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29.12.1992 *IL-ĠURNAL UFFIĊJALI TAL-KOMUNITAJIET EWROPEJ* L 383/113

ID-DIRETTIVA TAL-KUMMISSJONI 92/69/KEE

tat-31 ta' Lulju 1992

li tadatta għall-progress tekniku għas-sbatax-il darba d-Direttiva 67/548/KEE rigward l-approssimazzjoni tal-liġijiet, tar-regolamenti u tad-dispożizzjonijiet amministrattivi dwar il-klassifikazzjoni, l-imballaġġ u l-ittikkettjar ta' sustanzi perikoluż

IL-KUMMISSJONI TAL-KOMUNITAJIET EWROPEJ,

Wara li kkunsidrat it-Trattat li jistabbilixxi l-Komunità Ekonomika Ewropea

Wara li kkunsidrat id-Direttiva tal-Kunsill 67/548/KEE tas-27 ta' Ġunju 1967 rigward l-approssimazzjoni tal-liġijiet, tar-regolamenti u tad-dispożizzjonijiet amministrattivi dwar il-klassifikazzjoni, l-imballaġġ u l-ittikkettjar ta' sustanzi perikoluż [1], kif l-aħħar emendata bid-Direttiva 92/32/KEE [2], u partikolarment l-Artikoli 28 u 29 tagħha,

Billi l-Artikolu 3(1) tad-Direttiva 67/548/KEE u l-Artikolu 3 tad-Direttiva tal-Kunsill 88/379/KEE tas-7 ta' Ġunju 1988 rigward l-approssimazzjoni tal-liġijiet, tar-regolamenti u tad-dispożizzjonijiet amministrattivi dwar il-klassifikazzjoni, l-imballaġġ u l-ittikkettjar ta' sustanzi perikoluż [3], kif l-aħħar emendata bid-Direttivia tal-Kummissjoni 90/492/KEE [4], jipprovdu li l-propjetajiet fiżiko-kimiċi, it-tossiċità u l-ekotossiċità tas-sostanzi u preparazzjonijiet għandhom ikunu determinati skond il-metodi speċifikati fl-Anness V tad-Direttiva 67/548/KEE;

Billi t-test ta' l-Anness V tad-Direttiva 67/548/KEE huwa bħalissa ppubblikat f'żewġ partijiet, dawn huma rispettivament l-Anness għad-Direttiva tal-Kummissjoni 84/449/KEE [5], u l-Anness għad-Direttiva tal-Kummissjoni 88/302/KEE [6];

Billi, biex jitqiesu l-iżviluppi tekniċi jeħtieġ li jkunu riveduti l-metodi tal-prova li jinsabu fl-Anness tad-Direttiva tal-Kummissjoni 84/449/KEE;

Billi, biex jitqiesu l-iżviluppi tekniċi jeħtieġ li jkun rivedut il-metodu tat-test għat-test ta' l-inibizzjoni ta' l-alka li bħalissa tinsab fl-Anness tad-Direttiva tal-Kummissjoni 88/302/KEE u f'din l-okkażjoni biex ikun trasferit dan il-metodu ta' test fl-Anness tad-Direttiva 84/449/KEE;

Billi huwa xieraq li jitnaqqas għall-minimu n-numru ta' annimali użati għall-iskopijiet sperimentali, skond id-Direttiva tal-Kunsill 86/609/KEE fuq l-approssimazzjoni tal-liġijiet, ir-regolamenti u d-dispożizzjonijiet amministrattivi ta' l-Istati Membri dwar il-protezzjoni ta' annimali użati għall-finijiet sperimentali [7];

Billi d-dispożizzjonijiet ta' din id-Direttiva huma skond l-opinjoni tal-Kumitat għall-Adattament għall-Progress Tekniku tad-Direttivi fuq it-Tneħħija ta' Ostakoli Tekniċi fil-Kummerċ f'Sustanzi u Preparazzjonijiet Perikoluż,

ADOTTAT DIN ID-DIRETTIVA:

Artikolu 1

L-Anness tad-Direttiva 84/449/KEE huwa b'dan mibdul bl-Anness f'din id-Direttiva.

(1) ĠU C 196, tas-16.8.1967, p. 1.
(2) ĠU L 154, tal-5.6.1992, p. 1.
(3) ĠU L 187, tas-16.7.1988, p. 14.
(4) ĠU L 275, tal-5.10.1990, p. 35.
(5) ĠU L 251, tad-19.9.1984, p. 1.
(6) ĠU L 133, tat-30.5.1988, p. 1 u ĠU L 136, tat-2.6.1988, p. 20.
(7) ĠU L 358, tat-18.12.1986 p. 1

Artikolu 2

Il-metodu ta' prova ta' l-inibizzjoni ta' l-alka deskritt fl-Anness tad-Direttiva 88/302/KEE huwa b'dan imħassar.

Artikolu 3

L-Istati Membri għandhom idaħħlu fis-seħħ il-liġijiet, ir-regolamenti u d-dispożizzjonijiet amministrattivi meħtieġa biex iħarsu din id-Direttiva mhux aktar tard mit-30 ta' Ottubru 1993. L-Istati Membri għandhom jgħarrfu minnufih lill-Kummissjoni b'dan.

Meta l-Istati Membri jadottaw dawn id-dispożizzjonijiet dan għandhom ikollhom referenza għal din id-Direttiva jew ikunu akkumpanjati b'dik ir-riferenza fil-ħin tal-pubblikazzjoni uffiċjali agħhom.

Il-proċedura għal din ir-referenza għandha tkun adottata mill-Istati Membri.

Artikolu 4

Din id-Direttiva hija indirizzata lill-Istati Membri.

Magħmula fi Brussel, fil-31 ta' Lulju 1992.

Għall-Kummissjoni

Karel VAN MIERT

Membru tal-Kummissjoni

ANNEX

Annex to Commission Directive 92/69/EEC of 31 July 1992 adapting to technical progress for the seventeenth 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 [1]

[1] OJ No L 383, 29. 12. 1992, p. 113.

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INTRODUCTION

The Annex sets out test methods for the determination of physicochemical, toxicological and ecotoxicological properties listed in Annexes VII and VIII to Directive 79/831/EEC. The methods are based on those recognized and recommended by competent international bodies (in particular OECD).

When such methods were not available, national standards or scientific consensus methods have been adopted. Generally, tests should be performed with the substance as defined by the Directive. Attention should be given to the possible influence of impurities on the test results.

When the methods of this Annex are inappropriate for the investigation of a certain property, the notifier must justify the alternate method used.

Animal tests and studies shall be conducted in accordance with national regulations and shall take into account humane principles and international developments in the field of animal welfare.

Among equivalent testing methods, the method using the minimum number of animals is chosen.

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

A.1. MELTING/FREEZING TEMPERATURE

1. **METHOD**

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

1.1. INTRODUCTION

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

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

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

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

1.2. DEFINITIONS AND UNITS

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

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

Conversion of units (K to °C)

$t = T - 273{,}15$

t: Celsius temperature, degree Celsius (°C)

T: thermodynamic temperature, kelvin (K)

1.3. REFERENCE SUBSTANCES

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

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

1.4. PRINCIPLE OF THE TEST METHOD

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

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

1.4.1. **Capillary method**

1.4.1.1. *Melting temperature devices with liquid bath*

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

1.4.1.2. *Melting temperature devices with metal block*

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

1.4.1.3. *Photocell detection*

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

1.4.2. **Hot Stages**

1.4.2.1. *Kofler hot bar*

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

1.4.2.2. *Melt microscope*

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

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

1.4.2.3. *Meniscus method*

This method is specifically used for polyamides.

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

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

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

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

1.4.4. **Thermal analysis**

1.4.4.1 *Differential thermal analysis (DTA)*

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

1.4.4.2 *Differential scanning calorimetry (DSC)*

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

1.4.5. **Pour point**

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

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

1.5. QUALITY CRITERIA

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

TABLE: APPLICABILITY OF THE METHODS

A. Capillary methods

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

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

B. Hot stages and freezing methods

Method of Measurement	Substances which can be pulverized	Substances which are not readily pulverized	Temperature range	Estimated accuracy (1)	Existing Standard
Kofler hot bar	yes	no	283 to > 573 K	± 1,0 K	ANSI/ASTM D 3451-76
Melt microscope	yes	only to a few	273 to > 573 K	± 0,5 K	DIN 53736
Meniscus method	no	Specifically for polyamides	293 to > 573 K	± 0,5 K	ISO 1218 (E)
Freezing temperature methods	yes	yes	223 to 573 K	± 0,5 K	e.g. BS 4695
(1) Dependent on type of instrument and on degree of purity of the substance					

C. Thermal analysis

Method-of Measurement	Substances which can be pulverized	Substances which are not readily pulverized	Temperature range	Estimated accuracy (1)	Existing Standard
Differential Thermal Analysis	yes	yes	173 to 1 273 K	up to 600 K ± 0,5 K up to 1 273 K ± 2,0 K	ASTM E 537-76
Differential Scanning Calorimetry	yes	yes	173 to 1 273 K	up to 600 K ± 0,5 K up to 1 273 K ± 2,0 K	ASTM E 537-76
(1) Dependent on type of instrument and on degree of purity of the substance					

D. Pour point

Method of Measurement	Substances which can be pulverized	Substances which are not readily pulverized	Temperature range	Estimated accuracy (1)	Existing Standard
Pour point	for petroleum oils and oily substances	for petroleum oils and oily substances	223 to 323 K	± 3,0 K	ASTM D 97-66
(1) Dependent on type of instrument and on degree of purity of the substance					

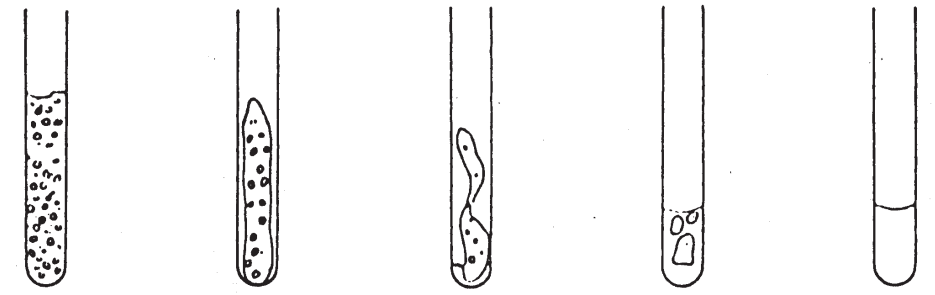
1.6. DESCRIPTION OF THE METHODS

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

1.6.1. **Methods with capillary tube**

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

Figure 1



Stage A (Beginning of melting): fine droplets adhere uniformly to the inside wall of the capillary tube.

Stage B a clearance appears between the sample and the inside wall due to shrinkage of the melt.

Stage C the shrunken sample begins to collapse downwards and liquefies.

Stage D a complete meniscus is formed at the surface but an appreciable amount of the sample remains solid.

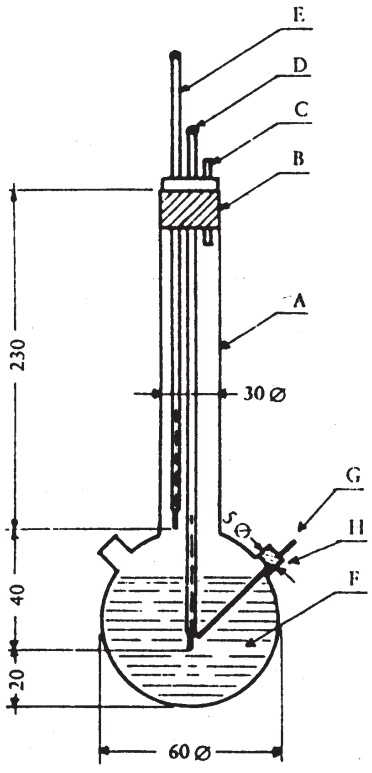
Stage E (Final stage of melting): there are no solid particles.

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

1.6.1.1. *Melting temperature devices with liquid bath apparatus*

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

Figure 2



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

Bath liquid:

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

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

Thermometer:

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

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

Procedure:

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

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

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

Calculation:

The calculation of the melting temperature is as follows:

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

where:

T = corrected melting temperature in K

T_D = temperature reading of thermometer D in K

T_E = temperature reading of thermometer E in K

n = number of graduations of mercury thread on thermometer D at emergent stem.

1.6.1.2. *Melting temperature devices with metal block*

Apparatus:

This consists of:

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

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

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

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

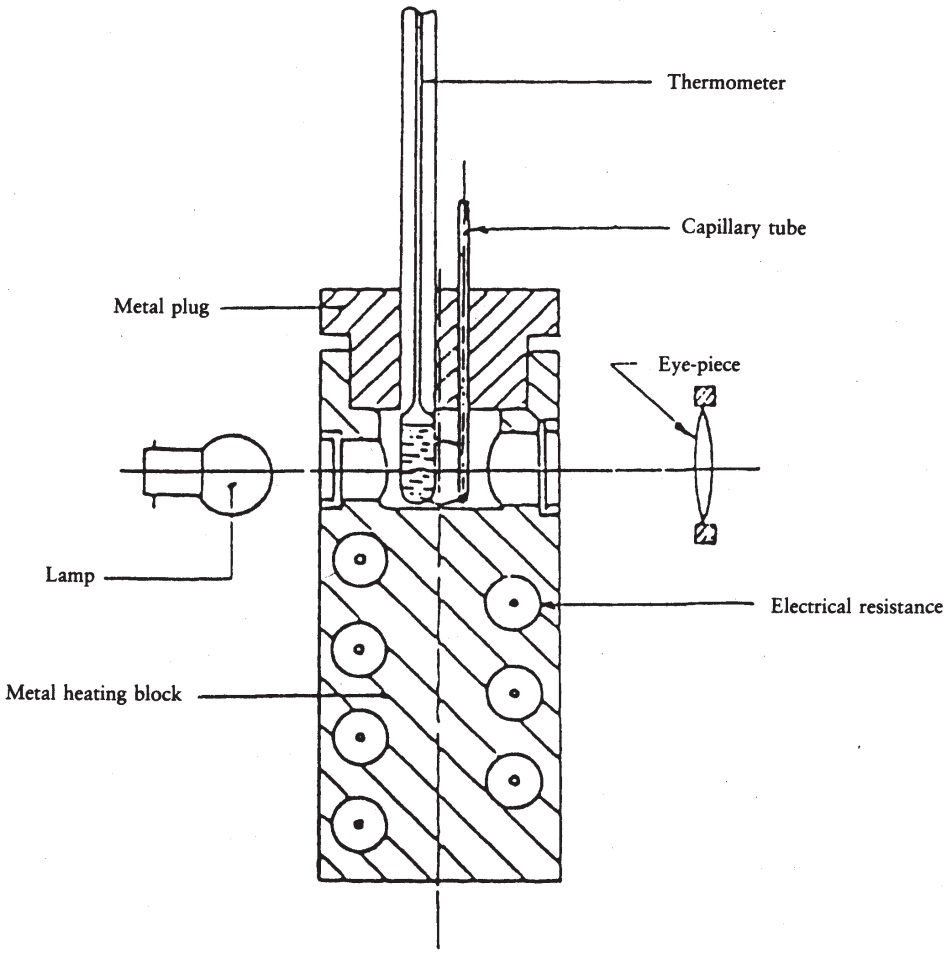
— four windows of heat-resistant glass on the lateral walls of the chamber, diametrically disposed at right-angles to each other. In front of one of these windows is mounted an eye-piece for observing the capillary tube. The other three windows are used for illuminating the inside of the enclosure by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

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

Thermometer:

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

Figure 3



1.6.1.3. *Photocell detection*

Apparatus and procedure:

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

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

1.6.2. **Hot stages**

1.6.2.1. *Kofler hot bar*

See Appendix.

1.6.2.2. *Melt microscope*

See Appendix.

1.6.2.3. *Meniscus method (polyamides)*

See Appendix.

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

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

See Appendix.

1.6.4. **Thermal analysis**

1.6.4.1. *Differential thermal analysis*

See Appendix.

1.6.4.2. *Differential scanning calorimetry*

See Appendix.

1.6.5. **Determination of the pour point**

See Appendix.

2. **DATA**

A thermometer correction is necessary in some cases.

3. **REPORTING**

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

— method used,

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

— an estimate of the accuracy.

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

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

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

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

4. **REFERENCES**

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

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

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

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

Appendix

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

1. **Capillary methods**

1.1. Melting temperature devices with liquid bath

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

1.2. Melting temperature devices with metal block

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

2. **Hot stages**

2.1. Kofler hot bar

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

2.2. Melt microscope

DIN 53736	Visuelle Bestimmung der Schmelztemperatur von teilkristallinen Kunststoffen.

2.3. Meniscus method (polyamides)

ISO 1218 (E)	Plastics — polyamides — determination of "melting point"
ANSI/ASTM D 2133-66	Standard specification for acetal resin injection moulding and extrusion materials
NF T 51-050	Résines de polyamides. Détermination du "point de fusion" Méthode du ménisque

3.	**Methods for the determination of the freezing temperature**	
	BS 4633	Method for the determination of crystallizing point
	BS 4695	Method for Determination of Melting Point of petroleum wax (Cooling Curve)
	DIN 51421	Bestimmung des Gefrierpunktes von Flugkraftstoffen, Ottokraftstoffen und Motorenbenzolen
	ISO 2207	Cires de pétrole: détermination de la température de figeage
	DIN 53175	Bestimmung des Erstarrungspunktes von Fettsäuren
	NF T 60-114	Point de fusion des paraffines
	NF T 20-051	Méthode de détermination du point de cristallisation (point de congélation)
	ISO 1392	Method for the determination of the freezing point
4.	**Thermal analysis**	
4.1.	Differential thermal analysis	
	ASTM E 537-76	Standard method for assessing the thermal stability of chemicals by methods of differential thermal analysis
	ASTM E 473-85	Standard definitions of terms relating to thermal analysis
	ASTM E 472-86	Standard practice for reporting thermoanalytical data
	DIN 51005	Thermische Analyse, Begriffe
4.2.	Differential scanning calorimetry	
	ASTM E 537-76	Standard method for assessing the thermal stability of chemicals by methods of differential thermal analysis
	ASTM E 473-85	Standard definitions of terms relating to thermal analysis
	ASTM E 472-86	Standard practice for reporting thermoanalytical data
	DIN 51005	Thermische Analyse, Begriffe
5.	**Determination of the pour point**	
	NBN 52014	Echantillonnage et analyse des produits du pétrole: Point de trouble et point d'écoulement limite — Monsterneming en ontleding van aardolieproducten: Troebelingspunt en vloeipunt
	ASTM D 97-66	Standard test method for pour point of petroleum oils
	ISO 3016	Petroleum oils — Determination of pour point.

A.2. BOILING TEMPERATURE

1. **METHOD**

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

1.1. INTRODUCTION

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

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

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

Remarks:

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

1.2 DEFINITIONS AND UNITS

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

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

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

where:

p = the vapour pressure of the substance in pascals

ΔH_v = its heat of vaporization in J mol^{-1}

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

T = thermodynamic temperature in K

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

Conversions

Pressure (units: kPa)

100 kPa = 1 bar = 0,1 MPa

("bar" is still permissible but not recommended)

133 Pa = 1 mm Hg = 1 Torr

(the units "mm Hg" and "Torr" are not permitted).

1 atm = standard atmosphere = 101 325 Pa

(the unit "atm" is not permitted).

Temperature (units: K)

$t = T - 273{,}15$

t: Celsius temperature, degree Celsius (°C)

T: thermodynamic temperature, kelvin (K)

1.3. REFERENCE SUBSTANCES

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

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

1.4. PRINCIPLE OF THE TEST METHOD

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

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

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

1.4.2. **Dynamic method**

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

1.4.3. **Distillation method for boiling temperature**

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

1.4.4. **Method according to Siwoloboff**

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

1.4.5. **Photocell detection**

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

1.4.6. **Differential thermal analysis**

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

1.4.7. **Differential scanning calorimetry**

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

1.5. QUALITY CRITERIA

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

TABLE 1: COMPARISON OF THE METHODS

Method of measurement	Estimated accuracy	Existing standard
Ebulliometer	± 1.4 K (up to 373 K) (1) (2) ± 2.5 K (up to 600 K) (1) (2)	ASTM D 1120-72 (1)
Dynamic method	± 0,5 K (up to 600 K) (2)	
Distillation process (boiling range)	± 0,5 K (up to 600 K)	ISO/R 918, DIN 53171, BS 4591/71
According to Siwoloboff	± 2 K (up to 600 K) (2)	
Photocell detection	± 0,3 K (at 373 K) (2)	
Differential thermal calorimetry	± 0,5 K (up to 600 K) ± 2,0 K (up to 1 273 K)	ASTM E 537-76
Differential scanning calorimetry	± 0,5 K (up to 600 K) ± 2,0 K (up to 1 273 K)	ASTM E 537-76

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

1.6. DESCRIPTION OF THE METHODS

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

1.6.1. **Ebulliometer**

See Appendix.

1.6.2. **Dynamic method**

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

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

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

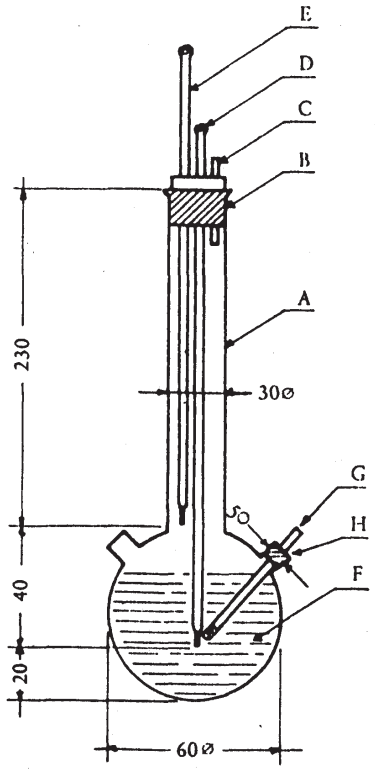
See Appendix.

1.6.4. **Method according to Siwoloboff**

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

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

Figure 1



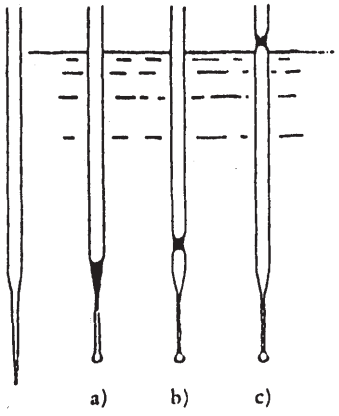
A: Measuring vessel
B: Stopper
C: Vent
D: Thermometer
E: Auxiliary thermometer
F: Bath liquid
G: Sample tube, maximum 5 mm outer diameter; containing a capillary tube, approximately 100 mm long, approximately 1 mm inner diameter and approximately 0,2 to 0,3 mm wall-thickness
H: Side tube

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

Figure 2
Principle according to Siwoloboff



Figure 3
Modified principle



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

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

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

1.6.5. **Photocell detection**

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

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

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

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

1.6.6. **Thermal analysis**

1.6.6.1. *Differential thermal analysis*

See Appendix.

1.6.6.2. *Differential scanning calorimetry*

See Appendix.

2. **DATA**

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

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

where:

Δp = (101,325 – p) [note sign]

p = pressure measurement in kPa

f_T = rate of change of boiling temperature with pressure in K/kPa

T = measured boiling temperature in K

T_n = boiling temperature corrected to normal pressure in K

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

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

TABLE 2: TEMPERATURE — CORRECTIONS FACTORS f_T

Temperature T (K)	Correction factor f_T (K/kPa)
323,15	0,26
348,15	0,28
373,15	0,31
398,15	0,33
423,15	0,35
448,15	0,37
473,15	0,39
498,15	0,41
523,15	0,44
548,15	0,45
573,15	0,47

3. **REPORTING**

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

— method used,

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

— an estimate of the accuracy.

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

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

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

4. **REFERENCES**

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

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

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

Appendix

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

1. **Ebulliometer**

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

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

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

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

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

A.3 RELATIVE DENSITY

1. **METHOD**

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

1.1. INTRODUCTION

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

1.2. DEFINITIONS AND UNITS

The relative density, D_4^{20}, of solids or liquids is the ratio between the mass of a volume of substance to be examined, determined at 20 °C, and the mass of the same volume of water, determined at 4 °C. The relative density has no dimension.

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

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

1.3. REFERENCE SUBSTANCES (1) (3)

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

1.4. PRINCIPLE OF THE METHODS

Four classes of methods are used.

1.4.1. **Buoyancy methods**

1.4.1.1. *Hydrometer (for liquid substances)*

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

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

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

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

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

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

1.4.2. **Pycnometer methods**

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

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

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

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

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

1.5. QUALITY CRITERIA

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

1.6. DESCRIPTION OF THE METHODS

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

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

2. **DATA**

See standards.

3. **REPORTING**

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

— method used,

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

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

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

TABLE: APPLICABILITY OF METHODS

Method of measurement	Density		Maximum possible dynamic viscosity	Existing Standards
	solid	liquid		
1.4.1.1. Hydrometer		yes	5 Pa s	ISO 387, ISO 649-2, NF T 20-050
1.4.1.2. Hydrostatic balance				
(a) solids	yes			ISO 1183 (A)
(b) liquids		yes	5 Pa s	ISO 901 and 758
1.4.1.3. Immersed body method		yes	20 Pa s	DIN 53217
1.4.2. Pycnometer				ISO 3507
(a) solids	yes			ISO 1183(B), NF T 20-053
(b) liquids		yes	500 Pa s	ISO 758
1.4.3. Air comparison pycnometer	yes			DIN 55990 Teil 3, DIN 53243
1.4.4. Oscillating densitimer		yes	5 Pa s	

4. **REFERENCES**

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

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

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

(4) Wagenbreth, H., Die Tauchkugel zur Bestimmung der Dichte von Flüssigkeiten, Technisches Messen tm, 1979, vol.11, 427-430.

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

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

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

Appendix

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

1. **BUOYANCY METHODS**

1.1. Hydrometer

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

1.2. Hydrostatic balance

For solid substances

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

For liquid substances

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

1.3. Immersed body method

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

2. **PYCNOMETER METHODS**

2.1. For liquid substances

ISO 3507	Pycnometers
ISO 758	Liquid chemical products; determination of density at 20 °C
DIN 12797	Gay-Lussac pycnometer (for non-volatile liquids which are not too viscous)
DIN 12798	Lipkin pycnometer (for liquids with a kinematic viscosity of less than $100 \cdot 10^{-6}$ m^2 s^{-1} at 15 °C)

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

2.2. For solid substances

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

3. **AIR COMPARISON PYCNOMETER**

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

A.4. VAPOUR PRESSURE

1. **METHOD**

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

1.1. INTRODUCTION

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

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

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

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

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

1.2. DEFINITIONS AND UNITS

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

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

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

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

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

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

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

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

where:

p = the vapour pressure of the substance in pascals

ΔH_v = its heat of vaporization in J mol^{-1}

R = the universal molar gas constant in J mol^{-1} K^{-1}

T = thermodynamic temperature in K

1.3. REFERENCE SUBSTANCES

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

1.4. PRINCIPLE OF THE TEST METHODS

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

1.4.1. **Dynamic method**

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

Recommended range:

10^3 up to 10^5 Pa.

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

1.4.2. **Static method**

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

Recommended range:

10 up to 10^5 Pa.

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

1.4.3. **Isoteniscope**

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

Recommended range:

from 100 to 10^5 Pa.

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

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

Recommended range:

10^{-3} to 1 Pa.

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

The method is based on the estimation of the mass of test substance flowing out per unit of time of a Knudsen cell (4) in the form of vapour, through a micro-orifice under ultra-vacuum conditions. The mass of effused vapour can be obtained either by determining the loss of mass of the cell or by condensing the vapour at low temperature and determining the amount of volatilized substance using chromatographic analysis. The vapour pressure is calculated by applying the Hertz-Knudsen relation.

Recommended range:

10^{-3} to 1 Pa.

1.4.6. **Gas saturation method**

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

Recommended range:

10^{-4} to 1 Pa.

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

1.4.7. **Spinning rotor**

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

Recommended range:

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

1.5. QUALITY CRITERIA

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

QUALITY CRITERIA

Measuring Method	Substances		Estimated Repeatability (1)	Estimated Reproducibility (1)	Recommended Range	Existing Standard
	solid	liquid				
1.4.1. Dynamic method	Low melting	yes	Up to 25%	Up to 25%	10^3 Pa to 2×10^3 Pa	—
			1 to 5%	1 to 5%	2×10^3 Pa to 10^5 Pa	—
1.4.2. Static method	yes	yes	5 to 10%	5 to 10%	10 Pa to 10^5 Pa (2)	NFT 20-048 (5)
1.4.3. Isoteniscope	yes	yes	5 to 10%	5 to 10%	10^2 Pa to 10^5 Pa	ASTM-D 2879-86
1.4.4. Effusion method Vap.Pres. balance	yes	yes	5 to 20%	Up to 50 %	10^{-3} Pa to 1 Pa	NFT 20-047(6)
1.4.5. Effusion method weight loss	yes	yes	10 to 30%	—	10^{-3} Pa to 1 Pa	—
1.4.6. Gas saturation method	yes	yes	10 to 30%	Up to 50 %	10^{-4} Pa to 1 Pa (2)	—
1.4.7. Spinning rotor method	yes	yes	10 to 20%	—	10^{-4} Pa to 0,5 Pa	—

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

1.6. DESCRIPTION OF THE METHODS

1.6.1. **Dynamic measurement**

1.6.1.1. *Apparatus*

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

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

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

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

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

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

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

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

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

Pressure is measured with a manometer.

1.6.1.2. *Measurement procedure*

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

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

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

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

1.6.2. **Static measurement**

1.6.2.1. *Apparatus*

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

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

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

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

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

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

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

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

1.6.2.2. *Measurement procedure*

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

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

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

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

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

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

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

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

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

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

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

   If either 1 or 2 is the case, the measurements must be repeated.

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

1.6.3. **Isoteniscope**

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

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

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

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

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

1.6.4.1. *Apparatus*

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

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

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

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

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

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

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

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

1.6.4.2. *Measurement procedure*

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

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

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

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

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

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

where

G = evaporation rate (kg s^{-1} m^{-2})

M = molar mass (g mol^{-1})

T = temperature (K)

R = universal molar gas constant (J mol^{-1} K^{-1})

p = vapour pressure (Pa)

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

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

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

1.6.5.1. *Apparatus*

The effusion apparatus consists of the following basic parts:

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

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

— a trap, using liquefied nitrogen or dry ice.

An electrically heated, aluminium vacuum tank with 4 stainless steel effusion cells is shown in figure 5 for example. The stainless steel foil of about 0,3 mm thickness has an effusion orifice of 0,2 to 1,0 mm diameter and is attached to the effusion cell by a threaded lid.

1.6.5.2. *Measurement procedure*

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

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

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

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

where

p = vapour pressure (Pa)

m = mass of the substance leaving the cell during time t (kg)

t = time (s)

A = area of the hole (m^2)

K = correction factor

R = universal gas constant (J mol^{-1} K^{-1})

T = temperature (K)

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

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

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

The above equation may be written:

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

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

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

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

where

p(r) = vapour pressure of the reference substance (Pa)

M(r) = molecular mass of the reference substance ($kg.mol^{-1}$)

1.6.6. **Gas saturation method**

1.6.6.1. *Apparatus*

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

Inert gas:

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

Flow control:

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

Traps to collect vapour:

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

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

Heat exchanger:

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

Saturator column:

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

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

1.6.6.2. *Measurement procedure*

Preparation of the saturator column:

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

Measurement:

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

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

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

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

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

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

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

1.6.6.3. *Calculation of vapour pressure*

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

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

where:

p = vapour pressure (Pa)

W = mass of evaporated test substance (g)

V = volume of saturated gas (m^3)

R = universal molar gas constant (J mol^{-1} K^{-1})

T = temperature (K)

M = molar mass of test substance (g mol^{-1})

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

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

1.6.7.1. *Apparatus*

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

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

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

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

1.6.7.2. *Measurement procedure*

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

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

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

where

$\bar{c}$ = average speed of the gas molecules

r = radius of the ball

ρ = mass density of the ball

σ = coefficient of tangential momentum transfer (ε = 1 for an ideal spherical surface of the ball)

t = time

v(t) = rotational speed after time t

v(o) = initial rotational speed

This equation may also be written:

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

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

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

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

where:

T = temperature

R = universal molar gas constant

M = molar mass

2. **DATA**

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

3. **REPORTING**

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

— method used,

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

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

— all of the raw data,

— a log p versus 1/T curve,

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

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

— nature of the change,

— temperature at which the change occurs at atmospheric pressure,

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

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

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Appendix 1

Estimation Method

INTRODUCTION

Calculated values of the vapour pressure can be used:

— for deciding which of the experimental methods is appropriate,

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

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

ESTIMATION METHOD

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

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

CALCULATION PROCEDURE

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

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

where:

T = temperature of interest

T_b = normal boiling point

P_{vp} = vapour pressure at temperature T

ΔH_{vb} = heat of vaporization

ΔZ_b = compressibility factor (estimated at 0,97)

m = empirical factor depending on the physical state at the temperature of interest

Further

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

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

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

$$\ln P_{vp} = \ln P_1 + \frac{\Delta H_{v1}}{\Delta Z_b R T_1}\left[1 - \left(3 - 2\frac{T}{T_1}\right)^{m}\frac{T_1}{T} - 2m\left(3 - 2\frac{T}{T_1}\right)^{m-1}\ln\frac{T}{T_1}\right]$$

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

REPORT

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

LITERATURE

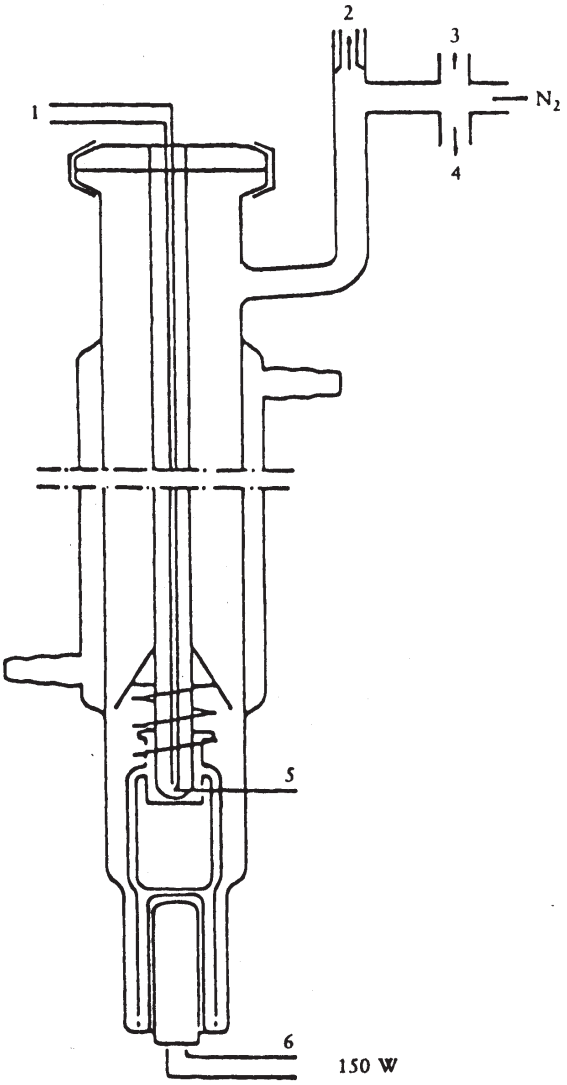
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Appendix 2

Figure 1

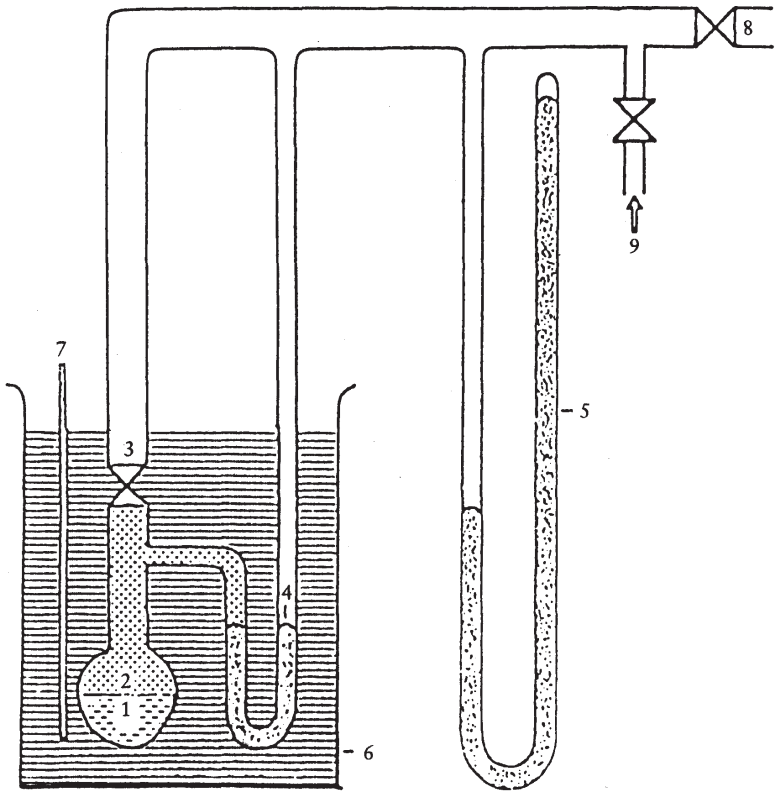
Apparatus for determining the vapour pressure curve according to the dynamic method



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

Figure 2a

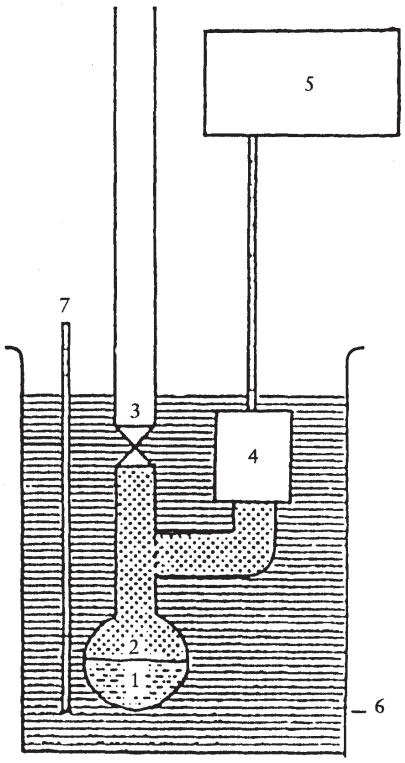
Apparatus for determining the vapour pressure curve according to the static ethod (using a U-tube manometer)



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

Figure 2b

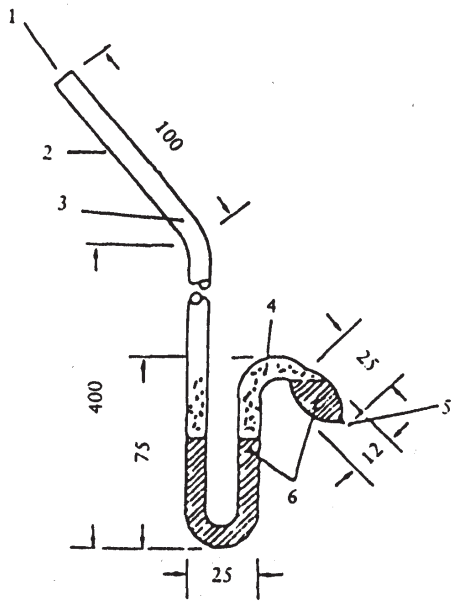
Apparatus for determining the vapour pressure curve according to the static method (using a pressure indicator)



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

Figure 3

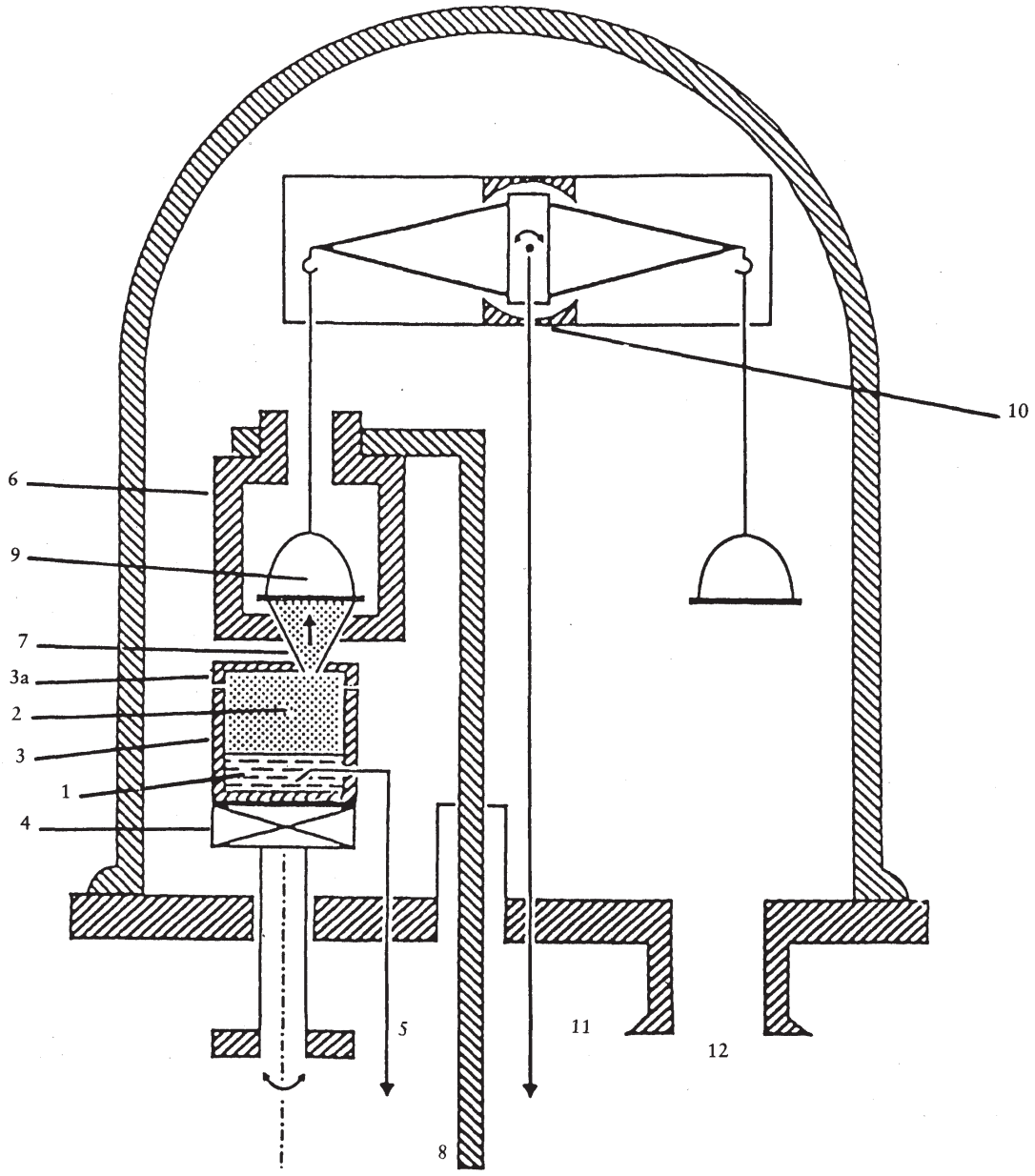
Isoteniscope (see reference 7)



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

Figure 4

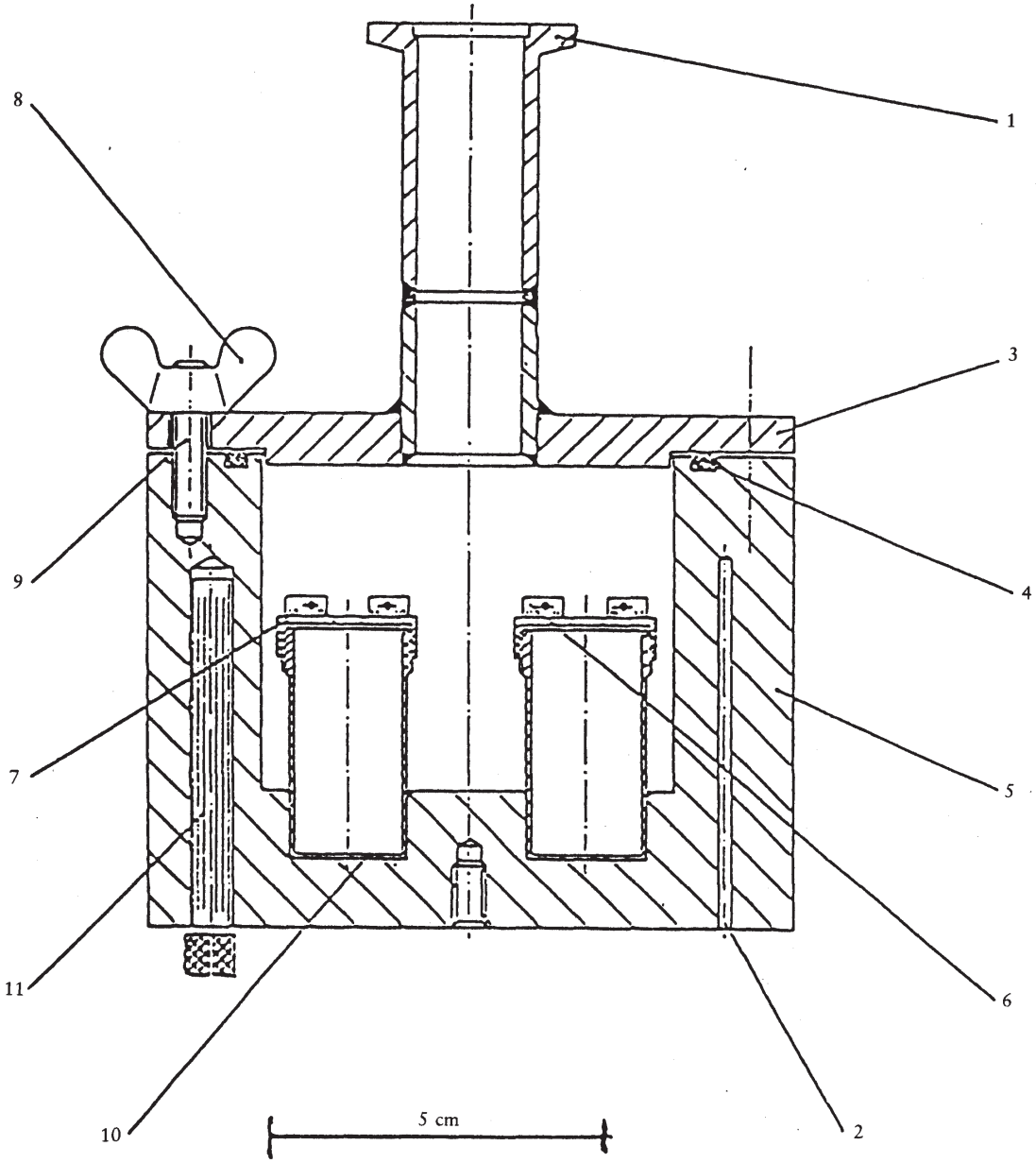
Apparatus for determining the vapour pressure curve according to the vapour pressure balance method



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

Figure 5

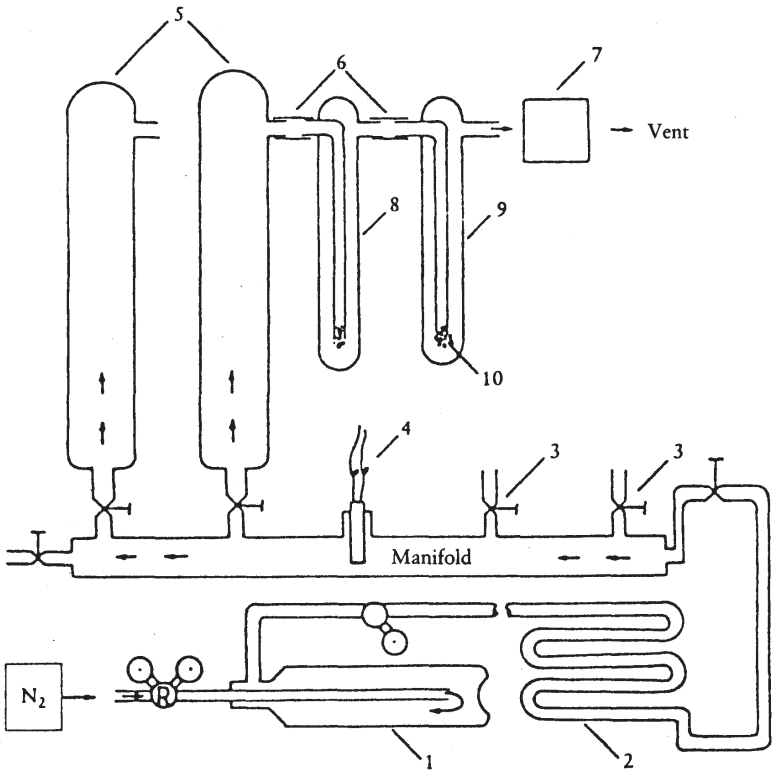
Example of apparatus for evaporation at low pressure by effusion methode, with an effusion cell volume of 8 cm^3



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

Figure 6a

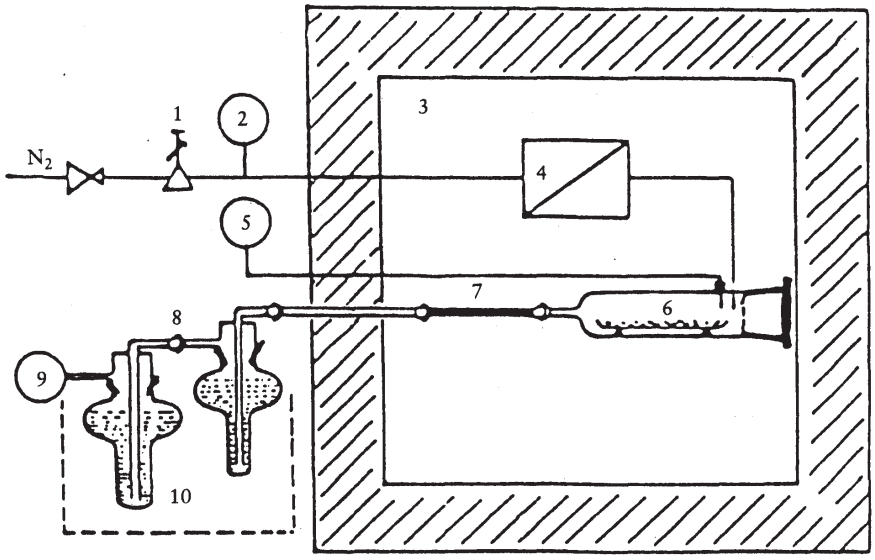
An example of a flow system for the determination of vapour pressure by the gas saturation method



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

Figure 6b

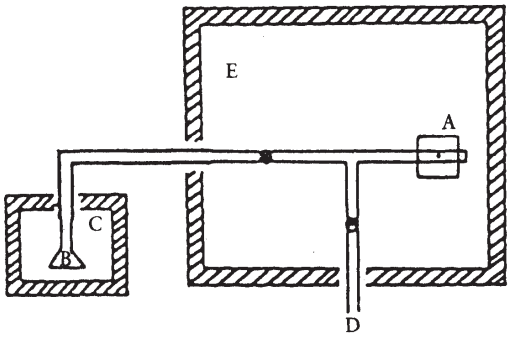
An example of a system for the determination of vapour pressure by the gas saturation method, with a capillary placed after the saturation chamber



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

Figure 7

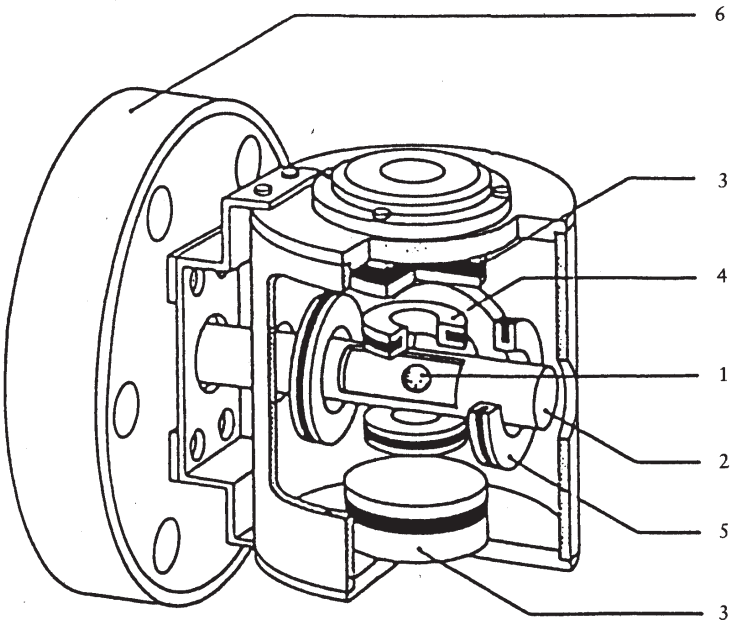
Example of the experimental set-up for spinning rotor method



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

Figure 8

Example of spinning rotor measuring head



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

A.5. SURFACE TENSION

1. **METHOD**

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

1.1. INTRODUCTION

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

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

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

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

1.2. DEFINITIONS AND UNITS

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

The surface tension is given as:

N/m (SI unit) or

mN/m (SI sub-unit)

1 N/m = 10^3 dynes/cm

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

1.3. REFERENCE SUBSTANCES

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

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

1.4. PRINCIPLE OF THE METHODS

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

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

1.5. QUALITY CRITERIA

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

1.6. DESCRIPTION OF THE METHODS

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

1.6.1. **Plate method**

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

1.6.2. **Stirrup method**

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

1.6.3. **Ring method**

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

1.6.4. **OECD harmonized ring method**

1.6.4.1. *Apparatus*

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

— mobile sample table,

— force measuring system,

— measuring body (ring),

— measurement vessel.

1.6.4.1.1. Mobile sample table

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

1.6.4.1.2. Force measuring system

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

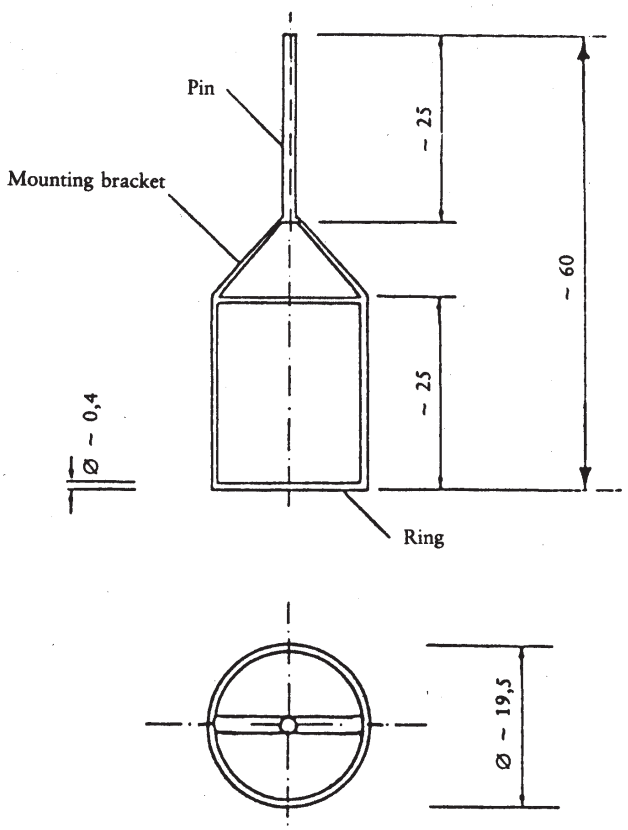
1.6.4.1.3. Measuring body (ring)

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

Figure

Measuring body

(All dimensions expressed in millimetres)



1.6.4.1.4. Measurement vessel

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

1.6.4.2. *Preparation of the apparatus*

1.6.4.2.1. Cleaning

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

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

Note:

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

1.6.4.2.2. Calibration of the apparatus

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

Mounting:

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

Zero point adjustment:

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

Calibrations:

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

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

$$\Phi_a = \frac{\sigma_r}{\sigma_a} \quad (1)$$

where:

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

m = mass of the rider (g)

g = gravity acceleration (981 cm s^{-2} at sea level)

b = mean circumference of the ring (cm)

σ_a = reading of the tensiometer after placing the rider on the ring (mN/m).

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

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

$$\Phi_b = \frac{\sigma_o}{\sigma_g} \qquad (2)$$

where:

σ_o = value cited in the literature for the surface tension of water (mN/m)

σ_g = measured value of the surface tension of the water (mN/m)

both at the same temperature.

1.6.4.3. *Preparation of samples*

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

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

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

1.6.5. **Test conditions**

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

1.6.6. **Performance of test**

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

2. **DATA**

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

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

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

TABLE: CORRECTION OF THE MEASURED SURFACE TENSION

Only for aqueous solutions, $\rho \approx 1$ g/cm³

R = 9,55 mm (average ring radius)

r = 0,185 mm (ring wire radius)

Experimental Value (mN/m)	Corrected Value (mN/m)	
	Weight calibration (see 1.6.4.2.2(a))	Water calibration (see 1.6.4.2.2(b))
20	16,9	18,1
22	18,7	20,1
24	20,6	22,1
26	22,4	24,1
28	24,3	26,1
30	26,2	28,1
32	28,1	30,1
34	29,9	32,1
36	31,8	34,1
38	33,7	36,1
40	35,6	38,2
42	37,6	40,3
44	39,5	42,3
46	41,4	44,4
48	43,4	46,5
50	45,3	48,6
52	47,3	50,7
54	49,3	52,8
56	51,2	54,9
58	53,2	57,0
60	55,2	59,1
62	57,2	61,3
64	59,2	63,4
66	61,2	65,5
68	63,2	67,7
70	65,2	69,9
72	67,2	72,0
74	69,2	—
76	71,2	—
78	73,2	—

This table has been compiled on the basis of the Harkins-Jordan correction. It is similar to that in the DIN Standard (DIN 53914) for water and aqueous solutions (density $\rho = 1$ g/cm³) and is for a commercially available ring having the dimensions R = 9,55 mm (mean ring radius) and r = 0,185 mm (ring wire radius). The table provides corrected values for surface-tension measurements taken after calibration with weights or calibration with water.

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

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

where:

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

R = the radius of the ring

f = the correction factor (1)

3. **REPORTING**

3.1. TEST REPORT

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

— method used,

— type of water or solution used,

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

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

— concentration of the solution,

— test temperature,

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

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

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

3.2. INTERPRETATION OF RESULTS

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

4. **REFERENCES**

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

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

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

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

A.6 WATER SOLUBILITY

1. **METHOD**

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

1.1. INTRODUCTION

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

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

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

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

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

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

1.2. DEFINITION AND UNITS

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

1.3. REFERENCE SUBSTANCES

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

1.4. PRINCIPLE OF THE TEST METHOD

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

1.4.1. **Column elution method**

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

1.4.2. **Flask method**

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

1.5. QUALITY CRITERIA

1.5.1. **Repeatability**

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

1.5.2. **Sensitivity**

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

1.6. DESCRIPTION OF THE METHOD

1.6.1. **Test conditions**

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

1.6.2. **Preliminary test**

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

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

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

1.6.3. **Column elution method**

1.6.3.1. *Support material, solvent and eluent*

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

Note:

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

1.6.3.2. *Loading of the support*

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

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

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

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

Test procedure:

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

— recirculating pump (see figure 1),

— levelling vessel (see figure 4).

1.6.3.3. *Column elution method with recirculating pump*

Apparatus

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

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

Measurement procedure

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

1.6.3.4. *Column elution method with levelling vessel*

Apparatus (see figures 4 and 3)

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

Measurement procedure

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

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

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

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

1.6.4. **Flask method**

1.6.4.1. *Apparatus*

For the flask method the following material is needed:

— normal laboratory glassware and instrumentation,

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

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

— equipment for analytical determination.

1.6.4.2. *Measurement procedure*

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

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

The pH of each sample should be recorded.

1.6.5. **Analysis**

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

2. **DATA**

2.1. COLUMN ELUTION METHOD

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

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

2.2. FLASK METHOD

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

3. **REPORTING**

3.1. COLUMN ELUTION METHOD

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

— the results of the preliminary test,

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

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

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

— the average of the two successive, acceptable runs,

— the temperature of the water during the saturation process,

— the method of analysis employed,

— the nature of the support material employed,

— loading of support material,

— solvent used,

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

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

3.2. FLASK METHOD

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

— the results of the preliminary test,

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

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

— the pH of each sample,

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

— the test temperature,

— the analytical method employed,

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

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

4. **REFERENCES**

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

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

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

Appendix

Figure 1

Column elution method with recirculating pump

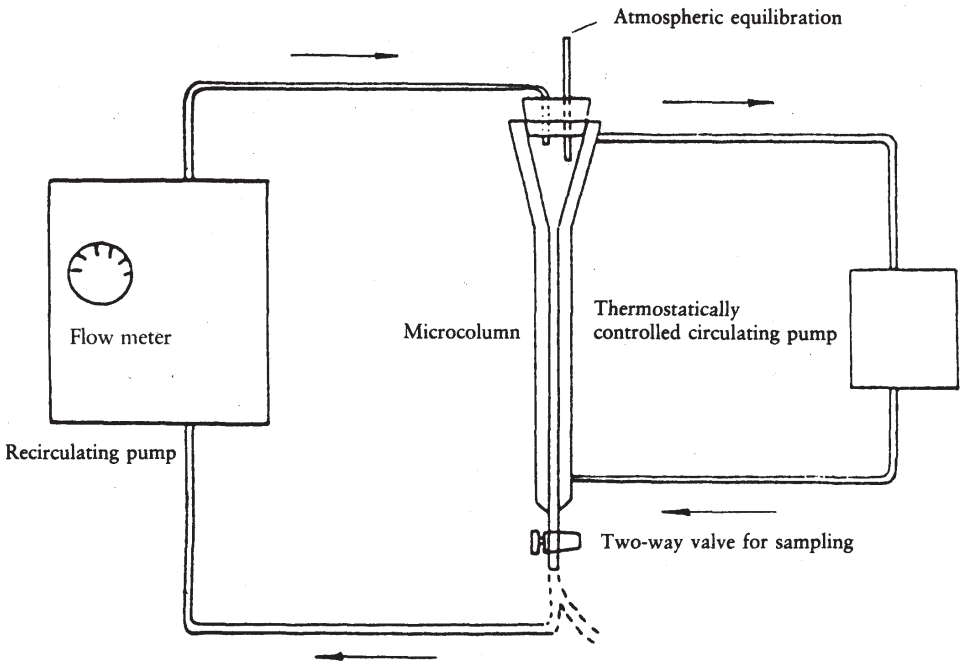


Figure 2

A typical microcolumn

(All dimensions in millimetres)

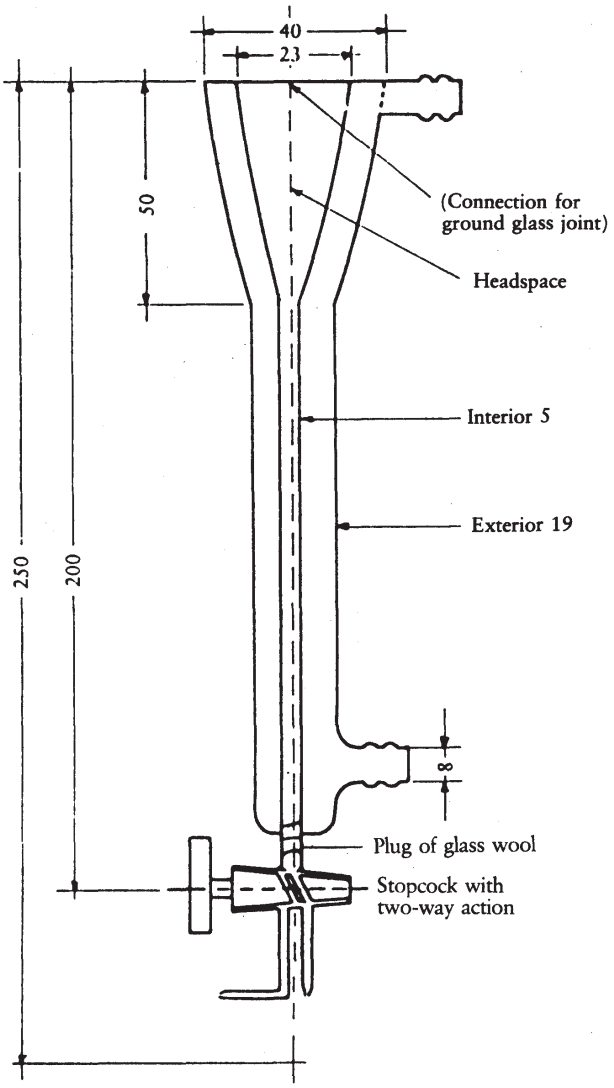


Figure 3

A typical microcolumn

(All dimensions in millimetres)

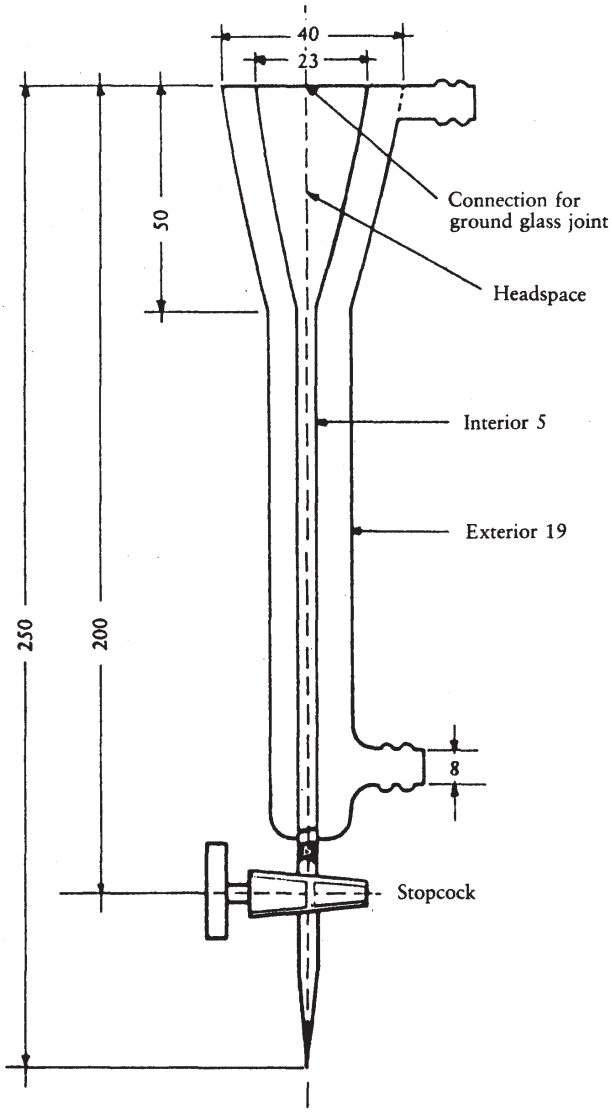
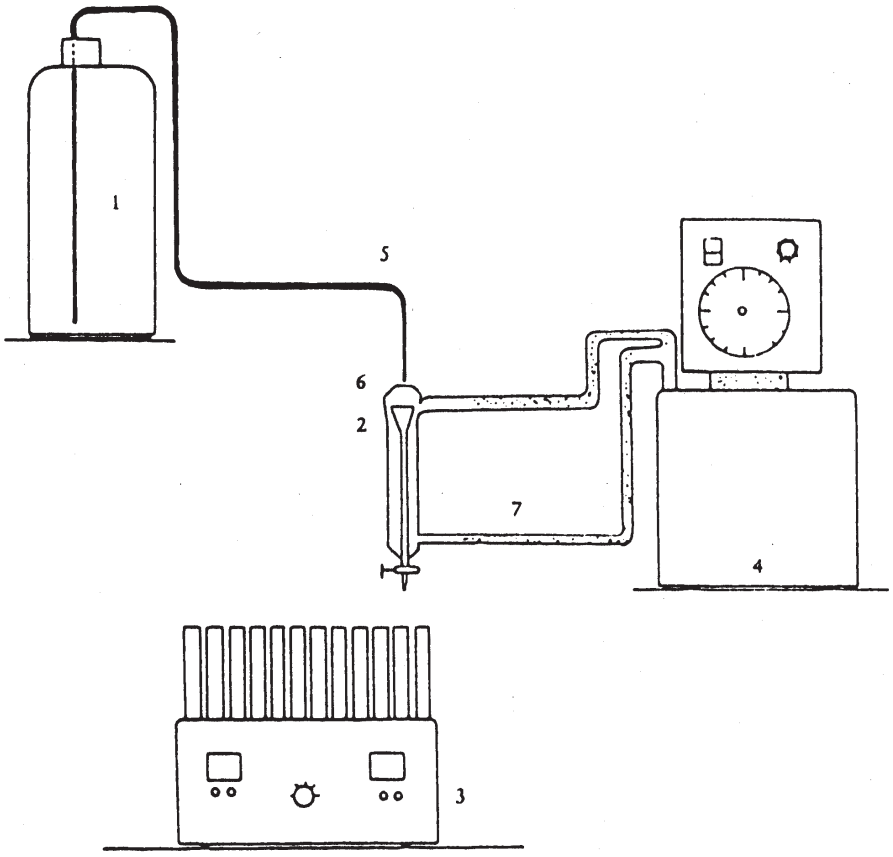


Figure 4

Column elution method with levelling vessel



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

A.8. PARTITION COEFFICIENT

1. **METHOD**

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

1.1. INTRODUCTION

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

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

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

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

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

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

1.2. DEFINITION AND UNITS

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

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

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

1.3. REFERENCE SUBSTANCES

Shake-flask method

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

HPLC method

In order to correlate the measured HPLC data of a compound with its P value, a calibration graph of log P vs. chromatographic data using at least 6 reference points has to be established. It is for the user to select the appropriate reference substances. Whenever possible, at least one reference compound should have a P_{ow} above that of the test substance, and another a P_{ow} below that of the test substance. For log P values less than 4, the calibration can be based on data obtained by the shake-flask method. For log P values greater than 4, the calibration can be based on validated literature values if these are in agreement with calculated values. For better accuracy, it is preferable to choose reference compounds which are structurally related to the test substance.

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

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

1.4. PRINCIPLE OF THE METHOD

1.4.1. **Shake-flask method**

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

1.4.2. **HPLC method**

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

$$k = \frac{t_R - t_0}{t_0}$$

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

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

1.5. QUALITY CRITERIA

1.5.1. **Repeatability**

Shake-flask method

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

HPLC method

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

1.5.2. **Sensitivity**

Shake-flask method

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

HPLC method

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

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

1.5.3. **Specificity**

Shake-flask method

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

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

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

1.6. DESCRIPTION OF THE METHOD

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

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

1.6.2. **Shake-flask method**

1.6.2.1. *Preparation*

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

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

Note:

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

1.6.2.1.1. Pre-saturation of the solvents

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

1.6.2.1.2. Preparation for the test

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

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

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

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

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

1.6.2.1.3. Test substance

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

1.6.2.2. *Test conditions*

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

1.6.2.3. *Measurement procedure*

1.6.2.3.1. Establishment of the partition equilibrium

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

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

1.6.2.3.2. Phase separation

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

1.6.2.4. *Analysis*

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

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

— photometric methods,

— gas chromatography,

— high-performance liquid chromatography.

1.6.3. **HPLC method**

1.6.3.1. *Preparation*

Apparatus

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

Mobile phase

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

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

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

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

Solutes

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

Test conditions

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

1.6.3.2. *Measurement*

Calculation of dead time t_o

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

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

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

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

Calibration graph

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

Determination of the capacity factor of the test substance

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

2. **DATA**

Shake-flask method

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

3. **REPORTING**

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

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

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

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

For shake-flask method:

— the result of the preliminary estimation, if any,

— temperature of the determination,

— data on the analytical procedures used in determining concentrations,

— time and speed of centrifugation, if used,

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

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

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

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

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

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

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

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

For HPLC method:

— the result of the preliminary estimation, if any,

— test and reference substances, and their purity,

— temperature range of the determinations,

— pH at which the determinations are made,

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

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

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

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

— description of equipment and operating conditions,

— elution profiles,

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

— dead-time and how it was measured.

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Appendix 1

Calculation/estimation Methods

INTRODUCTION

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

Calculated values of P_{ow} can be used:

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

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

— as a laboratory internal check on possible experimental errors,

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

ESTIMATION METHOD

Preliminary estimate of the partition coefficient

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

For this:

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

CALCULATION METHODS

Principle of the Calculation Methods

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

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

Quality Criteria

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

Calculation Procedures

Hansch π -Method

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

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

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

$$(\text{e.g. } \pi_{Cl} = \log P_{ow} (C_6H_5Cl) - \log P_{ow} (C_6H_6)$$

$$= 2,84 - 2,13 = 0,71).$$

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

Rekker Method

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

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

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

Hansch-Leo Method

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

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

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

Combined Method

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

Remarks

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

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

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

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

Report

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

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

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

— description of the calculation method,

— identification or supply of database,

— peculiarities in the choice of fragments,

— comprehensive documentation of the calculation.

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Appendix 2

Recommended Reference Substances for the HPLC-Method

No.	Reference Substance	log P_{ow}	pKa
1	2-Butanone	0,3	
2	4-Acetylpyridine	0,5	
3	Aniline	0,9	
4	Acetanilide	1,0	
5	Benzylalcohol	1,1	
6	p-Methoxyphenol	1,3	pKa = 10,26
7	Phenoxy acetic acid	1,4	pKa = 3,12
8	Phenol	1,5	pKa = 9,92
9	2,4-Dinitrophenol	1,5	pKa = 3,96
10	Benzonitrile	1,6	
11	Phenylacetonitrile	1,6	
12	4-Methylbenzyl alcohol	1,6	
13	Acetophenone	1,7	
14	2-Nitrophenol	1,8	pKa = 7,17
15	3-Nitrobenzoic acid	1,8	pKa = 3,47
16	4-Chloraniline	1,8	pKa = 4,15
17	Nitrobenzene	1,9	
18	Cinnamic alcohol	1,9	
19	Benzoic acid	1,9	pKa = 4,19
20	p-Cresol	1,9	pKa = 10,17
21	Cinnamic acid	2,1	pKa = 3,89 cis 4,44 trans
22	Anisole	2,1	
23	Methylbenzoate	2,1	
24	Benzene	2,1	
25	3-Methylbenzoic acid	2,4	pKa = 4,27
26	4-Chlorophenol	2,4	pKa = 9,1
27	Trichloroethylene	2,4	
28	Atrazine	2,6	
29	Ethylbenzoate	2,6	
30	2,6-Dichlorobenzonitrile	2,6	
31	3-Chlorobenzoic acid	2,7	pKa = 3,82
32	Toluene	2,7	
33	1-Naphthol	2,7	pKa = 9,34
34	2,3-Dichloroaniline	2,8	
35	Chlorobenzene	2,8	
36	Allyl-phenylether	2,9	
37	Bromobenzene	3,0	
38	Ethylbenzene	3,2	
39	Benzophenone	3,2	
40	4-Phenylphenol	3,2	pKa = 9,54
41	Thymol	3,3	
42	1,4-Dichlorobenzene	3,4	
43	Diphenylamine	3,4	pKa = 0,79
44	Naphthalene	3,6	
45	Phenylbenzoate	3,6	
46	Isopropylbenzene	3,7	
47	2,4,6-Trichlorophenol	3,7	pKa = 6
48	Biphenyl	4,0	
49	Benzylbenzoate	4,0	
50	2,4-Dinitro-6 sec. butylphenol	4,1	
51	1,2,4-Trichlorobenzene	4,2	
52	Dodecanoic acid	4,2	
53	Diphenylether	4,2	
54	n-Butylbenzene	4,5	
55	Phenanthrene	4,5	
56	Fluoranthene	4,7	
57	Dibenzyl	4,8	
58	2,6-Diphenylpyridine	4,9	
59	Triphenylamine	5,7	
60	DDT	6,2	
	Other reference substances of low log P_{ow}		
1	Nicotinic acid	− 0,07	

A.9. FLASH-POINT

1. **METHOD**

1.1. INTRODUCTION

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

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

1.2. DEFINITIONS AND UNITS

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

Units: °C

$t = T - 273{,}15$

(t in °C and T in K)

1.3. REFERENCE SUBSTANCES

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

1.4. PRINCIPLE OF THE METHOD

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

1.5. QUALITY CRITERIA

1.5.1. **Repeatability**

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

1.5.2. **Sensitivity**

The sensitivity depends on the test method used.

1.5.3. **Specificity**

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

1.6. DESCRIPTION OF THE METHOD

1.6.1. **Preparations**

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

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

1.6.2. **Test conditions**

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

1.6.3. **Performance of the test**

1.6.3.1. *Equilibrium method*

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

1.6.3.2. *Non-equilibrium method*

Abel apparatus:

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

Abel-Pensky apparatus:

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

Tag apparatus:

See ASTM D 56.

Pensky-Martens apparatus:

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

Remarks:

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

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

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

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

2. **DATA**

3. **REPORTING**

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

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

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

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

4. **REFERENCES**

None.

A.10. FLAMMABILITY (SOLIDS)

1. **METHOD**

1.1. INTRODUCTION

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

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

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

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

1.2. DEFINITION AND UNITS

Burning time expressed in seconds.

1.3. REFERENCE SUBSTANCES

Not specified.

1.4. PRINCIPLE OF THE METHOD

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

1.5. QUALITY CRITERIA

Not stated.

1.6. DESCRIPTION OF METHOD

1.6.1. **Preliminary screening test**

The substance is formed into an unbroken strip or powder train about 250 mm long by 20 mm wide by 10 mm high on a non-combustible, non-porous and low heat-conducting base plate.

A hot flame from a gas burner (minimum diameter 5 mm) is applied to one end of the powder train until the powder ignites or for a maximum of 2 minutes (5 minutes for powders of metals or metal-alloys). It should be noted whether combustion propagates along 200 mm of the train within the 4 minutes test period (or 40 minutes for metal powders). If the substance does not ignite and propagate combustion either by burning with flame or smouldering along 200 mm of the powder train within the 4 minutes (or 40 minutes) test period, then the substance should not be considered as highly flammable and no further testing is required. If the substance propagates burning of a 200 mm length of the powder train in less than 4 minutes, or less than 40 minutes for metal powders, the procedure described below (point 1.6.2. and following) should be carried out.

1.6.2. **Burning rate test**

1.6.2.1. *Preparation*

Powdery or granular substances are loosely filled into a mould 250 mm long with a triangular cross-section of inner height 10 mm and width 20 mm. On both sides of the mould in a longitudinal direction two metal plates are mounted as lateral limitations which project 2 mm beyond the upper edge of the triangular cross section (figure). The mould is then dropped three times from a height of 2 cm onto a solid surface. If necessary the mould is then filled up again. The lateral limitations are then removed and the excess substance scraped off. A non-combustible, non-porous and low heat-conducting base plate is placed on top of the mould, the apparatus inverted and the mould removed.

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

1.6.2.2. *Test conditions*

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

1.6.2.3. *Performance of the test*

Arrange the pile across the draught in a fume cupboard.

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

A hot flame from a gas burner (minimum diameter of 5 mm) is used to ignite the pile at one end. When the pile has burned a distance of 80 mm, the rate of burning over the next 100 mm is measured. The test is performed six times, using a clean cool plate each time, unless a positive result is observed earlier.

2. **DATA**

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

3. **REPORTING**

3.1. TEST REPORT

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

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

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

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

— all additional remarks relevant to the interpretation of results.

3.2. INTERPRETATION OF THE RESULT

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

4. **REFERENCES**

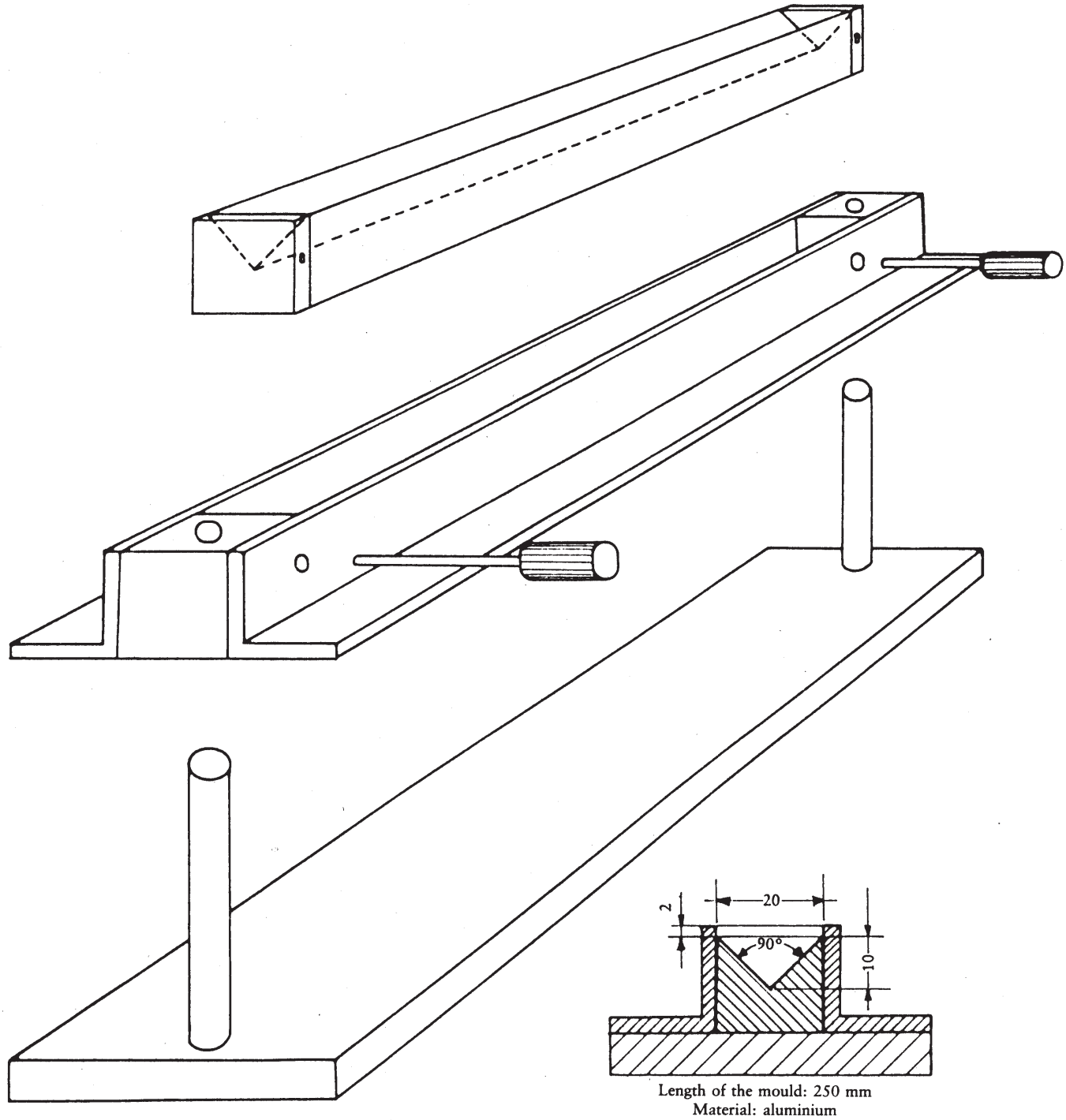
(1) NF T 20-042 (SEPT 85). Chemical products for industrial use. Determination of the flammability of solids.

Appendix

Figure

Mould and accessories for the preparation of the pile

(All dimensions in millimetres)



A.11. FLAMMABILITY (GASES)

1. **METHOD**

1.1. INTRODUCTION

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

1.2. DEFINITION AND UNITS

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

1.3. REFERENCE SUBSTANCES

Not specified.

1.4. PRINCIPLE OF THE METHOD

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

1.5. QUALITY CRITERIA

Not stated.

1.6. DESCRIPTION OF THE METHOD

1.6.1. **Apparatus**

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

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

1.6.2. **Test conditions**

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

1.6.3. **Performance of the test**

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

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

2. **DATA**

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

3. **REPORTING**

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

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

— a description, with dimensions, of the apparatus used

— the temperature at which the test was performed,

— the tested concentrations and the results obtained,

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

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

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

4. **REFERENCES**

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

(2) W.Berthold, D.Conrad, T.Grewer, H.Grosse-Wortmann, T.Redeker und H.Schacke. "Entwicklung einer Standard-Apparatur zur Messung von Explosionsgrenzen". Chem.-Ing.-Tech. 1984, vol 56, 2, 126-127.

A.12. FLAMMABILITY (CONTACT WITH WATER)

1. **METHOD**

1.1. INTRODUCTION

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

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

1.2. DEFINITIONS AND UNITS

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

1.3. PRINCIPLE OF THE METHOD

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

1.3.1. **Step 1**

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

1.3.2. **Step 2**

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

1.3.3. **Step 3**

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

1.3.4. **Step 4**

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

1.4. REFERENCE SUBSTANCES

Not specified.

1.5. QUALITY CRITERIA

Not stated.

1.6 DESCRIPTION OF METHODS

1.6.1. **Step 1**

1.6.1.1. *Test conditions*

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

1.6.1.2. *Performance of the test*

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

1.6.2. **Step 2**

1.6.2.1. *Apparatus*

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

1.6.2.2. *Test conditions*

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

1.6.2.3. *Performance of the test*

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

1.6.3. **Step 3**

1.6.3.1. *Test conditions*

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

1.6.3.2. *Performance of the test*

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

1.6.4. **Step 4**

1.6.4.1. *Apparatus*

The apparatus is set up as shown in the figure.

1.6.4.2. *Test conditions*

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

1.6.4.3. *Performance of the test*

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

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

2. **DATA**

The substance is considered hazardous if:

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

or

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

3. **REPORTING**

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

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

— details of any initial preparation of the test substance,

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

— the chemical identity of gas evolved,

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

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

4. **REFERENCES**

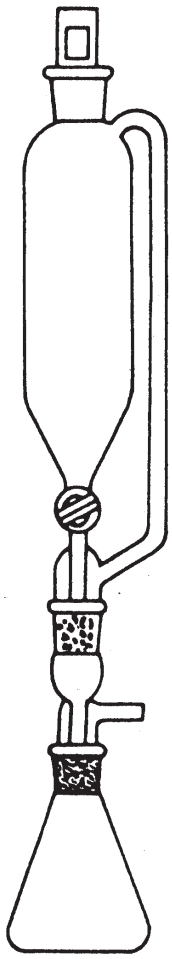
(1) Recommendations on the transport of dangerous goods, test and criteria, 1990, United Nations, New York.

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

Appendix

Figure

Apparatus



A.13. PYROPHORIC PROPERTIES OF SOLIDS AND LIQUIDS

1. **METHOD**

1.1. INTRODUCTION

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

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

1.2 DEFINITIONS AND UNITS

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

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

1.3. REFERENCE SUBSTANCES

Not specified.

1.4. PRINCIPLE OF THE METHOD

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

1.5. QUALITY CRITERIA

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

1.6. DESCRIPTION OF THE TEST METHOD

1.6.1. **Apparatus**

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

Note:

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

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

1.6.2. **Performance of the Test**

a) Powdery Solids

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

The test is performed six times unless ignition occurs.

b) Liquids

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

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

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

2. **DATA**

2.1. TREATMENT OF RESULTS

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

2.2. EVALUATION

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

3. **REPORTING**

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

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

— the results of the tests,

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

4. **REFERENCES**

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

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

A.14. EXPLOSIVE PROPERTIES

1. **METHOD**

1.1. INTRODUCTION

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

The method comprises three parts:

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

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

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

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

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

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

1.2. DEFINITIONS AND UNITS

Explosive:

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

1.3. REFERENCE SUBSTANCES

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

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

1.4. PRINCIPLE OF THE METHOD

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

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

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

1.4.2. **Thermal sensitivity**

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

1.4.3. **Mechanical sensitivity (shock)**

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

1.4.4. **Mechanical sensitivity (friction)**

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

1.5. QUALITY CRITERIA

Not stated.

1.6. DESCRIPTION OF METHOD

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

1.6.1.1. *Apparatus*

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

1.6.1.2. *Test conditions*

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

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

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

Heating is provided by propane taken from an industrial cylinder, fitted with a pressure regulator (60 to 70 mbar), through a meter and evenly distributed (as indicated by visual observation of the flames from the burners) by a manifold to four burners. The burners are located around the test chamber as shown in figure 1. The four burners have a combined consumption of about 3.2 litres of propane per minute. Alternative fuel gases and burners may be used but the heating rate must be as specified in figure 3. For all apparatus, the heating rate must be checked periodically using tubes filled with dibutyl phthalate as indicated in figure 3.

1.6.1.3. *Performance of the tests*

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

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

1.6.1.4. *Evaluation*

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

1.6.2. **Mechanical sensitivity (shock)**

1.6.2.1. *Apparatus (figure 4)*

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

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

1.6.2.2. *Test conditions*

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

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

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

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

1.6.2.3. *Performance of the tests*

A series of six tests are performed dropping the 10 kg mass from 0,40 m (40 J). If an explosion is obtained during the six tests at 40 J, a further series of 6 tests, dropping a 5 kg mass from 0,15 m (7,5 J), must be performed. In other apparatus, the sample is compared with the chosen reference substance using an established procedure (e.g. up-and-down technique etc.).

1.6.2.4. *Evaluation*

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

1.6.3. **Mechanical sensitivity (friction)**

1.6.3.1. *Apparatus (figure 5)*

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

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

1.6.3.2. *Test conditions*

The sample volume should be 10 mm^3 or a volume to suit any alternative apparatus.

Solid substances are tested in the dry state and prepared as follows:

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

(b) pressed, cast or otherwise condensed substances are broken into small pieces and sieved; the sieve fraction < 0,5 mm diameter is used for testing.

Substances normally supplied as pastes should be tested in the dry state where possible. If the substance cannot be prepared in the dry state, the paste (following removal of the maximum possible amount of diluent) is tested as a 0,5 mm thick, 2 mm wide, 10 mm long film, prepared with a former.

1.6.3.3. *Performance of the tests*

The porcelain peg is brought onto the sample under test and the load applied. When carrying out the test, the sponge marks of the porcelain plate must lie transversely to the direction of the movement. Care must be taken that the peg rests on the sample, that sufficient test material lies under the peg and also that the plate moves correctly under the peg. For pasty substances, a 0,5 mm thick gauge with a 2 × 10 mm slot is used to apply the substance to the plate. The porcelain plate has to move 10 mm forwards and backwards under the porcelain peg in a time of 0,44 seconds. Each part of the surface of the plate and peg must only be used once; the two ends of each peg will serve for two trials and the two surfaces of a plate will each serve for three trials.

A series of six tests are performed with a 360 N loading. If a positive event is obtained during these six tests, a further series of six tests must be performed with a 120 N loading. In other apparatus, the sample is compared with the chosen reference substance using an established procedure (e.g. up-and-down technique, etc.).

1.6.3.4. *Evaluation*

The test result is considered positive if an explosion (crepitation and/or a report or bursting into flame are equivalent to explosion) occurs at least once in any of the tests with the specified friction apparatus or satisfies the equivalent criteria in an alternative friction test.

2. **DATA**

In principle, a substance is considered to present a danger of explosion in the sense of the directive if a positive result is obtained in the thermal, shock or friction sensitivity test.

3. **REPORTING**

3.1. TEST REPORT

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

— identity, composition, purity, moisture content etc. of the substance tested,

— the physical form of the sample and whether or not it has been crushed, broken and/or sieved,

— observations during the thermal sensitivity tests (e.g. sample mass, number of fragments etc.),

— observations during the mechanical sensitivity tests (e.g. formation of considerable amounts of smoke or complete decomposition without a report, flames, sparks, report, crepitation etc.),

— results of each type of test,

— if alternative apparatus has been used, scientific justification as well as evidence of correlation between results obtained with specified apparatus and those obtained with equivalent apparatus must be given,

— any useful comments such as reference to tests with similar products which might be relevant to a proper interpretation of the results,

— all additional remarks relevant for the interpretation of the results.

3.2. INTERPRETATION AND EVALUATION OF RESULTS

The test report should mention any results which are considered false, anomalous or unrepresentative. If any of the results should be discounted, an explanation and the results of any alternative or supplementary testing should be given. Unless an anomalous result can be explained, it must be accepted at face value and used to classify the substance accordingly.

4. **REFERENCES**

(1) Recommendations on the Transport of Dangerous Goods: Tests and criteria, 1990, United Nations, New York.

(2) Bretherick, L., Handbook of Reactive Chemical Hazards, 4th edition, Butterworths, London, ISBN 0-750-60103-5, 1990.

(3) Koenen, H., Ide, K.H. and Swart, K.H., Explosivstoffe, 1961, vol.3, 6-13 and 30-42.

(4) NF T 20-038 (Sept. 85). Chemical products for industrial use — Determination of explosion risk.

Appendix

Example of Material specification for thermal sensitivity test (see DIN 1623)

(1) Tube: Material specification No 1.0336.505 g

(2) Orifice plate: Material specification No 1.4873

(3) Threaded collar and nut: Material specification No 1.3817

Figure 1

Thermal sensitivity test apparatus

(all dimensions in millimetres)

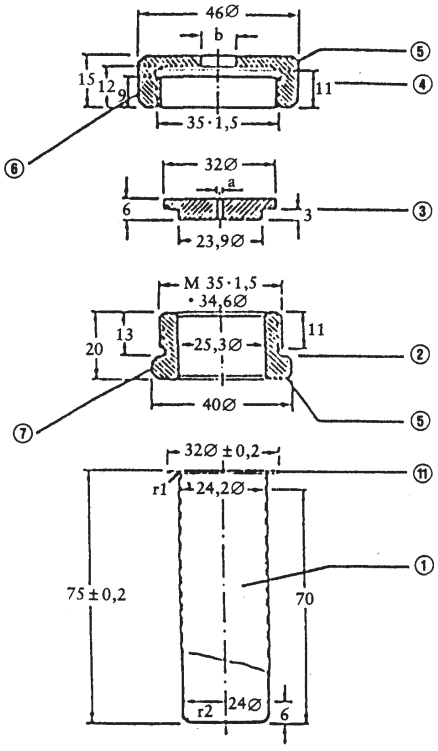


Fig. 1a Steel tube and accessories

(1) tube
(1a) outer flange
(2) threaded collar; low-friction thread
(3) orifice plate a = 2,0 or 6,0 mm diameter
(4) nut b = 10 mm diameter
(5) chamfered surface
(6) 2 flat for spanner size 41

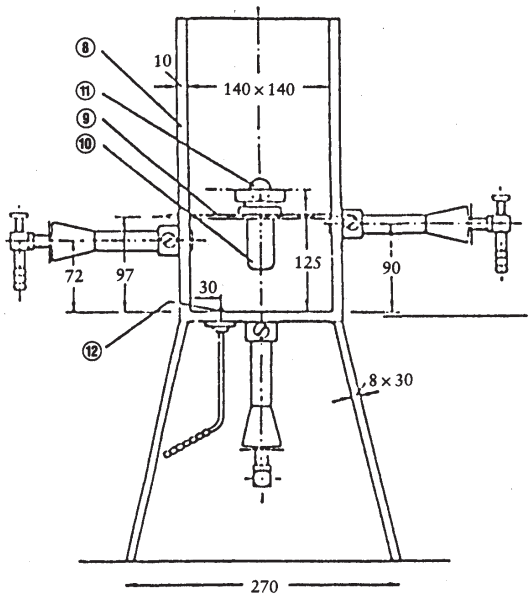


Fig. 1b Heating and protective device

(7) 2 flat for spanner size 36
(8) splinter-proof box
(9) 2 supporting rods for tube
(10) assembled tube
(11) position for rear burner; the other burners are visible
(12) pilot jet

Figure 2

Thermal sensitivity test

Examples of fragmentation

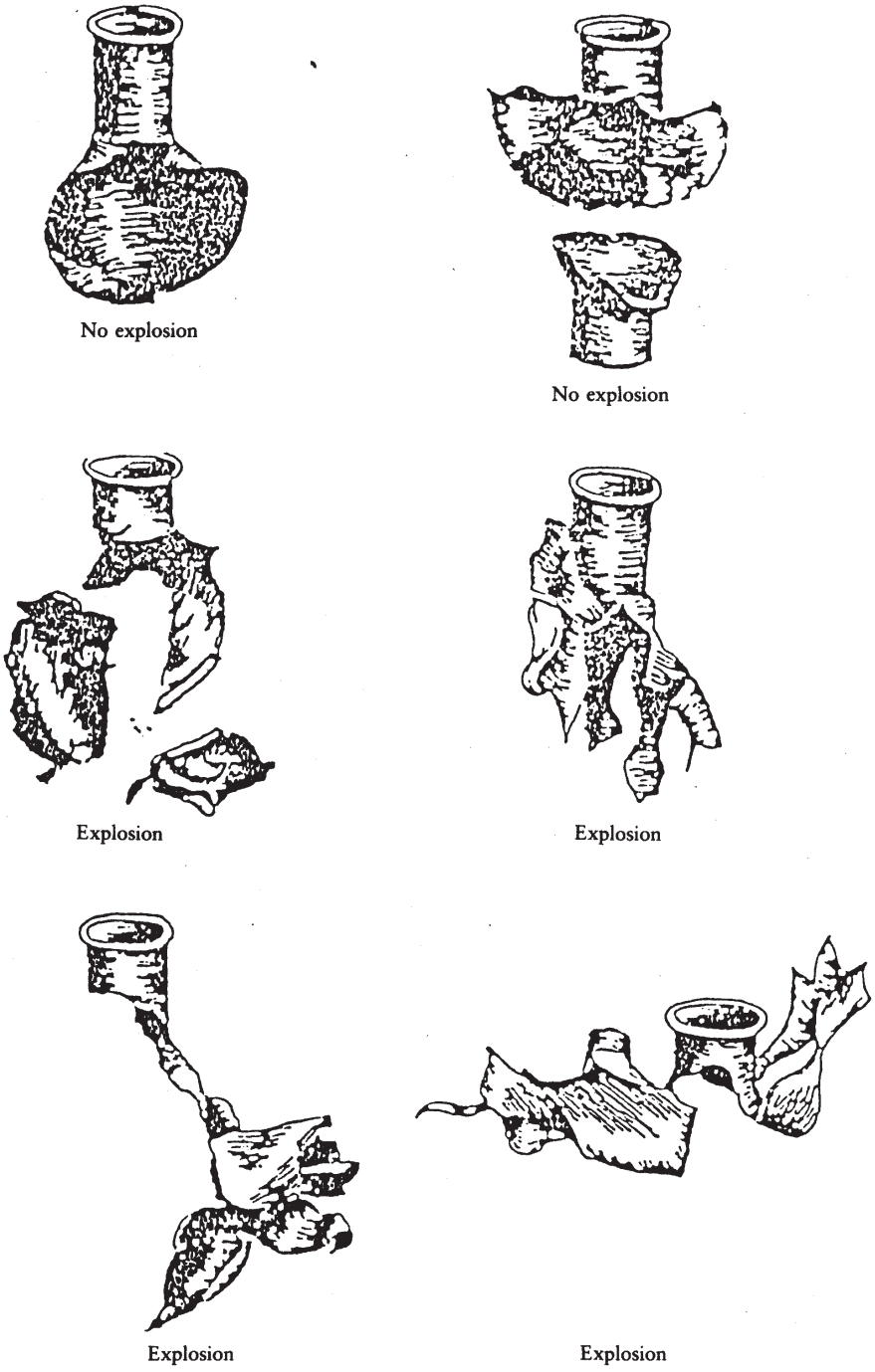
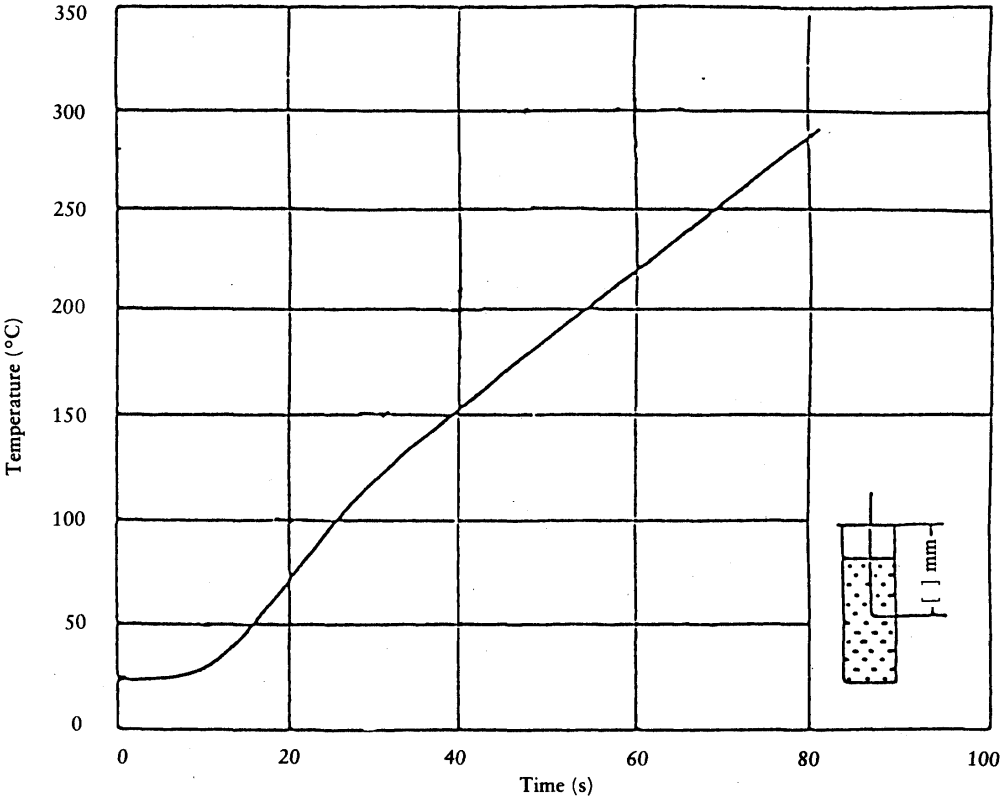


Figure 3

Heating rate calibration for thermal sensitivity test



Temperature/time curve obtained on heating dibutyl phthalate (27 cm3) in a closed (1,5 mm orifice plate) tube using a propane flow rate of 3,2 litre/minute. The temperature is measured with a 1 mm diameter stainless steel sheathed chromel/alumel thermocouple, placed centrally 43 mm below the rim of the tube. The heating rate between 135 °C and 285 °C should be between 185 and 215 K/minute.

Figure 4

Shock test apparatus

(all dimensions in millimetres)

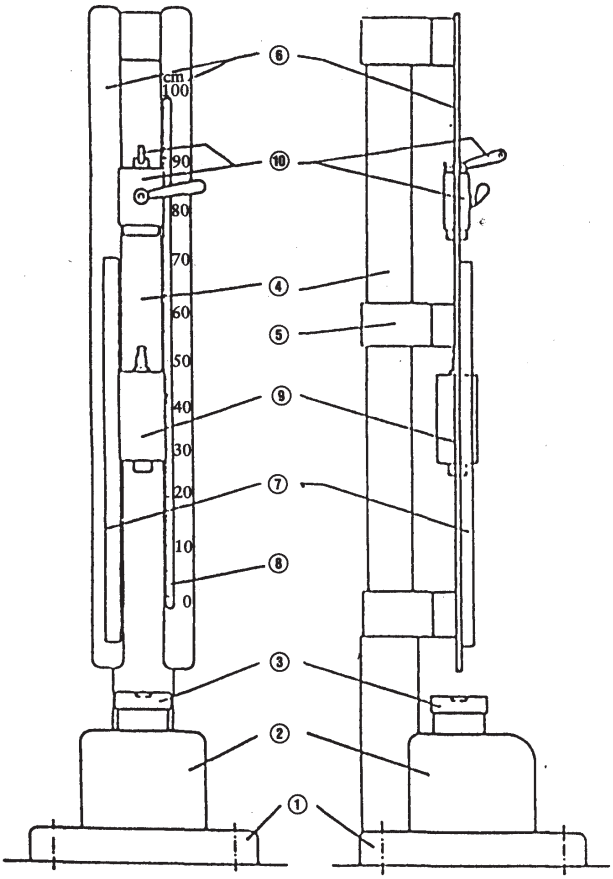


Fig. 4a Fall-hammer, front and side, general view

(1) base, 450 × 450 × 60
(2) steel block, 230 × 250 × 200
(3) anvil, 100 diameter × 70
(4) colum
(5) median cross-member
(6) 2 guides
(7) toothed rack

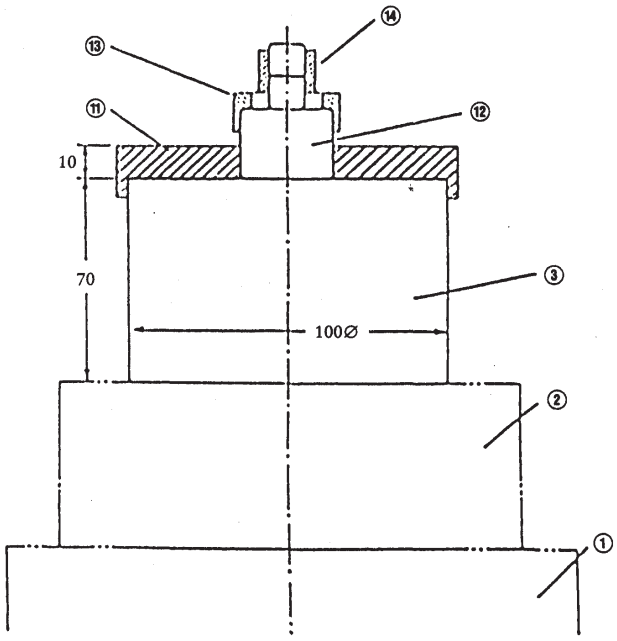


Fig. 4b Fall-hammer, lower part

(8) graduated scale
(9) fall-hammer(drop mass)
(10) holding and releasing device
(11) locating plate
(12) intermediate anvil (interchangeable), 26 diameter × 26
(13) locating ring with orifices
(14) impact device

Figure 4

Continued

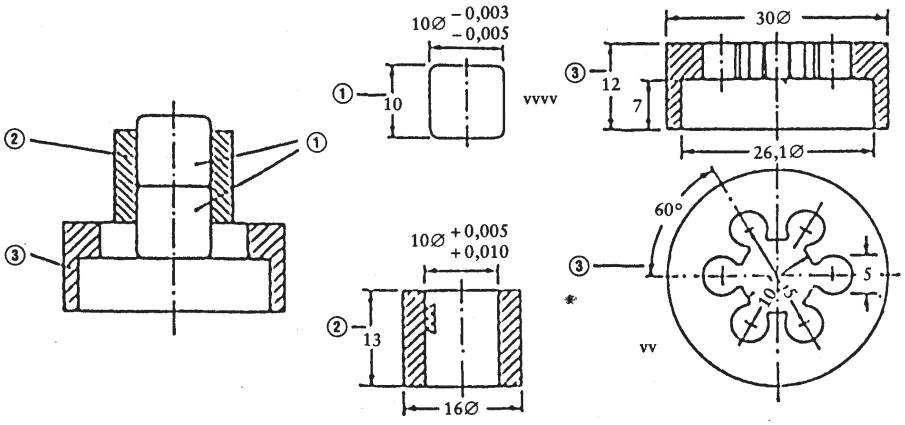
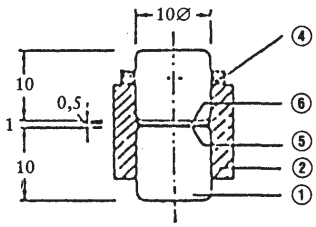


Fig. 4c Shock device for substances in powdered or paste-like form

(1) steel cylinders
(2) guide ring for steel cylinders
(3) locating ring with orifices
(a) vertical section
(b) plan
(4) rubber ring
(5) liquid substance (40 mm^3)
(6) space free from liquid

Fig. 4d Shock device for liquid substances



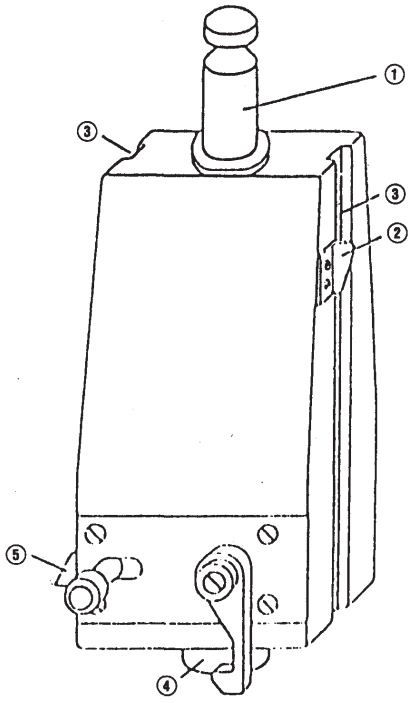


Fig. 4e Hammer (drop mass of 5 kg)

(1) suspension spigot
(2) height marker
(3) positioning groove
(4) cylindrical striking head
(5) rebound catch

Figure 5

Friction sensitivity apparatus

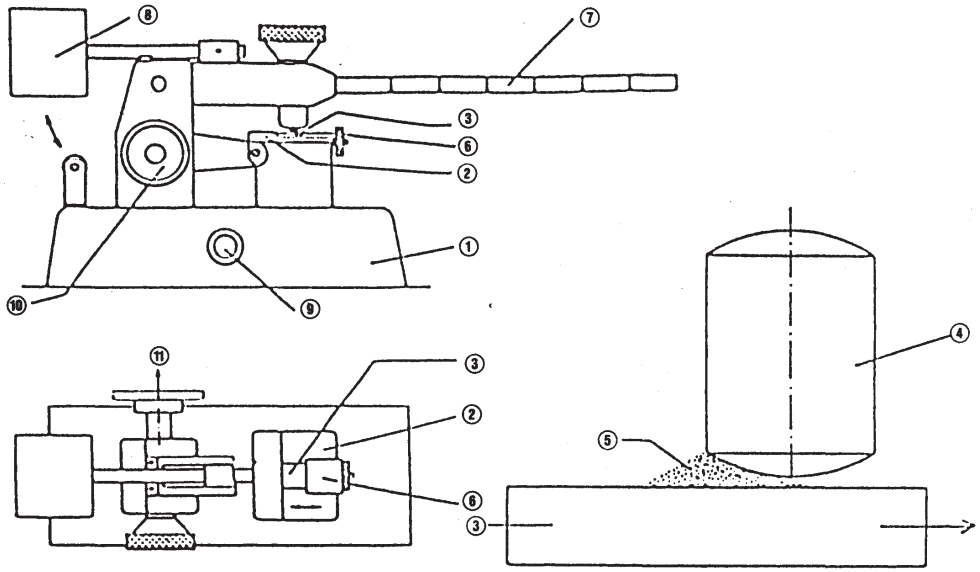


Fig. 5a Friction apparatus; elevation and plan view

(1) steel base
(2) movable carriage
(3) porcelain plate, 25 × 25 × 5 mm, hel on carriage
(4) fixed porcelain peg, 10 diameter × 15 mm
(5) sample under test, approximately 10 mm^3

Fig. 5b Starting position of peg on sample

(6) peg-holder
(7) loading arm
(8) counterweight
(9) switch
(10) wheel for setting carriage at starting position
(11) direction to electric drive motor

A.15. AUTO-IGNITION TEMPERATURE (LIQUIDS AND GASES)

1. **METHOD**

1.1. INTRODUCTION

Explosive substances and substances which ignite spontaneously in contact with air at ambient temperature should not be submitted to this test. The test procedure is applicable to gases, liquids and vapours which, in the presence of air, can be ignited by a hot surface.

The auto-ignition temperature can be considerably reduced by the presence of catalytic impurities, by the surface material or by a higher volume of the test vessel.

1.2. DEFINITIONS AND UNITS

The degree of auto-ignitability is expressed in terms of the auto-ignition temperature. The auto-ignition temperature is the lowest temperature at which the test substance will ignite when mixed with air under the conditions defined in the test method.

1.3. REFERENCE SUBSTANCES

Reference substances are cited in the standards (see 1.6.3). They should primarily serve to check the performance of the method from time to time and to allow comparison with results from other methods.

1.4. PRINCIPLE OF THE METHOD

The method determines the minimum temperature of the inner surface of an enclosure that will result in ignition of a gas, vapour or liquid injected into the enclosure.

1.5. QUALITY CRITERIA

The repeatability varies according to the range of auto-ignition temperatures and the test method used.

The sensitivity and specificity depend on the test method used.

1.6. DESCRIPTION OF THE METHOD

1.6.1. **Apparatus**

The apparatus is described in the method referred to in 1.6.3.

1.6.2. **Test conditions**

A sample of the test substance is tested according to the method referred to in 1.6.3.

1.6.3. **Performance of the test**

See IEC 79-4, DIN 51794, ASTM-E 659-78, BS 4056, NF T 20-037.

2. **DATA**

Record the test-temperature, atmospheric pressure, quantity of sample used and time-lag until ignition occurs.

3. **REPORTING**

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

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

— the quantity of sample used, atmospheric pressure,

— the apparatus used,

— the results of measurements (test temperatures, results concerning ignition, corresponding time-lags),

— all additional remarks relevant to the interpretation of results.

4. **REFERENCES**

None.

A.16. RELATIVE SELF-IGNITION TEMPERATURE FOR SOLIDS

1. **METHOD**

1.1. INTRODUCTION

Explosive substances and substances which ignite spontaneously in contact with air at ambient temperature should not be submitted to this test.

The purpose of this test is to provide preliminary information on the auto-flammability of solid substances at elevated temperatures.

If the heat developed either by a reaction of the substance with oxygen or by exothermic decomposition is not lost rapidly enough to the surroundings, self-heating leading to self-ignition occurs. Self-ignition therefore occurs when the rate of heat-production exceeds the rate of heat loss.

The test procedure is useful as a preliminary screening test for solid substances. In view of the complex nature of the ignition and combustion of solids, the self-ignition temperature determined according to this test method should be used for comparison purposes only.

1.2. DEFINITIONS AND UNITS

The self-ignition temperature as obtained by this method is the minimum ambient temperature expressed in °C at which a certain volume of a substance will ignite under defined conditions.

1.3. REFERENCE SUBSTANCE

None.

1.4. PRINCIPLE OF THE METHOD

A certain volume of the substance under test is placed in an oven at room temperature; the temperature/time curve relating to conditions in the centre of the sample is recorded while the temperature of the oven is increased to 400 °C, or to the melting point if lower, at a rate of 0,5 °C/min. For the purpose of this test, the temperature of the oven at which the sample temperature reaches 400 °C by self-heating is called the self-ignition temperature.

1.5. QUALITY CRITERIA

None.

1.6. DESCRIPTION OF THE METHOD

1.6.1. **Apparatus**

1.6.1.1. *Oven*

A temperature-programmed laboratory oven (volume about 2 litres) fitted with natural air circulation and explosion relief. In order to avoid a potential explosion risk, any decomposition gases must not be allowed to come into contact with the electric heating elements.

1.6.1.2. *Wire mesh cube*

A piece of stainless steel wire mesh with 0,045 mm openings should be cut according to the pattern in figure 1. The mesh should be folded and secured with wire into an open-topped cube.

1.6.1.3. *Thermocouples*

Suitable thermocouples.

1.6.1.4. *Recorder*

Any two-channel recorder calibrated from 0 to 600 °C or corresponding voltage.

1.6.2. **Test conditions**

Substances are tested as received.

1.6.3. **Performance of the test**

The cube is filled with the substance to be tested and is tapped gently, adding more of the substance until the cube is completely full. The cube is then suspended in the centre of the oven at room temperature. One thermocouple is placed at the centre of the cube and the other between the cube and the oven wall to record the oven temperature.

The temperatures of the oven and sample are continuously recorded while the temperature of the oven is increased to 400 °C, or to the melting point if lower, at a rate of 0,5 °C/min.

When the substance ignites the sample thermocouple will show a very sharp temperature rise above the oven temperature.

2. **DATA**

The temperature of the oven at which the sample temperature reaches 400 °C by self-heating is relevant for evaluation (see figure 2).

3. **REPORTING**

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

— a description of the substance to be tested,

— the results of measurement including the temperature/time curve,

— all additional remarks relevant for the interpretation of the results.

4. **REFERENCES**

(1) NF T 20-036 (September 85). Chemical products for industrial use. Determination of the relative temperature of the spontaneous flammability of solids.

Figure 1

Pattern of 20 mm test cube

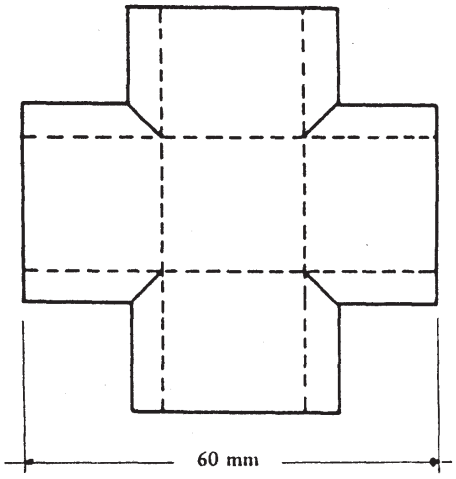
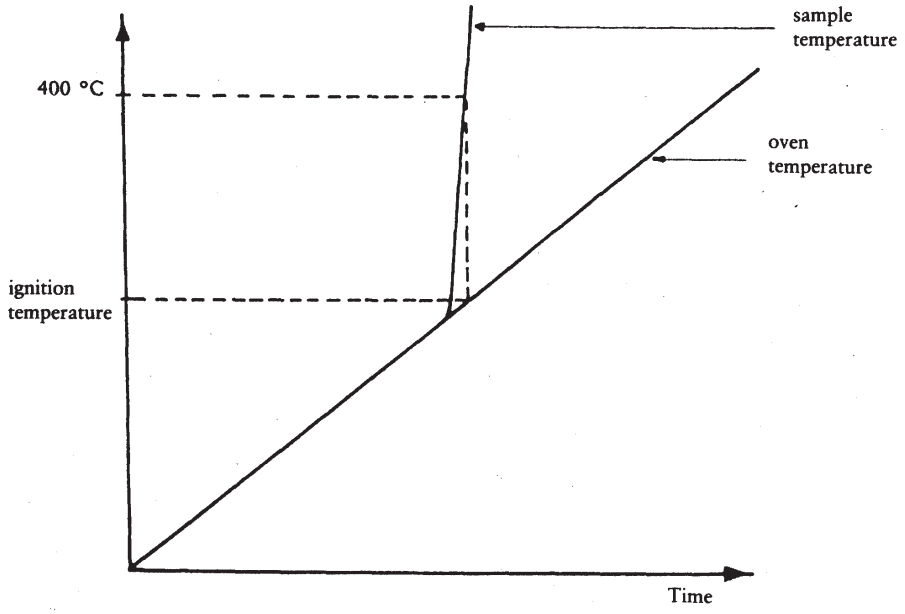


Figure 2

Typical temperature/time curve



A.17. OXIDIZING PROPERTIES (SOLIDS)

1. **METHOD**

1.1. INTRODUCTION

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

This test is not applicable to liquids, gases, explosive or highly flammable substances, or organic peroxides.

This test need not be performed when examination of the structural formula establishes beyond reasonable doubt that the substance is incapable of reacting exothermically with a combustible material.

In order to ascertain if the test should be performed with special precautions, a preliminary test should be performed.

1.2. DEFINITION AND UNITS

Burning time: reaction time, in seconds, taken for the reaction zone to travel along a pile, following the procedure described in 1.6.

Burning rate: expressed in millimetres per second.

Maximum burning rate: the highest value of the burning rates obtained with mixtures containing 10 to 90 % by weight of oxidizer.

1.3. REFERENCE SUBSTANCE

Barium nitrate (analytical grade) is used as reference substance for the test and the preliminary test.

The reference mixture is that mixture of barium nitrate with powdered cellulose, prepared according to 1.6, which has the maximum burning rate (usually a mixture with 60 % barium nitrate by weight).

1.4. PRINCIPLE OF THE METHOD

A preliminary test is carried out in the interests of safety. No further testing is required when the preliminary test clearly indicates that the test substance has oxidizing properties. When this is not the case, the substance should then be subject to the full test.

In the full test, the substance to be tested and a defined combustible substance will be mixed in various ratios. Each mixture is then formed into a pile and the pile is ignited at one end. The maximum burning rate determined is compared with the maximum burning rate of the reference mixture.

1.5. QUALITY CRITERIA

If required, any method of grinding and mixing is valid provided that the difference in the maximum rate of burning in the six separate tests differs from the arithmetic mean value by no more than 10 %.

1.6. DESCRIPTION OF THE METHOD

1.6.1. **Preparation**

1.6.1.1. *Test substance*

Reduce the test sample to a particle size < 0,125 mm using the following procedure: sieve the test substance, grind the remaining fraction, repeat the procedure until the whole test portion has passed the sieve.

Any grinding and sieving method satisfying the quality criteria may be used.

Before preparing the mixture the substance is dried at 105 °C, until constant weight is obtained. If the decomposition temperature of the substance to be tested is below 105 °C, the substance has to be dried at a suitable lower temperature.

1.6.1.2. *Combustible substance*

Powdered cellulose is used as a combustible substance. The cellulose should be a type used for thin-layer chromatography or column chromatography. A type with fibre-lengths of more than 85 % between 0,020 and 0,075 mm has proved to be suitable. The cellulose powder is passed through a sieve with a mesh-size of 0,125 mm. The same batch of cellulose is to be used throughout the test.

Before preparing the mixture, the powdered cellulose is dried at 105 °C until constant weight is obtained.

If wood-meal is used in the preliminary test, then prepare a soft-wood wood-meal by collecting the portion which passes through a sieve mesh of 1,6 mm, mix thoroughly, then dry at 105 °C for four hours in a layer not more than 25 mm thick. Cool and store in an air-tight container filled as full as practicable until required, preferably within 24 hours of drying.

1.6.1.3. *Ignition source*

A hot flame from a gas burner (minimum diameter 5 mm) should be used as the ignition source. If another ignition source is used (e.g. when testing in an inert atmosphere), the description and the justification should be reported.

1.6.2. **Performance of the test**

Note:

Mixtures of oxidizers with cellulose or wood-meal must be treated as potentially explosive and handled with due care.

1.6.2.1. *Preliminary test*

The dried substance is thoroughly mixed with the dried cellulose or wood-meal in the proportions 2 of test substance to 1 of cellulose or wood-meal by weight and the mixture is formed into a small cone-shaped pile of dimensions 3,5 cm (diameter of base) × 2,5 cm (height) by filling, without tamping, a cone-shaped former (e.g. a laboratory glass funnel with the stem plugged).

The pile is placed on a cool, non-combustible, non-porous and low heat-conducting base plate. The test should be carried out in a fume cupboard as in 1.6.2.2.

The ignition source is put in contact with the cone. The vigour and duration of the resultant reaction are observed and recorded.

The substance is to be considered as oxidizing if the reaction is vigorous.

In any case where the result is open to doubt, it is then necessary to complete the full train test described below.

1.6.2.2. *Train test*

Prepare oxidizer/cellulose-mixtures containing 10 to 90 % weight of oxidizer in 10 % increments. For borderline cases, intermediate oxidizer/cellulose mixtures should be used to obtain the maximum burning rate more precisely.

The pile is formed by means of a mould. The mould is made of metal, has a length of 250 mm and a triangular cross-section with an inner height of 10 mm and an inner width of 20 mm. On both sides of the mould, in the longitudinal direction, two metal plates are mounted as lateral limitations which project 2 mm beyond the upper edge of the triangular cross-section (figure). This arrangement is loosely filled with a slight excess of mixture. After dropping the mould once from a height of 2 cm onto a solid surface, the remaining excess substance is scraped off with an obliquely positioned sheet. The lateral limitations are removed and the remaining powder is smoothed, using a roller. A non-combustible, non-porous and low heat-conducting base plate is then placed on the top of the mould, the apparatus inverted and the mould removed.

Arrange the pile across the draught in a fume cupboard.

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

Due to hygroscopicity of cellulose and of some substances to be tested, the test should be carried out as quickly as possible.

Ignite one end of the pile by touching with the flame.

Measure the time of reaction over a distance of 200 mm after the reaction zone has propagated an initial distance of 30 mm.

The test is performed with the reference substance and at least once with each one of the range of mixtures of the test substance with cellulose.

If the maximum burning rate is found to be significantly greater than that from the reference mixture, the test can be stopped; otherwise the test should be repeated five times for each of the three mixtures giving the fastest burning rate.

If the result is suspected of being a false positive, then the test should be repeated using an inert substance with a similar particle size, such as kieselguhr, in place of cellulose. Alternatively, the test substance/cellulose mixture, having the fastest burning rate, should be retested in an inert atmosphere (< 2 % v/v oxygen content).

2. **DATA**

For safety reasons the maximum burning rate — not the mean value — shall be considered to be the characteristic oxidizing property of the substance under test.

The highest value of burning rate within a run of six tests of a given mixture is relevant for evaluation.

Plot a graph of the highest value of burning rate for each mixture versus the oxidizer concentration. From the graph take the maximum burning rate.

The six measured values of burning rate within a run obtained from the mixture with the maximum burning rate must not differ from the arithmetic mean value by more than 10 %; otherwise the methods of grinding and mixing must be improved.

Compare the maximum burning rate obtained with the maximum burning rate of the reference mixture (see 1.3).

If tests are conducted in an inert atmosphere, the maximum reaction rate is compared with that from the reference mixture in an inert atmosphere.

3. **REPORT**

3.1. TEST REPORT

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

— the identity, composition, purity, moisture content etc. of the substance tested;

— any treatment of the test sample (e.g. grinding, drying,…);

— the ignition source used in the tests;

— the results of measurements;

— the mode of reaction (e.g. flash burning at the surface, burning through the whole mass, any information concerning the combustion products,…);

— all additional remarks relevant for the interpretation of results, including a description of the vigour (flaming, sparking, fuming, slow smouldering, etc.) and approximate duration produced in the preliminary safety/screening test for both test and reference substance;

— the results from tests with an inert substance, if any;

— the results from tests in an inert atmosphere, if any.

3.2. INTERPRETATION OF THE RESULT

A substance is to be considered as an oxidizing substance when:

(a) in the preliminary test, there is a vigorous reaction;

(b) in the full test, the maximum burning rate of the mixtures tested is higher than or equal to the maximum burning rate of the reference mixture of cellulose and barium nitrate.

In order to avoid a false positive, the results obtained when testing the substance mixed with an inert material and/or when testing under an inert atmosphere should also be considered when interpreting the results.

4. **REFERENCES**

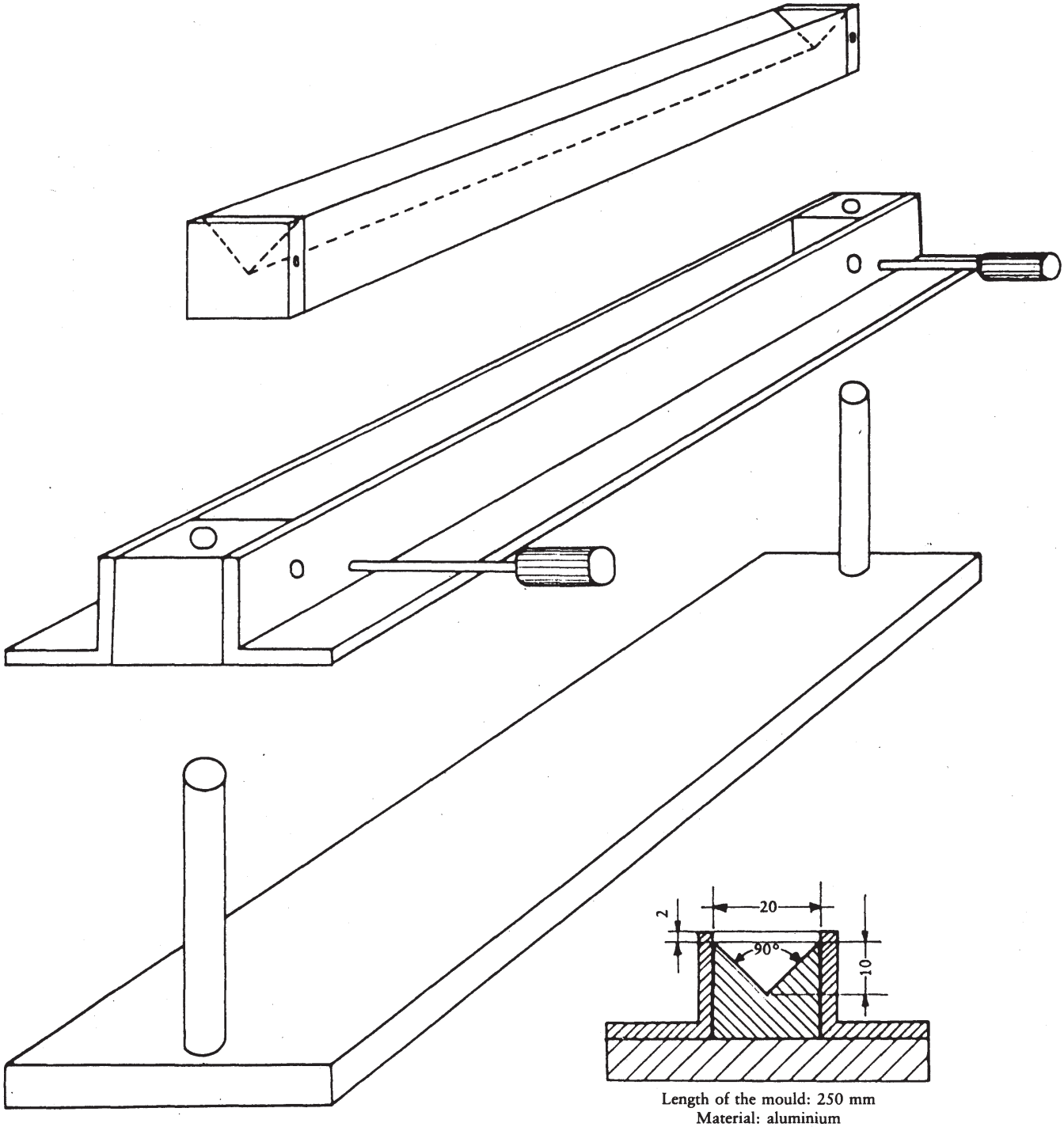
(1) NF T 20-035 (SEPT 85). Chemical products for industrial use. Determination of the oxidizing properties of solids.

Appendix

Figure

Mould and accessories for the preparation of the pile

(All dimensions in millimetres)



PART B: METHODS FOR THE DETERMINATION OF TOXICITY

GENERAL INTRODUCTION: PART B

A. INTRODUCTION

See General Introduction.

B. DEFINITIONS

(i) *Acute toxicity* comprises the adverse effects occurring within a given time (usually 14 days), after administration of a single dose of a substance

(ii) LD_{50} (median lethal dose) is a statistically derived single dose of a substance that can be expected to cause death in 50 % of dosed animals. The LD_{50} value is expressed in terms of weight of test substance per unit weight of test animal (milligrams per kilogram)

(iii) LC_{50} (median lethal concentration) is a statistically derived concentration of a substance that can be expected to cause death during exposure or within a fixed time after exposure in 50 % of animals exposed for a specified time. The LC_{50} value is expressed as weight of test substance per standard volume of air (milligrams per litre)

(iv) *No adverse effect level* is the maximum dose or exposure level used in a test which produces no detectable signs of toxicity

(v) *Sub-acute/Sub-chronic toxicity* comprises the adverse effects occurring in experimental animals as a result of repeated daily dosing with, or exposure to, a chemical for a short part of their expected lifespan

(vi) *Maximum Tolerated Dose (MTD)* is the highest dose level eliciting signs of toxicity in animals without having major effects on survival relative to the test in which it is used

(vii) *Skin irritation* is the production of reversible inflammatory changes in the skin following the application of a test substance

(viii) *Eye irritation* is the production of reversible changes in the eye following the application of a test substance to the anterior surface of the eye

(ix) *Skin sensitization* (allergic contact dermatitis) is an immunologically mediated cutaneous reaction to a substance

Specific definitions for inhalation toxicity

— an *aerosol* is defined as particles (solid and/or liquid) homogeneously dispersed in air

— the *aerodynamic diameter* is the diameter of a sphere of unit density (1 g cm^{-3}) having the same terminal settling velocity as the particle in question.

— the *mass median aerodynamic diameter* (MMAD) is the calculated aerodynamic diameter which divides the size distribution of the aerosol in half when measured by mass.

— the *geometric standard deviation* (GSD) is the ratio of the estimated 84 percentile to the 50 percentile and indicates the slope of the cumulative particle size distribution curve, assuming the size distribution to be log normal.

Specific definitions for the fixed dose procedure in the determination of acute oral toxicity

— *Evident toxicity* refers to toxic effects seen following administration of a test substance, which are of a severity such that administration at the next higher dose level could result in mortality.

— *Discriminating dose* is the highest out of the four fixed dose levels which can be administered without causing compound-related mortality (including humane kills).

C. MUTAGENICITY (including carcinogenicity pre-screening test)

For the preliminary assessment of mutagenic potential of a substance, it is necessary to obtain information on two categories of end point, namely, gene mutation and chromosomal abberrations.

These two end points are evaluated by the following tests:

(i) Tests on the production of gene (point) mutations in procaryotic cells such as *Salmonella typhimurium*; tests using *Escherichia coli* are also acceptable. The choice between these two test organisms may be determined by the nature of the chemical being tested.

(ii) Tests on the production of chromosomal aberrations in mammalian cells grown *in vitro*; an *in vivo* procedure (the micronucleus test or the metaphase analysis of bone marrow cells) is also acceptable. However, in the absence of any contraindications the *in vitro* methods are strongly to be preferred.

D. EVALUATION AND INTERPRETATION

There are limitations in the extent to which the results of animal and *in vitro* tests can be extrapolated directly to man and this must be borne in mind when tests are evaluated and interpreted.

Where available, evidence of adverse effects in humans may be of relevance in determining the potential effects of chemical substances on the human population.

E. LITERATURE REFERENCES

Toxicology is a developing experimental science and there is abundant literature for each topic. Relevant information can be found in the OECD Test Guidelines.

Additional remarks

Animal Care

Stringent control of environmental conditions and proper animal care techniques are essential in toxicity testing.

(i) Housing conditions

The environmental conditions in the experimental animal rooms or enclosures should be appropriate to the test species. For rats, mice and guinea pigs, suitable conditions are a room temperature of 22 ± 3 °C with a relative humidity of 30 to 70 %; for rabbits the temperature should be 20 ± 3 °C with a relative humidity of 30 to 70 %.

Some experimental techniques are particularly sensitive to temperature effects and, in these cases, details of appropriate conditions are included in the description of the test method. In all investigations of toxic effects, the temperature and humidity should be monitored, recorded, and included in the final report of the study.

When lighting is artificial, the sequence should normally be 12 hours light, 12 hours dark. Details of the lighting pattern should be recorded and included in the final report of the study.

In reports of animal experiments, it is important to indicate the type of caging used and the number of animals housed in each cage both during exposure to the chemical and any subsequent observation period.

(ii) Feeding conditions

Diets should meet all the nutritional requirements of the species under test. Where test substances are administered to animals in their diet the nutritional value may be reduced by interaction between the substance and a dietary constituent.

The possibility of such a reaction should be considered when interpreting the results of tests.

Dietary contaminants which are known to influence the toxicity should not be present in interfering concentrations.

Animal Welfare

When elaborating the test methods due consideration was given to animal welfare. Some examples are briefly given hereunder, this list is not exhaustive. The exact wording and/or conditions should be read in the text of the methods:

— For the determination of acute oral toxicity, an alternative method, the "Fixed Dose Procedure" is introduced. This procedure does not utilize death as specific endpoint. It uses fewer animals and results in less pain and distress than the classical determination of acute oral toxicity.

— The number of animals used is reduced to the scientifically acceptable minimum: only 5 animals of the same sex are tested per dose level for methods B.1 and B.3; only 10 animals (and only 5 for the negative control group) are used for the determination of the skin sensitisation by the guinea pig maximization test (method B.6); the number of animals needed for the positive control when testing mutagenicity *in vivo* is also lowered (methods B.11 and B.12)

— Pain and distress of animals during the tests are minimised: animals showing severe and enduring signs of distress and pain may need to be humanely killed; dosing test substances in a way known to cause marked pain and distress due to corrosive or irritating properties need not to be carried out (methods B.1, B.2 and B.3).

— Testing with irrelevantly high doses is avoided by the introduction of limit tests, not only in the acute toxicity tests (methods B.1, B.2 and B.3) but also in the *in vivo* tests for mutagenicity (methods B.11 and B.12).

— A strategy of testing for irritancy now allows the non-performance of a test, or its reduction to a single animal study, when sufficient scientific evidence can be provided.

Such scientific evidence can be based on the physico-chemical properties of the substance, the results of other tests already performed, or the results of well validated *in vitro* tests. For example, if an acute toxicity study by the dermal route has been conducted at the limit test dose with the substance (method B.3), and no skin irritation was observed, further testing for skin irritation (method B.4) may be unnecessary; materials which have demonstrated definite corrosion or severe skin irritancy in a dermal irritation study (method B.4) should not be further tested for eye irritancy (method B.5).

B.1 ACUTE TOXICITY (ORAL)

1. **METHOD**

1.1. INTRODUCTION

See General Introduction Part B (A).

1.2. DEFINITIONS

See General Introduction Part B (B).

1.3. REFERENCE SUBSTANCES

None.

1.4. PRINCIPLE OF THE TEST METHOD

The test substance is administered orally by gavage in graduated doses to several groups of experimental animals, one dose being used per group. The doses chosen may be based on the results of a range finding test. Subsequently, observations of effects and deaths are made. Animals which die during the test are necropsied and at the conclusion of the test surviving animals are necropsied. This method is directed primarily to studies in rodent species.

Animals showing severe and enduring signs of distress and pain may need to be humanely killed. Dosing test substances in a way known to cause marked pain and distress due to corrosive or irritating properties need not be carried out.

1.5. QUALITY CRITERIA

None.

1.6. DESCRIPTION OF THE TEST METHOD

1.6.1. **Preparations**

The animals are kept under the experimental housing and feeding conditions for at least five days prior to the test. Before the test, healthy young adult animals are randomized and assigned to the treatment groups. Where necessary, the test substance is dissolved or suspended in a suitable vehicle. It is recommended that wherever possible the use of an aqueous solution is considered first, followed by consideration of a solution in vegetable oil, then by possible solution in other vehicles, or in suspension. For non-aqueous vehicles the relevant toxic characteristics of the vehicle should be known or should be determined before or during the test. In rodents, normally the volume should not exceed 10 ml/kg body weight except in the case of aqueous solutions where 20 ml/kg may be used. Variability in test volume should be minimized by adjusting the concentration to ensure a constant volume at all dose levels.

1.6.2. **Test Conditions**

1.6.2.1. *Experimental Animals*

Unless there are contra-indications the rat is the preferred species.

Commonly used laboratory strains should be employed. For each sex, at the start of the test the range of weight variation in the animals used should not exceed ± 20 % of the appropriate mean value.

1.6.2.2. *Number and Sex*

At least five rodents are used at each dose level. They should all be of the same sex. If females are used, they should be nulliparous and non-pregnant. Where information is available demonstrating that a sex is markedly more sensitive, animals of this sex should be dosed.

Note: In acute toxicity tests with animals of a higher order than rodents, the use of smaller numbers should be considered.

Doses should be carefully selected, and every effort should be made not to exceed moderately toxic doses. In such tests administration of lethal doses of the test substance should be avoided.

1.6.2.3. *Dose Levels*

These should be sufficient in number, at least three, and spaced appropriately to produce test groups with a range of toxic effects and mortality rates. The data should be sufficient to produce a dose/response curve and, where possible, permit an acceptable determination of the LD_{50}.

1.6.2.4. *Limit Test*

When rodents are used, a limit test at one dose level of at least 2 000 mg/kg bodyweight may be carried out in a group of five males and five females using the procedures described above. If compound-related mortality is produced, a full study may need to be considered.

1.6.2.5. *Observation Period*

The observation period should be at least 14 days. However, the duration of observation should not be rigidly fixed. It should be determined by the toxic reactions, their rate of onset and the length of the recovery period; it may thus be extended when considered necessary. The time at which signs of toxicity appear and disappear and the time of death are important, especially if there is a tendency for deaths to be delayed.

1.6.3 **Procedure**

Animals should be fasted prior to substance administration. For the rat, food should be withheld overnight; for animals with higher metabolic rates a shorter period of fasting is appropriate; water is not restricted. The following day, the animals should be weighed and then the test substance administered by gavage in a single dose. If a single dose is not possible, the dose may be given in smaller fractions over a period not exceeding 24 hours. After the substance has been administered, food may be withheld for a further three to four hours. Where a dose is administered in fractions over a period it may be necessary to provide the animals with food and water depending on the length of the period.

Following administration, observations are made and recorded systematically, individual records should be maintained for each animal. Observations should be made frequently during the first day.

A careful clinical examination should be made at least once each working day, other observations should be made daily with appropriate actions taken to minimize loss of animals to the study, e.g. necropsy or refrigeration of those animals found dead and isolation or sacrifice of weak or moribund animals. Observations should include changes in the skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous systems, and somatomotor activity and behaviour pattern. Particular attention should be directed to obervation of tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma. The time of death should be recorded as precisely as possible.

Animals that die during the test and those surviving at the termination of the test are subjected to necropsy. All gross pathological changes should be recorded. Where indicated, tissues should be taken for histopathological examination.

Assessment of toxicity in the other sex

After completion of the study in one sex, at least one group of five animals of the other sex is dosed to establish that animals of this sex are not markedly more sensitive to the test substance. The use of fewer animals may be justified in individual circumstances. Where adequate information is available to demonstrate that animals of the sex tested are markedly more sensitive, testing in animals of the other sex may be dispensed with.

2. **DATA**

Data should be summarized in tabular form, showing for each test group the number of animals at the start of the test, time of death of individual animals, number of animals displaying other signs of toxicity, description of toxic effects and necropsy findings. Individual weights of animals should be determined and recorded shortly before the test substance is administered, weekly thereafter and at death. Changes in weight should be calculated and recorded when survival exceeds one day. Animals which are humanely killed due to compound-related distress and pain are recorded as compound-related deaths. The LD_{50} may be determined by a recognized method. Data evaluation should include the relationship, if any, between the animals' exposure to the test substance and the incidence and severity of all abnormalities, including behavioural and clinical abnormalities, gross lesions, body weight changes, mortality, and any other toxicological effects.

3. **REPORTING**

3.1. TEST REPORT

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

— species, strain, source, environmental conditions, diet, etc.,

— test conditions,

— dose levels (with vehicle, if used, and concentration),

— sex of animals dosed,

— tabulation of response data by sex and dose level (i.e. number of animals that died or were killed during the test, number of animals showing signs of toxicity, number of animals exposed),

— time of death after dosing, reasons and criteria used for humane killing of animals,

— all observations,

— LD_{50} value for the sex subjected to a full study determined at 14 days (with the method of determination specified),

— 95 % confidence interval for the LD_{50} (where this can be provided),

— dose/mortality curve and slope (where permitted by the method of determination),

— necropsy findings,

— any histopathological findings,

— results of any test on the other sex,

— discussion of the results (particular attention should be given to the effect that humane killing of animals during the test may have on the calculated LD_{50} value),

— interpretation of results.

3.2. EVALUATION AND INTERPRETATION

See General Introduction Part B (D).

4. **REFERENCES**

See General Introduction Part B (E).

B.1 bis ACUTE TOXICITY (ORAL) — FIXED DOSE METHOD

1. **METHOD**

1.1. INTRODUCTION

See General Introduction Part B (A).

1.2. DEFINITIONS

See General Introduction Part B (B).

1.3. REFERENCE SUBSTANCES

None.

1.4. PRINCIPLE OF THE TEST METHOD

The acute oral toxicity test provides information on the adverse effects which can follow, within a short period of time, the ingestion of a single dose of the test substance.

The fixed dose method is conducted in two stages.

In a preliminary sighting study, the effects of various doses administered orally by gavage to single animals of one sex are investigated in a sequential manner. The sighting study yields information on the dose-toxicity relationship, including an estimate of the minimum lethal dose. Normally, no more than five animals are used in this first stage.

In the main study, the substance is administered orally by gavage to groups of five male and five female animals at one of the pre-set dose levels (5, 50, 500 or 2 000 mg/kg). The dose used is derived from the sighting study and is that which is likely to produce "evident toxicity" (see 1.2. Definitions) but no deaths.

Following administration, observations for effects are made.

When the initial dose level chosen produces evident toxicity but no compound-related mortality, no further testing is needed.

Where evident toxicity is not seen at the chosen dose level, the substance should be re-tested at the next higher dose level. Where animals die, or where a severe toxic reaction requires humane killing of animals, the substance should be re-tested at the next lower dose level.

This procedure permits the identification of the "discriminating dose" (see 1.2. Definitions), that is the highest of the pre-set dose levels which can be administered without causing mortality (including humane kills).

Animals showing severe and enduring signs of distress and pain may need to be humanely killed. Dosing test substances in a way known to cause marked pain and distress due to corrosive or irritating properties need not be carried out.

1.5. QUALITY CRITERIA

None.

1.6. DESCRIPTION OF THE TEST METHOD

1.6.1. **Preparations**

1.6.1.1. *Experimental Animals*

Unless there are contra-indications the rat is the preferred species.

Commonly used laboratory strains should be employed. For each sex, at the start of the test, the range of weight variation in the animals used should not exceed ± 20 % of the appropriate mean value.

The animals are kept under the experimental housing and feeding conditions for at least five days prior to the test. Before the test, healthy young adult animals are randomized and assigned to the sighting study and main study treatment groups. In practice, only one group of each sex may be needed in the main study.

1.6.1.2. *Dose preparation and administration*

Where necessary, the test substance is dissolved or suspended in a suitable vehicle. It is recommended that wherever possible the use of an aqueous solution is considered first, followed by consideration of a solution in vegetable oil, then by possible solution in other vehicles, or in suspension. For non-aqueous vehicles the relevant toxic characteristics of the vehicle should be known or should be determined before or during the test. In rodents, normally the volume should not exceed 10 ml/kg body weight except in the case of aqueous solutions where 20 ml/kg may be used. Variability in test volume should be minimized by adjusting the concentration to ensure a constant volume at all dose levels.

Animals should be fasted prior to substance administration. For the rat, food should be withheld overnight; water is not restricted. The following day, the animals should be weighed and then the test substance administered by gavage in a single dose. If a single dose is not possible, the dose may be given in smaller fractions over a period not exceeding 24 hours. After the substance has been administered, food may be withheld for a further three to four hours. Where a dose is administered in fractions over a period it may be necessary to provide the animals with food and water depending on the length of the period.

1.6.2. **Procedure**

1.6.2.1. *Sighting study*

The effects of various doses are investigated in single animals. Normally female animals will be used in the absence of information indicating that males will be the more sensitive sex. Dosing is sequential, allowing at least 24 hours before dosing the next animal. All animals are carefully observed for signs of toxicity for at least seven days; if signs of moderate toxicity persist at seven days, the animal should be observed for up to an additional seven days. The following initial dose levels are considered: 5, 50, 500 and 2 000 mg/kg. If the initial dose chosen does not produce severe toxicity, and the next higher level produces mortality, then it will be necessary to investigate one or more intermediate dose levels as appropriate. In this way it should be possible to build up information on the dose level(s) that produce(s) some signs of toxicity and the minimum dose level that produces mortality.

An effort should be made to select the initial dose using evidence from related chemicals. In the absence of such information, it is suggested that the 500 mg/kg dose is used in the first instance. If no signs of toxicity are seen at the initial dose, then the next higher dose level is investigated. If no mortality occurs at 2 000 mg/kg, the sighting test is complete and the main study should be conducted at this dose level. If severe effects, necessitating humane killing are seen at the initial dose (e.g. 500 mg/kg), the next lower dose (e.g. 50 mg/kg) is given to another animal. If this animal survives, further animals may then be dosed with the appropriate intermediate dose levels between the fixed doses. Normally, one would not expect to use more than five animals in this procedure.

1.6.2.2. *Main study*

At least 10 animals (five female and five male) should be used for each dose level which is investigated. The females should be nulliparous and non-pregnant.

It is a principle of the fixed dose method that only moderately toxic doses are used in the main study. Administration of lethal doses of the test substance should be avoided.

The dose level to be used in the test should be selected from one of the four fixed dose levels, namely 5, 50, 500 or 2 000 mg/kg body weight. The initial dose level chosen should be that which is likely to produce evident toxicity but no compound-related mortality (including humane kills; accidental deaths are not included but should be recorded). No further testing is necessary when this dose level produces evident toxicity but no compound-related mortality.

Where evident toxicity does not result from administration of the chosen dose level, the substance should be re-tested at the next higher dose level. The animals, however, should continue to be kept under observation until the observation period is complete. Where a severe toxic reaction requires animals to be humanely killed or there is compound-related mortality, the substance should be retested at the next lower dose level. Again, animals that do not need to be humanely killed should be kept under observation for the full observation period.

Following administration, observations are made and recorded systematically. Individual records should be maintained for each animal.

The observation period should be at least 14 days. However, the duration of observation should not be rigidly fixed. It should be determined by the toxic reactions, their rate of onset and the length of the recovery period; it may thus be extended when considered necessary. The time at which signs of toxicity appear and disappear and the time of death are important, especially if there is a tendency for toxic signs to be delayed.

A careful clinical examination should be made at least twice on the day of dosing and at least once each day thereafter. Animals obviously in pain or showing severe signs of distress should be humanely killed. Additional observations will be necessary during the first few days after dosing if the animals continue to display signs of toxicity. The test may be terminated if it becomes apparent that the initial dose level chosen was too high.

Observations should include changes in the skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous systems, and somatomotor activity and behaviour pattern. Particular attention should be directed to observation of tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma.

Individual weights of animals should be determined shortly before the test substance is administered, daily for the next three days, and weekly thereafter. Animals that die during the test, and those surviving to termination of the test, are weighed and subjected to necropsy. All gross pathological changes should be recorded. Where indicated, tissues should be taken for histopathological examination.

The investigation of a second or, in exceptional circumstances, a third dose level may be required, dependent upon the results of the preceding dose level.

In the case in which a substance produces mortality at 5 mg/kg body weight (or where the sighting study indicates that mortality will result at that dose level) the acute toxicity of the substance may need to be further investigated.

2. DATA

Data from both the sighting study and the main study should be summarized in tabular form showing for each dose level tested the number of animals at the start of the test; the number of animals displaying signs of toxicity; the number of animals found dead during the test or killed for humane reasons; a description of the toxic effects and, for the main study, whether compound-related evident toxicity was observed; the time course of any toxic effects; and the necropsy findings. Changes in weight should be calculated and recorded when survival exceeds one day.

Animals which are humanely killed due to compound-related distress and pain are recorded as compound-related deaths.

3. REPORTING

3.1. TEST REPORT

The test report shall, if possible, include the following information, for both the sighting study and the main study, as appropriate:

— species, strain, source, environmental conditions, diet, etc.

— test conditions

— dose levels (with vehicle, if used, and concentration)

— full results of all dose levels investigated

— tabulation of response data by sex and dose level (i.e. number of animals used; changes in body weight; when applicable, number of animals that died or were killed during the test; number of animals showing signs of toxicity; nature, severity and duration of effects)

— time course of onset of signs of toxicity and whether these were reversible

— when animals died or were killed, time of death after dosing, reasons and criteria used for humane killing of animals

— necropsy findings

— any histopathological findings

— discussion of the results

— interpretation of results, including the signs of evident toxicity and the discriminating dose level identified in the test.

3.2. EVALUATION AND INTERPRETATION

DOSE	RESULTS	INTERPRETATION
5 mg/kg b.w.	Less than 100 % survival	Compounds which are VERY TOXIC.
	100 % survival; but evident toxicity	Compounds which are TOXIC.
	100 % survival; no evident toxicity	See results at 50 mg/kg.
50 mg/kg b.w.	Less than 100 % survival	Compounds which may be TOXIC or VERY TOXIC. See results at 5 mg/kg.
	100 % survival; but evident toxicity	Compounds which are HARMFUL.
	100 % survival; no evident toxicity	See results at 500 mg/kg.
500 mg/kg b.w.	Less than 100 % survival	Compounds which may be TOXIC or HARMFUL. See results at 50 mg/kg.
	100 % survival; but evident toxicity	Compounds considered as having no significant acute toxicity.
	100 % survival; no evident toxicity	See results at 2 000 mg/kg.
2 000 mg/kg b.w.	Less than 100 % survival	See results at 500 mg/kg.
	100 % survival; with or without evident toxicity	Compounds which do not have significant acute toxicity.

See also General Introduction Part B (D).

4. **REFERENCES**

See General Introduction Part B (E).

B.2. ACUTE TOXICITY (INHALATION)

1. **METHOD**

1.1. INTRODUCTION

It is useful to have preliminary information on the particle size distribution, the vapour pressure, the melting point, the boiling point, the flash point and explosivity (if applicable) of the substance.

See also General Introduction Part B (A).

1.2. DEFINITIONS

See General Introduction Part B (B).

1.3. REFERENCE SUBSTANCES

None.

1.4. PRINCIPLE OF THE TEST METHOD

Several groups of experimental animals are exposed for a defined period to the test substance in graduated concentrations, one concentration being used per group. Subsequently observations of effects and deaths are made. Animals which die during the test are necropsied and at the conclusion of the test surviving animals are necropsied.

Animals showing severe and enduring signs of distress and pain may need to be humanely killed. Dosing test substances in a way known to cause marked pain and distress due to corrosive or severe irritating properties need not be carried out.

1.5. QUALITY CRITERIA

None.

1.6. DESCRIPTION OF THE TEST METHOD

1.6.1. **Preparations**

The animals are kept under the experimental housing and feeding conditions for at least five days prior to the experiment. Before the test healthy young animals are randomized and assigned to the required number of groups. They need not be subjected to simulated exposure unless this is indicated by the type of exposure apparatus being used.

Solid test substances may need to be micronised in order to achieve particles of an appropriate size.

Where necessary a suitable vehicle may be added to the test substance to help generate an appropriate concentration of the test substance in the atmosphere and a vehicle control group should then be used. If a vehicle or other additives are used to facilitate dosing, they should be known not to produce toxic effects. Historical data can be used if appropriate.

1.6.2. **Test Conditions**

1.6.2.1. *Experimental Animals*

Unless there are contra-indications the rat is the preferred species. Commonly used laboratory strains should be employed. For each sex, at the start of the test the range of weight variation in the animals used should not exceed ± 20 % of the appropriate mean value.

1.6.2.2. *Number and Sex*

At least 10 rodents (five female and five male) are used at each concentration level. The females should be nulliparous and non-pregnant.

Note:

In acute toxicity tests with animals of a higher order than rodents, the use of smaller numbers should be considered. Doses should be carefully selected, and every effort should be made not to exceed moderately toxic doses. In such tests administration of lethal doses of the test substance should be avoided.

1.6.2.3. *Exposure Concentrations*

These should be sufficient in number, at least three, and spaced appropriately to produce test groups with a range of toxic effects and mortality rates. The data should be sufficient to produce a concentration mortality curve and, where possible, permit an acceptable determination of an LC_{50}.

1.6.2.4. *Limit Test*

If an exposure of five male and five female test animals to 5 mg per litre of a gas, or aerosol of liquid or solid substance (or, where this 2s not possible due to the physical or chemical, including explosive, properties of the test substance, the maximum attainable concentration) produces, after a four-hour exposure, no compound-related mortality within 14 days, further testing may not be considered necessary.

1.6.2.5. *Exposure Time*

The period of exposure should be four hours.

1.6.2.6. *Equipment*

The animals should be tested with inhalation equipment designed to sustain a dynamic airflow of at least 12 air changes per hour, to ensure an adequate oxygen content and an evenly distributed exposure atmosphere. Where a chamber is used its design should minimize crowding of the test animals and maximize their exposure by inhalation to the test substance. As a general rule to ensure stability of a chamber atmosphere the total "volume" of the test animals should not exceed 5 % of the volume of the test chamber. Oro-nasal, head only, or whole body individual chamber exposure may be used; the first two will help to minimize the uptake of the test substance by other routes.

1.6.2.7. *Observation Period*

The observation period should be at least 14 days. However, the duration of observations should not be rigidly fixed. It should be determined by the toxic reactions, their rate of onset and the length of the recovery period; it may thus be extended when considered necessary. The time at which signs of toxicity appear and disappear and the time of death are important, especially if there is a tendency for deaths to be delayed.

1.6.3. **Procedure**

Shortly before exposure, the animals are weighed, and then exposed to the test concentration in the designated apparatus for a period of four hours, after equilibration of the chamber concentration. Time for equilibration should be short. The temperature at which the test is performed should be maintained at 22 ± 3 °C. Ideally the relative humidity should be maintained between 30 % and 70 %, but in certain instances (e.g. tests of some aerosols) this may not be practicable. Maintenance of a slight negative pressure inside the chamber (≥ 5 mm of water) will prevent leakage of the test substance into the surrounding area. Food and water should be withheld during exposure. Suitable systems for the generation and monitoring of the test atmosphere should be used. The system should ensure that stable exposure conditions are achieved as rapidly as possible. The chamber should be designed and operated in such a way that a homogeneous distribution of the test atmosphere within the chamber is maintained.

Measurements or monitoring should be made:

— of the rate of air flow (continuously).

— of the actual concentration of the test substance measured in the breathing zone at least three times during exposure (some atmospheres, e.g. aerosols at high concentrations, may need more frequent monitoring). During the exposure period the concentration should not vary by more than ± 15 % of the mean value. However in the case of some aerosols, this level of control may not be achievable and a wider range would then be acceptable. For aerosols, particle size analysis should be performed as often as necessary (at least once per test group).

— of temperature and humidity, continuously if possible.

During and following exposure, observations are made and recorded systematically; individual records should be maintained for each animal. Observations should be made frequently during the first day. A careful clinical examination should be made at least once each working day, other observations should be made daily with appropriate actions taken to minimize loss of animals from the study, e.g. necropsy or refrigeration of those animals found dead and isolation or sacrifice of weak or moribund animals.

Observations should include changes in the skin and fur, eyes, mucous membranes, respiratory, circulatory, autonomic and central nervous systems, and somatomotor activity and behaviour pattern. Particular attention should be directed to observation of respiratory behaviour, tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma. The time of death should be recorded as precisely as possible. Individual weights of animals should be determined weekly after exposure, and at death.

Animals that die during the test and those surviving at the termination of the test are subjected to necropsy with particular reference to any changes in the upper and lower respiratory tract. All gross pathological changes should be recorded. Where indicated, tissues should be taken for histopathological examination.

2. **DATA**

Data should be summarized in tabular form showing for each test group the number of animals at the start of the test, time of death of individual animals, number of animals displaying other signs of toxicity, description of toxic effects and necropsy findings. Changes in weight must be calculated and recorded when survival exceeds one day. Animals which are humanely killed due to compound-related distress and pain are recorded as compound-related deaths. The LC_{50} should be determined by a recognized method. Data evaluation should include the relationship, if any, between the animal's exposure to the test substance and the incidence and severity of all abnormalities, including behavioural and clinical abnormalities, gross lesions, body weight changes, mortality and any other toxic effects.

3. **REPORTING**

3.1. TEST REPORT

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

— species, strain, source, environmental conditions, diet etc.;

— test conditions: description of exposure apparatus

including design, type, dimensions, source of air, system for generating aerosols, method of conditioning air and the method of housing animals in a test chamber when this is used. The equipment for measuring temperature, humidity, and aerosol concentrations and particle size distribution should be described.

Exposure data

These should be tabulated and presented with mean values and a measure of variability (e.g. standard deviation) and shall, if possible, include:

(a) airflow rates through the inhalation equipment;

(b) temperature and humidity of the air;

(c) nominal concentrations (total amount of test substance fed into the inhalation equipment divided by volume of air);

(d) nature of vehicle, if used;

(e) actual concentrations in test breathing zone;

(f) The mass median aerodynamic diameter (MMAD) and the geometric standard deviation (GSD);

(g) equilibration period;

(h) exposure period;

— tabulation of response data by sex and exposure level (i.e. number of animals that died or were killed during the test; number of animals showing signs of toxicity; number of animals exposed);

— time of death during or following exposure, reasons and criteria used for humane killing of animals;

— all observations;

— LC_{50} value for each sex determined at the end of the observation period (with method of calculation specified);

— 95 % confidence interval for the LC_{50} (where this can be provided);

— dose/mortality curve and slope (where permitted by the method of determination);

— necropsy findings;

— any histopathological findings;

— discussions of the results (particular attention should be given to the effect that humane killing of animals during the test may have on the calculated LC_{50} value);

— interpretation of the results.

3.2. EVALUATION AND INTERPRETATION

See General Introduction Part B (D).

4. **REFERENCES**

See General Introduction Part B (E).

B.3. ACUTE TOXICITY (DERMAL)

1. **METHOD**

1.1. INTRODUCTION

See General Introduction Part B (A).

1.2. DEFINITION

See General Introduction Part B (B).

1.3. REFERENCE SUBSTANCES

None.

1.4. PRINCIPLE OF THE TEST METHOD

The test substance is applied to the skin in graduated doses to several groups of experimental animals, one dose being used per group. Subsequently, observations of effects and deaths are made. Animals which die during the test are necropsied and at the conclusion of the test surviving animals are necropsied.

Animals showing severe and enduring signs of distress and pain may need to be humanely killed. Dosing test substances in a way known to cause marked pain and distress due to corrosive or irritating properties need not be carried out.

1.5. QUALITY CRITERIA

None.

1.6. DESCRIPTION OF THE TEST METHOD

1.6.1. **Preparations**

The animals are kept in their experimental cages under the experimental housing and feeding conditions for at least five days prior to the experiment. Before the test, healthy young adult animals are randomized and assigned to the treatment groups. Approximately 24 hours before the test, fur should be removed by clipping or shaving from the dorsal area of the trunk of the animals. When clipping or shaving the fur, care must be taken to avoid abrading the skin which could alter its permeability. Not less than 10 % of the body surface should be clear for the application of the test substance. When testing solids, which may be pulverized if appropriate, the test substance should be moistened sufficiently with water or, where necessary, a suitable vehicle to ensure good contact with the skin. When a vehicle is used, the influence of the vehicle on penetration of skin by the test substance should be taken into account. Liquid test substances are generally used undiluted.

1.6.2. **Test Conditions**

1.6.2.1. *Experimental Animals*

The adult rat or rabbit may be used. Other species may be used but their use would require justification. Commonly used laboratory strains should be employed. For each sex, at the start of the test the range of weight variation in the animals used should not exceed ± 20 % of the appropriate mean value.

1.6.2.2. *Number and Sex*

At least 5 animals are used at each dose level. They should all be of the same sex. If females are used, they should be nulliparous and non-pregnant. Where information is available demonstrating that a sex is markedly more sensitive, animals of this sex should be dosed.

Note: In acute toxicity tests with animals of a higher order than rodents, the use of smaller numbers should be considered. Doses should be carefully selected, and every effort should be made not to exceed moderately toxic doses. In such tests, administration of lethal doses of the test substance should be avoided.

1.6.2.3. *Dose Levels*

These should be sufficient in number, at least three, and spaced appropriately to produce test groups with a range of toxic effects and mortality rates. Any irritant or corrosive effects should be taken into account when deciding on dose levels. The data should be sufficient to produce a dose/response curve and, where possible, permit an acceptable determination of the LD_{50}.

1.6.2.4. *Limit Test*

A limit test at one dose level of at least 2 000 mg/kg bodyweight may be carried out in a group of 5 male and 5 female animals, using the procedures described above. If compound-related mortality is produced, a full study may need to be considered.

1.6.2.5. *Observation Period*

The observation period should be at least 14 days. However, the duration of observation should not be rigidly fixed. It should be determined by the toxic reactions, their rate of onset and the length of the recovery period; it may thus be extended when considered necessary. The time at which signs of toxicity appear and disappear, their duration and the time of death are important, especially if there is a tendency for deaths to be delayed.

1.6.3. **Procedure**

Animals should be caged individually. The test substance should be applied uniformly over an area which is approximately 10 % of the total body surface area. With highly toxic substances the surface area covered may be less but as much of the area should be covered with a layer as thin and uniform as possible.

Test substances should be held in contact with the skin with a porous gauze dressing and non-irritating tape throughout a 24-hour exposure period. The test site should be further covered in a suitable manner to retain the gauze dressing and test substance and ensure that the animals cannot ingest the test substance. Restrainers may be used to prevent the ingestion of the test substance but complete immobilisation is not a recommended method.

At the end of the exposure period, residual test substance should be removed, where practicable, using water or some other appropriate method of cleansing the skin.

Observations should be recorded systematically as they are made. Individual records should be maintained for each animal. Observations should be made frequently during the first day. A careful clinical examination should be made at least once each working day, other observations should be made daily with appropriate actions taken to minimize loss of animals to the study, e.g. necropsy or refrigeration of those animals found dead and isolation or sacrifice of weak or moribund animals.

Observations should include changes in fur, treated skin, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous systems, and somatomotor activity and behaviour pattern. Particular attention should be directed to observations of tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma. The time of death must be recorded as precisely as possible. Animals that die during the test and those surviving at the termination of the test are subjected to necropsy. All gross pathological changes should be recorded. Where indicated, tissues should be taken for histopathological examination.

Assessment of toxicity in the other sex

After completion of the study in one sex, at least one group of 5 animals of the other sex is dosed to establish that animals of this sex are not markedly more sensitive to the test substance. The use of fewer animals may be justified in individual circumstances. Where adequate information is available to demonstrate that animals of the sex tested are markedly more sensitive, testing in animals of the other sex may be dispensed with.

2. **DATA**

Data should be summarized in tabular form, showing for each test group the number of animals at the start of the test, time of death of individual animals, number of animals displaying other signs of toxicity, description of toxic effects and necropsy findings. Individual weights of animals should be determined and recorded shortly before the test substance is applied, weekly thereafter, and at death; changes in weight should be calculated and recorded when survival exceeds one day. Animals which are humanely killed due to compound-related distress and pain are recorded as compound-related deaths. The LD_{50} should be determined by a recognized method.

Data evaluation should include an evaluation of relationships, if any, between the animal's exposure to the test substance and the incidence and severity of all abnormalities, including behavioural and clinical abnormalities, gross lesions, body weight changes, mortality, and any other toxicological effects.

3. **REPORTING**

3.1. TEST REPORT

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

— species, strain, source, environmental conditions, diet, etc.;

— test conditions (including method of skin cleansing and type of dressing: occlusive or not occlusive);

— dose levels (with vehicle, if used, and concentrations),

— sex of animals dosed;

— tabulation of response data by sex and dose level (i.e. number of animals that died or were killed during the test; number of animals showing signs of toxicity; number of animals exposed);

— time of death after dosing, reasons and criteria used for humane killing of animals;

— all observations;

— LD_{50} value for the sex subjected to a full study, determined at 14 days with the method of determination specified;

— 95 % confidence interval for the LD_{50} (where this can be provided);

— dose/mortality curve and slope where permitted by the method of determination;

— necropsy findings;

— any histopathological findings;

— results of any test on the other sex;

— discussion of results (particular attention should be given to the effect that humane killing of animals during the test may have on the calculated LD_{50} value);

— interpretation of the results.

3.2. EVALUATION AND INTERPRETATION

See General Introduction Part B (D).

4. **REFERENCES**

See General Introduction Part B (E).

B.4. ACUTE TOXICITY (SKIN IRRITATION)

1. **METHOD**

1.1. INTRODUCTION

See General Introduction Part B (A).

1.2. DEFINITION

See General Introduction Part B (B).

1.3. REFERENCE SUBSTANCES

None.

1.4. PRINCIPLE OF THE TEST METHOD

Initial considerations

Careful consideration needs to be given to all the available information on a substance to minimize the testing of substances under conditions that are likely to produce severe reactions. The following information may be useful when considering whether a complete test, a single-animal study, or no further testing is appropriate.

i) Physicochemical properties and chemical reactivity. Strongly acidic or alkaline substances (demonstrated pH of 2 or less or 11,5 or greater, for example) may not require testing for primary dermal irritation if corrosive properties can be expected. Alkaline or acidic reserve should also be taken into account.

ii) If convincing evidence of severe effects in well validated *in vitro* tests is available, a complete test may not be required.

iii) Results from acute toxicity studies. If an acute toxicity test by the dermal route has been conducted with the substance at the limit test dose level (2 000 mg/kg body weight), and no skin irritation was observed, further testing for skin irritation may be unnecessary. In addition, testing of materials which have been shown to be highly toxic by the dermal route is unnecessary.

The substance to be tested is applied in a single dose to the skin of several experimental animals, each animal serving as its own control. The degree of irritation is read and graded after a specific interval, and is further described to provide a complete evaluation of the effects. The duration of the observations should be sufficient to evaluate fully the reversibility of the effects observed.

Animals showing severe and enduring signs of distress and pain may need to be humanely killed.

1.5. QUALITY CRITERIA

None.

1.6. DESCRIPTION OF THE TEST METHOD

1.6.1. **Preparations**

Approximately 24 hours before testing, fur should be removed, by clipping or shaving, from the dorsal area of the trunk of the animal.

When clipping or shaving the fur, care should be taken to avoid abrading the skin. Only animals with healthy intact skin should be used.

Some strains of rabbit have dense islets of hair which are more prominent at certain times of the year. Test substances should not be applied to these zones of dense hair growth.

When testing solids (which may be pulverized if considered necessary) the test substance should be moistened sufficiently with water or, where necessary, a suitable vehicle, to ensure good contact with the skin. When vehicles are used, the influence of the vehicle on irritation of skin by the test substance should be taken into account. Liquid test substances are generally used undiluted.

1.6.2. **Test Conditions**

1.6.2.1. *Experimental Animals*

Although several mammalian species may be used, the albino rabbit is the preferred species.

1.6.2.2. *Number of Animals*

If it is suspected from *in vitro* screening results or other considerations that the substance might produce necrosis (i.e. be corrosive) a single-animal test should be considered. If the results of this test do not indicate corrosivity, the test should be completed using at least two additional animals.

For the complete test, at least three healthy adult animals are used. Separate animals are not required for an untreated control group. Additional animals may be required to clarify equivocal responses.

1.6.2.3. *Dose Level*

Unless there are contra-indications 0,5 ml of liquid or 0,5 g of solid or semi-solid is applied to the test site. Adjacent areas of untreated skin of each animal serve as controls for the test.

1.6.2.4. *Observation Period*

The duration of the observation period should not be fixed rigidly. It should be sufficient to evaluate fully the reversibility or irreversibility of the effects observed, but need not normally exceed 14 days after application.

1.6.3. **Procedure**

Animals should be caged individually. The test substance should be applied to a small area (approximately 6 cm^2) of skin and covered with a gauze patch, which is held in place with non-irritating tape. In the case of liquids or some pastes it may be necessary to apply the test substance to the gauze patch and then apply that to the skin. The patch should be loosely held in contact with the skin by means of a suitable occlusive or semi-occlusive dressing for the duration of the exposure period. Access by the animal to the patch and resultant ingestion/inhalation of the test substance should be prevented.

At the end of the exposure period, residual test substance should be removed, where practicable, using water or an appropriate solvent, without altering the existing response or the integrity of the epidermis.

Exposure duration normally is four hours.

If it is suspected that the substance might produce necrosis (i.e. be corrosive), the duration of exposure should be reduced (e.g. to one hour or three minutes). Such testing may also employ a single animal in the first instance and, if not precluded by the acute dermal toxicity of the test compound, three patches may be applied simultaneously to this animal. The first patch is removed after three minutes. If no serious skin reaction is observed, the second patch is removed after one hour. If the observations at this stage indicate that a four-hour exposure is necessary and can be humanely conducted, the third patch is removed after four hours and the responses are graded. In this case (i.e. when a four-hour exposure has been possible), the test should then be completed using at least two additional animals, unless it is not considered humane to do so (e.g. if necrosis is observed following the four hour exposure).

If a serious skin reaction (e.g. necrosis) is observed at either three minutes or one hour, the test is immediately terminated.

Longer exposures may be indicated under certain conditions, e.g. expected pattern of human use and exposure.

1.6.3.1. *Observation and Grading*

Animals should be observed for signs of erythema and oedema and the response graded at 60 minutes, and then at 24, 48 and 72 hours after patch removal. Dermal irritation is graded and recorded according to the system in table 1. Further observations may be needed if reversibility has not been fully established within 72 hours. In addition to the observation of irritation, any serious lesions such as corrosion (irreversible destruction of skin tissue) and other toxic effects should be fully described.

Techniques such as histopathological examination or measurement of skin-fold thickness may be used to clarify doubtful reactions or responses masked by staining of the skin by test substance.

2. **DATA**

Data should be summarized in tabular form, showing for each individual animal the irritation gradings for erythema and oedema throughout the observation period. Any serious lesions, a description of the degree and nature of irritation, reversibility or corrosion and any other toxic effect observed should be recorded.

3. **REPORTING**

3.1. TEST REPORT

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

— species, strain, source, environmental conditions, diet, etc.;

— test conditions (including the relevant physicochemical properties of the chemical, the technique of skin preparation and cleansing, and the type of dressing: occlusive or semi-occlusive);

— tabulation of irritation response data for each individual animal for each observation time period (e.g. 1, 24, 48 and 72 hours, etc., after patch removal);

— description of any serious lesions observed, including corrosivity;

— description of the degree and nature of irritation observed and any histopathological findings;

— description of any toxic effects other than dermal irritation;

— discussion of the results;

— interpretation of the results.

3.2. EVALUATION AND INTERPRETATION

See General Introduction Part B (D).

4. **REFERENCES**

See General Introduction Part B (E).

Appendix

TABLE: GRADING OF SKIN REACTION

Erythema and Eschar Formation

	Value
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness) or eschar formation (injuries in depth) preventing erythema reading	4

Oedema Formation

No oedema	0
Very slight oedema (barely perceptible)	1
Slight oedema (edges of area well defined by definite raising)	2
Moderate oedema (edges raised approximately 1 mm)	3
Severe oedema (raised more than 1 mm and extending beyond the area of exposure)	4

B.5. ACUTE TOXICITY (EYE IRRITATION)

1. **METHOD**

1.1. INTRODUCTION

See General Introduction Part B (A).

1.2. DEFINITION

See General Introduction Part B (B).

1.3. REFERENCE SUBSTANCES

None.

1.4. PRINCIPLE OF THE TEST METHOD

Initial Considerations

Careful consideration needs to be given to all the available information on a substance to minimize the testing of substances under conditions that are likely to produce severe reactions. The following information may be useful in this regard.

i) Physicochemical properties and chemical reactivity. Strongly acidic or alkaline substances which, for example, can be expected to result in a pH in the eye of 2 or less, or 11,5 or greater, may not require testing if severe lesions can be expected. Alkaline or acidic reserve should also be taken into account.

ii) Results from well-validated alternative studies; materials which have been shown to have potential corrosive or severe irritant properties should not be further tested for eye irritation, it being presumed that such substances will produce severe effects on the eyes in a test using this method.

iii) Results from skin irritation studies. Materials which have demonstrated definite corrosive or severe skin irritancy in a dermal irritation study should not be further tested for eye irritancy, it being presumed that such substances might produce severe effects on the eyes.

The substance to be tested is applied in a single dose to one of the eyes in each of several experimental animals; the untreated eye is used to provide control information. The degree of irritation is evaluated and graded at specific intervals and is further described to provide a complete evaluation of the effects. The duration of the observations should be sufficient to evaluate fully reversibility or irreversibility of the effects observed.

Animals showing severe and enduring signs of distress and pain may need to be humanely killed.

1.5. QUALITY CRITERIA

None.

1.6. DESCRIPTION OF THE TEST METHOD

1.6.1. **Preparations**

Both eyes of each experimental animal provisionally selected for testing should be examined within 24 hours before testing starts. Animals showing eye irritation, ocular defects or pre-existing corneal injury should be not used.

1.6.2. **Test Conditions**

1.6.2.1. *Experimental Animals*

Although a variety of experimental animals have been used it is recommended that testing be performed using healthy adult albino rabbits.

1.6.2.2. *Number of Animals*

A single-animal test should be considered if marked effects are anticipated. If the results of this test in one rabbit suggest the substance to be severely irritant (reversible effect) or corrosive (irreversible effect) to the eye using the procedure described, further testing for ocular irritancy in subsequent animals may not need to be carried out. Occasionally, further testing in additional animals may be appropriate to investigate specific aspects.

In cases other than a single-animal test at least 3 animals should be used. Additional animals may be required to clarify equivocal responses.

1.6.2.3. *Dose Level*

For testing liquids, a dose of 0,1 ml is used. In testing solids, pastes, and particulate substances, the amount used should have a volume of 0,1 ml, or weigh approximately 0,1 g (the weight must always be recorded). If the test material is solid or granular it should be ground to a fine dust. The volume of particulates should be measured after gently compacting them, e.g. by tapping the measuring container.

For substances contained in pump sprays or pressurized aerosol containers the liquid should be expelled and 0,1 ml collected and instilled into the eye as described for liquids.

1.6.2.4. *Observation Period*

The duration of the observation period should not be rigidly fixed. It should be sufficient to evaluate the reversibility or irreversibility of the effects observed, but normally need not exceed 21 days after instillation.

1.6.3. **Procedure**

Animals should be caged individually. The test substance should be placed in the conjunctival sac of one eye of each animal after gently pulling the lower lid away from the eyeball. The lids are then gently held together for about one second to prevent loss of the material. The other eye, which remains untreated, serves as a control.

If it is thought that the substance could cause unreasonable pain, a local anaesthetic may be used prior to instillation of the test substance. The type, concentration, and the time of application of the local anaesthetic should be carefully selected to ensure that no significant differences in reaction to the test substance will result from its use. The control eye should be similarly anaesthetized.

The eyes of the test animals should not be washed out for 24 hours following instillation of the test substance. At 24 hours a washout may be used if considered appropriate.

For some substances shown to be irritating by this test, additional tests using rabbits with eyes washed soon after instillation of the substance may be indicated. In these cases it is recommended that three rabbits be used. Half a minute after instillation the eyes of the rabbits are washed for half a minute using a volume and velocity of flow which will not cause injury.

1.6.3.1. *Observation and Grading*

The eyes should be examined at 1, 24, 48 and 72 hours. If there is no evidence of ocular lesions at 72 hours the study may be ended.

Extended observation may be necessary if there is persistent corneal involvement or other ocular irritation in order to determine the progress of the lesions and their reversibility or irreversibility. In addition to the observations of the cornea, iris and conjunctiva, any other lesions which are noted should be recorded and reported. The grades of ocular reaction (table) should be recorded at each examination. (The grading of ocular responses is subject to various interpretations. To assist testing laboratories and those involved in making and interpreting the observations an illustrated guide to eye irritation may be used.)

Examination of reactions can be facilitated by use of a binocular loupe, hand slit-lamp, biomicroscope, or other suitable device. After recording the observations at 24 hours, the eyes of any or all rabbits may be further examined with the aid of fluorescein.

2. **DATA**

Data should be summarized in tabular form, showing for each individual animal the irritation grades at the designated observation time. A description of the degree and nature of irritation, the presence of serious lesions and any effects other than ocular which were observed, shall be reported.

3. **REPORTING**

3.1. TEST REPORT

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

— animal data (species, strain, source, environmental conditions, diet, etc.);

— test conditions (including relevant physicochemical properties of the test substance);

— tabulation of irritant/corrosive response data for each individual animal at each observation time point (e.g. 1, 24, 48 and 72 hours);

— description of any serious lesions observed;

— narrative description of the degree and nature of irritation or corrosion observed, including the area of the cornea involved, and the reversibility;

— description of the method used to grade the irritation at 1, 24, 48 and 72 hours (e.g. hand slit-lamp, biomicroscope, fluorescein);

— description of any non-ocular topical effects noted;

— discussion of the results;

— interpretation of the results.

3.2. EVALUATION AND INTERPRETATION

See General Introduction Part B (D).

4. **REFERENCES**

See General Introduction Part B (E).

Appendix

GRADING OF OCULAR LESIONS

Cornea

Opacity: degree of density (area most dense taken for reading)

No ulceration or opacity	0
Scattered or diffuse areas of opacity (other than slight dulling of normal lustre), details of iris clearly visible	1
Easily discernible translucent area, details of iris slightly obscured	2
Nacreous area, no details of iris visible, size of pupil barely discernible	3
Opaque cornea, iris not discernible through the opacity	4

Iris

Normal	0
Markedly deepened rugae, congestion, swelling, moderatecircumcorneal hyperaemia, or injection, any of these or combination of any thereof, iris still reacting to light (sluggish reaction is positive)	1
No reaction to light, haemorrhage, gross destruction (any or all of these)	2

Conjunctivae

Redness (refers to the most severe reading of palpebral and bulbar conjunctivae as compared to the control eye)

Blood vessels normal	0
Some blood vessels definitely hyperaemic (injected)	1
Diffuse, crimson colour, individual vessels not easily discernible	2
Diffuse beefy red	3

Chemosis: lids and/or nictitating membranes

No swelling	0
Any swelling above normal (including nictitating membranes)	1
Obvious swelling with partial eversion of lids	2
Swelling with lids about half closed	3
Swelling with lids more than half closed	4

B.6. SENSITIZZAZZJONI TAL-ĠILDA

1. **METODU**

1.1. INTRODUZZJONI

Rimarki:

Is-sensittività u l-abbiltà tat-testijiet li jintebħu b'sensitizzaturu potenzali għall-ġilda umana huma meqjusa bħala importanti fis-sistema tal-klassifikazzjoni dwar it-tossiċità relevanti għas-saħħa umana.

Ma hemm l-ebda metodu wieħed tat-test li b'mod edekwat jidentifika s-sustanzi kollha bil-potenzal għas-sensitizzazzjoni tal-ġilda umana u li huwa relevanti għas-sustanzi kollha.

Fatturi bħal ma huma l-karatteristċi fiżiċi ta' sustanza, inkluża l-abbiltà tagħha li tippenetra l-ġilda, għandhom ikunu meqjusa fil-għażla tat-test.

It-testijiet bl-użu tal-fniek ta' l-indi jistgħu ikunu sud-diviżi f'testijiet tat-tip adġuvanti, li fihom l-istat ellerġiku jkun ippotenzat bit-taħlil jew bis-sospenzjoni tas-sustanza tat-test fi *Freunds Complete Adjuvant* (FCA), u f'testijiet mhux adġuvanti.

Testijiet tat-tip adġuvanti pjuttost li jkunu aktar eżatti fil-predizzjoni ta' effett sensitizzanti probabbli tal-ġilda ta' sustanza fuq il-bniedem milli dawk il-metodi li ma jutilizzawx il-*Freunds Complete Adjuvant* u huma għalhekk il-metodi preferiti.

It-test tal-Massimizzazzjoni tal-Fenek ta' l-Indi (GPMT) huwa test tat-tip adġuvanti ferm użat. Għalkemm diversi metodi oħrajn jistgħu jkunu wżati għas-sejbien tal-potenzal ta' sustanza li tikkapuna reazzjoni ta' sensitizzazzjoni tal-ġilda, il-GPMT huwa meqjusa bħala li huma t-teknika adġuvinanti preferuta.

B'ħafna mill-klassijiet kimiċi, testijiet mhux-adġuvinanti (dak preferut huwa t-0est Buehler) huma meqjusa bħala li huma anqas sensittivi.

F'ċerti każi jista jkun hemm raġunijiet tajbin għall-għażla tat-test Buehler li jinvolvi applikazzjoni topika pjuttost milli l-injezzjoni intradermali użata fit-Test tal-Massimizzazzjoni tal-Fenek ta' l-Indi. Ġustifikazzjoni xjentifika għandha tkun mogħtija meta t-test Buehler ikun użat.

It-Test tal-Massimizzazzjoni tal-fenek ta' l-Indi (GPMT) u t-test Buehler huma deskritti f'dan il-metodu. Metodi oħrajn jistgħu ikunu wżati basta li dawn ikunu ben-validati u l-ġustifikazzjoni xjentifika tkun mogħtija.

Independentement mill-metodi wżati, s-sensittività tar-razza tal-fenek ta' l-indi użata għat-testijiet tas-sensitizzazzjoni tal-ġilda għandha tkun ivverifikata matul intervalli regolari (sitt xhur) bl-użu ta' sensitizzatur magħruf li huwa ħafif jew moderat u numru sodisfaċenti sabiex ikunu akkwistati reazzjonijiet pożittivi.

Ara wkoll l-Introduzzjoni Ġenerali Parti B (A).

1.2. DEFINIZZJONI

Ara l-Introduzzjoni Ġenerali Parti B (B).

1.3. SUSTANZI TA' REFERENZA

Is-sustanzi li ġejjin, imdewba kif meħtieġ, huma rakkomandati, kif ukoll xi sustanza sensitizzanti oħra magħrufa jew mil-letteratura jew li tappartieni għal grupp tat-sustanza li tkun ittestjata.

- p-phenylenediamine	CAS N 106-50-3
- 2,4-dinitrochlorobenzene	CAS N 97-00-7
- dikromat tal-putassa	CAS N 7778-50-9
- sulfat neomiċin	CAS N 1405-10-3
- sulfat tan-nikil	CAS N 7786-81-4

1.4. PRINCIPJU TAL-METODI TAT-TEST

B'segwiment ta' l-esposizzjoni inizjali lejn sustanza tat-test (il-perijodu ta' l-"induzzjoni", l-annimali huma suġġetti għal madwar ġimgħatejn wara l-aħħar esposizzjoni ta' l-induzzjoni għal esposizzjoni "imħaqqa" tas-sustanza tat-test Sabiex ikun stabbilit jekk l-istat ta' l-ipersensittività jkunx ġie mibdi. Is-sensitizzazzjoni hija determinata bl-eżami tar-reazzjoni tal-ġilda għall-esposizzjoni mħaqqa.

1.5. KRITERJA TAL-KWALITÀ

Xejn

1.6. DESKRIZZJONI TAL-METODI TAT-TEST

1.6.1. **It-Test tal-Massimazzjoni tal-Fenek ta' l-Indi (GPMT)**

1.6.1.1. *Preparazzjonijiet*

Fniek ta' l-indi albino, żgħażagħ u b'saħħithom huma miġbura kif jinzerta u assenjati għat-trattament u l-gruppi ta' kontroll. Qabel ma jirċievu d-doża, il-pil huwa mneħħi, bl-imqass jew bit-tqaxxir, miż-żona ta' fuq l-ispalla. Attenzjoni għandha tingħata Sabiex tkun evitata li ssir xi ħsara lill-ġilda.

1.6.1.2. *Kundizzjonijiet tat-test*

1.6.1.2.1. Annimali sperimentali

Razzez tal-fniek ta' l-indi albino normalment użati fil-laboratorju huma utilizzati, li jkunu jiżnu anqas minn 500 g.

1.6.1.2.2. Numru u sess.

Annimali maskili u/jew femminili jistgħu ikunu wżati. Jekk ikunu użati femminili, dawn għandhom ikunu femminili li qatt ma weldu u li ma humiex tqal. Minimu ta' 10 annimali huma wżati fil-grupp ittrattat u mill-anqas 5 fil-grupp tal-kontroll. L-użu ta' l-anqas annimali għandu jkun ġustifikat. Fil-każ ta' riżultati ekwivoċi, eżaminazzjoni ħistopatoloġika tista tgħin saviex ikun deċiż jekk it-test għandhum ikun imtenni bl-użu ta' sett ieħor ta' annimali.

Meta ma jkunx pssobbli li jkun konkluż definittivament li s-sustanza tat-test ma tkunx sensitizzatur, l-ittestjar ta' annimali addizzjonali sabiex twassal għal total ta' mill-anqas 20 annimali tat-test u 10 annimali tal-kontroll hija rakkomandata.

1.6.1.2.3. Livell tad-doża

Il-konċentrazzjoni tas-sustanza tat-test hija aġġustata lejn livell li jipproduċi xi ftit ta' l-evidenza ta' l-irritazzjoni tal-ġilda, imma li tkun ben tollerata mill-annimali matul kull stadju ta' l-induzzjoni.

Il-konċentrazzjoni mħaqqa għandha tkun il-massimu li ma jipproduċi l-ebda evidenza ta' l-irritazzjoni tal-ġilda f'annimali m'humiex sensitizzati.

Dawn il-konċentrazzjoninijiet jistgħu jkunu determinati fi skala żgħira (tnejn jew tlett annimali) waqt studju pilota.

1.6.1.2.4. Perijodu ta' l-Osservazzjoni

Matul il-perijodu ta' l-induzzjoni, l-osservazzjomni huma mwettqa sabiex tkun verifikata l-possibbiltà ta' l-effetti irritanti. Wara l-esposizzjoni imħaqqa, ir-reazzjonijiet tal-ġilda huma reġistrati 24 u 48 siegħa wara t-tneħħija tal-biċċa.

1.6.1.3. *Proċedura*

L-annimali huma miżuna qabel ma jibda t-test u fit-tmiem tat-test. Iż-żona tal-ispalla għandha tkun imnaddfa mix-xagħar. Hemm żewġ stadju f'din il-proċedura:

1.6.1.3.1. Induzzjoni

Jum 0 – il-grupp ittrattat

Il-pari segwenti ta' injezzjonijiet intradermali, kull waħda ta' 0,1 ml, huma mogħtija fiż-żona tal-ispalla hekk li kull wieħed mill-pari jkun fuq kull naħa taċ-ċentru ta' l-annimal:

Injezzjoni 1:	0,1 ta' *Freunds Complete Adjuvant* (FCA) hija mħalta ma l-ilma jew ma salin fiżjoliġiku 1:1,
Injezzjoni 2:	0,1 tas-sustanza tat-test, meta meħtieġ bil-mezz xieraq,
Injezzjoni 3:	0,1 tas-sustanza tat-test fi FCA.

Fl-injezzjoni 3, sustanzi li jinħallu fl-ilma huma mdewba fi 0,05 ml ilma u fi 0,05 ml DCA mhux imdewweb. Jekk sustanzi liposolubbli jew insolubbli għandhom ikunu ittestjati, dawn huma mħalta ma FCA mhux imdewweb.

Fl-injezzjoni 3, il-konċentrazzjonini finali tas-sustanza tat-test għandha tkun egwali għal dik fl-injezzjoni 2.

Injezzjonijiet 1 u 2 huma mogħti malajr waħda wara l-oħra u l-aktar qrib lejn ir-ras, waqt li l-injezzjoni 3 tkun lejn il-parti kawdjali taż-żona tat-test.

Jum 0 – il-grupp tal-kontroll

Il-pari segwenti ta' injezzjonijiet intradermali huma mogħtija fl-istess żoni bħal hawn fuq:

Injezzjoni 1:	0,1 ta' *Freunds Complete Adjuvant* (FCA) hija mħallta ma l-ilma jew ma salin fiżjoliġiku 1:1,
Injezzjoni 2:	0,1 ml biss tal-mezz,
Injezzjoni 3:	0,1 tal-mezz fi FCA.

Jum 6 – il-gruppi tal-kontroll u trattati

Jekk is-sustanza ma tkunx irritanti għall-ġilda, iż-żona tat-test, wara l-qtugħ u/jew it-tqaxxir, tkun miżbuha bi 0,5 ml ta' 10% sulfat lawrju tas-sodju fil-vażellina, Sabiex toħloq irritazzjoni lokali.

Jum 7 – il-grupp ittrattat

Iż-żona tat-test għandha tkun imnaddfa għal darba oħra mix-xagħar. Is-sustanza tat-test f'mezz xieraq (l-għażla tal-mezz għandha tkun ġustifikata, is-solidi jkunu polverizzati fini ħafna u inkorporati f'mezz xieraq; il-likwidi jekk xieraq, jistgħu jkunu applikati direttament) hija mdelka fuq karta tal-filtru (2 × 4 cm) u applikata maż-żona tat-test u miżmumha f'kuntatt b'faxxa okklussiva għal 48 siegħa.

Jum 7 – il-grupp tal-kontroll

Iż-żona tat-test għandha tkun imnaddfa għal darba oħra mix-xagħar. Il-mezz biss huwa applikat bl-istess manjera maż-żona tat-test u miżmumha f'kuntatt b'faxxa okklussiva għal 48 siegħa.

1.6.1.3.2. Tħaqqieq

Jum 21

Il-ġembejn huma ittrattati u l-annimali tal-kontroll huma mnaddfa mix-xagħar. Biċċa jew kapsula li jkun fiha s-sustanza tat-test hija applikata ma wieħed mill-ġnub ta' l-annimali ittratati u l-biċċa jew il-kapsula mil-mezz biss mal-ġemb l-ieħor.

Il-biċċiet huma miżmuma b'faxxa okklussiva għal 24 siegħa.

Il-grupp tal-kontroll huwa espost b'manjiera identika.

Jiem 23 u 24

— 21 siegħa wara t-tneħħija tal-biċċa, iż-żona mħaqqa hija mnaddfa u x-xagħar ikun imneħħi, jekk hekk ikunu meħtieġ,

— tlett siegħat aktar tard (fi 48 siegħa mill-bidu ta' l-applikazzjoni tat-tħaqqieq) ir-reazzjoni tal-ġilda hija osservata u reġistrata,

— 24 siegħa wara din l-osservazzjoni, it-tieni osservazzjoni (72 siegħa) hija mwettqa u reġistrata.

Sabiex ikunu kjarifikati r-riżultati akkwistati fl-ewwel tħaqqieqa, t-tieni tħaqqieqa, jekk meħtieġ b'mezz gdid mal-grupp tal-kontroll, għandha tkun meqjusa madwar ġimgħa minn wara l-ewwel waħda.

1.6.1.3.3. Osservazzjoni u Gradar

Ir-reazzjoni kollha tal-ġilda f'xi sejbiet mhux tas-soltu li jirriżultaw mill-proċeduri ta' l-induzzjoni u tat-tħaqqieq għandhom ikunu irreġistrati u rapportati.

It-teknika ta' tali eżami jew kejl ħispatoloġiċi tal-ħxuna tal-qabda tal-ġilda tista tkun użata sabiex ikunu kjarifikati r-reazzjonijiet dubjużu jew dawk moħbija bit-tebgħa tal-ġilda bis-sustanza tat-test.

1.6.2. **It-test Buehler**

1.6.2.1. *Preparazzjonijiet*

Fniek ta' l-indi albino, żgħażagħ u b'saħħithom huma miġbura kif jinzerta u assenjati għat-trattament u l-gruppi ta' kontroll. Qabel ma jirċievu d-doża, il-pil huwa mneħħi, bl-imqass jew bit-tqaxxir, miż-żona ta' fuq ispalla waħda. Attenzjoni għandha tingħata Sabiex tkun evitata li ssir xi ħsara lill-ġilda.

1.6.2.2. *Il-kondizzjoni tat-test*

1.6.2.2.1. Annimali sperimentali

Razez tal-fniek ta' l-indi albino nurmalment użati fil-labortatorju huma utlizzati, li jkunu jiżnu anqas minn 500 g.

1.6.2.2.2. Numru u sess.

Annimali maskili u/jew femminili jistgħu ikunu wżati. Jekk ikunu użati femminili, dawn għandhom ikunu femminili li qatt ma welldu u li ma humiex tqal. Minimu ta' 20 annimal huma wżati fil-grupp ittrattat u mill-anqas 10 fil-grupp tal-kontroll. L-użu ta' anqas annimali għandu jkun ġustifikat. Fil-każ ta' riżultati ekwivoċi, eżaminazzjoni ħistopatoloġika tista tgħin sabiex ikun deċiż jekk it-test għandhum ikun imtenni bl-użu ta' sett ieħor ta' annimali.

1.6.2.2.3. Livell tad-doża

Għal kull stadju ta' l-induzzjoni, il-konċentrazzjonini tas-sustanza tat-test hija aġġustata għal-ogħla livell li jista jkun ittollerat b'mod sistematiku u li, għal sustanzi irritanti, jipproduċi irritazzjoni ħafifa jew moderata fil-maġġoranza ta' l-annimali tat-test. Il-konċentrazzjoni mħaqqa għandha tkun il-massimu li ma jipproduċi l-ebda evidenza ta' l-irritazzjoni tal-ġilda f'annimali mhux-sensitizzati. Dawn il-konċentrazzjoninijiet jistgħu jkunu determinati fi skala żgħira (tnejn jew tlett annimali) waqt studju.

1.6.2.2.4. Perijodu ta' l-Osservazzjoni

Matul il-perijodu ta' l-induzzjoni, l-osservazzjomni huma mwettqa sabiex tkun verifikata l-possibbiltà ta' l-effetti irritanti. Wara l-esposizzjoni mħaqqa, r-reazzjonijiet tal-ġilda huma reġistrati 24 u 48 siegħa wara t-tneħħija tal-biċċa, i.e. 30 sa 54 siegħa wara l-bidu ta' l-applikazzjoni.

1.6.2.3. *Il-proċedura*

L-annimali huma miżuna qabel ma jibda t-test u fit-tmiem tat-test.

Hemm żewġ stadju f'din il-proċedura:

1.6.2.3.1. Induzzjoni

Jum 0 – il-grupp ittrattat

Ġenb wieħed huwa mnaddaf mix-xagħar. 0,5 ml tas-sustanza tat-test f'mezz xieraq (il-għażla tal-mezz għandha tkun iġġustifikata; likwidi, jekk xierqa, jistgħu jkunu applikati direttament) hija mxerrda fuq biċċa tajjar. Dan huwa applikat fiż-żona tat-test u miżmum f'kuntatt mal-ġilda b'biċċa jew kapsula okklussiva u faxxa xierqa għal 6 siegħat.

Jum 0 – il-grupp tal-kontroll

Ġenb wieħed huwa mnaddaf mix-xagħar. Il-mezz biss huwa applikat b'manjera simili bħal dik taż-żona tat-test. Dan huwa miżmum f'kuntatt mal-ġilda b'biċċa jew kapsula oklussiva u faxxa xierqa għal 6 siegħat.

Jiem 7 u 14

L-istess applikazzjoni bħal fil-jum 0 hija mwettqa fuq l-istess żona tat-test (ix-xagħar imnaddaf jekk ikun meħtieġ) fil-jum 7 u fil-jum 14.

1.6.2.3.2. Tħaqqieq

Jum 28

Il-ġenb l-ieħor huwa trattat u l-annimali tal-kontroll huma mnaddfa mix-xagħar. Biċċa jew kapsula oklussiva li jkun fihom 0,5 ml tas-sustanza tat-test huma applikati, f'konċentrazzjonini massima mhux irritanti, mannaħa ta' wara tal-koxxa ta' l-annimali itrattati. Biċċa jew kapsula oklusiva bil-mezz biss hija wkoll applikata mal-parti anterjuri tal-koxxa.

Il-biċċiet oklusivi huma miżmuma f'kuntatt b'faxxa oklussiva għal 6 siegħa.

Il-grupp tal-kontroll huwa espost b'manjiera identika.

Jiem 29 u 30

— 21 siegħa wara t-tneħħija tal-biċċa, ż-żona mħaqqa hija mnaddfa u x-xagħar ikun imneħħi, jekk hekk ikunu meħtieġ,

— tlett siegħat aktar tard (fi 30 siegħa mill-bidu ta' l-applikazzjoni tat-tħaqqieq) ir-reazzjoni tal-ġilda hija osservata u reġistrata,

— 24 siegħa wara din l-osservazzjoni, it-tieni osservazzjoni (54 siegħa) hija mwettqa u reġistrata.

1.6.2.3.3. Osservazzjoni u Gradar

Ir-reazzjoni kollha tal-ġilda f'xi sejbiet mhux tas-soltu li jirriżultaw mill-proċeduri ta' l-induzzjoni u tat-tħaqqieq għandhom ikunu reġistrati u rapportati.

It-teknika ta' tali eżami jew kejl ħispatoloġiċi tal-ħxuna tal-qabda tal-ġilda jista jkun użat sabiex ikunu kjarifikati r-reazzjonijiet dubjużu jew dawk moħbija bit-tebgħa tal-ġilda bis-sustanza tat-test.

2. **DATA (GPMT u t-test Buehler)**

Id-*data* għandha tkun riassunta f'għamla tabulari, li turi għal kull annimal individwali r-reazzjonijiet tal-ġilda għal kull perijodu ta' l-osservazzjoni.

3. **RAPPURTAGG (GPMT u t-test Buehler)**

3.1. RAPPORT TAT-TEST (GPMT u t-test Buehler)

Ir-rapport tat-test għandu, jekk possibbli, jinkludi l-informazzjoni li ġejja:

— ir-razza tal-fniek ta' l-indi wżata;

— il-kondizzjonijiet tat-test, il-konċentrazzjoni tal-mezz u tas-sustanza tat-test użata għall-induzzjoni u t-tħaqqieq;

— in-numru, l-età u s-sess ta' l-annimali;

— il-piż individwali ta' l-annimali fil-bidu u fil-konklużjoni tat-test;

— kull osservazzjoni magħmula fuq kull annimali, inkluża s-sistema ta' gradjazzjoni, jekk waħda tkun użata.

— diskussjoni tar-riżultati;

— interpretazzjoni tar-riżultati.

3.2. EVALWAZZJONI U INTERPRETAZZJONI (GPMT u t-test Buehler)

Ara l-Introduzzjoni Ġenerali Parti B (D).

4. **REFERENZI**

Ara l-Introduzzjoni Ġenerali Parti B (E).

B.7. DOŻI REPETUTI (28 jum) TOSSICITÀ (ORALI)

1. **METODU**

1.1. INTRODUZZJONI

Ara l-Introduzzjoni Ġenerali Parti B (A).

1.2. DEFINIZZJONIJIET

Ara l-Introduzzjoni Ġenerali Parti B (B).

1.3. SUSTANZI TA' REFERENZA

Xejn

1.4. PRINCIPJU TAL-METODU TAT-TEST

Is-sustanza tat-test hija amministrata b'mod orali, ta' kuljum, b'dożi meqjusa lill-diversi gruppi ta' annimali sperimentali, b'doża waħda tkun użata ma kull grupp matul perijodu ta' 28 jum. Matul il-perijodu ta' l-amministrazzjoni, l-annimali huma osservati kuljum sabiex ikunu skoperti sinjali tat-tossiċità. Annimali li jmutu matul it-test ikollhom awtopsija tal-mewt u l-konklużjoni tat-test li l-annimali li jibqgħu ħajjin ikollhom ukoll l-awtopsija ta' wara l-mewt.

1.5. KRITERJA TAL-KWALITÀ

Xejn

1.6. DESKRIZZJONI TAL-METODU TAT-TEST

1.6.1. **Preparazzjonijiet**

L-annimali huma miżmuma f'fondi sperimentali u f'kondizzjonijiet tal-għalf għal mill-anqas ħamest ijiem. Qabel it-test, annimali adulti żgħażagħ b'saħħithom huma magħżula b'mod kif jinzerta u assenjati lejn gruppi għat-trattament. Is-sustanza tat-test tista tkun amministrata ma l-ikel, bil-mod tat-tmiegħ sfurzat, f'kapsoli, jew fl-ilma tax-xorb. L-annimali kollha għandhom jirċievu d-doża bl-istess metodu matul il-perijodu kollu ta' l-esperiment. Jekk xi materjal jew additivi oħrajn ikunu wżati għall-iffaċilitar tad-doża, dawn għandhom ikun u magħrufa li ma jipproduċu l-ebda effetti tossiċi. *Data* storika tista tkun użata, jekk xieraq.

1.6.2. **Kondizzjonijiet tat-test**

1.6.2.1. *Annimali sperimentali*

Sakemm ma jkunx hemm indikazzjonijiet mod ieħor, il-far huwa l-ispeċi preferita. Razzez komuni wżati fil-laboratorju ta' annimali żgħażagħ b'saħħithom għandhom ikunu wżati u d-dożi għandhom, idealment, jibdew qabel mal-firien ikollha aktar minn sitt ġimgħat, u fi kwalunkwe każ mhux aktar minn tmin ġimgħat.

Fil-bidu ta' kull test, il-medda tal-varjazzjoni fil-piż ta' l-annimali wżati m'għandhiex teċċedi ± 20 % tal-valur medju kif xieraq.

1.6.2.2. *Numru u sess.*

Mill-anqas 10 annimali gerriema (ħamsa femminili u ħamsa maskili) għandhom ikunu wżati f'kull livell ta' doża. Jekk ikunu użati femminili, dawn għandhom ikunu femminili li qatt ma welldu u li ma humiex tqal. Jekk sagrifikar interim huwa ippjanat, in-numru ta' l-annimali għandu jkun miżjud bin-numru taa' l-annimali skedati li jkunu sagrifikati qabel it-tkomplija ta' l-istudju. B'żieda, grupp satellita ta' 10 annimali (ħames annimali kull sess) jistgħu jkunu trattati b'doża ta' livell għoli għal 28 jum u osservati għar-riversabbiltà, l-persistenza, jekk l-okkurrenza mdewma ta' l-effetti tossiċi għal 14 il-jum ta' wara t-trattament. Grupp satellita ta' 10 annimali ta' kontroll (ħames annimali kull sess) huwa wkoll użat.

1.6.2.3. *Livelli tad-doża*

Mill-anqas tlett livelli ta' doża u kontroll, għandhom ikunu wżati. Appart milli t-trattament tas-sustanza tat-test, l-annimali fil-grupp tal-kontroll għandhom ikunu trattati b'mod identiku bħal dawk suġġetti għat-test. Meta mezz ikun użat sabiex jiffaċilita l-għoti tad-doża, l-kontrolli għandhom ikollhom doża bil-mezz bl-istess mod bħal gruppi trattati, u jirċievu l-istess ammont ta' mezz bħal dak li jkunu ireċevew il-grupp bil-livell ta' l-ogħla doża. Il-livell ta' l-ogħla doża għandu jirriżulta f'effetti tossiċi imma ma jipproduċi l-ebda, jew ftit, imwiet. Il-livell tad-doża l-aktar baxxa m'għandux jipproduċi xi evidenza ta' tossiċità. Meta jkun hemm estimi użabbli ta' l-esposizzjoni umana, l-livell l-aktar baxx għandu jeċċedi dan. Idealment, il-livell medju tad-doża għandu jipproduċi effetti tossiċi minimi osservabbli. Jekk aktar minn doża intermedja waħda tkun użata, l-livelli tad-doża għandhom ikunu spazjati bi gradjazzjoni ta' l-effetti tossiċi. Fil-gruppi baxxi jew intermedji u f'dawk tal-kontroll, l-inċidenza ta' xi fatalitajiet għandha tkun baxxa sabiex tippermetti evalwazzjoni sensibbli tar-riżultati.

Meta s-sustanza tat-test tkun amministrata ma l-ikel, jew b'konċentrazzjoni kostanti ma l-ikel (ppm jew mg/kg ta' l-ikel) jew livell ta' doża kostanti f'termini tal-piż tal-ġisem ta' l-annimal, jistgħu jkunu wżati; l-użu alternattiv għandu jkun speċifikat. Għal sustanzi amministrati bit-tmiegħ sfurzat, id-doża għandha tkun mogħtija f'ħinijiet simili f'kull jum u l-livelli tad-doża għandhom ikunu aġġustati matul l-intervalli (ta' kull ġimgha jew ta' darbtejn fil-gimgħa) Sabiex jinżamm livell ta' doża kostanti f'termini tal-piż ta' l-annimal.

1.6.2.4. *Test ta' limitazzjoni*

Jekk studju ta' 28 jum imwettaq b'konformità mal-metodu dettaljat hawn taħt, f'livell ta' doża waħda ta' 1000 mk/kg il-piż tal-ġisem/kuljum jew livell ta' doża ogħla relattata ma l-esposizzjoni umana possibbli meta dan ikunu magħruf, ma jipproduċi l-ebda effetti tossiċi, aktar testijiet ma jkunux aktar meqjusa bħala meħtieġa. Għal sustanzi b'tossiċità baxxa, huma importanti li jkun assigurat li meta amministrati ma l-ikel, il-kwantitajiet u l-proprjetajiet l-oħrajn tas-sustanza tat-test involuta ma jkunux jinterferixxu mal-ħtiġiet nutrizzjonali normali.

1.6.2.5 *Perijodu ta' l-osservazzjoni*

L-annimali kollha għandhom ikunu osservati kuljum u s-sinjali tossiċi reġistrati jkunu inklużi fil-waqt tat-tfaqqiegħ, tal-grad u tat-tul ta' żmien. Il-ħin tal-mewt u ż-żmien li fih tidher jew tisparixxi t-tossiċità għandhom ikunu registrati.

1.6.3. **Il-proċedura**

L-annimali jirċievu d-doża bis-sustanza tat-test idealment fis-sebat ijiem tal-ġimgħa għal perijodu ta' 28 jum. L-annimali f'xi grupp satellita skedat għall-osservazzjoni b'segwiment għandhom jinżammu għal 14 il-jum ieħor mingħajr trattament sabiex tkun żvelata xi irkupru minn, jew il-persistenza ta', l-effetti tossiċi.

L-osservazzjonijiet għandhom jinkludi tibdil fil-ġilda u l-pil, fl-għajnejn u membrani mukożji, u wkoll fis-sistemi respiratorji, ċirkolatorji, awtonomiċi u tas-sistema ċentrali tan-nervituri, l-attività somatomotori u l-istil ta' l-imġieba. Kejl ta' kull ġimgħa għandu jsir tal-konsum ta' l-ikel (u l-konsum ta' l-ima meta s-sustanza tat-test tkun amministrata fl-ilma tax-xorb) u l-annimali għandhom ikunu mwieżna kull ġimgħa.

Osservazzjonijiet regolari ta' l-annimali huma meħtieġa sabiex jassiguraw li l-annimali jkunu, sakemm ikun possibbli, mhux mitlufa mill-istudju minħabba kawżi oħrajn bħalma huma kannibaliżmu, awtoloż tan-nisġa jew mitlufa. Fit-tmiem tal-perijodu ta' l-istudju, dawk kolla li jkunu għadhom ħajjin fil-gruppi tat-trattament mhux-satellita, jkunu maqtula u awtopsija tkun imwettqa immedjetament. Annimali moribondi u annimali f'uġiegħ jew strapazz sever għandhom ikunu mneħħija meta dan ikun innutat, u maqtula sabiex tkun evitata l-moħqrija u titwettaq awtopsija ta' wara l-mewt.

L-eżami li ġejja għandha sseħħ fit-tmiem tal-perijodu tat-test għall-annimali kollha, inklużi dawk tal-kontrolli:

1. ħematoloġija, inkluża mill-anqas il-ħematokrità, il-konċentrazzjoni tal-ħemaglobulini, l-għadd ta' l-eritroċiti, il-għadd totali u differenzali tal-lewtoċiti, u l-kejl tal-potenzal ta' boċċi fid-demm;

2. biokemistrija klinika tad-demm, inkluż mill-anqas parametru wieħed tal-fwied u tal-funzjoni tal-kliewi: Aminotransferaži ta' l-alanina (qabel kienet magħrufa bħala t-transaminaži piruvika glutamika), l-aminotransferaži aspartata tas-serum (qabel kienet magħrufa bħala t-transaminaži ossalo-aċetika glutamika), in-nitroġenu ta' l-urea, l-albumina, il-kreatinin tad-demm, il-bilirubin totali u l-protein totali tas-serun.

Determinazzjonijiet oħrajn li jistgħu jkunu meħtieġa għal evalwazzjoni tossikologika adekwata, inkluż il-kalċju, l-fosfru, l-klorid, is-sodju, l-putassa, l-glokożju tas-sawm, l-analiżi tal-lipidi, l-ormoni, il-bilanċ tal-aċidu/bażi, il-metaemoglobina, l-attività kolinesteraži.

Biokemistrija klinika addizzjonali tista tkun użata, meta meħtieġ, Sabiex tkun estiża l-investigazzjoni ta' l-effetti osservati.

1.6.3.1. *Nekropsija Grossa (awtopsija ta' wara l-mewt)*

L-annimali kollha fl-istudju għandhom ikunu suġġetti għall-awtopsja sħiħa grossa ta' wara l-mewt. Mill-anqas il-fwied, il-kliewi, l-adrenali, u t-testi għandhom ikunu mwieżna noiedja malajr kemm jista jkun possibbli wara d-dissikazzjoni sabiex ikun evitat li jinxfu. Organi u ċerti nsiġ tal-ġisem (fwied, kliewi, il-milsa (l-marrara), it-testi, l-adrenali, l-qalb, u xi organi oħrajn li juru feriti kbar jew tibdil fid-daqs) għandhom ikunu preservati f'medja xierqa għall-possibbiltà ta' eżami fuut ħistopatoloġiku.

1.6.3.2. *Eżaminazzjoni ħistopatoloġika*

Fil-grupp ta' doża għolja u fil-grupp ta' kontroll, l-eżaminazzjoni ħistoloġika għandha tkun imwettqa fuq l-organi u l-insiġ ippreservati. Organi u nsiġ li juru difetti attribwibbli għas-sustanza tat-test fil-livell ta' l-ogħla doża għandhom ikunu eżaminati fil-gruppi kollha tad-doża l-baxxa. L-annimali f'xi grupp satellita għandhom ikunu eżaminati ħistoloġikalment b'enfasi partikolari fuq l-organi u nsiġ identifikati bħala li juru effetti fil-gruppi l-oħrajn trattati.

2. **DATA**

Id-*data* għandha tkun riassunta f'għamla tabulari, li turi għal kull grupp tat-test, in-numru ta' l-annimali fil-bidu tat-test u n-numru ta' l-annimali li jiżvelaw kull tip ta' ferita.

Ir-riżultati osservati kollha għandhom ikunu evalwati b'metodu statistiku xieraq. Kwalunkwe metodu statistiku rikonoxxut jista jkun użat.

3. **RAPPURTAĠĠ**

3.1. RAPPORT TAT-TEST

Ir-rapport tat-test għandu, jekk possibbli, jinkludi l-informazzjoni li ġejja:

— l-ispeċi, s-silta, s-sorsi, il-kondizzjonijiet ambjentali, d-dieta, etc.,

— il-kondizzjoni tat-test,

— il-livelli tad-doża (inkluż il-mezz, jekk użat), u l-konċentrazzjonijiet;

— dettalji dwar ir-reazzjoni tossika bis-sess u d-doża;

— il-livell ta' l-ebda effett, meta possibbli;

— il-ħin tal-mewt matul l-istudju jew meta l-annimali jkunu baqgħu ħajjin fit-terminazzjoni tat-test;

— l-effetti tossiċi jew oħrajn;

— il-ħin ta' l-osservazzjoni u kull sinjal abnormali u l-korsa sussegwenti tiegħu;

— dettalji dwar l-ikel u piż tal-ġisem;

— testijiet ħematoloġiċi wżati u r-riżultati kollha;

— testijiet ħematoloġiċi kliniċi użati u r-riżultati kollha;

— is-sejbiet ta' l-awtopsja ta' wara l-mewt,

— deskrizzjoni dettaljata tas-sejbiet ħistopatoloġiċi kollha;

— trattament statistiku tar-riżultati, meta xieraq;

— diskussjoni tar-riżultati;

— interpretazzjoni tar-riżultati.

3.2. EVALWAZZJONI U INTERPRETAZZJONI

Ara l-Introduzzjoni Ġenerali Parti B (D).

4. **REFERENZI**

Ara l-Introduzzjoni Ġenerali Parti B (E).

B.8. DOŻI REPETUTI (28 jum) TOSSICITÀ (TEĦID MAN-NIFS)

1. **METODU**

1.1. INTRODUZZJONI

Huwa utili li jkun hemm informazzjoni preliminarja dwar id-distribuzzjoni tad-daqs tal-partiċelli, l-pressjoni tal-fwar, il-punt tat-taħlil, il-punt tat-togħlija, il-punt tal-leħħa jew tal-isplussività (jekk applikabbli) tas-sustanza.

Ara wkoll l-Introduzzjoni Ġenerali Parti B (A).

1.2. DEFINIZZJONI

Ara l-Introduzzjoni Ġenerali Parti B (B).

1.3. SUSTANZI TA' REFERENZA

Xejn

1.4. PRINCIPJU TAL-METODU TAT-TEST

Diversi gruppi ta' annimali sperimentali huma esposti għal perijodu definittiv lejn is-sustanza tat-test f'konċentrazzjonini bi gradwazzjonijiet, b'konċentrazzjoni waħda tkun użata għal kull grupp, għall-perijodu ta' 28 jum. Meta mezz ikun użat sabiex jgħin fil-ġenerazzjoni b'konċentrazzjoni xierqa tas-sustanzi tat-test fl-atmosfera, mezz ta' grupp ta' kontroll għandu jkun użat. Matul il-perijodu ta' l-amministrazzjoni, l-annimali huma osservati kuljum sabiex ikunu skoperti sinjali tat-tossiċità. Annimali li jmutu matul it-test ikollhom awtopsija tal-mewt u l-konklużjoni tat-test li l-annimali li jibqgħu ħajjin ikollhom ukoll l-awtopsija ta' wara l-mewt.

1.5. KRITERJA TAL-KWALITÀ

Xejn

1.6. DESKRIZZJONI TAL-METODU TAT-TEST

1.6.1. **Preparazzjonijiet**

L-annimali huma miżmuma b'fondi sperimentali u f'kondizzjonijiet tal-għalf għal mill-anqas ħamest ijiem qabel l-esperiment. Qabel it-test, annimali adulti żgħażagħ b'saħħithom huma magħżula b'mod kif jinzerta u assenjati lejn in-numru meħtieġ ta' gruppi. Meta meħtieġ, materjal adattat jista jkun miżjud mas-sustanza tat-test sabiex jgħin fil-ġenerazzjoni ta' konċentrazzjonini xierqa tas-sustanza tat-test fl-atmosfera. Jekk xi materjal jew addittivi oħrajn ikunu wżati għall-faċilizzazzjoni tad-doża, dawn għandhom ikunu magħrufa li ma jipproduċu l-ebda effetti tossiċi. *Data* storika tista tkun użata, jekk xieraq.

1.6.2. **Il-kondizzjonijiet tat-test**

1.6.2.1. *Annimali sperimentali*

Sakemm ma jkunx hemm indikazzjonijiet mod ieħor, il-far huwa l-ispeċi preferita. Is-siltiet normalment użati fil-laboratorju ta' l-annimali żgħażagħ b'saħħithom għandhom ikunu wżati.

Fil-bidu ta' kull test, il-medda tal-varjazzjoni fil-piż ta' l-annimali użati m'għandhiex teċċedi ± 20 % tal-valur medju kif xieraq.

1.6.2.2. *Numru u sess.*

Mill-anqas 10 annimali (ħamsa femminili u ħamsa maskili) għandhom ikunu wżati f'kull livell ta' doża. Jekk ikunu wżati femminili, dawn għandhom ikunu femminili li qatt ma welldu u li ma humiex tqal. Jekk sagrifikar interim huwa pjanat, in-numru ta' l-annimali għandu jkun miżjud bin-numru ta' l-annimali skedati li jkunu sagrifikati qabel it-tkomplija ta' l-istudju. B'żieda, grupp satellita ta' 10 annimali (ħames annimali kull sess) jistgħu jkunu trattati b'doża ta' livell għoli għal 28 jum u osservati għar-riversabbiltà, l-persistenza, jekk l-okkurenza mdewma ta' l-effetti tossiċi tkun għal 14 il-jum wara t-trattament. Grupp satellita ta' 10 annimali ta' kontroll (ħames annimali kull sess) huwa wkoll użat.

1.6.2.3. *Konċentrazzjoni ta' l-esposizzjoni*

Mill-anqas tlett konċentrazzjoninijiet huma meħtieġa, b'kontroll jew mezz ta' kontroll (korrespondenti għall-konċentrazzjoni tal-mezz fil-livell l-aktar għoli) tal-mezz jekk użat. Appart milli t-trattament tas-sustanza tat-test, l-annimali fil-grupp tal-kontroll għandhom ikunu trattati b'mod identiku bħal dawk suġġetti għat-test. Il-livell ta' l-ogħla doża għandu jirriżulta f'effetti tossiċi imma ma jipproduċi l-ebda, jew ftit, imwiet. Il-livell tad-doża l-aktar baxxa m'għandux jipproduċi xi evidenza ta' tossiċità. Meta jkun hemm estimi użabbli ta' l-esposizzjoni umana, il-livell l-aktar baxx għandu jeċċedi dan. Idealment, il-livell medju tad-doża għandu jipproduċi effetti tossiċi minimi osservabbli. Jekk aktar minn doża intermedja waħda tkun użata, l-livelli tad-doża għandhom ikunu spazjati bi gradjazzjoni ta' l-effetti tossiċi. Fil-gruppi baxxi jew intermedji u f'dawk tal-kontroll, l-inċidenza ta' xi fatalitajiet għandha tkun baxxa sabiex tippermetti ewalwazzjoni sensibbli tar-riżultati.

1.6.2.4. *Ħin ta' l-esposizzjoni*

It-tul ta' l-esposizzjoni ta' kuljum għandu jkun ta' sitt siegħat imma perijodi oħrajn jistgħu ikunu meħtieġa sabiex jilħqu l-ħtiġiet speċifiċi.

1.6.2.5. *Apparat:*

L-annimali għandhom ikunu testjati bl-apparat tan-nifs iddisinjat għas-sostenn ta' nixxija ta' arja dinamika ta' mill-anqas 12 il-kambjamenti ta' l-arja fis-siegħa, sabiex ikun assigurat kontenut adekwat ta' l-ossiġenu u esposizzjoni ta' l-atmosfera mqassma b'mod egwali. Meta kabina tkun użata, d-disinn tagħha għandu jimminimizza l-iffullar ta' l-annimali tat-test u jimminimizzaw l-esposizzjoni tagħhom man-nifs tas-sustanza tat-test. Bħala regola ġenerali sabiex tkun assigurata l-istabbilità ta' l-atmosfera fil-kabina il-"volum" totali ta' l-annimali tat-test m'għandux jeċċedi 5 % tal-volum tal-kabina tat-test. Ħalq-nażali, ir-ras biss, jew l-ispazji tal-kabina ta' l-esposizzjoni jista jkun użat; bl-ewwel tnejn għandhom jgħinu li jimminimizzaw l-ammont tas-sustanza tat-test b'mezzi oħrajn.

1.6.2.6. *Perijodu ta' l-Osservazzjoni*

L-annimali sperimentali għandhom ikunu osservati ta' kuljum għal sinjali ta' tossiċità matul il-perijodu kollu tat-trattament u ta' l-irkupru. Il-ħin tal-mewt u ż-żmien li fih tidher jew tisparixxi t-tossiċità għandhom ukoll ikunu reġistrati.

1.6.3. **Il-proċedura**

L-annimali jirċievu d-doża bis-sustanza tat-test idealment fis-sebat ijiem tal-ġimgħa għal perijodu ta' 28 jum. L-annimali f'xi grupp satellita skedat għall-osservazzjoni b'segwiment għandhom jinżammu għal 14 il-jum ieħor mingħajr trattament sabiex tkun żvelata xi rkupru minn, jew il-persistenza ta', l-effetti tossiċi. It-temperatura li fiha t-test ikun imwettaq għandha tinżamm fi 22 ± 3 °C.

Idealment, l-umdità relattiva għandha tinżamm bejn 30 u 70%, imma f'ċerti kazi (e.g. it-testijiet ta' uħud mill-*aerosols*) dan jista ma jkunx prattikabbli. Il-manteniment ta' pressjoni kemxejn negattiva ġewwa l-kabina (= 5 mm ta' ilma) tkun tipprevjeni l-ħruġ tas-sustanza tat-test fiż-żona tal-madwar. L-ikel u l-ilma għandhom ikunu miċħuda matul l-esposizzjoni.

Sistema ta' inħalazzjoni dinamika b'konċentrazzjoni analitika xierqa tas-sistema tal-kontroll għandha tkun użata. Sabiex tkun stabbilita esposizzjoni xierqa tal-konċentrazzjoni, test bi prova huwa rakkomandat. Il-kurrent ta' l-arja għandu jkun aġġustat sabiex jassigura li l-kondizzjonijiet matul il-kabina ta' l-esposizzjoni jkunu omoġenji. Is-sistema għandha tassigura li kondizzjonijiet stabbli ta' l-esposizzjoni jintlaħqu malajr kemm jista jkun possibbli.

Il-kejl u l-moniteraġġ għandu jsir:

(a) tar-rata tal-kurrent ta' l-arja (b'mod kontinwu).

(b) il-konċentrazzjoni attwali tas-sustanza tat-test imkejla fiż-żona ta' fejn jittieħed in-nifs. Matul il-perijodu ta' l-esposizzjoni, l-konċentrazzjonijiet m'għandhomx ivarjaw b'aktar minn ± 15 % tal-medja tal-valur. B'danakollu, fil-każ ta' uħud mill-*aerosols*, dan il-livell ta' kontroll ma jistax ikun milħuq u medda aktar wiesa tkun għalhekk aċċettabbli. Matul iż-żmien totali ta' l-istudju, l-konċentrazzjonijiet minn jum għal jum għandhom jinżammu kostanti kemm jista jkun possibbli. Għall-*aerosols*, mill-anqas analiżi ta' partikola waħda għandha tkun imwettqa darba fil-ġimgha għal kull grupp;

(c) tat-temperatura u l-umidità, kontinwament jekk possibbli.

Matul u wara l-esposizzjoni, osservazzjoni hija mwettqa u reġistrata b'mod sistematiku; dettalji individwali għandhom jinżammu għal kull annimal. L-annimali kollha għandhom ikunu osservati kuljum u s-sinjali tossiċi reġistrati jkunu inklużi, meta jsir it-tfaqqiegħ, il-grad u t-tul ta' żmien tagħhom. L-osservazzjonijiet għandhom jinkludu tibdil fil-ġilda u l-pil, fl-għajnejn u membrani mukożji, u anki s-sistemi respiratorji, ċirkolatorji, awtonomiċi u tas-sistema ċentrali tan-nervaturi, l-attività somatomotori u l-istil ta' l-imġieba. Il-kejl għandu jsir ta' kull ġimgħa dwar il-piż ta' l-annimali. Huwa wkoll rakkomandat li l-konsum ta' l-ikel ikun imkejjel ta' kull ġimgħa. Osservazzjonijiet regolari ta' l-annimali huma meħtieġa sabiex jassiguraw li l-annimali jkunu, sakemm ikun possibbli, m'humiex mitlufa mill-istudju minħabba kawżi oħrajn bħalma huma kannibaliżmu, awtolożi tan-nisġa jew mitlufa. Fit-tmiem tal-perijodu ta' l-istudju, dawk kollha li jkunu għadhom ħajjin fil-gruppi tat-trattament mhux-satellita, jkunu maqtula u awtopsija tkun imwettqa immedjatament. Annimali moribondi u annimali f'uġiegħ jew li għaddejjin minn strapazz sever għandhom ikunu mneħħija meta dan ikun innutat, u maqtula sabiex tkun evitata l-moħqrija, waqt li titwettaq awtopsija ta' wara l-mewt.

L-eżami li ġejja għandha sseħħ fit-tmiem tal-perijodu tat-test għall-annimali kollha, inklużi dawk tal-kontrolli:

(i) ħematoloġija, inkluża mill-anqas il-ħematokritä, l-konċentrazzjonini tal-ħemaglobulini, l-għadd ta' l-eritroċiti, l-għadd totali u differenzali tal-lewtoċiti, u l-kejl tal-potenzal ta' boċċi fid-demm;

(ii) biokemistrija klinika tad-demm, inkluż mill-anqas parametru wieħed tal-fwied u tal-funzjoni tal-kliewi: aminotransferaži ta' l-alanina (qabel kienet magħrufa bħala t-transaminaži piruvika glutamika), l-aminotransferaži aspartata tas-serum (qabel kienet magħrufa bħala t-transaminaži ossalo-aċetika glutamika), in-nitroġenu ta' l-urea, l-albumina, l-kreatinin tad-demm, il-bilirubin totali u l-protein totali tas-serun.

Determinazzjonijiet oħrajn li jistgħu jinħtieġu għal evalwazzjoni tossikoloċika adekwata, inkluż il-kalċju, l-fosfru, l-klorid, is-sodju, l-putassa, l-glokożju tas-sawm, l-analiżi tal-lipidi, l-ormoni, l-bilanċ tal-aċidu/bażi, il-metaemoglobina, l-attività kolinesteraži.

Biokemistrija klinika addizzjonali tista tkun użata, meta meħtieġ, sabiex tkun estiża l-investigazzjoni ta' l-effetti osservati.

1.6.3.1. *Nekropsija Grossa (awtopsija ta' wara l-mewt)*

L-annimali kollha fl-istudju għandhom ikunu suġġetti għall-awtopsija sħiħa grossa ta' wara l-mewt. Mill-anqas il-fwied, il-kliewi, l-adrenali, u t-testi għandhom ikunu mwieżna niedja malajr kemm jista jkun poissibbli wara d-dissikazzjoni sabiex ikun evitat li jinxfu. Organi u ċerti nsiġ tal-ġisem (fwied, kliewi, il-milsa (l-marrara), t-testi, l-adrenali, l-qalb, u xi organi oħrajn li juru feriti kbar jew tibdil fid-daqs) għandhom ikunu preservati f'medja xierqa għall-possibbiltà ta' eżami fuut ħistopatoloġiku. Il-pulmuni għandhom jinqalgħu intatti, miżuna u ittrattati b;'fissattiv xieraq Sabiex ikun assigurat li l-istruttura tal-pulmun tkun miżmumha.

1.6.3.2. *Eżaminazzjoni ħistopatoloġika*

Fil-grupp ta' doża għolja u fil-grupp ta' kontroll, l-eżaminazzjoni ħistoloġika għandha tkun imwettqa fuq l-organi u l-insiġ ippreservati. Organi u nsiġ li juru difetti attribwibbli għas-sustanza tat-test fil-livell ta' l-għola doża għandhom ikunu eżaminati fil-gruppi kollha tad-doża l-baxxa. L-annimali f'xi grupp satellita għandhom ikunu eżaminati ħistoloġikalment b'enfasi partikolari fuq l-organi u nsiġ identifikati bħala li juru effetti fil-gruppi l-oħrajn ittrattati.

2. **DATA**

Id-*data* għandha tkun riassunta f'għamla tabulari, li turi għal kull grupp tat-test, in-numru ta' l-annimali fil-bidu tat-test u n-numru ta' l-annimali li jiżvelaw kull tip ta' ferita.

Ir-riżultati osservati kollha għabdhom ikunu evalwati b'metodu statistiku xieraq. Kwalunkwe metodu statistiku rikonoxxut jista jkun użat.

3. **RAPPORTAĠĠ**

3.1. RAPPORT TAT-TEST

Ir-rapport tat-test għandu, jekk possibbli, jinkludi l-informazzjoni li ġejja:

— l-ispeċi, s-silta, s-sorsi, l-kondizzjonijiet ambjentali, id-dieta, etc.,

— il-kondizzjoni tat-test,

Deskrizzjoni ta' l-apparat ta' l-esposizzjoni, inkluż id-disinn, it-tip, il-qisien, is-sorsi ta' l-arja, is-sistema għall-ġenerazzjoni ta' l-aerosols, metodu għall-kondizzjonar ta' l-arja u l-metodu taż-żamma ta' l-annimali fil-kabina tat-test, meta din tkun użata. L-apparat għall-kejl tat-temperatura, l-umidità, il-koncentrazzjonijiet ta' l-*aerosol* u d-distrubuzzjoni tad-daqs tal-particelli, għandhom ikunu deskritti.

Data ta' l-esposizzjoni:

Dawn għandhom ikunu tabulati u preżentati mal-valuri medji u l-kejl tal-varjabbiltà (e.g. devjazzjoni normali) u għandhom, meta possibbli, jinkludu:

(a) ir-rati tal-kurrent ta' l-arja matul l-apparat tat-teħid man-nifs;

(b) it-temperatura u l-umidità ta' l-arja;

(c) konċentrazzjonijiet nominali (ammont totali tas-sustanza ta' kif infuża fl-apparat tat-teħid man-nifs, diviża bil-volum ta' l-arja);

(d) in-natura tal-mezz, jekk użat;

(e) il-konċentrazzjonijiet attwali fiż-żona tat-teħid tan-nifs waqt it-test;

(f) Il-massa tad-dijametru erodinamiku medjan (MMAD) u d-devjazzjoni ġeometrika normali (GSD);

— dettalji dwar ir-reazzjoni tossika bis-sess u d-doża;

— il-ħin tal-mewt matul l-istudju jew meta l-annimali jkunu baqgħu ħajjin fit-terminazzjoni tat-test;

— deskrizzjoni ta' l-effetti tossiċi u oħrajn; il-livell ta' l-ebda effett;

— il-ħin ta' l-osservazzjoni u kull sinjal annormali u l-korsa sussegwenti tiegħu;

— dettalji dwar l-ikel u piż tal-ġisem;

— testijiet ħematoloġiċi użati u r-riżultati kollha;

— testijiet ħematoloġiċi kliniċi użati u r-riżultati kollha;

— is-sejbiet ta' l-awtopsja ta' wara l-mewt,

— deskrizzjoni dettaljata tas-sejbiet ħistopatoloġiċi kollha;

— trattament statistiku tar-riżultati, meta xieraq;

— diskussjoni tar-riżultati;

— l-interpretazzjoni tar-riżultati.

3.2. EVALWAZZJONI U INTERPRETAZZJONI

Ara l-Introduzzjoni Ġenerali Parti B (D).

4. **REFERENZI**

Ara l-Introduzzjoni Ġenerali Parti B (E).

B.9. DOŻI REPETUTI (28 jum) TOSSICITÀ (DERMALI)

1. **METODU**

1.1. INTRODUZZJONI

Ara l-Introduzzjoni Ġenerali Parti B (A).

1.2. DEFINIZZJONIJIET

Ara l-Introduzzjoni Ġenerali Parti B (B).

1.3. SUSTANZI TA' REFERENZA

Xejn

1.4. PRINCIPJU TAL-METODU TAT-TEST

Is-sustanza tat-test hija amministrata b'mod orali, ta' kuljum, b'dożi meqjusa lil diversi gruppi ta' annimali sperimentali, b'doża waħda tkun użata ma kull grupp matul perijodu ta' 28 jum. Matul il-perijodu ta' l-amministrazzjoni, l-annimali huma osservati ta' kuljum sabiex ikunu skoperti sinjali tat-tossiċità. Annimali li jmutu matul it-test ikollhom awtopsija tal-mewt u l-konklużjoni tat-test li l-annimali li jibqgħu ħajjin ikollhom ukoll l-awtopsija ta' wara l-mewt.

1.5. KRITERJA TAL-KWALITÀ

Xejn

1.6. DESKRIZZJONI TAL-METODU TAT-TEST

1.6.1. **Preparazzjonijiet**

L-annimali huma miżmuma f'fondi sperimentali u f'kondizzjonijiet tal-għalf għal mill-anqas ħamest ijiem. Qabel it-test, annimalu adulti żgħażagħ b'saħħithom huma magħżula b'mod kif jinzerta u assenjati lejn gruppi għat-trattament. Xi ftit qabel it-testijiet, il-pil huwa maqtugħ miż-żona dorsali tad-dahar ta' l-annimali tat-test. It-tqaxxir jista jkun użat imma għandu jkun imwettaq madwar 24 siegħa qabel it-test. Ir-repetizzjoni tal-qtugħ jew tat-tqaxxir normalment ikun meħtieġ f'intervalli ta' madwar darba fil-ġimgħa. Meta jsir il-qtugħ jew it-tqaxxir tal-pil, kura għandha tittieħed sabiex ikun evitat xi grif fil-ġilda li jkunu jistgħu jibdlu l-permeabbiltà tagħha. Mhux anqas minn 10% tal-wiċċ tal-ġisem għandu jkun imnaddaf għall-applikazzjoni tas-sustanza tat-test. Il-piż ta' l-annimali għandu jkun meqjus meta tkun deċiża ż-żona li għandha tkun imnaddfa u l-qisien kollha tal-koperta. Meta jsiru testijiet tas-solidi, li jistgħu ikunu pulverizzati jekk xieraq, is-sustanza tat-test għandha tkun b'umidità suffiċjenti bl-ilma, jew, meta meħtieġ, apparat adattat sabiex jassigura kuntatt tajjeb mal-ġilda. Sustanzi likwidi tat-test huma ġeneralment użati mingħajr ma jkunu mrattba. Applikazzjoni odjerna bħala bażi ta' minn ħamsa sa sebat ijiem fil-ġimgħa hija wżata.

1.6.2. **Kundizzjonijiet tat-test**

1.6.2.1. *Annimali sperimentali*

Firien jew fniek, jew fniek ta' l-indi adulti jistgħu ikunu wżati. Speċje oħrajn jistgħu ikunu wżati imma l-użu tagħhom ikun jeħtieġ ġustifikazzjoni.

Fil-bidu ta' kull test, il-medda tal-varjazzjoni fil-piż ta' l-annimali użati m'għandhiex teċċedi ± 20 % tal-valur medju kif xieraq.

1.6.2.2. *Numru u sess.*

Mill-anqas 10 annimali gerriema (ħamsa femminili u ħamsa maskili) għandhom ikunu wżati f'kull livell ta' doża. Jekk ikunu użati femminili, dawn għandhom ikunu femminili li qatt ma weldu u li ma humiex tqal. Jekk sagrifikar interim huwa ippjanat, in-numru ta' l-annimali għandu jkun miżjud bin-numru ta' l-annimali skedati li jkunu sagrifikati qabel it-tkomplija ta' l-istudju. B'żieda, grupp satellita ta' 10 annimali (ħames annimali kull sess) jistgħu ikunu ittrattati b'doża ta' livell għoli għal 28 jum u osservati għar-riversabbiltà, persistenza, jew għal okkurrenza mdewma ta' l-effetti tossiċi għal 14 il-jum ta' wara t-trattament. Grupp satellita ta' 10 annimali ta' kontroll (ħames annimali kull sess) huwa wkoll użat.

1.6.2.3. *Livell tad-doża*

Mill-anqas tlett livelli ta' dożi huma meħtieġa bil-kontroll u mezz ta' kontroll, jekk mezz ikun użat. Il-perijodu ta' l-osservazzjoni għandu jkun ta' mill-anqas ta' sitt siegħat kuljum. Għal sustanzi amministrati bi tmiegħ sfurzat, id-doża għandha tkun mogħtija f'ħinijiet simili f'kull jum u l-livelli tad-doża għandhom ikunu aġġustati matul l-intervalli (ta' kull ġimgħa jew ta' darbtejn fil-gimgħa) sabiex jinżamm livell ta' doża kostanti f'termini tal-piż ta' l-annimal. Apparti milli t-trattament tas-sustanza tat-test, l-annimali fil-grupp tal-kontroll għandhom ikunu trattati b'mod identiku bħal dawk suġġetti għat-test. Meta mezz ikun użat sabiex jiffaċilita l-għoti tad-doża, l-kontrolli għandhom ikollhom doża bil-mezz bl-istess mod bħal gruppi ittrattati, u jirċievu l-istess ammont ta' mezz bħal dak li jkunu irċevew il-grupp bil-livell ta' l-ogħla doża. Il-livell ta' l-ogħla doża għandu jirriżulta f'effetti tossiċi imma ma jipproduċi l-ebda, jew ftit, imwiet. Il-livell tad-doża l-aktar baxxa m'għandux jipproduċi xi evidenza ta' tossiċità. Meta jkun hemm estimi użabbli ta' l-esposizzjoni umana, il-livell l-aktar baxx għandu jeċċedi dan. Idealment, il-livell medju tad-doża għandu jipproduċi effetti tossiċi minimi osservabbli. Jekk aktar minn doża intermedja waħda tkun użata, l-livelli tad-doża għandhom ikunu spazzjati bi gradjazzjoni ta' l-effetti tossiċi. Fil-gruppi baxxi jew intermedji u f'dawk tal-kontroll, l-inċidenza ta' xi fatalitajiet għandha tkun baxxa sabiex tippermetti ewalwazzjoni sensibbli tar-riżultati.

Jekk l-applikazzjoni tas-sustanzi tat-test tipproduċi irritazzjoni severa tal-ġilda, l-konċentrazzjonijiet għandhom ikunu mnaqqsa u dan jista jirriżulta fi tnaqqis fil, jew fl-assenza ta', l-effetti tossiċi l-oħrajn, fil-livell għoli tad-doża. Aktar minn hekk, jekk il-ġilda tkon sofriet ħsara qawwija, jista jkun meġtieġ li jkun imwaqqaf l-istudju u li jinbeda studju ġdid b'konċentrazzjoni aaktar baxxa.

1.6.2.4. *Test ta' limitazzjoni*

Jekk studju preliminari fil-livell ta' doża ta' 1 000 mg/kg, jew livell ta' doża ogħla relattat ma l-esposizzjoni umana possibbli meta dan ikunu magħruf, ma jipproduċi l-ebda effetti tossiċi, aktar testijiet ma jkunu aktar meqjusa bħala meħtieġa.

1.6.2.5. *Perijodu ta' l-Osservazzjoni*

L-annimali sperimentali għandhom ikunu osservati ta' kuljum għal sinjali tat-tossiċità. Il-ħin tal-mewt u ż-żmien li fih tidher jew tisparixxi t-tossiċità għandhom ukoll ikunu irreġistrati.

1.6.3. **Il-proċedura**

L-annimali għandhom jinżammu f'gaġġeġ individwali. L-annimali jirċievu d-doża bis-sustanza tat-test idealment fis-sebat ijiem tal-ġimgħa għal perijodu ta' 28 jum. L-annimali f'xi grupp satellita skedat għall-osservazzjoni b'segwiment għandhom jinżammu għal 14 il-jum ieħor mingħajr trattament sabiex tkun żvelata xi irkupru minn, jew il-persistenza ta', l-effetti tossiċi. Il-perijodu ta' l-osservazzjoni għandu jkun ta' mill-anqas ta' sitt sieghat kuljum.

Is-sustanza tat-test għandha tkun applikata b'mod uniformi fuq żona li hija madwar 10% taż-żona totali tal-wiċċ tal-ġisem. B'sustanzi ferm tossiċi, iż-żona tal-wiċċ koperta tista tkun anqas imma kemm jista jkun miż-żona għandha tkun koperta b'saff irqieq u uniformi kemm jista jkun possibbli.

Matul l-esposizzjoni s-sustanza tat-test tinżamm f'kuntatt mal-ġilda b'garża poruża u faxxa mhux irritanti. Is-sit tat-test għandu jkun aktar kopert b'mod xieraq sabiex iżomm il-faxxex bil-garża u s-sustanza tat-test u jassiguraw li l-annimali ma jkunux jistgħu jilgħaqu u jieklu s-sustanza tat-test. Restrizzjonijiet jistgħu jintużaw sabiex jipprevjenu l-inġestjoni tas-sustanza tat-test, imma immobilizzazzjoni kompleta mhix metodu rakkommandat. Bħala alternattiva, "apparat b'għonq protettiv" jista jkun użat.

Fit-tmiem tal-perijodu ta' l-esposizzjoni, ir-residwu tas-sustanza tat-test għandu jkun imneħħi, meta prattikabbli, bl-użu ta' ilma jew xi metodu xieraq ieħor tat-tindif tal-ġilda.

L-annimali kollha għandhom ikunu osservati ta' kuljum u s-sinjali tossiċi irreġistrati jkunu inklużi, meta jsir it-tfaqqiegħ, il-grad u t-tul ta' żmien tagħhom. L-osservazzjonijiet għandhom jinkludu tibdil fil-ġilda u l-pil, fl-għajnejn u membrani mukożji, u anki fis-sistemi respiratorji, ċirkolatorji, awtonomiċi u tas-sistema ċentrali tan-nervituri; l-attività somatomotori u l-istil ta' l-imġieba. Il-kejl għandu jsir ta' kull ġimgħa dwar il-piż ta' l-annimali. Huwa wkoll rakkommandat li l-konsum ta' l-ikel ikun imkejjel ta' kull ġimgħa. Osservazzjonijiet regolari ta' l-annimali huma meħtieġa sabiex jassiguraw li l-annimali jkunu, sakemm ikun possibbli, mhux mitlufa mill-istudju minħabba kawżi oħrajn bħalma huma kannibaliżmu, awtolożi tan-nisġa jew mitlufa. Fit-tmiem tal-perijodu ta' l-istudju, dawk kollha li jkunu għadhom ħajjin fil-gruppi tat-trattament mhux-satellita, jkunu maqtula u awtopsija tkun imwettqa immedjatament. Annimali moribondi u annimali f'uġiegħ jew strapazz sever għandhom ikunu mneħħija meta dan ikun innutat, u maqtula sabiex tkun evitata l-moħqrija u titwettaq awtopsja ta' wara l-mewt.

L-eżami li ġejja għandha sseħħ fit-tmiem tal-perijodu tat-test għall-annimali kollha, inklużi dawk tal-kontrolli:

1) ħematoloġija, inkluża mill-anqas il-ħematokrità, l-konċentrazzjonini tal-ħemaglobulini, l-għadd ta' l-eritroċiti, l-għadd totali u differenzali tal-lewtoċiti, u l-kejl tal-potenzal ta' boċċi fid-demm;

2) biokemistrija klinika tad-demm, inkluż mill-anqas parametru wieħed tal-fwied u tal-funzjoni tal-kliewi: aminotransferaży ta' l-alanina tas-serum (qabel kienet magħrufa bħala t-transaminaży piruvika glutamika), il-aminotransferaży aspartata tas-serum (qabel kienet magħrufa bħala t-transaminaży ossalo-aċetika glutamika), in-nitroġenu ta' l-urea, l-albumina, il-kreatinin tad-demm, il-bilirubin totali u l-protein totali tas-serun.

Determinazzjonijiet oħrajn li jistgħu jkunu meħtieġa għal evalwazzjoni tossikologika adekwata, inkluż il-kalċju, l-fosfru, l-klorid, is-sodju, il-putassa, l-glokożju tas-sawm, l-analiżi tal-lipidi, l-ormoni, l-bilanċ tal-aċidu/bażi, l-metaemoglobina, l-attività kolinesteraży.

Biokemistrija klinika addizzjonali tista tkun użata, meta meħtieġ, sabiex tkun estiża l-investigazzjoni ta' l-effetti osservati.

1.6.4. **Nekopsija Grossa (awtopsija ta' wara l-mewt)**

L-annimali kollha fl-istudju għandhom ikunu suġġetti għall-awtopsja sħiħa grossa ta' wara l-mewt. Mill-anqas il-fwied, il-kliewi, l-adrenali, u t-testi għandhom ikunu miżuna niedja malajr kemm jista jkun poissibbli wara d-dissikazzjoni sabiex ikun evitat li jinxfu. Organi u ċerti nsiġ tal-ġisem (fwied, kliewi, il-milsa (l-marrara), it-testi, l-adrenali, l-qalb, u xi organi oħrajn li juru feriti kbar jew tibdil fid-daqs) għandhom ikunu ippreservati f'medja xierqa għall-possibbiltà ta' eżami futura ħistopatoloġika.

1.6.5. **Eżaminazzjoni ħistopatoloġika**

Fil-grupp ta' doża għolja u fil-grupp ta' kontroll, l-eżaminazzjoni ħistoloġika għandha tkun imwettqa fuq l-organi u l-insiġ ippreservati. Organi u nsiġ li juru difetti attribwibbli għas-sustanza tat-test fil-livell ta' l-ogħla doża għandhom ikunu eżaminati fil-gruppi kollha tad-doża l-baxxa. L-annimali f'xi grupp satellita għandhom ikunu eżaminati ħistoloġikalment b'enfasi partikolari fuq l-organi u nsiġ identifikati bħala li juru effetti fil-gruppi l-oħrajn ittrattati.

2. **DATA**

Id-*data* għandha tkun riassunta f'għamla tabulari, li turi għal kull grupp tat-test, in-numru ta' l-annimali fil-bidu tat-test u n-numru ta' l-annimali li jiżvelaw kull tip ta' ferita.

Ir-riżultati osservati kollha għabdhom ikunu evalwati b'metodu statistiku xieraq. Kwalunkwe metodu statistikali rikonoxxut jista jkun użat.

3. **RAPPORTAĠĠ**

3.1. RAPPORT TAT-TEST

Ir-rapport tat-test għandu, jekk possibbli, jinkludi l-informazzjoni li ġejja:

— id-dettalji dwar l-annimal (l-ispeċje, silta, sorsi, il-kondizzjonijiet ambjentali, dieta, etc.,);

— kondizzjonijiet tat-test (inklużu t-tip ta' faxex: okklussivi jew mhux okklussivi);

— il-livelli tad-doża (inkluż il-mess, jekk użat), u l-konċentrazzjoninijiet;

— il-livell ta' l-ebda effett, meta possibbli;

— dettalji dwar ir-reazzjoni tossika bis-sess u d-doża;

— il-ħin tal-mewt matul l-istudju jew meta l-annimali jkunu baqgħu ħajjin fit-terminazzjoni tat-test;

— l-effetti tossiċi jew oħrajn;

— il-ħin ta' l-osservazzjoni u kull sinjal annormali u l-korsa sussegwenti tiegħu;

— dettalji dwar l-ikel u piż tal-ġisem;

— testijiet ħematoloġiċi użati u r-riżultati kollha;

— testijiet ħematoloġiċi kliniċi użati u r-riżultati kollha;

— is-sejbiet ta' l-awtopsja ta' wara l-mewt,

— deskrizzjoni dettaljata tas-sejbiet ħistopatoloġiċi kollha;

— trattament statistiku tar-riżultati, meta xieraq;

— diskussjoni tar-riżultati;

— interpretazzjoni tar-riżultati.

3.2. EVALWAZZJONI U INTERPRETAZZJONI

Ara l-Introduzzjoni Ġenerali Parti B (D).

4. **REFERENZI**

Ara l-Introduzzjoni Ġenerali Parti B (E).

B.10. MUTAĠENICITÀ (TEST SITOĠENETIKU MAMMALJAN "IN VITRO")

1. **METODU**

1.1. INTRODUZZJONI

Ara l-Introduzzjoni Ġenerali Parti B (A).

1.2. DEFINIZZJONI

Ara l-Introduzzjoni Ġenerali Parti B (C).

1.3. SUSTANZI TA' REFERENZA

Xejn

1.4. PRINCIPJU TAL-METODU TAT-TEST

It-test sitoġenetiku *in vitro* huwa test ta' mutaġenċità f'terminu qasir għas-sejbien ta aberrazzjonijiet kromosomali strutturali fil-ċelloli imkabbra tal-mammaljani. Kulturi ta' linji ta' ċelloli stabbiliti, kif ukoll kulturi ta' ċelloli primarji, jistgħu ikunu wżati. Wara l-esposizzjoni lejn il-kimika tat-test u mingħajr sistema xierqa ta' attivazzjoni metabolika, l-kulturi tat-test huma trattati b'inibituri bobini bħal ma hija l-kolċinikina sabiex takkumula ċ-ċelloli fi stadji simili għall-metafaži ta' mitosis (c-metafaži). Iċ-ċelloli huma miġbura matul żminijiet xierqa u l-preparazzjonijiet kromosomatiċi huma mħejjija. Il-preparazzjonijiet huma msoffija u ċ-ċelloli tal-metafaži huma analizzati għall-annormalitajiet.

1.5. KRITERJA TAL-KWALITÀ

Xejn

1.6. DESKRIZZJONI TAL-METODU TAT-TEST

1.6.1. **Preparazzjonijiet**

1.6.1.1. *Celloli*

Linji ta' ċelloli stabbiliti jew kulturi ta' ċelloli primarji huma wżati, e.g., ċelloli tal-*hamster* Ciniż u l-limfoċiti umani. Il-kimika tat-test tkun ippreparata f'medja ta' kultura jew imdewba f'mezz xieraq qabel ma jseħħ it-trattament taċ-ċelloli.

1.6.1.2. *Sistema ta' attivazzjoni metabolika*

Iċ-ċelloli għandhom ikunu esposti għas-sustanza tat-test kemm fil-preżenza u fin-nuqqas tas-sistema xierqa ta' l-attivazzjoni metabolika. Is-sistema komuni l-aktar użata hija l-frazzjoni kofattur-supplementata post-mitokondrjali preparat mill-fwied ta' annimali gerriema trattati bl-aġenti li jinduċu l-enżimi.

1.6.2. **Kondizzjonijiet tat-test**

Numru ta' kulturi:

Mill-anqas kultru doppji huma wżati għal kull wieħed mill-punti sperimentali.

L-użu ta' kontroll negattiv u pożittiv:

Solvent (meta s-solvent ma jkunx il-medja tal-kultura jew l-ilma), taħlita ta' l-attivazzjoni ta' l-enżimi tal-fwied, taħlita u solvent ta' l-attivazzjoni ta' l-enżimi tal-fwied, u kontrolli mhux ittrattati huma wżati bħala kontrolli negattivi.

Fi kwalunkwesperiment, kontroll pożittix huwa nkluż; meta t-taħlita ta' l-attivazzjoni ta' l-enżimi tal-fwied tkun użata sabiex twettaq l-attivazzjoni tal-kimika tat-test, kompost magħruf li jeħtieġ l-attivazzjoni metabolika għandu jkun użat bħala kontroll pożittiv.

Livell tad-doża:

Mill-anqas tlett dożi tal-kompost tat-test matul mill-anqas medda *one-log* tad-doża huma wżati. L-ogħla doża għandha jkollha l-effett ta' inibizzjoni mitotika ta' l-attività b'madwar 50% jew li tesebixxi uħud mill-indikazzjonijiet l-oħrajn tas-sitotossiċità. Jekk ma tkunx tossika, s-sustanza tal-prova għandha tkun ittestjata sal-limitu tas-solubbiltà, jew sa konċentrazzjonini massima ta' 5 mg/ml.

Kondizzjonijiet tal-kultura:

Medja xierqa tal-kultura, kondizzjonijiet ta' l-inkubazzjoni (e.g. temperatura, mezzi tal-kultura użati, konċentrazzjonijiet ta' CO_2 u umidità) huma wżati.

1.6.3. **Il-proċedura**

1.6.3.1. *Preparazzjoni tal-kulturi*

Linji ta' ċelloli stabbiliti: Iċ-ċelloli huma ġenerati minn kulturi tal-ħażna (e.g. bit-tripsinizzazzjoni jew biċ-ċekċik 'il barra), miżruha f'mezzi tal-kultura f'densità xierqa, u inkubati fi 37°C.

Limfociti umani: Demm sħiħ ħeparinizzat huwa miżjud mal-medja tal-kultura li jkun fiha *phytohaemagglutinin*, serum tal-fetu ta' l-għoġol u antibiotiċi u inkubat fi 37°C.

1.6.3.2. *Trattament tal-kulturi bil-kompost tat-test*

(i) Trattament mingħajr it-taħlita ta' l-attivazzjoni ta' l-enżimi tal-fwied

It-trattamenti kollha għandhom, meta possibbli, jkopru mill-anqas il-perijodu ta' waħda miċ-ċikli sħaħ taċ-ċellola u l-iskemi tal-fissazzjoni, għandhom jassiguraw l-analiżi ta' l-ewwel mitożi post-trattament ta' ċelloli trattati fi stadji differenti matul iċ-ċiklu.

Meta t-trattament ma jkoprix it-tul taċ-ċiklu kollu ta' waħda miċ-ċelloli, il-ħinijiet tal-fissazzjoni huma magħżula Sabiex iwettqa l-kampjunar taċ-ċelloli li jkunu fi stadji differenti taċ-ċiklu taċ-ċellola matul it-trattamet, i.e. G1, S u G2.

Il-kimika tat-tyest hija miżjuda mal-kulturi ta' linji ta' ċelloli stabbiliti meta dawn ikunu fl-istadju espotenzali tat-tkabbir. Il-kulturi tal-limfokite umana huma trattati waqt li jkunu f'kondizzjoni semi-sinkronizzata.

(ii) Trattament bit-taħlita ta' l-attivazzjoni ta' l-enżimi tal-fwied. Għat-trattament, il-kompost tat-test f'ġemgħa mas-sistema ta' l-attivazzjoni għandu jkun preżenti sakemm ikun possibbli mingħajr ma jkun eżerċitat l-effett tossiku fuq iċ-ċelloli. Jekk għal raġunijiet ta' tossiċità dan it-trattament ma jkoprix it-tul taċ-ċiklu kollu ta' waħda miċ-ċelloli, l-ħinijiet tal-fissazzjoni huma magħżula sabiex iwettqu l-kampjunar taċ-ċelloli li jkunu fi stadji differenti taċ-ċiklu taċ-ċellola matul it-trattamet, i.e. G_1, S u G_2.

Il-ħsad taċ-ċelloli:

Kulturi taċ-ċelloli huma trattati b'inibutur bobinu għal ħin xieraq qabel ma jseħħ il-ħsad. Kull kultura hija maħsuda u proċessata separatament għall-preparazzjoni tal-kromożomi.

Mill-anqas żewġ ħinijiet tal-ħsad huma meħtieġa. Huwa rakkomandat li wieħed ikun madwar ta' ċiklu ta' ċellola waħda, u l-ieħor aktar tard. Dan huwa sabiex jassigura li l-istadji kollha taċ-ċiklu taċ-ċellola jkunu koperti u jippermetti għad-dewmien fiċ-ċiklu taċ-ċellola.

1.6.3.3. *Preparazzjoni tal-kromożomju*

Il-preparazzjonijiet tal-kromożomju jinvolvu t-trattament ħipotoniku taċ-ċelloli, fissazzjoni, tixrid fuq il-lametti, u l-għoti tal-kulur.

Analiżi:

Mill-anqas 100 metafaži imxerrda sewwa għal kull kultura huma analizzati għal aberrazzjonijiet kromożomali. Il-lametti jkunu kodifikati qabel l-analiżi. Fil-limfotici umani, l-metafżijiet biss li jkun fihom 16 ċentromeri huma analizzati.

F'linji ta' ċelloli stabbiliti l-metafaži biss li jkun fih ± 2 ċentromeri tan-numru modali huma analizzati.

B'żieda, l-indiċi mitotiċi, jew xi indikazzjoni oħra tas-sitotossiċità meta xieraq, għandhom ikunu assessjati matul it-test għal kull livell ta' doża.

2. **DATA**

Id-*data* hija preżentata f'għamla tabulari. Aberrazzjonijiet tat-tip kromatidji (spazji, qsim, interkambjalità), aberrazzjoni tat-tip kromosomju (e.g. spazji, qsim, minuti, ċrieki, diċentriċi, poliċentriċi) u n-numru tal-metafaži aberrenti (inklużu u esklużu l-ispazji) huma elenkati separatament għall-kulturi kollha, kemm ittrattati u tal-kontroll.

Id-*data* hija evalwata bil-metodi statistiċi xierqa.

Ir-riżultati tat-test għandhom ikunu mqabbla ma kontrolli konkorrenti negattivi.

Mill-anqas żewġ esperimenti independenti huma mwettqa. B'danakollu, jekk jista jkun xjentifikament ġustifikat, esperiment singolu jista jkun suffiċjenti. Mhux meċessarju li jkun imwettaq it-tieni test bl-istess mod identiku ta' l-esperiment inizjali. Tabilhaqq, jista jkun preferibbli li jkunu mibdula l-kondizzjonijiet tat-test sabiex tkun akkwistata *data* aktar utili.

3. **RAPPORTAĠĠ**

3.1. RAPPORT TAT-TEST

Ir-rapport tat-test għandu, jekk possibbli, jinkludi l-informazzjoni li ġejja:

— iċ-ċelloli użati;

— il-kondizzjoni tat-test, komposizzjoni tal-medja, konċentrazzjonini CO_2, temperatura ta' l-inkubazzjoni, ż-żmien ta' l-inkubazzjoni, l-livelli tad-doża, l-ħin tat-trattament, it-tul tat-trattament bil, u l-konċentrazzjoni ta' l-inibitur bobin kif użat, it-tip tat-tahlita ta' l-attivazzjoni ta' l-inżimi tal-fwied kif użati, kontrolli pożittivi u negattivi;

— in-numru ta' kulturi taċ-ċelloli:

— in-numru tal-metafaži analizzati (dettalji mogħtija għal kull kultura);

— indiċi mitotika jew indikazzjoni oħra tas-sitotossiċità;

— it-tip u n-numru ta' l-aberrazzjonijiet mogħtija separatament għal kull kultura ittrattata u tal-kontroll, in-numru modali tal-kromożomi fil-linji ta' ċelloli stabbiliti kif użati;

— evalwazzjoni statistika;

— diskussjoni tar-riżultati;

— interpretazzjoni tar-riżultati.

3.2. EVALWAZZJONI U INTERPRETAZZJONI

Ara l-Introduzzjoni Ġenerali Parti B (D).

4. **REFERENZI**

Ara l-Introduzzjoni Ġenerali Parti B (E).

B.11. MUTAĠENICITÀ (TEST SITOĠENETIKU MAMMALJAN "IN VITRO", ANALIŻI KROMOSOMALI)

1. **METODU**

1.1. INTRODUZZJONI

Ara l-Introduzzjoni Ġenerali Parti B (A).

1.2. DEFINIZZJONI

Ara l-Introduzzjoni Ġenerali Parti B (C).

1.3. SUSTANZI TA' REFERENZA

Xejn

1.4. PRINCIPJU TAL-METODU TAT-TEST

It-test sitoġenetiku *in vitro* huwa test ta' mutaġenċità f'terminu qasir għas-sejbien ta aberrazzjonijiet kromosomali strutturali fil-ċelloli imkabbra tal-mammaljani. Aberrazzjonijiet kromosomali huma ġeneralment evalwati fl-ewwel mitoži post-trattament. Bil-mitoġeni kimika, l-maġġoranza ta' l-aberrazzjonijiet indotti huma tat-tip kromatidi.

Il-metodu jutilizza ċ-ċelloli tal-mudullun tal-għadam ta' mammiferi li huma esposti għall-kimika tat-test birrottot xierqa u huma sagrifikati matul intervalli sekwenzali. L-annimali huma aktar itrattati, qabel ma jkunu sagrifikati, bl-inibitur bobin bhalma huwa l-kolċikin sabiex jakkumula ċ-ċelloli fi stadju ta' mitoži li jixbaħ il-metafaži (c-metafaži). Il-preparazzjonijiet tal-kromożomi mnixxfa fl-arja miċ-ċelloli huma magħmula u mogħtija l-kulur u l-metafaži huma analizzati b'mod mikroskopikali għall-aberrazzjonijiet kromosomali.

1.5. KRITERJA TAL-KWALITÀ

Xejn

1.6. DESKRIZZJONI TAL-METODU TAT-TEST

1.6.1. **Preparazzjonijiet**

Il-kimika tat-test hija mdewba f'salmura normali. Jekk ma tinħallx, dan ikun imdewweb jew sospiż fil-mezz xieraq.

Soluzzjonijiet ippreparati friski tal-kompost tat-test huma utilizzati. Jekk xi mezzi jintużaw għall-faċilizzazzjoni tad-doża, dawn għandhom ikunu magħrufa li ma jipproduċu l-ebda effetti tossiċi.

1.6.2. **Kundizzjonijiet tat-test**

1.6.2.1. *Annimali sperimentali*

Speċi ta' annimali gerriema, bħalma huma firien, ġrieden jew *hamsters* Ciniżi, huma wżati. Annimali adulti żgħażagħ adulti u b'saħħithom huma miġbura kif jinzerta u assenjati għat-trattament u l-gruppi ta' kontroll.

1.6.2.2. *Numru u sess*

Mill-anqas ħames femminili u ħames maskili ghal kull esperiment u grupp tal-kontroll huma utilizzati. B'hekk, 10 annimali jkunu sagrifikati f'kull perijodu ta' żmien ghal kull grupp jekk diversi ħinijiet tat-test wara t-trattament huma inklużi fl-iskeda sperimentali.

Għal grupp ta' kontroll pożittiv, ħin singolu tal-kampjunar huwa suffiċjenti.

1.6.2.3. *Rotta ta' l-amministrazzjoni*

Għaxar komposti għandhom ikunu ġeneralment amministrati darba biss. Ibbażata fuq l-informazzjoni tossikoloġika skeda ta' trattament repetuta tista tkun utilizzata. B'danakollu, l-iskeda tat-trattament imtenni tista tkun applikata biss jekk il-kompost tat-test ma jkunx jesebixxi l-effetti ċitotossiċi fil-mudullun ta' għadam. Ir-rottot normali ta' l-amministrazzjoni huma orali u l-injezzjoni intraperitonali. Rottot oħrajn ta' l-amministrazzjoni jistgħu jkunu xierqa.

1.6.2.4. *L-użu ta' kontrolli negattivi u pożittivi*

Kompost magħruf li jipproduċi aberrazzjonijiet kromsomali *in vivo* li hu utilizzat bħala kontroll pożittiv u grupp ta' kontroll negattiv (solvent) huwa wkoll inkluż fid-disinn ta' kull esperiment.

1.6.2.5. *Livell tad-doża*

Għal sett bażi, doża waħda tal-kompost tat-test huwa wżat, id-doża tkun il-massimu tollerat tad-doża jew dik li tipproduċu xi indikazzjoni tas-sitotossiċità (e.g. inibizzjonali parzjali tal-mitożi).

Għal komposti "m'humiex tossiċi", d-doża (limitu) massimu li jeħtieġ li jkun investigat b'segwiment ta' doża singola, l-amministrazzjoni hija 2 000 mg/kg il-piż tal-ġisem.

Jekk skeda ta' doża repetura tkun utilizzata, il-limiru tad-doża 1 000 mg/kg tal-piż tal-ġisem kuljum.

Livelli ta' doża addizzjonali jistgħu ikunu utilizzati meta dawn huma indikati b'raġunijiet xjentifiċi.

Jekk it-test utilizzat bħala metodu għall-verifika mill-anqas żewġ livelli ta' doži addizzjonali għandhom ikunu wżati.

1.6.3. **Il-proċedura**

It-test jista jkun imwettaq b'żewġ metodi:

(i) L-annimali huma trattati bil-kompost tat-test darba, fl-ogħla doża tollerata. Fl-ewwel istanza, kampjuni huma meħuda fi 24 siegħa wara t-trattament. Jekk ir-riżultati huma kjarament pożittivi f'dan l-istadju, aktar kampjunar jista ma jkunx meħtieġ. B'danakollu, jekk ir-riżultati huma negattivi jew ekwivoċi, ladarba ċ-ċiklu kinetiku taċ-ċellola jista jkun influwenzat bil-kimika tat-test, wieħed aktar kmieni u ieħor aktar tard fl-intervall tal-kampjunar, adekwat spazjati fi ħdan il-medda ta' minn sitta sa 48 siegħat, huma applikati.

Meta doži addizzjonali huma wżati, kampjuni għandhom ikunu meħuda fl-intervalli partikolarment sensittivi jew, jekk dak ma jkunx magħruf, 24 siegħa wara t-trattament.

(ii) Jekk l-informazzjoni farmaċokinetika u metabolitika tindika skeda ta' trattament repetut, doża repetuta tista tkun utilizzata u l-kampjuni għandhom ikun u miġbura sitta u 24 siegħa wara l-aħħar trattament.

Preparazzjoni tal-mudullun tal-għadam:

Qabel ma jkunu sagrifikati, l-annimali huma injettati b'mod intraperizjonali b'doża xierqa ta' l-inibitur bobin sabiex ikun akkwistat numru adekwat taċ-ċelloli f'c-metafaži. Il-mudullun tal-għadam huwa akkwistat kemm mill-femora ta' annimali maqtula friski bit-tlaħliħ b'taħlita iżotonika. Wara trattament ipotoniku xieraq, iċ-ċelloli huma iffissati u mbagħad imxerrda fuq lametti. Wara t-tnixxif fl-arja l-lametti jingħataw il-kulur.

Analiżi:

Il-lametti jkunu kodifikati qabel l-analiżi mikroskopika. Mill-anqas 50 metafażijiet ben spazzjali bin-numru sħiħ ta' ċentromeri huma analizzati għal kull annimal dwar l-aberrazzjonijiet strutturali kromosomalji. B'mod addizzjonali, l-indiċi mitotiċi jistgħu ikunu stabbiliti għal kull annimal.

2. **DATA**

Id-*data* hija preżentata f'għamla tabulari. Aberrazzjonijiet tat-tip kromatidiku u isokromatidiku (spazju, qsim, interkambjabbilità), u l-indiċi mitotiċi, meta stabbiliti, huma elenkati separatament għall-annimali kollha kemm ittrattati u tal-kontroll. Il-medja tan-numru u d-devjazzjonijiet normali għal kull grupp sperimentali u ta' kontroll huma wkoll elenkati. Id-*data* hija evalwata bil-metodi statistiċi xierqa.

3. **RAPPORTAĠĠ**

3.1. RAPPORT TAT-TEST

Ir-rapport tat-test għandu, jekk possibbli, jinkludi l-informazzjoni li ġejja:

— speċi, siltiet u l-età ta' l-annimali użati;

— in-numru ta' annimali ta' kull sess fi gruppi sperimentali u tal-kontroll;

— il-kondizzjoni tat-test, deskrizzjoni dettaljata tat-trattament u l-iskeda tal-kampjunar, livelli tad-doża, it-tul tat-trattament bi u l-konċentrazzjoni ta' l-inibitur bobin kif utilizzat;

— in-numru ta' metafaži analizzati kull annimal;

— indiċi mitotiċi, meta stabbiliti;

— it-tip u n-numru ta' aberrazzjonijiet mogħtija separatament għal kull annimal ittrattat u tal-kontroll;

— sinjali tat-tossiċità matul il-korsa tal-istudju;

— evalwazzjoni statistika;

— diskussjoni tar-riżultati;

— interpretazzjoni tar-riżultati.

3.2. EVALWAZZJONI U INTERPRETAZZJONI

Ara l-Introduzzjoni Ġenerali Parti B (D).

4. **REFERENZA**

Ara l-Introduzzjoni Ġenerali Parti B (E).

B.12. MUTAĠENICITÀ (TEST MIKRONUKLEU)

1. **METODU**

1.1. INTRODUZZJONI

Ara l-Introduzzjoni Ġenerali Parti B (A).

1.2. DEFINIZZJONI

Ara l-Introduzzjoni Ġenerali Parti B (C).

1.3. SUSTANZI TA' REFERENZA

Xejn

1.4. PRINCIPJU TAL-METODU TAT-TEST

It-test mikronukleu huwa test mammaliku f'terminu-qasir in vivo għas-sejbien tal-ħsara kromosomali jew ħsara ta' l-apparat mitotiku bil-kimika. Il-bażi ta' dan l-assaġġ hija ż-żieda fil-mikronukleji fil-ertroċiti polikromatiċi ta' l-annimali ittrattati versus il-kontrolli.

Il-mikronukleji huma furmati minn fragmenti kromosomali, jew tal-kromosomiċi sħaħ neqsin fil-mitoży. Meta l-eritroblasti jiżviluppaw fi eritroċiti, in-nukleu ewlieni huwa mormi waqt li l-mikronukleu jista jkun miżmum fis-sitoplażma. Eritroċiti polikromatiċi żgħażagħ fil-mudullun ta' l-għadam tal-mammiferi tal-laboratorju li kienu esposti għas-sustanzi bir-rotott xierqa huma wżati f'dan it-test. Il-mudullun tal-għadam ikun estruż u l-preparazzjonijiet tat-tiċpis magħmula u mogħtija l-kulur. Eritroċiti polikromatiċi huma mnaqqxa għall-mikronuklei taħt il-mikroskopju u r-relattività tal-polikromatiċi ma' l-eritroċiti mormokromatiċi hija stabbilita.

1.5. KRITERJA TAL-KWALITÀ

Xejn

1.6. DESKRIZZJONI TAL-METODU TAT-TEST

1.6.1. **Preparazzjonijiet**

Il-kimika tat-test hija mdewba f'taħlita iżotonika. Jekk ma tinħallx, dan ikun imdewweb jew sospiż fil-mezz xieraq. Jekk xi mezzi jkunu wżati għall-faċilitazzjoni tad-doża, dawn għandhom ikunu magħrufa li ma jipproduċu l-ebda effetti tossiċi. Soluzzjonijiet ippreparati friski tal-kompost tat-test huma utilizzati.

1.6.2. **Kondizzjonijiet tat-test**

1.6.2.1. *Annimali sperimentali*

Il-ġreden huma rakkommandati, imma mammiferi oħrajn jistgħu ikunu wżati. Annimali adulti żgħażagħ b'saħħithom huma miġbura kif jinzerta u assenjati għat-trattament u l-gruppi ta' kontroll.

1.6.2.2. *Numru u sess.*

Mill-anqas ħames femminili u ħames maskili għal kull esperiment u grupp tal-kontroll huma utilizzati. B'ħekk, 10 annimali jkunu sagrifikati f'kull perijodu ta' żmien għal kull grupp jekk diversi ħinijiet tat-test wara t-trattament huma inklużu fl-iskeda sperimentali. Għal grupp ta' kontroll pożittiv, ħin singolu tal-kampjunar huwa suffiċjenti.

1.6.2.3. *Rotta ta' l-amministrazzjoni*

Għaxar komposti għandhom ikunu ġeneralment amministrati darba biss. Ibbażata fuq l-informazzjoni tossikoloġika skeda ta' trattament repetut tista tkun utilizzata. B'danakollu, l-iskeda tat-trattament imtenni tista tkun applikata biss jekk il-kompost tat-test ma jkunx jesebixxi l-effetti ċitotossiċi fil-mudullun ta' għadam. Ir-rotott normali ta' l-amministrazzjoni huma orali u l-injezzjoni intraperitonali. Rottot oħrajn ta' l-amministrazzjoni jistgħu jkunu xierqa.

1.6.2.4. *L-użu ta' kontrolli negattivi u pożittivi*

Il-kontrolli pożittivi u negattivi (solventi) għandhom ikunu użati f'kull esperiment.

1.6.2.5. *Livell tad-doża*

Għal sett bażi, doża waħda tal-kompost tat-test huwa wżat, id-doża tkun il-massimu tollerat tad-doża jew dik li tipproduċi xi indikazzjoni tas-sitotossiċità, e.g. bil-kambjament tar-relattività tal-polikromatiċi ma l-eritrokati tal-normokromatiċi.

Għal komposti "mhux tossiċi", id-doża (limitu) massimu li jeħtieġ li jkun investigat b'segwiment ta' doża singola, l-amministrazzjoni hija 2 000 mg/kg il-piż tal-ġisem.

Jekk skeda ta' doża repetura tkun utilizzata, l-limitu tad-doża hu 1 000 mg/kg tal-piż tal-ġisem kuljum.

Livelli ta' doża addizzjonali jistgħu jkunu utilizzati meta huma indikati b'raġunijiet xjentifiċi.

Jekk it-test utilizzat bħala metodu għall-verifika mill-anqas żewġ livelli ta' doži addizzjonali għandhom ikunu wżati.

1.6.3. **Il-proċedura**

It-test jista jkun imwettaq b'żewġ metodi:

(i) L-annimali huma trattati bil-kompost tat-test darba. Il-ħinijiet tal-kampjunar għandhom ikunu koinċidenti mar-reazzjoni massima ta' l-assaġġ, li jvarjaw mal-kompost tat-test. Għalhekk, il-kampjuni tal-mudullun tal-għadam għandhom ikunu miġbura mill-anqas darbtejn li jibdew mhux aktar kmieni minn 12 il-siegħa minn wara t-trattament u ma jestendux 'il hinn minn 48 siegħa.

Meta doži addizzjonali huma wżati, kampjuni għandhom ikunu meħuda fl-intervalli partikolarment sensittivi jew, jekk dak ma jkunx magħruf, 24 siegħa wara t-trattament.

(ii) Jekk l-informazzjoni farmaċokinetika u metabolitika tindika skeda ta' trattament repetut, doża repetuta tista tkun utilizzata u l-kampjuni għandhom ikunu miġbura sitta u 12 siegħa wara l-aħħar trattament.

Preparazzjoni tal-mudullun tal-għadam:

Il-mudullun tal-għadam huwa akkwistat kemm mill-femora ta' annimali maqtula friski bit-tlaħliħ b'taħlita iżotonika. Iċ-ċelloli huma sedimentati biċ-ċentrifugazzjoni u s-supernatant huwa mwarrab. Taqtiriet tas-sospenzjoni taċ-ċellola omoġenika huma mqiegħda fuq lastri u mxerda bħala tiċpisa. Wara t-tnixxif fl-arja il-lametti jingħataw il-kulur.

Analiżi:

Il-lametti jkunu kodifikati qabel l-analiżi mikroskopika. Mill-anqas 1 000 eritroċiti polikromatiċi għal kull annimal huma mnaqqxa għall-inċidenza ta' mikronuklej.

Ir-relattività tal-normokromatika ma l-eritrokatiċi polikromatiċi hija determinata għal kull annimali bil-għadd ta' total ta' 1 000 eritroktiċi.

2. **DATA**

Id-*data* hija preżentata f'għamla tabulari. Għalhekk in-numru ta' eritroċiti polikromatiċi inċiżi, n-numru ta' eritroċiti bil-mikronuklej, u l-persentaġġ taċ-ċelloli mikronukleati huma elenkati separatament għal kull annimali sperimentali u tal-kontroll, kif ukoll tar-relattività tan-normokromatika ma l-eritroċiti polikromati. Il-medja tan-numru u d-devjazzjonijiet normali għal kull grupp sperimentali u ta' kontroll huma wkoll elenkati. Id-*data* hija evalwata bil-metodi statistiċi xierqa.

3. **RAPPORTAĠĠ**

3.1. RAPPORT TAT-TEST

Ir-rapport tat-test għandu, jekk possibbli, jinkludi l-informazzjoni li ġejja:

— speċi, siltiet u l-età ta' l-annimali użati;

— in-numru ta' annimali ta' kull sess fi gruppi sperimentali u tal-kontroll;

— il-kondizzjoni tat-test, deskrizzjoni dettaljata tat-trattament għall-iskeda tal-kampjunar, livelli tad-doża, dettalji tat-tossiċità. Kontrolli negattivi u pożittivi;

— kriterja għat-tnaqqix mikronukleju;

— relazzjoni tad-doża/effett, meta possibbli;

— sinjali tat-tossiċità matul il-korsa ta' l-istudju;

— evalwazzjoni statistika;

— diskussjoni tar-riżultati;

— interpretazzjoni tar-riżultati.

3.2. EVALWAZZJONI U INTERPRETAZZJONI

Ara l-Introduzzjoni Ġenerali Parti B (D).

4. **REFERENZI**

Ara l-Introduzzjoni Ġenerali Parti B (E).

B.13. MUTAĠENICITÀ (*ESCHERICHIA COLI* – ASSAĠĠ TA' MUTAZZJONI B'LURA)

1. **METODU**

1.1. INTRODUZZJONI

Ara l-Introduzzjoni Ġenerali Parti B (A).

1.2. DEFINIZZJONI

Ara l-Introduzzjoni Ġenerali Parti B (C).

1.3. SUSTANZI TA' REFERENZA

Xejn

1.4. PRINCIPJI TAL-METODU TAT-TEST

Is-sistema ta' reviżjoni tripotam *Eskerika coli* (trp) hija assaġġ mikrobjali li tkejjel il-trp. trp+ treġġiegħ lura bil-kimiċi li jikkaġunaw tibdil bażiku fil-ġenomi ta' l-organiżmi.

Il-bakterja huma esposti għall-kimiċi tat-test bi, jew mingħajr, l-attivazzjoni metabolika. Wara perijodu xieraq ta' l-inkubazzjoni fuq medja minima, kolomji revertanti huma magħduda u mqabbla man-numru ta' revertanti spontanji fuq il-kultura mhux ittrattata u/jew tas-solvent tal-kontroll.

1.5. KRITERJA TAL-KWALITÀ

Xejn

1.6. DESKRIZZJONI TAL-METODU TAT-TEST

Il-metodi li ġejjin jistgħu jkunu wżati sabiex iwettqu l-assaġġ: (1) il-metodu tal-pre-inkubazzjoni; u (2) il-metodu ta' l-inkorporazzjoni diretta, li fih il-bakterja u l-aġent tat-test huma mħallta buq kisja ta' l-agar u mferrha fuq il-wiċċ tal-pjanċa selettiva ta' l-agar.

1.6.1. **Preparazzjoni**

1.6.1.1. *Bakterja*

Il-bakterja huma mkabbra fi 37 °C sa tard fil-fażi tat-tkabbir esponenzali jew dik bikrija stazzjonarja. Id-densità approssimattiva tac-ċellola għandha tkun 108-109 ċelloli kull millilitru.

1.6.1.2. *Attivazzjoni metabolika*

Il-bakterja għandhom ikunu esposti għas-sustanza tat-test kemm fil-preżenza u fin-nuqqas tas-sistema xierqa ta' l-attivazzjoni metabolika. Is-sistema komuni l-aktar użata hija l-frazzjoni kofattur-supplementata post-mitokondrjali ippreparata mill-fwied ta' annimali gerriema trattati bl-aġenti li jinduċu l-enżimi.

1.6.2. **Kondizzjoni tat-test**

1.6.2.1. *Siltiet tat-testijiet*

Tlett siltiet, WP2, WP2 uvr A u WP2 uvr A pKM 101 għandhom ikunu wżati. Metodi rikonoxxuti tal-preparazzjonijiet tal-kultura tal-massa u mill-ħażna għandhom ikunu wżati. Il-ħtiġiet tat-tkabbir u l-identità ġenetika tas-siltiet, is-sensittività tagħhom għar-radjazzjoni UV jew il mitomiċin °C u r-reżistenza għall-ampiċillin fis-silta WP2 uvr A pKM 101 għandhom ikunu verifikati. Is-siltiet għandhom ukoll jipprovdu revertanti spontanji fi ħdan il-medda tal-frekwenza mistennija.

1.6.2.2. *Medja*

Medja xierqa għall-esprezzjoni u l-għażla tal-mutanti hija wżata b'kisja adekwata ta' l-agar.

1.6.2.3. *L-użu ta' kontrolli negattivi u pożittivi*

Kontrolli konkorrenti, mhux-trattati u s-solvent tal-kontroll għandhom ikunu mwettqa. Kontrolli pożittivi għandhom ikunu mwettqa wkoll għal żewġ skopijiet:

(i) Sabiex joffru konformità tas-sensittività tas-siltiet tal-bakterja.

Sulfonat metil metanju, ossidu 4-nitrokwinolin jew etilnitrosowrea jistgħu ikunu wżati bħala kontrolli pożittivi għat-testijiet mingħajr l-attivazzjoni metabolika.

(ii) Sabiex jassiguraw l-attività tas-sistemi metabolizzanti xierqa.

Kontroll pożittiv għall-attività ta' sistema metabolizzanti waħda għas-siltiet kollha hu 2-amino-antraċena. Meta disponibbli, kontroll pożittiv ta' l-istess klassi ta' kimika bħal dak tal-kimika fit-test għandu jkun użat.

1.6.2.4. *Ammont tas-sustanza tat-test għal kull pjanċa*

Mill-anqas ħames ammonti differenti tal-kimika tal-prova huma ittestjati, bl-intervalli ta' nofs-log bejn il-pjanċi. Is-sustanzi huma ittestjati sal-limitu tas-solubbilità jew tat-tossiċità. It-tossiċità tkun tidher bit-tnaqqis fin-numru ta; revertanti spontanji, tindif tal-medda fl-isfond, jew bi grad tas-sopravivenza tal-kulturi trattati. Kimiki mhux-tossiċi għandhom ikunu ittestjati sa 5 mg kull pjanċa qabel ma s-sustanza tat-test tkun meqjusa bħala negattiva.

1.6.2.5. *Kondizzjonijiet ta' l-inkubazzjoni*

Il-pjanċi huma inkubati għal 48 sa 72 siegħa fi 37 °C.

1.6.3. **Il-proċedura**

Għall-metodu ta' l-inkorporazzjoni diretta tal-pjanċa mingħajr l-attivazzjoni ta' l-enżima, l-kimika u 0,1 ml tal-kultura batterika friska huma miżjuda ma 2 ml tal-kisja ta' l-agar. Għal testijiet bl-attivazzjoni metabolika, 0,5 ml tat-taħlita ta' l-attivazzjoni ta' l-enżima tal-fwied li jkun fiha ammont adekwat tal-frazzjoni post-mitokondrjali hija miżjuda mal-kisja ta' l-agar wara ż-żieda tal-kimika tat-test u tal-bakterja. Il-kontenuti ta' kull tubu huma mħallta u mferrgħa fuq il-wiċċ ta' pjanċa selettiva ta' l-agar. Il-kisja ta' l-agar tkun imħollija li tissolidifika u l-pjanċi huma inkubati fi 37 °C għal 48 sa 72 siegħa. Fit-tmiem tal-perijodu ta' l-inkubazzjoni, kolonji revetanti ghal kull pjanċa huma magħduda.

Għall-metodu tal-pre-inkubazzjoni, taħlita tal-kimika tat-test 0,1 ml ta' kultura batterika friska u ammont adekwat tat-taħlita ta' l-attivazzjoni ta' l-enżima tal-fwied jew l-istess ammont tal-kuxxin hija pre-inkubata qabel iż-żieda ta' 2 ml tal-kisja ta' l-agar. Il-proċeduri l-oħrajn kollha huma l-istess għall-metodu ta' l-inkorporazzjoni.

Il-pjanċi kollha għaż-żewġ metodi huma mlestija mill-anqas fi tlieta.

2. **DATA**

In-numri tal-kolonji revertanti għal kull pjanċa huma irrapportati kemm għas-serje tal-kontroll u wkoll ta' dawk ittrattati. L-għadd ta' pjanċi individwali, n-numru medju tal-kolonji revertanti kull pjanċa u d-devjazzjonijiet normali għandhom ikunu preżentati għall-kimika tat-test u tal-kontrolli.

Id-*data* hija evalwata bl-użu tal-metodi statistiċi xierqa.

Mill-anqas żewġ esperiment independenti huma mwettqa. Mhux neċessarju li jkun imwettaq it-tieni test bl-istess mod identiku ta' l-esperiment inizjali. Tabilħaqq, jista jkun preferibbli li jkunu mibdula l-kondizzjonijiet tat-test sabiex tkun akkwistata *data* aktarutili.

3. **RAPPORTAĠĠ**

3.1. RAPPORT TAT-TEST

Ir-rapport tat-test għandu, jekk possibbli, jinkludi l-informazzjoni li ġejja:

— il-bakterja, siltiet użati;

— il-kondizzjoni tat-test, livelli tad-doża, tossiċità, komposizzjoni tal-medja, proċeduri tat-trattament (pre-inkubazzjoni, inkubazzjoni); sistema ta' l-attivazzjoni metabolika; sustanzi ta' referenza, kontrolli negattivi;

— għadd tal-pġjanċi individwali, in-numru medju tal-kolonji revertanti kull pjanċa, id-devjazzjoni normali, ir-relazzjoni doża/effett, meta possibbli;

— diskussjoni tar-riżultati;

— interpretazzjoni tar-riżultati.

3.2. EVALWAZZJONI U INTERPRETAZZJONI

Ara l-Introduzzjoni Ġenerali Parti B (D).

4. **REFERENZI**

Ara l-Introduzzjoni Ġenerali Parti B (E).

B.14. MUTAĠENICITÀ (*SALMONELLA TYPHIMURIUM* – ASSAĠĠ TA' MUTAZZJONI B'LURA)

1. **METODU**

1.1. INTRODUZZJONI

Ara l-Introduzzjoni Ġenerali Parti B (A).

1.2. DEFINIZZJONI

Ara l-Introduzzjoni Ġenerali Parti B (C).

1.3. SUSTANZI TA' REFERENZA

Xejn

1.4. PRINCIPJU TAL-METODU TAT-TEST

Is-sistema ta' revirsjoni tas-*Salmonella tifimurjum* ħistidina (his) hija assaġġ mikrobjali li tkejjel il-trp. trp+ treġġiegħ lura bil-kimiċi li jikkaġunaw tibdil bażiku fil-ġenomi ta' l-organiżmi.

Il-bakterja huma esposti għall-kimiċi tat-test bi, jew mingħajr, l-attivazzjoni metabolika u mqiegħda fuq pjanċi ta' medja minimali. Wara perijodu xieraq ta' l-inkubazzjoni fuq medja minima, kolomji revertanti huma magħduda u mqabbla man-numru ta' revertanti spontanji fuq il-kultura mhux ittrattata u/jew tas-solvent tal-kontroll.

1.5. KRITERJA TAL-KWALITÀ

Xejn

1.6. DESKRIZZJONI TAL-METODU TAT-TEST

1.6.1. **Preparazzjonijiet**

1.6.1.1. *Bakterja*

Kulturi friski tal-bakterja huma mkabbra fi 37 °C sa tard fil-faži tat-tkabbir esponenzali jew dik bikrija stazzjonarja. Id-densità approssimattiva tal-ċellola għandha tkun 108 sa 109 ċelloli kull millilitru.

1.6.1.2. *Attivazzjoni metabolika*

Il-bakterja għandhom ikunu esposti għas-sustanza tat-test kemm fil-preżentza u fin-nuqqas tas-sistema xierqa ta' l-attivazzjoni metabolika. Is-sistema komuni l-aktar użata hija l-frazzjoni kofattur-supplementata post-mitokondrjali ippreparat mill-fwied ta' annimali gerriema trattati bl-aġenti li jinduċu l-enżimi.

1.6.2. **Kondizzjonijiet tat-test**

1.6.2.1. *Siltiet tat-testijiet*

Mill-anqas erba' siltiet TA 1535, TA 1537 jew TA 97, TA 98 u TA 100 huma wżati; il-siltiet l-oħrajn bħalma huma TA 1538 u TA 102 jistgħu ikunu wżati bħala żidea. Metodi rikonoxxuti tal-preparazzjonijiet tal-kultura tal-massa u mill-ħażna għandhom ikunu wżati. Il-ħtiġiet tat-tkabbir u l-identità ġenetika tas-siltiet, is-sensittività tagħhom għar-radjazzjoni UV u l-vjola kristalliku, u tar-reżistenza tagħhom għall-ampiċillin għandhom ikunu verifikati. Is-siltiet għandhom ukoll jipprovdu revertanti spontanji fi ħdan il-medda tal-frekwenza mistennija.

1.6.2.2. *Medja*

Medja selettiva xierqa hija wżata b'kisja adekwata ta' agar.

1.6.2.3. *L-użu ta' kontrolli negattivi u pożittivi*

Kontrolli konkorrenti, mhux-trattati u s-solvent tal-kontroll għandhom ikunu mwettqa. Kontrolli pożittivi għandhom ikunu mwettqa wkoll għal żewġ skopijiet:

(i) Sabiex joffru kormità tas-sensittività tas-siltiet tal-bakterja.

Il-komposti li ġejjin jistgħu jkunu wżati għat-testijiet mingħajr l-attivazzjoni metabolika:

Siltiet	Imorru lura bi
TA 1535, TA 100	Ażid tas-sodju
TA 1538, TA 98, TA 97	2-nitroflworin
TA 1537	9-aminoaċridin
TA 102	idroperossidu JUMenju

(ii) Sabiex jassiguraw l-attività tas-sistemi metabolizzanti xierqa.

Kontroll pożittiv għall-attività ta' sistema metabolizzanti waħda għas-siltiet kollha hija 2-amino-antraċena. Meta disponibbli, kontroll pożittiv ta' l-isrtess klassi ta' kimika bħal dak tal-kimika fit-test għandu jkun użat.

1.6.2.4. *Ammont tas-sustanza tat-test għal kull pjanċa.*

Mill-anqas ħames ammonti differenti tal-kimika tal-prova huma ittestjati, bl-intervalli ta' nofs-log bejn il-pjanċi. Is-sustanzi huma ittestjati sal-limitu tas-solobbilità jew tat-tossiċità. It-tossiċità tkun tidher bit-tnaqqis fin-numru ta; revertanti spontanji, tindif tal-medda fl-isfond, jew bi grad tas-sopravivenza tal-kulturi ittrattati. Kimiki mhux-tossiċi għandhom ikunu ittestjati sa 5 mg kull pjanċa qabel is-sustanza tat-test tkun meqjusa bħala negattiva.

1.6.2.5. *Kondizzjonijiet ta' l-inkubazzjoni*

Il-pjanċi huma inkubati għal 48 sa 72 siegħa fi 37 °C.

1.6.3. **Il-proċedura**

Għall metodu ta' l-inkorporazzjoni diretta tal-pjanċa mingħajr l-attivazzjoni ta' l-enżima, il-kimika u 0,1 ml tal-kultura batterika friska huma miżjuda ma 2 ml tal-kisja ta' l-agar. Għal testijiet bl-attivazzjoni metabolika, 0,5 ml tat-taħlita ta' l-attivazzjoni ta' l-enżima tal-fwied li jkun fiha ammont adekwat tal-frazzjoni post-mitokondrjali hija miżjuda mal-kisja ta' l-agar wara ż-żieda tal-kimika tat-test u tal-bakterja. Il-kontenuti ta' kull tubu huma mħallta u mferrgħa fuq il-wiċċ ta' pjanċa selettiva ta' l-agar. Il-kisja ta' l-agar tkun imħollija li tissolidifika u l-pjanċi huma inkubati fi 37 °C għal 48 sa 72 siegħa. Fit-tmiem tal-perijodu ta' l-inkubazzjoni, kolonji revetanti kull pjanċa huma magħduda. Għamm-metodu tal-pre-inkubazzjoni, taħlita tal-kimika tat-testm 0,1 ml ta' kultura batterika friska u ammont adekwat tat-taħlita ta' l-attivazzjoni ta' l-enżima tal-fwied jew l-istess ammont tal-kuxxin hija pre-inkubata qabel iż-żieda ta' 2 ml tal-kisja ta' l-agar. Il-proċeduri l-oħrajn kollha huma l-istess għall-metodu ta' l-inkorporazzjoni.

Il-pjanċi kollha għaż-żeġ metodi huma mlestija mill-anqas fi tlieta.

2. **DATA**

In-numri tal-kolonji revertanti kull pjanċa huma irraportati kemm għas-serje tal-kontroll u wkoll ta' dawk ittrattati.

L-għadd ta' pjanċi individwali, n-numru medju tal-kolonji revertanti kull pjanċa u d-devjazzjonijiet normali għandhom ikunu preżentati għall-kimika tat-test u tal-kontrolli.

Id-*data* hija evalwata bl-użu tal-metodi statistiċi xierqa.

Mill-anqas żewġ esperiment independenti huma mwettqa. Mhux neċessarju li jkun imwettaq it-tieni test bl-istess mod identiku ta' l-esperiment inizjali. Tabilħaqq, jista jkun preferibbli li jkunu mibdula l-kondizzjonijiet tat-test sabiex tkun akkwistata *data* aktar utili.

3. **RAPPORTAĠĠ**

3.1. RAPPORT TAT-TEST

Ir-rapport tat-test għandu, jekk possibbli, jinkludi l-informazzjoni li ġejja:

— il-bakterja, siltiet użati;

— il-kondizzjoni tat-test, livelli tad-doża, tossiċità, komposizzjoni tal-medja, proċeduri tat-trattament (pre-inkubazzjoni, inkubazzjoni); sistema ta' l-attivazzjoni metabolika; sustanzi ta' referenza, kontrolli negattivi;

— għadd tal-pjanċi individwali, n-numru medju tal-kolonji revertanti kull pjanċa, id-devjazzjoni normali, r-relazzjoni doża/effett, meta possibbli;

— diskussjoni tar-riżultati;

— interpretazzjoni tar-riżultati.

3.2. EVALWAZZJONI U INTERPRETAZZJONI

Ara l-Introduzzjoni Ġenerali Parti B (D).

4. **REFERENZI**

Ara l-Introduzzjoni Ġenerali Parti B (E).

PARTI C: METODI GĦAD-DETERMINAZZJONI TAT-TOSSICITÀ

C.1. TOSSICITÀ AKUTA GĦALL ĦUT

1. **METODU**

1.1. INTRODUZZJONI

L-iskop ta' dan it-test huwa sabiex jiddetermina t-tossiċita letali akuta ta' sustanza għall-ħut fl-ilma ħelu. Huwa mixtieq li jkun hemm, sa kemm ikun possibbli, informazzjoni dwar is-solubbiltà fl-ilma, il-pressjoni tal-fwar, l-istabbilità kimika, ilpkostanti tad-disassoċjazzjoni u tal-biodegrabbilità tas-sustanza sabiex tgħin fil-għażla tal-metodu tat-test l-aktar xieraq (statiku, semi-statiku jew ta' kurrent kontinwu) sabiex ikunu assigurati l-konċentrazzjoninijiet kostanti sodisfaċenti tas-sustanza tat-test matul il-perijodu tat-test.

Informazzjoni addizzjonali (per eżempju l-formula strutturali, il-grad tal-purità, n-natura u l-persentaġġ ta' impuritajiet sinifikanti, l-preżenza u l-ammonti ta' addittivi, u l-ko-effiċjent tal-qasma n-oktonal/ilma) għandhom ikunu meqjusa kemm fl-ippjanar tat-test u fl-interpretazzjoni tar-riżultati.

1.2. DEFINIZZJONIJIET U UNITAJIET

Tossiċità akuta hija l-effett ħażin apparenti intiż f'organiżmu matul perijodu qasir (jiem) ta' l-esposizzjoni tiegħu għal sustanza. Fit-test preżenti, tossiċità akuta hija espressa bħala l-konċentrazzjonini medjana letali LD50), jiġifieri l-konċentrazzjoni fl-ima li toqtol 50% f'lott ta' prova ta' ġut matul perijodu kontinwu ta' l-esposizzjoni li għandu jkun mistqarr.

Il-konċentrazzjoninijiet kollha tas-sustanza fit-test huma mogħtija bil-piż/volum (milligrammi kull litru). Dawn jistgħu ikunu espressi wkoll bħala piż bil-piż (mg.kg-1).

1.3. SUSTANZI TA' REFERENZA

Sustanza ta' referenza tista tkun ittestjata bħala mezz sabiex turi li l-kondizzjonijiet waqt it-test fil-laboratorju, ir-reazzjoni tal-ispeċi ittestjati ma jkunux inbidlu b'mod sinifikanti.

L-ebda sustanzi ta' referenza ma huma speċifikati f'din il-kitba.

1.4. PRINCIPJU TAL-METODU TAT-TEST

Test limitat jista' jkun imwettaq fi 100 mg kull litru sabiex tkun determinata li il-LC50 tkun akbar minn din il-konċentrazzjoni.

Il-ħut hu espost għas-sustanza tat-test miżjuda ma' l-ilma fil-medda tal-konċentrazzjonijiet għal perijodu ta' 96 siegħa. Il-mortalitajiet huma irreġistrati fi mill-anqas intervalli ta' 24 siegħa, u l-konċentrazzjoninijiet li joqtlu 50% tal-ħut (LC_{50}) f'kull żmien ta' l-osservazzjoni huma ikkalkolati, meta dan ikun possibbli

1.5. KRITERJA TAL-KWALITÀ

Il-kriterja tal-kwalità għandha tkun applikabbli għal-limitu tat-test kif ukoll għall-metodu tat-test kollu.

Il-mortalità fil-kontrolli m'għandhiex teċċedi 10% (jew ħuta waħda jekk anqas minn għaxa jkunu wżati) sat-tmiem tat-test.

Il-konċentrazzjoni ta' l-ossiġenu mdewweb għandha tkun ta' aktar monn 60% tal-valur tas-saturazzjoni ta' l-arja matul iż-żmien kollu.

Il-konċentrazzjonijiet tas-sustanza tat-test għandhom jinżammu sa fil-limitu ta' 80% tal-konċentrazzjoninijiet inizjali matul iż-żmien kollu tat-test.

Għal sustanzi li jdubu faċilment fil-medja tat-test, b'hekk ifornu taħlitiet stabbli, i.e. dawk li ma jkollhomx reazzjoni volatili sinifikanti, jkollhom degradazzjoni, ikunu idrolizzati jew adsorbiti, l-konċentrazzjoni inizjali tista tkun meqjusa bħala ekwivalenti tal-konċentrazzjoni nominali. Evidenza għandha tkun ippreżentata li l-livelli tal-konċentrazzjonijiet ikunu nżammu matul it-test u li l-kriterja tal-kwalità kienet ġiet milħuqa.

Għal sustanzi li huma:

(i) ftit li xejn jinħallu fil-medja tat-test, jew

(ii) kapaċi li jiffurmaw emulsjonijiet jew dispersjonijiet stabbli, inkella

(iii) ma humiex stabbli f'taħlitiet akweji,

l-konċentrazzjonini inizjali għandha tkun meqjusa bħala l-konċentrazzjoni mkejla fit-taħlita (jew, jekk teknikament dan ma jkunx possibbli, imkejla f'kolonna ta' l-ilma) fil-bidu tat-test. Il-konċentrazzjoni għandha tkun determinata wara perijodu ta' ekwilibriju imma qabel l-introduzzjoni tal-ħut tat-test.

Fi kwalunkwe minn dawn il-każi, aktar kejl għandu jsir matul it-test sabiex jikkonferma l-konċentrazzjoni attwali ta' l-esposiżżjoni jew li l-kriterja tal-kwalità tkun ġiet milħuqha.

Il-pH m'għandux ivarja b'aktar minn unità waħda.

1.6. DESKRIZZJONI TAL-METODU TAT-TEST

Tlett tipi ta' proċedura jistgħu ikunu wżati:

Test statiku:

Test tat-tossiċità li fih l-ebda ċaqlieq ma jseħħ tat-taħlita tat-test. (It-taħlitiet jinqgħu mingħajr tibdil matul iż-żmien kollu tat-test.)

Test semi-statiku:

Test mingħajr ċaqlieq tat-taħlita tat-test, imma bit-tiġdid regolari tal-lottijiet tat-taħlitiet tat-test eara perijodi mtawwla (e.g. 24 siegħa).

Test biċ-ċaqlieq kontinwu:

Test tat-tossiċità li fih l-ilma jkun imġedded regolarment fil-kabini tat-test, il-kimika li tkun ittestjata tiġi trasportata ma l-ilma użat għat-tiġdid tal-medja tat-test.

1.6.1. **Reaġenti**

1.6.1.1. *Taħlitiet tas-sustanzi tat-test*

Taħlitiet diġa lesti tas-saħħa meħtieġa huma preparati bit-tidwib tas-sustanza f'ilma de-jonizzat jew f'ilma skond 1.6.1.2.

Il-konċentrazzjoni tat-test kif magħżula huma ippreparati bit-tidwib tat-taħlita diġa lest. Jekk konċentrazzjoninijiet għoljin huma ittestjati, is-sustanza tista tkun imdewba direttament mat-trattib ta' l-ilma.

Is-sustanza għandha normalment tkun ittestjata biss sal-limitu tas-solubbilità tagħha. Għal uħud mis-sustanzi (e.g. sustanzi li jkollhom solubbilità baxxa fl-ilma, jew P_{ow9} għoli, jew dawk li jiffurmaw tixriq stabbli pjuttost milli taħlita vera fl-ilma), huwa aċċettabbli li tintuża l-konċentrazzjonini tat-test aktar għolja mil-limitu tal-solubbilità tas-sustanza sabiex ikun assigurat li s-solubbilità massima/stabbilità tal-konċentrazzjoni tkun intlaħqet. Huwa mportanti, b'danakollu, li din il-konċentrazzjoni ma tkunx b'mod ieħor tħawwad is-sistema tat-test (e.g, li jkun hemm rita tas-sustanza fil-wiċċ ta' l-ilma li ma tħallix li jseħħ l-ossiġinazzjoni ta' l-ilma, etc.).

Tixrid ultrasoniku, solventi organiċi, emulsifikaturi jew dispersanti jistgħu jintużaw bħala għajnuna għat-tħejjija tat-taħlitiet bil-lest ta' sustanzi b'solubbilità baxxa fl-ilma jew sabiex jgħinu fit-tixrid ta' dawn is-sustanzi fil-medja tat-test. Meta tali sustanzi awżiljarji huma wżati, il-konċentrazzjoninijiet kollha tat-test għandhom jinkludu l-istess ammont tas-sustanza awżiljarja, u ħut addizzjonali tal-kontroll għandhom ikunu esposti għall-istess konċentrazzjoni tas-sustanza awżiljarja bħal dik użata fis-serje tat-test. Il-konċentrazzjoni ta' tali awżiljarji għandha tkun minimizzata, imma fl-ebda każ m'għandha teċċedi 100 mg kull litru fil-medja tat-test.

It-test għandu jkun imwettaq mingħajr l-aġġustament tal-pH. Jekk ikun hemm evidenza ta' tibdil qawwi fil-pH, huwa rakkomandat li t-test għandu jkun repetut bi pH aġġustat u r-riżultati inklużu fir-rapport. F'dak il-każ, il-valur tal-pH tat-taħlita bil-lest għandu jkun aġġustat għall-valur tal-pH ta' l-ilma tat-taħlit, sakemm ma jkunx hekk raġunijiet speċifiċi il-għala dan m'għandux isir. HCI u NaOH huma preferuti għal dan l-iskop. Dan l-aġġustament tal-pH għandu jsir b'tali mod hekk li l-konċentrazzjoni tas-sustanza tat-test fit-taħlita bil-lest ma tkunx mibdula sa xi medda sinifikanti. Jekk ikun hemm xi reazzjoni kimika jew preċipitazzjoni fiżika tal-kompost tat-test ikkaġunata bl-aġġustament, dan għandu jkun mistqarr.

1.6.1.2. *Ilma taż-żamma u tat-trattib*

Forniment ta' ilma tax-xorb (mhux kontaminat b'konċentrazzjonijiet potenzalment ta' ħsara mill-klorin, metalli tqal jew sustanzi oħrajn), ilma naturali ta kwalità tajba jew ilma rikostitwita (ara l-Appendiċi I) jista jkun użat. Ilmijiet b'ebusija totali ta' bejn 10 u 250 mg kull litru (bħala $CaCO_3$) u bi pH minn 6,0 sa 8,5 huma preferuti.

1.6.2. **Apparat**

L-apparat kollu għandu jkun magħmul minn materjal kemikalment inert.

— sistema ta' taħlil awtomatiku (għal test b'ċaqlieq kontinwu),

— arloġġ ta' l-ossiġenu,

— apparat għad-determinazzjoni ta' l-ebusija ta' l-ilma,

— apparat adekwat għall-kontroll tat-temperatura,

— arloġġ pH.

1.6.3. **Ħut tat-test**

Il-ħut għandu jkun f'kondizzjoni tajba tas-saħħa u ħieles minn xi malformazzjoni apparenti.

L-ispeċi użati għandhom ikunu magħżula fuq il-bażi ta' kriterja prattika, bħalma hija d-disponibbiltà faċli tagħhom matul is-sena, l-faċilità tal-manutenzjoni, l-konvenjenza għat-testijiet, is-sensittività relattiva għall-kimika u xi fatturi ekonomiċi, bioloġiċi u ekoloġiċi li jistgħu jaffettwaw ir-riżultati. Il-ħtieġa tal-kompattibbiltà tad-*data* akkwistat u l-armonizzazzjoni internazzjonali eżistenti (referenza 1) għandha wkoll tkun imfakkra meta ssir l-għażla tal-ispeċi tal-ħut.

Lista tal-ispeċi tal-ħut li huma rakkommandati għat-twettieq ta' dan it-test hija mogħtija fl-Appendiċi 2; il-ħut irrigat (taż-żebra) u t-trotta ikkulurita huma l-ispeċi preferuti.

1.6.3.1. *Iż-zamma*

Il-ħut tat-test preferibbilment jiġu minn lott wieħed ta' l-istess tul u età. Il-ħut għandu jinżamm għal mill-anqas 12 il-jum, fil-kondizzjonijiet kif ġejjin:

tagħbija:

xierqa għas-sistema (riċirkolazzjoni jew kurrent kontinwu) u l-ispeċi tal-ħut,

l-ilma:

ara 1.6.1.2,

dawl:

12 sa 16 il-siegħa ta' dawl kuljum,

konċentrazzjoni ta' ossiġenu mdewweb:

mill-anqas 80% tal-valur tas-saturazzjoni ta' l-arja,

ikel:

tlett darbiet fil-ġimgħa jew kull jum, jieqaf 24 siegħa qabel il-bidu tat-test.

1.6.3.2. *Mortalità*

B'segwiment tal-perijodu ta' 48 siegħa sakem joqgħodu, il-mortalitajiet huma reġistrati u din il-kriterja li ġejja għandha tkun applikata.

— aktar minn 10% tal-popolazzjoni matul sebat ijiem:

ċaħda tal-lott kollu kemm hu,

— bejn 5 u 15% tal-popolazzjoni:

perijodu taż-żamma ikompli għal sebat ijiem addizzjonali. Jekk l-ebda mortalità ma sseħħ, il-lott huwa aċċettabbli, altrimentri għandu jkun miċħud,

— anqas minn 5% tal-popolazzjoni:

aċċettazzjoni tal-lott.

1.6.4. **Adattazzjoni**

Il-ħut kollu għandu jkun espost għall-ilma tal-kwalità u tat-temperatura li jkunu wżati fit-test għal mill-anqas sebat ijiem qabel ma jintużaw

1.6.5. **Proċedura tat-test**

Test tas-sejba tal-medda jista jipprekedi t-test definittiv, sabiex tkun akkwistata l-informazzjoni dwar il-medda tal-konċentrazzjonijiet li għandhom ikunu wżati fit-test ewlieni.

Kontroll wieħed mingħajr is-sustanza tat-test għandu jkun magħmul u, jekk relevanti, kontroll wieħed li jkun fih sustanza awżiljarja għandu wkoll ikun magħmul, b'żieda mas-serje tat-testijiet.

Jiddependi mill-proprjetajiet fiżiċi u kimiċi tal-kompost tat-test, test statiku, semi-statiku jew biċ-ċaqlieq kontinwu għandu jkun magħżul skond kif xieraq, sabiex iwettaq il-kriterja tal-kwalità.

Il-ħut huwa espost għas-sustanza kif deskritt hawn taħt:

— tul ta' żmien: 96 siegħa

— numru ta' l-annimali: mill-anqas 7 kull konċentrazzjoni,

— tankijiet: ta' daqs adattat b'relazzjoni mat-tagħbija rakkommandata,

— tagħbija: tagħbija massima ta' 1 g kull litru għal testijiet statiċi u semi-statiċi hija rakkommandata; għal sistemi ta' ċaqlieq kontinwu, tagħbija għola hija aċċettabbli

— konċentrazzjoni tat-test: Mill-anqas ħames konċentrazzjoninijiet li jiddifferixxu b'fattur kostanti li ma jeċċedix 2,2 u kemm jista jkun possibbli li jkopri l-medda ta' 0 sa 100% mortalità.

— ilma: ara 1.6.1.2,

— dawl: 12 sa 16 il-siegħa ta' dawl kuljum,

— temperatura: xierqa għall-ispeċi (l-Appendiċi 2) imma fi ħdan ± 1 °C skond it-test partikolari,

— konċentrazzjoni ta' ossiġenu mdewweb: mhux anqas minn 60% tal-valur tas-saturazzjoni ta' l-arja fit-temperatura magħżula,

— ikel: xejn.

Il-ħut ikunu spezzjonati wara l-ewwel 2 sa 4 siegħat u mill-anqas matul intervalli ta' 24 siegħa. Il-ħut huma meqjusa bħala mejta jekk meta jkunu minsusa fil-pedunkula kawdjali ma jipproduċu l-ebda reazzjoni u l-ebda movimenti tan-nifs ma jkunu viżibbli. Il-ħut mejta jkunu mneħħija malli dan ikun osservat u l-mortalitajiet huma reġistrati.

Reġistri jinżammu ta' annormalitajiet viżibbli (e.g. talf ta' l-ekwilibriju, tibdil fl-imġieba ta' l-għawm, funzjoni respiratorja, pigmentazzjoni, etc.).

Il-kejl tal-pH, l-ossiġenu mdewweb u tat-temperatura għandhom jitwettqu ta' kuljum.

Test ta' limitazzjoni

Bl-użu tal-proċeduri deskritti f'dan il-metodu tat-test, test tal-limitazzjoni jista jkun imwettaq fi 100 mg kull litru sabiex ikun muri li l-LC50 iku akbar minn din il-konċentrazzjoni.

Jekk in-natura tas-sustanza tkun tali li konċentrazzjoni ta' 100 mg kull litru fl-ima tat-test ma tistax tkun milħuqha, it-test tal-limitazzjoni għandu jkun imwettaq f'konċentrazzjoni egwali għas-solubbilità tas-sustanza (jew tal-konċentrazzjoni massima li tifforma tixrid stabbli) fil-medja użata (ara wkoll il-punt 1.6.1.1).

It-test tal-limitazzjoni għandu jkun imwettaq bl-użu ta' minn 7 sa 10 ħutiet, bl-istess numru ta' kontroll(i). (It-teorija binominali tiddetta li meta 10 ħutiet huma wżati b'mortalità żero, ikun hemm 99,9% affidabbilità li l-LC_{50} huwa akbar mill-konċentrazzjonini użata f'dak it-test tal-limitazzjoni. Bi 7, 8 jew 8 ħutiet, l-assenza tal-mortalità tipprovdi mill-anqas 99% affidabbilità li l-LC_{50} huwa akbar mill-konċentrazzjoni użata.)

Jekk ikun hemm mortalijiet, studju sħiħ għandu jkun imwettaq. Jekk effetti sub-letali huma osservati, dawn għandhom ikunu reġistrati.

2. **DATA U EVALWAZZJONI**

Għal kull perijodu ta' meta l-osservazzjonijiet kienu reġistrati (24, 48, 72 u 96 siegħa), pinġi l-persentaġġ tal-mortalità għal kull perijodu ta' l-esposizzjoni kif rakkommandat kontra l-konċentrazzjoni fuq karta tal-probabbiltà logaritmika,

Meta possibbli u għal ħin ta' l-osservazzjoni, l-LC_{50} u l-limiti tal-kunfidenza (p = 0,05) għandhom ikunu ikkalkolati bl-użu tal-proċeduri normali; dawn il-valuri għandhom ikunu mqarrba lejn wieħed, l-aktar lejn tnejn, figuri sinifikanti (eżempji tat-tqarrib lejn żewġ figuri: 170 għal 173,5; 0,13 għal 0,127; 1,2 għal 1,21).

F'dawk il-każi meta l-linja magħwġa bil-kurva tal-konċentrazzjoni/persentaġġ tar-reazzjoni tkun wieqfa wisq sabiex tippermetti l-kalkolazzjoni tal-LC_{50}, estimi grafiċi ta' dan il-valur huma suffiċjenti.

Meta żewġ konċentrazzjonijiet konsekuttivi, b'relattività ta' 2,2 jagħtu biss 0 u 100% mortalità, dawn iż-żewġ valuri huma suffiċjenti sabiex jindikaw il-medda li fiha jkun jidħol il-LC_{50}.

Jekk ikun osservat li l-istabbilità jew l-omoġenità tas-sustanza tat-test ma jistgħux jinzammu dan għandu jkun irrapportat u qies għandu jittieħed fl-interpretazzjoni tar-riżultati.

3. **RAPPORTAĠĠ**

Ir-rapport tat-test għandu, jekk possibbli, jinkludi l-informazzjoni li ġejja:

— informazzjoni dwar il-ħut tat-test (isem xjentifiku, razza, fornitur, xi pre-trattament, daqs u numru użat f'kull konċentrazzjoni tat-test);

— is-sorsi tat-trattib - ilma u l-karatteristiċi kimiċi maġġuri (pH, ebusija, temperatura);

— fil-każ ta' sustanza b'solubilità akwea baxxa, l-metodu tal-preparazzjoni tat-taħlitiet kemm dawk imħejjija lesti u tat-test;

— konċentrazzjoni ta' xi sustanzi awżiljarji;

— lista tal-konċentrazzjonijiet użati u xi informazzjoni disponibbli dwar l-istabbilità tal-konċentrazzjonijiet tal-kimika ittestjata fit-taħlita tat-test;

— jekk analiżi kimika titwettaq, metodi wżati u r-riżultati akkwistati;

— r-riżultati tat-test tal-limitazzjoni, jew imwettaq;

— ir-raġunijiet tal-għażla u d-dettalji tal-proċedura tat-test kif użata (e.g. statiku, semi-statiku, rata tad-dożar, rata taċ-ċaqlieq kontinwu, jekk huma mogħti l-arja, t-tagħbija tal-ħut, etc.);

— deskrizzjoni ta' l-apparat tat-test;

— l-organizzazzjoni tad-dawl;

— il-konċentrazzjonijiet ta' l-ossiġenu mdewweb, il-valuri pH u t-temperaturi tat-taħlitiet tat-test kull 24 siegħa;

— evidenza tal-kriterja tal-kwalità li tkun ġiet imwettqa;

— tabella li turi il-mortalità kumulattiva ta' kull konċentrazzjoni u l-kontroll (u l-kontroll bis-sustanza awżiljarja, jekk meħtieġ) f'kull wieħed mill-ħinijiet rakkommandati ta' l-osservazzjoni;

— grafika tal-konċentrazzjoni/persentaġġ tal-kurva tar-reazzjoni fit-tmiem ta' kull test;

— jekk possibbli, l-valuri LC_{50} f'kull wieħed mill-ħinijiet rakkommandati ta' l-osservazzjoni (bi 95% tal-limiti ta' l-affidibbiltà);

— proċedura statistiċi wżati għad-determinazzjoni tal-valuri LC_{50};

— jekk tkun użata sustanza ta' referenza, r-riżultati akkwistati,

— l-ogħla konċentrazzjoni tat-test li ma toħloq l-ebda mortalità matul il-perijodu tat-test;

— l-anqas konċentrazzjoni tat-test li toħloq 100% mortalità matul il-perijodu tat-test.

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Appendiċi 1

Ilma rikostitwit

Eżempju ta' trattib xieraq ta' l-ilma

Il-kimiċi kollha għandhom ikunu ta' grad analitiku.

L-ilma għandu jkun distillat ta' kwalità tajba, jew ilma dejonizzat b'konduttività ta' anqas minn 5 μScm^{-1}.

Apparat għad-distillazzjoni ta' l-ilma m'għandux ikun fih xi partijiet magħmula mir-ramm.

Taħlitiet bil-lest

$CaCI_2$ $2H_20$ (Diħidrat tal-klorid tal-kalċju):	11,76 g
Dewweb fi, u wassal għal litru bl-ilma.	
$MgSO_4$ $7H_20$ (ħeptaidrat tas-sulfat tal-magneżju):	4,93 g
Dewweb fi, u wassal għal litru bl-ilma.	
$NaHCO_3$ (karbonat ta' l-idroġenu tas-sodju):	2,59 g
Dewweb fi, fl-ilma u wassal għal litru bl-ilma.	
KC1 (klorid tal-putassa):	0,23 g
Dewweb fi, u wassal għal litru bl-ilma.	

Ilma rikostitwit għat-tidwib

Ħawwad 25 ml ta' kull wieħed ta' l-erba taħlitiet bil-lest u wassal sa litru bl-ilma.

Qawwi bl-arja sakemm il-konċentrazzjonini ta' l-ossiġenu mdewweb tkun egwali għall-valur tas-saturizzazzjoni ta' l-arja.

Il-pH għandu jkun 7,8 ± 0,2.

Jekk meħtieġ, aġġusta il-pH bi NaOH (idrossidu tas-sodju) jew HCI (aċidu idrokloriku).

L-ilma tat-trattib hekk ippreparat huwa mwarrab għal madwar 12 il-siegħa u l-ebda arja aktar ma tkun miżjuda.

Is-somma tal-jonji Ca u Mg f'din it-taħlita hija ta' 2,5 mmol kull litru. Ir-relattività tal-jonji Ca:Mg hija 4:1 u tal-joni Na:K hija 10:1. L-alkalinità totali ta' din it-taħlita hija 0,8 mmol kull litru.

Kwalunkwe devjazzjoni fil-preparazzjoni ta' l-ilma tat-trattib m'għandhiex tibdel il-komposizzjoni jew il-proprjetajiet ta' l-ilma.

Appendiċi 2

Speċi ta' ħut rakkommandat għat-testijiet

Speċje rakkommandati	Medda rakkommandata tat-Temperatura tat-Test (°C)	Tul total rakkommandat ta' l-annimal tat-test (cm)
Brachydanio rerio (Teleostei, Cyprinidae) (Hamilton—-Buchanan) Ħut-Zebrab	20 sa 24	3,0 ± 0,5
Pimephales promelas (Teleostei, Cyprinidae) (Rafinesque) Fathead minnow (ħut ta' l-ilma ħelu)	20 sa 24	5,0 ± 2,0
Cyprinus carpio (Teleostei, Cyprinidae) (Linneaus 1758) Karpjun komuni	20 sa 24	6,0 ± 2,0
Oryzias latipes (Teleostei, Poeciliidae) Cyprinodontidae (Tomminck and Schiegel 1850) Killifish ħamrani	20 sa 24	3,0 ± 1,0
Poecilia reticulata (Teleostei, Poeciliidae) (Peters 1859) Guppy	20 sa 24	3,0 ± 1,0
Lepomis macrochirus (Teleostei, Centrarchidae) (Rafinesque Linneaus 1758) Bluegill	20 sa 24	5,0 ± 2,0
Onchorhynchus mykiss (Teleostei, Salmonidae) (Walbaum 1988) Trota kulurita	12 sa 17	6,0 ± 2,0
Leuciscus idus (Teleostei, Cyprinidae) (Linneaus 1758) Orfe dehbi	20 sa 24	6,0 ± 2,0

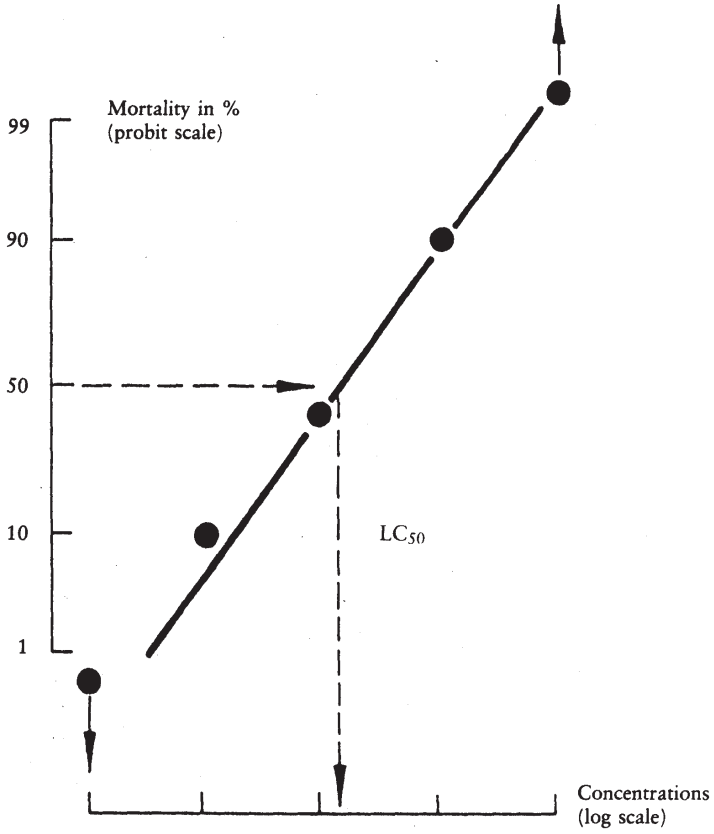
Ġabra

Il-ħut elenkat hawn fuq huma faċli sabiex jitkabbru u/jew faċilment disponibbli matul is-sena kollha. Dawn jistgħu ikunu mkabbra u ikkultivati jew fl-irzizet tal-ħut jew fil-laboratorju, f'kondizzjonijiet ikkontrollati dwar il-mard u l-parassiti, hekk li l-annimal tat-test ikunu b'saħħtu u ta' nisel magħruf. Dan il-ħut huwa disponibbli f'ħafna partijiet tad-dinja.

Appendiċi 3

Eżempju tal-konċentrazzjonini: persentaġġ tal-mortalità

Eżempju tad-determinazzjoni ta' LC_{50} bl-użu tal-karta *log-probit*



C.2. TOSSICITÀ AKUTA GĦAL DAPHNIA

1. **METODU**

1.1. INTRODUZZJONI

L-iskop ta' dan it-test huwa sabiex jiddetermina l-medja effettiva tal-konċentrazzjoni għall-immobbilizzazzjoni (EC_{50}) tas-sustanza għad-*Daphnia* fl-ilma frisk. Huwa mixtieq li jkun hemm, sakemm ikun possibbli, informazzjoni dwar is-solubbilità, pressjoni tal-fwar, stabbilità kimika, limitazzjonijiet tad-disassoċjazzjoni u l-biodegradabbiltà tas-sustanzi qabel ma jibda t-test.

Informazzjoni addizzjonali (per eżempju l-formula strutturali, l-grad tal-purità, n-natura u l-persentaġġ ta' impuritajiet sinifikanti, l-preżenza u l-ammonti ta' addittivi, u l-ko-effiċjent tal-qasma n-oktonal/ilma) għandhom ikunu meqjusa kemm fl-ippjanar tat-test u fl-interpretazzjoni tar-riżultati.

1.2. DEFINIZZJONIJIET U UNITAJIET

Il-ħtieġa tad-Direttiva għall-LC_{50} dwar id-*Daphnia* hija meqjusa li tkun imwettqa bid-determinazzjoni tal-LC_{50} kif deskritta f'dan il-metodu tat-test.

Tossiċità akuta hija espressa f'dan it-test bħala l-medja tal-konċentrazzjonini effettiva (EC_{50}) għall-immobilizzazzjoni. Din hija l-konċentrazzjoni, f'termini tal-valuri inizjali, li timmobilizza 50% tad-*Daphnia* f'lott tat-test fi ħdan perijodu kontinwu ta' l-esposizzjoni li għandu jkun mistqarr.

Immobilizazzjoni:

Dawk l-annimali ma jkunux jistgħu jgħumu fi żmien 15 il-sekonda minn wara aġitazzjoni ħafifa fil-kontenitur tat-test huma meqjusa bħala immobbli.

Il-konċentrazzjoninijiet kollha tas-sustanza fit-test huma mogħtija bil-piż/volum (milligrammi kull litru). Dawn jistgħu jkunu espressi wkoll bħala piż bil-piż ($mg.kg^{-1}$).

1.3. SUSTANZI TA' REFERENZA

Sustanza ta' referenza tista tkun ittestjata bħala mezz sabiex turi li l-kondizzjonijiet waqt it-test fil-laboratorju, ir-reazzjoni tal-ispeċi ittestjati ma jkunux inbidlu b'mod sinifikanti.

Is-sommarju tar-riżultati ta' test-f'ċirku KEE, li jutilizza erba' sustanzi differenti, huwa mogħti fl-Appendiċi 2.

1.4. PRINCIPJU TAL-METODU TAT-TEST

Test limitat jista jkun imwettaq fi 100 mg kull litru sabiex tkun determinata li il-LC_{50} tkun akbar minn din il-konċentrazzjoni.

Id-*Daphnia* huwa espost għas-sustanza tat-test miżjuda ma l-ilma fil-medda tal-konċentrazzjonijiet għal perijodu ta' 48 siegħa. Jekk test aktar qasir ikun użat, ġustifikazzjoni għandha tkun mogħtija fir-rapport tat-test.

Taħt kondizzjonijiet ta' test altrimenti identiċi, u medda adekwata tal-konċentrazzjonijiet tas-sustanza tat-test, konċentrazzjonijiet differenti tas-sustanza tat-test eżerċitati f'medd ta' gradi differenti dwar l-effett ta' l-abbiltà ta' l-għawm tad-*Daphnia*. Konċentrazzjonijiet differenti jirriżultaw f'persentaġġi differenti tad-*Daphnia* li ma jkunux aktar jistgħu jgħumu fit-tmiem tat-test. Il-konċentrazzjonijiet li jikkaġunaw żero jew 100% immobilizzazzjoni huma derivati dirett mill-osservazzjonijiet tat-test, filwaqt li l-48-siegħa EC_{50} hija determinata bil-kalkolu, meta dan ikun possibbli.

Sistema statika hija wżata għal dan il-metodu, għalhekk is-soluzzjonijiet tat-test ma humiex imġedda matul il-perijodu ta' l-esposizzjoni.

1.5. KRITERJA TAL-KWALITÀ

Il-kriterja tal-kwalità għandha tkun applikabbli għal-limitu tat-test kif ukoll għall-metodu tat-test kollu.

L-immobilizzazzjoni fil-kontrolli m'għandhiex, fit-tmien tat-test, teċċedi 10%.

Id-*Daphnia* tat-test fil-gruppi ta' kontroll m'għandhiex li tkun ġiet maqbuda mill-wiċċ ta' l-ilma.

Huwa mixtieq li l-konċentrazzjonijiet ta' l-ossiġenu mdewweb fir-reċipjenti tat-test jibqgħu aktar minn 3 mg 1^{-1} matul il-korsa tat-test. B'danakollu, fl-ebda ċirkostanzi m'għandha l-konċentrazzjonini ta' l-ossiġenu mdewweb tinżel taħt 2 mg 1-1.

Il-konċentrazzjoninijiet tas-sustanza tat-test għandhom jinżammu sa fil-limitu ta' 80% tal-konċentrazzjoninijiet inizjali matul iż-żmien kollu tat-test.

Għal sustanzi li jdubu faċilment fil-medja tat-test, b'hekk ifornu taħlitiet stabbli, i.e. dawk li ma jkollhomx reazzjoni volatili sinifikanti, jkollhom degradazzjoni, ikunu idrolizzati jew assorbiti, l-konċentrazzjoni inizjali tista tkun meqjusa bħala li hija l-ekwivalenti tal-konċentrazzjoni nominali. Evidenza għandha tkun ippreżentata li l-livelli tal-konċentrazzjonijiet ikunu nżammu matul it-test u li l-kriterja tal-kwalità kienet ġiet milħuqa.

Għal sustanzi li:

(i) ftit li xejn jinħallu fil-medja tat-test, inkella

(ii) huma kapaċi li jiffurmaw emulsjonijiet jew dispersjonijiet stabbli, inkella

(iii) m'humiex stabbli f'taħlitiet akweji,

il-konċentrazzjoni inizjali għandha tkun meqjusa bħala l-konċentrazzjoni mkejla fit-taħlita (jew, jekk teknikament dan ma jkunx possibbli, imkejla f'kolonna ta' l-ilma) fil-bidu tat-test. Il-konċentrazzjoni għandha tkun determinata wara perijodu ta' ekwilibriju imma qabel l-introduzzjoni ta' l-organiżmi tat-test.

Fi kwalunkwe minn dawn il-każi, aktar kejl għandu jsir matul it-test sabiex jikkonferma l-konċentrazzjoni attwali ta' l-esposiżżjoni jew li l-kriterja tal-kwalità tkun ġiet milħuqha.

Il-pH m'għandux ivarja b'aktar minn unità waħda.

1.6. DESKRIZZJONI TAL-METODU TAT-TEST

1.6.1. **Reaġenti**

1.6.1.1. *Taħlitiet tas-sustanzi tat-test*

Taħlitiet diġa lesti tas-saħħa meħtieġa huma ippreparati bit-tidwib tas-sustanza f'ilma de-jonizzat jew f'ilma skond 1.6.1.2.

Il-konċentrazzjonijiet tat-test kif magħżula huma preparati bit-tidwib tat-taħlita diġa lest. Jekk konċentrazzjonijiet għoljin huma testjati, s-sustanza tista tkun imdewba direttament mat-trattib ta' l-ilma.

Is-sustanza għandha normalment tkun ittestjata biss sal-limitu tas-solubbilità tagħha. Għal uħud mis-sustanzi (e.g. sustanzi li jkollhom solubbilità baxxa fl-ilma, jew P_{ow} [9] għoli, jew dawk li jiffurmaw tixriq stabbli pjuttost milli taħlita vera fl-ilma), huwa aċċettabbli li tintuża l-konċentrazzjoni tat-test aktar għolja mil-limitu tal-solubbilità tas-sustanza sabiex ikun assigurat li s-solubbilità massima/stabbilità tal-konċentrazzjoni tkun intlaħqet. Huwa importanti, b'danakollu, li din il-konċentrazzjoni ma tkunx b'mod ieħor tħawwad is-sistema tat-test (e.g, li jkun hemm rita tas-sustanza fil-wiċċ ta' l-ilma li ma tħallix li jseħħ l-ossiġinazzjoni ta' l-ilma, etc.).

Tixrid ultrasoniku, solventi organiċi, emulsifikaturi jew dispersanti jistgħu jintużaw bħala għajnuna għat-tħejjija tat-taħlitiet bil-lest ta' sustanzi b'solubbilità baxxa fl-ilma jew sabiex jgħinu fit-tixrid ta' dawn is-sustanzi fil-medja tat-test. Meta tali sustanzi awżiljarji huma wżati, l-konċentrazzjonijiet kollha tat-test għandhom jinkludu l-istess ammont tas-sustanza awżiljarja, u ħut addizzjonali tal-kontroll għandhom ikunu esposti għall-istess konċentrazzjoni tas-sustanza awżiljarja bħal dik użata fis-serje tat-test. Il-konċentrazzjoni ta' tali awżiljarji għandha tkun minimizzata, izda fl-ebda każ m'għandha teċċedi 100 mg kull litru fil-medja tat-test.

It-test għandu jkun imwettaq mingħajr l-aġġustament tal-pH. Jekk ikun hemm evidenza ta' tibdil qawwi fil-pH, huwa rakkomandat li t-test għandu jkun repetut bi pH aġġustat u r-riżultati inklużi fir-rapport. F'dak il-każ, il-valur tal-pH tat-taħlita bil-lest għandu jkun aġġustat għall-valur tal-pH ta' l-ilma tat-taħlit u sakemm ma jkunx hekk raġunijiet speċifiċi il-għala dan m'għandux isir. HCI u NaOH huma preferuti għal dan l-iskop. Dan l-aġġustament tal-pH għandu jsir b'tali mod hekk li l-konċentrazzjoni tas-sustanza tat-test fit-taħlita bil-lest ma tkunx mibdula sa xi medda sinifikanti. Jekk ikun hemm xi reazzjoni kimika jew preċipitazzjoni fiżika tal-kompost tat-test ikkaġunata bl-aġġustament, dan għandu jkun mistqarr.

1.6.1.2. *Ilma tat-test*

Ilma rikostitwita hija wżata f'dan it-test (ara l-Appendiċi 1 u r-refgerenza (2): ISO 6341). Sabiex tkun evitata l-ħtieġa għall-akklimatizzazzjoni qabel it-test, huwa rakkommandat li l-ilma tal-kultura għandha tkun ta' kwalità simili (pH, ebusija) bħall-ilma użat fit-test.

1.6.2. **Apparat**

Apparat u tagħmir normali tal-laboratorju għandu jkun użat. Apparat li kien ġie f'kuntatt ma taħlitiet oħrajn tat-test għandu preferibbilment ikun magħmul kompletament mill-ħġieġ:

— Arloġġ ta' l-ossiġenu (bil-mikro-elettrodu jew b'tagħmir xieraq għall-kejl ta' l-ossiġenu mdewwb f'kampjuni ta' volum baxx),

— apparat adekwat għall-kontroll tat-temperatura,

— arloġġ pH,

— apparat għad-determinazzjoni ta' l-ebusija ta' l-ilma.

1.6.3. **Organiżmi tat-test**

Daphnia magna hija l-ispeċi preferita għat-test, għalkemm *Daphnia pulex* hija wkoll permissibbli. L-annimali tat-test għandhom ikunu ta' età ta' anqas minn 22 siegħa, fil-bidu tat-test, imwielda fil-laboratorju, ħielsa minn xi mard estern u bi storja magħrufa (e.g. tnissil – xi pre-trattamenti, etc.).

1.6.4. **Proċedura tat-test**

Test tas-sejba tal-medda jista jipprekedi t-test definittiv, sabiex tkun akkwistata l-informazzjoni dwar il-medda tal-konċentrazzjonijiet li għandhom ikunu wżati fit-test ewlieni.

Kontroll wieħed mingħajr is-sustanza tat-test għandu jkun magħmul u, jekk relevanti, kontroll wieħed li jkun fih sustanza awżiljarja għandu wkoll ikun magħmul, b'żieda mas-serje tat-testijiet.

Id-*Daphnia* hija esposta għas-sustanza kif deskritt hawn taħt:

— *tul ta' żmien*: preferibbilment 48 siegħa,

— *numru ta' l-annimali*: mill-anqas 20 annimal għal kull konċentrazzjonini tat-test, preferibbilment diviżi f'erba' lottijiet ta' ħames annimali 'l wieħed jew żewġ lottijiet ta' 10,

— *tagħbija*: mill-anqas 2 ml fit-taħlita tat-test għandha tkun ipprovduta għal kull annimal,

— *konċentrazzjoni tat-test*: It-taħlita tat-test għandha tkun ippreparata immedjatament qabel l-introduzzjoni tad-*Daphnia*, preferibbilment mingħajr l-użu ta' xi solvent apparti milli l-ilma. Il-konċentrazzjonijiet huma magħmula minn serje ġeometrika, fir-relattività tal-konċentrazzjoni li ma teċċedix 2.2. Konċentrazzjonijiet suffiċjenti sabiex jagħtu 0 u 100% immobilizzazzjoni wara 48 siegħa u medda ta' gradi intermedji ta' l-immobilizzazzjoni li jippermetti l-kalkolu tal-EC_{50} tal-48 siegħa għandhom ikunu ipprovati flimkien mal-kontrolli,

— *ilma*: ara 1.6.1.2,

— *dawl*: iċ-ċiklu ta' dawl/dlam huwa voluntarju,

— *temperatura*: it-temperatura tat-test għandha tkun bejn 18 u 22 °C, imma għal test wieħed din għanda tkun kostanti fil-limitu ta' ± 1 °C,

— *arjazzjoni*: it-taħlitiet tat-test m'għandhomx ikunu arjata bil-bżieżaq,

— *ikel*: xejn.

Il-pH u l-konċentrazzjoni ta' l-ossiġenu tal-kontrolli u tal-konċentrazzjonijiet kollha tat-test għandhom ikunu mkejla fit-tmiem tat-test; il-pH tat-taħlitiet tat-test m'għandux ikunu modifikat.

Komposti volatili għandhom ikunu testjati fl-kontenitur magħluq u mimlija sa fuq, kbar biż-żejjed sabiex jipprevjenu n-nuqqas ta' ossiġenu.

Id-*Daphnia* huma spezzjonati mill-anqas wara 24 siegħa ta' l-esposizzjoni u mill-ġdid wara 48 siegħa.

Test ta' limitazzjoni

Test limitat jista' jkun imwettaq fi 100 mg kull litru sabiex tkun determinata li il-EC_{50} tkun akbar minn din il-konċentrazzjoni.

Jekk in-natura tas-sustanza tkun tali li konċentrazzjoni ta' 100 mg kull litru fl-ima tat-test ma tistax tintlaħaq, it-test tal-limitazzjoni għandu jitwettaq f'konċentrazzjoni ugwali għas-solubbilità tas-sustanza (jew tal-konċentrazzjoni massima li tifforma tixrid stabbli) fil-medja użata (ara wkoll il-punt 1.6.1.1).

It-test tal-limitazzjoni għandu jkun imwettaq bl-użu ta' 20 *Daphnia*, bl-istess numru ta' kontroll(i). Jekk ikun hemm mortalijiet, studju sħiħ għandu jitwettaq.

2. **DATA U EVALWAZZJONI**

Għal kull perijodu ta' meta l-osservazzjonijiet kienu reġistrati (24 u 48 siegħa), pinġi l-persentaġġ tal-mortalità għal kull perijodu ta' l-esposizzjoni kif rakkommandat kontra l-konċentrazzjonini fuq karta tal-probabbiltà logaritmika,

Meta possibbli u għal ħin ta' l-osservazzjoni, il-EC_{50} u l-limiti tal-kunfidenza (p = 0,05) għandhom ikunu kalkolati bl-użu tal-proċeduri normali; dawn il-valuri għandhom ikunu mqarrba lejn wieħed, l-aktar lejn tnejn, figuri sinifikanti (eżempji tat-tqarrib lejn żewġ figuri: 170 għal 173,5; 0,13 għal 0,127; 1,2 għal 1,21).

F'dawk il-każi meta l-linja magħwġa bil-kurva tal-konċentrazzjoni/persentaġġ tar-reazzjoni tkun wieqfa wisq sabiex tippermetti l-kalkolazzjoni tal-EC_{50}, estimi grafiċi ta' dan il-valur huma suffiċjenti.

Meta żewġ konċentrazzjonijiet konsekuttivi, b'relattività ta' 2,2, jagħtu biss 0 u 100% mortalità, dawn iż-żewġ valuri huma suffiċjenti sabiex jindikaw il-medda li fiha jkun jidħol il-EC_{50}.

Jekk ikun osservat li l-istabbilità jew l-omoġenità tas-sustanza tat-test ma tistax tkun miżmuma, dan għandu jkun irrapportat u qis għandu jittieħed fl-interpretazzjoni tar-riżultati.

3. **RAPPURTAĠĠ**

Ir-rapport tat-test għandu, jekk possibbli, jinkludi l-informazzjoni li ġejja:

— informazzjoni dwar l-organiżmi tat-test (isem xjentifiku, siltiet, fornitur jew sorsi, xi pre-trattamemnt, metodu tat-tnissil - inkluż is-sorsi, t-tip u l-ammont ta' ikel, il-frekwenza ta' l-għalf);

— is-sorsi tat-trattib-ilma u l-karatteristiċi kimiċi maġġuri (pH, ebusija, temperatura);

— fil-każ ta' sustanza b'solubilità akwea baxxa, l-metodu tal-preparazzjoni tat-taħlitiet kemm dawk imħejjija lesti u tat-test;

— konċentrazzjoni ta' xi sustanzi awżiljarji;

— lista tal-konċentrazzjonijiet użati u xi informazzjoni disponibbli dwar l-istabbilità tal-konċentrazzjonijiet tal-kimika ittestjata fit-taħlita tat-test;

— jekk analiżi kimika tkun imwettqa, l-metodi użati u r-riżultati akkwistati;

— ir-riżultati tat-test tal-limitazzjoni, jew imwettaq;

— deskrizzjoni ta' l-apparat tat-test;

— l-organizzazzjoni tad-dawl;

— il-konċentrazzjonijiet ta' l-ossiġenu mdewweb, il-valuri pH u t-temperaturi tat-taħlitiet tat-test;

— evidenza tal-kriterja tal-kwalità li tkun ġiet imwettqa;

— tabella li turi il-mortalità kumulattiva ta' kull konċentrazzjoni u l-kontroll (u l-kontroll bis-sustanza awżiljarja, jekk meħtieġ) f'kull wieħed mill-aħjnijiet rakkommandati ta' l-osservazzjoni (24 u 48 siegħa);

— grafika tal-konċentrazzjoni/persentaġġ tal-kurva tar-reazzjoni fit-tmiem ta' kull test;

— jekk possibbli, il-valuri EC_{50} f'kull wieħed mill-ġinijiet rakkommandati ta' l-osservazzjoni (bi 95% tal-limiti ta' l-affidabbiltà);

— proċedura statistiċi użati għad-determinazzjoni tal-valuri EC_{50};

— jekk tkun użata ta' sustanza ta' referenza, ir-riżultati akkwistati,

— l-għola konċentrazzjoni tat-test li ma toħloq l-ebda mortalità matul il-perijodu tat-test;

— l-anqas konċentrazzjoni tat-test li toħloq 100% mortalità matul il-perijodu tat-test.

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Appendiċi 1

Ilma rikostitwit

Eżempju ta' trattib xieraq ta' l-ilma (skond ISO 6341)

Il-kimiċi kollha għandhom ikunu ta' grad analitiku.

L-ilma għandu jkun distillat ta' kwalità tajba, jew ilma dejonizzat b'konduttività ta' anqas minn 5 μScm^{-1}.

Apparat għad-distillazzjoni ta' l-ilma m'għandux ikun fih xi partijiet magħmula mir-ramm.

Taħlitiet bil-lest

$CaCl_2 \cdot 2H_2O$ (Diħidrat tal-klorid tal-kalċju): dewweb fi, u wassal għal litru bl-ilma.	11,76 g
$MgSO_4 \cdot 7H_2O$ (ħeptaidrat tas-sulfat tal-magneżju): dewweb fi, u wassal għal litru bl-ilma.	4,93 g
$NaHCO_3$ (karbonat ta' l-idroġenu tas-sodju): dewweb fi, u wassal għal litru bl-ilma.	2,59 g
KC1 (klorid tal-putassa): dewweb fi, u wassal għal litru bl-ilma.	0,23 g

Ilma rikostitwit għat-tidwib

Ħawwad 25 ml ta' kull wieħed ta' l-erba taħlitiet bil-lest u wassal sa litru bl-ilma.

Qawwi bl-arja sakemm il-konċentrazzjoni ta' l-ossiġenu mdewweb tkun egwali għall-valur tas-saturizzazzjoni ta' l-arja.

Il-pH għandu jkun 7,8 ± 0,2.

Jekk meħtieġ, aġġusta il-pH bi NaOH (idrossidu tas-sodju) jew HCI (aċidu idrokloriku).

L-ilma tat-trattib hekk ippreparat huwa mwarrab għal madwar 12 il-siegħa u l-ebda arja aktar ma tkun miżjuda.

Is-somma tal-jonji Ca u Mg f'din it-taħlita hija ta' 2,5 mmol kull litru. Ir-relattività tal-jonji Ca:Mg hija 4:1 u ta' jonji Na:K hija 10:1. L-alkalinità totali ta' din it-taħlita hija 0,8 mmol kull litru.

Kwalunkwe devjazzjoni fil-preparazzjoni ta' l-ilma tat-trattib m'għandhiex tibdel il-komposizzjoni jew il-proprjetajiet ta' l-ilma.

Appendiċi 2

Sommarju tar-riżultati tat-test-ċirku KEE imwettaq fil-1978 (ċitat ukoll fir-referenza 2)

Attenzjoni: l-iskop ta' dan it-test-ċirku kien għad-determinazzjoni tal-EC_{50} *24 siegħa.*

Sustanzi wżati:

1) Dikromat tal-putassa

2) Aċidu tetrapropilbenżenesulfoniku

3) Aċidu tetrapropilbenżenesulfoniku, melħ tas-sodju

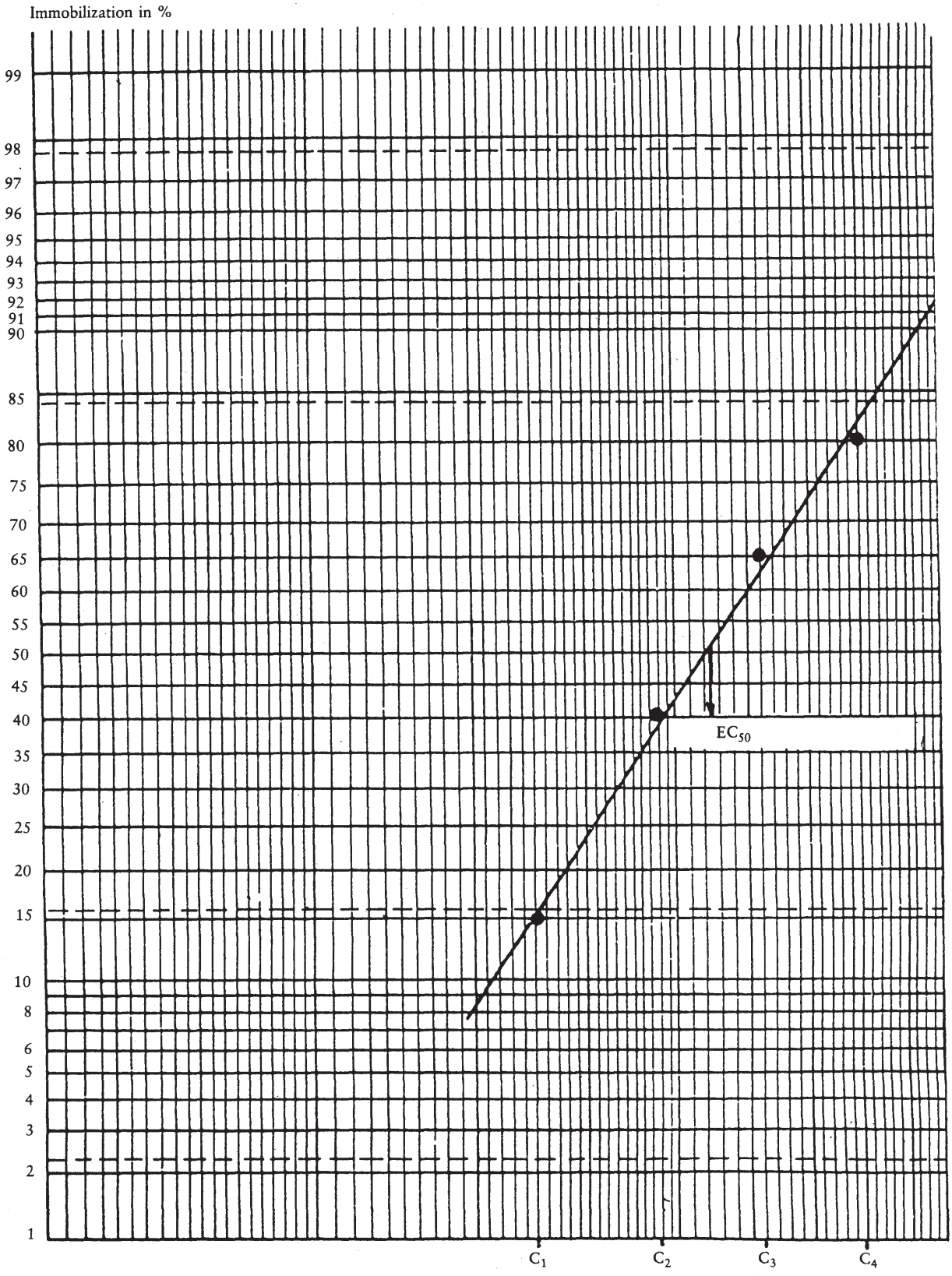
4) melħ trikloro-2,4,5-fenoksaċetiku, melħ tal-putassa

Sustanza	Numru ta' laboratorji parteċipanti	Numru ta' riżultati għall-kalkolazzjoni	KE siegħa mg/l medja
1	46	129	1,5
2	36	108	27
3	31	84	27
4	32	72	770

Appendiċi 3

Eżempju tal-konċentrazzjoni: persentaġġ ta' l-immobilizzazzjoni

Eżempju tad-determinazzjoni ta' EC_{50} bl-użu tal-karta *log-probit*



C.3. TEST TA' L-INIBIZZJONI ALGAL

1. **METODU**

1.1. INTRODUZZJONI

L-iskop ta' dan it-test huwa sabiex jiddetermina l-effetti ta' sustanza fit-tkabbir ta' speċi algali ħodor uniċ̀llulari. Testijiet relattivament qosra (72 siegħa) jistgħu jassessjaw l-effetti matul diversi ġenerazzjonijiet. Dan il-metodu jista jkun adattat għall-użu ma diversi speċi ta' l-algar uniċellulari, f'liema każ deskrizzjoni tal-metodu użat għandha tkun ipprovduta mar-rapport tat-test.

Dan il-metodu huwa l-aktar faċli li jkun applikat ma sustanzi li jinħallu fl-ilma, skond il-kondizzjonijiet tat-test, li pjuttost jibqgħu fl-ilma.

Dan il-metodu jista jkun użat għal sustanzi li ma jfixklux direttament il-kejl tat-tkabbir ta' l-algal.

Huwa mixtieq li jkun hemm, sakemm ikun possibbli, informazzjoni dwar is-solubbilità, pressjoni tal-fwar, stabbilità kimika, limitazzjonijiet tad-disassoċjażżjoni u l-biodegradabbiltà tas-sustanzi qabel ma jibda t-test.

Informazzjoni addizzjonali (per eżempju l-formula strutturali, il-grad tal-purità, in-natura u l-persentaġġ ta' impuritajiet sinifikanti, il-preżenza u l-ammonti ta' additтivi, u l-ko-effiċjent tal-qasma n-oktonal/ilma) għandhom ikunu meqjusa kemm fl-ippjanar tat-test u fl-interpretazzjoni tar-riżultati.

1.2. DEFINIZZJONIJIET U UNITAJIET

Densità taċ-ċellola: in-numru ta' ċelloli kull millilitru;

Tkabbir: iż-żieda fid-densità tal-ċelloli matul il-perijodu tat-test;

Rata tat-tkabbir: iż-żieda fid-densità tal-ċelloli matul l-unità taż-żmien;

EC_{50}: f'dan il-metodu, il-konċentrazzjoni tas-sustanza tat-test li tirriżulta fi 50% tnaqqis jew fit-tkabbir (E_bC_{50}) jew fir-rata tat-tkabbir (E_rC_{50}) relattiva mal-kontroll;

NOEC (*no observed effect concentration*) (l-ebda effett ta' konċentrazzjoni m'hu osservat): f'dan il-metodu, l-għola konċentrazzjoni testjata li fiha inobizzjoni sinifikati tat-tkabbir hija osservata b'relattività mal-kontroll.

Il-konċentrazzjonijiet kollha tas-sustanza fit-test huma mogħtija bil-piż/volum (milligrammi kull litru). Dawn jistgħu jkunu espressi wkoll bħala piż bil-piż ($mg.kg^{-1}$).

1.3. SUSTANZI TA' REFERENZA

Sustanza ta' referenza tista' tkun ittestjata bħala mezz sabiex turi li l-kondizzjonijiet waqt it-test fil-laboratorju, r-reazzjoni tal-ispeċje ittestjati ma jkunux inbidlu b'mod sinifikanti.

Jekk test aktar qasir ikun użat, ġustifikazzjoni għandha tkun mogħtija fir-rapport tat-test. Dikromat tal-putassa jista' jkun użat bħala sustanza ta' referenza, imma l-kulur tiegħu jista jaffettwa l-kwalità u l-intensità tad-dawl disponibbli għaċ-ċelloli u wkoll id-determinazzjonijiet spektrofotometriċi, jekk użati. Id-dokromat tal-putassa kien ġie użat fit-test inter-laboratorju internazzjonali (ara ref. (3) u Appendiċi 2).

1.4. PRINCIPJU TAL-METODU TAT-TEST

Test limitat jista' jkun imwettaq fi 100 mg kull litru sabiex tkun determinata li il-LC_{50} tkun akbar minn din il-konċentrazzjoni.

Il-kulturi tat-tkabbir-expotenzali ta' l-algar ħodor magħżula huma esposti għal diversi konċentrazzjonijiet tas-sustanza tat-ptest matul diversi ġenerazzjonijiet taħt kondizzjonijiet definiti.

It-taħlitiet tat-test huma inkunati għal perijodu ta' 72 siegħa, li matulhom id-densità taċ-ċelloli f'kull taħlita hija mkejjla mill anqas kull 24 siegħa. L-inibizzjoni tat-tkabbir b'relazzjoni mal-kultura tal-kontroll hija determinata.

1.5. KRITERJA TAL-KWALITÀ

Il-kriterja tal-kwalità għandha tkun applikabbli għal-limitu tat-test kif ukoll għall-metodu tat-test kollu.

Id-densità taċ-ċellola fil-kulturi tal-kontroll għandha li tkun żdiedet b'fattur ta' mill-anqas 16 matul tlett ijiem.

Il-konċentrazzjonijiet tas-sustanza tat-test għandhom jinżammu sa fil-limitu ta' 80% tal-konċentrazzjonijiet inizjali matul iż-żmien kollu tat-test.

Għal sustanzi li jdubu faċilment fil-medja tat-test, b'hekk ifornu taħlitiet stabbli, i.e. dawk li ma jkollhomx reazzjoni volatili sinifikanti, jkollhom degradazzjoni, ikunu idrolizzati jew adsorbiti, l-konċentrazzjonini inizjali tista tkun meqjusa bħala li hija l-ekwivalenti tal-konċentrazzjoni nominali. Evidenza għandha tkun ippreżentata li l-livelli tal-konċentrazzjonijiet ikunu nżammu matul it-test u li l-kriterja tal-kwalità kienet ġiet milħuqa.

Għal sustanzi li huma:

(i) ftit li xejn jinħallu fil-medja tat-test, jew

(ii) kapaċi li jiffurmaw emulsjonijiet jew dispersjonijiet stabbli, inkella

(iii) mhux stabbli f'taħlitiet akweji,

il-konċentrazzjoni inizjali għandha tkun meqjusa bħala l-konċentrazzjoni mkejla fil-bidu tat-test. Il-konċentrazzjoni għandha tkun determinata wara perijodu ta ekwilibrazzjoni.

Fi kwalunkwe minn dawn il-każi, aktar kejl għandu jsir matul it-test sabiex jikkonferma l-konċentrazzjoni attwali ta' l-esposiżżjoni jew li l-kriterja tal-kwalità tkun ġiet milħuqha.

Huwa rikonoxxut li ammonti sinifikanti tas-sustanza tat-test jistgħu jkunu inkorporati bil-biomassa ta' l-algal matul il-perijodu tat-test. Għalhekk, għall-iskop li tkun murija l-konformità mal-kriterja tal-kwalità ta' hawn fuq, kemm l-ammont tas-sustanza inkorporata fil-biomassa ta' l-algal u wkoll tas-sustanza fit-taħlita (jew, jekk mhux teknikament possibbli, imkejla fil-kolonna ta' l-ilma) għandhom ikunu meqjusa. B'dana-kollu, minħabba li d-determinazzjoni tal-konċentrazzjoni tas-sustanza fil-biomassa ta' l-algal tista toħloq problemi tekniċi sinifikanti, konformità mal-kriterja tal-kwalità tista tkun murija billi reċipjent taat-test ikun suġġett għall-ogħla konċentrazzjoni tas-sustanza mingħajr l-algal u l-kejl tal-konċentrazzjonijiet fit-taħlita (jew, jekk mhux teknikament possibbli, imkejla fil-kolonna ta' l-ilma) fil-bidu u fit-tmiem tal-perijodu tat-test.

1.6. DESKRIZZJONI TAL-PROCEDURA TAT-TEST

1.6.1. **Reaġenti**

1.6.1.1. *Taħlitiet tas-sustanzi tat-test*

Taħlitiet diġa lesti tas-saħħa meħtieġa huma preparati bit-tidwib tas-sustanza f'ilma de-jonizzat jew f'ilma skond 1.6.1.2.

Il-konċentrazzjonijiet tat-test kif magħżula huma ippreparati biż-żieda ta' alikwota xierqa mal-pre-kulturi ta' l-algal (ara Appendiċi 1). Is-sustanza għandha normalment tkun ittestjata biss sal-limitu tas-solubbilità tagħha. Għal uħud mis-sustanzi (e.g. sustanzi li jkollhom solubbilità baxxa fl-ilma, jew P_{ow} [9] għoli, jew dawk li jiffurmaw tixriq stabbli pjuttost milli taħlita vera fl-ilma), huwa aċċettabbli li tintuża l-konċentrazzjoni tat-test aktar għolja mil-limitu tal-solubbilità tas-sustanza sabiex ikun assigurat li s-solubbilità massima/s-tabbilità tal-konċentrazzjoni tkun intlaħqet. Huwa importanti, b'danakollu, li din il-konċentrazzjoni ma tkunx b'mod ieħor tħawwad is-sistema tat-test (e.g, li jkun hemm rita tas-sustanza fil-wiċċ ta' l-ilma li ma tħallix li jseħħ l-ossiġinazzjoni ta' l-ilma, etc.).

Tixrid ultrasoniku, solventi organiċi, emulsifikaturi jew dispersanti jistgħu jintużaw bħala għajnuna għat-tħejjija tat-taħlitiet bil-lest ta' sustanzi b'solubbilità baxxa fl-ilma jew sabiex jgħinu fit-tixrid ta' dawn is-sustanzi fil-medja tat-test. Meta tali sustanzi awżiljarji huma wżati, il-konċentrazzjonijiet kollha tat-test għandhom jinkludu l-istess ammont tas-sustanza awżiljarja, u ħut addizzjonali tal-kontroll għandhom ikunu esposti għall-istess konċentrazzjoni tas-sustanza awżiljarja bħal dik użata fis-serje tat-test. Il-konċentrazzjoni ta' tali awżiljarji għandha tkun minimizzata, imma fl-ebda każ m'għandha teċċedi 100 mg kull litru fil-medja tat-test.

It-test għandu jkun imwettaq mingħajr l-aġġustament tal-pH. Jekk ikun hemm evidenza ta' tibdil qawwi fil-pH, huwa rakkomandat li t-test għandu jkun repetut bi pH aġġustat u r-riżultati inklużi fir-rapport. F'dak il-każ, il-valur tal-pH tat-taħlita bil-lest għandu jkun aġġustat għall-valur tal-pH ta' l-ilma tat-taħlit, sakemm ma jkunx hekk raġunijiet speċifiċi il-għala dan m'għandux isir. HCI u NaOH huma preferuti għal dan l-iskop. Dan l-aġġustament tal-pH għandu jsir b'tali mod hekk li l-konċentrazzjoni tas-sustanza tat-test fit-taħlita bil-lest ma tkunx mibdula sa xi medda sinifikanti. Jekk ikun hemm reazzjoni kimika jew preċipitazzjoni fiżika tal-kompost tat-test ikkaġunata bl-aġġustament, dan għandu jkun mistqarr.

1.6.1.2. *Medja tat-test*

L-ilma għandu jkun distillat ta' kwalità tajba, jew ilma dejonizzat b'konduttività ta' anqas minn 5 ìScm^{-1}. Apparat għad-distillazzjoni ta' l-ilma m'għandux ikun fih xi partijiet magħmula mir-ramm.

Il-medja li ġejja hija rakkommandata.

Erba' taħlitiet bil-lest huma preparati, skond it-tabella li ġejja. It-taħlitiet bil-lest huma sterilizzati b'filtrazzjoni tal-membrana jew bl-awtoklavar, u maħżuna fid-dlam fi 4 °C. It-taħlita bil-lest Nru 4 għandha tkun sterilizzata biss bil-filtrazzjoni tal-membrana. Dawn it-taħlitiet bil-lest huma mdewwba sabiex ikunu milħuqa l-konċentrazzjonijiet finali tan-nutrijent fit-taħlitiet tat-test.

Nutrient	Konċentrazzjoni fit-taħlita bil-lest		Konċentrazzjoni finali fit-taħlita tat-test	
Taħlita bil-lest *1: makro-nutrienti*				
NH_4Cl	1,5	g/l	15	mg/l
$MgCl_2.6H_2O$	1,2	g/l	12	mg/l
$CaCl_2.2H_2O$	1,8	g/l	18	mg/l
$MgSO_4.7H_2O$	1,5	g/l	15	mg/l
$KH_2\ PO_4$	0,16	g/l	1,6	mg/l
Taħlita bil-lest *2:Fe-EDTA*				
$FeCl_3.6H_2$	80	mg/1	0,08	mg/l
$Na_2EDTA.2H_2O$	100	mg/l	0,1	mg/l
Taħlita bil-lest *3: elementi taċċa*				
H_3BO_3	185	mg/l	0,185	mg/l
$MnCl_2.4H_2O$	415	mg/l	0,415	mg/l
$ZnCl_2$	3	mg/l	3×10^{-3}	mg/l
$CoCl_2.6H_2O$	1,5	mg/l	$1,5 \times 10^{-3}$	mg/l
$CuCl_2.2H_2O$	0,01	mg/l	10^{-5}	mg/l
$Na_2MoO_4.2H_2O$	7	mg/l	7×10^{-3}	mg/l
Taħlita bil-lest *4: $NaHCO_3$*				
$NaHCO_3$	50	g/l	50	mg/l

Il-pH tal-medja wara l-ekwilibrazzjoni bl-arja huwa ta' madwar 8.

1.6.2. **Apparat**

— Apparat normali tal-laboratorju,

— Garafini tat-test ta' volum xieraq (e.g. garafini koniċi 250 ml huma adatti meta l-volum tat-taħlita tat-test huwa 100 ml). Il-garafini tat-test għandhom ikunu kollha identiċi f'dak li jirrigwardja l-materjal u l-qisien.

— Apparat tal-kultura: armarju jew kabina li fiha it-temperatura fil-medda ta' 21 °C to 25 °C tkun tista tinżamm fi ± 2 C, u illuminazzjoni kontinwa uniformi ipprovduta fil-medda spettrali 400 sa 700 nm. Jekk l-algae fil-kultru tal-kontrol ikunu laħqu r-rati tat-tkabbir rakommandat, jista jkun assumit li kondizzjonijiet tat-tkabbir, inkluża l-intensità tad-dawl, kienu adekwati.

Huwa rakkomandat li jsir użu. Fil-livell medju tat-taħlitiet tat-test, ta' intensità tad-dawl fil-medda ta' 60 sa 120 $\mu E.m^{-2}.s^{-1}$ (35 sa 70 × 1018 photons.m-2.s-1) meta mkejla fil-medda 400 sa 700 nm bl-użu tar-riċevitur xieraq. Għall-istrumenti tal-kejl tad-daawl kalibrati fi lux, medda ekwivalenti ta' 6 000 sa 10 000 lx hija aċċettabbli.

L-intensità tad-dawl tista tkun akkwistata bl-użu ta' minn erba' sa seba lampi florixxenti ta' 30 W tat-tip abjad universali (it-temperatura tal-kulur ta' madwar 4 300 K), f'distanza ta' 0,35 m mill-kultura ta' l-algal.

— Il-kejl tat-densità taċ-ċellola għandu jsir bl-użu tal-metodu ta' l-għadd dirett taċ-ċelloli ħajjin, e.g. mikroskopju bil-qsim ta' l-għadd. B'danakollu, proċeduri oħrajn (fotometrija, turbidimetria,...) jistgħu ikunu wżati jekk ta' sensittività suffiċjenti u jekk ikun muri li jkunu sew korrelati b'mod sodisfaċenti mad-densità taċ-ċellola.

1.6.3. **Organiżmi tat-test**

Huwa suġġerit li l-ispeċje ta' l-algal ħodor użati jkunu tal-ispeċi li jikbru malajr li huma konvenjenti għall-preparatazzjoni tal-kultura u tat-testijiet. L-ispeċi li ġejjin huma preferuti:

— *Selenastrum capricornutum*, e.g. ATCC 22662 jew CCAP 278/4,

— *Scenedesmus subspicatus*, e.g. 86.81 SAG,

Nota:

ATCC = American Type Culture Collection (U.S.A.)

CCAP = Culture Centre of Algae and Protozoa (U.K.)

SAG = Ġbir tal-kultura ta' l-algal (Göttingen, F.R.G.)

Jekk speċi oħrajn huma wżati, is-silta għandha tkun inkluża fir-rapport.

1.6.4. **Proċedura tat-test**

Il-medda tal-konċentrazzjoni li fiha l-effetti pjuttost li jseħħu hija determinata fuq il-bażi tar-riżultati mit-testijiet tas-sejbin tal-medda.

Iż-żewġ kejl tat-tkabbir (biomassa u r-rata tat-tkabbir) jistgħu jirriżultaw minn kejl ferm differenti ta' l-inibizzjoni tat-tkabbir, it-tnejn għandhom ikunu wżati fit-test tas-sejbien tal-medda sabiex jassiguraw li l-progressjoni ġeometrika tal-konċentrazzjoninijiet tkun tippermetti l-estimi kemm tal-E_bC_{50} u wkoll tal-E_rC_{50}.

Densità taċ-ċellola inizjali

Huwa rakommandat li d-densità tal-ċellola inizjali fil-kultru tat-test għandha tkun madwar 104 ċelloli/ml għal *Selenastrum capricornutum* u *Scenedesmus subspicatus*. Jekk speċje oħrajn huma wżati, il-biomassa għandha tkun komparabbli.

Konċentrazzjonijiet tas-sustanza tat-test

Għat-test, mill-anqas ħames konċentrazzjoninijiet huma magħmula minn serje ġeometrika, fir-relattività tal-konċentrazzjonini li ma teċċedix 2,2. Il-konċentrazzjoni l-aktar baxxa kif ittestjata m'għandha jkollha l-ebda effett fuq it-tkabbir ta' l-algae. L-ogħla konċentrazzjoni ittestjata għandha tnaqqas it-tkabbir b'mill-anqas 50% b'relattività mal-kontroll u, preferibbilment, twaqqaf it-tkabbir kompletament.

Repliki u kontrolli

Id-disinn tat-test għandu jinkludi tlett replikati għal kull konċentrazzjoni tat-test. It-tlett kontrolli mingħajr is-sustanza tat-test huma esegwiti u, jekk relevanti, tlett kontrolli li jkun fihom is-sustanza awżiljarja huma wkoll esegwiti. Jekk ġustifikat, id-disinn tat-test jista jkun mibdul sabiex jiżdiedu n-numru ta' konċentrazzjonijiet u jitnaqqsu n-numru ta' repliki f'kull konċentrazzjoni.

L-imġieba tat-test

Il-kulturi tat-test li jkun fihom il-konċentrazzjonijiet mixtieqa tas-sustanza tat-test u l-kwantità mixtieqa ta' l-algal inoculum huma preparati biż-żieda ta alikwoti ta' taħlitiet bil-lest tas-sustanza tat-test sa ammonti xierqa tal-pre-kulturi ta' l-algal (ara Appendiċi 1).

Il-garfina tal-kultura huma mċekkċa u mqiegħda fl-apparat tal-kulturar. Iċ-ċelloli algar huma miżmuma f'sospenzjoni biċ-ċekċik, taħwid jew tbaqbieq bl-arja, sabiex jittejjeb l-iskambju tal-gass u titnaqqas il-varjazzjoni pH fit-taħlitiet tat-test. Il-kulturi għandhom ikunu miżmuma f'temperatura fil-medda ta' 21 sa 25 °C, ikkontrollata fi ± 2 °C.

Id-densità taċ-ċellola f'kull garafina hija determinata fi mill-anqas 24, 48 u 72 siegħa wara l-bidu tat-test. Medja ta' l-algal iffiltrata li jkun fiha il-konċentrazzjoni xierqa tal-kimika tat-test hija wżata sabiex tiddetermina l-isfond meta jkunu wżati kejl tad-densità taċ-ċellola apparti mill-metodi ta' l-għadd direct.

Il-pH huwa mkejjel fil-bidu tat-test u wara 72 siegħa.

Il-pH tal-kontrolli m'għandux normalment jiddevja b'aktar minn 1,5 unitajiet matul it-test.

L-ittestjar ta' sustanza volatili

Sa issa ma hemm l-ebda mod ġeneralment aċċettabbli għat-testijiet ta' sustanzi volatili. Meta sustanza tkun magħrufha bħala li għandha tendenzi għall-vaporizzazzjoni, garafini magħluqa tat-test bi spazju miżjud fil-wiċċ jistgħu ikunu wżati. Il-possibbiltà ta' nuqqas ta' CO_2 għandha tkun meqjusa meta jkun ikkalkolat l-ispazju fil-wiċċ tal-garafini magħluqa. Varjazzjonijiet ta' dan il-metodu huma proponuti (ara referenza (4))

Tentattivi għandhom isiru għad-determinazzjoni ta' l-ammont tas-sustanza li tibqa' fit-taħlita, u attenzjoni estrema hija rakkomandata meta jkunu interpretati r-riżultati tat-testijiet b'kimiċi volatili bl-użu ta' sistemi magħluqa.

Test ta' limitazzjoni

Test limitat jista' jkun imwettaq fi 100 mg kull litru sabiex tkun determinata li il-EC_{50} tkun akbar minn din il-konċentrazzjoni.

Jekk in-natura tas-sustanza tkun tali li konċentrazzjonini ta' 100 mg kull litru fl-ima tat-test ma tistax tkun milħuqha, t-test tal-limitazzjoni għandu jkun imwettaq f'konċentrazzjoni egwali għas-solubbilità tas-sustanza (jew tal-konċentrazzjoni massima li tifforma tixrid stabbli) fil-medja użata (ara wkoll il-punt 1.6.1.1).

It-test tal-limitazzjoni għandu jkun imwettaq mill-anqas fi tlett darbiet, bl-istess numru ta' kontrolli. Iż-żewġ kejl tat-tbabbir (biomassa u r-rata tat-tkabbir) għandhom ikunu wżati għat-test tal-limitazzjoni.

Jekk, fit-test tal-limitazzjoni, medja ta' tnaqqis ta' 25% jew aktar tkun misjuba jew bil-biomassa inkella fir-rata tat-tkabbir bejn it-test tal-limitazzjoni u tal-kontroll, test sħiħ għandu jkun imwettaq.

2. **DATA U EVALWAZZJONI**

Id-densità mkejla taċ-ċelloli fil-kulturi tat-test u tal-kontrolli huma tabulati flimkien mal-konċentrazzjonijiet tas-sustanza tat-test u l-ħinijiet tal-kejl. Il-valur medju tad-densità taċ-ċellola għal kull konċentrazzjoni tas-sustanza tat-test u għall-kontrolli huwa mpinġi kontra l-ħin (0-72 siegħa) sabiex jipproduċu l-kurva tat-tkabbir.

Sabiex tkun determinata r-relazzjoni tal-konċentrazzjonini/effett, iż-żewġ resqien li ġejjin għandhom ikunu wżati. Uħud mis-sustanzi jistgħu jistimulaw it-tkabbit f'konċentrazzjoninijiet baxxi. Dawk il-punti biss tad-*data* li jindikaw l-inibizzjoni bejn 0 u 100% għandhom ikunu meqjusa.

2.1. TQABBIL TAŻ-ŻONI PERMEZZ TAL-KURVI TAT-TKABBIR

Iż-żona bejn il-kurvi tat-tkabbir u l-linja orizzonataali $N = N_0$ tistatkun ikkaalkolata skond din il-formula:

$$A = \frac{N_1 - N_0}{2} \times t_1 + \frac{N_1 + N_2 - 2N_0}{2} \times (t_2 - t_1) + \ldots + \frac{N_{n-1} + N_n - 2N_0}{2} \times (t_n - t_{n-1})$$

meta

A = il-medda taż-żona,

N_0 = in-numru ta' ċelloli/ml fil-ħin ta' t0 (il-bidu tat-test),$_0$,

N_1 = in-numru mkejjel ta' ċelloli/ml fi t_1,

N_n = in-numru mkejjal ta' ċelloli/ml fil-ħin t_n,

t_1 = il-ħin ta' l-ewwel kejl wara l-bidu tat-test,

t_n = il-ħin tal-kejl applikabbli wara l-bidu tat-test,

n = in-numru ta' kejl meħuda wara l-bidu tat-test.

Il-persentaġġ ta' l-inibizzjoni tat-tkabbir taċ-ċellola f'kull konċentrazzjonini tas-sustanza (I_A) huwa ikkalkolat skond din il-formula:

$$I_A = \frac{A_c - A_t}{A_c} \times 100$$

meta

A_c = il-medda taż-żona bejn il-kurva tal-kontroll tat-tkabbir u l-linja orizzontali $N = N_0$.

A_t = il-medda taż-żona bejn il-kurva tat-tkabbir fil-konċentrazzjoni t u l-linja orizzontali $N = N_0$.

Il-valuri I_A huma mpinġija fuq karta semilogaritmika jew fuq karta probit semilogaritmika kontra l-konċentrazzjonijiet korrespondenti. Jekk pinġuti fuq karta probit, il-punti huma mdaħħla b'linja dritta, jew bl-għajnejn inkella b'regressjoni komputata.

Il-EC_{50} hija ikkalkolata bil-linja tar-regressjoni bil-qari mill-konċentrazzjoni li hija ekwivalenti għall-inibizzjoni ta' 50% (IA = 50%). Sabiex ikun muri dan il-valur mingħajr ebda ambigwità b'relazzjoni ma dan il-metodu tal-kalkolazzjoni, huwa proponut li jsir użu mis-simbolu E_bC_{50}. Huwa essenzali li l-E_bC_{50} ikun ikkwotat mal-perijodu xieraq ta' l-esposizzjoni, e.g. E_bC_{50}(0-72h).

2.2. TQABBIL TAR-RATI TAT-TKABBIR

Il-medja tar-rata speċifika tat-tkabbir (μ) għall-kulturi li jikbru b'mod esponenzali tista tkun ikkalkolata bħala

$$\mu = \frac{\ln N_n - \ln N_0}{t_n - t_0}$$

meta t_0 = hija il-ħin fil-bidu tat-test

B'mod alternattiv, il-medja tar-rata speċifika tat-tkabbir tista tkun akkwistata mil-linja mżerzqa tar-regressjoni fit-tpinġija ta' 1n N *versus* il-ħin.

Il-persentaġġ ta' l-inibizzjoni speċifika tat-tkabbir f'kull konċentrazzjoni tas-sustanza ($I_\mu t$) huwa ikkalkolat skond din il-formula:

$$I_{\mu t} = \frac{\mu_c - \mu_t}{\mu_c} \times 100$$

meta

μ_c = medja tal-kontroll speċifiku tat-tkabbir

μ_t = medja tar-rata speċifika tat-tkabbir għall-konċentrazzjoni tat-test t

It-tnaqqis perċentwali fil-medja speċifika tat-tkabbir f'kull konċentrazzjoni tas-sustanza tat-test imqabbel mal-valur tal-kontroll huwa mpinġi kontra l-logaritmu tal-konċentrazzjoni. Il-EC_{50} jista jkun moqri mill-grafika riżultanti. Sabiex tkun murija n-nuqqas ta' ambigwità, il-EC_{50} akkwistat b'dan il-metodu, huwa proponut li jsir użu mis-simbolu ErC_{50}. Il-ħinijiet tal-kejl għandhom ikunu indikati, e.g. jekk il-valuri jirrigwardjaw il-ħinijiet 0 u 21 siegħa, is-simbolu isir E_rC_{50} (0-72h).

Nota: rata speċifika tat-tkabbir hija t-terminu logaritmiku, u tibdiliet żgħar fir-rata tat-tkabbir tista' twassal għal tibdiliet kbar fil-biomassa. Il-valuri E_bC u E_rC huma għalhekk mhux numerikament komparabbli.

2.3. KALKOLU TAL-NOEC

Il-Konċentrazzjoni ta' l-Effett Osservat N hija determinata bi proċedura statistikali xierqa għat-tqabbil tal-multi-kampjun (e.g. analaiżi tal-varjanza u t-test Dunnett), bl-użu tal-valur ta' repliki individwali taż-żoni fil-kurvi tat-tkabbir A (ara l-unt 2.1) jew fir-rati tat-tkabbir μ (ara l-punt 2.2).

3. **RAPPORTAĠĠ**

Ir-rapport tat-test għandu, jekk possibbli, jinkludi l-informazzjoni li ġejja:

— sustanza tat-test: *data* ta' l-identifikazzjoni tal-kimika;

— organiżmi tat-test: oriġini, kultura tal-laboratorju, numru tas-silta, metodu tal-kultivazzjoni;

— il-kondizzjoni tat-test:

  — il-jum tal-bidu u tat-tmiem tat-test u t-tul taż-żmien tiegħu,

  — temperatura,

  — il-komposizzjoni tal-medja,

  — l-apparat tal-kultura,

  — il-pH tat-taħlitiet fil-bidu u fit-tmiem tat-test (bħala spjegazzjoni għandhom ikunu ipprovduti jekk id-devjazzjonijiet tal-pH ikunu aktar minn 1,5 unità ta' kif osservati),

  — il-mezz u l-metodu użat għat-taħlil tas-sustanza tat-test u l-konċentrazzjoni tal-mezz fit-taħlitiet tat-test,

  — l-intensità u l-kwalità tad-dawl,

  — il-konċentrazzjonijiet ittestjati (imkejla jew minimi).

— riżultati:

  — id-densita) taċ-ċellola għal kull garafina f'kull punt tal-kejl u l-metodu għall-kejl tad-densità taċ-ċellola,

  — valuri medji tad-densità taċ-ċellola,

  — kurvi tat-tkabbir,

  — preżentazzjoni grafika tal-konċentrazzjoninijiet li jaffetwaw il-relattività,

  — il-valuri KE u l-metodu tal-kalkolazzjoni,

  — NOEC,

  — effetti oħrajn osservati.

4. **REFERENZI**

(1) OECD, Pariġi, 1981, Linji ta' Gwida tat-Testijiet 201, Deċiżjoni tal-Kunsill C(81) 30 Finali.

(2) Umweltbundesamt, Berlin, 1984, Verfahrensvorschlag 'Hemmung der Zellvermehrung bei der Grünalge *Scenedesmus subspicatus*', in: Rudolph/Boje: Ökotoxikologie, ecomed, Landsberg, 1986.

(3) ISO 8692 – Kwalità ta' l-ilma – Test ta' l-inibizzjoni tat-tkabbir ta' l-algal fl-ilma frisk bil *Scenedesmus subspicatus* u *Selenastrum capricornutum*.

(4) S.Galassi and M.Vighi - Chemosphere, 1981, vol.10, 1123-1126.

Appendiċi 1

Eżempju tal-proċedura għall-kultura ta' l-algae

Oservazzjonijiet ġenerali

L-iskop tal-kultura fuq il-bażi tal-proċedura li ġejja huwa li jkunu akkwistati kulturi ta' l-algal għat-testijiet tat-tossiċità.

Metodi xierqa għandhom ikunu wżati sabiex jassiguraw li l-kulturi ta' l-algal ma jkunux infettati bil-bakterja (ISO 4833). Kulturi asseniċi jistgħu jkunu mixtieqa imma kulturi unjagali huma essenzali.

L-operazzjonijiet kollha għandhom jitwettqu f'kondizzjonijiet sterili sabiex tkun evitata l-kontaminazzjoni mal-bakterja minn algae oħrajn. Kulturi kontaminati għandhom ikunu miċħuda.

Proċeduri għall-akkwista tal-kulturi algal

Preparazzjoni tat-taħlitiet tan-nutrient (medja):

Il-medja tista tkun ippreparata bit-trattib tat-taħlitiet konċentrati bil-lest tan-nutrijenti. Għal medja solida, 0,8% agar huwa miżjud. Il-medja wżata għandha tkun sterili. Sterlizzazzjoni bl-awtoklavar tista twassal għat-telf ta' NH_3.

Kultura bil-lest:

Il-kulturi bil-lest huma kulturi algali żgħar li huma regolarment trasferiti lejn medja friska sabiex jaġixxu bħala l-materjal tat-test inizjali. Jekk il-kulturi ma jkunux użati regolarment, dawn għandhom ikunu mferrgħin bilmod mit-tubi agar. Dawn huma trasferiti lejn medja friska mill-anqas darba kull xahrejn.

Il-kulturi bil-lest huma mkabbra f'garafini koniċi li jkun fihom il-medja xierqa (volum ta' madwar 100 ml). Meta l-algae huma inkubati fi 20°C b'illuminazzjoni kontinwa, trasferiment ta' kull ġimgħa huwa meħtieġ.

Matul it-trasferiment, ammont tal-kultura "qadima" huwa trasferit b'pipetti sterili għal ġewwa garafina bil-medja friska, hekk li l-ispeċi li jikbru bil-ħeffa, l-konċentrazzjoni inizjali tkun ma madwar 100 darba iżgħar minn dik tal-kultura l-qadima.

Ir-rata tat-tkabbir ta' speċje tista tkun determinata mill-kurva tat-tkabbir. Jekk dan ikun magħruf, ikun possibbli li tkun ikkalkolata d-densità li fiha l-kultura għandha tkun trasferita lejn medja ġdida. Dan għandu jseħħ qabel il-kultura tilħaq il-fażi tal-mewt.

Pre-kultura:

Il-pre-kultura hija ntiża li tagħti ammont ta' algae adatti għall-innokulazzjoni fil-kulturi tat-test. Il-pre-kultura hija inkubata fl-istess kondizzjonijiet bħal tat-test u użata meta tkun għadha esponenzalment tikber, normalment wara perijodu ta' inkubazzjoni ta' madwar tlett ijiem. Meta l-kulturi ta' l-algal ikun fihom ċelloli deformati jew annormali, dawn għandhom ikunu mwarrba.

Appendiċi 2

L-ISO 8692 - Kwalità ta' l-ilma - Test ta' l-inibizzjoni tat-tkabbir ta' l-algar f'ilma frisk bi *Scenedesmus subspicatus* u *Selenastrum capricornutum*, jirraportaw ir-riżultati li ġejjin għal test inter-laboratorju fost 16 il-baoratorju, testijiet bid-dikromat tal-putassa:

	Mezzi (mg/l)	Medda (mg/l)
E_rC_{50} (0-72 h)	0,84	0,60 to 1,03
E_bC_{50} (0-72 h)	0,53	0,20 to 0,75

C.4. DETERMINAZZJONI TAL-BIODEGRADABBILITÀ "LESTA"

PARTI I. KONSIDERAZZJONIJIET ĠENERALI

I.1. INTRODUZZJONI

Sitt metodi tat-ġtestijiet huma deskritti li jippermettu l-għażla tal-kimiċi għal biodegradabbilità lesta f;medja akwea arobika:

(a) Dissolved Organic Carbon (DOC) Die-Away (Method °C.4-A) (Karbonju Organiku Mdewweb (DOC) Bit-Tneħħija tal-Kulur (Metodu °C.4-A)

(b) Modified OECD Screening - DOC Die-Away (Method °C.4-A) (Għażla Mofifikata OECD - DOC Bit-Tneħħija tal-Kulur (Metodu °C.4-B)

(c) Carbon dioxide (CO_2) Evolution (Modified Sturm Test) (Method °C.4-C) (Evoluzzjoni tad-diossidu tal-karbonju (CO_2) (Test Sturm modifikat (Metodu °C.4-C)

(d) Manometric Respirometry (Method °C.4-D) (Respirometrija Manjometrika (Metodu °C.4-D)

(e) Flixkun magħluq (Metodu °C.4-E)

(f) MITI (Ministeru tal-Kummerċ Internazzjonali u l-Industrija – Ġappun) (Metodu °C.4-F)

Konsiderazzjonijiet ġenerali u komuni għas-sitt testijiet kollha huma mogħtija fil-Parti I tal-metodu. Punti speċifiċi għal metodi individwali huma mogħtija fil-Partijiet II sa VII. L-annessi fihom definizzjonijiet, formuli u materjal ta' gwida.

Eżerċizzju ta' tqabbil inter-laboratorju OECD, magħmul fil-1988, kien wera li l-metodi jagħtu riżultati konsistenti. B'danakollu, jiddependi mill-karatteristiċi fiżiċi tal-sustanza tat-test, wieħed jew ieħor mill-metodi jista jkun preferut.

I.2. SELEZZJONI TAL-METODU XIERAQ

Sabiex ikun magħżul il-metodu l-aktar xieraq, informazzjoni dwar is-solubbiltà tal-kimika, pressa tal-fwar u l-karatteristiċi ta' l-assorbazzjoni huma essenzali. L-istruttura jew il-formula tal-kimika għandhom ikunu magħrufa sabiex ikun ikkalkolati l-valuri teoretiċi u/jew il-verifika tal-valuri mkejla tal-parametri, e.g. ThOD, $ThCO_2$, DOC, TOC, COD (ara l-Annessi I u II).

Kimiki tat-test li jinħallu fl-ilma għal mill-anqas 100 mg/l jistgħu ikunu assessjati bil-metodi kollha, basta li dawn ma jkunux volatili u mhux-assorbanti. Għal dawk il-kimiki li jinħallu bilmod fl-ilma, li huma volatili jew assorbanti, metodi xierqa huma indikati fit-Tabella 1. Il-manjera li biha l-kimiċi li jinħallu bilmod fl-ilma u kimiki volatili jistgħu ikunu ittratti kif deskritt fl-Anness III. Kimiki moderatament volatili jistgħu ikunu ittestjati bil-metodu DOC tat-Tneħħija tal-Kulur jekk ikun hemm spazzju biż-żejjed tal-gass fir-reċipjenti tat-test (li għandhom ikunu magħluqa sewwa). F'dan il-każ, kontrol abjotiku jista jkun stabbilit sabiex jippermetti għal xi telf fiżiku.

Tabella 1: Applikabbiltà tal-metodi tat-test

Test	Metodu analitiku	Adattabbilità għal tahlitiet li huma:		
		Jinhallu ftit	volatili	assorbenti
DOC Tneħħija tal-kulur	Karbonju organiku mdewweb	—	—	+/–
Mod. OECD Tneħħija tal-kulur	Karbonju organiku mdewweb	—	—	+/–
Evoluzzjoni CO_2	Respirometrija: Evoluzzjoni CO_2	+	—	+
Respirometrija manometrika	Respirometrija manometrika: konsum ta' l-ossiġenu	+	+/–	+
Flixkun magħluq	Respirometrija: ossiġenu mdewweb	+/–	+	+
MITI	Respirometrija: konsum ta' l-ossiġenu	+	+/–	+

Informazzjoni dwar il-purità jew il-proporzjonijiet relattivi ta' komponenti maġġuri tal-materjal tat-test hija meħtieġa għall-interpretazzjoni tar-riżultati akkwistati, speċjalment meta r-riżultati huma baxxi jew marġinali.

Informazzjoni dwar it-tossiċità tal-kimika tat-test mal-bakterja (l-Anness IV) tista tkun ferm utili għall-għażla tal-konċentrazzjonijiet xierqa tat-test u tista tkun essenzjali għall-interpretazzjoni tajba tal-valuri tal-biodegradazzjoni baxxa.

I.3. SUSTANZI TA' REFERENZA

Sabiex tkun verifikata l-proċedura, kimiċi ta' referenza li jilħqu l-kriterja għal biodegradabbilità faċli huma ittestjati billi jkun hemm garafina xierqa b'mod parallel mal-provi nomali tat-test.

Kimiċi xierqa huma l-anilin (iddistillat frisk), l-aċetat tas-sodju u l-benżoat tas-sodju. Dawn il-kimiċi ta' referenza kollhom jkollhom degradazzjoni b'dawn il-metodi meta l-ebda *inoculum* ma jkun deliberatament miżjud.

Huwa suġġerit li kika ta' referenza għandha tkun imfittxija li jkollha biodegradabbilità faċli imma meħtieġa b'żieda ma l-*inoculum*. Il-ftalat ta' l-idroġenu tal-putassa kien proponut imma aktar evidenza jeħtieġ li tkun akkwistata b'din is-sustanza qabel ma tista tkun aċċettata bħal sustanza ta' referenza.

Fit-testijiet respirometriċi, il-komposti li jkun fihom in-nitroġenu jistgħu jaffettwaw l-akkwist ta' l-ossiġenu minħabba n-nitrifikazzjoni (ara l-Annessi II u V).

I.4. PRINCIPJU TAL-METODI TAT-TEST

Taħlita, jew sospenzjoni, tas-sustanza tat-test f'medja minerali, hija innokulata u inkubata permezz tal-kondizzjonijiet erobiċi, fid-dlam jew f'dawl baxx hafna. L-ammont ta' DOC fit-taħlita tat-test minħabba l-*inoculum* għandu jinżamm baxx kemm jista jkun possibbli imqabbel ma l-ammont ta' DOC minħabba s-sustanza tat-test. Għandu jkun hemm marġini għall-attività endoġenea ta' l-*inoculum* billi jkunu mwettqa testijiet parallelli fl-imbjank bl-*inoculum* imma mingħajr is-sustanza tat-test, għalkemm l-attività endoġenea taċ-ċelloli fil-preżenta tas-sustanza ma tkunx eżattament taqbel ma dik fil-kontroll endoġeneu. Sustanza ta' referenza tkun utilizzata b'mod parallel għall-verifika ta' l-operazzjoni tal-proċeduri.

Ġeneralment, degradazzjoni hija segwita bid-determinazzjoni tal-parametri, bħalma huma DOC, produzzjoni CO_2 bl-akkwist ta' l-ossiġenu, u l-kejl ikun meħud f'intervalli ta' spiss suffiċjenti sabiex jippermettu l-identifikazzjoni tal-bidu u tat-tmiem tal-biodegradazzjoni. B'respirometri awtomatiċi, il-kejl ikun kontinwu. DOC ta' kultant huwa mkejjel b'żieda ma parametru ieħor, imma dan normalment iseħħ biss fil-bidu u fit-tmiem tat-test. Analiżi kimika speċifika tista wkoll tkun użata għall-assessjar tad-degradazzjoni primarja tas-sustanza tat-test, u sabiex tkun determinata l-konċentrazzjoni ta' xi sustanzi intermedji iffurmati (obligatorja fit-test MITI).

Normalment, it-test idum 28 jum. B'danakollu, t-testijiet jistgħu jieqfu qabel it-28 jum, i.e. malli l-kurva tal-bijodegredazzjoni tkun laħqet il-quċċata lixxa għal mill-anqas 3 determinazzjonijiet. It-testijiet jistgħu wkoll ikunu mtawla lill hinn minn 28 jum meta l-kurva turi li l-biodegradazzjoni tkun bdiet imma l-quċċata lixxa ma tkunx intlaħqet fil-jum 28.

I.5. KRITERJA TAL-KWALITÀ

I.5.1. **Reproduċibbiltà**

Minħabba n-natura tal-biodegradazzjoni u tal-popolazzjoni mħallta tal-bakterja użata bħala *inocula*, determinazzjonijiet għandhom jitwettqu mill-anqas b'mod doppju.

Hija esperjenza komuni li akbar ma tkun il-konċentrazzjoni tal-mikro-organiżmi inizjalment miżjuda mal-medja tat-test, aktar tkun żgħira l-varjazzjoni bejn ir-repetizzjonijiet. Testijiet biċ-ċrieki kienu wkoll urew li kien hemm varjazzjonijiet kbar bejn ir-riżultati akkwistati minn laboratorji differenti, imma ftehim tajjeb huwa normalment akkwistat b'komposti faċilment biodegradabbli.

I.5.2. **Validità tat-test**

Test huwa meqjus bħala validu jekk id-differenza ta' l-estremi fil-valuri tar-repetizzjoni tat-tneħħija tal-kimika tat-test fil-quċċata lixxa, fit-tmiem tat-test jew fit-tmiem tas-sessjoni ta' 10 t'ijiem, kif xieraq, hija anqas minn 20% u jekk il-perċentwal tad-degradazzjoni tas-sustanza ta' referenza jkun laħaq il-livell għal biodegradabbiltà faċli sa 14 il-jum. Jekk waħda minn dawk il-kondizzjonijiet ma tkunx milħuqha, it-test għandu jkun repetut. Minħabba l-limitazzjoni stretta tal-metodi, valuri baxxi mhux neċessarjament ifissru li s-sustanza tat-test ma tkun biodegradabbli permezz tal-kondizzjonijiet ambjentali, imma tindika li aktar xogħol għandu jsir sabiex tkun stabbilita l-biodegradabbiltà.

Fit-test tat-tossiċità, li jkun fih kemm it-test tas-sustanza u kimika ta' referenza, anqas minn 35% degradazzjoni (ibbażat fuq DOC) jew anqas minn 25% (ibbażata fuq RhOD jew $ThCO_2$) li tkun seħħet matul l-14 il-jum, il-kimika tat-test tista tkun meqjusa bħala inibitriċi (ara wkoll l-Anness IV). Is-serje ta' testijiet għandu jkun repetut, jekk possibbli bl-użu ta' konċentrazzjoni aktar baxxa tal-kimika tat-test u/jew ta' konċentrazzjoni aktar għolja ta' l-*inoculum*, imma mhux aktar minn 30 mg solidi/litru.

I.6. PROCEDURA ĠENERALI U PREPARAZZJONIJIET

Il-kondizzjonijiet ġenerali applikabbli għat-testijiet huma riassunti fit-Tabella 2. Apparat u l-kondizzjonijiet sperimentali tal-kondizzjonijiet li jappartieni speċifikament għal test individwali huma deskritti aktar tard taħi l-intestatura għall-dak it-test.

Tabella 2: Kundizzjonijiet tat-test

Test	DOC Tneħħija tal-kulur	Evoluzzjoni CO_2	Respirometrija manometrika	Għażla modifikati OECD	Flixkun maghluq	MITI (1)
Konċentrazzjoni tas-Sustanza tat-test hija bħala mg/l					2-10	100
10-40	10-20	10-40	100	mg DOC/l		
mg ThOD/l			50-100		5-10	
Konċentrazzjoni ta' l-*Inoculum* (f'ċelloli/I, b'mod approssimattiv)	= 30 mg/1 SS jew = 100 ml effluwent/1 (10^7- 10^8)			0,5 ml affluwent sekondarju/l (10^5)	= 5 ml ta' l-effluwent/1 (10^4 – 10^6)	30 mg/l SS (10^7 – 10^8)
Konċentrazzjoni ta' l-elementi fil-medja minerali (fi mg/I)						
P	116				11,6	29
N	1,3				0,13	1,3
Na	86				8,6	17,2
K	122				12,2	36,5
Mg	2,2				2,2	6,6
Ca	9,9				9,9	29,7
Fe	0,05-0,1				0,05-0,1	0,15
pH	7,4 ± 0,2					preferibbilment 7,0
Temperatura	22 ± 2 °C					25 + 1 °C

DOC = Dissolved Organic Carbon (Karbonju organiku mdewweb) ThOD = Theoretical Oxygen Demand (Talba teoretika ta' l-ossiġenu) SS = Solidi sospiżi

I.6.1. **Ilma tat-trattib**

Ilma deonizzat jew distillat, ħieles minn konċentrazzjonijiet inibitorji ta' sustanzi tossiċi (e.g. Cu^{++} jonji) huwa wżat. Dan għandu jkun fih mhux aktar minn 10% tal-kontenut tal-karbonju organiku introdott bil-materjal tat-test. Il-purità għolja ta' l-ilma tat-test hija meħtiega sabiex telimina l-valuri għolja fl-imbjank.

Kontaminazzjoni tista tirriżulta minn impuritajiet inerenti u wkoll minn reżini ta' l-iskambju tal-jonji u l-materjal *lysed* mill-bakterja u l-algae. Għal kull serje ta' testijiet għandhek tuża lott wieħed biss ta' ilma, ivverifikat bil-quddiem bl-analiżi DOC. Tali verifika ma tkunx meħtieġa għat-test tal-flixkun magħluq, imma l-konsum ta' l-ossiġenu ta' l-ilma għndu jkun baxx.

I.6.2. **Taħlitiet bil-lest ta' komposti minerali**

Sabiex isiru t-taħlitiet tat-test, taħlitiet bil-lest tal-konċentrazzjonijiet xierqa jkunu magħmula minn komponenti minerali. Dawn it-taħlitiet bil-lest li ġejjin jistgħu jkunu wżati (bil-fatturi differenti tat-trattib) għall-metodu DOC Tneħħija bil-kulur, Għażla OECD Modifikata, Evoluzzjoni CO_2, Respirometrija Manometrika, it-test tal-Flixkun Magħluq. Il-fatturi tat-trattib u, għat-test MITI, il-preparazzjoni speċifika tal-medja minerali, huma mogħtija taht l-intestaturi tat-testijiet speċifiċi.

Taħlitiet bil-lest: Ipprepara t-taħlitiet bil-lest li ġejjin, bl-użu ta' reaġenti ta' grad analitiku.

(a)	Monopotassi*um dihydrogen orthophosphate*, KH_2PO_4	8,50 g
	Dipotassium monohydrogen orthophosphate, KH_2PO_4	21,75 g
	Disodium monohydrogen orthophosphate dihydrate $Na_2HPO_4 . 2 H_2O$	33,40 g
	Klorid ta' l-ammonium NH_4Cl	0,50 g
	Dewweb fl-ilma u wassal sa 1 litru. Il-pH tat-taħlita għandu jkun 7,4.	
(b)	Klorid tal-kalċji, anidriju, $CaC1_2$	27,50 g
	jew dihidrat tal-klorid tal-kalċju $CaCl_2 . 2 H_2O$	36,40 g
	Dewweb fl-ilma u wassal sa litru	
(c)	Eptaidrat tas-sulfat tal-magneżju, $MgSO_4 7 H_20$	22,50 g
	Dewweb fl-ilma u wassal għal-litru	
(d)	Ħadid (III) eksaidrat tal-klorid, $FeCl_3 . 6 H_2O$	0,25 g
	Dewweb fl-ilma u wassal għal-litru.	

Nota: Sabiex tkun evitata l-ħtieġa li tkun ippreparata din is-soluzzjonu immedjetament qabel l-użu, żid qatra ta' HCL ikkonċentrat, jew 0,4 g melħ disodju ta' l-aċidu *ethylenediaminetetra*-aċetiku (EDTA) kull litru.

I.6.3. **Taħlitiet bil-lest ta' kimiċi**

Per eżempju, dewweb 1-10 g, kif xieraq, tal-kimika tat-test jew ta' referenza, f'ilma de-jonizzata u wassal sa litru meta s-solubbiltà teċċedi 1 g/l. Altrimenti, ipprepara t-taħlitiet bil-lest fil-medja minerali jew żid il-kimika direttament mal-medja minerali. Għat-trattament ta' kimiċi anqas solubbli, ara l-Anness III, imma fit-test MITI (il-Metodu °C.4-F), la s-solventi u l-anqas l-aġenti emulsifikanti m'għandhom ikunu wżati.

I.6.4. **Inocula**

L-*inoculum* jista jkun akkwistat minn varjeta ta' sorsi: ħama' attivata, effluwenti tad-drenaġġ (mhux klorinati), ilmijiet minn wiċċ l-art u ħamrija jew minn taħlita ta' dawn. Għal testijiet tar-Repirometrija ta' l-Evoluzzjoni jew Manjometika DOC Tneħħija bil-Kulur, CO_2, jekk il-ħama' attivata tkun użata, dan għandu jkun meħud minn impjant tat-trattament jew unità ta' skala ta' laboratorju li tirċieci predominantament drenaġġ domestiku. Kien instab li *Inocula* minn sorsi oħrajn tagħti tixrid aktar għoli tar-riżultati. Għall-għażla OECD Modifikata u t-testijiet tal-Flixkun Magħluq, *inoculum* mingħajr il-frak tal-ħama' aktar imdewwb huwa meħtieġ u s-sorsi preferita huwa l-effluwent sekondarju mill-impjant tat-trattament ta' l-ilma ta' l-iskart domestiku jew minn unita ta' skala ta' laboratorju. Għat-test MITI, l-*inoculum* huwa akkwistat minn taħlita ta' sorsi u huwa deskritt taħt l-intestatura ta' dan it-test speċifiku.

I.6.4.1. *Inoculum minn ħama' attivita*

Iġbor kampjun minn ħama' attivata friska mit-tank ta' l-arjazzjoni ta' l-impjant tat-trattament tad-drenaġġ jew minn unit´ta' skala tal-laboratorju li predominantament jitratta d-drenaġġ domestiku. Warral il-partiċelli rozzi, jekk meħtieġ bil-filtrazzjoni minn passatur fin u wara dan zomm il-ħama f'mod erobiku.

B'mod alternattiv, ħalli joqgħod, jew inkella għaddi miċ-ċentrufuga (e.g. fi 1 100 g għal 10 min.) wara t-tneħħija ta' xi partiċelli rozzi. Warrab is-supernatant. Il-ħama tista tkun maħsula f'medja minerali. Issospendi l-ħama ikkonċentrata f'medja minerali sabiex takkwista konċentrazzjoni ta' 3-5 g solidi sospiżi kull litru u imla bl-arja sakemm meħtieġ.

Il-ħama' għandha tkun miġbura proporzjonalment minn impjant ta' xogħol konvenzjonali. Jekk il-ħama jkun jeħtieġ li tingħabar minn impjant ta' trattament qawwi, jew ikun maħsub li jkun fiha inibituri, din għandha tkun maħsula. Ħalliha toqgħod jew għaddi l-ħama' sospiża mill-ġdid minn ċentrifugu wara li tkun imħallta sewwa, warrab is-supernatant u mill-ġdid erġa issospendi l-ħama' maħsula f'volum ieħor tal-medja minerali. Irrepeti din il-proċedura sakemm il-ħama' tkun meqjusa bħala ħielsa minn substrata jew inibitur eċċessivi.

Wara li s-sopenzjoni shiħa mill-ġdid tkun akkwistata, jew b'ħama' mhux trattata, warrab il-pamkun immedjetament qabel l-użu għad-determinazzjoni tal-piż xott tas-solidi sospiżi.

Alternattiva oħra hija li tkun omoġenizzata l-ħama' attivata (3-5 g solidi sospiżi kull litru). Il-ħama' hija trattata f'ħawwadi mekkaniku għal 2 minuti f'veloċità medja. Ħalli l-ħama' tal-ħawwadi toqgħod għal 30 minuta jew aktar jekk hekk meħtieġ u ferra l-likwidu għall-użu bħala *inoculum* bir-rata ta' 10 ml/l tal-medja minerali.

I.6.4.2. *Sorsi oħrajn ta' l-inoculum*

Dan jista jkun akkwistat minn effluwent sekondarju ta' l-impjant tat-trattament jew minn unità tal-iskala tal-laboratorju li jirċievi b'mod predominanti d-drenaġġ domestiku. Iġbor kampjun frisk u żommu erobiku waqt it-trasport. Ħallieh joqgħod għal siegħa jew iffiltra minn karta rozza tal-filtru u żomm l-effluwent imferra jew il-filtrat erobiku sakemm ikunu meħtieġa. Sa 100 ml ta' dan it-tip ta' *inoculum* jista jkun użat ma kull litru ta' medja.

Sors ieħor għall-*inoculum* hija l-ilma tal-wiċċ ta' l-art. F'dan il-każ, iġbor kampjun xieraq mill-ilma tal-wiċċ, e.g. xmara, għadira, u żommu erobiku sakemm ikun meħtieġ. Jekk meħtieġ, ikkonċentra l-*inoculum* bil-fitrazzjoni jew biċ-ċentrifugazzjoni.

I.6.5. **Pre-kondizzjonat ta' l-inocula**

L-*inocula* tista tkun pre-kondizzjonata għall-kondizzjonijiet sperimentali,, imma mhux pre-afattata għall-kimika tat-test. Il-pre-kondizzonjar jikkonsisti mill-arjazzjoni tal-ħama' attvita f'medja minerali jew effluwent sekondarju għal 5-7 tijiem fit-temperatura tat-test. Il-pre-kondizzjonar ta' kull tant itejjeb il-prċiżjoni tal-metodi tat-test bit-tnaqqis tal-valuri vojta. Huma meqjus bħala mhux neċessarju il-pre-kondizzjonar ta' l-*inoculum* MITI.

I.6.6. **Kontrolli abjotiċi**

Meta meħtieġ, għandek tivverifika d-degradazzjoni abjotika possibbli tas-sustanza tat-test bid-determinazzjoni tat-tneħħija ta' DOC, l-akkwist ta' l-ossiġenu jew ta' l-evoluzzjoni tad-diossidu tal-karbonju f'kontrolli sterili li ma jkun fihom l-ebda *inoculum*. Sterlizza bil-filtrazzjoni minn membrana (0,2-0,45 mikrometri) jew biż-żieda ta' sustanza tossika xierqa fil-konċentrazzjoni adatta. Jekk tkun użata l-membrana tal-filtrazzjoni, iġbor kampjuni b'mod axettiku għaż-żamma tal-isterilità. Sakemm l-assorbazzjoni tal-kimika tat-test ma tkunx diġa ġiet imwarrba minn qabel, testijiet li jkejlu l-biodegradazzjoni bħala t-tneħħija ta' DOC, speċjalment bl-*inocula* tal-ħama' attivita, għandhom jinkludu kontroll abjotiku li huwa innokulat u ivvelenat.

I.6.7. **Numru ta' garafini**

In-numru ta' garafini fi prova tipika huwa deskritt taħt l-intestaturi ta' kull wieħed mit-testijiet.

It-tip ta' garafina li ġej jista jkun użat:

Sospenzjoni tat-test: li jkun fiha s-sustanza tat-test u l-*inoculum*

Inoculumi fl-imbjank: li jkun fih biss l-*inoculum*

Proċedura tal-kontroll: li jkun fiha s-sustanza ta' referenza u l-*inoculum*

Kontroll ta' abjotiku sterili: sterili, li jkun fiha s-sustana tat-test (ara I.6.6)

Kontroll ta' l-adsorbazzjoni: li jkun fiha s-sustanza, l-*inoculum* u l-aġent sterilizzanti

Kontroll tat-tossiċità: li jkun fiha s-sustanza tat-test, is-sustanza ta' referenza u l-*inoculum*

Huwa mandatorju li d-determinazzjoni tas-sospenzjoni tat-test u l-imbjank ta' l-*inoculum* iseħħ b'mod paralell. Huwa rakommandabbli li jsiru d-determinazzjonijiet fil-garafini l-oħrajn b'mod parallel ukoll.

Dan jista, b'danakollu, mhux dejjem ikun possibbli. Assigura li kampjoni jew qari suffiċjenti jkunu meħuda sabiex jippermettu t-tneħħija perċentwali fl-ispażju ta' l-10 tijiem ta' l-assessjar.

I.7. DATA U EVALWAZZJONI

Fil-kalkolu ta' D_t, id-degradazzjoni preċentwali, il-valuri medji tal-kejl duplikat tal-parametru fiż-żewġ reċipjenti tat-test u l-imbjank ta' l-*inoculum*, ikunu wżati. Dawn il-formuli huma stabbiliti fis-sezzjonijiet ta' hawn taħt fit-testijiet speċifiċi. Il-korsa tad-degradazzjoni hija murija b'mod grafiku u l-perijodu ta' 10 tijiem huwa indikat. Ikkalkola u irrapporta l-perċentwal tat-tneħħija milħuq fit-tmiem tal-perijodu ta' 10 tijiem u l-valur tal-quċċata lixxa jew fit-tmiem tat-test, liema minnhom huwa xieraq.

Fit-testijiet respirometriċi, l-komposti li jkun fihom in-nitroġenu jistgħu jaffettwaw l-akkwist ta' l-ossiġenu minħabba n-nitrifikazzjoni (ara l-Annessi II u V).

I.7.1. **Degradazzjoni mkjela permezz tad-determinazzjoni DOC**

Id-degredazzjoni perċentwali D_t kull darba li kampjun ikun miġbur għandha tkun ikkalkolata b'mod separat għal kull waħda mill-garafini li jkun fihom is-sustanza tat-test bl-użu tal-valuri medji tal-kejn duplikat DOC sabiex il-validità tat-test tista tkun assessjata (ara I.5.2.). Hija ikkalkolata bl-użu ta' din l-ekwazzjoni li ġejja:

$$D_t = \left(1 - \frac{C_t - C_{bt}}{C_o - C_{bo}}\right) \times 100$$

meta:

D_t = % degradazzjoni fil-ħin t,

C_o = il-medja tal-bidu tal-konċentrazzjoni ta' DOC tal-kultura inokulata fil-medju li jkun fih is-sustanza tat-test (mg DOC/l),

C_t = il-medja tal-konċentrazzjoni ta' DOC tal-kultura innokulata fil-medju li jkun fih is-sustanza fil-ħin t (mg DOC/l),

C_{bo} = il-medja tal-bidu tal-konċentrazzjonini ta' DOC fl-imbjank inokulata fil-medja minerali (mg DOC/l),

C_{bt} = il-medja tal-konċentrazzjoni ta' DOC fl-imbjank innokulata fil-medja minerali fil-ħin t (mg DOC/l),

Il-konċentrazzjoninijiet kollha huma mkejla b'mod sperimentali.

I.7.2. **Degradazzjoni mkjela permezz ta' analiżi speċifika**

Meta *data* analitika speċifika tkun disponibbli, wettaq il-kalkolazzjoni tal-biodegredazzjoni primarja minn:

$$D_t = \frac{S_b - S_a}{S_b} \times 100$$

meta:

Dt = % degradazzjoni fil-ħin t, normalment 28 jum,

S_a = ammont residwu tas-sustanza tat-test fil-medja inokulata fit-tmiem tat-test (mg),

S_b = l-ammont residwu tas-sustanza tat-test fit-test fl-imbjank bl-ima/medja li magħha tkun ġiet miżjuda biss is-sustanza tat-test (mg).

I.7.3. **Degredazzjoni abjotika**

Meta jkun użat il-kontroll abjotiku sterili, wettaq il-kalkolazzjoni tal-perċentali tad-degradazzjoni abjotika bl-użu:

$$\% \text{ degradazzjoni abjotika} = \frac{C_{s(o)} - C_{s(t)}}{C_{s(o)}} \times 100$$

Meta:

$C_{s(o)}$ = DOC Il-konċentrazzjonini sterili tal-kontroll fil-jum 0

$C_{s(t)}$ = DOC Il-konċentrazzjonini sterili tal-kontroll fil-jum t

I.8. RAPPURTAĠĠ

Ir-rapport tat-test għandu, jekk possibbli, jinkludi l-informazzjoni li ġejja:

— is-sustanzi tat-test u tar-referenza, u il-purità tagħhom,

— il-kondizzjoni tat-test,

— *inoculum*: is-sit(i) tan-natura u l-kampjunar, konċentrazzjonini u xi trattament ta' pre-kondizzjonar;

— il-proporzjon u n-natura tal-iskart industriali preżenti fid-drenaġġ, jekk magħruf;

— iż-żmien u t-temperatura tat-test;

— fil-każ ta' kimika tat-test li ma tantx tinħall, it-trattament mogħti;

— il-metodu tat-test applikat; ir-raġinijiet xjentifiċi u l-ispejagazzjoni għandha tkun mogħtija għal xi tibdil tal-proċedura;

— il-folja tad-*data*;

— kwalunkwe fenomena ta' l-inibizzjoni;

— xi degradazzjoni abjotika osservata;

— id-*data* speċifika kimika analitika, jekk disponibbli;

— id-*data* analitika dwar l-intermedjati, jekk disponibbli;

— il-grafika tad-degradazzjoni perċentwali kontra l-ħin għat-test u s-sustanzi ta' referenza; il-faži li ddewwem il-process, il-faži tad-degradazzjoni, il-perijodu ta' 10 t'ijiem u l-linja għandha kjarament tkun indikata (l-Anness I). Jekk it-test ikun konformi mal-kriterja tal-validità, l-medja tal-perċentwali tad-degradazzjoni fil-garafini li jkun fihom is-sustanza tat-test tista tkun użata għall-grafika.

— perċentwal tat-tneħħija eata l-perijodu ta' 10 t'ijiem, u l-linja lixxa fil-quċċata jew fit-tmiem tat-test.

PARTI II. DOC TEST TAT-TNEĦĦIJA TAL-KULUR (MetodU °C.4-A)

II.1. PRINCIPJU TAL-METODU

Volum imkejjel tal-medja minerali inokulata li jkun fiha konċentrazzjonini magħrufa tas-sustanza tat-test 10-40 mg DOC/l) bħala l-unika sorsi nominali ta' karbonju organiku jkun mogħti l-arja fid-dlam jew fid-dawl baxx fi 22 ± 2 °C.

Id-degradazzjoni hija segwita bl-analiżi DOC matul intervalli frekwenti matul perijodu ta' 28 jum. Il-grad tal-biodegradazzjoni huwa ikkalkolat bl-espressjoni tal-konċentrazzjoni DOC imneħħija (ikkorreġuta għal dak fil-kontroll ta' l-*inoculum* fl-imbjank) bħala perċentwali tal-konċentrazzjoni inizjali preżenti. Il-grad tal-biodegradazzjoni primarja jista wkoll ikun ikkalkolat mill-analiżi kimika supplemetari mwettqa fil-bidu u fit-tmiem ta' l-inkubazzjoni.

II.2. DESKRIZZJONI TAL-METODU

II.2.1. **Apparat**

(a) Garafini koniċi, e.g. 250 ml sa 2 1, jiddependi mill-volum meħtieġ għall-analiżi DOC;

(b) Magna taċ-ċekċik li tista takkomoda l-garafini koniċi, jew bil-kontroll awtomatiku tat-temperatura jew użata fit-temperatura kostanti tal-kamra, u forza suffiċjenti tal-potenza għaż-żamma tal-kondizzjonijiet etobiċi fil-garafini kollha;

(c) Apparat tal-filtrazzjoni, b'membrani xierqa;

(d) Analiżattur DOC;

(e) Apparat għad-determinazzjoni ta' l-ossiġenu mdewweb;

(f) Centrifuga.

II.2.2. **Preparazzjoni tal-medja minerali**

Għall-preparazzjoni tat-taħlitiet bil-lest, ara I.6.2.

Ħawwad 10 ml tat-taħlita (a) ma 800 ml ilma tat-trattib, żid 1 ml tat-taħlitiet (b) sa (d) u wassal sa litru bl-ilma tat-trattib.

II.2.3. **Preparazzjoni u pre-kondizzjonar ta' l-*inoculum***

L-*inoculum* jista jkun akkwistat minn varjeta ta' sorsi: ħama' attivata, effluwenti tad-drenaġġ (mhux klorinati), ilmijiet minn wiċċ l-art u ħamrija jew minn taħlita ta' dawn.

Ara I.6.4., I.6.4.1., I.6.4.2. u I.6.5.

II.2.4. **Preparazzjoni tal-garafini**

Bħala eżempju, introduċi 800 ml porzjonijiet tal-medja minerali ġewwa garafini koniċi 2 l u żid volumi suffiċjenti tat-taħlitiet bil-lest tat-test u tas-sustanzi ta' referenza mal-garafini separati sabiex takkwista konċentrazzjoni kemikali ekwivalenti għal 10-40 mg DOC/1. Ivverifika l-valuri pH u aġġusta, jekk meħtieġ, għal 7,4. Inokula l-garafini b'ħama' attivita jew b'sorsi oħrajn ta' l-*inocula* (ara I.6.4.), sabiex takkwista konċentrazzjoni mhux aktar minn 30 mg solidi sospiżi fil-litru. Ippreapara wkoll il-kontrolli ta' l-*inoculum* fil-medja minerali imma minghajr il-kimika tat-test jew ta' referenza.

Jekk meħtieġ, uża reċipjent wieħed sabiex tivverifika l-possibbiltà ta' l-effett inibitorju tal-kimika tat-test bl-inokulazzjoni tat-taħlita li jkun fiha, fil-medja minerali, konċentrazzjonijiet komparabbli kemm tal-kimika tat-test u dik ta' referenza.

Ukoll, jekk meħtieġ, organizza garafina sterili oħra sabiex tivverifika jekk il-kimika tat-test tkun degradata abjotikament bl-użu tat-taħlita mhux inokulata tal-kimika (ara I.6,6.).

B'mod addizzjonali, jekk ikun hemm suspett li l-kimika tat-test tkun sinifikament assorbita fuq il-ħġieġ, ħama' etc., wettaq assessjar preliminari sabiex tkun determinata l-medda possibbli ta' l-assorbazzjoni u b'hekk l-adattabbiltà tat-test għall-kimika (ara t-Tabella 1). Organizza garafina li jkun fiha s-sustanza tat-test, l-*inculum* u l-aġent sterilizzanti.

Wassal il-volumi fil-garafini kollha għal 1:1 bil-medja minerali u, wara t-taħwib, ħu kampjun minn kull garafina sabiex tiddetermina l-konċentrazzjonini inizjali ta' DOC (ara l-Anness II,4). Għatti l-ftugħ tal-garafini, e.g. b'liega ta' l-aluminju, b'tali mod hekk li tippermetti l-iskambju ħieles ta' l-arja bejn il-garafina u l-atmosfera tal-madwar. Imbagħad deffes ir-reċipjenti fil-magna taċ-ċekċik għall-bidu tat-test.

II.2.5. **Numru ta' garafini fi prova tipika**

Il-garafini 1 u 2: Sospenzjoni tat-test,

Il-garafini 3 u 4: L-imbjank ta' l-*inoculum,*

Garafina 5: Proċedura tal-kontroll preferibbilment

u meta meħtieġ:

Garafina 6: Kontroll ta' abjotiku sterili

Garafina 7: Kontroll ta' l-adsorbazzjoni

Garafina 8: Kontroll tat-tossiċità

Ara wkoll I.6.7.

II.2.6. **It-twettieq tat-test**

Matul it-test, iddetermina l-konċentrazzjonijiet ta' DOC f'kull garafina b'mod doppju f'intervalli tal-ħin magħruf, ta' spiss b'mod suffiċjenti sabiex tkun tista tiddetermina l-bidu tal-perijodu ta' 10 tijiem u l-persentaġġ tat-tneħħija fit-tmiem tal-perijodu ta' 10 tijiem. Ħu biss il-volum minimu tas-sospenzjoni tat-test meħtieġ għal kull determinazzjoni.

Qabel il-kampjunar għamel tajjeb għat-telf ta' l-evaporazzjoni mill-garafini biż-żieda ta' ilma tat-trattib (I.6.1) fl-ammont meħtieġ, jekk ikun neċessarju. Ħawwad il-medja tal-kultura sewwa qabel ma tiġbed il-kampjun u assigura li l-materjal li jeħel mal-ġnub tar-reċipjenti jkun imdewweb jew sospiż qabel il-kampjunar. Immedjetament iffiltra mill-membrana jew b'ċentrifuga (ara l-Anness II.4) wara li jkun inġabar il-kampjun. Analizza l-kampjuni tal-filtrat jew taċ-ċentrifuga l-istess jum, altrimentri aħżen fi 2-4 °C għal massimu ta' 48 siegħa, jew f'anqas minn 18 °C għal perijodu twil.

II.3. DATA U RAPPORTAĠĠ

II.3.1. **Trattament tar-riżultati**

Ikkalkola l-persentaġġ tad-degradazzjoni fil ħin kif mogħti taħt I.7.1. (determinazzjoni DOC) u, preferibbilment, taħt I.7.2. (analiżi speċifika).

Irreġistra r-riżultati kollha fuq il-karti tad-*data* kif ipprovduta.

II.3.2. **Validità tar-riżultati**

Ara I.5.2.

II.3.3. **Rappurtaġġ**

Ara I.8.

II.4. FOLJA TAD-DATA

Eżempju tal-folja tad-*data* huwa mogħti aktar il-quddiem.

TEST DOK TAT-TNEĦĦIJA TAL-KULUR

1. **LABORATORJU**

2. **IL-JUM TAL-BIDU TAT-TEST**

3. **IS-SUSTANZA TAT-TEST**

Isem:

Il-konċentrazzjoni tat-taħlita bil-lest: mg/l bħala kimika

Konċentrazzjoni inizjali fil-medja, għal: mg/l bħala kimika

4. **INOCULUM**

Sorsi:

Trattament mogħti:

Pre-kondizzjonar, jekk ikun hemm:

Konċentrazzjoni ta' solidi sospiżi fit-taħlita tar-reazzjoni: mg/l

5. **DETERMINAZZJONI TAL-KARBONJU**

Analizzatur tal-karbonju:

	Garafina Nru		DOC wara jiem n (mg/l)				
			0	n_1	n_2	n_3	n_x
Kimika tat-test flimkien ma l-*inoculum*	1	a_1					
		a_2					
		a, medja $C_{a(t)}$					
	2	b_1					
		b_2					
		b, medja $C_{b(t)}$					
Inoculum fl-imbjank mingħajr il-kimika tat-test	3	C_1					
		C_2					
		c, medja $C_{c(t)}$					
	4	d_1					
		d_2					
		d, medja. $C_{d(t)}$					
	$C_{bl(t)} = \frac{C_{c(t)} + C_{d(t)}}{2}$						

6. **EVALWAZZJONI TAD-*DATA* MHIX ITTRATTATA**

Garafina Nru		% degradazzjoni wara n jiem				
		0	n_1	n_2	n_3	n_x
1	$D_1 = \left(1 - \frac{C_{a(t)} - C_{bl(t)}}{C_{a(o)} - C_{bl(o)}}\right) \times 100$	0				
2	$D_2 = \left(1 - \frac{C_{b(t)} - C_{bl(t)}}{C_{b(o)} - C_{bl(o)}}\right) \times 100$	0				
Medja ([1])	$D = \frac{D_1 - D_2}{2}$	0				

([1]) D u D_2 m'għandhomx ikunu miġbura f'medja jekk ikun hemm differenza konsiderevoli.

Nota: formati simili jistgħu ikunu wżati għall-kimika tar-referenza u l-kontrolli tat-tossiċità

7. **KONTROLLI ABJOTICI** (voluntarji)

	Żmien (jiem)	
	0	t
Konċ. DOC (mg/l) f'kontroll sterili	$C_{s(o)}$	$C_{s(t)}$

$$\% \text{ degradazzjoni abjotika} = \frac{C_{s(o)} - C_{s(t)}}{C_{s(o)}} \times 100$$

8. **ANALŻI TA' KIMIKA SPECIFIKA** (voluntarja)

	ammont residwu tal-kimika tat-test fit-tmiem tat-test (mg/l)	% degradazzjoni primarja
Kontroll sterili	S_b	
Medja tat-test inokulat	S_a	$\frac{S_b - S_a}{S_b} \times 100$

PARTI III. TEST TA' L-GĦAŻLA OECD MODIFIKATA (Metodu °C.4-B)

III.1. PRINCIPJU TAL-METODU

Volum imkejjel tal-medja minerali inokulata li jkun fiha konċentrazzjoni magħrufa tas-sustanza tat-test (10-40 mg DOC/l) bħala l-unika sorsi nominali ta' karbonju huwa inokulat b' 0,5 ml effluwent kull litru tal-medja. It-taħlita hija mogħtija l-arja fid-dlam jew f'dawl baxx ħafna fi 22 ± 2 °C.

Id-degradazzjoni hija segwita bl-analiżi DOC matul intervalli frekwenti matul perijodu ta' 28 jum. Il-grad tal-biodegradazzjoni huwa ikkalkolat bl-espressjoni tal-konċentrazzjonini DOC imneħħija (ikkorreġuta għal dak fil-kontroll ta' l-*inoculum* fl-imbjank) bħala perċentwali tal-konċentrazzjonini inizjali preżenti. Il-grad tal-biodegradazzjoni primarja jista wkoll ikun ikkalkolat mill-analiżi kimika supplemetari mwettqa fil-bidu u fit-tmiem ta' l-inkubazzjoni.

III.2. DESKRIZZJONI TAL-METODU

III.2.1. **Apparat**

(a) Garafini koniċi, e.g. 250 ml sa 2 1itri, jiddependi mill-volum meħtieġ għall-analiżi DOC;

(b) Magna taċ-ċekċik - li tista takkomoda l-garafini koniċi, jew bil-kontroll awtomatiku tat-temperatura jew użata fit-temperatura kostanti tal-kamra, u forza suffiċjenti tal-potenza għaż-żamma tal-kondizzjonijiet etobiċi fil-garafini kollha;

(c) Apparat tal-filtrazzjoni, b'membrani xierqa;

(d) Analiżattur DOC;

(e) Apparat għad-determinazzjoni ta' l-ossiġenu mdewweb;

(f) Centrifuga.

III.2.2. **Preparazzjoni tal-medja minerali**

Għall-preparazzjoni tat-taħlitiet bil-lest, ara I.6.2.

Ħawwad 10 ml tat-taħlita (a) ma 800 ml ilma tat-trattib, żid 1 ml tat-taħlitiet (b) sa (d) u wassal sa litru bl-ilma tat-trattib.

Dan il-metodu jiża biss 0,5 ml effluwent/litru bħala *inoculum* u għalhekk il-medja jista jkun li tkun teħtieġ li tkun imsaħħa b'elementi taċċa u fatturi tat-tkabbir. Dan huwa magħmul biż-żieda ta' 1 ml kull wieħed mat-taħlitiet li ġejjin għal kull litru tal-medja finali:

Taħlita ta' l-element taċċa:

Tetraidrat tas-sulfat tal-magneżja, $MnSO_4$ $4H_20$	39,9 mg
Aċidu Boriku, H_3B0_3	57,2 mg
Ħepaidrat tas-sulfat taż-żingu, $ZnSO_4$. $7H_20$	42,8 mg
Ammonium heptamolybdate $(NH_4)_6$ $M_{O7}O_{24}$	34,7 mg
Fe-ċelat ($FeCl_3$ aċidu *ethylenediamine*-tetra-aċetiku)	100,0 mg

Dewweb fi, u wassal għal 1 000 ml bl-ilma tat-trattib.

Taħlita tal-vitamina:

Estratt tal-ħmira	15,0 mg

Dewweb l-estratt tal-ħmira fi 100 ml ilma. Sterlizza bil-passaġġ minn membrana 0,2 mikronji, jew għamilha friska.

III.2.3. **Preparazzjoni u pre-kondizzjonar ta' l-*inoculum***

L-*inoculum* jkun akkwistat minn effluwent sekondarju ta' l-impjant tat-trattament jew minn unità tal-iskala tal-laboratorju li jirċievi b'mod predominanti d-drenaġġ domestiku. Ara I.6.4.2. u I.6.5.

0,5 ml kull litru tal-medja minerali hija wżata.

III.2.4. **Preparazzjoni tal-garafini**

Bħala eżempju, introduċi 800 ml porzjonijiet tal-medja minerali ġewwa garafini koniċi 2 l u żid volumi suffiċjenti tat-taħlitiet bil-lest tat-test u tas-sustanzi ta' referenza mal-garafini separati sabiex takkwista konċentrazzjoni kemikali ekwivalenti għal 10-40 mg DOC/1. Ivverifika l-valur pH u aġġusta, jekk meħtieġ, għal 7,4. Innokula l-garafini fl-effluwent tad-drenaġġ fi 0,5 ml/litru (ara I.6.4.2.). Ippreparata wkoll il-kontrolli ta' l-*inoculum* fil-medja minerali mingħajr il-kimika tat-test jew ta' referenza.

Jekk meħtieġ, uża reċipjent wieħed sabiex tivverifika l-possibbiltà ta' l-effett inibitorju tal-kimika tat-test bl-inokulazzjoni tat-taaħl;ita li jkun fiha, fil-medja minerali, konċentrazzjonijiet komparabbli kemm tal-kimika tat-test u dik ta' referenza.

Ukoll, jekk meħtieġ, organizza garafina sterili oħra sabiex tivverifika jekk il-kimika tat-test tkun degradat abjotikament bl-użu tat-taħlita mhix inokulata tal-kimika (ara I.6,6.).

B'mod addizzjonali, jekk ikun hemm suspett li l-kimika tat-test tkun sinifikament adsorbita fuq il-ħġieġ, ħama' etc., wettaq assessjar preliminari sabiex tkun determinata l-medda possibbli ta' l-adsorbazzjoni u b'hekk l-adattabbiltà tat-test għall-kimika (ara t-Tabella 1). Organizza garafina li jkun fiha s-sustanza tat-test, l-*inculum* u l-aġent sterilizzanti.

Wassal il-volumi fil-garafini kollha għal 1:1 bil-medja minerali u, wara t-taħwib, ħu kampjun minn kull garafina sabiex tiddetermina l-konċentrazzjonini inizjali ta' DOC (ara l-Anness II,4). Għatti l-ftugħ tal-garafini, e.g. b'liega ta' l-aluminju, b'tali mod hekk li tippermetti l-iskambju ħieles ta' l-arja bejn il-garafina u l-atmosfera tal-madwar. Imbagħad deffes ir-reċipjenti fil-magna taċ-ċekċik għall-bidu tat-test.

III.2.5. **Numru ta' garafini fi prova tipika**

Il-garafini 1 u 2: Sospenzjoni tat-test

Il-garafini 3 u 4: *Inoculumi* fl-imbjank

Garafina 5: Proċedura tal-kontroll

Preferibbilment u meta meħtieġ:

Garafina 6: Kontroll ta' abjotiku sterili

Garafina 7: Kontroll ta' l-adsorbazzjoni

Garafina 8: Kontroll tat-tossiċità

Ara wkoll I.6.7.

III.2.6. **It-twettieq tat-test**

Matul it-test, iddetermina l-konċentrazzjoninijiet ta' DOC f'kull garafina b'mod doppju f'intervalli tal-ħin magħruf, ta' spiss b'mod suffiċjenti sabiex tkun tista tiddetermina l-bidu tal-perijodu ta' 10 tijiem u l-ersentaġġ tat-tneħħija fit-tmiem tal-perijodu ta' 10 tijiem. Ħu biss il-volum minimu tas-sospenzjoni tat-test meħtieġ għal kull determinazzjoni.

Qabel il-kampjunar għamel tajjeb għat-telf ta' l-evaporazzjoni mill-garafini biż-żieda ta' ilma tat-trattib (I.6.1) fl-ammont meħtieġ, jekk ikun neċċessarju. Ħawwad il-medja tal-kultura sewwa qabel ma tiġbed il-kampjun u assigura li l-materjal li jeħel mal-ġnub tar-reċipjenti jkun imdewweb jew sospiż qabel il-kampjunar. Immedjatament iffiltra mill-membrana jew b'ċentrifuga (ara l-Anness II.4) wara li jkun inġabar il-kampjun. Analizza l-kampjuni tal-filtrat jew taċ-ċentrifuga l-istess jum, altrimentri aħżen fi 2-4 °C għal massimu ta' 48 siegħa, jew f'anqas minn 18 °C għal perijodu twil.

III.3. DATA U RAPPORTAĠĠ

III.3.1. **Trattament tar-riżultati**

Ikkalkola l-persentaġġ tad-degradazzjoni fil ħin ta' kif mogħti taħt I.7.1. (determinazzjoni DOC) u, voluntarjament, taħt I.7.2. (analiżi speċifika).

Irreġistra r-riżultati kollha fuq il-karti tad-*data* kif ipprovduta.

III.3.2. **Validità tar-riżultati**

Ara I.5.2.

III.3.3. **Rappurtaġġ**

Ara I.8.

III.4. FOLJA TAD-*DATA*

Eżempju tal-folja tad-*data* huwa mogħti aktar il-quddiem.

TEST MODIFIKAT TAL-GĦAŻLA OECD

1. **LABORATORJU**

2. **IL-JUM TAL-BIDU TAT-TEST**

3. **IS-SUSTANZA TAT-TEST**

Isem:

Il-konċentrazzjoni tat-taħlita bil-lest: mg/litru bħala kimika

Konċentrazzjoni inizjali fil-medja, għal: mg/litru bħala kimika

4. **INOCULUM**

Sorsi:

Trattament mogħti:

Pre-kondizzjonar, jekk ikun hemm:

Konċentrazzjoni ta' solidi sospiżi fit-taħlita tar-reazzjoni: mg/l

5. **DETERMINAZZJONI TAL-KARBONJU**

Analizzatur tal-karbonju:

	Garafina Nru		DOC wara jiem n (mg/l)				
			0	n_1	n_2	n_3	n_x
Kimika tat-test flimkien ma l-*inoculum*	1	a_1					
		a_2					
		a, medja $C_{a(t)}$					
	2	b_1					
		b_2					
		b, medja $C_{b(t)}$					
Inoculum fl-imbjank mingħajr il-kimika tat-test	3	C_1					
		C_2					
		c, medja $C_{c(t)}$					
	4	d_1					
		d_2					
		d, mean. $C_{d(t)}$					
	$C_{bl(t)} = \frac{C_{c(t)} + C_{d(t)}}{2}$						

6. **EVALWAZZJONI TAD-DATA MHIX TRATTATA**

Garafina Nru		% degradazzjoni wara n jiem				
		0	n_1	n_2	n_3	n_x
1	$D_1 = \left(1 - \frac{C_{a(t)} - C_{bl(t)}}{C_{a(o)} - C_{bl(o)}}\right) \times 100$	0				
2	$D_2 = \left(1 - \frac{C_{b(t)} - C_{bl(t)}}{C_{b(o)} - C_{bl(o)}}\right) \times 100$	0				
Medja [1]	$D = \frac{D_1 - D_2}{2}$	0				

[1] D u D m'għandhomx ikunu miġbura f'medja jekk ikun hemm differenza konsiderevoli.

Nota: formati simili jistgħu ikunu wżati għall-kimika tar-referenza u l-kontrolli tat-tossiċità

7. **KONTROLLI ABJOTICI** (voluntarji)

	Żmien (jiem)	
	0	t
Konċ. DOC (mg/litru) Kontroll sterili	$C_{s(o)}$	$C_{s(t)}$

$$\% \text{ degradazzjoni abjotika} = \frac{C_{s(o)} - C_{s(t)}}{C_{s(o)}} \times 100$$

8. **ANALIŻI TA' KIMIKA SPECIFIKA** (volontarja)

	Ammont residwu tal-kimika tat-test fit-tmiem tat-test	% degradazzjoni primarja
Kontroll sterili	S_b	
Medja tat-test inokulat	S_a	$\frac{S_b - S_a}{S_b} \times 100$

PARTI IV. TEST TA' L-EVOLUZZJONU CO_2 (Metodu °C.4-C)

IV.1. PRINCIPJU TAL-METODU

Volum imkejjel tal-medja minerali innokulata li jkun fiha konċentrazzjoni magħrufa tas-sustanza tat-test 10-40 mg DOC/l) bħala l-unika sorsi nominali ta' karbonju organiku jkun mogħti l-arja fid-dlam jew fid-dawl baxx. Id-degradazzjoni hija segwita matul 28 jiem bid-determinazzjoni tad-diossidu tal-karbonju prodott, li huwa marbut fil-barju jew fl-idossidu tas-sodju u li huma mkejjel bit-titrazzjoni ta' l-idrossu residwu bħala karbonju mhux organiku. L-ammont tad-diossidu tal-karbonju prodott mit-test kimiku (ikkorreġut għal dak derivat mill-*inoculum* fl-imbjank(huwa espress bħala persentaġġ ta $ThCO_2$. Il-grad tal-biodegradazzjoni primarja jista wkoll ikun ikkalkolat mill-analiżi kimika supplemetari mwettqa fil-bidu u fit-tmiem ta' l-inkubazzjoni.

IV.2. DESKRIZZJONI TAL-METODU

IV.2.1. **Apparat**

(a) Garafini, 2-5 litri, kull waħda mgħammra b'tubu ta' l-arjazzjoni li jwassal sa kważi il-qiegħ tar-reċipjent u bi hruġ;

(b) Ħawwada manjetiċi, għal meta jsir l-assessjar tal-kimika li ma tantx tinħall;

(c) Flixkien ta' assorbazzjoni tal-gass;

(d) Apparat għal kontroll u l-kejl tan-nixxija ta' l-arja;

(e) Apparat għat-tindig tad-diossidu tal-karbonju, għall-preparazzjoni ta' l-arja li hija ħielsa mid-dossidu tal-karbonju; jew b'mod alternattiv, taħlita ta' CO_2 ħieled mill-ossiġenu u CO_2 ħieles min-nitroġenu, minn ċilindri ta=gass fil-proporzjonijiet korretti (20 % O2:80 % N_2) jistgħu ikunu wżati;

(f) Apparat għad-determinazzjoni tad-diossidu tal-karbonju, jew b'mod titrimetriku jew inkella f'xi għala ta' l-analizzatur tal-karbonju mhux-organiku;

(g) Apparat tal-filtrazzjoni bil-membrana (voluntarju);

(h) Analiżattur DOC (voluntarju).

IV.2.2. **Preparazzjoni tal-medja minerali**

Għall-preparazzjoni tat-taħlitiet bil-lest, ara I.6.2.

Ħawwad 10 ml tat-taħlita (a) ma 800 ml ilma tat-trattib, żid 1 ml tat-taħlitiet (b) sa (d) u wassal sa litru bl-ilma tat-trattib.

IV.2.3. **Preparazzjoni u pre-kondizzjonar ta' l-inoculum**

L-*inoculum* jista jkun akkwistat minn varjeta ta' sorsi: ħama' attivata, effluwenti tad-drenaġġ (mhux klorinati), ilmijiet minn wiċċ l-art u ħamrija jew minn taħlita ta' dawn.

Ara I.6.4., I.6.4.1., I.6.4.2. u I.6.5.

IV.2.4. **Preparazzjoni tal-garafini**

Bħala eżempju, il-volumi u l-piżijiet li ġejjin jindikaw il-valuri għal garafini ta' 5-litri li jkun fihom 3 litri ta' sospensjoni. Jekk volumi iżgħar ikunu wżati, immodifika l-valuri kif meħtieġ, imma assigura li d-diossidu tal-karbonju iffurmat jista jkun imkejjel bl-eżatezza.

Ma kull garafina ta' 5 litri żid 2 400 ml tal-medja minerali. Żid volum xieraq tal-ħama' attivata ippreparata (ara I.6.4.1 u I.6.5.) sabiex takkwista konċentrazzjoni ta' solidi sospiżi ta' mhux aktar minn 30 mg/l fit-taħlita finali 3 l ta' l-inokulat.

B'mod alternattiv, l-ewwel dewweb il-ħama' ippreparata sabiex takkwista sospensjoni ta' 500-1 000 mg/l fil-medja minerali qabel ma żżid alikwott mal-kontenuti tal-garafina ta' 5-litri sabiex takkwista konċentrazzjoni ta' 30 mg/l; dan jassigura preċiżjoni akbar. Sorsi oħrajn ta' l-*inoculum* jistgħu ikunu wżati (ara I.6.4.2.).

Daħħal l-arja f'dawn it-taħlitiet tal-CO_2 ħieles mill-arja matul il-lejl sabiex titnaddaf is-sistema mid-diossidu tal-karbonju.

Żid il-materjal tat-test u s-sustanza ta' referenza, separatament, bħala volum magħruf tat-taħlitiet lesti, f'garafini kopji sabiex takkwista l-konċentrazzjonijiet, kontribwiti biż-żieda tal-kimika, ta' 10 sa 20 mg DOC jew TOC/l; ħalli wħud mill-garafini mingħajr iż-żieda tal-kimika bħala kontrolli ta' l-*inoculum*. Żid sustanzi tat-test li jinħallu bilmod direttament fil-garafini u iżen jew ikkalkula il-bażi tal-volum u opera kif deskritt fl-Anness III.

Jekk meħtieġ, uża garafina waħda sabiex tivverifika l-effett inibitorju possibbli tat-kimika tat-test biż-żieda kemm tal-kimika tat-test u tar-referenza fl-istess konċentrazzjonijiet kif preżenti fil-garafini l-oħrajn.

Ukoll, jekk meħtieġ, organizza garafina sterili oħra sabiex tivverifika jekk il-kimika tat-test tkun degradat abjotikament bl-użu tat-taħlita mhux inokulata tal-kimika (ara I.6,6.). Sterilizza biż-żieda ta' sustanza tossika fil-konċentrazzjoni xierqa.

Wassal il-volumi tas-sospenzi fil-garafina kollha għal 3 litri biż-żueda ta' medja minerali li preċedentament mogħtija l-arja bi CO_2 ħieles mill-arja. B'mod operattiv, il-kampjuni jistgħu ikunu miġbura għall-analiżi DOC (ara l-Anness II.4(u/jew analiżi speċifika. Waħħal il-flixkien ta' l-assorbazzjoni mal-ħruġ tal-ġarafini.

Jekk ikun użat l-idrossidu tal-barju, waħħal tlett fliexken ta' l-assorbazzjoni, b'kull wieħed ikun fih 100 ml ta, 0,0125 M taħlita ta' l-idrossidu tal-barju, f'serje ta' garafini ta' 5-litri 'l wieħed. It-taħlita għandha tkun ħielsa mis-sulfat u l-karbonat preċipitat u s-saħħa tagħha għandhom ikunu determinati immedjatament qabel l-użu. Jekk ikun użat l-idrossidu tas-sodju, waħħal żewġ nasses, it-tieni taġixxi bħala kontroll għad-demostrazzjoni li d-diossidu tal-karbonju kollu kien assorbit fl-ewwel wieħed. Fliexken ta' l-assorbazzjoni mgħammra b'għeluq tal-flixkun tas-serum huma adatti. Żid 200 ml 0,05 M idrossidu tas-sodju ma kull flixkun, li huwa suffiċjenti sabiex jassorbixxi l-kwantità totali tad-diossidu tal-karbonju evolut meta l-kimika tat-test tkun kompletament degradata. It-taħlita ta' l-idrossi tas-sodju, anki meta ippreparata friska, ikun fiha traċċi tal-karbonati, dan huma ikkorreġut bit-tnaqqis tal-karbonat fl-imbjank.

IV.2.5. **Numru ta' garafini fi prova tipika**

Il-garafini 1 u 2: Sospenzjoni tat-test

Il-garafini 3 u 4: *Inoculumi* fl-imbjank

Garafina 5: Proċedura tal-kontroll u,

preferibbilment u meta meħtieġ:

Garafina 6: Kontroll ta' abjotiku sterili

Garafina 7: Kontroll tat-tossiċità

Garafina 8: Kontroll tat-tossiċità

Ara wkoll I.6.7.

IV.2.6. **It-twettieq tat-test**

Ibda t-test bit-tbaqbieq ta' CO_2 ħieles mill-arja matul is-sospenzjoni bir-rata ta' 30-1—ml/min. Iġbor il-kampjuni ta' l-assorbent diossidu tal-karbonju perjodikament għall-analiżi tal-kontenut CO_2. Matul l-ewwel għaxart ijiem huwa rakkomndat li l-analiżi għandha tkun imwettqa fit-tieni jew fit-tielet jum u imbagħad kull ħames jum sat-28 jum ħekk li l-perijodu ta' l-10 tijiem jista jkun identifikat.

Fit-28 jum, iġbor il-kampjuni (voluntarja) għall-analiżi DOC u/jew speċifika, kejjel il-pH tas-sospenzjoni u żid 1 ml ta' l-aċidu idrokloriku ikkonċentrar ma kull garafina; agħti l-arja lill-garafini matul il-lejn sabiex twarrab id-diossidu tal-karbonju preżenti fis-sospenzjonijiet tat-test. Fil-jum 29 agħmel l-aħħar analiżi tad-diossidu tal-karbonju evolut.

Fil-jiem tal-kejl ta' CO_2, aqla l-assorbatur ta' l-idrossidu tal-barju l-ebreb lejn il-garafina, ittitra t-taħlita ta' l-idrossidu bi HCl 0,05 M bl-użu tal-fenilftalejn bħala indikatur. Mexxi l-assorbaturi li jkun fadat post aktar qrib tal-garafina u poġġi assorbatur ġdid li jkun fih 100 ml ta' l-idrossidu tal-barju frisk 0,0125 M fit-tarf imbiegħed tas-serje. Wettaq titrazzjonijiet kif meħtieġ, per eżempju, meta l-preċipitazzjoni sostanzjali hija murija fl-ewwel nassa u qabel ma jkun evidenti fit-tieni, jew mill-anqas darba fil-ġimgħa. B'mod alternattiv, bi NaOH bħala assorbatur, iġbed b'siringa kampjun żgħir (jiddependi mill-karatteristiċi ta' l-analizzatur tal-karbonju użat) tat-taħlita ta' l-idrossidu tas-sodju fl-assorbatur l-eqreb lejn il-garafina. Injetta direttament il-kampjun fil-parti IC ta' l-anilizzatur tal-karbonju għall-analiżi tad-diossidu tal-karbonju evolut.

Analizza l-kontenuti tat-tieni nassa biss fit-tmiem tat-test sabiex tikkorieġi għal xi ammont żejjed tad-diossidu tal-karbonju.

IV.3. *DATA* U RAPPORTAĠĠ

IV.3.1. **Trattament tar-riżultati**

L-ammont ta' CO_2 fin-nassa ta' l-assorbatur meta ittrat huwa mogħti bi:

$$mgCO_2 = (100 \times C_B - 0,5 \times V \times C_A) \times 44$$

meta:

V = il-volum ta' HC1 użat għat-titrazzjoni tal-100 ml fl-assorbatur (ml).

CB = il-konċentrazzjoni tat-taħlita ta' l-idrossidu tal-barju (M),

CA = il-konċentrazzjoni tat-taħlita ta' l-aċidu idrokloriku (M),

jekk CB jkun 0,0125 M u CA huwa 0,05 M, it-titrazzjoni għal 100 ml idrossidu tal-barju hija 50 ml u l-piż ta' CO_2 huwa mogħti bi:

$$\frac{0,05}{2} \text{ x } 44 \text{ x ml HC1 titrat} = 1.1 \text{ x ml HC1}$$

Hekk, f'dan il-każ, sabiex il-volum ta' HC1 titrat ikun ikkopnvertit għal mg ta-CO_2 prodott, il-fattur huwa 1,1.

Ikkalkola l-piżijiet ta' CO_2 prodott biss mill-*inoculum* u mill-*iconulum* flimkien mal-kimika tat-test bl-użu tal-valuri rispettivi tat-titrazzjoni u d-differenza fil-piż ta' CO_2 prodott biss mill-kimika tat-test.

Per eżempju, jekk l-*inoculum* biss jagħti titrazzjoni ta' 48 ml u l-*inoculum* flimkien mal-kimika tat-test jagħti 45 ml,

CO_2 mill-*inoculum* = 1,1 × (50-48) = 2,2 mg

CO_2 mill-*inoculum* flimkien mal-kimika tat-test = 1,1 × (50-45) = 5,5 mg u b#hekk il-piż ta'

CO_2 prodott mill-kimika tat-test hija 3,3 mb.

Il-persentaġġ tal-biodegradazzjoni huwa ikkalkolat minn:

$$\% \text{ degradażżjoni} = \frac{\text{Mg } CO_2 \text{ prodott x } 100}{\text{Th}CO_2 \text{ x mg kimika tat-test miżjuda } 6}$$

jew,

$$\% \text{ degradazzjoni} = \frac{\text{Mg } CO_2 \text{ prodott x } 100}{\text{mg TOC miżjud mit-test} \times 3.67\text{mg}}$$

3,67 ikun il-fattur tal-konverżjoni (44/12) għall-karbonju tad-diossidu tal-karbonju.

Akkwista l-persentaġġ tad-degradazzjoni wara kwalunkwe intervall ta' ħin biż-żieda tal-persentaġġ tal-valuri $ThCO_2$ ikkalkolat għal kull waħda mill-jiem, sa dak il-ħin, li fih ikun imkejjel.

Għall-assorbaturi idrossidu tas-sodju, ikkalkola l-ammont tad-diossidu tal-karbonju prodott, espressa bħala IC (mg), bil-moltiplikazzjoni tal-konċentrazzjoni ta' IC fl-assorbent bil-volum ta' l-assorbenr.

Ikkalkula l-persentaġġ tad-degradazzjoni minn:

$$\% \text{ ta' } ThCO_2 = \frac{\text{mg IC mill-garafina tat-test - mg IC mill-imbjank}}{\text{mg TOC miżjud bħala kimika tat-test}} \times 100$$

Ikkalkola DOC imneħħija (voluntarja) kif deskritt taħt 1.7. Irreġistra dawn u r-riżultati l-oħrajn kollha fuq il-karti tad-*data* kif ipprovduta.

IV.3.2. **Validità tar-riżultati**

Il-kontenut IC tas-sospenzjoni tal-kimika tat-test fil-medja minerali fil-bidu tat-test għandu jkun anqas minn 5% tal-TC, u t-total CO_2 ta' l-evoluzzjoni ta' l-*inoculum* fl-imbjank fit-tmiem tat-test għandu normalment jeċċedi 40 mg/l medja. Jekk valuri akbar minn 70 mg CO_2/litru jkunu akkwistati, id-*data* u t-teknika sperimentali għandha tkun eżaminata b'mod kritiku.

Ara wkoll I.5.2.

IV.3.3. **Rapportaġġ**

Ara I.8.

IV.4. FOLJA TAD-DATA

Eżempju tal-folja tad-*data* huwa mogħti aktar il-quddiem.

TEST TA' L-EVOLUZZJONI TAD-DIOSSIDU TAL-KARBONJU

1. **LABORATORJU**

2. **IL-JUM TAL-BIDU TAT-TEST**

3. **IS-SUSTANZA TAT-TEST**

Isem:

Il-konċentrazzjoni tat-taħlita bil-lest: mg/litru bħala kimika

Konċentrazzjoni inizjali fil-medja: mg/litru bħala kimika

Total °C miżjud mal-garafina: mg C

$ThCO_2$: mg CO_2

4. **INOCULUM**

Sorsi:

Trattament mogħti:

Pre-kondizzjonar, jekk ikun hemm:

Konċentrazzjoni ta' solidi sospiżi fit-taħlita tar-reazzjoni: mg/litru

5. **PRODUZZJONI U DEGRADABBILTÀ TAD-DIOSSIDU TAL-KARBONJU**

Metodas: Ba(OH)2/NaOH/kitas

Żmien (jiem)	Test ta' CO2 iffurmat (mg)		CO2 iffurmat bl-imbjank (mg)		CO2 iffurmat kumulattiv (mg) test nieqes mill-medja ta' l-imbjank)		ThCO2 $\frac{\text{kumulattiv dawdzumi}}{ThCO_2} \times 100$		
	1	medja	3	medja					
	1		2		medja	2	4	1	2
0									
n1									
n2									
n3									
28									

Nota: formati simili jistgħu ikunu wżati għall-kimika tar-referenza u l-kontrolli tat-tossiċità

6. **ANALIŻI TAL-KARBONJU** (voluntarju)

Analizzatur tal-karbonju:

Żmien (jiem)	Imbjank mg/l	Kimika tat-test mg/l
0	$C_{b(o)}$	C_o
28 ([1])	Cb(t)	C_t
([1]) jew fit-tmiem ta' l-inkubazzjoni		

$$\% \text{ DOC - removed} = \left(1 - \frac{C_t - C_{b(t)}}{C_o - C_{b(o)}}\right) \times 100$$

7. **DEGRADAZZJONI ABJOTIKA** (voluntarja)

$$\% \text{ abiotic degradatio n} = \frac{CO_2 - CO_2 \text{ formation in terile flask after 28 day (mg)}}{ThCO_2 \text{ (mg)}} \times 100$$

PARTI V. TEST TAR-RESPIROMETRIJA MANJOMETRIKA (Metodu °C.4-D)

V.1. PRINCIPJU TAL-METODU

Volum imkejjel tal-medja minerali inokulata, li jkun fiha konċentrazzjoni magħrufa tal-kimika tat-test (100 mg/litru tas-sustanza tat-test, li tagħti mill-anqas 50-100 mg ThOD/litru) bħala s-sorsi unika nominali tal-karbonju organiku, hija mħawwda f'garafina magħluqa f'temperatura kostanti (± 1 °C jew eqreb) għal sa 28 jum. Il-konsum ta' l-ossiġenu huwa determinat jew bil-kejl tal-kwantità ta' ossiġenu (prodott b'mod elettolitiku) meħtieġ għaż-żamma tal-volum tal-gass fil-garafina respirimetru, jew bit-tibdil fil-volum jew fil-pressjoni (jew ġemgħa tat-tnejn) fl-apparat. Id-diossidu tal-karbonju evolu huwa assorbit fit-taħlita ta' l-idrossidu tal-putassa jew f'xi assorbent xieraq ieħor. L-ammont ta' ossiġenu akkwistat bil-kimika tat-test (ikkorreġut għall-akwista mill-*inoculim* fl-imbjank, imwettaq b'mod parallel) huwa espress bħala persentaġġ ta' ThOD jew COD. B'mod voluntarju, il-grad tal-biodegradazzjoni primarja jista wkoll ikun ikkalkolat mill-analiżi kimika supplemetari mwettqa fil-bidu u fit-tmiem ta' l-inkubazzjoni, u fl-aħħar mill-aħħar il-biodegredazzjoni bl-analiżi DOC..

V.2. DESKRIZZJONI TAL-METODU

V.2.1. **Apparat**

(a) respirometru xieraq;

(b) kontroll tat-temperatura, żamma ta' ± 1 °C jew aħjar;

(c) Apparat tal-filtrazzjoni bil-membrana (voluntarju);

(d) analizzatur tal-karbonju (voluntarju).

V.2.2. **Preparazzjoni tal-medja minerali**

Għall-preparazzjoni tat-taħlitiet bil-lest, ara I.6.2.

Ħawwad 10 ml tat-taħlita (a) ma 800 ml ilma tat-trattib, żid 1 ml tat-taħlitiet (b) sa (d) u wassal sa litru bl-ilma tat-trattib.

V.2.3. **Preparazzjoni u pre-kondizzjonar ta' l-inoculum**

L-*inoculum* jista jkun akkwistat minn varjeta ta' sorsi: ħama' attivata, effluwenti tad-drenaġġ (mhux klorinati), ilmijiet minn wiċċ l-art u ħamrija jew minn taħlita ta' dawn.

Ara I.6.4., I.6.4.1., I.6.4.2. u I.6.5.

V.2.4. **Preparazzjoni tal-garafini**

Ipprepara t-taħlitiet għat-test u l-kimika tar-referenza, f'lottijiet separati, f'medja minerali ekwivalenti għal konċentrazzjoni, normalment, ta' 100 mg kimika/litru (li tagħti mill-anqas 50-100 mg ThOD/litru), bl-użu tat-taħlitiet bil-lest.

Ikkalkola il-ThOD fuq il-bażi tal-formazzjoni tal-imluħa ta' l-ammonium, sakemm nitrifikazzjoni tkun antiċipata, meta l-kalkolazzjoni għandha tkun ibbażata fuq il-formazzjoni nitrika (ara l-Anness II.2.)

Iddetermina l-valuri pH u aġġusta, jekk meħtieġ, għal 7,4. ± 0,2.

Sustanzi li ma tantx jinħallu għandhom ikunu miżjuda fi stadju aktar tard (ara hawn taħt).

Jekk it-tossiċita tal-kimika tat-test għandha tkun determinata, ipprepara taħlita oħra fil-medja minerali li jkun fiha kemm il-kimika tat-test u tar-referenza fl-istess konċentrazzjonijiet bħal fit-taħlitiet individwali.

Jekk il-kejl tat-teħid fiżiku-kimiku ta' l-ossiġenu huwa meħtieġ, ipprepara taħlita tal-kimika tat-test fi, mormalment, 100 mh ThOD/litru li tkun ġiet sterilizzata biż-żieda ta' sustanza tassika adatta (ara I.6,6.).

Introduċi l-volum meħtieġ tat-taħlitiet tal-kimika tat-test u ta' referenza, rispettivament, mill-anqas fil-garafini duplikati. Żid ma garafini oħrajn il-medja minerali biss (għal kontrolli ta' l-*inoculum*) u, jekk meħtieġ, it-taħlita tal-kimika tat-test imħallta/ta' referenza, u t-taħlita sterili.

Jekk il-kimika tat-test ma tantx tkun tinħall, żid sustanzi tat-test li jinħallu bil-mod direttament fil-garafini u iżen jew ikkalkula l-bażi tal-volum u opera kif deskritt fl-Anness III. Żid l-idrossidu tal-putazza, gerbubi tal-ġir tas-soda jew assorbent ieħor mal-kompartimenti assorbatur CO_2.

V.2.5. **Numru ta' garafini fi prova tipika**

Il-garafini 1 u 2: Sospenzjoni tat-test

Il-garafini 3 u 4: *Inoculumi* fl-imbjank

Garafina 5: Proċedura tal-kontroll preferibbilment,

u meta meħtieġ:

Garafina 6: Kontroll sterili

Garafina 7: Kontroll tat-tossiċità

Garafina 8: Kontroll tat-tossiċità

Ara wkoll I.6.7.

V.2.6. **It-twettieq tat-test**

Ħalli l-garafini jilħqu t-temperatura mixtieqa u inokula r-reċipjenti xierqa bil-ħama' attivata ippreparata jew b'sors ieħor ta' l-*inoculum* sabiex takkwista konċentrazzjoni ta' solidi sospiżi ta' mhux aktar minn 30 mg/litru. Ħejji l-apparat, qabbad il-ħawwadi u ivverifika li ma tgħaddix arja, u ibda bil-kejl tat-teħid ta' l-ossiġenu. Normalment l-ebda attenzjoni oħra m'hija meħtieġa apparti milli t-teħid tal-qari u tal-virifika odjerna sabiex tara li t-temperatura korretta u t-taħwis adekwat ikun mantenut.

Ikkalkola t-teħid ta' l-ossiġenu mill-qari meħuda f'intervalli regolati u ta' spiss, bl-użu tal-motodi rakkomandati mill-manifattur ta' l-apparat. Fit-tmiem ta' l-inkubazzjoni, normalment 28 jum, kejjel il-pH tal-kontenuti tal-garafini, speċjalment jekk it-teħid ta' l-ossiġenu jkun baxx jew aktar minn $ThODNH_4$ (għal komposti li jkun fihom in-nitroġenu).

Jekk meħtieġ, neħħli l-kampjuni mill-garafini respirometri, inizjalment u finalment, għall-analiżi ta' DOC jew kimika speċifika (ara l-Anness II.4). Fit-tneħħija inizjali, assigura li l-volum tas-sospensjoni tat-test li jkun baqa' fil-garafina, jkun magħruf. Meta l-ossiġenu jkun meħud mis-sustanza tat-test li jkun fiha l-N, iddetermina ż-żieda fil-konċentrazzjoni tan-nitrat u wettaq in-nitrazzjoni matul 28 jum u ikkalkola l-korrezzjoni għall-ossiġenu ikkunsmat bin-nitrifikazzjoni (l-Anness V).

V.3. DATA U RAPPORTAĠĠ

V.3.1. **Trattament tar-riżultati**

Iddividi l-akkwista ta' l-ossiġenu (mg) tal-kimika tat-test wara ħin pre-determina (korreġut għal dak ta' l-*inoculum* tal-kontroll fl-imbjank wara l-istess ħin) bil-piż tat-kimika tat-test kif użata. Dan iforni il-BOD espress bħala mg ossiġenu/mg kimika tat-test, iġiefiri

$$\text{BOD} = \frac{\text{mg } 0_2 \text{ teħid bil-kimika tat-test -- mg } 0_2 \text{ teħid bl-imbjank}}{\text{Mg kimika tat-test fil-garafina}}$$

= mg 02 kull mg kimika tat-test.

ikkalkola l-persentaġġ tal-biodegradazzjoni jew minn:

$$\%\ \text{biodegradazzjoni} = \%\ \text{ThOD} = \%\text{ThOD} = \frac{\text{BOD } (O_2/\text{mg kimika})}{\text{ThOD } (O_2/\text{mg kimika})} \times 100$$

Jew inkella minn:

$$\%\ \text{COD} = \frac{\text{BOD } (0_2/\text{mg kimika})}{\text{COD } (0_2/\text{mg kimika})} \times 100$$

Għandu jkun innotat li dawn iż-żewġ metodi mhux neċessarjament jagħtu l-istess valur, huwa preferibbli li jsir użu mill-metodu preċedenti.

Għal sustanzi tat-test li jkun fihom in-nitroġenu, għamel użu mill-ThOD xieraq (NH_4 jew NO_3) skond ma jkun magħruf jew mistenni dwar l-okkorrenza tan-nitrifikazzjoni (l-Anness II.2). Jekk in-nitrifikazzjoni isseħħ imma ma tkun kompluta, ikkalkola korrezzjoni għall-ossiġenu ikkunsmat bin-nitrifikazzjoni mit-tibdil fil-konċentrazzjoni tan-nitrit u n-nitrat (l-Anness V).

Meta determinazzjonijiet voluntarji tal-karbonju organiku u/jew ta' kimika speċifika jkun saru, ikkalkola il-persentaġġ tad-degradazzjoni, kif deskritt taħt I.7.

Irreġistra r-riżultati kollha dwara id-*data* fuq il-karti tad-*data* kif ipprovduta.

V.3.2. **Validità tar-riżultati**

It-teħid ta' l-ossiġenu ta' l-*inoculum* fl-imbjank normalment huwa 20-30 mg O_2/litru u m'għandux ikun aktar minn 60 mg/litru fi 28 jum. Valuri għola minn 60 mg/litru jkunu jeħtieġu eżaminazzjoni kritika tad-*data* u tat-tekniki sperimentali. Jekk il-valur tal-pH huwa l-barra mill-medda 6-8,5 u l-konsum ta' l-ossiġenu bil-kimika tat-test huwa anqas minn 60%, it-test għandu jkun repetut b'konċentrazzjoni aktar baxxa tal-kimika tat-test.

Ara wkoll I.5.2.

V.3.3. **Rapportaġġ**

Ara I.8.

V.4. FOLJA TAD-DATA

Eżempju tal-folja tad-*data* huwa mogħti aktar il-quddiem.

TEST RESPIRATORJU MANOMETRIKU

1. **LABORATORJU**

2. **IL-JUM TAL-BIDU TAT-TEST**

3. **IS-SUSTANZA TAT-TEST**

Isem:

Il-konċentrazzjoni tat-taħlita bil-lest: mg/litru

Konċentrazzjoni inizjali fil-medja, Co: mg/litru

Volum fil-garafina tat-test (V): ml

ThOD jew COD: mg O_2/mg sustanza tat-test (NH_4, NO_3)

4. **INOCULUM**

Sorsi:

Trattament mogħti:

Pre-kondizzjonar, jekk ikun hemm:

Konċentrazzjoni ta' solidi sospiżi fit-taħlita tar-reazzjoni: mg/l

5. **TEĦID TA' L-OSSIĠENU BIODEGRADABBILITÀ**

		Zeit (Tage) (jum)											
		0		7		14			21			28	
O_2 upt. (mg), kimika tat-test	1												
	2												
	a, medja												
O_2 upt.l (mg) imbjank	3												
	4												
	b, medja												
BOD(mg) ikkorreġut	(a_1-b_m)												
	(a_2-b_m)												
BOD kull mg tal-kimika tat-test	$\frac{(a_1 - b)}{C_oV}$												
	$\frac{(a_2 - b)}{C_oV}$												
% degradazzjoni $\frac{BOD}{ThOD} \times 100$	D_1 (a_1)												
	D_2 (a_2)												
	Medja (1)												
V = Volum tal-medja fil-garafina tat-test													

(1) D_1 u D_2 m'ghandhomx ikunu miġbura f'medja jekk ikun hemm differenza konsiderevoli.

N.B.: Formati simili jistgħu ikunu wżati għall-kimika tar-referenza u l-kontrolli tat-tossiċità

6. **KORREZZJONI GĦAN-NITRIFIKAZZJONI** (ara l-Anness V)

Jum	0	28	Differenza
(i) Konċentrazzjoni tan-nitrat (mg N/litru)			(N)
(ii) Ossiġenu ekwivamenti (4,57 x N x V) (mg)	—	—	
(iii) Konċentrazzjoni tan-nitrat (mg N/litru)			(N)
(iv) Ossiġenu ekwivamenti (3,43 x N x V) (mg)	—	—	
(ii + iv) Ossiġenu ekwivamenti totali	—	—	

7. **ANALIŻI TAL-KARBONJU** (volontarju)

Analizzatur tal-karbonju:

Żmien (jiem)	mg/litru fl-imbjank	Kimika tat-test mg/litru
0	(C_{blo})	(C_o)
28 (1)	(C_{blt})	(C_t)
(1) jew fit-tmiem ta' l-inkubazzjoni		

$$\% \text{ DOC removed} = \left(1 - \frac{C_t - C_{blt}}{C_o - C_{blo}}\right) \times 100$$

8. **KIMIKA SPECIFIKA** (volontarja)

Sb = konċentrazzjoni fil-fiżiko0kimika (sterili) mal-kontroll ta' 28 jum

Sa = konċentrazzjoni fil-garafina inokulta fi 28 jum.

$$\% \text{ biodegradazzjoni} = \frac{S_b - S_a}{S_b} \times 100$$

9. **DEGRADAZZJONI ABJOTIKA** (voluntarja)

a = konsum ta' l-ossiġenu f'garafini sterili wara 28 jum, (mg)

$$\text{konsum ta' l-ossiġenu għal kull mg tal-kimika tat-test} = \frac{a}{C_o V}$$

(ara s-sezzjonijiet 1 u 3)

$$\% \text{ abiotic degradation} = \frac{a \times 100}{C_o V \times \text{ThOD}}$$

PARTI VI. TEST TAL-FLIXKUN MAGĦLUQ (Metodu °C.4-E)

VI.1. PRINCIPJU TAL-METODU TAT-TEST

It-taħlita tal-kimika tat-test f'medja minerali, normalment fi 2-5 mg/litru, hija inokulata b'relattivament numru żgħir ta' mikro-organiżmi minn popolazzjoni mħallta u miżmuma fi fliexken, kompletament mimlija u magħluqa, fid-dlam u f'temperatura kostanti. Id-degradazzjoni hija segwita bl-analiżi ta' l-ossiġneu maħlul matul perijodu ta' 28 jum. L-ammont ta' ossiġenu akkwistat bil-kimika tat-test, ikkorreġut għall-akwista mill-*inoculim* fl-imbjank, imwettaq b'mod parallel, huwa espress bħala persentaġġ ta' ThOD jew COD.

VI.2. DESKRIZZJONI TAL-METODU

VI.2.1. **Apparat**

a) Fliexken BOD, b'tappini tal-ħġieġ, e.g. 250-300 ml;

b) Banju ta' l-ilma jew inkubatur, għaż-żamma tal-fliexken f'temperatura kostanti (± 1 °C jew aħjar) bl-esklużjoni tad-dawl;

c) Fliexken tal-ħġieġ kbar (2-5 litri) għall-preparazzjoni tal-medja u għall-mili tal-fliexken BOD;

d) Elettrodu u arloġġ ta' l-ossiġenu, jew apparat u reaġenti għat-titrazzjoni *Winker*.

VI.2.2. **Preparazzjoni tal-medja minerali**

Għall-preparazzjoni tat-taħlitiet bil-lest, ara I.6.2.

Ħawwad 1 (wieħed) tat-taħlita (a) sa (d) u wassal sa litru bl-ilma tat-taħwid.

VI.2.3. **Preparazzjoni ta' l-inoculum**

L-*inoculum* huwa normalment akkwistat minn effluwent sekondarju ta' l-impjant tat-trattament jew minn unità tal-iskala tal-laboratorju li jirċievi b'mod predominanti d-drenaġġ domestiku. Sors alternattiv ieħor għall-*inoculum* hija l-ilma tal-wiċċ ta' l-art. Normalment l-użu huwa ta' taqtira waħda (0,05 ml) ma 5 ml tal-filtrat għal kull litru tal-medja; il-provi jistgħu jeħtieġ li jiskopru l-aħjar volum għal effluwent partikolari (ara I.6.4.2. u I.6.5.).

VI.2.4. **Preparazzjoni tal-garafini**

B'mod qawwi daħħal l-arja f'medja minerali għal mill-anqas 20 minuta. Wettaq kull waħda mis-serje tat-testijiet bil-medja akkwistata mill-istess lott. Ġeneralment, il-medja hija lesta għall-użu wara li tkun qagħdet għal 20 siegħa, fit-temperatura tat-test. Iddetermina l-konċentrazzjoni ta' l-ossiġenu mdewweb għall-iskopijiet tal-kontroll; il-valur għandu jkun madwar 0 mg/litru fi 20 °C. Wettaq l-operazzjonijiet kollha tat-trasferiment u tal-mili tal-medja saturata bl-arja imma mingħajr bżieżaq, per eżempju, bl-użu ta' sifuni.

Ipprepara grupp paralelli ta' fliexken BOD għad-determinazzjoni tat-test u l-kimika ta' referenza f'serje sperimentali simultanji. Organizza numru suffiċjenti tal-flixkien BOD, inklużi l-*inoculum* fl-imbjank, sabiex tippermetti mill-anqas kejl duplikat tal-konsum ta' l-ossiġenu li jsir matul l-intervalli mixtieqa tat-test, per eżempju, wara 0, 7, 14, 21 u 28 jum. Sabiex tassigura li tkun tista tidentifika l-perijodu ta' l-10 tijiem, aktar fliexken jistgħu ikunu meħtieġa.

Żid medja minerali mimlija kollha bl-arja ma fliexken kbar hekk li jkunu mimlija għal madwar terz. Imbagħad żid taħlitiet bil-lest suffiċjenti tal-kimika tat-test u tal-kimika ta' referenza sabiex tissepara l-flixken il-kbar hekk l-konċentrazzjoni finali tal-kimika tkun normalment mhux akbar minn 10 mg/litru. M'għandhek iżżid l-ebda kimika ma l-imbjank fil-medja tal-kontroll li tkun fi flixkun kbir ieħor.

Sabiex tassigura li l-attività ta' l-*inoculum* ma tkunx limitata, il-konċentrazzjoni ta' l-ossiġenu mdewweb m'għandhiex tinżel aktar baxxa minn 0,5 mg/litru fil-fliexken BOD. Dawn jillimitaw il-konċentrazzjoni tal-kimika tat-test għal madwar 2 mg/litru. B'dana kollu, għal komposti b'degradibbiltà baxxa u dawk bi ThOD baxxa, 5-10 mg/litru jistgħu ikunu wżati. F'uħud mill-każi, ikun rakkommandabbli li twettaq b'mod parallel serje tal-kimika tat-test f'żewġ konċentrazzjonijiet differenti, per eżempju, 2 u 5 mg/litru. Normalment, għandhek twettaq il-kalkolu tal-ThOD fuq il-bażi tal-formazzjoni tal-melħ ta' l-ammonium, imma, jekk in-nitrifikazzjoni tkun mistennija jew ikun magħruf li sseħħ, agħmel il-kalkolu fuq il-bażi tal-formazzjoni tan-nitrat (ThODNO3: ara l-Anness II.2). B'danakollu, jekk in-nitrifikazzjoni ma tkunx kompluta imma sseħħ, għamel korrezzjoni għat-tibdil fil-konċentrazzjoni tan-nitrit u n-nitrat, determinati fl-analiżi, (ara l-Anness V).

Jekk it-tossiċità tal-kimika tat-test għandha tkun investigata (fil-każ, per eżempju, tal-valur preċedenti baxx tal-biodegradabbiltà li jkun ġie misjub), serje oħra ta' flixkien hija neċessarja.

Ipprepara flixkun kbir ieħor li jkun fih medja minerali bl-arja (sa madwar terz tal-volum tiegħu) flimkien mal-kimika tat-test u l-kimika ta' referenza fil-konċentrazzjoni finali normalment l-istess bħal dawk fil-fliexken kbar l-oħrajn.

Inokula t-taħlitiet fil-fliexken il-kbar b'effluwent sekondarju (qatra waħda jew madwar 0,05 ml, ma 5 ml/litru) jew b'sorsi oħra bħal ma hu l-ilma tax-xmara (ara I.6.4.2.). Finalment, wassal it-taħlitiet għall-volum b'medja minerali bl-arja bl-użu ta' manka li tilħaq sal-qiegħ tal-flixkun sabiex takkwista taħlita adekwata.

VI.2.5. **Numru ta' garafini fi prova tipika**

Fi prova tipika, il-fliexken li ġejjin huma wżati:

mill-anqas 10 li jkun fihom il-kimika tat-test u ta' l-*inoculum* (sospenzjoni tat-test),

mill-anqas 10 li jkun fihom biss l-*inoculum* (*inoculum* fl-imbjank),

mill-anqas 10 li jkun fihom il-kimika ta' referenza u ta' l-*inoculum* (kontroll tal-proċedura),

u, meta meħtieġ, 6 fliexken li jkun fihom il-kimika tat-test, kimika ta' referenza u l-*inoculum* (kontroll tat-tossiċità). B'dana kollu, sabiex tassigura li tkun tista tidentifika l-perijodu ta' l-10 t'ijiem, madwar id-doppju ta' fliexken jistgħu ikunu meħtieġa.

VI.2.6. **It-twettieq tat-test**

Forni kull taħlita ippreparata immedjetament ġewwa kull grupp rispettiv tal-fliexken BOD bil-manka mill-kwart tl-isfel (mhux mill-qiegħ) tal-flixkun kbir xieraq, hekk li l-fliexken BOD kollha jkunu kompletament mimlija. Taptap bil-ġentilizza sabiex tneħħi xi bżieżaq ta' l-arja. Analiżża il-fliexken ħin-żero immedjetament għall-ossiġenu mdewweb bil-metodi *Winkles* jew bl-elettrodi. Il-kontenuti tal-fliexken jistgħu jkunu preservati għal analiżi aktar tard bil-metodu *Winkles* biż-żieda tal-manganiż (II) sulfat jew idrossidu tas-sodju (l-ewwel reaġent *Winkler*). Aħżen bil-galbu l-fliexken magħluqa bl-istopponi, li jkun fihom l-ossiġentu iffissat bħala manganiż kannella (III) ossidu idrat, fid-dlam fi 10-20 °C għal mhux aktar minn 24 siegħa qabel ma tipproċedi bil-kumplament tal-passi tal-metodu *Winkler*. Agħmel it-tappini fuq il-kumplament tal-flixkien, tassigura li l-ebda bżieżaq ta' l-arja ma jkunu magħluqa, u inkuba fi 20 °C fid-dlam. Kull serja għandha tkun akkumpanjata b'serje paralella sħiħa għad-determinazzjoni tal-medja in-bjank inokulata. Neħħi mill-anqas il-flixkien duplikati tas-serje kollha għall-abaliżi ta' l-ossiġenu mdewweb matul l-intervalli tal-ħin (mill-anqas kull ġimgħa) matul it-28 jum ta' l-inkubazzjoni.

Kampjuni ta' kull ġimgħa għandhom jippermettu l-assessjar tat-tneħħija perċentwali fil-perijodu ta' 14 il-jum, fil-waqt li l-kmpjunar ta' kull 3-4 jiem għandu jippermetti li l-perijodu ta' 10 tijiem li jkun identifikat, li jkun jeħtieġ madwar id-doppju ta' tant fliexken.

Għal sustanzi tat-test li jkun fihom N, korrezzjonijiet għat-teħid ta' l-ossiġenu minn xi nitrifikazzjoni li sseħħ, għandu jsir. Sabiex isir dan, uża l-metodu ta' l-elettrodi-O_2 għad-determinazzjoni tal-konċentrazzjoni ta' l-ossiġenu mdewweb u imbagħad warrab il-kampjun mill-flixkun BOD għall-analiżi tan-nitrit u n-nitrat. Miż-żieda fil-konċentrazzjoni tan-nitrit u n-nitrat, wettaq il-kalkolu ta' l-ossiġenu użat (ara l-Anness V).

VI.3. DATA U RAPPORTAĠĠ

VI.3.1. **Trattament tar-riżultati**

L-ewwel agħmel il-kalkolu tal-BID eżerċitat wara kull perijodu ta' żmien billi tnaqqas l-eżawriment ta' l-ossiġenu (mg O_2/litru) ta' l-*inoculum* fl-imbjank minn dak esibit bit-test kimiku. Iddividi dan l-eżawriment ikkorreġut bil-konċentrazzjoni (mg/litru) tal-kimika tat-test, sabiex takkwista l-BOD speċifika vġama mg ossiġenu kull mg tal-kimika tat-test. Wettaq il-kalkolu tal-persentaġġ tal-biodegradabbiltà billi tiddividi l-BOD speċifika bil-ThOD speċifika (ikkalkolata skond l-Anness II,2) jew il-COS (determinata bl-analiżi, ara l-Anness II.3),

$$\text{b'hekk : BOD} = \frac{\text{mg } 0_2 \text{ teħid bil-kimika tat-test -- mg } 0_2 \text{ teħid bl-imbjank}}{\text{Mg kimika tat-test fil-garafina}}$$

= mg O2 kull mg tal-kimika tat-test

$$\text{\% biodegradazzjoni} = \frac{\text{BOD (mg } O_2\text{/mg kimika tat-test)}}{\text{ThOD (mg } O_2\text{/mg kimika tat-test)}} \times 100$$

jew

$$\text{\% biodegradazzjoni} = \frac{\text{BOD (mg } O_2\text{/mg kimika tat-test)}}{\text{COD (mg } O_2\text{/mg kimika tat-test)}} \times 100$$

Għandu jkun innotat li dawn iż-żewġ metodi mhux neċessarjament jagħtu l-istess valur, huwa preferibbli li jsir użu mill-metodu preċedenti.

Għal sustanzi tat-test li jkun fihom in-nitroġenu, għamel użu mill-ThOD xieraq (NH_4 jew NO_3) skon ma jkun magħruf jew mistenni dwar l-okkorrenza tan-nitrifikazzjoni (l-Anness II.2). Jekk in-nitrifikazzjoni sseħħ imma ma tkun kompluta, ikkalkola korrezzjoni għall-ossiġenu ikkunsmat bin-nitrifikazzjoni mit-tibdil fil-konċentrazzjoni tan-nitrit u n-nitrat (l-Anness V).

VI.3.2. **Validità tar-riżultati**

L-eżawriment ta' l-ossiġenu mill-imbjank ta' l-*inoculum* m'għandux jeċċedi 1,5 mg ossiġenu mdewwb/litru wara 28 jum. Valuri għola minn dan jeħtieġu investigazzjoni tat-tekniki sperimental. Il-konċentrazzjoni residwa ta' l-ossiġenu fil-fliexken tat-test m'għandhiex tinżel għal anqas minn 0,5 mg/litru fi kwalunkwe żmien. Livelli hekk baxxi ta' l-ossiġenu huma validi biss jekk il-metodu għad-determinazzjoni ta; l-ossiġenu mdewweb tkun tista tkejjel tali livella bl-akbar eżatezza.

Ara wkoll I.5.2.

VI.3.3. **Rapportaġġ**

Ara I.8.

VI.4. **FOLJA TAD-*DATA***

Eżempju tal-folja tad-*data* huwa mogħti aktar il-quddiem.

TEST TAL-FLIXKUN MAGĦLUQ

1. **LABORATORJU**

2. **IL-JUM TAL-BIDU TAT-TEST**

3. **IS-SUSTANZA TAT-TEST**

Isem:

Il-konċentrazzjoni tat-taħlita bil-lest: mg/litru

Konċentrazzjoni inizjali fil-flixkun: mg/litru

ThOD jew COD: mg O_2/mg sustanza tat-test

4. **INOCULUM**

Sorsi:

Trattament mogħti:

Pre-kondizzjonar, jekk ikun hemm:

Konċentrazzjoni fit-taħlita tar-reazzjoni: mg/litru

5. **DETERMINAZZJONI DO**

Metodu: Winkler/elettrodu

Flask Analyses

Iż-żmien ta' l-inkubazzjoni (d)			DO (mg/1)			
			0	n_1	n_2	
Imbjank (min-għajr kimika)	1	C_1				
	2	C_2				
Medja	$m_b = \frac{C_1 + C_2}{2}$					
Kimika tat-test	1	a_1				
	2	a_2				
Medja	$m_t = \frac{a_1 + a_2}{2}$					

Nota: Formati simili jistgħu ikunu wżati għall-kompost tar-referenza u l-kontrolli tat-tossiċità

6. **KORREZZJONI GĦAN-NITRIFIKAZZJONI** (ara l-Anness V)

Iż-żmien ta' l-inkubazzjoni (d)	0	n_1	n_2	n_3
(i) Konċentrazzjoni tan-nitrat (mg N/litru)				
(ii) Tibdil tal-konċentrazzjoni tan-nitrat (mg N/litru)	—			
(iii) Ossiġenu ekwivalenti (mg/litru)	—			
(iv) Konċentrazzjoni tan-nitrat (mg N/litru)				
(v) Tibdil tal-konċentrazzjoni tan-nitrat (mg N/litru)	—			
(vi) Ossiġenu ekwivalenti (mg/litru)	—			
(iii + vi) Ossiġenu totali ekwivalenti (mg/litru)	—			

7. **EŻAWRIMENT DO: % DEGRADAZZJONI**

	Degradazzjoni wara jiem n (mg/litru)			
	n_1	n_2	n_3	
GARAFINA 1: $(m_{to} - m_{tx}) - (m_{bo} - m_{bx})$				
GARAFINA 2: $(m_{to} - m_{tx}) - (m_{bo} - m_{bx})$				
GARAFINA 1: $\%\ D_1 = \frac{[(m_{to} - m_{tx}) - (m_{bo} - m_{bx})] \times 100}{\text{conc. of test x ThOD chemical} \times \text{ThOD}}$				
2. GARAFINA: $\%\ D_2 = \frac{[(m_{to} - m_{tx}) - (m_{bo} - m_{bx})] \times 100}{\text{conc. of test x ThOD chemical} \times \text{ThOD}}$				
$\%\ D\ \text{mean}(^1) == \frac{D_1 + D_2}{2}$				

(1) Tieħux din il-medja jekk ikun hemm differenza konsiderevoli bejn ir-replikati.

m_{to} = valur fil-garafina tat-test fil-ħin 0

m_{tx} = valur fil-garafina tat-test fil-ħin x

m_{bo} = il-valur medju ta' imbjank fil-ħin 0

m_{bx} = il-valur medju ta' imbjank fil-ħin x

Applika wkoll il-korrezzjoni għan-nitrifikazzjoni minn iii+vi fis-sezzjoni 6.

8. **EŻAWRIMENT DO IMBJANK**

Konsum ta' l-ossiġenu fl-imbjank: (mbo mb28) mg/litru Dan il-konsum huwa importanti għall-validità tat-test. Dan għandu jkun anqas minn 1,5 mg/litru.

PARTI VII. TEST M.I.T.I. (Metodu °C.4-F)

VII.1. PRINCIPJU TAL-METODU

It-teħid ta' l-ossiġenu bit-taħlita mħawda, jew bis-sospensjoni, tal-kimika tat-test f'medja minerali, inokulata b'mikro-organiżmi speċjalment imkabbra, mhux adattati, hija mkejla awtomatikament matul perijodu ta' 28 jum f'respirometri magħluq u fid-dlam fi 25 ± 1 °C. Id-disossidu tal-karbonju evolut huwa assorbit bil-ġir tas-soda. Il-biodegradabbiltà hija espressa bħala persentaġġ tat-teħid ta' l-ossiġenu (ikkorreġut għat-teħit fl-imbjank) tat-teħid teoretiku (ThOD). Il-persentaġġ tal-biodegradazzjoni primarja jista wkoll ikun ikkalkolat mill-analiżi kimika supplemetari mwettqa fil-bidu u fit-tmiem ta' l-inkubazzjoni, u fl-aħħar mill-aħħar il-biodegredazzjoni bl-analiżi DOC..

VII.2. DESKRIZZJONI TAL-METODU

VII.2.1. **Apparat**

(a) Arloġġ BOD elettroliku awtomatiku jew respirometru normalment mgħammar b'6 fliexken, 300 ml il-wieħed, u mgħammar b'kikkri sabiex ikun fihom l-assorbent CO_2;

(b) Kamra ta' temperatura kostanti u/jew banju ta' l-ilma fi 25 °C ± 1 °C jew aħjar;

(c) Apparat tal-filtrazzjoni bil-membrana (voluntarju);

(d) analiżattur tal-karbonju (voluntarju).

VII.2.2. **Preparazzjoni tal-medja minerali**

Ipprepara t-taħlitiet bil-lest li ġejjin, bl-użu ta' reaġenti ta' grad analitiku u l-ilma (I.6.1.):

(a)	Monopotassium dihydrogen ortho phosphate, KH_2PO_4	8,50 g
	Dipotassium monohydrogen ortho phosphate, K_2HPO_4	21,75 g
	Disodium monohydrogen ortho phosphate dodecahydrate Na_2HPO_4 12 H_20	44,60 g
	Klorid ta' l-ammonium, NH_4C_1	1,70 g
	Dewweb fl-ilma u wassal għal-litru.	
	Il-valur pH tat-taħlita għandu jkun 7,2	
(b)	Eptaidrat tas-sulfat tal-magnežju, $MgSO_4$ 7 H_2O	22,50 g
	Dewweb fl-ilma u wassal għal-litru	
(c)	Klorid tal-kalċji, anidriju, CaC12	27,50 g
	Dewweb fl-ilma u wassal għal-litru.	
(d)	Ħadid (III) eksaidrat tal-klorid, FeCl3 6 H_2O	0,25 g
	Dewweb fl-ilma u wassal għal-litru.	

Ħu 3 ml ta' kull taħlita (a), (b), (c) u (d) u wassal sa litru.

VII.2.3. **Preparazzjoni ta' l-inoculum**

Iġbor kampjuni friski minn mhux anqas minn għaxar siti, l-aktar minn żoni ta' fejn varjetà ta' kimiċi huma użati u mormija. Minn siti bħalma huma l-impjanti tat-trattament tad-drenaġġ, trattament ta' l-iskart industrijali, xmajjar, għadajjer, ibħra, iġbor litru tal-kampjun u tal-ħama', ħamrija tal-wiċċ, ilma, etc., u ħallat sewwa flimien. Wara li tneħħi l-materja li jkun hemm fil-wiċċ u tħalli li joqgħod, aġġusta s-supernatant għal pH 7 ± 1 bl-idrossidu tas-sodju u l-aċidu fosforiku.

Uża l-volum xieraq mas-supernayant iffiltrat sabiex timla reċipjent tal-"mili u ġbir" tal-ħama' attivata u agħti l-arja lil-likwidu għal madwar 2 3½ siegħat. Tletin minuta wara li tkun waqfet l-arjazzjoni, warrab madwar terż tal-volum kollu tas-supernatant u żid volum egwali tat-taħlita (pH 7) li jkun fiha 0,1 % kull wieħed tal-glukożju, peptun, u monoputassa orto fosfat, mal-materja li tkun qagħdet u erġa ibda bl-arjazzjoni. Irrepeti din il-proċedura darba kuljum. L-unità tal-ħama' għandha tkun operata skond il-prattika tajba: l-effluwenti għandhom ikunu ċari, t-temperatura għandha tinżamm fi 25 ± 2 C, il-pH għandu jkun 7 ± 1, il-ħama' għandha tkun qagħdet sewwa, b'arjazzjoni suffiċjenti sabiex iżżomm it-taħlita erobika matul il-ħin kollu, il-protażoa għandha tkun preżenti u l-attività tal-ħama għandha tkun ittestjata kontra sustanza ta' referenza mill-anqas darba kull tlett xhur. Tutilizzax il-ħama' bħala *inoculum* sa mill-anqas xahar wieħed mill-operazzjoni, imma mhux aktar tard minn erba' xhur. Minn hemm 'il quddiem, iġbor kampjun minn mill-anqas 10 siti matul intervelli regolari, darba kull tlett xhur.

Sabiex iżżomm kemm il-ħama' friska u dik antika fl-istess attività, ħawwad is-supernatant iffiltrat ta' ħama' attivata bl-użu ta' volum egwali tas-supernatant iffiltrat mat-taħlita ta' l-għaxar sorsi miġbura friski u wettaq il-kultura ta' dak il-likwir miġmugħ kif deskritt hawn fuq. Iġbor il-ħama' għall-użu bħala *inoculum* 18-24 siegħa wara liu l-unita tkun ġiet mimlija.

VII.2.4. **Preparazzjoni tal-garafini**

Ipprepara dawn is-sitt garafini kif ġejjin:

Nru. 1: kimika tat-test f'ilma mdewweb fi 100 mg/litru

Nru. 2, 3 u 4: kimika tat-test f'minerali mdewweb fi 100 mg/litru

Nru. 5: kimika tat-test (e.g. analin) f'medja minerali mdewwba fi 100 mg/litru

Nru. 6: medja minerali biss

Żid il-kimika tat-test li ma tantx tinħall fuq il-bażi ta' piż jew volum jew ittratta kif deskritt fl-Anness III, apparti li la s-solventi u l-anqas l-aġenti emulsifikanti m'għandhom ikunu wżati. Żid l-assorbent tal-CO_2 mal-garafini kollha fil-kikkri speċjali kif fornuti. Aġġusta l-pH fil-garafini nri. 2, 3 u 4 għal 7,0.

VII.2.5. **It-twettieq tat-test**

Inokulta l-garafini nri. 2, 3 u 4 (sospensjoni tat-test, nru. 5 (kontroll ta' l-attività) u nru. 6 (*inoculum fl-imbjank*) b'volum żgħir ta' l-*inoculum* sabiex ikun hemm konċentrazzjoni ta' 30 mg/litru ta' solidi sospiżi. *Inoculum* N huwa miżjud mal-garafina nru. 1 li sservi bħala kontroll abjotiku. Ħejji l-apparat, qabbad il-ħawwadi u ivverifika li ma tgħaddix arja, u ibda bil-kejl tat-teħid ta' l-ossiġenu taħt kondizzjonijiet fid-dlam. Ivverifika ta' kuljum, it-temperatura, l-ħawwadi u r-reġistratur kowlometriku tat-teħid ta' l-ossiġenu, u innota xi kambjamenti fil-kulur tal-kontenuti tal-garafini. Aqra l-akkwist ta' l-ossiġenu għas-sitt garafini direttament bil-metodu xieraq, per eżempju, mill-grafika tar-reġistrazzjoni ta' sitt punti, li tipproduċi kurva BOD. Fit-tmiem ta' l-inkubazzjoni, normalment 28 jum, kejjel il-pH tal-kontenuti tal-garafini u iddetermina l-konċentrazzjoni tal-kimika tat-test residwa u xi intermedju u, fil-każ ta' sustanza li tinħall fl-ilma, il-konċentrazzjoni ta' DOC (l-Anness II.4). Agħti attenzjoni speċjali fil-każ ta' kimika volatili. Jekk nitrifikazzjoni tkun antiċipata, iddetermina l-konċentrazzjoni tan-nitrat u tan-nitrit, jekk dan ikun possibbli.

VII.3. DATA U RAPPORTAĠĠ

VII.3.1. **Trattament tar-riżultati**

Iddividi l-akkwista ta' l-ossiġenu (mg) tal-kimika tat-test wara ħin pre-determina (korreġut għal dak ta' l-*inoculum* tal-kontroll fl-imbjank wara l-istess ħin) bil-piż tat-kimika tat-test kif użata. Dan iforni il-BOD espress bħala mg ossiġenu/mg kimika tat-test, jiġifieri

$$BOD = \frac{\text{mg } O_2 \text{ teħid bil-kimika tat-test -- mg } O_2 \text{ teħid bl-imbjank}}{\text{mg kimika tat-test fil-garafina}}$$

$$= \text{mg } 0_2/\text{mg tal-kimika tat-test}$$

Il-persentaġġ tal-biodegradazzjoni huwa imbagħad akkwistat minn:

$$\% \text{ biodegradazzjoni} = \% \text{ ThOD} = \frac{\text{BOD } ((O_2/\text{mg kimika}))}{\text{ThOD } ((O_2/\text{mg kimika}))} \times 100$$

Għat-taħlitiet, ikkalkola il-ThOD mill-analiżi elementali, bħal fil-każ ta' kompost sempliċi. Uża il-ThOD ($ThODNH_4$ jew $ThODNO_3$) xierqa skond ta' jekk in-nitrifikazzjoni tkunx assenti jew kompluta (l-Anness II.2). Jekk b'danakollu, n-nitrifikazzjoni isseħħ imma ma tkunx kompluta, ikkalkola korrezzjoni għall-ossiġenu ikkunsmat bin-nitrifikazzjoni mit-tibdil fil-konċentrazzjoni tan-nitrit u n-nitrat (l-Anness V).

Ikkalkola l-persentaġġ tal-biodegredazzjoni primarja mit-telf ta' kimika speċifika (paterna) (ara I.7,2).

$$D_t = \frac{S_b - S_a}{S_b} \times 100\ \%$$

Jekk issib li kien hemm xi telf tal-kimika tat-test fil-garafina nru. 1, kejjel it-tneħħija fiżiku-kimika, agħmel rapport dwar dan u uża l-konċentrazzjoni tal-kimika tat-test (Sb) wara 28 jum f'din il-garafina sabiex tikkalkola l-persentaġġ tal-biodegredazzjoni.

Meta jsiru d-determinazzjonijiet ta' DOC (voluntarji), ikkalkola il-persentaġġ aħħari tal-biodegredazzjoni, minn:

$$D_t = \left(1 - \frac{C_t - C_{bt}}{C_o - C_{bo}}\right) \times 100\ \%$$

kif deskritt taħt il-punt I.7.1. Jekk issib li kien hemm xi telf tal-DOC fil-garafina nru. 1, kejjel it-tneħħija fiżiku-kimika, uża l-konċentrazzjoni DOC f'din il-garafina sabiex tikkalkola l-persentaġġ tal-biodegredazzjoni.

Irreġistra r-riżultati kollha dwara id-*data* fuq il-karti tad-*data* kif ipprovduta.

VII.3.2. **Validità tar-riżultati**

It-teħid ta' l-ossiġenu ta' l-*inoculum* fl-imbjank normalment huwa 20-30 mg O2/litru u m'għandux ikun aktar minn 60 mg/litru fi 28 jum. Valuri għola minn 60 mg/litru jkunu jehtieġu eżaminazzjoni kritika tad-*data* u tat-tekniki sperimentali. Jekk il-valur tal-pH huwa 'l barra mill-medda 6-8,5 u l-konsum ta' l-ossiġenu bil-kimika tat-test huwa anqas minn 60%, it-test għandu jkun repetut b'konċentrazzjoni aktar baxxa tal-kimika tat-test.

Ara wkoll I.5.2.

Jekk il-presentaġġ tad-degradazzjoni ta' l-anilin ikkalkolat mill-konsum ta' l-ossiġenu ma jkunx jeċċedi 40 % wara 7 tijiet u 65 % wara 14 il-jum, it-test ikun meqjus bħala invalidu.

VII.3.3. **Rapportaġġ**

Ara I.8.

VII.4. FOLJA TAD-*DATA*

Eżempju tal-folja tad-*data* huwa mogħti hawn taħt.

TEST MITI (1)

1. **LABORATORJU**

2. **IL-JUM TAL-BIDU TAT-TEST**

3. **IS-SUSTANZA TAT-TEST**

Isem:

Il-konċentrazzjoni tat-taħlita bil-lest: mg/l bħala kimika

Konċentrazzjoni inizjali fil-medja, Co: mg/l bħala kimika

Volum tat-taħlita ta' reazzjoni, V: ml

ThOD: mg O_2/l

4. **INOCULUM**

Siti tal-kampjunar tal-ħama':

1) ... 8) ...

6) ... 4) ...

2) ... 9) ...

7) ... 5) ...

3) ... 10) ...

Konċentrazzjonijiet ta' solidi sospiżi fil-ħama' attivata wara l-akklamitizzazzjoni bid-drenaġġ sintetiku +... mg/litru

Volum tal-ħama' attivata kull litru tal-medja finali =... ml

Konċentrazzjoni tal-ħama' fil-medja finali =... mg/litru

5. **TEĦID TA' L-OSSIĠENU BIODEGRADABBILITÀ**

Tip ta' respirometru użat:

			Zeit (Tage) (jum)				
			0	7	14	21	28
O_2 upt. (mg), kimika tat-test	a_1						
	a_2						
	a_3						
O_2 upt. (mg), imbjank	b						
Ikkorreġut O_2 upt. (mg)	$(a_1 - b)$ $(a_2 - b)$ $(a_3 - b)$						
BOD kull mg tal-kimika tat-test	$\frac{(a-b)}{C_oV}$	Garafina 1.					
		Garafina 2.					
		Garafina 3.					
% degradazzjoni $\frac{BOD}{ThOD} \times 100$		1					
		2					
		3					
		Medja [1]					

Nota: Formati simili jistgħu ikunu wżati għall-kompost ta' referenza.

[1] Tieħux din il-medja jekk ikun hemm differenza konsiderevoli bejn ir-replikati.

6. **ANALIŻI TAL-KARBONJU** (voluntarju)**:**

Analizzatur tal-karbonju:

Lombik	DOC				% DOC imnehhi	Medja
	Mért		Ikkorreġut			
Víz + sustanza tat-test	a				—	—
Iszap + sustanza tat-test	b1		b1-c			
Iszap + sustanza tat-test	b2		b2-c			
Iszap + sustanza tat-test	b3		b3-c			
Kontroll fl-imbjank	c		—		—	—

$$\% \text{ DOC removed} : \frac{a - (b - c)}{a} \times 100$$

7. **DATA SPECIFIKA KUMIKA ANALITIKA**

	Ammont residwu tal-kimika tat-test fit-tmiem tat-test	% degradazzjoni
Test imbjank bi Waterzel	Sb	
meda inokulata	S_{a1}	
	S_{a2}	
	S_{a3}	

$$\% \text{ biodegradazzjoni} = \frac{S_b - S_a}{S_b} \times 100$$

Ikkalkola il-% tad-0degradazzjoni għall-garafini a1, a2 u a3 rispettivament

8. **RIMARKI**

Il-kurva BOD kontra l-ħin, jekk disponibbli, għandha tkun mehmuża.

ANNESS I

ABBREVJAZZJONIJIET U DEFINIZZJONIJIET

DO: Ossiġenu mdewweb (mg/l) hija l-konċentrazzjoni ta' l-ossiġenu mdewweb f'kampjun akweju.

BOD: Talba biokimika ta' l-ossiġenu (g) hija l-ammont ta' l-ossiġenu ikkunsmat mill-mikro-organiżmi meta ssir il-metabolizzazzjoni tal-kompost tat-test; espress wkoll bħala g teħid ta' l-ossiġenu kull g tal-kompost tat-test. (ara l-metodu °C.5).

COD: Talba ta' l-ossiġenu Kimiku (g) hija l-ammont ta' orriġenu ikkunsmat matul l-assidizzazzjoni ta' kompost tat-test bl-aċidu dikromatiku jaħraq; din tipprovdi għall-ammont tal-materja ossidizzabbli preżenti; ukoll espressa bħala g ossiġenu ikkunsmat kull g tal-kompost tat-test. (ara l-metodu °C.6).

DOC: Karbonju organiku mdewweb huwa l-karbonju organiku preżenti fit-taħlita jew li jgħaddi minn filtru ta' 0,45 mikrometri jew jibqa fis-supernatant wara ċ-ċentrifigazzjoni fi 40 000 m.s-2 (± 4 000 g) għal 15 il-minuta.

ThOD: Talba teoretika ta' l-ossiġenu (mg) hija l-ammont totali ta' l-ossiġenu meħtieġ għall-ossidizzazzjoni kompleta ta'kimika; hija ikkalkolata mill-formula molekulati (ara l-Anness II.2) u hija wkoll espress bħala mg ossiġenu meħtieġ kull mg tal-kompost tat-test.

$ThCO_2$: Diossidu karbonju teoretiku (mg) hija l-kwantita ta' diossidu tal-karbonju ikkalkolat li jkun prodott mill-kontenut tal-karbonju magħruf jew imkejjel tal-kompost tat-test meta kompletament mineralizzat, ukoll espress bħala mg diossidu tal-karbonju kull mg tal-kompost tat-test.

TOC: Karbonju organiku totali ta' kampjun huwa s-somma tal-karbonju organiku fit-taħlita u fis-sospenzjoni.

IC: Karbonju inorganiku

TC: Karbonju totali huwa s-somma tal-karbonju organiku u inorganiku preżenti f'kampjun.

Biodegredazzjoni primarja:

hija t-tibdil fl-istruttura kimika ta' sustanza, imwassla bl-azzjoni bjoloġika, li tirriżulta mit-telf ta' proprjetà speċifika ta' dik is-sustanza.

Biodegredazzjoni finalissima (erobika):

hija l-livell tad-degradazzjoni akkwistata meta l-kompost tat-test ikun totalment utilizzat bil-mokro-organiżmi li jirriżulta fil-produzzjoni tad-diossidu tal-karbonju, ilma, melħ minerali u kostitwenti ġodda mikrobjali ċellulari (biomassa).

Biodegrabbillità Pronta:

klassifikazzjoni arbitrarja tal-kimika li tkun għaddiet minn ċerti testijiet speċifiċi tal-għażla għall-biodegradabbilità finalissima; dawn it-testijiet huma hekk strinġenti li huma assumiti li tali komposti jkunu malajr jew kompletament biodegradabbli f'ambjenti akweji permezz ta' kondizzjonijiet erobiċi.

Biodegrabbillità Inerenti:

klassifikazzjoni tal-kimika li dwarha jkun hemm evidenza mingħajr ekwivoku tal-biodegradazzjoni (primarja jew finalissima) fi kwalunkwe test rikonoxxut tal-biodegradabbilità.

Trattabbilità:

hija l-amenabbilità ta' komposti għat-tneħħija matul it-trattament bioloġiku ta' l-ilma ta' l-iskart mingħajr ma tkun affettwata b'mod ħażin l-operazzjoni normali tal-proċessi tat-trattament. Ġeneralment, komposti faċilment biodegradabbli jistgħu jkunu trattati, imma mhux il-komposti ineretament biodegradabbli huma hekk. Proċessi abjotiċi jistgħu ukoll ikunu operabbli.

L-ispazju

taż-żmien mill-innokulazzjoni, fit-test tat-tneħħija bil-kulur, sal-persentaġġ tad-degradazzjoni, jkun żdied b'mill anqas 10 %. L-ispazju taż-żmien huwa ta' spiss varjabbli u riproduċibbli ħażin ħafna.

Il-ħin tad-degradazzjoni

huwa l-ħin mit-tmiem ta' l-ispazju tal-ħin sal-ħin li 90% tal-livell massimu tad-degradazzjoni tkun ġiet milħuqha.

Il-peridjou ta' 10 tijiem

huma d-19 il-jum li immedjetament isegwu l-akkwist ta' l-10% degradazzjoni.

ANNESS II

KALKOLU U DETERMINAZZJONI TA' PARAMETRI SOMMARJI XIERQA

Jiddependi mill-metodu magħżul, ċerti parametri sommarji jkunu meħtieġa. Is-sezzjoni li ġejja tiddeskrivi d-deriva ta' dawn il-valuri. L-użu ta' dawn il-parametri hija deskritta fil-metodi individwali.

1. **Kontenut tal-Karbonju**

Il-kontenut tal-karbonju huwa ikkalkolat minn komposizzjoni elementali magħrufa jew determinata bl-analiżi elementali tas-sustanza tat-test.

2. **ThOD =** *Theoretical Oxygen Demand* **(Talba teoretika ta' l-ossiġenu)**

It-Talba teoretika ta' l-ossiġenu (ThOD) tista tkun ikkalkolata jekk il-komposizzjoni elementali tkun magħrufa jew determinata b'analiżi elementali. Hija għal kompost:

$$C_hH_hCl_{cl}N_nNa_{na}O_oP_pS_s$$

mingħajr nitrifikazzjoni,

$$ThOD_{NH4} = \frac{16[2\ c + 1/2\ (h - cl - 3\ n) + 3\ s + 5/2\ p + 1/2\ na - o]}{MW} mg/mg$$

jew bin-nitrifikazzjoni,

$$ThOD_{NH3} = \frac{16[2\ c + 1/2\ (h - cl) + 5/2\ n + 3\ s + 5/2\ p + 1/2\ na - o]}{MW} mg/mg$$

3. **Talba ta' l-Ossiġenu Kimiku (COD)**

It-Talba ta' l-Ossiġenu Kimiku (COD) hija determinata skond il-metodu °C.6.

4. **DOC =** *Dissolved Organic Carbon* **(Karbonju organiku mdewweb)**

Il-Karbonju organiku mdewweb (DOC) huwa bid-definizzjoni l-karbonju organiku ta' kwalunkwe kimika jew taħlita fl-ilma li tgħaddi minn filtru 0,45 mikrometri.

Kampjuni mir-reċipjenti tat-test huma miġbura u iffiltrati immedjatament minn apparat tal-filtrazzjoni bl-użu ta' membrana filtranti xierqa. L-ewwel 20 ml (l-ammont jista jkun imnaqqas meta jsir użu minn filtranti żgħar) tal-filtrat ikunu mwarrba. Volumi ta' 10-20 ml jew anqas, jekk injettati (il-volum jiddependi mill-ammont meħtieġ għall-analizzatur tal-karbonju) huma miżmuma għall-analizzar tal-karbonju. Il-konċentrazzjoni DOC hija determinata permezz ta' l-analizzatur tal-karbonju organiku li huwa kapaċi li jkejjel b'eżatezza l-konċentrazzjoni tal-karbonju ekwivalenti għal, jew aktar baxxa minn 10 % tal-konċentrazzjoni DOC inizjali użat fit-test.

Kampjuni iffiltrati li ma jkunux jistgħu jiġu analizzati fl-istess jum utili jistgħu ikunu preservati bil-ħażna fir-refriġeratur fi 2-4 °C għal 48 h, jew f'anqas minn 18 °C għal perijodu twil.

Rimarki:

Filtri tal-membra huma ta' spiss impregnati b'surfaktanti għall-idrofilizzazzjoni. B'hekk il-filtru jista jkun fih sa diversi mg ta' karbonju organiku solubbli li jkun jinterferixxi fid-determinazzjonijiet tal-biodegradabbilità. Surfaktanti u komposti organiċi solubbli oħrajn huma mneħħija mill-filtri billi jkunu mogħllija f'ilma dejonizzat għal tlett perijodi ta' sieġħa kull wieħed. Il-filtri jistgħu mbagħad ikunu maħżuna fl-ilma għal ġimgħa waħda. Jekk ikunu wżati l-kartuċċi tal-filtri, kull lott għandu jkun ivverifikat dwar il-konformità li dan ma jkunx jirrilaxxa karbonju organiku li jinħall.

Jiddependi mit-tip tal-filtri tal-membrana, l-kimika tat-test tista' tkun miżmuma bl-assorbazzjoni. Jista jkun għalhekk rakkomandabbli li jkun assigurat li l-kimika tat-test ma tkunx miżmuma bil-filtru.

Centrifugazzjoni fi 40 000 m.sek 2 (4 000 g) għal 15 il-minuta tista tkun użata għad-differenzazzjoni ta' TOC versus DOC minflok il-filtrazzjoni. Il-metodu mhux affidabbli fil-konċentrazzjoni inizjali ta' < 10 mg DOC/l ladarba jew mhux il-bakterja kollha tkun imneħħija jew inkella l-karbonju bħala parti tal-bakterja, l-plażma tkun imdewba mill-ġdid.

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ANNESS III

EVALWAZZJONI TAL-BIODEGRADABBILITÀ TA' SUSTANZI LI FTIT LI XEJN JINĦALLU

Fit-testijiet tal-biodegradabbilità b'sustanzi li ftit li xejn jinħallu, l-aspetti li ġejjin għandhom jirċievu attenzjoni speċjali.

Waqt li l-likwidi omoġenji rari jippreżentaw problemi tal-kampjunar, huwa rakommandabbli li materjali solidi jkunu omoġenizzati bil-mezzi xierqa sabiex ikunu evitati żbalji minħabba n-nuqqa ta' omoġenità. Attenzjoni speċjali għandha tkun mogħtija meta kampjuni rappreżentattivi ta' ftit milligrammi huma meħtieġa minn taħlitiet ta' kimika jew sustanzi b'ammonti żgħar ta' impuritajiet.

Diversi għamliet ta' aġitazzjoni matul it-testijiet tista tkun użata. Attanzjoni għandha tingħata għall-użu biss ta' aġitazzjoni suffiċjenti sabiex il-kimika tinżamm imxerda, u sabiex ikunu evitati sħana żejda, rgħawwa eċċessiva u forzi qawwija eċċessivi.

Jista jkun użat emulsifikatur li jagħti tixrid stabbli tal-kimika. Dan m'għandux ikun tossiku għall-bakterja u m'għandux ikun biodegradabbli jew li joħloq ir-rgħawwa bil-kondizzjonijiet tat-test.

L-istess kriterja hija applikabbli għal solventi bħalma hija applikabbli għall-emulsifikanti.

Mhux rakkomandat li trasportaturi solidi jkunu wżati għal sustanzi solidi tat-test imma dawn jistgħu ikunu adatti għal sustanzi żejtnija.

Meta sustanzi awżiljarji bħalma huma l-emulsifikanti, solventi u trasportaturi jkunu wżati, prova fl-imbjank li jkun fiha s-sustanza awżiljarja, għandha tkun imwettqa.

Kwalunkwe mit-tlett testijiet respirometriċi $C0_2$, BOD, MITI jistgħu ikunu wżati fl-istudju tal-biodebradabbilità tal-komposti li ftit li xejn jinħallu.

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ANNESS IV

EVALWAZZJONI TAL-BIODEGRADABBILITÀ TA' KIMIKA SUSPETTATA LI TKUN TOSSIKA GĦALL-*INOCULUM*

Meta kimika hija suġġetta għal testijiet ta' biodegradabbilità faċli u tidher li ma tkunx biodegradabbli, l-proċedura li ġejja hija rakkommandabbli jew distinzjoni bejn l-inibizzjoni u l-inenità tkun mixtieqa (Reynolds et al., 1987).

Inocula simili jew identika għandha tkun użata għat-testijiet tat-tossiċità u l-biodegredazzjoni.

Sabiex tkun assessjata t-tossiċità tal-kimika studjata f'testijiet tal-biodegradibbiltà faċli, l-applikazzjoni ta' ġemgħa waħda ta' l-inibizzjoni tar-rata tar-Respirazzjoni tal-Ħama' (test ta' l-inibizzjoni tar-respirazzjoni tal-ħama attivata — Dir 88/302/EEC), BOD u/jew il-metodi ta' l-Inibizzjoni tat-Tkabbir, għandhom ikunu jidħru jidhru xierqa.

Jekk l-inibizzjoni minħabba t-tossiċità għandha tkun evitata, huwa suġġerit li l-konċentrazzjonijiet tas-sustanza tat-test użata fit-testijiet tal-biodegradabbiltà faċli għandha tkun mhux anqas minn 1/10 tal-valuri EC50 (jew anqas mill-valuri EC20) akkwistati fit-testijiet tat-tossiċità. Komposti b'valur EC50 jew akbar minn 300 mg/l aktarx li ma jkollhomx effetti tossiċi fit-testijiet tal-biodehradabbiltà faċli.

Il-valuri EC50 ta;' anqas minn 20 mg/l huma aktarx li jimponu problemi serji għal testijiet sussegwenti. Konċentrazzjoni tat-test baxxi għandhom ikunu użati, li joħolqu l-ħtiega ta' l-użu tat-test strinġenti u sensittiv tal-Flixkun Magħluq jew l-użu ta' 14C — materjal bit-tikketta. B'mod alternattiv, *inoculum* akklamitizzat jista jippermetti li jkunu użati konċentrazzjonijiet ogħla tas-sustanza tat-test. F'dan il-każ ta' l-aħħar, b'danakollu, l-kriterja speċifika tat-test tal-biodegradabbilità faċli li tintilef.

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ANNESS V

KORREZZJONI TAT-TEĦID TA' L-OSSIĠENU GĦALL-INTERFERENZA BIN-NITRIFIKAZZJONI

Zbalji minħabba li ma tkunx ikkonsidrata n-nitrifikazzjoni fil-kalkolazzjoni tat-teħid ta' l-ossiġenu bil-biodegradabbilità tas-sustanzi tat-test li ma jkun fihom N huma marġinali (mhux akbar minn 5%), anki jekk l-ossidazzjoni ta' l-ammonju-N fil-medja isseħħ b'mod erratiku bħal bejn ir-reċipjenti tat-test u l-imbjank. B'danakollu, għal sustanzi tat-test li jkun fihom N, żbalji serji jistgħu iseħħu.

Jekk in-nitrifikazzjoni tkun sehhet imma ma tkunx kompluta, t-teħid osservat ta' l-ossiġenu bit-taħlita tar-reazzjoni jista' jkun ikkoerruġut għall-ammont ta' l-ossiġenu użat fl-ammonju ossidizzanti għan-nitrit u n-nitrat, jekk il-bidliet fil-konċentrazzjoni matul l-inkubazzjoni tan-nitrit u n-nitrat huma determinati bil-konsiderazzjoni ta' dawn l-ekwazzjonijiet li ġejjin:

$$2\ NH_4CI + 3\ 0_2 = 2\ HNO_2 + 2\ HC1 + 2\ H_20 \quad (1)$$

$$2\ HNO_2 + 0_2 = 2\ HNO_3 \quad (2)$$

Globalment:

$$2\ NH_4C1 + 4\ 0_2 = 2\ HNO_3 + 2\ HCI + 2\ H_2O \quad (3)$$

Mill-ekwazzjoni (1), l-akkwist ta' l-ossiġenu bit-28 g ta' nitroġenu li jkun jinsab fil-klorid ta' l-ammonium (BH_4C1) li jkun ossidizzat għan-nitrit huwa 96 g, i.e. fattur ta' 3,43 (96/28). Bl-istess mod, mill-ekwazzjoni (3), l-akkwist ta' l-ossiġenu bit-28 g ta' nitroġenu li jkun ossidizzat għan-nitrit huwa 128 g, i.e. fattur ta' 4,57 (128/28).

Ladarba r-reazzjonijiet huma sekwenzali, li jkunu esegwiti minn speċje ta' bakterja distinta u differenti, huwa possibbli għall-konċentrazzjoni tan-nitrit li tiżdied jew tonqos; f'dan il-każ ta' l-aħħar, konċentrazzjoni ekwivalenti tan-nitrat ikun iffurmat. Għalhekk, l-ossiġenu ikkunsmat fil-formazzjoni ta' ornitrat hija 4,75 multiplikata biż-żieda fil-konċentrazzjoni tan-nitrat, filwaqt li l-ossiġenu assoċjaat mal-formazzjoni tan-nitri hija 3,43 multiplikata biż-żieda fil-konċentrazzjoni tan-nitrit jew bit-tnaqqis fil-konċentrazzjoni tagħha, it-telf ta' l-ossiġenu huwa —3,43 moltiplikat bit-tnaqqis fil-konċentrazzjoni.

Dan ifisser:

0_2 ikkunsmat fil-formazzjoni tan-nitrat = 4,57 x iż-żieda fil-konċentrazzjoni tan-nitrat (4)

u

0_2 ikkunsmat fil-formazzjoni tan-nitrit = 3,43 x iż-żieda fil-konċentrazzjoni tan-nitrit (5)

u

O_2 mitluf fl-għebien tan-nitrit = — 3,43 x it-tnaqqis fil-konċentrazzjoni tan-nitrat (6)

Sabiex

0_2 akkwistat minħabba n-nitrifikazzjoni = ± 3,43 x il-kambjament fin-nitrit ikkonċentrat + 4,57 x iż-żidea fin-nitrat ikkonċentrat. (7)

u għalhekk

0_2 akkwistat minħabba l-ossidizzazzjoni C = l-akkwist totali osservat — l-akkwist minħabba n-nitrifikazzjoni (8)

B'mod alternattiv, jekk it-total biss ta' l-N ossidizzat huwa determinat, l-akkwista ta' l-ossiġenu minħabba n-nitrifikazzjoni jista' jkun meqjus bħala, fl-ewwel approssimazzjoni, 4,57 x iż-żieda fl-N ossidizzat.

Il-valur ikkorreġut għall-konsum ta' l-ossiġenu minħabba l-ossidizzazzjoni ta' C huwa mbagħad imqabbel mal-ThOD NH3, kif ikkalkolat fl-Anness II.

C.5 DEGRADAZZJONI — TALBA TA' L-OSSIĠENU BIOKEMIKALI

1. **METODU**

1.1. INTRODUZZJONI

L-iskop tal-metodu huwa l-kejl tat-talba ta' l-ossiġenu biokemikali (BOD) ta' sustanzi organiċi solidi u likwidi.

L-informazzjoni elaborata b'dan it-test tappartjeni għal komposti li jinħallu fl-ilmal b'dana kollu, komposti volatili u dawk b'solubbiltà baxxa fl-ilma jistgħu wkoll, għall-anqas fil-prinċipju, jkunu ittestjati.

Il-metodu huwa applikabbli biss għal dawk il-materja organiċi tat-test li ma humiex inibitorji għall-bakterja fil-konċentrazzjoni wżata fit-test. Jekk il-materjal tat-test ma jkunx jinħall fil-konċentrazzjoni tat-test, miżuri speċjali, bħalma hu l-użu ta' dispersjoni ultrasonika, jistgħu jkunu wżati sabiex tkun akkwistata dispersjoni tajba tal-materjal tat-test.

Informazzjoni dwar it-tossiċità tal-kimika tista tkun utili għall-interpretazzjoni ta' riżultati baxxi u fil-għażla tal-konċentrazzjonijiet xierqa tat-test.

1.2. DEFEINIZZJONI U UNITAJIET

Il-BOD huwa definit bħala l-massa ta' ossiġenu mdewweb meħtieġ minn volum speċifikat tat-taħlita tas-sustanza għall-proċess ta' l-ossidizzazzjoni biokimika skond il-kondizzjonijiet preskritti.

Dawn ir-riżultati huma espressi bħala grammi tal-BOD f'kull gramma tas-sustanza ittestjata.

1.3. SUSTANZI TA' REFERENZA

L-użu ta' sustanza ta' referenza xierqa għall-verifika ta' l-attività ta' l-*inoculum* hija mixtieqa.

1.4. PRINCIPJU TAL-METODU TAT-TEST

Ammont predominanti tas-sustanza, mdewweb jew imxerred b'medja adatta sew-arjata, huwa innokulat mal-mikro-organiżmi u inkubat f'temperatura kostanti ambjenti definita, f'post fid-dlam.

Il-BOD huwa determinat bid-differenza tal-kontenut ta' l-ossiġenu mdewweb fil-bidu u fit-tmien tat-test. It-tul ta' żmien tat-test għandu jkun mill-anqas ħamest ijiem u mhux aktaar minn 28 jum.

Imbjank għandu jkun determinat f'assaġġ parallel li ma jkun fih l-ebda sustanza tat-test.

1.5. KRITERJA TAL-KWALITÀ

Id-determinazzjoni BOD ma tistgħax tkun meqjusa bħala determinazzjoni valida tal-biodegradabbiltà ta' sustanza. Dan it-test jista biss ikun meqjus bħala test ta' prova.

1.6. DESKRIZZJONI TAL-METODU TAT-TEST

Taħlita jew tixrid preliminari tas-sustanza huwa ippreparat sabiex tkun akkwista l-konċentrazzjoni BOD kompatibbli mal-metodu użat. Il-BOD huwa mbagħad determinat skond kwalunkwe metodu standardizzat nazzjonali jew internazzjonali.

2. **DATA U EVALWAZZJONI**

Il-BOD li jkun jinsab fit-taħlita preliminari huwa kalkolat skond il-metodu normalizzat magħżul, u konvertit fi grammi ta' BOD kull gramma tas-sustanza tat-test.

3. **RAPPORTAĠĠ**

Il-metodu użat għandu jkun mistqarr.

It-talba għall-ossiġenu biokemikali għandha tkun il-medja ta' mill-anqas tlett kejl validi.

L-informazzjoni u r-rimarki kollha relevanti għall-interpretazzjoni tar-riżultati għandhom ikunu irrappurtati, speċjalment f'dak li jirrigwardja l-impuritajiet, l-istat fiżiku, l-effetti tossiċi u l-komposizzjoni inerenti tas-sustanza.li jkunu jaffettwaw ir-riżultati.

L-użu ta' addittiv għall-inibizzjoni tan-nitrifikazzjoni bioloġika għandu jkun irrappurtat.

4. **REFERENZI**

Lista ta' metodi standardizzati, per eżempju:

NF T 90 - 103: Determinazzjoni tat-talba ta' l-ossiġenu biokemikali.

NBN 407: Talba ta' l-ossiġenu biokemikali.

NEN 3235 5.4: Bepaling van het biochemish zuurstofverbruik (BZV).

Id-determinazzjoni tat-talba ta' l-ossiġenu biokemikali, il-Metodu S għall-eżaminazzjoni ta' l-ilma u l-materjali assoċjati, HMSO, Londra.

ISO 5815: Determinazzjoni tat-talba ta' l-ossiġenu biokemikali wara n jiem.

C.6. DEGRADAZZJONI — TALBA TA' L-OSSIĠENU KEMIKALI

1. **METODU**

1.1. INTRODUZZJONI

L-iskop tal-metodu huwa l-kejl tat-talba ta' l-ossiġenu kemikali (COD) ta' sustanzi organiċi solidi u likwidi f'manjera normali, arbitrarja, skond kondizzjonijiet fissi tal-laboratorju.

Informazzjoni dwar il-formula tas-sustanza tkun utili għat-twettieq tat-test u l-interpretazzjoni tar-riżultat akkwistat (e.g. imluħa aloġenizzati, imluħa ferrużi ta' komposti organiċi, komposti organoklorini).

1.2. DEFINIZZJONIJIET U UNITAJIET

It-talba ta' l-ossiġenu kemikali hija l-kejl ta' l-ossidabbilità ta' sustanza, espressa fl-ammont ekwivalenti ta' ossiġenu ta' reaġent ossidizzanti ikkunsmat mis-sustanza skond kondizzjonijiet fiss-tal-laboratorju.

Ir-riżultat huwa espress bħala grammi tal-COD kull gramma tas-sustanza ittestjata.

1.3. SUSTANZI TA' REFERENZA

Sustanzi ta' referenza ma jeħtieġux li jkunu wżati fil-każi kollha meta tkun investigata sustanza ġdida. Dawn għandhom primarjament iservu għall-kalibrazzjoni tal-metodu minn żmien għal żmien u sabiex jippermettu l-paragun mar-riżultati tal-metodu applikat.

1.4. PRINCIPJU TAL-METODU TAT-TEST

Ammont predeterminat tas-sustanza, imdewba jew imxerda fl-ilma, huwa ossidizzat bid-dikromat tal-putass f'medja qawwija ta' l-aċidu sulfuriku bis-sulfat tal-fidda bħala katalist, taħt reflus għal sagħtejn. Id-dikromat residwu huwa determinat bit-titrazzjoni mas-sulfat ta' l-ammonium ferruż standardizzat.

Fil-każ ta' sustanzi li jkun fihom il-klorin, (*) sulfat tal-merkurju huwa miżjud sabiex titnaqqas l-interferenza tal-klorid.

1.5. KRITERJA TAL-KWALITÀ

Minħabba l-manjiera arbitrarja tad-determinazzjoni, il-COD huwa "indikatur ossidizzabbli" u bħala tali użat bħala metodu prattiku fil-kejl tal-materja organika.

Il-klorid jista jinterferixxi f'dan it-test, aġenti inorganiċi tat-tnaqqis jew ta' l-ossidizzazzjoni jistgħu ukoll jinterferixxu mad-determinazzjoni tal-COD.

Uħud mill-kompost ċikliċi u ħafna mis-sustanzi volaiti (e.g. aċidi xaħmija baxxi) ma humiex kompletament ossidizzati b'dan it-test.

1.6. DESKRIZZJONI TAL-METODU TAT-TEST

Taħlita jew tixrid preliminarji tas-sustanza huma ippreparati sabiex ikun akkwistat COD bejn 250 u 600 mg kull litru.

Rimarki:

Fil-każ ta' sustanza li ma tantx tinħall u mhux-dispersibbli, ammont ta' sustanza miġjuba trab fin jew sustanza likwida korrespondenti għal madwar S mg ta' COD tista tkun miżuna u poġġuta fl-apparat esperimentali bl-ilma.

It-talba ta' l-ossiġeni kemikali (COD) hija ta' spiss u speċjalment fil-każ ta' sustanzi li ma tantx jinħallu, determinata b'mod vantaġġuż ġewwa varjant tal-metodu, i.e., f'sistema magħluqa bl-ekwalizzatur tal-pressjoni (H. Kelkenberg, 1975). F'din il-modifika, l-komposti huma determinati biss b'diffikulta bil-metodu konvenzjonali. — e.g. aċidu aċetiku — jista ta' spiss ikun kwantifikat b'suċċess. Il-metodu wkoll jonqos, b'dana kollu, fil-każ tal-pridin Jekk il-konċentrazzjoni tad-dikromat tal-putassa, kif preskritt fir-ref. (1), huma mtella għal 0,25 N (0,0416 M), l-użin dirett ta' 5-10 mg tas-sustanza huwa faċilitat, li huwa essenzali għad-determinazzjoni tal-COD ta' sustanzi li ma tantx jinħallu fl-ilma (ref. (2)).

Altrimentri l-BOD huwa mbagħad determinat skond kwalunkwe metodu xieraq standardizzat nazzjonal;i jew internazzjonali.

2. **DATA U EVALWAZZJONI**

Il-COD li jkun jinsab fil-garafina sperimentali huwa ikkalkolat skond il-metodu normalizzat magħżul, u ikkonvertit fi grammi ta' COD kull gramma tas-sustanza tat-test.

(*) Wara l-u\u, it-ta]litiet li jkun fihom l-imlu]a tal-merkurju g]andhom ikunu ittrattati sabiex tkun evitata d-dissiminazzjoni tal-merkurju fl-ambjent.

3. **RAPPORTAĠĠ**

Il-metodu użat għandu jkun mistqarr.

It-talba għall-ossiġenu kemikali għandha tkun il-medja ta' mill-anqas tlett kejl validi. L-informazzjoni u r-rimarki kollha relevanti għall-interpretazzjoni tar-riżultati għandhom ikunu irrappurtati, speċjalment f'dak li jirrigwardja l-impuritajiet, l-istat fiżiku, l-effetti tossiċi u l-komposizzjoni inerenti tas-sustanza.li jkunu jaffetwaw ir-riżultati.

L-użu tas-sulfat tal-merkurju għal minimizzar ta' l-interferenza tal-klorid għandu jkun rapportat.

4. **REFERENZI**

(1) Kelkenberg, H.,Z. von Wasser und Abwasserforschung, 1975, vol. 8, 146.

(2) Gerike, P. *The biodegradability testing of poorly water soluble compounds*. Chemosphere, 1984, vol. 13, 169.

Lista ta' metodi standardizzati, per eżempju:

NBN T 91-201 *Determination of the chemical oxygen demand.*

ISBN 0 11 7512494 *Chemical oxygen demand (dichromate value) of polluted and waste waters.*

NFT 90-101 *Determination of the chemical oxygen demand.*

DS 217 = *Water analysis determination of the chemical oxygen demand.*

DIN 38409-H-41 *Determination of the chemical oxygen demand (COD) within the range above 15 mg per litre.*

NEN 3235 5.3 Bepaling van het chemisch zuurstofverbruik.

ISO 6060 Kwalità ta' l-ilma: talba ta' l-ossiġenu kemikali metodi dikromati.

C.7. DEGRADAZZJONI — IDROLEŻI TAD-DEGRADAZZJONI ABJOTIKA BĦALA FUNZJONI TA' pH

1. **METODU**

Il-metodu huwa ibbażat fuq il-Linja ta' Gwida tat-Testijiet OECD (1).

1.1. INTRODUZZJONI

L-idroleżi hija reazzjoni importanti għall-kontroll tad-degradazzjoni abjotika. Din ir-reazzjoni hija partikolarment relevanti għal sustanzi b'diedegradabbiltà baxxa, u tista tinfluwenza l-persistenza ta' sustanza fl-ambjent.

Ħafna mir-reazzjonijiet idroliċi huma psewdo ta' l-ewwel ordni u, għalhekk, "in-nofs żmien" huwa independenti mill-konċentrazzjoni. Dan normalment jippermetti l-estrapolazzjoni tar-riżultati misjuba f'konċentrazzjoni tal-laboratorju mal-kondizzjonijiet ambjentali.

Aktar minn hekk, diversi eżempji kienu ġew irrapportati (2), li juru ftehim sodisfaċenti bejn ir-riżultati misjuba f'ilmijiet puri u naturali għal diversi tipi ta' kimika.

Huwa utili li jkun hemm informazzjoni preliminarja dwar il-fjammabbiltà tas-sustanza qabel ma jitwettaq it-test.

Dan il-metodu huwa applikabbli biss għal sustanzi li jinħallu fl-ilma. L-impuritajiet jistgħu jaffettwaw ir-riżultati.

L-imġieba idrolitika tal-kimika għandha tkun eżaminata fil-valuri pH li normalment aktar jinsabu fl-ambjent (pH 4 sa 9).

1.2. DEFINIZZJONIJIET U UNITAJIET

L-idroleżi tirreferi għar-reazzjoni ta' RX kimiku ma l-ilma Din ir-reazzjoni tista tkun rapreżentata bl-iskambju nett tal-grupp X bi OH:

$$RX + HOH \rightarrow ROH + HX \quad (1)$$

Ir-rata li fiha l-konċentrazzjonijiet ta' RX tonqos hija mogħtija bi:

$$\text{rata} = k\ [H_2O] \cdot [RX] \quad (2)$$

Minħabba li l-ilma huwa preżenti b'eċċess kbir meta mqabbel mal-kimika, dan it-tip ta' reazzjoni huwa normalment deskritt bħala reazzjoni ta' psewdo ta' l-ewwel ordni, li fiha rata kostanti tkun osservata, hija mogħtija bir-relazzjoni

$$k_{obs} = k \times [H_2O] \quad (3)$$

Dan il-kostant jista jkun determinat għal valur pH wieħed u temperatura waħda, Y, bl-użu ta' l-espressjoni:

$$k_{obs}\ \frac{2{,}303}{t} \times \log \frac{C_0}{C_t} \quad (4)$$

meta:

t = il-ħin,

C0 = il-konċentrazzjoni tas-sustanza fil-ħin 0,

C = il-konċentrazzjoni tas-sustanza fil-ħin t, u

2,303 = il-farttur tal-konverżjoni bejn il-lagarittmi 10 naturali u bażiċi.

Il-konċentrazzjonijiet huma espressi fi grammi kull litru jew moli kull litru.

Il-qisien ta' dan il-konstant k0b huwa (ħin) — I

"Il-perijodu ta' nofs-ħajja" $t_{1/2}$ Huwa definit bħala ż-żmien meħtieġ għat-tnaqqis tal-konċentrazzjoni tas-sustanza tat-test bi; 5O %, jiġifieri:

$$C_t = 1/2 \cdot C_0 \quad (5)$$

Mill-espressjonijiet (4) u (5) wieħed jista juri li:

$$t_{1/2} = 0{,}693/k_{obs} \quad (6)$$

1.3. SUSTANZI TA' REFERENZA

Mhux meħtieġ li jsir użu minn sustanzi ta' referenza fil-każi kollha meta tkun investigata sustanza ġdida. Dawn għandhom primarjament iservu sabiex jivverifikaw l-imġieba tal-metodu minn żmien għal żmien u sabiex jippermettu l-paragun mar-riżultati minn metodi oħrajn.

Is-sustanzi li ġejjin kienu ġew użati bħala sustanzi ta' referenza (1):

Aċidu aċetilsalikliku (aspirina)

Aċidu fosforptiku *0,0-diethyl O-(6-methyl-2-(1-methylethyl)4-pyrimidinyl) ester.* (Dinpilat, Djażinon)

1.4. PRINCIPJU TAL-METODU TAT-TEST

Is-sustanza hija mdewba fl-ilma f'konċentrazzjoni baxxa; il-pH u t-temperatura huma ikkontrollati.

It-tnaqqis tal-konċentrazzjoni tas-sustanza mal-ħin huwa segwit bi kwalunkwe proċedura analitika xierqa.

Il-logaritmu tal-konċentrazzjoni huwa mpġinġi kontra l-ħin u, jekk dan jirriżulta f'linja dritta, il-kostant ta' l-ewwel ordni jista jkun akkwistat miż-żurżieqa tiegħu (ara l-punt 2).

Meta ma jkunx prattiku li tkun determinata r-rata kostanti direttament minn temperatura partikolari, huwa normalment possibbli li jkun ikkalkolat il-kostant permezz ta' l-użu tar-relazzjoni Arrhenius, li tagħti dependenza mat-temperatura tar-rata kostanti. Mit-tpinġija lineari tal-logaritmu tar-rata kostanti, kif determinat fit-temperatura xierqa, bħala funzjoni tar-reċiprokal tat-temperatura assoluta, K, huwa possibbli li tkun estrapolata r-rata ta' valur kostanti li ma tkunx direttament akwistabbli.

1.5. KRITERJA TAL-KWALITÀ

Huwa irrappurtat fir-referenza (2) li l-kjel ta' l-idroleżi rata-kostanti fi 13 il-klassi ta' strutturi organiċi jistgħu ikunu ta' preċiżjoni għolja.

Ir-repetabbiltà tiddependi partikolarment mill-kontroll tal pH u tat-temperatura u tista tkun affettwata bil-preżenza ta' mikro-organiżmi u f'każi speċjali, bil-konċentrazzjoni ta' l-ossiġenu mdewweb.

1.6. DESKRIZZJONI TAL-METODU TAT-TEST

1.6.1. **Reaġenti**

1.6.1.1. *Taħlitiet kuxxin*

It-test huwa mwettaq fi tlett valuri pH: 4,0, 7,0 u 9,0.

Għal dan l-iskop, taħlitiet kuxxin għandhom ikunu preparati bl-użu ta' kimika tal-grad ta' reaġent u ilma distillat jew dejonizzat, sterili. Uħud mill-eżempji tas-sistema kuxxin huma preżenti fl-Appendiċi.

Is-sistema kuxxin użata tista wkoll tinfluwenza r-rata ta' l-idroleżi; jekk ikun hemm evidenza ta' dan, sistema kuxxin alternattiva għandha tkun użata. L-użu tal-kuxxini tal-borat jew ta' l-aċetat huwa rakkommandabbli fir-referenza (2) minflok il-fosfat.

Jekk il-valur pH tat-taħlitiet kuxxini ma jkun magħruf fit-temperatura użata matul it-test, dan jista jkun determinat bl-arloġġ pH kalibrat fit-temperatura magħżula bi preċiżjoni ta' unitajiet pH ± 0,1.

1.6.1.2. *Soluzjoni tat-test*

Is-sustanza tat-test għandha tkun imdewba fil-kuxxin magħżul u l-konċemtrazzjoni m'għandhiex teċċedi 0,01 M jew nofs il-konċentrazzjoni tas-saturazzjoni, liema minnhom tkun l-aktar baxxa.

L-użu ta' solventi organiċi li jithaltu ma l-ilma huwa rakommandabbli biss għal sustanzi b'solubbilità baxxa fl-ilma.

L-ammont ta' solvent għandu jkun anqas minn 1 %, u m'għandux jinterferixxi mal-proċess idrolitiku.

1.6.2. **Apparat**

Garafini bl-istoppini tal-ħġieġ għandhom ikunu wżati, imma l-*grease* għandu jkun evitat fuq il-ħġieġ magħruk.

Jekk il-kimika jew is-sistema tal-kuxxin tkun volatili, jew jekk it-test ikun imwettaq f'temperaturi elevati, tubi issiġillati jew magħluqa bis-septum huma preferibbli u spazju taħt it-tappin għandu jkun evitat.

1.6.3. **Il-metodu analitiku**

Il-metodu għandu jkun speċifiku sabiex jippermetti d-determinazzjoni tas-sustanza tat-test fil-konċentrazzjonijiet tat-taħlita tat-test u jistgħu jikkonsistu f'ġemgħa ta' teknika analitika xierqa..

Il-metodu analitiku użat ikun jiddependi min-natura tas-sustanza u għandu jkun preċiż b'mod suffiċjenti għas-sensittività sabiex jintebaħ bi tnaqqis ta' 10% tal-konċentrazzjoni inizjali.

1.6.4. **Il-kondizzjonijiet tat-test**

It-testijiet għandhom ikunu mwettqa bl-użu ta' għeluq termostatikament ikkontrollat jew f'banju b'temperatura kostanti irregolat fi ± 0,5 °C tat-temperatu magħżulha. It-temperatura għandha tinżamm u tkun imkejla fil-limitu ta' ± 0,1 °C. Interferenza fotolitika għandha tkun evitata bill-mezzi xierqa.

Għal sustanzi li huma faċilment ossidabbli, jkun meħtieġ li jkun eskluż l-ossiġenu mdewweb (e.g. bit-tbaqbieq bin-nitroġenu jew bl-argon għal ħames minuti qabel il-preparatazzjoni tat-taħlita).

1.6.5. **Proċedura tat-test**

1.6.5.1. *Test prelimenari*

Għal sustanzi kollha. it-test preliminari għandu jkun imwettaq fi 50 ± 0,5 °C fit-tlett valuri pH: 4,0, 7,0 u 9,0. Numru suffiċjenti ta' kejl għandu jsir, sabiex jistgħu jsiru l-estimi ta' jekk, għal kull valur pH u fi 50 °C, iż-żmien ta' nofs-ħajja (t!) ikun anqas minn 2,4 siegħat jew anqas minn 10% ta' l-idroleżi, jkun osservat wara ħamest ijiem. (Wieħed jista jwettaq l-estimi li dawn il-valuri jkunu jikkorrespondu rispettivament maż-żmien ta' nofs-ħajja ta' anqas minn jum wieħed jew aktar minn sena skond il-kondizzjonijiet aktar rappreżentattivi minn dawk ambjentali (25 °C)).

Jekk it-test preliminari jindika li 50 % jew aktar tas-sustanza tat-test tkun ġiet idrolizzata fi 2,4 siegħat fi 50 °C, jekk anqas minn 10% jkun ġie idrolizzat wara ħamest ijiem f'kull wieħed mit-tlett valuri pH (4, 7 u 9), l-ebda testijiet oħrajn ma jkunu meħtieġa.

Fil-każi l-oħrajn, għal valuri pH individwali li dwarhom din ilk-kondizzjoni ma tkun sodisfatta, it-Test 1 huwa mwettaq.

1.6.5.2. *Test 1*

It-Test 1 huwa mwettaq f'temperatura waħda, preferibbilment fi 50 ± 0,5 °C, u, jekk possibbli, f'kondizzjonijiet sterili f'dawk il-valuri pH li dwarhom it-test preliminarju jkun wera l-ħtieġa ta' aktar testijiet.

Numru suffiċjenti ta' kampjuni (mhux anqas minn erba') għandhom ikunu magħżula sabiex ikopru l-medda 20 sa 70 % ta' l-idroleżi tat-test għall-imġieba ta' psewdo l-ewwel ordni fil-valuri pH speċifikati.

Għal kull valur pH li fih ikun imwettaq it-test, l-ordni tar-reazzjoni għandha tkun determinata.

Estimi tar-rata kostanti fi 25 °C:

Id-deċiżjoni dwar kif għandhek tippoċiedi b'mod sperimentali jiddependi minn jekk jistax ikunu konkluż mit-Test 1 li r-reazzjoni tkun psewdo l-ewwel ordni jew le.

Jekk ma jistax ikun konkluż b'ċertezza mit-Test 1 li r-reazzjoni tkun psewdo l-ewwel ordni, aktar esperimenti għandhom ikunu mwettqa kif deskritt fit-Test 2.

Jekk ma jistax ikun konkluż b'ċertezza mit-Test 1 li r-reazzjoni tkun psewdo l-ewwel ordni, aktar esperimenti għandhom ikunu mwettqa kif deskritt fit-test 3. (B'mod alternattiv, jista' skond ċirkostanzi speċjali, jkun possibbli li ssir il-kalkolazzjoni tal-kostanti tar-rata fi 25 °C minn kostanti fi 50 °C, ikkalkolati bl-użu tar-riżultati mit-test 1, (ara 3.2)).

1.6.5.3. *Test 2*

Dan it-test huwa mwettaq, għal kull valur pH li dwar r-riżultati tat-test 1 ikunu werw il-ħtieġa li jsir dan:

— jew f'temperatura waħda ta' anqas minn 40 °C,

— jew f'żewġ temperaturi ta' aktar mnn 50 °C li jiddifferixxu minn xulxin b'mill-anqas 10 °C.

Għal kull valur pH u temperatura meta t-test 2 ikun imwettaq, mill-anqas sitta punti tad-*data* adekwatament spazzjali, għandhom isiru hekk li l-gradi ta' l-idroleżi jkunu fil-medda 20 sa 70 %.

Għal valur pH wieħed u temperatura waħda, determinazzjoni hija mwettqa b'mod duplikat. Meta t-test 2 isir f'żewġ temperaturi ta' aktar minn 50 °C, il-mod duplikat huwa preferibbilment imwettaq fl-anqas minn dawn iż-żewġ temperaturi.

Għal kull valur pH u temperatura meta t-test 2 ikun imwettaq, estimi grafiċi taż-żmien ta' nofs-ħajja (t1) għandu jkun mogħti, meta dan ikun possibbli. 2

1.6.5.4. *Test 3*

Dan it-test huwa mwettaq, għal kull valur pH li dwar r-riżultati tat-test 1 ikunu werw il-ħtieġa li jsir dan:

— jew f'temperatura waħda ta' anqas minn 40 °C,

— jew f'żewġ temperaturi ta' aktar inn S0 °C li jiddifferixxu minn xulxin b'mill-anqas 10 °C.

Għal kull pH u temperatura meta t-Test 3 ikun imwettaq, tlett punti tad-*data* huma magħżula, l-ewwel fil-ħin 0 u t-tieni u t-tielet meta l-grad ta' l-idroleżi jkun akbar minn 30 %, il-kostant k0b, u t, għandhom ikunu ikkalkolati.

2. **DATA**

Għall-imġieba tal-psewdo l-ewwel ordni l-valuri ta' k0b, għal kull valur pH u kull temperatura tat-testijiet tista tkun akkwistata mit-tpinġija tal-logaritmi tal-konċentrazzjoni versus il-ħin bl-użu ta' l-espressjoni:

$$k_{obs} = - \text{żurieqa} \times 2{,}303 \quad [7]$$

Aktar t½ jista jkun ikkalkolat skond l-ekwazzjoni (6).

Wettaq l-evalwazzjoni k25°C bl-applikazzjoni ta' l-ekwazzjoni Arrhenius meta xieraq.

Għall-imġieba mhux pseudo ta' l-ewwel ordni ara 3.1.

3. **RAPPORT**

3.1. RAPPORTAĠĠ

Ir-rapport tat-test għandu, jekk possibbli, jinkludi l-informazzjoni li ġejja:

— speċifikazzjoni tas-sustanza;

— kwalunkwe riżultati akkwistati bis-sustanzi ta' referenza;

— il-prinċipju u d-dettalji tal-metodu analitiku użat;

— għal kull wieħed mit-testijiet: it-temperatura, il-valur pH, il-posizzjoni tal-kuxxin u tabella tal-konċentrazzjonijiet tal-punti kollha-ħin-*data*;

— għar-reazzjoni tal-psewdo l-ewwel ordni, il-valuri ta' kOb ta' ti u l-proċedura tal-kalkolazzjoni tagħha;

— għal reazzjoni ta' mhux-psewdo ta' l-ewwel ordni, pinġi r-tiżultati bħala logaritmu tal-konċentrazzjoni *versus* il-ħin;

— l-informazzjoni kollha u l-osservazzjonijiet meħtieġa għall-interpretazzjoni tar-riżultati.

3.2. INTERPRETAZZJONI TAR-RIŻULTATI

Jista jkun possibbli li jkunu kalkolati l-valuri aċċettabbli tal-kostant tar-rata (fi 25 °C) tas-sustanzi tat-test, basta li l-valuri sperimentali ta' l-attivazzjoni ta' l-enerġija huma diġa eżistenti għall-omologwi tas-sustanza tat-test u basta li jkun jkun assumit raġjonevolment li l-attivazzjoni ta' l-eneġija tas-sustanza tat-test ma tkun ta' l-istess ordni ta' magnitudini.

4. **REFERENZI**

(1) OECD, Pariġi, 1981, Linji ta' Gwida tat-Testijiet 111, Deċiżjoni tal-Kunsill C(81) 30 finali.

(2) W. Mabey and T. Mill, *'Critical Review of Hydrolysis of Organic Compounds in Water Under Environmental Conditions,'* J. Phys. Chem. Ref. *Data*, 1978, vol. 7 (2), 383-415.

Appendiċi

TAĦLIT TA' KUXXIN

A. **CLARK U LUBS**

Il-valuri pH irrapportati f'dawn it-tabelli kienu ġew ikkalkolati mill-kejl potenzali bl-użu tal-ekwazzjonijiet normal Sorensen. Il-valur pH attwali huwa 0.04 unità ogħla mill-valuri tabulati.

Komposizzjoni	pH
0,1 M ftalat ta' l-idroġenu tal-putassa + 0,1 N HC1 fi 20 °C	
2,63 ml 0,1 N HC1 + 50 ml ftalat sa 100 ml	3,8
0,1 M ftalat ta' l-idroġenu tal-putassa + 0,1 NaOH fi 20 °C	
0,40 ml 0,1 N HC1 + 50 ml ftalat sa 100 ml	4,0
3,79 ml 0,1 N HC1 + 50 ml ftalat sa 100 ml	4,2
0,1 M fosfat monopotassju + 0,1 N NaOH fi 20 °C	
23,45 ml 0,1 N NaOH + 50 ml fosfat sa 100 ml	6,8
29,63 ml 0,1 N NaOI + 50 ml fosfat sa 100 ml	7,0
35,00 ml 0,1 N NaOH + 50 ml fosfat sa 100 ml	7,2

0,1 M H3B03 fi 0,1 M KC1 + 0,1 N NaOH fi 20 °C	
16,30 ml 0,1 NNaOH + 50 ml aċidu boriku sa 100 ml	8,8
21,30 ml 0,1 N NaOH + 50 ml aċidu boriku sa 100 ml	9,0
26,70 ml 0,1 N NaOH + 50 ml aċidu boriku sa 100 ml	9,2

B. **KOLTHOFF U VLEESHOUWER**

Komposizzjoni	pH
0,1 M ċitrat monopotassju u 0,1 N NaOH fi 18 °C (żid kristal żgħir tat-tirnol sabiex tipprevjenti t-tkabbir tal-moffa)	
2,0 ml 0,1 N NaOH + 50 ml ċitrat sa 100 ml	3,8
9,0 ml 0,1 N NaOH + 50 ml ċitrat sa 100 ml	4;0
16,3 ml 0,1 N NaOH + 50 ml ċitrat sa 100 ml	4,2

C. **SORENSEN**

0,05 M boraxx + 0,1 N HC1

Komposizzjoni		pH			
ml Borazz	ml HCI	Sörensen 18 °C	Walbum		
			10 °C	40 °C	70 °C
8,0	2,0	8,91	8,96	8,77	8,59
8,5	1,5	9,01	9,06	8,86	8,67
9,0	1,0	9,09	9,14	8,94	8,74
9,5	0,5	9,17	9,22	9,01	8,80
10,0	0,0	9,24	9,30	9,08	8,86

0,05 M boraxx + 0,1 N NaOH

Komposizzjoni		pH			
ml Borazz	ml HCI	Sörensen 18 °C	Walbum		
			10 °C	40 °C	70 °C
10,0	0,0	9,24	9,30	9,08	8,86
9,0	1,0	9,36	9,42	9,18	8,94
8,0	2,0	9,50	9,57	9,30	9,02
7,0	3,0	9,68	9,76	9,44	9,12