preparations,
- Criteria for use:
 - normally limited to special cases involving the use of very toxic or toxic substances and preparations in industry or in agriculture.

S 39 Wear eye/face protection

- Applicability:
 - organic peroxides,
 - corrosive substances and preparations, including irritants which give rise to risk of serious damage to the eyes.
 - very toxic and toxic substances and preparations,
- Criteria for use:
 - obligatory for those substances and preparations to which R 34, R 35 or R 41 have been ascribed,
 - obligatory for organic peroxides,
 - recommended when it is necessary to draw the attention of the user to eye contact risks not mentioned in the risk phrases which have to be ascribed,
 - normally limited to exceptional cases for very toxic and toxic substances and preparations, where there is a risk of splashing and they are likely to be easily absorbed by the skin.

S 40 To clean the floor and all objects contaminated by this material use ... (to be specified by the manufacturer)

- Applicability:
 - all dangerous substances and preparations,
- Criteria for use:
 - normally limited to those dangerous substances and preparations for which water is not considered to be a suitable cleansing agent (e.g. where absorption by powdered material, dissolution by solvent, etc. is necessary) and where it is important for health and/or safety reasons to provide a warning on the label.

S 41 In case of fire and/or explosion do not breathe fumes

- Applicability:
 - dangerous substances and preparations which on combustion give off very toxic or toxic gases,
- Criteria for use:
 - normally limited to special cases.

S 42 During fumigation/spraying wear suitable respiratory equipment (appropriate wording to be specified by the manufacturer)

- Applicability:
 - substances and preparations intended for such use but which may endanger the health and safety of the user unless proper precautions are taken,
- Criteria for use:
 - normally limited to special cases.

S 43 In case of fire use ... (indicate in the space the precise type of fire-fighting equipment. If water increases the risk add: Never use water)

- Applicability:
 - extremely flammable, highly flammable and flammable substances and preparations,
- Criteria for use:
 - obligatory for substances and preparations which, in contact with water or damp air, evolve extremely flammable gases,
 - recommended for extremely flammable, highly flammable and flammable substances and preparations, particularly when they are immiscible with water.

S 45 In case of accident or if you feel unwell seek medical advice immediately (show the label where possible).

- Applicability:
 - very toxic substances and preparations,
 - toxic and corrosive substances and preparations,
 - substances and preparations causing sensitisation by inhalation,
- Criteria for use:
 - obligatory for the substances and preparations mentioned above.

S 46 If swallowed, seek medical advice immediately and show this container or label

- Applicability:
 - all dangerous substances and preparations other than those which are very toxic, toxic, corrosive or dangerous to the environment,
- Criteria for use:
 - obligatory for all dangerous substances and preparations mentioned above which are likely to be used by the general public, unless there is no reason for fear any danger from swallowing, particularly by children.

S 47 Keep at temperature not exceeding ... °C (to be specified by the manufacturer)

- Applicability:
 - substances and preparations which become unstable at a certain temperature,
- Criteria for use:
 - normally limited to special cases (e.g. certain organic peroxides).

S 48 Keep wetted with ... (appropriate material to be specified by the manufacturer)

- Applicability:
 - substances and preparations which may become very sensitive to sparks, friction or impact if allowed to dry out,
- Criteria for use:
 - normally limited to special cases, for example nitrocelluloses.

S 49 Keep only in the original container

- Applicability:
 - substances and preparations sensitive to catalytic decomposition,
- Criteria for use:
 - substances and preparations sensitive to catalytic decomposition, for example certain organic peroxides.

S 50 Do not mix with ... (to be specified by the manufacturer)

- Applicability:
 - substances and preparations which may react with the specified product to evolve very toxic or toxic gases,
 - organic peroxides,
- Criteria for use
 - recommended for substances and preparations mentioned above which are likely to be used by the general public, when it is a better alternative to R 31 or R 32,
 - obligatory with certain peroxides which may give violent reaction with accelerators or promoters.

S 51 Use only in well-ventilated areas

- Applicability:
 - substances and preparations likely to or intended to produce vapours, dusts, sprays, fumes, mists, etc. which give rise to inhalation risks or to a fire or explosion risk,
- Criteria for use:
 - recommended when use of S 38 would not be appropriate. Thus important when such substances and preparations are likely to be used by the general public.

S 52 Not recommended for interior use on large surface areas

- Applicability:
 - volatile, very toxic, toxic and harmful substances and preparations containing them,
- Criteria for use:
 - recommended when damage to health is likely to be caused by prolonged exposure to these substances by reason of their volatilisation from large treated surfaces in the home or other enclosed places where persons congregate.

S 53 Avoid exposure — Obtain special instructions before use

- Applicability:
 - substances and preparations that are carcinogenic, mutagenic and/or toxic to reproduction,
- Criteria for use:
 - obligatory for the abovementioned substances and preparations to which at least one of the following R-phrases has been assigned: R 45, R 46, R 49, R 60 or R 61.

S 56 Dispose of this material and its container to hazardous or special waste collection point

- Applicability:
 - all dangerous substances and preparations,
- Criteria for use:
 - recommended for all dangerous substances and preparations likely to be used by the general public for which special disposal is required.

S 57 Use appropriate containment to avoid environmental contamination

- Applicability:
 - substances which have been assigned the symbol "N",
- Criteria for use:
 - normally limited to substances not likely to be used by the general public.

S 59 Refer to manufacturer/supplier for information on recovery/recycling

- Applicability:
 - all dangerous substances and preparations,
- Criteria for use:
 - obligatory for substances dangerous for the ozone layer,
 - recommended for other substances and preparations for which recovery/recycling is recommended.

S 60 This material and its container must be disposed of as hazardous waste

- Applicability:
 - all dangerous substances and preparations,
- Criteria for use:
 - recommended for substances and preparations not likely to be used by the general public and where S 35 is not assigned.

S 61 Avoid release to the environment. Refer to special instructions/safety data sheet

- Applicability:
 - substances dangerous for the environment,
- Criteria for use:
 - normally used for substances which have been assigned the symbol "N",
 - recommended for all substances classified dangerous for the environment not covered above.

S 62 If swallowed, do not induce vomiting: seek medical advice immediately and show this container or label.

- Applicability:
 - substances and preparations classified as harmful with R 65 in accordance with the criteria in section 3.2.3,
 - not applicable to substances and preparations which are placed on the market in aerosol containers (or in containers fitted with a sealed spray attachment), see sections 8 and 9,
- Criteria for use:
 - obligatory for substances and preparations mentioned above, if sold to, or likely to be used by the general public, except when S 45 or S 46 are obligatory,
 - recommended for the substances and preparations mentioned above when used in industry, except where S 45 or S 46 are obligatory,

S 63 In case of accident by inhalation: remove casualty to fresh air and keep at rest

- Applicability:
 - very toxic and toxic substances and preparations (gases, vapours, particulates, volatile liquids),
 - substances and preparations causing respiratory sensitisation,

- Criteria for use:
 - obligatory for substances and preparation to which R 26, R 23 or R 42 has been assigned which are likely to be used by the general public in a way which could result in inhalation

S 64 If swallowed, rinse mouth with water, (only if the person is conscious).

- Applicability:
 - corrosive or irritant substances and preparations,
- Criteria for use:
 - recommended for the above substances and preparations which are likely to be used by the general public and where the above treatment is suitable.

7.5.2. *Choice of safety phrases*

The final choice of safety phrases must have regard to the risk phrases indicated on the label and to the intended use of the substance or preparation:

- as a general rule, a maximum of four S-phrases shall suffice to formulate the most appropriate safety advice; for this purpose the combined phrases listed in Annex IV shall be regarded as single phrases,
- in the case of S-phrases concerning disposal, one S-phrase shall be used, unless it is clear that disposal of the material and its container does not present a danger for human health or the environment. In particular, advice on safe disposal is important for substances and preparations sold to the general public,
- some R-phrases become superfluous if a careful selection is made of S-phrases and vice versa; S-phrases which obviously correspond to R-phrases will appear on the label only if it is intended to emphasise a specific warning,
- particular attention must be given, in the choice of safety phrases, to the foreseen conditions of use of certain substances and preparations, for example spraying or other aerosol effects. Phrases should be chosen with the intended use in view,
- the safety phrases S 1, S 2 and S 45 are obligatory for all very toxic, toxic and corrosive substances and preparations sold to the general public,
- the safety phrases S 2 and S 46 are obligatory for all other dangerous substances and preparations (except those only classified as dangerous to the environment) sold to the general public.

Where the phrases selected according to the strict criteria in 6.2 result in redundancy or ambiguity or are clearly unnecessary given the specific product/package then some phrases may be deleted.

8. SPECIAL CASES: SUBSTANCES

8.1. **Mobile gas cylinders**

For mobile gas cylinders the requirements concerning labelling are considered to be satisfied when they are in agreement with Article 23 or Article 24(6)b.

However, by way of derogation from Article 24(1) and 82), one of the following alternatives can be used for gas cylinders with a water capacity of less than or equal to 150 litres:

- the format and dimensions of the label can follow the prescriptions of the ISO standard ISO/DP 7225,
- the information specified in Article 23(2) may be provided on a durable information disc or label held captive on the cylinder.

8.2. Gas containers intended for propane, butane or liquefied petroleum gas (LPG)

These substances are classified in Annex I. Although classified in accordance with Article 2, they do not present a danger to human health when they are placed on the market in closed refillable cylinders or in non-refillable cartridges within the scope of EN 417 as fuel gases which are only released for combustion.

These cylinders or cartridges must be labelled with the appropriate symbol and the R- and S-phrases concerning flammability. No information concerning the effects on human health is required on the label. However, the information concerning effects on human health which should have appeared on the label shall be transmitted to the professional user by the person responsible for placing the substance on the market in the format foreseen in Article 27 of the Directive. For the consumer, sufficient information shall be transmitted to enable them to take all necessary measures for health and safety as foreseen in Article 1(3) of Directive 91/155/EEC, as modified by Directive 93/112/EEC.

8.3. Metals in massive form

These substances are classified in Annex I or shall be classified in accordance with Article 6. However, some of these substances, although classified in accordance with Article 2 do not present a danger to human health by inhalation, ingestion or contact with skin or to the aquatic environment in the form in which they are placed on the market. Such substances do not require a label according to Article 23. However, all the information which should have appeared on the label shall be transmitted to the user by the person responsible for placing the metal on the market, in a format foreseen in Article 27.

8.4. Substances classified with R 65

Substances classified as harmful on the basis of an aspiration hazard need not be labelled as harmful with R 65 when placed on the market in aerosol containers or in containers fitted with a sealed spray attachment. '
