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Acts whose titles are printed in light type are those relating to day-to-day management of agricultural matters, and are generally valid for a limited period.
The titles of all other Acts are printed in bold type and preceded by an asterisk.

II

(Acts whose publication is not obligatory)

COMMISSION

COMMISSION DIRECTIVE

of 25 April 1984

adapting to technical progress for the sixth 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

(84/449/EEC)

THE COMMISSION OF THE EUROPEAN
COMMUNITIES,

the Removal of Technical Barriers to Trade in
Dangerous Substances and Preparations,

Having regard to the Treaty establishing the European
Economic Community,

HAS ADOPTED THIS DIRECTIVE:

Having regard to Council Directive 67/548/EEC of
27 June 1967 on the approximation of laws, regulations
and administrative provisions relating to the
classification, packaging and labelling of danger-
ous substances ⁽¹⁾, as amended for the sixth time by
Council Directive 79/831/EEC ⁽²⁾, and in particular
Articles 19, 20 and 21 thereof,

Article 1

The text of Annex V to Directive 67/548/EEC is
hereby replaced by the text of the Annex to this
Directive.

Article 2

Whereas Article 3 (1) of Directive 79/831/EEC
provides that the physico-chemical properties, toxicity
and ecotoxicity of the substances and preparations shall
be determined according to the methods specified in
Annex V;

Member States shall adopt and publish before 1 July
1985 the measures needed to comply with this Directive
and immediately inform the Commission thereof. They
shall apply these measures by 1 July 1986 at the
latest.

Article 3

Whereas Article 19 of Directive 79/831/EEC of
18 September 1979 provides that Annex V comes under
the procedure, involving the Committee for Adaptation
to Technical Progress; whereas due regard should
be given, in particular, to any methods recognized
and recommended by competent international
organizations;

This Directive is addressed to the Member States.

Done at Brussels, 25 April 1984.

Whereas the provisions of this Directive are in
accordance with the opinion of the Committee for the
Adaptation to Technical Progress of the Directives on

For the Commission

Karl-Heinz NARJES

Member of the Commission

⁽¹⁾ OJ No L 196, 16. 8. 1967, p. 1.

⁽²⁾ OJ No L 259, 15. 10. 1979, p. 10.

ANNEX

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 marketed. 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.

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PART A: METHODS FOR THE DETERMINATION OF PHYSICO-CHEMICAL PROPERTIES**A. 1. MELTING POINT/MELTING RANGE****1. METHOD**

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

1.1. Introduction

The methods and devices described are to be applied for the determination of the melting point of chemical 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 the substance can be pulverized easily, with difficulty, or not at all.

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

1.2. Definitions and units

The melting point is defined as the temperature at which the phase transition from solid to liquid state at normal atmospheric pressure takes place.

This temperature ideally corresponds to the temperature at the solidification point or the freezing point.

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

Conversion of units (K to °C):

$$t = T - 273,15$$

with t in °C, T in K.

1.3. Reference substances

Reference substances do not need to be employed in all cases when investigating a new substance. This should primarily serve to calibrate the method from time to time and to allow a comparison of results when another method is applied.

Some calibration substances are listed in the references (2).

1.4. Principle of the test method

The temperature (temperature range) of the phase transition from the solid to the liquid state is determined. In practice while heating a sample of the test substance at atmospheric pressure the temperatures of the initial melting and the final stage of melting are determined. Three types of methods are described, namely capillary method, hot stages method and freezing point determinations.

1.4.1. *Capillary method*

1.4.1.1. Melting point devices with liquid bath

A small amount of the finely ground substance is charged 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. 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 heat 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 543 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 point, 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 points 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 also used. A typical microscope hot stage melting point 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 cut off air from the working area.

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

1.4.2.3. Meniscus method

This method is specifically used for polyamides.

Determination of 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 observed visually.

1.4.3. *Method to determine the freezing point*

The sample is filled in a special test tube and placed in an apparatus for the determination of the crystallizing point. The sample is stirred gently and continuously during cooling and the temperature is read and recorded at 30-second intervals. As soon as the temperature remains constant for a few readings this temperature (corrected for thermometer error) is recorded as the crystallizing point.

1.5. **Quality criteria**

The applicability and accuracy of the different methods used for the determination of the melting point/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	Approximate maximum accuracy ⁽¹⁾	Remarks
Melting point devices with liquid bath	Yes	Only to a few	273 to 573 K	± 0,3 K	Standard existing JIS K 0064
Melting point devices with metal block	Yes	Only to a few	293 to 573 K	± 0,5 K	Standard existing ISO 1218 (E)
Photocell detection	Yes	Several with appliance devices	253 to 573 K	± 0,1 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	Approximate maximum accuracy ⁽¹⁾	Remarks
Kofler hot bar	Yes	No	283 to 543 K	± 1,0 K	Standard existing ANSI/ASTM D 3451-76
Melt microscope	Yes	Only to a few	273 to 573 K (up to 1 773 K)	± 0,2 K	Standard existing DIN 53736
Meniscus method	No	Specifically for polyamides	293 to 573 K	± 0,5 K	Standard existing ISO 1218 (E)
Freezing point methods	For liquid substances	For liquid substances	223 to 573 K	± 0,5 K	Standard existing such as BS 4699

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

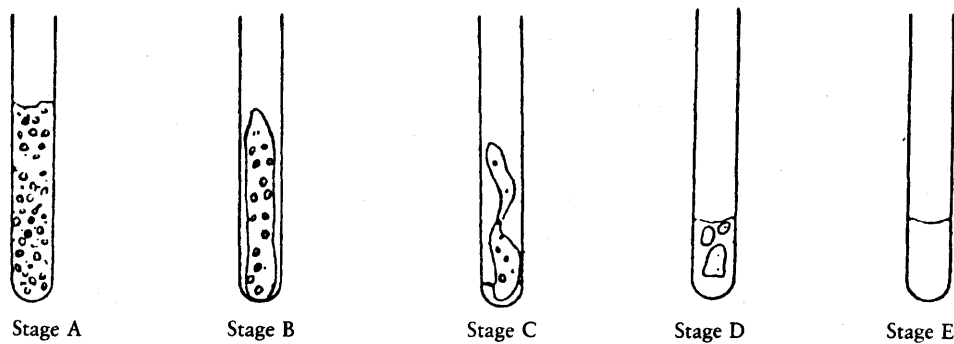
1.6. Description of the methods

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

Finely pulverized substances usually show the stages of melting shown in figure 1 during a slow temperature rise.

Figure 1



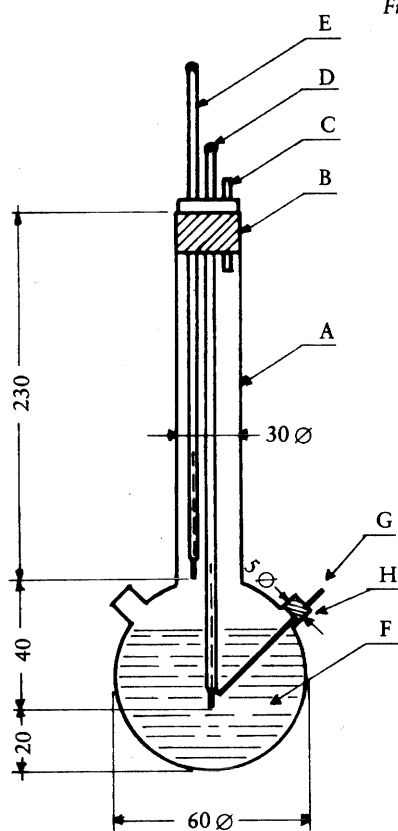
- Stage A (Beginning of melting; wet point): fine droplets adhere uniformly to the inside wall of the capillary tube;
- Stage B (Shrinkage point): a clearance appears between the sample and the inside wall due to shrinkage of the melt;
- Stage C (Collapse point): the shrunken sample begins to collapse downwards and liquefies;
- Stage D (Liquefying point): 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 point the temperatures are recorded at the beginning of melting and at the final stage.

1.6.1.1. Melting point devices with liquid bath apparatus

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

Figure 2



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

Bath liquid:

The suitable liquid should be chosen from below depending on the melting point. Liquid paraffin for melting points not higher than 473 K, concentrated sulphuric acid or silicone oil for melting points not higher than 573 K.

For melting points above 523 K, a mixture consisting of three parts sulphuric acid and two parts potassium sulphate (in weight ratio) can be used.

Thermometer:

Only those thermometers should be used which fulfil the requirements of the following or equivalent standards: ASTM E 1-71, DIN 12770, JIS K 8001.

Procedure:

The dry substance is finely 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 tubes are 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 point.

The bath liquid is heated so that the temperature rise is approximately 3 K/min. The liquid must 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 point 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. Metal block

Apparatus:

This consists of:

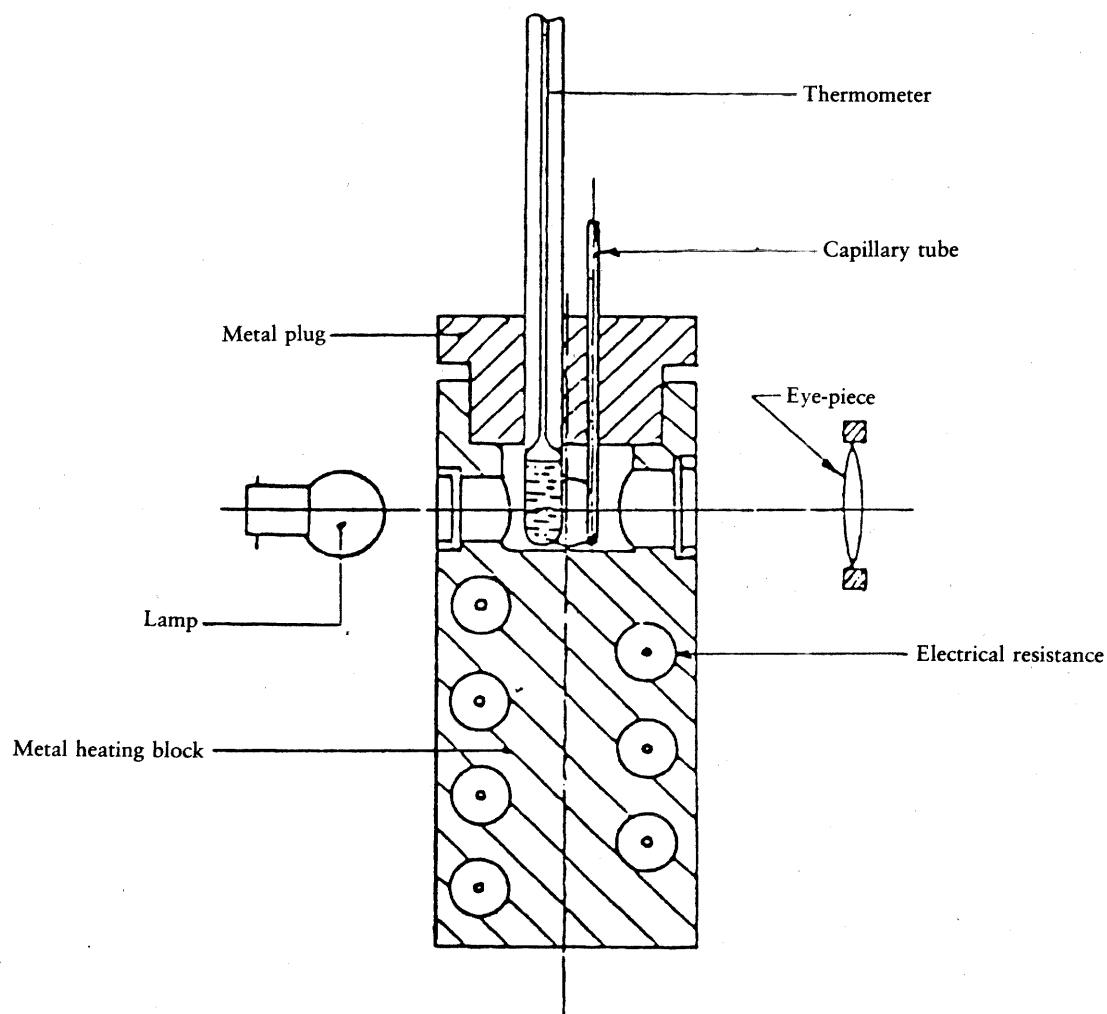
- a cylindrical metal block, the upper part of which is hollow and forms a chamber (see figure 3),
- a metal plug, with two or more holes, allowing tubes to be mounted into the metal block,
- a heating system, for the metal block, provided for example by an electrical resistance enclosed in the block,
- 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 1.6.1.1.

Thermoelectrical instruments with comparable accuracy are also applicable.

Figure 3



Procedure:

See 1.6.1.1. A thermometer correction must not be applied in this case. The recorded temperature indicates the melting point.

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.

Five 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 melting point 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 point should be less than 1 K/min.

1.6.3. Methods for the determination of the freezing point

See Appendix.

2. DATA

A thermometer correction is necessary in some cases.

3. REPORTING

The method used shall be stated.

The reported melting point is the mean of at least two measurements which are in the range of the approximate accuracy (see table). An estimate of the accuracy should be provided. 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 point, otherwise the two temperatures are reported.

Some substances will decompose or sublime before the melting point is reached. In these circumstances this should 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, Physicochemical measurements: Catalogue of reference materials from national laboratories, *Pure and applied chemistry*, vol. 48, 1976, pp. 505 to 515.

Appendix

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

1. **Capillary methods**

1.1. *Melting point 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 Kapillerverfahren
JIS K 00-64	Testing methods for melting point of chemical products

1.2. *Melting point 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
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2.2. *Melt microscope*

DIN 53736	Visuelle Bestimmung der Schmelztemperatur von teilkristallinen Kunststoffen
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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 du polyamides. Détermination du « point de fusion » Méthode du ménisque

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

BS 4633	Method for the determination of crystallizing point
BS 4695	Method for Determination of Melting Point of Petroleum Wax (Cooling Curve)
DIN 10319	Bestimmung des Gefrierpunktes von Milch

DIN 51421	Bestimmung des Gefrierpunktes von Flugkraftstoffen, Ottokraftstoffen und Motorenbenzolen
DIN 51556	Bestimmung des Erstarrungspunktes am rotierenden Thermometer
DIN 53175	Bestimmung des Erstarrungspunktes von Fettsäuren
NF T 60-114	Point de fusion des paraffines

A. 2. BOILING POINT/BOILING RANGE

1. METHOD

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

1.1. Introduction

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

Emphasis is put on the method using photocell detection, because this method allows the determination of melting as well as boiling points. 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 standard pressure can be adjusted during the measurement. But this method is not at present automated.

Remarks

The influence of impurities on the determination of the boiling point depends greatly upon the nature of the impurity. The effect can be considerable if a highly volatile solvent is present in the sample.

The composition of the investigated sample changes from measurement to measurement due to the volatilization of low boiling components: continuously increasing values are obtained in these circumstances.

1.2. Definitions and units

The standard boiling temperature is described as the temperature at which the pressure of the saturated vapour of a liquid is the same as the standard pressure.

The measured boiling point is dependent on the atmospheric pressure. This dependence can be described quantitatively by the Clausius-Clapeyron equation as follows:

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

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
= $8,31 \text{ J mol}^{-1} \text{ K}^{-1}$

(The temperature T is expressed in K).

The temperature at the boiling point (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' units are still permissible but not recommended);

133 Pa = 1 mm Hg = 1 Torr (the units 'mm Hg' and 'Torr' are not permitted).

Temperature (units: K)

$t = T - 273,15$

(t in $^{\circ}\text{C}$, and T in K).

1.3. Reference substances

Reference substances do not need to be employed in all cases when investigating a new substance. This should primarily serve to calibrate the method from time to time and to allow a comparison of results.

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

1.4. Principle of the test method

All methods for the determination of the boiling point (boiling range) are based on the measurement of the boiling temperature. Five methods are described.

1.4.1. *Determination by use of the Ebulliometer*

Ebullimeters were originally developed for the determination of the molecular weight by boiling point elevation, but they are also suited for exact boiling point 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 a thermocouple in the reflux while boiling. The pressure can be varied in this method.

1.4.3. *Distillation method for boiling point and boiling range*

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.

The temperature at which a regular string of bubbles escapes from the capillary or the temperature at which, on momentary cooling, the string of bubbles stops and the fluid suddenly starts rising in the capillary (Siwoloboff) is determined.

1.4.5. *Photocell detection*

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

1.5. Quality criteria

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

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.

TABLE 1: COMPARISON OF THE METHODS

Method of measurement	Approximate accuracy	Remarks
Ebulliometer	$\pm 1,4$ K (up to 373 K) ⁽¹⁾ ⁽²⁾ $\pm 2,5$ K (above 373 K) ⁽¹⁾ ⁽²⁾	Existing standard ASTM D 1120-72 ⁽¹⁾
Dynamic method	$\pm 0,5$ K ⁽²⁾	
Distillation process (boiling range)	$\pm 0,5$ K	Existing standard e.g. ISO/R 918 DIN 53171 BS 4591/71
According to Siwoloboff	± 1 K to ± 2 K ⁽²⁾	
Photocell detection	$\pm 0,3$ K (at 373 K) ⁽²⁾	

⁽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.

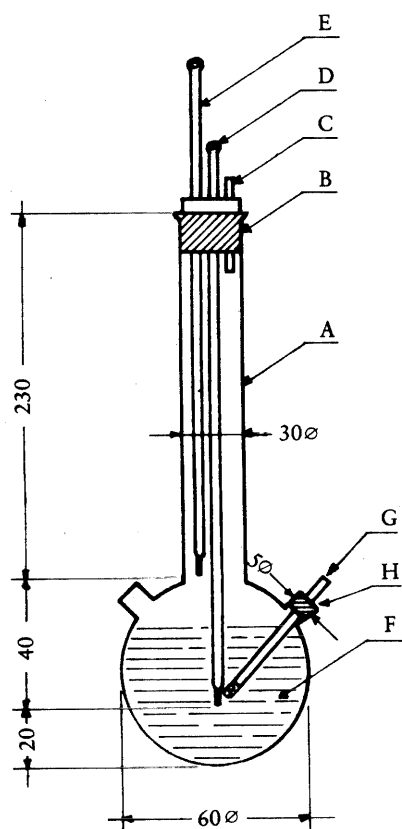
⁽²⁾ Only valid for pure substances.

1.6.4. *Method according to Siwoloboff*

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

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

Figure 1



- A: Measuring tube
 B: Stopper
 C: Vent
 D: Thermometer
 E: Auxiliary thermometer
 F: Bath liquid
 G: Sample tube, maximum 5 mm outer diameter; 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

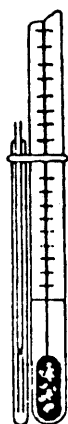
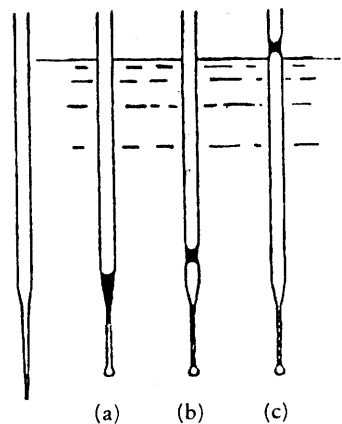


Figure 3

Modified principle



The bath liquid is chosen according to boiling temperature. At temperatures up to 573 K, sulphuric acid or 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 point, 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 from the boiling capillary.

The boiling point is that point 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 point is determined in a melting point 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 point apparatus (b), the air bubble expands. The boiling point 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 immensely. 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 cold room or cooling bath. The exact execution of the boiling point determination can be obtained from the instrument manual.

2. DATA

At small deviations from the normal pressure (maximum ± 5 kPa) the boiling point 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:

$\Delta p = (101,325 - p)$ note sign

p = pressure measurement in kPa

f_T = rate of change of boiling point 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-CORRECTION 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 method used shall be stated. The reported boiling point is the mean of at least two measurements, which are in the range of approximate accuracy indicated in table 1. If determinations are not reproducible, other methods shall be considered.

The measured boiling points 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 pressure. Where a test substance boils over a temperature range this range should be indicated. Estimates of accuracy shall be provided for all results.

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.

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 Field 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

A. 3. RELATIVE DENSITY

1. METHOD

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

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

1.3. Reference substances (1) (2)

Reference substances do not need to be employed in all cases when investigating a new substance.

They should primarily serve to calibrate the method from time to time and to allow a comparison of results.

1.4. Principle of the methods

Four 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 floating hydrometers, which allow the density of a liquid to be deduced from the depth of immersion by reading of 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 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 ball method (for liquid substances) (3)

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 ball 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 densitometer* (4) (5) (6)

The density of a liquid can be measured by an oscillating densitometer. A mechanical oscillator constructed in the form of a U-tube is vibrated at a specific frequency that depends on the mass of the oscillator. 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.

The accuracy as cited in the ISO standard refers only to pure substances.

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 method/standard used shall be reported.

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	Remarks
	Solid	Liquid		
1.4.1.1. Hydrometer		×	5 Pa s	ISO 387 ISO/R 649
1.4.1.2. Hydrostatic balance (a) solids (b) liquids	×	×	5 Pa s	ISO/R 1185 (A) ISO/R 91 and R 758
1.4.1.3. Immersed ball method		×	20 Pa s	DIN 53217
1.4.2. Pycnometer (a) solids (b) liquids	×	×	500 Pa s	ISO/R 3507 ISO/R 1183 (B) ISO/R 758
1.4.3. Air comparison pycnometer	×			DIN 55990 Teil 3 DIN 53243
1.4.4. Oscillating densitometer		×	5 Pa s	

4.

REFERENCES

- (1) OECD, Paris, 1981, *Test Guideline 109*, Decision of the Council C(81) 30 final.
- (2) IUPAC, Recommended reference materials for realization of physico-chemical properties, *Pure and applied chemistry*, vol. 48, 1976, pp. 508.
- (3) Wagenbreth, H., Die Tauchkugel zur Bestimmung der Dichte von Flüssigkeiten, *Technisches Messen tm*, vol. 11, 1979, pp. 427 to 430.
- (4) Leopold, H., Die digitale Messung von Flüssigkeiten, *Elektronik*, vol. 19, 1970, p. 297 to 302.
- (5) Baumgarten, D., Füllmengenkontrolle bei vorgepackten Erzeugnissen — Verfahren zur Dichtebestimmung bei flüssigen Produkten und ihre praktische Anwendung, *Die Pharmazeutische Industrie*, vol. 37, 1975, pp. 717 to 726.
- (6) Riemann, J., Der Einsatz der digitalen Dichtemessung im Brauereilaboratorium, *Brauwissenschaft*, vol. 9, 1976, pp. 253 to 255.

Appendix

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

1. **Buoyancy methods**

1.1. *Hydrometer*

- | | |
|-----------|--|
| DIN 12790 | Hydrometer; general instructions |
| ISO 387 | |
| DIN 12791 | Part I: Density hydrometers; construction, adjustment and use
Part II: Density hydrometers; standardized sizes, designation |
| ISO/R 649 | |
| DIN 12793 | Laboratory glassware: range find hydrometers |

1.2. *Hydrostatic balance*

For solid substances:

- | | |
|------------|--|
| ISO R 1183 | Method A: Methods for determining the density and relative density of plastics excluding cellular plastics |
| 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 R 91 | ISO R 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 ball method*

- | | |
|-----------|--|
| DIN 53217 | Testing of paints, varnishes and similar products; determination of density by pycnometer (enlargement for immersed ball method to be published in 1981) |
|-----------|--|

2. **Pycnometer methods**

2.1. *For liquid substances*

- | | |
|-----------|---|
| ISO 3507 | Pycnometers |
| ISO/R 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 $100 \cdot 10^{-6} \text{ m}^2 \text{ s}^{-1}$ 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 $100 \cdot 10^{-6} \text{ m}^2 \text{ s}^{-1}$ 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 a too high 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.

2.2. *For solid substances*

ISO/R 1183	Method B: Methods for determining the density and relative density of plastics excluding cellular plastics
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 methods described are based on the OECD Test Guideline (1).

1.1. Introduction

It is useful to have preliminary information on the structure, the melting point and the boiling point 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 $< 10^{-3}$ to 10^5 Pa.

Impurities will usually affect the vapour pressure. The influence of impurities on the determination of the vapour pressure depends greatly upon the kind of impurity. The effect can be considerable if a highly volatile solvent is present in the sample.

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 (Newton/m²).

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

1 Torr (mm Hg) = $1,333 \times 10^2$ Pa

1 atmosphere (physical atm) = $1,013 \times 10^5$ Pa

1 atmosphere (technical at) = $9,81 \times 10^4$ Pa

1 bar = 10^5 Pa

The SI unit of temperature is the degree 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 offer the possibility to compare results when another method is applied.

1.4. Principle of the test methods

For determining the vapour pressure, five 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, between 20 and 100 °C.

This method has also been recommended for boiling point determination and is useful for that purpose up to 350 °C.

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, between 0 and 100 °C.

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-2879-75.

Recommended range:

from 100 to 10^5 Pa between 0 and 100 °C.

1.4.4. *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, between 0 and 100 °C.

1.4.5. *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 and the vapour is then collected in a suitable trap. Measurement of the amount of material transported by a known amount of carrier gas is used to calculate the vapour pressure at a given temperature.

Recommended range:

up to 1 Pa.

1.5. *Quality criteria*

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

TABLE: QUALITY CRITERIA

Measuring method	Substances		Estimated repeatability (¹)	Estimated reproducibility (¹)	Recommended range	Existing standard
	Solid	Liquid				
1.4.1. Dynamic method		×	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	×	×	5 to 10 %	5 to 10 %	10 Pa to 10 ⁵ Pa	—
1.4.3. Isoteniscope	×	×	5 to 10 %	5 to 10 %	10 ² Pa to 10 ⁵ Pa	ASTM-D 2879-75
1.4.4. Vapour pressure balance	×	×	5 to 20 %	Up to 50 %	10 ⁻³ Pa to 1 Pa	—
1.4.5. Gas saturation method	×	×	10 to 30 %	Up to 50 %	< 10 ⁻³ Pa to 1 Pa	—

(¹) Dependent of the degree of purity.

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 regulating and 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 by using a thermocouple or resistance thermometer which is immersed in a small quantity of oil. It is inserted into the charging tube which has a male ground joint and is sealed on the bottom.

The crosspiece makes the necessary connections to the pressure regulation and measurement equipment.

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

A cartridge heater, which is inserted into the glass apparatus externally from below, is used for heating the boiling vessel. The desired heating current is set by means of a voltage-regulating transformer, and is monitored by means of an ammeter.

An oil pump is used for setting the desired vacuum between 10² Pa and roughly 10⁵ Pa.

A nitrogen cylinder is used for setting a desired pressure and is connected via a valve which is also used for ventilating the apparatus.

A precision pressure gauge which is connected to the crosspiece is used for pressure measurement.

1.6.1.2. Measurement procedure

The vapour pressure is measured by determining the boiling point 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 point (boiling equilibrium in case of a mixture) has been reached. Frothing substances cannot be measured using this method.

In order to perform the measurement, all glass parts are first thoroughly cleaned and dried and then evacuated under a gas ballast. The substance is then introduced into the apparatus. If solids are not in a powdered form, problems may occur during the filling process, but they can be circumvented by heating the cooling water jacket. After filling, the apparatus is flanged together and the substance degassed. The lowest desired pressure is then set, and the heating system is switched on. Simultaneously the thermocouple or the resistance thermometer is connected to a recorder. Equilibrium is reached when a constant boiling temperature can be read off at a given constant pressure. 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

A typical measuring apparatus (figure 2) includes a heating and a cooling system consisting of glass and metal, for bringing the sample to a regulated temperature, as well as equipment for setting and measuring the pressure and temperature.

The sample chamber has on one side a high-vacuum valve made of stainless steel, and on the other side a U-tube containing a suitable manometric fluid. The other end of the U-tube is joined to a crosspiece, one branch of which leads to the vacuum pump, another to the nitrogen cylinder and the third to the pressure gauge.

For bringing the substance to a regulated temperature, the entire sample chamber including the valve poppet and a sufficiently large section of the U-tube (for practical purposes, up to the height of the valve poppet) is placed in an appropriate constant temperature bath. The temperature is measured using a thermocouple or resistance-thermometer very close to the outside of the sample chamber and can be recorded.

Liquid nitrogen or a dry-ice/alcohol mixture is suitable for supercooling the sample. An ultracryostat is used for measuring at low temperatures.

A suitable pump is used to evacuate the apparatus to the required pressure.

The vapour pressure of a substance is usually measured indirectly via a zero indicator. This zero indicator can be a U-tube containing a liquid, as specified below, or, among others, a membrane manometer. In a temperature-controlled bath, the vapour pressure moves the liquid in the U-tube out of equilibrium. Nitrogen is now let into the apparatus from a connected nitrogen cylinder via a valve to compensate the effect of the vapour pressure and bring the pressure gauge back to zero. The nitrogen pressure required for this is read off at a precision pressure gauge which is at ambient temperature and corresponds to the vapour pressure of the substance at the corresponding steady temperature. Various liquids such as mercury, silicone oils, phthalates, according to pressure range and the chemical behaviour of a substance, can be used as U-tube liquids for zero balancing.

Mercury can be used from atmospheric pressure down to 10^2 Pa, silicone oils and phthalates also below 10^2 Pa down to 10 Pa; the membrane manometer can even be applied below 10^{-1} Pa.

1.6.2.2. Measurement procedure

Before measurement, all parts of the apparatus in figure 2 are thoroughly cleaned with solvents and then dried in a vacuum. The U-tube is then filled with the desired fluid, which should be degassed at elevated temperature prior to filling.

After filling with the substance, the apparatus is flanged together and the sample chamber sufficiently supercooled. Then with the valve open above the sample chamber, the enclosed air is pumped out of the apparatus for several minutes. The valve above the substance is then closed, the sample is brought to the selected temperature, and during this, the resulting displacement of the columns is observed and compensated to the zero position with nitrogen, if necessary, until temperature constancy is reached. The sample chamber is then again supercooled. If residual pressure is observed in the supercooled condition, it is caused either by air contained in the sample which is released during the heating process which can be drawn off, or by the cooling temperature not being low enough. Liquid nitrogen must then be used as a coolant.

After the sample has been sufficiently degassed, the temperature dependency of the vapour pressure is determined at sufficiently small temperature intervals.

1.6.3. *Isoteniscope*

A complete description of this method can be found in reference 2. 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. In the case of solids, depending on the pressure and temperature range, the manometer liquids listed in 1.6.2 are used. For liquids, the sphere of the isoteniscope is filled with the substance to be investigated which is degassed at elevated temperature during boiling.

Simultaneously, a part of the liquid is distilled out of the sphere and is condensed in the upper cooled sphere and returns to the U-tube. When this is filled sufficiently with degassed liquid, the lower sphere with the U-tube in a thermostated bath is brought up to the chosen temperature and the resulting vapour pressure is indirectly measured just as in the method described in 1.6.2.

In the case of solids the degassed manometer liquid is filled into the bulge on the long arm of the isoteniscope. Then the solid to be investigated is filled into the lower sphere 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. *Vapour pressure balance*

1.6.4.1. *Apparatus*

There are several different designs available in the literature (1). The one described here illustrates the principles involved. A number of parts are shown in figure 4. These are base plate and bell jar, a pump with a vacuum measuring device and equipment for measuring the vapour pressure by a pointer deflection. The following built-in equipment is mounted on the base plate:

- An evaporator furnace with flange and rotary feedthrough. The evaporator furnace is a flat, cylindrical copper vessel. (The furnace can also be made of glass surrounded by a copper wall.) It sits in a copper retainer which is screwed onto a piece of stainless steel at its lower protruding edge. The piece of stainless steel, in turn, is mounted on the base plate by means of a flange so that it can be rotated about the axis of the furnace. Heating is provided by a heater coil which sits inside the piece of stainless steel, and is thus closed off from the vacuum chamber.
- The furnace lid is made of copper, and has three evaporation openings of various cross-sections which are located at 90 degrees to one another. By rotating the furnace, the desired furnace opening or an intermediate position can be placed under the slot in the cooler which is positioned eccentrically to the furnace, thus aiming the molecular beam at the balance pan or diverting it. A thermocouple or resistance thermometer is mounted in the furnace wall for temperature measurement.
- The balance is a moving-coil instrument. The pointer is replaced by a small tube on which are mounted the balance beam and counterweight. The balance beam has a replaceable pan made of a thin piece of gold-plated aluminium. A 0,1 mm thick constantan wire, onto which calibration weights can be set, is attached to the approximate centre of the balance beam. The vapour pressure can be recorded using a photoelectric zero-point instrument.

- A cylindrical brass pot surrounds the balance pan on all sides with the exception of the two slots for the movement of the balance beam and a narrow opening for the entrance of the molecular beam. Heat dissipation to the outside is provided by a copper bar on top of the brass pot and is routed via a stainless steel tube through the base plate and thermally insulated from it. The bar is immersed in a Dewar flask containing liquid nitrogen under the base plate.

1.6.4.2. Measurement procedure

The copper furnace is filled with the substance, the lid closed, and the plate orifice, shield and cooler slid over the furnace. The bell is mounted, and the vacuum pumps are switched on. The final pressure before beginning measurement is roughly 10^{-4} Pa. From 10^{-2} Pa downwards, the cooling of the refrigeration box is started.

After a period of time, the balance will have attained a sufficiently low temperature so that the escaping vapour jet can condense on the scale pan. This condensation produces a signal on the connected recorder. This signal can be used in two ways: for the particular apparatus described here the vapour pressure is determined directly from the pressure on the scale pan (the molecular mass is not required). At the same time the mass condensed is determined and the evaporation rate can therefore be calculated from the time of deposition. This latter applies to more general apparatus. The vapour pressure may also then be calculated from the evaporation rate and molecular mass using the Herz relationship:

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

where:

G = evaporation rate (kg/s m²)

M = molar mass (g/mol)

T = temperature (K)

R = universal molar gas constant (J/mol K)

p = vapour pressure (Pa)

After the necessary vacuum is reached, the series of measurements commence at the lowest desired measuring temperature. The necessary orifice is opened and the vapour jet passes the shield directly mounted above the cover and then strikes the cooled scale pan. The scale pan is dimensioned so that the entire jet is collected in its cosinal distribution. The momentum of the vapour jet causes a force onto the scale pan where the condensation takes place on its cooled surface. By the force of the vapour jet the scale beam will be deflected from the equilibrium state. At the end of the scale beam is a small tab which is registered optically via a prism system and two photodiodes. A connected control circuit then momentarily regulates and resets the scale beam to the balanced state. The required torque is recorded and after a counterweighting it corresponds to the vapour pressure of the substance.

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. The two series will only be reproducible if the sample being measured is sufficiently pure. If the third run fails to confirm the results of the second run, then it is possible that the substance may be decomposing in the temperature range being measured.

1.6.5. Gas saturation method

1.6.5.1. Apparatus

A typical apparatus used to perform this test comprises a number of components given in figure 5 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. The gas employed must be dry (see figure 5, 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.

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 solution coated 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 at temperatures above 20 °C, the region between the saturator column and the traps should be heated to prevent condensation of the test substance.

1.6.5.2 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 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 because of increased dead space and 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 (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. 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.

1.6.5.3. 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 adsorbed test substance (g)

V = volume of saturated gas (m^3)

R = universal molar gas constant (J/mol K)

T = temperature (K)

M = molar mass (g/mol)

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).

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 following information shall if possible be included in the report:

Precise specification of the substance (identity and impurities):

- At least two vapour pressure and temperature values, preferably in the range 0 to 50 °C. It should also include all of the raw data and a $\log p$ versus $1/T$ curve. In addition, an estimate of the vapour pressure at 20 or 25 °C should be given.

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.

The method used shall be stated.

4. REFERENCES

- (1) OECD, Paris, 1981, *Test Guideline 104*, Decision of the Council C(81) 30 final.
- (2) OECD, Paris, 1981, *Test Guideline 104*, reference 4, Decision of the Council C(81) 30 final.

Appendix

Figure 1

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

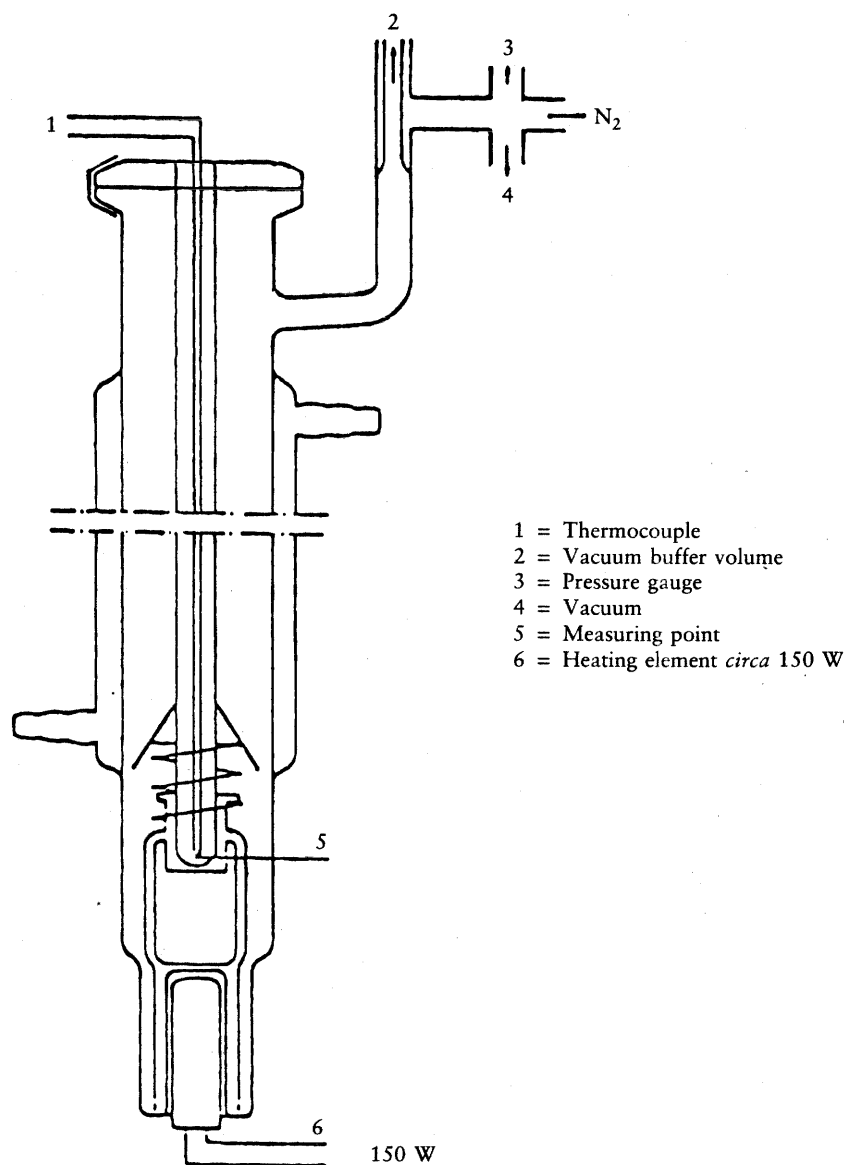


Figure 2

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

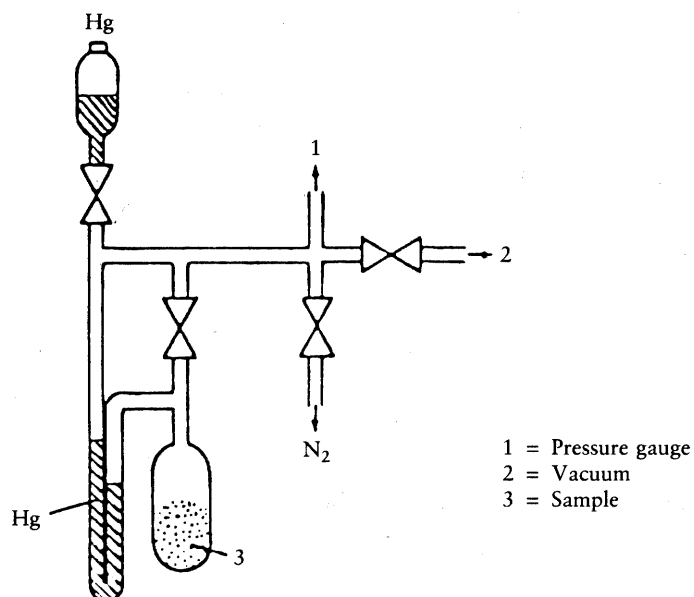


Figure 3

Isoteniscope (see reference 2)

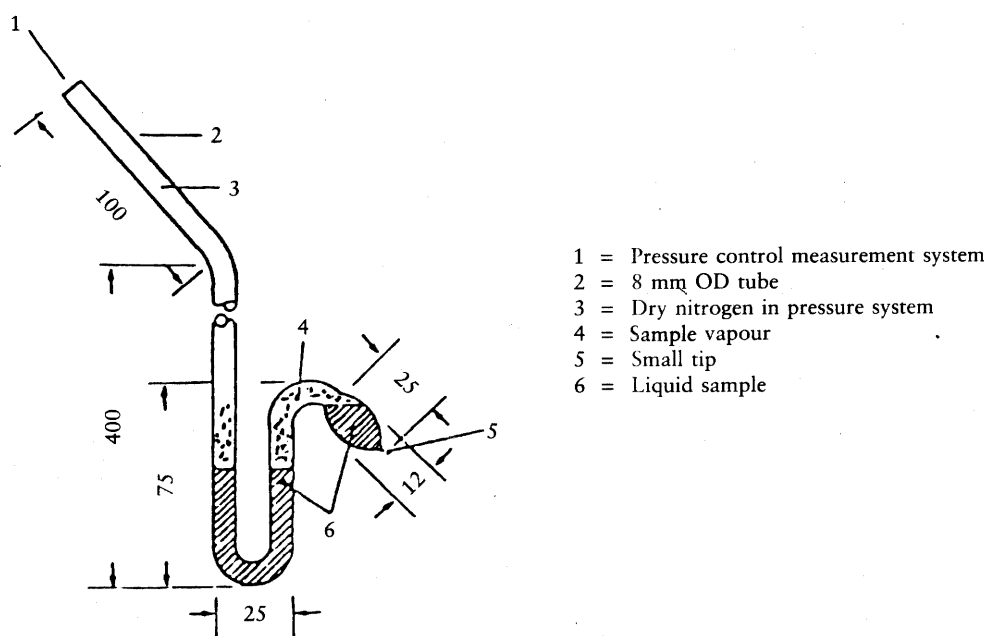
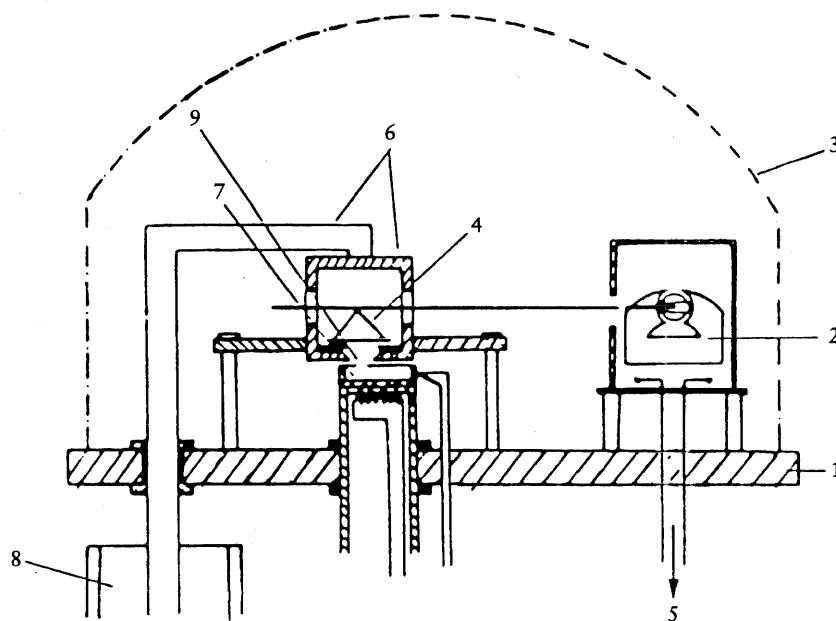


Figure 4

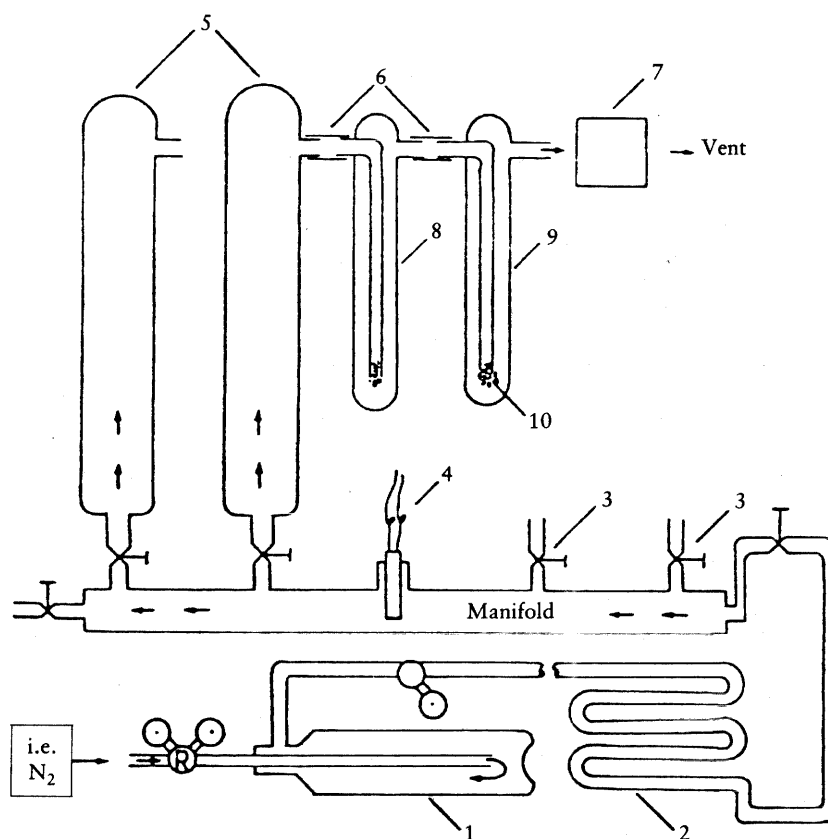
Apparatus for determining the vapour pressure curve according to the vapour pressure balance method



- 1 = Base plate
- 2 = Moving coil instrument
- 3 = Bell jar
- 4 = Balance with scalepan
- 5 = Vacuum measuring device
- 6 = Refrigeration box and cooling bar
- 7 = Evaporator furnace
- 8 = Dewar flask with liquid nitrogen
- 9 = Shield

Figure 5

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

A. 5. SURFACE TENSION

1. METHOD

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

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 dyn/cm

1 mN/m = dyn/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. This shall primarily serve to calibrate the method from time to time and to offer a chance to compare results when another method is applied.

Reference substances which cover a wide range of surface tensions are given in references 1 and 2.

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.

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

1.6.1. Plate method

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

1.6.2. *Stirrup method*

See ISO 304 (Surface active agents — determination of surface tension by drawing up liquid films).

1.6.3. *Ring method*

See ISO 304 (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 1) is located above the sample table. The error of the force measurement shall not exceed $\pm 10^{-6}$ N, corresponding to an error limit of $\pm 0,1$ 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.

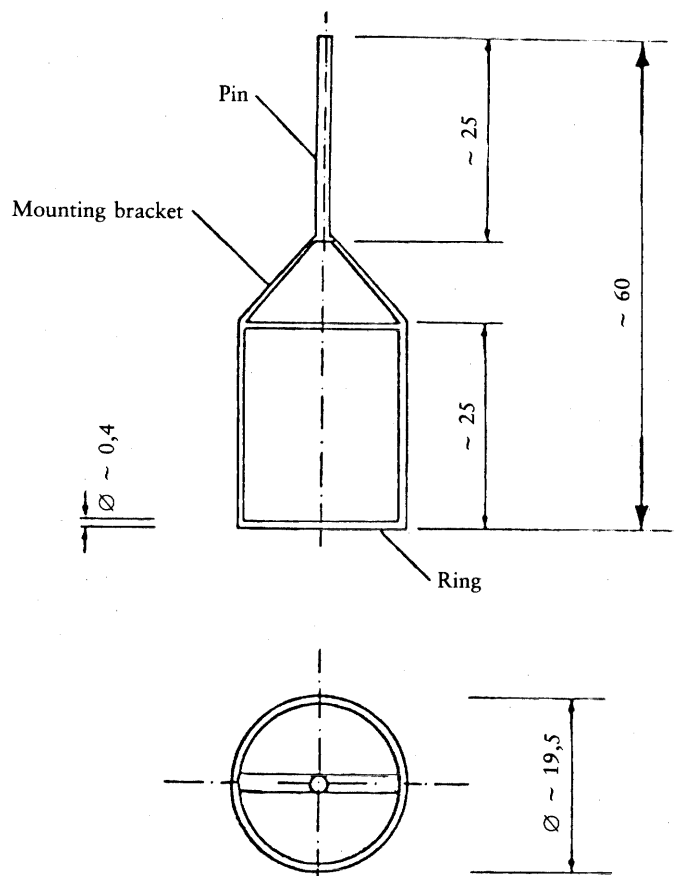
1.6.4.1.3. *Measuring body (ring)*

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

Mounting:

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

Zero point adjustment:

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

Calibrations:

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

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

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

where:

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

m = mass of the rider (g)

g = gravity acceleration (981 cm s⁻² at sea level)

b = mean circumference of the ring (cm)

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

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

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

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

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 ($\pm 0,5$ °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 $\pm 0,5$ °C.

1.6.6. *Performance of test*

The solutions to be measured shall be fixed into the carefully cleaned measuring vessel, taking care to avoid foaming, and subsequently the measuring vessel shall be placed onto the table of the test apparatus. The table-top with measuring 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 in 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 (3) 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 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, $\rho \approx 1 \text{ g/cm}^3$ $R = 9,55 \text{ mm}$ (average ring radius) $r = 0,185 \text{ mm}$ (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 and is similar to that in the DIN Standard (DIN 53914) for water and aqueous solutions (density $\rho = 1 \text{ g/cm}^3$) and is for a commercially available ring having the dimensions $R = 9,55 \text{ mm}$ (mean ring radius) and $r = 0,185 \text{ mm}$ (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

The test report shall if possible contain at least the following information:

- method used: ISO or OECD harmonized ring tensiometer method,
- 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.

4. REFERENCES

- (1) OECD, Paris, 1981, *Test Guideline 115*, Decision of the Council C(81) 30 final.
 - (2) *Pure and applied chemistry*, vol. 48, 1976, p. 511.
 - (3) Harkins, W. D., Jordan, H. F., *J. Amer. Chem. Soc.*, vol. 52, 1930, p. 1751.
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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 method is not applicable to volatile substances.

The two test methods described below cover the whole range of solubilities:

- 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 tested 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 offer the possibility to compare results when another method is applied.

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, silica gel or sand and 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 (2). 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 % is 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 at least 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 $20 \pm 0,5$ °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 $\pm 0,1$ °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
Approximate 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 contents of the measuring cylinder are transferred to a 100 ml measuring cylinder which is then filled up with water to 100 ml and shaken. At lower solubilities the time required to dissolve a substance can be considerably longer (24 hours 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, 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 silica. A suitable volatile solvent of analytical reagent quality should be used to apply the test substance to the support material. Double distilled water from glass or quartz apparatus should be employed as the eluent or solvent.

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 the test substance 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.

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 plus 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/hr. The pump is connected with polytetrafluoroethylene 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 (care must be taken that no contamination and/or adsorption occurs with the tube material) or a membrane pump.

Measurement procedure

The flow through the column is started. It is recommended that a flow rate of approximately 25 ml/hr be used (approximately 10 bed volumes/hr for the described column). 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 $\pm 30\%$ 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

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

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 compound 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 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, voltametric 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.

2.2. Flask method

The individual results should be given for each of the three flasks and those results deemed to be constant (repeatability 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 report shall if possible contain an indication of the results of the preliminary test plus the following information:

- 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.

3.2. Flask method

The report shall if possible include the following information:

- 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.

4. REFERENCES

- (1) OECD, Paris, 1981, *Test Guideline 105*, Decision of the Council C(81) 30 final.
- (2) OECD, Paris, 1981, *Test Guideline 116*, Decision of the Council C(81) 30 final.

Appendix

Figure 1

Schematic test arrangement

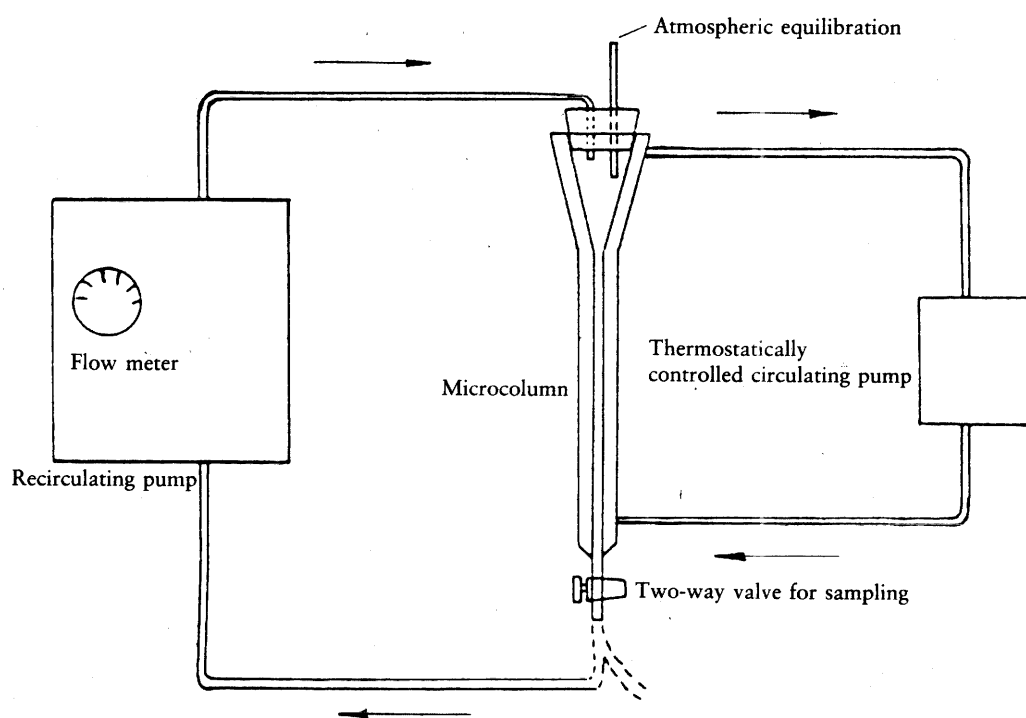


Figure 2

A typical microcolumn

(All dimensions in millimetres)

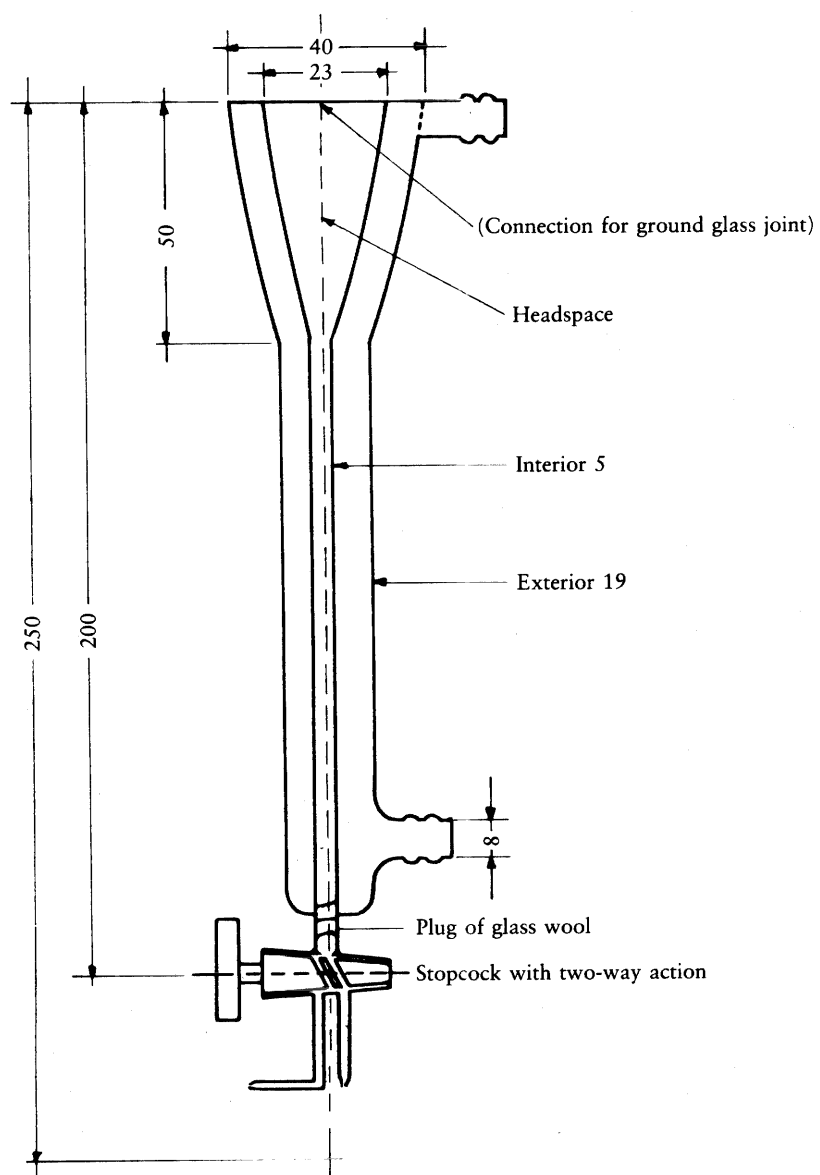


Figure 3

A typical microcolumn

(All dimensions in millimetres)

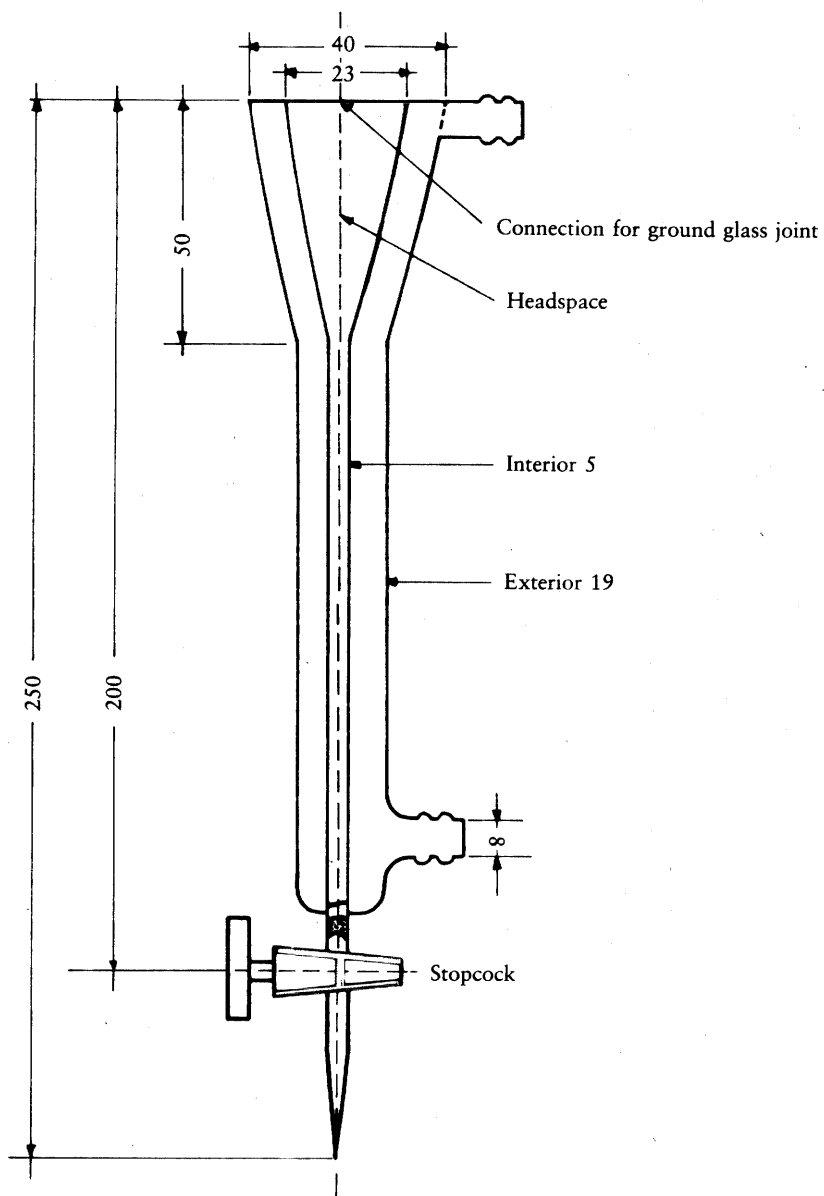
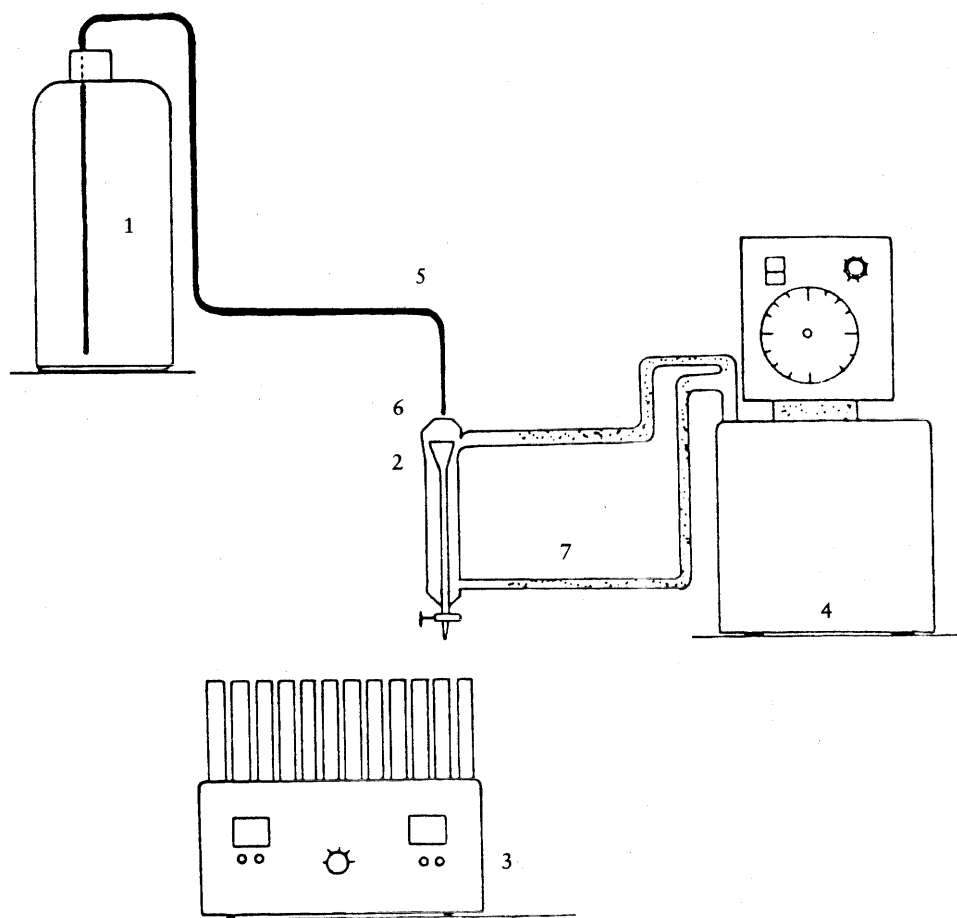


Figure 4

Test arrangement for the determination of solubility in water of slightly soluble, low volatile substances



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

A. 7. FAT SOLUBILITY

1. METHOD

The method described is based on the OECD Test Guideline (1)

1.1. Introduction

It is useful to have preliminary information on the partition coefficient, water solubility, structural formula and stability at 50 °C of the substance to perform this test. This method is applicable only to those substances which are essentially pure, which are stable at 50 °C and which are not appreciably volatile under the same conditions.

The method is not suitable for test substances which are reactive with triglycerides.

1.2. Definitions and units

The mass fraction of a substance which forms a homogeneous phase with a liquid fat (oil) without giving rise to chemical reactions is defined as fat solubility. The maximum of such mass fraction is called the saturation mass fraction, and this is a function of temperature.

The saturation mass fraction of a substance should be given in milligrams per 100 g of standard fat at $37 \pm 0,5$ °C.

The following relationship exists between the solubility in grams per 100 g of solution (S') and the solubility in grams per 100 g of solvent (S):

$$S = \frac{100 \times S'}{100 - S'} \text{ grams per 100 g of standard fat}$$

Multiplication of the S value with 1 000 gives milligrams per 100 g of standard fat.

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 offer the possibility to compare results when another method is applied.

1.4. Principle of the method

The substance is added to a liquid 'standard fat' and stirred. A sufficient amount of substance is added to ensure an excess. The dissolved amount of test substance should be determined by a suitable analytical method.

1.5. Quality criteria

1.5.1. Specificity

The repeatability of the measurement is unknown at present.

Results should apply to standard fats and are appropriate for relatively pure substances. Even at 37 °C, fats may form emulsions or fine suspension of solid substances. Since these will interfere with subsequent determination of mass fraction, the formation of these must be avoided.

1.6. Description of the method

1.6.1. Preparation

1.6.1.1. Apparatus

The following items of equipment are required:

- normal laboratory glassware,
- balance,
- centrifuge with thermostat,
- a stirrer which can be used in combination with a temperature control system,
- thermostat.

1.6.1.2. Standard fats

The use of standard fats is necessary. These standard fats should be easily definable and an example of such a standard fat is given in the Appendix.

1.6.1.3. Preliminary test

A simplified preliminary test should be run to determine the approximate amount of substance necessary for the establishment of the saturation mass fraction at the test temperature (37 °C).

Note

The rate of establishment of the saturation equilibrium may be greatly dependent upon the particle size in the case of solid substances. For this reason, such materials should be pulverized.

1.6.1.4. Preparation of the substance

Weigh eight samples into 50 ml flasks. Normally each should be of twice the quantity necessary for saturation as determined in the preliminary test.

After adding a weighed amount of approximately 25 g of liquefied and mixed standard fat, the flasks, fitted with the stirrers, are tightly closed with ground glass stoppers. One half (group I) is stirred at 30 °C, and the other half (group II) at approximately 50 °C, each for at least one hour.

1.6.2. Test conditions

The determination of fat solubility is carried out at $37 \pm 0,5$ °C.

1.6.3. Measurement procedure

Stir the contents of the flasks in both groups at $37 \pm 0,5$ °C, until thoroughly mixed.

The stirring time required to establish equilibrium cannot be predicted in general. In the case of liquid substances, saturation may be reached within minutes; in the case of solid substances it may take hours. In general no more than three hours of stirring will be needed; after this time, stirring should be stopped for two of the flasks in both groups and these two flasks allowed to stand for at least one hour at 37 °C in order to separate the undissolved amount of substance and to allow the formation of homogeneous phase. In the event of emulsion or suspension formation (e.g. Tyndall effect), this must be eliminated by a suitable method such as thermostated centrifugation.

The third and fourth flask in both groups should be stirred for at least 24 hours before standing for one hour at $37 \pm 0,5$ °C.

Note

If no bottom sediment (for solid substances) has formed or no phase separation (for liquid substances) has occurred after this period, the test must be repeated with a greater amount of substance.

1.6.4. *Analysis*

One sample is taken from each saturated fat phase for analysis. This sample is weighed, and the mass fraction is determined.

Any suitable analytical method may be applied either directly or after extraction with water or an organic solvent or any other separation technique.

Examples of such methods are:

- spectrophotometry
- gas or liquid chromatography
- voltammetry

2. **DATA**

If there are significant differences in results from either under- or over-saturation or short and long time periods, the test should be repeated with longer stirring times.

3. **REPORTING**

The following information shall if possible be included in the test report:

- precise specification of the substance (identity and impurities),
- precise specification of the fat (e.g. description, characteristics, origin, composition),
- method of analysis, deviations and special features.

The results must be evaluated as described above and they are part of the test report. If there were no significant differences between the various observed values in milligrams per 100 g, the individual values, the mean value and the standard deviation should be reported. If there are significant differences, even after retesting, then only the individual results should be reported.

All information and remarks relevant for the interpretation of results have to be reported.

4. **REFERENCE**

- (1) OECD, Paris 1981, *Test Guideline 116*, Decision of the Council C(81) 30 final.
-

Appendix

EXAMPLE FOR STANDARD FATS

The following table shows the composition of a typical standard fat.

Fatty acid distribution

Number of C-atoms in the fatty acid moiety	6	8	10	12	14	16	18	others
GLC areas (%)	0,5	7,5	10,3	50,4	13,9	7,6	8,6	1

Glyceride distribution

Total number of C-atoms in the fatty acid moieties	22	24	26	28	30	32	34	36	38	40	42	44	46	48	50
GLC areas (%)	0,1	0,3	1,0	2,3	4,9	10,9	13,9	21,1	16,1	11,7	9,8	4,4	2,2	1,1	0,2

Purity

Monoglyceride content (enzymatic)	≤ 0,1 %
Diglyceride content (enzymatic)	≤ 0,4 %
Unaponifiable content	≤ 0,1 %
Wijs number	≤ 0,5
Acid number	0,02
Water content (K. Fischer)	≤ 0,1 %
Clear melting point	28,5 °C

Typical absorption spectrum (layer thickness d = 1 cm, comparison: water, 35 °C)

Wavelength (nm)	290	310	330	350	370	390	430	470	510
Transmission (%)	2	15	37	64	80	88	95	97	98

At least 10 % light transmission at 303 nm.

This fat simulant is a synthetic mixture of saturated triglycerides with a fatty acid and triglyceride distribution similar to that of a coconut fat.

A. 8. PARTITION COEFFICIENT

1. METHOD

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

1.1. Introduction

It is useful to have preliminary information on dissociation constant, water solubility, and surface tension of the substance to perform this test.

This method applies only to essentially pure substance soluble in water and octanol. It is not applicable to surface active material.

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:

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

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

1.3. Reference substances

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 offer the possibility to compare results when another method is applied.

1.4. Principle of the 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 there are many different techniques which 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.

1.5. Quality criteria

1.5.1. Repeatability

In order to assure the precision 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 $\pm 0,3 \log$ units.

1.5.2. Sensitivity

The measuring range of the method is determined by the limit of detection of the analytical procedure. This should be sufficient to permit the assessment of values of P_{ow} up to 10^5 when the concentration of the solute in either phase is not more than 0,01 mol per litre.

1.5.3. *Specificity*

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 correction. (The use of buffer solutions in place of water should be considered for such compounds.)

1.6. *Description of the method*

1.6.1. *Preliminary estimate of the partition coefficient*

The value of the partition coefficient can be estimated either by means of a simple calculation (2) or by use of the solubilities of the test substance in the pure solvents (1):

For this:

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

Alternatively, it may be roughly determined by performing a simplified preliminary test.

1.6.2. *Preparation*

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

Water: Distilled or double distilled water from glass or quartz apparatus should be employed.

Note

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

1.6.2.1. *Presaturation 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. For doing 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.

1.6.2.2. *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 is added; in the second, twice the volume of n-octanol is added; and in the third, half the volume of n-octanol is added.

1.6.2.3. Test substance

For a material balance during the test, a stock solution is prepared in n-octanol with a mass concentration between 1 and 100 mg/ml. The actual mass 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 stable conditions.

1.6.3. Test Conditions

The test temperature should be kept constant ($\pm 1^\circ\text{C}$) and lie in the range of 20 to 25 $^\circ\text{C}$.

1.6.4. Measurement procedure

1.6.4.1. 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 octanol parts should be measured by volume. The test vessels should either be placed in a suitable shaker or shaken by hand. A recommended method is to rotate the centrifuge tube quickly through 180° about its transverse axis so that any trapped air rises through the two phases.

1.6.4.2. Phase separation

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 re-equilibrated at the test temperature for at least one hour before analysis.

1.6.5. 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 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 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 physico-chemical determinations which may be appropriate are:

- photometric methods,
- gas chromatography,
- high-pressure liquid chromatography.

2. DATA

If the measured P_{ow} is greater than 10^4 , it is recommended that the results be compared with a calculated P_{ow} value, as for example one obtained by the method given in reference 3.

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 following information shall if possible be included in the report:

- Precise specification of the substance (identity and impurities).
- Temperature of the determination.
- Data on the analytical procedures used in determining concentrations.
- 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 aqueous phase during the experiment.
- All information and remarks relevant for the interpretation of results.

4. REFERENCES

- (1) OECD, Paris, 1981, *Test Guideline 107*, Decision of the Council C(81) 30 final.
 - (2) OECD, Paris, 1981, *Test Guideline 107*, reference 2, Decision of the Council C(81) 30 final
 - (3) OECD, Paris, 1981, *Test Guideline 107*, reference 10, Decision of the Council C(81) 30 final.
-

A. 9. FLASH POINT

1. METHOD

1.1. Introduction

It is useful to have preliminary information on flammability of the substance to perform this test. The test procedure is applicable to liquid substances, as marketed, whose vapours can be ignited by ignition sources. The test methods described in this text are only reliable for flashpoint ranges which are specified in the individual methods.

1.2. Definitions and units

The flashpoint is the lowest temperature, corrected at a pressure of 101,325 kPa, at which the test liquid in a closed test vessel evolves vapours, under the conditions defined in the test method, in such 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 used in all cases when investigating a new substance. They should primarily serve to calibrate the method from time to time and to offer a chance to compare results when another method is applied.

1.4. Principle of the method

The substance is placed in a test vessel which is progressively heated until the vapour reaches a sufficiently high concentration in air to produce a flammable mixture which can be ignited.

1.5. Quality criteria

1.5.1. Repeatability

The repeatability varies according to flashpoint 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 flashpoint 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.

1.6.2. *Test conditions*

The apparatus is preferably 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 temperature from 5 to 65 °C), DIN 51755 part 2 (for temperature below 5 °C), NF M07-036.

Tag apparatus:

See ASTM D 56, ISO 2719.

Pensky-Martens apparatus:

See ISO 2719, (EN 11), DIN 51758, ASTM 8013, ASTM D 93, BS 2000-34, NF M07-019.

Remarks

When the flashpoint, determined by a non-equilibrium method in 1.6.3.2, is found to be: 0 ± 2 °C, 21 ± 2 °C, 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 flashpoint may be used for a notification.

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

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

2. **DATA**

3. **REPORTING**

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

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 powder, granular and pasty 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. Moreover, metal powders which can incandesce should also be considered to be highly flammable, if incandescence propagates throughout the sample. Such incandescence, and the associated difficulties in extinguishing a fire, are the main reason why metal powders are especially dangerous. Normal extinguishing agents such as carbon dioxide and/or water can increase the hazard considerably.

1.2. Definition and units

Burning time expressed in seconds.

1.3. Reference compounds

Not specified.

1.4. Principle of the method

The substance in its commercial form is made into a pile of 250 mm length. An attempt is then made to ignite the sample under the conditions defined in 1.6.3, and the burning time is measured.

1.5. Quality criteria

1.6. Description of method

1.6.1. Preparation

In the case of powder and granular substances the product in its commercial form is loosely filled into 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 a longitudinal direction two metal sheets 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. Then the lateral limitations are removed and the excess substance scraped off. A non-combustible and non-porous plate is placed on top of the mould, the apparatus inverted and the mould removed.

Pasty substances are spread on a non-combustible surface in the form of a rope 250 mm length with a cross section of about 1 cm².

Any suitable ignition source such as a small flame or a hot wire of minimum temperature of 1 000 °C is used to ignite the pile at one end.

1.6.2. *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.

1.6.3. *Performance of the test*

Ignite one end of the pile. When the pile has burned a distance of 80 mm, measure the rate of burning over the next 100 mm. The test is performed six times, using a clean cool plate each time.

2. **DATA**

The values of burning time determined within six tests are necessary for the evaluation.

3. **REPORTING**

3.1. **Test report**

The 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.
- The measurement results.
- All additional remarks relevant to the interpretation of results.

3.2. **Interpretation of the result**

Powders, granular or pasty substances are to be considered as highly flammable when the time of burning in one of the six tests carried out according to the test procedure described in 1.6 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.

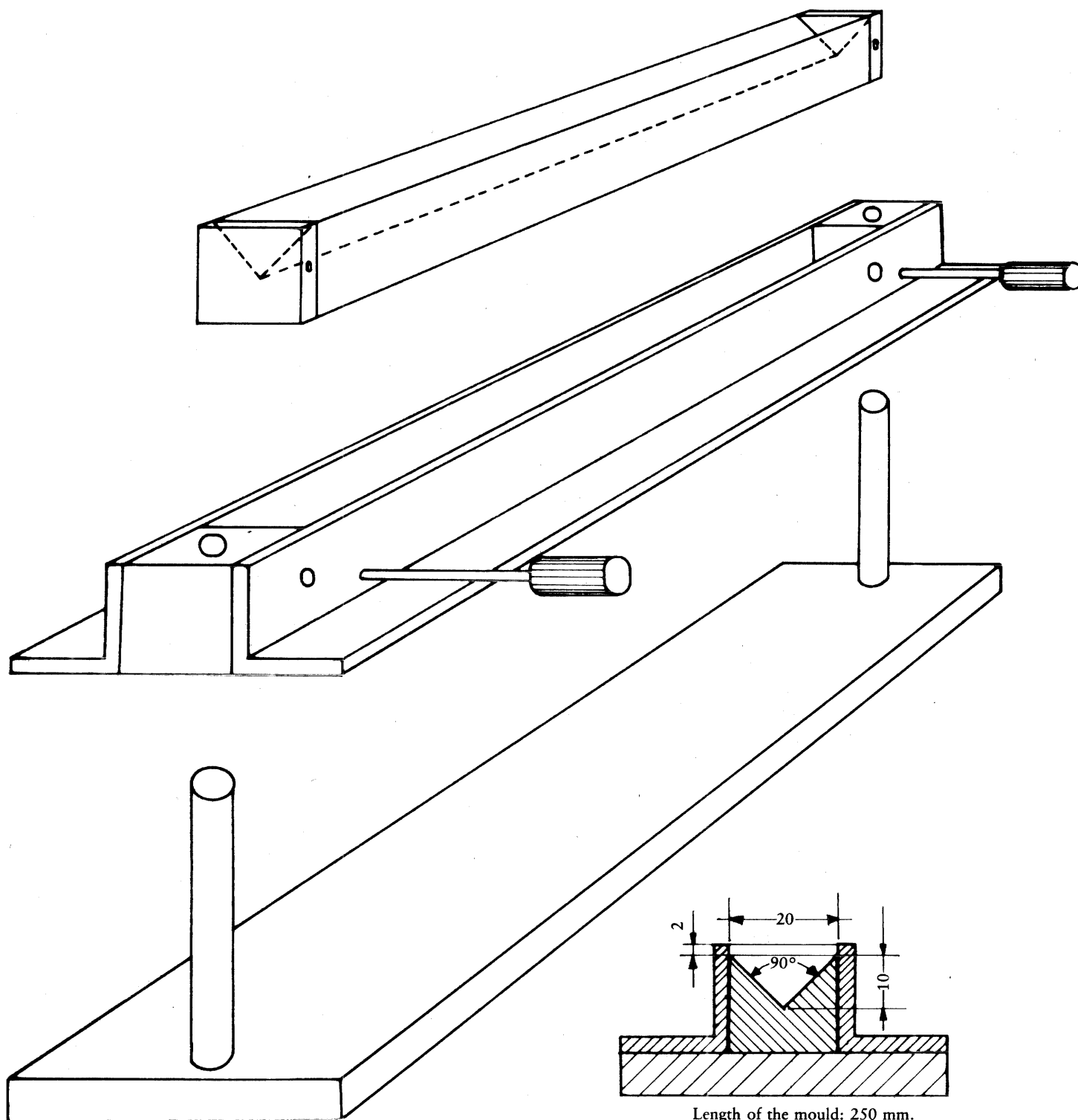
4. **REFERENCES**

None

Appendix

Figure

Mould and accessories for the preparation of the pile
(All dimensions in millimetres)



Length of the mould: 250 mm.
Material: aluminium.

A. 11. FLAMMABILITY (GASES)

1. METHOD

1.1. Introduction

This method allows a determination of whether gases mixed with air at room temperature and atmospheric pressure have a range of flammability. 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 substance

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.

1.6.2. Test conditions

The test must be performed at room temperature.

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 a flame detaches itself from the ignition source and propagates independently. The concentration of gas is varied in steps of 1 % vol. until ignition occurs as described above.

2. DATA

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

3. REPORTING

The 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 room temperature at which the test is performed.
- The tested concentrations and the results obtained.
- The result of the test: non-flammable gas or highly flammable gas.
- In the case of a 'non-flammable' conclusion, it should be stated that all concentrations were tested by raising the concentrations by 1 % steps, from 0 to 100 %.
- All information and remarks relevant to the interpretation of results have to be reported.

4. REFERENCES

None.

A. 12. FLAMMABILITY (SUBSTANCES AND PREPARATIONS WHICH, IN CONTACT WITH WATER OR DAMP AIR, EVOLVE HIGHLY FLAMMABLE GASES IN DANGEROUS QUANTITIES)

1. METHOD

1.1. Introduction

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

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 and preparations 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. This limit does not take the toxicity of a gas into account.

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.

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 substance

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 substance is used as marketed and 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 substance is used as marketed and 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 substance is used as marketed and the test is performed at room temperature.

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 figure (see appendix).

1.6.4.2. Test conditions

Inspect the container of the test substance for any powder $< 500 \mu\text{m}$ (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 used in the commercial state. The test should be performed at room temperature (20 °C) and atmospheric pressure.

1.6.4.3. Performance of the test

Water is put into the dropping funnel of the apparatus and enough of the substance, up to a maximum weight of 25 g, to produce between 100 and 250 cm³ of gas, is weighed out and placed 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 time taken for all the gas to be evolved is noted and, where possible, intermediate readings should be taken. This test should be performed in triplicate.

If the chemical identity of the gas is unknown, the gas has to 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 test method (A. 11).

2. DATA

One ignition or evolution of highly flammable gas at the rate greater than 1 litre/kg per hour in three attempts is sufficient to consider a test substance hazardous (1.6.1, 1.6.2 and 1.6.3).

3. REPORTING

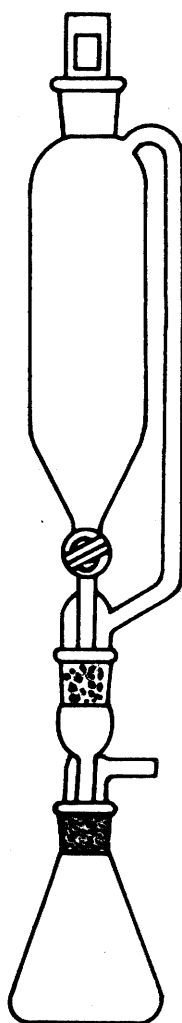
The report shall if possible include the following information:

- The precise specification and description of the substance as received (e.g. colour, particle size and physical state).
- Any initial preparation of the test substance.
- The results of the tests.
- The chemical identity of gas evolved.
- The rate of evolution of gas (1.6.4).
- Any additional remarks relevant to the interpretation of the results.

4.

REFERENCES

- (1) ISO 1773
- (2) OECD, Paris, *Preliminary test guideline for the determination of substances which give off highly inflammable gases in dangerous amounts on contact with water*, A 80/28, final report of the OECD chemical testing programme.
- (3) UN Doc. No ST/SG/AC10/1 rev. 1.

*Appendix**Figure***Apparatus**

A. 13. FLAMMABILITY (SOLIDS AND LIQUIDS)

1. METHOD

1.1. Introduction

It is useful to have preliminary information on the auto-flammability of a substance. The test procedure is applicable to solid and liquid substances as marketed, which will ignite spontaneously in small amounts, a short time after coming into contact with air, at room temperature.

Substances not covered by this test method are those which need hours or days at room temperature before self-ignition occurs, or those which need to be exposed to considerably higher temperature before self-ignition occurs.

1.2. Definitions and units

Liquids and solids are considered to be highly flammable if they ignite at least once out of six tests under the conditions described in 1.6.

The auto-flammability of liquids may also need to be tested following the method A. 15: Auto-flammability: determination of the temperature of self-ignition of volatile liquids and gases.

1.3. Reference substances

Not specified.

1.4. Principle of the method

The substance is brought into contact with air at a temperature of 25 ± 10 °C for a period of five minutes. If ignition occurs the substance is considered to be highly flammable.

1.5. Quality criteria

Repeatability: because of the importance in relation to safety only one positive result out of six tests is sufficient to deem the substance to be highly flammable.

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.

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.

1.6.2. Performance of the test

(a) Powdery solids

1 to 2 cm³ 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.

(b) Liquids

Circa 5 cm³ of the liquid to be tested is poured into the prepared porcelain cup and it is observed whether the substance ignites within five minutes.

2. DATA

The results of six tests are relevant for the evaluation.

3. REPORTING

The report shall if possible include the following information

- A description of the substance to be tested.
- The results of the test.

4. REFERENCES

- (1) OECD, Paris, *Preliminary test guideline for the determination of pyrophoric behaviour of solids and liquids*, A 80/23, final report of the OECD chemical testing programme.
-

A. 14. EXPLOSIVE PROPERTIES

1. METHOD

1.1. Introduction

The method provides a scheme of testing to determine whether or not a solid, a liquid or a pasty substance or preparation presents a danger of explosion when submitted to the effect of a flame (thermal sensitivity) or to impact or friction (sensitivity to mechanical stimuli).

The method comprises three parts:

- (a) a test of thermal sensitivity;
- (b) a test of mechanical sensitivity with respect to shock;
- (c) a test of mechanical sensitivity with respect to friction.

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 or not a substance or preparation is capable of exploding under any conditions; nor is it intended to determine the extent to which initial decomposition may be propagated to cause explosion of the whole sample.

The method is appropriate for determining whether a substance or preparation will present a danger of explosion (thermal and mechanical sensitivity) in the particular conditions specified in the directive. The tests are irrelevant when available thermodynamic information (heat of formation, heat of decomposition, absence of certain reactive groups (1) in the structural formula) establishes beyond reasonable doubt that the substance or preparation is incapable of decomposing, forming gases and releasing heat very rapidly (i.e. the material does not present any risk of explosion). It is recognized that the method is not definitive. It comprises a number of chosen types of specified apparatus which are widely used internationally and which usually give meaningful results.

The person conducting the tests may elect to use alternative apparatus in the three methods specified, providing it can be justified scientifically and the apparatus is recognized internationally. In this case he must determine the correlation of his results with those obtained with the specified apparatus.

1.2. Definitions and units

Explosive:

Substances and preparations which may explode under the effect of flame or which are more sensitive to shocks or friction than dinitrobenzene.

1.3. Reference substance

Meta-dinitrobenzene, technical crystalline product, for friction and shock method.

1.4. Principle of the method

A preliminary screening test is necessary to establish safe conditions for the performance of the three tests of sensitivity:

1.4.1. *Preliminary screening test*

Very small samples (*circa* 10 mg) of the substance or preparation are subjected to heating without confinement in a Bunsen flame, to impact 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 should be performed with special precautions, to avoid injury to the experimenter.

1.4.2. *Thermal sensitivity*

The method involves heating the substance or preparation in a steel tube, with various degrees of confinement being provided by nozzle-plates with different diameters of orifice, to determine whether the substance or preparation is liable to explode under conditions of thermal stress.

1.4.3. *Mechanical sensitivity (shock)*

The method involves subjecting the substance or preparation to the shock of a falling hammer on a steel anvil.

1.4.4. *Mechanical sensitivity (friction)*

The method involves subjecting the substance or preparation 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. *Apparatus*

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

The steel tube is made from deep drawn sheet (see Appendix) by a drawing process. It has an internal diameter of 24 mm, a length of 75 mm and wall thickness of 0,5 mm. At the open end, the tube is provided with a flange for closing the tube (figure 1). The tube is provided with a pressure-resistant, circular nozzle plate with a central hole and this plate is firmly secured to the tube with the aid of a two-part screw joint (nut and cap-nut). The nozzle plate (see figure 1) is 6 mm thick and made from heat-resistant chromium steel (see Appendix).

A range of nozzle-plates of various diameters of opening (1, 1,5, 2, 2,5, 3, 4, 5, 6, 8, 10, 12, 14, 16, 18, 20 ... mm) are available to the experimenter to determine the degree of danger of explosion presented by the substance or preparation. The nut and cap-nut (figure 1) consist of chromium-manganese steel (see Appendix) which is spark free up to 800 °C. The steel tubes are used only for one experiment.

1.6.1.2. *Mechanical sensitivity (shock)*

A typical falling-mass apparatus consists essentially of a cast steel block (grey casting) with foot and anvil, column, guides, drop weight and a release mechanism. The steel block 230 mm (depth) × 250 mm (width) × 200 mm (height) with a cast foot 450 mm (depth) × 450 mm (width) × 60 mm (height) carries the steel anvil 100 mm (diameter) × 70 mm (height) which is screwed on. At the back of the steel block, the holder is screwed on and in this the column, which consists of the seamless, drawn steel tube 90 mm (outer diameter) and 70 mm (inner diameter), is fastened. Four screws anchored in a solid concrete block 60 × 60 × 60 cm hold the drop hammer in such a manner that the rails are absolutely

vertical and the drop weight is easily guided. The mass of the drop hammer weight has to be 10 kilograms. The weight consists of solid steel. It must have an impact surface of tempered steel, HRC 60 to 63, and a minimum diameter of 25 mm. The tests are to be conducted with a drop height of 0,4 m.

The sample to be investigated is placed in a die device consisting of two solid coaxial steel cylinders, one above the other, and a hollow steel cylinder as the guiding ring. The solid steel cylinders must 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. If explosion occurs the steel cylinders and the hollow cylinder are not to be used for further tests. The die device is erected on an intermediate anvil 26 mm (diameter) $\times$ 26 mm (height) made of steel and centred by a centering ring with a locating ring to take away the fumes of the explosion.

1.6.1.3. Mechanical sensitivity (friction)

The friction apparatus consists of a cast steel base plate (grey cast) on which is mounted the friction device itself consisting of a fixed porcelain peg and moveable porcelain plates. The porcelain plate is fixed in a sliding cradle, moving between two rails. The cradle is driven via a driving rod, an eccentric pulley and transmission gear by an electric motor, in such a manner that the porcelain plate is moved under the porcelain rod with a backwards and forwards motion of 10 mm. The porcelain peg is to be loaded with about 360 newtons.

The porcelain plates are made from white technical porcelain and have the dimensions 25 mm (length) $\times$ 25 mm (width) $\times$ 5 mm (height). Both the friction surfaces of the plates are coarsened (rough depth 9 μ m to 32 μ m) before firing, by rubbing with a sponge.

The cylindrical porcelain peg is also made of white technical porcelain. It is 15 mm long, has a diameter of 10 mm and roughened spherical end surfaces with a radius of curvature of 10 mm.

1.6.2. Test conditions

1.6.2.1. Thermal sensitivity (effect of a flame)

The substance, in the physical form in which it is to be supplied, is filled into the tube to a height of 60 mm in three equal increments. Each increment is gently consolidated by applying a force of 80 newtons to the surface by means of a suitable wooden piston slightly smaller than the tube. In the case of a gelatinous substance, care is taken to avoid air bubbles during filling.

1.6.2.2. Mechanical sensitivity (shock)

The substance is tested in the dry state. The sample must have a volume of 40 mm³, or a volume to suit the specified apparatus. For solid substances, with the exception of pastes, the following applies:

- (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 disintegrated and sieved; the sieve fraction from 0,5 to 1 mm diameter is used for testing.

With liquid substances the upper steel cylinder is pressed down until it is at a distance of 1 mm from the lower cylinder and it is held in this position.

1.6.2.3. Mechanical sensitivity (friction)

The substance is tested in the dry state. The sample must have a volume of 10 mm³. For solid substances, with the exception of pastes, the following applies:

- (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 disintegrated and sieved; the sieve fraction < 0,5 diameter is used for testing.

1.6.3. *Performace of the tests*

1.6.3.1. Thermal sensitivity (effect of a flame)

Heating is provided by propane, taken from an industrial cylinder fitted with a pressure regulator (500 mbar), through a meter and distributed by a manifold to the four burners. The four burners consume 3,2 litres of propane per minute. If other heating gases are used, appropriate burners, gas consumption, inflow of air have to be chosen so that, for comparative measurements with inert substances (sand, dibutylphtalate), similar temperature/time curves as for the heating with propane in the filled tubes are registered.

Burners are located around the test chamber as shown in figure 2.

The burners are adjusted in such a way that the tip of the internal blue cone of the flame almost touches the tube. The test is to be carried out in a steel chamber with the dimension given in figure 2.

The dimensions of the burners for propane are given in figures 3a and 3b. Two series of three tests are mandatory, the first series using a nozzle-plate with a hole of 2 mm diameter, the second using a hole greater than 2 mm (e.g. 6 mm) diameter.

If an explosion occurs during the first series (2 mm hole) it is unnecessary to proceed to the next series. If an explosion does not occur after five minutes the test is terminated.

1.6.3.2. Mechanical sensitivity (shock)

In the specified impact apparatus, six tests are performed dropping the 10 kilogram mass from 0,4 m. In other apparatus the sample is compared with m-dinitrobenzene using the established procedure (up-and-down technique, etc.).

1.6.3.3. Mechanical sensitivity (friction)

The porcelain peg is brought onto the sample under test and the weight is hung on. 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. The porcelain plate has to be moved under the porcelain peg, to and fro, over a distance of 10 mm each way in a time of 0,44 seconds. Each part of the surface may be used only for one test.

2. DATA

2.1. Treatment of results

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

2.2. Evaluation

In principle, a substance or preparation is considered to present a danger of explosion in the sense of the directive if:

- (a) an explosion occurs (i.e. the tube bursts into three or more fragments) within the fixed number of tests of thermal sensitivity; or
- (b) an explosion (bursting into flame is equivalent to explosion) occurs at least once in six tests with the specified impact apparatus or the sample is more sensitive than m-dinitrobenzene in an alternative impact test; or
- (c) an explosion (crepitation or bursting into flame is equivalent to explosion) occurs at least once in six tests with the specified friction apparatus or the sample is more sensitive than m-dinitrobenzene in an alternative friction 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 or preparation tested,
- the physical form of the sample and whether or not it has been sieved,
- observations during the tests (type of reaction, sparks, flame, explosion, number of fragments, etc.),
- results of each 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.

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.

Sometimes the result may be an artefact which relates to the physical form or the volatile nature of the substance or preparation during the test; in such an instance it is useful to know what result one would obtain when the substance or preparation is in the form in which it is to be placed on the market. Alternative testing may be able to determine this information. Unless an anomalous result can be explained in this manner, it must be accepted at face value and used to classify the substance or preparation accordingly.

4.

REFERENCES

- (1) Bretherick, L., Handbook of Reactive Chemical Hazards, London, Butterworths, 1979, pp. 60 to 63.
- (2) Koenen, H., Ide, K. H., Über die Prüfung explosiver Stoffe, I. Ermittlung der Reibempfindlichkeit, Explosive Stoffe, vol. 3, 1955, pp. 57 to 65 and 89 to 93.
- (3) Koenen, H., Ide, K. H., Über die Prüfung explosiver Stoffe, III. Ermittlung der Empfindlichkeit explosiver Stoffe gegen thermische Beanspruchung in einer Erhitzungskammer mit verschiedenen definierten Öffnungen (Stahlhusenverfahren), Explosive Stoffe, vol. 4, 1956, pp 119 to 125, and 143 to 148.
- (4) Koenen, H., Ide, K. H., Haupt, W., Über die Prüfung explosiver Stoffe, IV. Ermittlung der Schlagempfindlichkeit explosiver Stoffe von fester, flüssiger und gelatinöser Beschaffenheit, Explosive Stoffe, vol. 6, 1958, pp. 178 to 189, 202 to 214 and 223 to 235.
- (5) ONU, 1980, December, United Nations Committee of Experts on the Transport of Dangerous Goods (Doc. ST/SG/AC/.10/5/Add. 3, table 4.3).

*Appendix***Example of Material specification**

- (1) Material specification No 1.0336.505 g, in accordance with DIN 1623, sheet 1.
 - (2) Material specification No 1.4873, in accordance with sheet 'Stahl-Eisen-Werkstoff' 490-52.
 - (3) Material specification No 1.3817, in accordance with sheet 'Stahl-Eisen-Werkstoff' 490-52.
-

Figure 1

(All dimensions in millimetres)

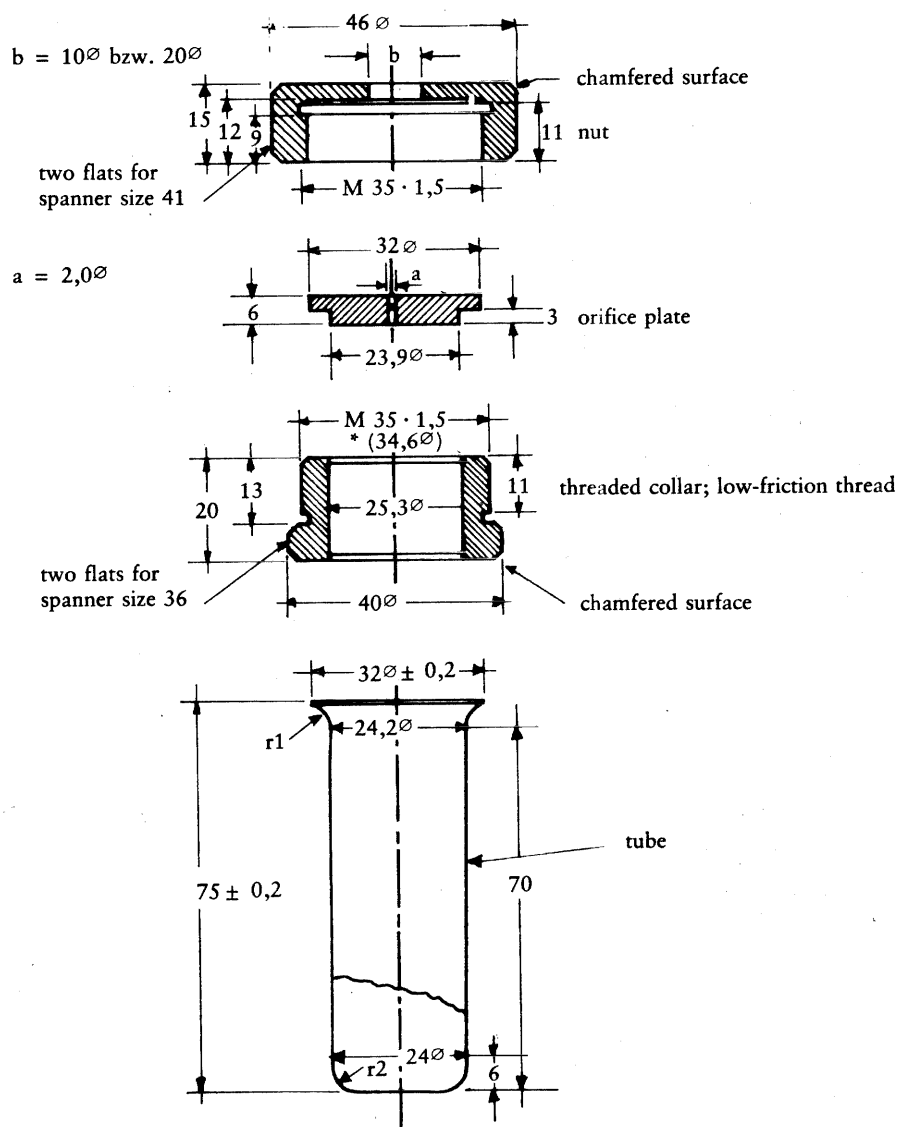


Figure 2

(All dimensions in millimetres)

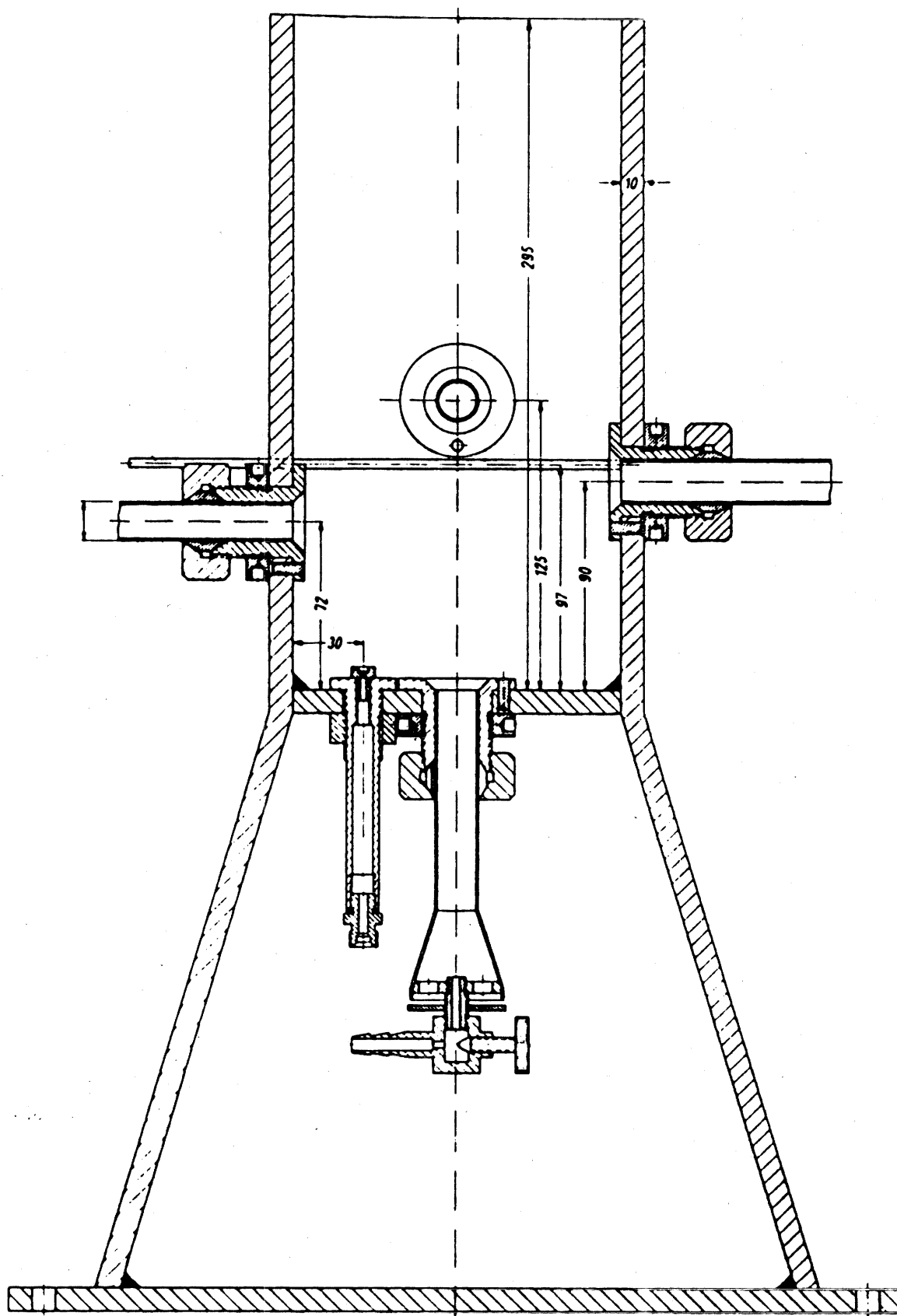


Figure 3a

Material: brass

(All dimensions in millimetres)

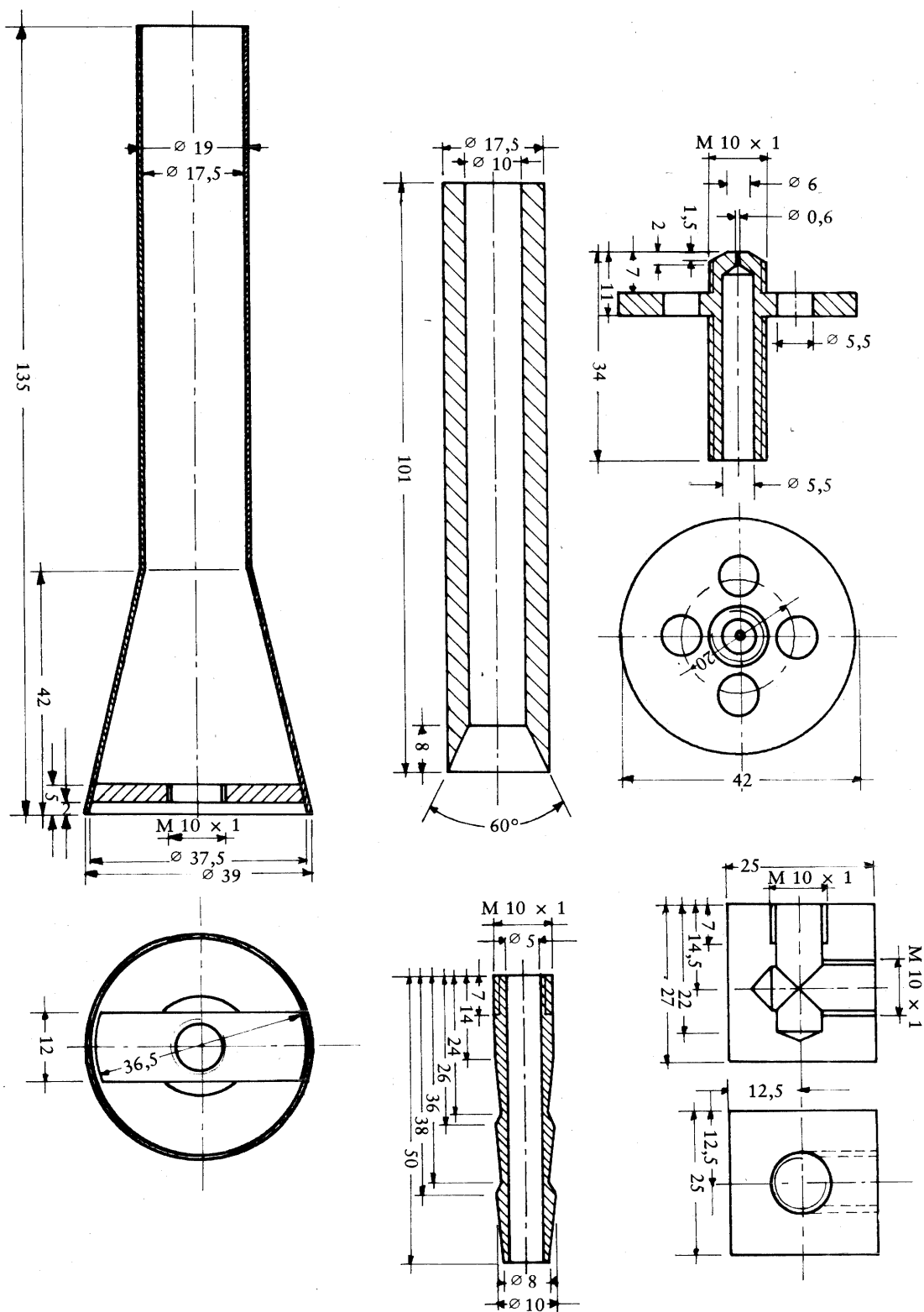
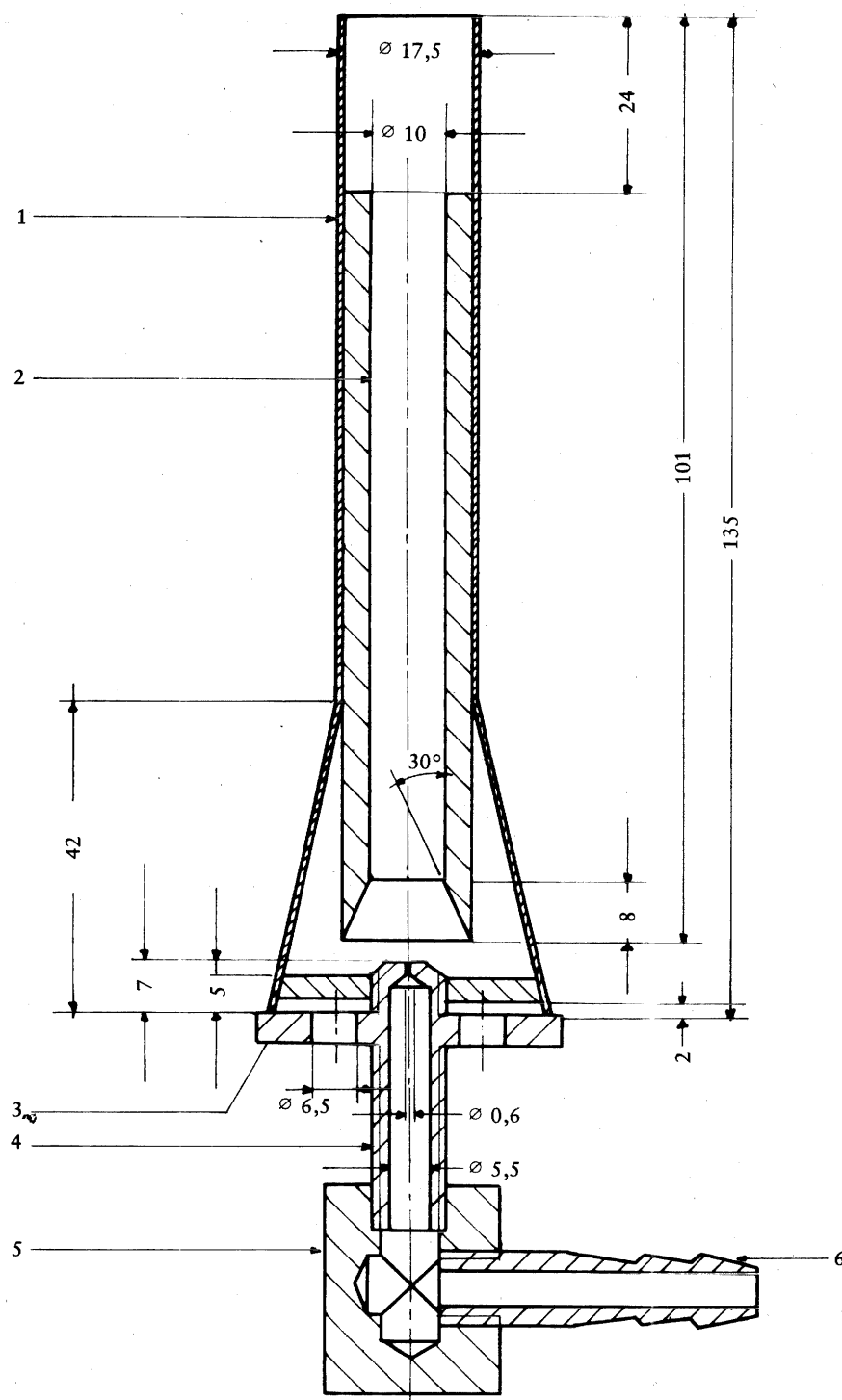


Figure 3b

Material: brass

(All dimensions in millimetres)



A. 15. AUTO-FLAMMABILITY (DETERMINATION OF THE TEMPERATURE OF SELF-IGNITION OF VOLATILE LIQUIDS AND OF GASES)

1. METHOD

1.1. Introduction

It is useful to have preliminary information on the auto-flammability of a substance. The test procedure is applicable to gaseous and volatile liquid substances as marketed, which, or whose vapours, can be ignited in the presence of air, by a hot surface. The self-ignition temperature can be considerably reduced by the presence of catalytic impurities.

1.2. Definitions and units

The degree of auto-flammability is expressed in terms of self-ignition temperature. The self-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

Not specified.

1.4. Principle of the method

Auto-flammability behaviour of gases and vapours is determined using the apparatus described in IEC 79-4.

1.5. Quality Criteria

The repeatability varies according to the range of self-ignition temperatures and the test method used (max. ± 5 °C).

The sensitivity depends on the test method used.

The specificity depends 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

2. DATA

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

3. REPORTING

The 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 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. AUTO-FLAMMABILITY (SOLIDS — DETERMINATION OF RELATIVE SELF-IGNITION TEMPERATURE)

1. METHOD

1.1. Introduction

Explosive substances and substances which ignite spontaneously in contact with air at ambient temperature should 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 at a rate of 0,5 °C/min. The temperature of the oven at which the sample temperature reaches 400 °C by self-heating is called the self-ignition temperature for the purpose of this test.

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. It must be ensured that any decomposition gases cannot come into contact with the electric heating elements, to avoid a potential explosion risk.

1.6.1.2. Wire mesh cube

A piece of stainless steel wire mesh with 0,045 mm openings will be cut according to the pattern in figure 1 (see Appendix). The mesh will be folded and secured with wire into open topped cubes.

1.6.1.3. Thermocouples

Suitable thermocouples.

1.6.1.4. Recorder

Any two-channel recorder calibrated 0 to 600 °C or corresponding voltage.

1.6.2. Test conditions

Substances are tested in their commercial form.

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 sample 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 of the solid, 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 in the Appendix).

3. REPORTING

The 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

None.

Appendix

Figure 1

Pattern of 20 mm test cube

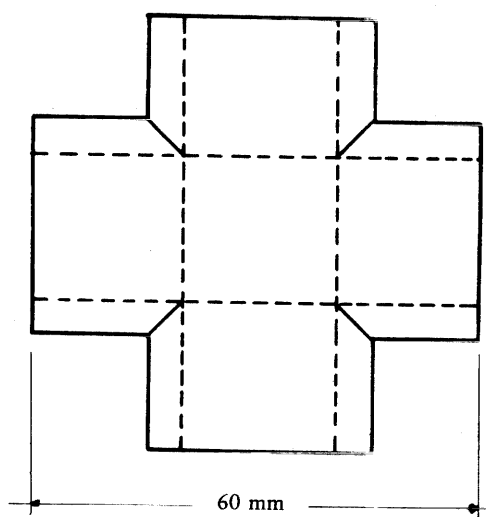
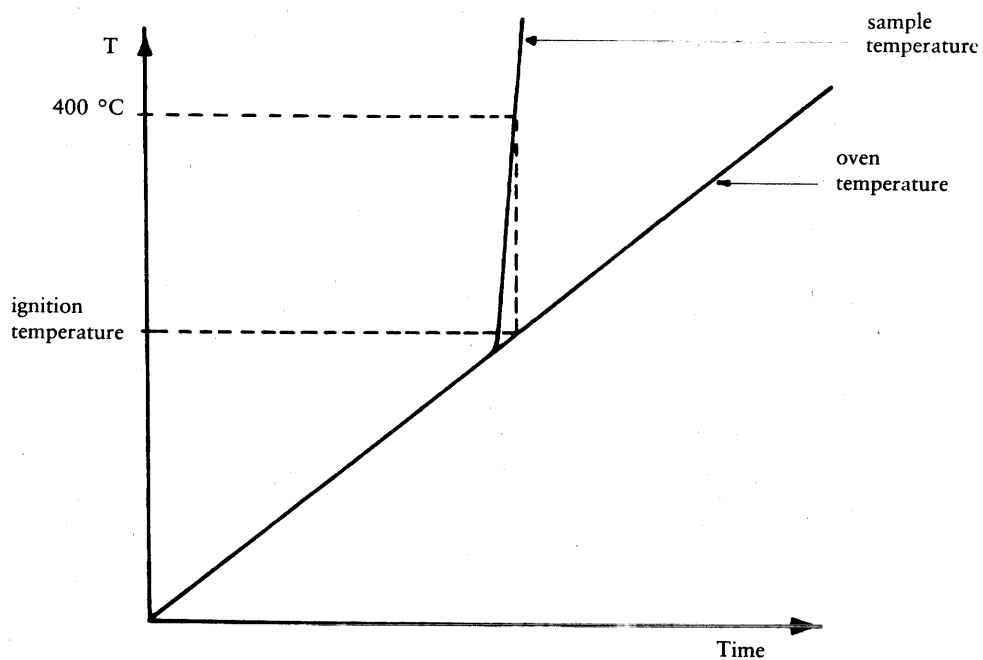


Figure 2

Typical temperature/time curve



A. 17. OXIDIZING PROPERTIES

1. METHOD

1.1. Introduction

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

This test is not applicable to liquids and gases, explosive or highly flammable substances, organic peroxides or to combustible solids liable to melt under the conditions of the test.

This test is irrelevant when examination of the structural formula establishes beyond reasonable doubt that the substance or preparation 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 values of 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.

Potassium dichromate could also be used in the preliminary tests.

Special precautions should be taken when handling potassium dichromate.

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. The test will be sufficient when the preliminary test clearly indicates that the test substance or preparation has oxidizing properties. When this is not the case, the substance or preparation is then to be subject to a further test.

In the further 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 not more than 10 %.

1.6. Description of the method

1.6.1. Preliminary test

The substance, in its commercial forms, is dried. The dried substance is roughly 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) $\times$ 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 impervious non-conducting surface over the ignition source, which comprises an inert metal wire such as platinum or nickel (which can be electrically heated to approximately 1 000 °C) located approximately 1 mm above the test surface and which spans the base of the cone shape pile. The test should be carried out in a fume cupboard as in 1.6.3.

The ignition source is switched on and left on. The vigour and duration of the resultant reaction are observed and recorded.

The substance or preparation 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. Preparation

1.6.2.1. Test substance

Reduce the test sample to a particle size $< 0,125$ mm using the following procedure:

Sieve the test substance in its commercial state; 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 decomposition temperature of the substance to be tested is below 105 °C, substance has to be dried at a suitable lower temperature.

1.6.2.2. 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.

Before preparing the mixture the powdered cellulose will be 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 1 600 μ m, 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.

1.6.2.3. Mixture

Prepare oxidizer/cellulose-mixture 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.

Note

Mixtures of oxidizers with cellulose must be treated as potentially explosive and handled with due care.

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 longitudinal direction, two metal sheets are mounted as lateral limitations which overlook 2 mm the upper edge of the triangular cross-section (figure). This arrangement is loosely filled with mixture in a slight excess. After dropping the mould once from a height of 2 cm on to a solid surface, the remaining excess substance is scraped off with an oblique positioned sheet. Then the lateral limitations are removed and the remaining powder is smoothed, using a roller. A non-combustible plate is then placed on the top of the mould, the apparatus inverted and the mould removed.

1.6.2.4. Ignition source

The flame of a gas burner or a platinum wire electrically heated to approximately 1 000 °C is used as ignition source.

1.6.3. 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.

Due to hygroscopicity of cellulose and of 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 or the glowing platinum wire.

Measure the time of reaction over 200 mm after the reaction zone has propagated an initial distance of 30 mm.

The test is performed with the reference substance. The test is then carried out at least once with each one of the range of mixtures of the test substance with cellulose.

If a maximum burning rate is found significantly greater than that of the reference substance, the test can be stopped, otherwise the three mixtures having produced the highest burning rate must have the test repeated five times.

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 highest value of burning rate for each mixture versus the contents of oxidizer.

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 for 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).

3. REPORT

3.1. Test report

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

- a description of the substance to be tested,
- any treatment of the test sample (e.g. grinding, drying, ...),
- 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.

3.2. Interpretation of the result

A substance is to be considered as oxidizing substance when:

- (a) in the preliminary test, there is a vigorous reaction;
- (b) in the test, the maximum burning rate of the mixtures to be tested is higher than or equal to the maximum burning rate of the reference mixture of cellulose and barium nitrate.

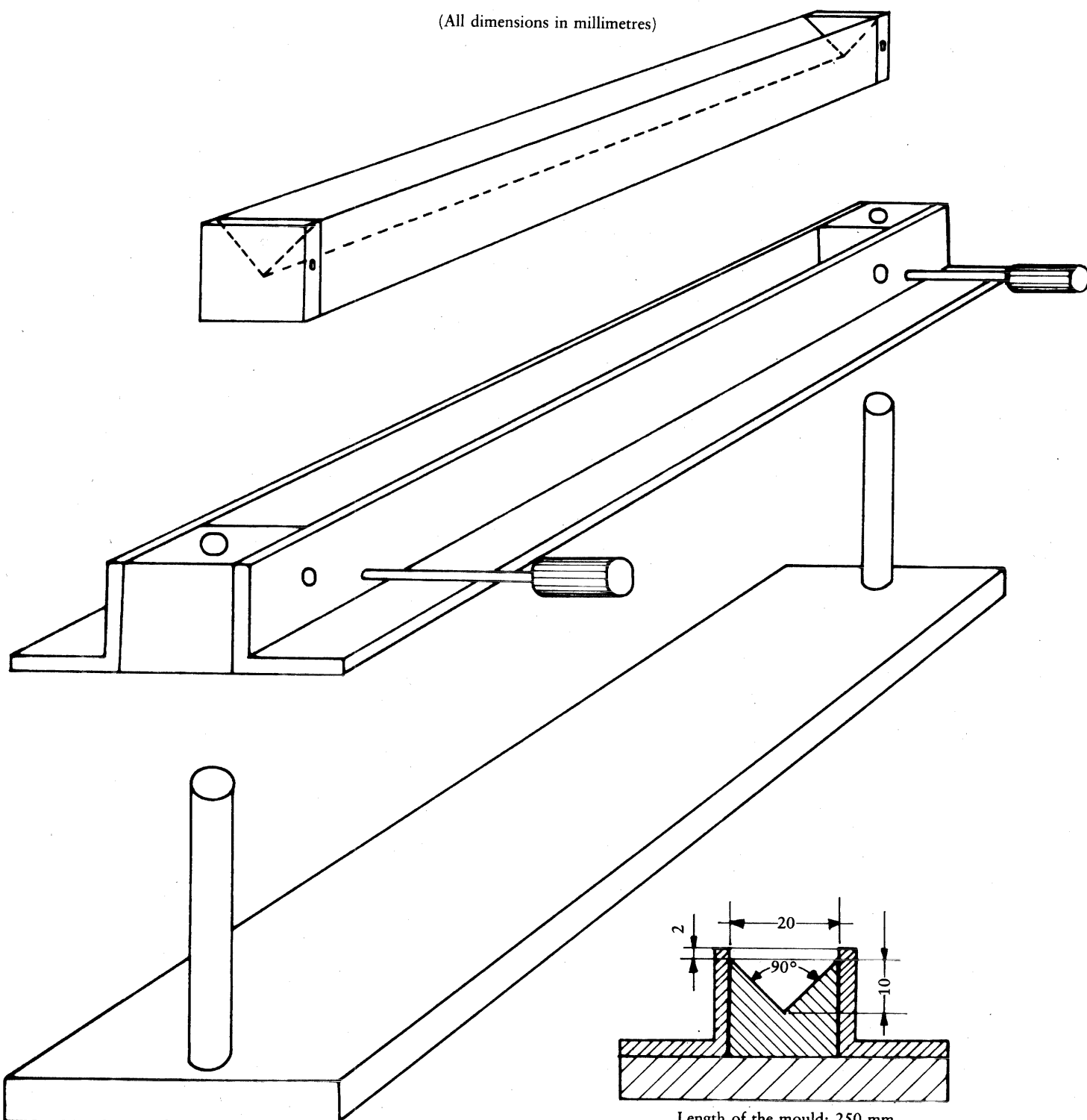
4. REFERENCES

None.

*Appendix**Figure*

Mould and accessories for the preparation of the pile

(All dimensions in millimetres)



Length of the mould: 250 mm.
Material: aluminium

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) *LD₅₀* (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 *LD₅₀* value is expressed in terms of weight of test substance per unit weight of test animal (milligrams per kilogram).
- (iii) *LC₅₀* (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 *LC₅₀* value is expressed as weight of test substance per standard volume of air (milligrams per litre).
- (iv) *No toxic effect level* is the maximum dose or exposure level used in a test which produces no detectable adverse effects.
- (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 life-span.
- (vi) *Maximum tolerated dose* (MTD) is the highest dose level eliciting signs of toxicity without having major effects on survival relative to the test in which it is used, e.g. in carcinogenicity studies, due to effects other than tumors.
- (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.

C. 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, human data are considered to be of more relevance in determining the potential effects of chemical substance on the human population.

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 aberrations.

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.

D. 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 rodents suitable conditions are a room temperature of 22 ± 3 °C with a relative humidity of 30 to 70 %; for rabbits and guinea pigs 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.

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. Subsequently, observations of effects and deaths are made. Animals which die during the test are necropsied, and at the conclusion of the test the surviving animals are necropsied. This method is directed primarily to studies in rodent species.

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 the range of the weight variation in animals used in a test should not exceed $\pm 20\%$ 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 dose level. The females should be nulliparous and non-pregnant.

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 LD₅₀.

1.6.2.4. Limit test

An adequate estimate of acute oral toxic potential for most purposes is obtained if there is no compound-related mortality in a treated group (five animals per sex) within 14 days following a dose of 5 000 mg/kg.

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 in a single dose to animals by groups by gavage.

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. Cageside 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. 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.

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. The LD₅₀ 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),
- tabulation of response data by sex and dose level (i.e. number of animals dying, number of animals showing signs of toxicity, number of animals exposed),
- time of death after dosing,
- cageside observations,
- LD₅₀ value for each sex determined at 14 days (with the method of determination specified),
- 95 % confidence interval for the LD₅₀,
- dose/mortality curve and slope (where permitted by the method of determination),
- necropsy findings,
- any histopathological findings,
- discussion of the results,
- interpretation of results.

3.2. Evaluation and interpretation

See General Introduction Part B (C).

4. REFERENCES

See General Introduction Part B (D).

B. 2. ACUTE TOXICITY (INHALATION)

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

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.

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.

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 the weight variation in animals used in a test should not exceed $\pm 20\%$ 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.

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 20 mg per litre of a gas or 5 mg per litre of an aerosol or a particulate for four hours (or, where this is not possible due to the physical or chemical, including explosive, properties of the test substance, the maximum attainable concentration) produces no compound related mortality within 14 days further testing may not be considered necessary.

1.6.2.5. Exposure time

The minimum 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 minimum 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 aerosols) this may not be practicable. Food and water should be withheld during exposure. A dynamic inhalation system with a suitable analytical concentration control system should be used. To establish suitable exposure concentrations a trial test is recommended. The system should ensure that stable exposure conditions are achieved as rapidly as possible. The rate of airflow should be adjusted to ensure that conditions throughout the exposure chamber are homogeneous.

Measurements or monitoring should be made:

- (a) of the rate of air flow (continuously);
- (b) of the actual concentration of the test substance measured in the breathing zone. During the exposure period the concentration should not vary by more than $\pm 15\%$ of the mean value. However in the case of dusts and some aerosols, this level of control may not be achievable and a wider range would then be acceptable. For particulates and aerosols, analysis should be made as often as necessary, at least once, to determine particle size distribution,
- (c) of temperature and humidity,
- (d) 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 to the study, e.g. necropsy or refrigeration of those animals found dead and isolation or sacrifice of weak or moribund animals.

Cageside 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. 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 particulates and 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 particulate aerosol concentrations and size should be described.

Exposure data

These should be tabulated and presented with mean values and a measure of variability (e.g. standard deviation) and 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 the volume of air);
- (d) nature of vehicle, if used;
- (e) actual concentrations in test breathing zone;
- (f) median particle sizes;
- (g) equilibration period;
- (h) exposure period;
- tabulation of response data by sex and exposure level (i.e. number of animals dying; number of animals showing signs of toxicity; number of animals exposed),
- time of death during or following exposure,
- cageside observations,
- LC_{50} for each sex determined at the end of the observation period (with method of calculation specified),
- 95 % confidence interval for the LC_{50} ,
- dose/mortality curve and slope (where permitted by the method of determination),
- necropsy findings,
- any histopathological findings,
- discussions of the results,
- interpretation of the results.

3.2. EVALUATION AND INTERPRETATION

See General Introduction Part B (C).

4. REFERENCES

See General Introduction Part B (D).

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 the surviving animals are necropsied.

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 the range of the weight variation in animals used in a test should not exceed $\pm 20\%$ of the appropriate mean value.

1.6.2.2. Number and sex

At least 10 animals (five female and five male) with healthy intact skin should be used at each dose level. The females should be nulliparous and non-pregnant. The use of a smaller number of animals may sometimes be justified, especially in the case of the rabbit.

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 LD₅₀.

1.6.2.4. Limit test

If, in a preliminary test, a dose of 2 000 mg/kg or more, applied to the intact skin of at least five animals per sex, produces no compound related mortality within 14 days, further testing at other dose levels may not be considered necessary.

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. Restraint may be used to prevent the ingestion of the test substance but complete immobilization 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.

Cageside 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.

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. The LD₅₀ 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),
- dose levels (with vehicle, if used, and concentrations),
- tabulation of response data by sex and dose level (i.e. number of animals dying; number of animals showing signs of toxicity; number of animals exposed),
- time of death after dosing,
- cageside observations,
- LD₅₀ value for each sex determined at 14 days with the method of determination specified,
- 95 % confidence interval for the LD₅₀ (where this can be provided),
- dose/mortality curve and slope where permitted by the method of determinations,
- necropsy findings,
- any histopathological findings,
- discussion of results,
- interpretation of the results.

3.2. Evaluation and interpretation

See General Introduction Part B (C).

4. REFERENCES

See General Introduction Part B (D).

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

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.

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.

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.

Test substances which are strongly acidic or alkaline need not be tested for primary dermal irritation, owing to their predictable corrosive properties. The testing of materials which have been shown to be very toxic by the dermal route may be unnecessary.

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

At least three healthy adult animals are used. 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. Separate animals are not required for an untreated control group. 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 cm²) 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 semi-occlusive dressing for the duration of the exposure period. However, the use of an occlusive dressing may be considered appropriate in certain cases. Access by the animal to the patch and resultant ingestion/inhalation of the test substance should be prevented.

Exposure duration is four hours. If it is suspected that the substance might produce a severe skin reaction, (i.e. be corrosive), the duration of exposure should be reduced (e.g. to one hour or three minutes).

If an exposure period shorter than four hours is used, and a serious skin reaction is observed, the experiment need not be repeated using a four hour exposure period. Longer exposures may be indicated under certain conditions, e.g. expected pattern of human use and exposure. 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.

1.6.3.1. Observation and grading

Animals should be observed for signs of erythema and oedema and the response graded at 30 to 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, as necessary, to establish reversibility. 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.

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 chemical, and the technique of skin preparation and cleansing),
- 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 (C).

4. REFERENCES

See General Introduction Part B (D).

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) to slight eschar formation (injuries in depth)	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

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.

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. Strongly alkaline or acidic test substances and substances which have demonstrated definite corrosivity in a dermal irritation study or in other tests need not be tested for eye irritation.

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

At least three 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.

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.

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 substances shown to be irritating by this test the value of irrigating the eye as a means of reducing irritant or other damaging effects may be examined. In these cases it is recommended that six rabbits be used. Four seconds after instillation of the test substance, the eyes of three rabbits are washed and 30 seconds after instillation of the test substance the eyes of the other three rabbits are washed. For both groups, the eyes are washed for five minutes 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 irritation 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 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 (C).

4. REFERENCES

See General Introduction Part B (D).

Appendix

TABLE: 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, moderate circumcorneal 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 palpebral and bulbar conjunctivae, cornea and iris)

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 nictating membranes

No swelling	0
Any swelling above normal (including nictating 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. ACUTE TOXICITY**SKIN SENSITIZATION****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/Guinea-Pig Maximization Test

Following initial exposure to a test substance (the 'induction' period) the animals are subjected two weeks after the last induction exposure to a 'challenge' exposure to the test substance in order to establish if a hypersensitive state has been induced. Sensitization is determined by examining the skin reaction to the challenge exposure.

1.5. Quality criteria

None.

1.6. Description of the test method**1.6.1. Preparations**

Healthy young animals (less than one year old) are randomized and assigned to the treatment and control groups. Prior to dosing, the hair is removed, by clipping or shaving, from the shoulder region. Care should be taken to avoid damaging the skin.

1.6.2. Test conditions**1.6.2.1. Experimental animals**

The albino guinea-pig is used.

1.6.2.2. Number and sex

Male and/or female animals can be used. If females are used, they should be nulliparous and non-pregnant. 20 animals are used in the treatment group and at least 10 in the control group. The use of fewer animals must be justified.

1.6.2.3. Dose levels

The concentration of the test substance is adjusted to the highest level that can be well tolerated in each induction stage.

The challenge concentration should produce no evidence of skin irritation in non-sensitized animals. These concentrations can be determined by a small scale (two to three animals) pilot study.

1.6.2.4. Observation period

During the induction period skin observations are carried out to check for possible irritation effects. After the challenge exposure, skin reactions are recorded at 48 and 72 hours.

1.6.3. Procedure

The animals are weighed before the test commences and at the end of the test. The shoulder region is cleared of hair. There are two stages in the procedure:

1.6.3.1. Induction

Day 0 — treated group

The following pairs of intradermal injections are given in the shoulder region so that one of each pair lies on each side of the midline.

1. 0,1 ml Freund's Complete Adjuvant (FCA),
2. 0,1 ml test substance, in vehicle when appropriate,
3. 0,1 ml test substance in FCA.

Injectons 1 and 2 are given close to each other and nearest the head, while 3 is towards the caudal part of the test area.

Day 0 — control group

The following pairs of intradermal injections are given in the same sites as above.

1. 0,1 ml FCA,
2. 0,1 ml vehicle alone,
3. 0,1 ml vehicle in FCA,

Day 7 — treated group

The test area is again cleared of hair. The test substances in a suitable vehicle (liquids if appropriate can be applied directly) is spread over a filter paper and applied to the test area and held in contact by a suitable dressing for 48 hours.

Day 7 — control group

The test area is again cleared of hair. The vehicle only is applied in a similar manner to the test area and held in contact by a suitable dressing for 48 hours.

1.6.3.2. Challenge

Day 21

The flanks of treated and control animals are cleared of hair. A patch or chamber containing the test substance is applied to the left flank of treated animals and a patch or chamber with vehicle only to the right flank.

The patches are held in contact by a suitable dressing for 24 hours.

The control group is exposed in an identical manner.

Day 23 and 24

- 21 hours after removing the patch the challenge area is cleaned and shaved if necessary,
- three hours later (at 48 hours from the start of challenge application) the skin reaction is observed and recorded,
- 24 hours after this observation a second observation (72 hours) is made and recorded.

To clarify the results obtained in the first challenge, a second challenge, if necessary with a new vehicle control group, should be considered approximately one week after the first one.

1.6.3.3. Observation and grading

All skin reactions and any unusual findings resulting from induction and challenge procedures should be recorded and reported.

2. DATA

Data should be summarized in tabular form, showing for each animal the skin reactions at each observation.

3. REPORTING

3.1. Test report

The test report shall if possible contain the following information:

- strain of guinea-pig used,
- test conditions,
- number, age and sex of animals,
- individual weights of animals at the start and at the conclusion of the test,
- each observation made on each animal including grading system if one is used,
- discussion of the results,
- interpretation of the results.

3.2. Evaluation and interpretation

See General Introduction Part B (C).

4. REFERENCES

See General Introduction Part B (D).

Remarks

The Guinea-Pig Maximization Test (GPMT) is a widely used adjuvant-type test. Although several other methods, listed in the Appendix, can be used to detect the potential of a substance to provoke skin sensitization reaction, the GPMT is considered to be the preferred (reference) method. Its sensitivity and ability to detect potential human skin sensitizers are considered important in a classification system for

toxicity relevant to public health. The choice of any alternative test must be governed by criteria which ensure that it is valid. Among these criteria is an expected response to standard allergens such as 2,4-dinitro-chlorobenzene or p-phenylenediamine or another potent sensitizer appropriate to the class of substance being tested.

There is no single test method which will adequately identify all substances with a potential for sensitizing human skin and which is relevant for all substances.

Factors such as the physical characteristics of a substance, including its ability to penetrate the skin, and its mode of contact with humans likely to be exposed must be considered in the selection of a test. Tests using guinea-pigs can be subdivided into the adjuvant-type tests, in which an allergic state is potentiated by dissolving or suspending the test substance in Freund's Complete Adjuvant (FCA), and the non-adjuvant tests. In certain cases there may be good reasons for choosing a test involving topical application rather than the intradermal injection used in the Guinea-Pig Maximization Test. The considerations will again include the physical characteristics of the test substance and the conditions of likely human exposure.

Regardless of the method used, the sensitivity of the strain of guinea-pig being used for skin sensitization testing must be checked at regular intervals (six months) using known strong and moderate sensitizers and a satisfactory number of positive responses obtained.

Appendix

	Draize	Freunds Complete Adjuvant (FCA)	Mauer optimization	Buehler	Open epicutaneous test	Split adjuvant
<i>Species</i>	guinea-pig	guinea-pig	guinea-pig	guinea-pig	guinea-pig	guinea-pig
<i>Route</i>	intradermal (id)	intradermal (id)	intradermal (id)	epicutaneous (ec)	epicutaneous (ec)	(id and ec)
<i>Number in test group</i>	20	8—10	10—10	10—20	6—8	10—20
<i>Number of test groups</i>	1	1	1	1	up to 6	1
<i>Number in control group</i>	20	8—10	10—10	10—20	6—8	10—20
<i>Induction exposure route</i>	id	id	id	dermal	dermal	id and dermal
<i>Number of exposures</i>	10	5	9	3	20 or 21	4
<i>Exposure period</i>	—	—	24 hr	6 hr each	continuous	48 hr each
<i>Patch type</i>	—	—	—	closed	open	closed
<i>Test group(s)</i>	TS ⁽¹⁾	TS in FCA	TS in FCA	TS	TS	TS
<i>Control group</i>	—	FCA only	—	—	vehicle (v) only	—
<i>Site</i>	left flank	right flank	back	left flank	right flank	shoulder
<i>Frequency</i>	every 2nd day	every 2nd day	every 2nd day	every 7th day	daily	0, 2, 4, 7 d
<i>Duration</i>	0—18 d	0—8 d	0—21 d	0—14 d	0—20 d	0—7 d
<i>Concentration</i>	2—10 times that of first	same throughout	0,1 ml of 0,1 %	same throughout	same per group different between groups	same throughout
<i>Challenge exposure</i>						
<i>Route</i>	id	dermal	id	dermal	dermal	dermal
<i>Number of exposure(s)</i>	1	2	2	1	2	1
<i>Day(s)</i>	35	22 and 35	14 and 28	28	21 and 35	20
<i>Exposure period</i>	—	—	24 hr	6 hr	—	24 hr
<i>Patch type</i>	—	open	—	closed	open	closed
<i>Test group(s)</i>	TS	TS	TS	TS	TS	TS
<i>Control group</i>	TS	TS	TS	TS	TS	TS
<i>Site</i>	right flank	left flank	back, new site	right flank	left flank	shoulder
<i>Concentration</i>	same as first	4 different	0,1 ml of 0,1 %	same as induction	4 different	half induction
<i>Evaluation (hr after challenge)</i>	24, 48	24, 48, 72	24	24, 48	24, 48 and/or 72	24, 48

⁽¹⁾ TS = test substance.

B. 7. SUB-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, daily, in graduated doses to several groups of experimental animals, one dose per group for a period of 28 days. During the period of administration the animals are observed daily to detect signs of toxicity. Animals which die during the test are necropsied, and at the conclusion of the test surviving animals are necropsied.

1.5. Quality criteria

None.

1.6. Description of the test method**1.6.1. Preparations**

The animals are kept under the experimental housing and feeding conditions for at least five days prior to the test. Before the test, healthy young animals are randomized and assigned to the treatment groups. The test substances may be administered in the diet, by gavage, in capsules, or in the drinking water. All animals should be dosed by the same method during the entire experimental period. 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 preferred species is the rat. Commonly used laboratory strains of young healthy animals should be employed and dosing should begin ideally before the rats are six weeks old, and in any case not more than eight weeks old. At the commencement of the study, the weight variation in the animals used should not exceed $\pm 20\%$ of the mean value.

1.6.2.2. Number and sex

At least 10 animals (five female and five male) should be used at each dose level. The females should be nulliparous and non-pregnant. If interim sacrifices are planned, the number of animals should be increased by the number of animals scheduled to be sacrificed before the completion of the study. In addition, a satellite group of 10 animals (five animals per sex) may be treated with the high dose level for 28 days and observed for reversibility, persistence, or delayed occurrence of toxic effects for 14 days post treatment.

1.6.2.3. Dose levels

At least three dose levels and a control should be used. Except for treatment with the test substance, animals in the control group should be handled in an identical manner to the test-group subjects. Where a vehicle is used to facilitate dosing, the controls should be dosed with the vehicle in the same way as the treated groups, and receive the same amount of vehicle as that received by the highest dose level group. The highest dose level should result in toxic effects but produce no, or few, fatalities. The lowest dose level should not produce any evidence of toxicity. Where there is a usable estimation of human exposure, the lowest level should exceed this. Ideally, the middle dose level should produce minimal observable toxic effects. If more than one intermediate dose is used the dose levels should be spaced to produce a gradation of toxic effects. In the low and intermediate groups and in the controls, the incidence of any fatalities should be low in order to permit a meaningful evaluation of results.

When the test substance is administered in the diet, either a constant dietary concentration (ppm or mg/kg of food) or a constant dose level in terms of the animal's body weight may be used; the alternative used must be specified. For a substance administered by gavage, the dose should be given at similar times each day and dose levels should be adjusted at intervals (weekly or bi-weekly) to maintain a constant dose level in terms of animal body weight.

1.6.2.4. Limit test

If a 28-day study conducted in accordance with the method detailed below, at one dose level of 1 000 mg/kg body weight/day or a higher dose level related to possible human exposure where this is known, produces no evidence of toxic effects, further testing may not be considered necessary. For substances of low toxicity it is important to ensure that when administered in the diet the quantities and other properties of the test substance involved do not interfere with normal nutritional requirements.

1.6.2.5. Observation period

All the animals should be observed daily and signs of toxicity recorded including their time of onset, degree and duration. The time of death and the time at which signs of toxicity appear and disappear should be recorded.

1.6.3. Procedure

The animals are dosed with the test substance ideally on seven days per week for a period of 28 days. Animals in any satellite group scheduled for follow-up observations should be kept for a further 14 days without treatment to detect recovery from, or persistence of, toxic effects.

Cageside observations should include changes in skin and fur, eyes and mucous membranes as well as respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behaviour pattern. Weekly measurements should be made of food consumption (and water consumption when the test substance is administered in the drinking water) and the animals weighed weekly.

Regular observation of the animals is necessary to ensure that animals are as far as possible not lost from the study due to causes such as cannibalism, autolysis of tissues or misplacement. At the end of the study period, all survivors in the non-satellite treatment groups are necropsied. Moribund animals should be removed and necropsied when noticed.

The following examination shall be made at the end of the test period for all animals including controls:

1. haematology including at least haematocrit, haemoglobin concentration, erythrocyte count, total and differential leucocyte count, and a measure of clotting potential;
2. clinical biochemistry in blood including at least one parameter of liver and kidney function: alanine aminotransferase (formerly known as glutamic pyruvic transaminase), serum aspartate aminotransferase (formerly known as glutamic oxaloacetic transaminase), urea nitrogen, albumin, blood creatinine, total bilirubin and total serum protein.

Other determinations which may be necessary for an adequate toxicological evaluation include calcium, phosphorus, chloride, sodium, potassium, fasting glucose, analysis of lipids, hormones, acid/base balance, methaemoglobin, cholinesterase activity.

Additional clinical biochemistry may be employed, where necessary, to extend the investigation of observed effects.

1.6.3.1. Gross necropsy

All animals in the study should be subjected to a full gross necropsy. The liver, kidney, adrenals and testes should be weighed wet as soon as possible after dissection to avoid drying. Organs and tissues (liver, kidney, spleen, adrenals and heart, and any organs showing gross lesions or changes in size) should be preserved in a suitable medium for possible future histopathological examination.

1.6.3.2. Histopathological examination

In the high-dose group and in the control group, histological examination should be performed on preserved organs and tissues. Organs and tissues showing defects attributable to the test substance at the highest dosage level should be examined in all lower-dosage groups. Animals in any satellite group should be examined histologically with particular emphasis on those organs and tissues identified as showing effects in the other treated groups.

2. DATA

Data should be summarized in tabular form, showing for each test group the number of animals at the start of the test and the number of animals displaying each type of lesion.

All observed results should be evaluated by an appropriate statistical method. Any recognized statistical method may be used.

3. REPORTING

3.1. Test report

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

- species, strain, source, environmental conditions, diet, etc.,
- test conditions,
- dose levels (including vehicle, if used) and concentrations,
- toxic response data by sex and dose,
- no-effect level, when possible,
- time of death during the study or whether animals survived to termination,
- toxic or other effects,
- the time of observation of each abnormal sign and its subsequent course,
- food and body-weight data,
- haematological tests employed and all results,
- clinical biochemistry tests employed and all results,
- necropsy findings,
- a detailed description of all histopathological findings,
- statistical treatment of results where appropriate,
- discussion of the results,
- interpretation of the results.

3.2. Evaluation and interpretation

See General Introduction Part B (C).

4. REFERENCES

See General Introduction Part B (D).

B. 8. SUB-ACUTE TOXICITY (INHALATION)**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

Several groups of experimental animals are exposed daily for a defined period to the test substance in graduated concentrations, one concentration being used per group, for a period of 28 days. Where a vehicle is used to help generate an appropriate concentration of the test substance in the atmosphere, a vehicle control group should be used. During the period of administration the animals are observed daily to detect signs of toxicity. Animals which die during the test are necropsied and at the conclusion of the test surviving animals are necropsied.

1.5. Quality criteria

None.

1.6. Description of the test method**1.6.1. Preparations**

The animals are kept under the experimental housing and feeding conditions for at least five days prior to the experiment. Before the test, healthy young animals are randomized and assigned to the required number of groups. Where necessary, a suitable vehicle may be added to the test substance to help generate an appropriate concentration of the substance in the atmosphere. If a vehicle or other additive is used to facilitate dosing, it should be known not to produce toxic effects. Historical data can be used if appropriate.

1.6.2. Test conditions**1.6.2.1. Experimental animals**

Unless there are contra-indications, the rat is the preferred species. Commonly used laboratory strains of young healthy animals should be employed.

At the commencement of the study, the range of weight variation of animals used should not exceed $\pm 20\%$ of the appropriate mean value.

1.6.2.2. Number and sex

At least 10 animals (five female and five male) should be used for each test group. The females should be nulliparous and non-pregnant. If interim sacrifices are planned, the numbers should be increased by the number of animals scheduled to be sacrificed before the completion of the study. In addition, a satellite

group of 10 animals (five animals per sex) may be treated with the high concentration level for 28 days and observed for reversibility, persistence, or delayed occurrence of toxic effects for 14 days post treatment.

1.6.2.3. Exposure concentration

At least three concentrations are required, with a control or a vehicle control (corresponding to the concentration of vehicle at the highest level) if a vehicle is used. Except for treatment with the test substance, animals in the control group should be handled in an identical manner to the test-group animals. The highest concentration should result in toxic effects but no, or few, fatalities. The lowest concentration should not produce any evidence of toxicity. Where there is a usable estimation of human exposure, the lowest concentration should exceed this. Ideally, the intermediate concentration should produce minimal observable toxic effects. If more than one intermediate concentration is used the concentrations should be spaced to produce a gradation of toxic effects. In the low and intermediate groups and in the controls, the incidence of fatalities should be low to permit a meaningful evaluation of the results.

1.6.2.4 Exposure time

The duration of daily exposure should be six hours but other periods may be needed to meet specific requirements.

1.6.2.5. Equipment

The animals should be tested in inhalation equipment designed to sustain a dynamic airflow of at least 12 air changes per hour to ensure an adequate oxygen content and an evenly distributed exposure atmosphere. Where a chamber is used, its design should minimize crowding of the test animals and maximize their exposure by inhalation of the test substance. As a general rule to ensure stability of a chamber atmosphere the total 'volume' of the test animals should not exceed 5 % of the volume of the test chamber. Oro-nasal, head only, or individual whole body chamber exposure may be used; the first two will minimize uptake by other routes.

1.6.2.6. Observation period

The experimental animals should be observed daily for signs of toxicity during the entire treatment and recovery period. The time of death and the time at which signs of toxicity appear and disappear should be recorded.

1.6.3. Procedure

The animals are exposed to the test substance daily, five to seven days per week, for a period of 28 days. Animals in any satellite groups scheduled for follow-up observations should be kept for a further 14 days without treatment to detect recovery from, or persistence of, toxic effects. The temperature at which the test is performed should be maintained at 22 ± 3 °C.

Ideally, the relative humidity should be maintained between 30 and 70 %, but in certain instances (e.g. tests of aerosols) this may not be practicable. Food and water should be withheld during exposure.

A dynamic inhalation system with a suitable analytical concentration control system should be used. To establish suitable exposure concentrations a trial test is recommended. The airflow should be adjusted to ensure that conditions throughout the exposure chamber are homogenous. The system should ensure that stable exposure conditions are achieved as rapidly as possible.

Measurements or monitoring should be made:

- (a) of the rate of airflow (continuously);
- (b) of the actual concentration of the test substance measured in the breathing zone. During the daily exposure period the concentration should not vary by more than $\pm 15\%$ of the mean value. However, in the case of dusts and aerosols, this level of control may not be achievable and a wider range would then be acceptable. During the total duration of the study, the day-to-day concentrations should be held as constant as practicable. For particulates and aerosols, in order to assess the consistency of the particle size distribution, measurement of the latter should be made as often as necessary;
- (c) of temperature and humidity;
- (d) during and following exposure; observations are made and recorded systematically; individual records should be maintained for each animal. All the animals should be observed daily and signs of toxicity recorded including the time of onset, their degree and duration. Cageside observations should include changes in the skin and fur, eyes, mucous membranes, respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behaviour pattern. Measurements should be made weekly of the animals' weights. It is also recommended that food consumption is measured weekly. Regular observation of the animals is necessary to ensure that animals are not lost from the study due to causes such as cannibalism, autolysis of tissues or misplacement. At the end of the study period, all survivors in the non-satellite treatment groups are necropsied. Moribund animals should be removed and necropsied when noticed.

The following examinations shall be made at the end of the test on all animals including the controls:

- (i) haematology, including at least haematocrit, haemoglobin concentration, erythrocyte count, total and differential leucocyte count and a measure of clotting potential;
- (ii) clinical biochemistry determinations in blood including at least one parameter of liver and kidney function: serum alanine aminotransferase (formerly known as glutamic pyruvic transaminase), serum aspartate aminotransferase (formerly known as glutamic oxaloacetic transaminase), urea nitrogen, albumin, blood creatinine, total bilirubin and total serum protein measurements.

Other determinations which may be necessary for an adequate toxicological evaluation include calcium, phosphorus, chloride, sodium, potassium, fasting glucose analysis of lipids, hormones, acid/base balance, methaemoglobin and cholinesterase activity.

Additional clinical biochemistry may be employed, where necessary, to extend the investigation of observed toxic effects.

1.6.3.1. Gross necropsy

All animals in the study should be subjected to a full gross necropsy. The liver, kidneys, adrenals and testes should be weighed wet as soon as possible after dissection to avoid drying. Organs and tissues (the respiratory tract, liver, kidneys, spleen, adrenals, heart and any organs showing gross lesions or changes in size) should be preserved in a suitable medium for possible future histopathological examination. The naso-pharynx and lungs should be removed intact, weighed and treated with a suitable fixative to ensure that lung structure is maintained. Perfusion with a fixative is considered to be an effective procedure.

1.6.3.2. Histopathological examination

In the high-concentration group and in the control(s), histological examination should be performed on preserved organs and tissues. Organs and tissue showing defects attributable to the test substance at the highest dosage level should be examined in all lower-dosage groups. Animals in any satellite groups should be examined histologically with particular emphasis on those organs and tissues identified as showing effects in the other treated groups.

2. DATA

Data should be summarized in tabular form, showing for each test group the number of animals at the start of the test and the number of animals displaying each type of lesion.

All observed results should be evaluated by an appropriate statistical method. Any recognized statistical method may be used.

3. REPORTING

3.1. Test report

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

- species, strain, source, environmental conditions, diet, etc.,
- test conditions:

Description of exposure apparatus including design, type, dimensions, source of air, system for generating particulates and aerosols, method of conditioning air, treatment of exhaust air and the method of housing animals in a test chamber when this is used. The equipment for measuring temperature, humidity and, where appropriate, stability of aerosol concentrations or particle size, should be described.

Exposure data:

These should be tabulated and presented with mean values and a measure of variability (e.g. standard deviation) and shall, if possible, include:

- (a) airflow rates through the inhalation equipment;
- (b) temperature and humidity of air;
- (c) nominal concentrations (total amount of test substance fed into the inhalation equipment divided by the volume of air);
- (d) nature of vehicle, if used;
- (e) actual concentrations in test breathing zone;
- (f) median particle sizes (where appropriate);
- toxic response data by sex and concentration,
- time of death during the study or whether animals survived to termination,
- description of toxic or other effects; no-effect level,
- the time of observation of each abnormal sign and its subsequent course,
- food and body-weight data,
- haematological tests employed and results,

- clinical biochemistry tests employed and results,
- necropsy findings,
- a detailed description of all histopathological findings,
- a statistical treatment of results where possible,
- discussion of the results,
- interpretation of results.

3.2. **Evaluation and interpretation**

See General Introduction Part B (C).

4. **REFERENCES**

See General Introduction Part B (D).

B. 9. SUB-ACUTE TOXICITY (DERMAL)**1. METHOD****1.1. Introduction**

See General Introduction Part B (A),

1.2. Definitions

See General Introduction Part B (B),

1.3. Reference substances

None.

1.4. Principle of the test method

The test substance is applied daily to the skin in graduated doses to several groups of experimental animals, one dose per group, for a period of 28 days. During the period of application, the animals are observed daily to detect signs of toxicity. Animals which die during the test are necropsied, and at the conclusion of the test the surviving animals are necropsied.

1.5. Quality criteria

None.

1.6. Description of the test method**1.6.1. Preparations**

The animals are kept under the experimental housing and feeding conditions for at least five days prior to the test. Before the test, healthy young animals are randomized and assigned to the treatment and control groups. Shortly before testing, fur is clipped from the dorsal area of the trunk of the test animals. Shaving may be employed but it should be carried out approximately 24 hours before the test. Repeat clipping or shaving is usually needed at approximately weekly intervals. When clipping or shaving the fur, care must be taken to avoid abrading the skin. Not less than 10 % of the body surface area should be clear for the application of the test substance. The weight of the animal should be taken into account when deciding on the area to be cleared and on the dimensions of the covering. When testing solids, which may be 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. Liquid test substances are generally used undiluted. Daily application on a five- to seven-day per week basis is used.

1.6.2. Test conditions**1.6.2.1. Experimental animals**

The adult rat, rabbit or guinea-pig may be used. Other species may be used but their use would require justification. At the commencement of the test, the range of the weight variation should not exceed ± 20 % of the mean weight.

1.6.2.2. Number and sex

At least 10 animals (five female and five male) with healthy skin should be used at each dose level. The females should be nulliparous and non-pregnant. If interim sacrifices are planned, the numbers should be increased by the number of animals scheduled to be sacrificed before the completion of the study. In addition, a satellite group of 10 animals (five animals per sex) may be treated with the high dose level for 28 days and observed for reversibility, persistence, or delayed occurrence of toxic effects for 14 days post treatment.

1.6.2.3. Dose levels

At least three dose levels are required with a control or a vehicle control if a vehicle is used. The exposure period should be at least six hours per day. The application of the test substance should be made at similar times each day, and adjusted at intervals (weekly or bi-weekly) to maintain a constant dose level in terms of animal body-weight. Except for treatment with the test substances, animals in the control group should be handled in an identical manner to the test group subjects. Where a vehicle is used to facilitate dosing, the vehicle control group should be dosed in the same way as the treated groups, and receive the same amount as that received by the highest dose level group. The highest dose level should result in toxic effects but produce no, or few, fatalities. The lowest dose level should not produce any evidence or toxicity. Where there is a usable estimation of human exposure, the lowest level should exceed this. Ideally, the intermediate dose level should produce minimal observable toxic effects. If more than one intermediate dose is used the dose levels should be spaced to produce a gradation of toxic effects. In the low and intermediate groups and in the controls, the incidence of fatalities should be low in order to permit a meaningful evaluation of the results.

If application of the test substance produces severe skin irritation, the concentrations should be reduced and this may result in a reduction in, or absence of, other toxic effects at the high dose level. Moreover if the skin has been badly damaged it may be necessary to terminate the study and undertake a new study at lower concentrations.

1.6.2.4. Limit test

If a preliminary study at a dose level of 1 000 mg/kg, or a higher dose level related to possible human exposure where this is known, produces no toxic effects, further testing may not be considered necessary.

1.6.2.5. Observation period

The experimental animals should be observed daily for signs of toxicity. The time of death and the time at which signs of toxicity appear and disappear should be recorded.

1.6.3. Procedure

Animals should be caged individually. The animals are treated with the test substance, ideally on seven days per week, for a period of 28 days. Animals in any satellite groups scheduled for follow-up observations should be kept for a further 14 days without treatment to detect recovery from or persistence of toxic effects. Exposure time should be at least six hours per day.

The test substance should be applied uniformly over an area which is approximately 10 % of the total body surface area. With highly toxic substances, the surface area covered may be less but as much of the area as possible should be covered with as thin and uniform a layer as possible.

During exposure the test substance is held in contact with the skin with a porous gauze dressing and non-irritating tape. The test site should be further covered in a suitable manner to retain the gauze dressing and test substance and ensure that the animals cannot ingest the test substance. Restrainers may

be used to prevent the ingestion of the test substance but complete immobilization is not a recommended method.

At the end of the exposure period, residual test substance should be removed, where practicable, using water or some other appropriate method of cleansing the skin.

All the animals should be observed daily and signs of toxicity recorded including the time of onset, their degree and duration. Cageside observations should include changes in skin and fur, eyes and mucous membranes as well as respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behaviour pattern. Measurements should be made weekly of the animals' weights. It is also recommended that food consumption is measured weekly. Regular observation of the animals is necessary to ensure that animals are not lost from the study due to causes such as cannibalism, autolysis of tissues or misplacement. At the end of the study period, all survivors in the non-satellite treatment groups are necropsied. Moribund animals should be removed and necropsied when noticed.

The following examinations shall be made at the end of the test on all animals including the controls:

1. haematology, including at least haematocrit, haemoglobin concentration, erythrocyte count, total and differential leucocyte count, and a measure of clotting potential;
2. clinical biochemistry in blood including at least one parameter of liver and kidney function: serum alanine aminotransferase (formerly known as glutamic pyruvic transaminase), serum aspartate aminotransferase (formerly known as glutamic oxaloacetic transaminase), urea nitrogen, albumin, blood creatinine, total bilirubin and total serum protein.

Other determinations which may be necessary for an adequate toxicological evaluation include calcium, phosphorus, chloride, sodium, potassium, fasting glucose, analysis of lipids, hormones, acid/base balance, methaemoglobin and cholinesterase activity.

Additional clinical biochemistry may be employed, where necessary, to extend the investigation of observed effects.

1.6.4. *Gross necropsy*

All animals in the study should be subjected to a full gross necropsy. The liver, kidneys, adrenals and testes should be weighed wet as soon as possible after dissection, to avoid drying. Organs and tissues, i.e. normal and treated skin, liver, kidney and target organs (that is those organs showing gross lesions or changes in size), should be preserved in a suitable medium for possible future histopathological examination.

1.6.5. *Histopathological examination*

In the high dose group and in the control group, histological examination should be performed on the preserved organs and tissues. Organs and tissues showing defects attributable to the test substance at the highest dosage level should be examined in all lower-dosage groups. Animals in the satellite group should be examined histologically with particular emphasis on those organs and tissues identified as showing effects in the other treated groups.

2. DATA

Data should be summarized in tabular form, showing for each test group the number of animals at the start of the test and the number of animals displaying each type of lesion.

All observed results should be evaluated by an appropriate statistical method. Any recognized statistical method may be used.

3. REPORTING

3.1. Test report

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

- animal data (species, strain, source, environmental conditions, diet, etc.),
- test conditions,
- dose levels (including vehicle, if used) and concentrations,
- no-effect level, where possible,
- toxic response data by sex and dose,
- time of death during the study or whether animals survived to termination,
- toxic or other effects,
- the time of observation of each abnormal sign and its subsequent course,
- food and body-weight data,
- haematological tests employed and results,
- clinical biochemistry tests employed and results,
- necropsy findings,
- a detailed description of all histopathological findings,
- statistical treatment of results where possible,
- discussion of the results,
- interpretation of the results.

3.2. Evaluation and interpretation

See General Introduction Part B (C).

4. REFERENCES

See General Introduction Part B (D).

B. 10. OTHER EFFECTS — MUTAGENICITY**IN VITRO MAMMALIAN CYTOGENIC TEST****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 *in vitro* cytogenetic test is a short-term mutagenicity test for the detection of structural chromosomal aberrations in cultured mammalian cells. Cultures of established cell lines as well as primary cell cultures may be used. After exposure to test chemicals with and without a liver enzyme activation mixture (a cofactor supplemented post-mitochondrial fraction), cell cultures are treated with spindle inhibitors, such as colchicine to accumulate cells in a metaphase-like stage of mitosis (c-metaphase). Cells are harvested at appropriate times and chromosome preparations are made. Preparations are stained and metaphase cells are analyzed for chromosomal abnormalities.

1.5. Quality criteria

None.

1.6. Description of the test method**1.6.1. Preparations**

Test chemicals are prepared in culture medium or dissolved in appropriate vehicles prior to treatment of the cells. Established cell lines or cultures of primary cells are used, e.g. Chinese hamster cells and human lymphocytes.

1.6.2. Test conditions

Number of cultures:

At least duplicate cultures are used for each experimental point.

Use of negative and positive control:

Solvent (when the solvent is not the culture medium or water), liver enzyme activation mixture, liver enzyme activation mixture and solvent, and untreated controls are used as negative controls.

In each experiment a positive control is included; when liver enzyme activation mixture is used to activate the test chemical, a compound known to require metabolic activation must be used as a positive control.

Dose level:

At least three doses of the test compound over at least a one-log dose range are employed, the highest dose suppressing the mitotic activity by approximately 50 %.

Culture conditions:

Appropriate culture medium, incubation conditions (e.g. temperature, culture vessels used, CO₂ concentrations and humidity) are used.

1.6.3. Procedure**1.6.3.1. Preparation of cultures**

Established cell lines: Cells are generated from stock cultures (e.g. by trypsinization or by shaking off), seeded in culture vessels at appropriate density, and incubated at 37 °C.

Human lymphocytes: Heparinized whole blood is added to culture medium containing phytohaemagglutinin, fetal calf serum and antibiotics and incubated at 37 °C.

1.6.3.2. Treatment of the cultures with the test compound**(i) Treatment without liver enzyme activation mixture**

All treatments shall cover the period of one whole cell cycle and fixation schemes shall ensure the analysis of first post treatment mitoses of cells treated at different stages in the cycle.

When the treatment does not cover the length of one whole cell cycle, multiple fixation times are chosen to sample cells that are in different stages of the cell cycle during the treatment i.e. G₁, S and G₂.

The test chemical is added to cultures of established cell lines when they are in the exponential stage of growth. Human lymphocyte cultures are treated while they are in a semi-synchronous condition.

If the test chemical changes the cell cycle length the fixation interval has to be changed accordingly.

(ii) Treatment with liver enzyme activation mixture

For the treatment, the test compound in combination with the activation system should be present for as long as possible without exerting a toxic effect on the cells. If for toxicity reasons this treatment does not cover the length of a whole cell cycle, multiple fixation times are chosen to sample cells that are in different stages of the cell cycle during the treatment, i.e. G₁, S and G₂.

Harvesting cells:

Cell cultures are treated with the spindle inhibitor one to two hours prior to harvesting. Each culture is harvested and processed separately for the preparation of chromosomes.

1.6.3.3. Chromosome preparation

Chromosome preparations involve hypotonic treatment of the cells, fixation, spreading on slides, and staining.

Analysis:

At least 100 well-spread metaphases per culture are analyzed for chromosomal aberrations. Slides are coded before analysis. In human lymphocytes only metaphases containing 46 centromeres are analyzed.

In established cell lines only metaphases containing ± 2 centromeres of the modal number are analyzed.

2. DATA

Data are presented in a tabular form. Chromatid-type aberrations (gaps, breaks, interchanges), chromosome-type aberrations (gaps, breaks, minutes, rings, dicentrics, polycentrics) and the number of aberrant metaphases (including and excluding gaps) are listed separately for all treated and control cultures. The data are evaluated by appropriate statistical methods.

3. REPORTING**3.1. Test report**

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

- cells used,
- test conditions: composition of medium, CO₂ concentration, incubation temperature, incubation time, dose levels, treatment time, duration of treatment with and concentration of the spindle inhibitor used, type of liver enzyme activation mixture used, positive and negative controls,
- number of cell cultures,
- number of metaphases analyzed (data given separately for each culture),
- mitotic index,
- type and number of aberrations given separately for each treated and control culture, modal number of chromosomes in established cell lines used,
- statistical evaluation,
- discussion of the results,
- interpretation of the results.

3.2. Evaluation and interpretation

See General Introduction Part B (C).

4. REFERENCES

See General Introduction Part B (D).

B. 11. OTHER EFFECTS — MUTAGENICITY**IN VIVO MAMMALIAN BONE-MARROW — CYTOGENETIC TEST, CHROMOSOMAL ANALYSIS****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

This *in vivo* cytogenetic test is a short-term mutagenicity test for the detection of structural chromosomal aberrations. Chromosomal aberrations are generally evaluated in first post-treatment mitoses. With chemical mutagens, the majority of the induced aberrations are of the chromatid type.

The method employs bone-marrow cells of mammals which are exposed to test chemicals by appropriate routes and are sacrificed at sequential intervals. Animals are further treated, prior to sacrifice, with a spindle inhibitor such as colchicine to accumulate cells in a metaphase-like stage of mitosis (c-metaphase). Air-dried chromosome preparations from the cells are made and stained and metaphases are analyzed microscopically for chromosomal aberrations.

1.5. Quality criteria

None.

1.6. Description of the test method**1.6.1. Preparations**

Test chemicals are dissolved in normal saline. If insoluble, they are dissolved or suspended in appropriate vehicles.

Freshly prepared solutions of the test compound are employed. If a vehicle is used to facilitate dosing, it must not interfere with the test compound or produce toxic effects.

1.6.2. Test conditions**1.6.2.1. Experimental animals**

Rodent species, such as rats, mice or Chinese hamsters, are used. Healthy young adult animals are randomized and assigned to treatment and control groups.

1.6.2.2. Number and sex

At least five female and five male animals per experimental and control group are employed. Thus, 10 animals would be sacrificed per time period per group if several test times after treatment are included in the experimental schedule.

1.6.2.3. Route of administration

Test compounds should generally be administered only once. Based on toxicological information a repeated treatment schedule can be employed. However, the repeated treatment schedule can only be applied if the test compound does not exhibit cytotoxic effects in bone-marrow. The usual routes of administration are oral and intraperitoneal injection. Other routes of administration may be appropriate.

1.6.2.4. Use of negative and positive controls

A compound known to produce chromosomal aberrations *in vivo* is employed as a positive control and a negative (solvent) control group is also included in the design of each experiment.

1.6.2.5. Dose level

For the base set, one dose of the test compound is used, the dose being the maximum tolerated dose or that producing some indication of cytotoxicity (e.g. partial inhibition of mitosis).

Additional dose levels may be used where these are indicated by scientific reasons.

If the test is being used as a method for verification at least two additional dose levels should be used.

1.6.3. Procedure

The test may be performed in two ways:

- (i) Animals are treated with the test compound once, at the highest tolerated dose. Samples are taken three times after treatment. The central sampling interval is 24 hours. Since cell cycle kinetics can be influenced by the test chemical, one earlier and one later sampling interval, adequately spaced within the range of six to 48 hours, is applied.

When additional dose levels are used samples should be taken at the particularly sensitive intervals or, if that is not known, 24 hours after treatment.

- (ii) If pharmacokinetic and metabolic information indicate a repeated treatment schedule, repeated dosage can be employed and samples should be taken six and 24 hours after the last treatment.

Bone-marrow preparation:

Prior to sacrifice, animals are injected intraperitoneally with an appropriate dose of the spindle inhibitor to obtain an adequate number of cells in c-metaphase. Bone-marrow is obtained from both femora of freshly killed animals by rinsing with an isotonic solution. After appropriate hypotonic treatment the cells are fixed and then spread on slides. After air-drying the slides are stained.

Analysis:

Slides are coded before microscopic analysis. At least 50 well-spread metaphases with the complete number of centromeres are analyzed per animal for structural chromosomal aberrations. Additionally, the mitotic indexes may be established for each animal.

2. DATA

Data are presented in a tabular form. Chromatid- and isochromatid-type aberrations (gaps, breaks, interchanges), and the mitotic indexes, where established, are listed separately for all treated and control animals. Mean numbers and standard deviations for each experimental and control group are also listed. The data are evaluated by appropriate statistical methods.

3. REPORTING**3.1. Test report**

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

- species, strain and age of animals used,
- number of animals for each sex in experimental and control groups,
- test conditions: detailed description of treatment and sampling schedule, dose levels, duration of treatment with and concentration of the spindle inhibitor used,
- number of metaphases analyzed per animal,
- mitotic indexes, where established,
- type and number of aberrations given separately for each treated and control animal,
- statistical evaluation,
- discussion of the results,
- interpretation of the results.

3.2. Evaluation and interpretation

See General Introduction Part B (C).

4. REFERENCE

See General Introduction Part B (D).

B. 12. OTHER EFFECTS — MUTAGENICITY**MICRONUCLEUS TEST****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 micronucleus test is a mammalian short-term *in vivo* test for the detection of chromosomal damage or damage of the mitotic apparatus by chemicals. The basis of this assay is an increase in micronuclei in the polychromatic erythrocytes of treated animals versus the controls.

Micronuclei are formed from chromosomal fragments or whole chromosomes lagging in mitosis. When erythroblasts develop into erythrocytes, the main nucleus is expelled while the micronucleus may be maintained in the cytoplasm. Young polychromatic erythrocytes in the bone-marrow of laboratory mammals which were exposed to test substances by appropriate routes are used in this test. The bone-marrow is extracted and smear preparations are made and stained. Polychromatic erythrocytes are scored for micronuclei under the microscope and the ratio of polychromatic to normochromatic erythrocytes is established.

1.5. Quality criteria

None.

1.6. Description of the test method**1.6.1. Preparations**

Test chemicals are dissolved in isotonic solution. If insoluble, they are dissolved or suspended in appropriate vehicles. If a vehicle is used, it must not interfere with the test compound or produce toxic effects. Normally, freshly prepared solutions of the test compound are employed.

1.6.2. Test conditions**1.6.2.1. Experimental animals**

Mice are recommended, but other mammals may be used. Healthy young adult animals are randomized and assigned to treatment and control groups.

1.6.2.2. Number and sex

At least five female and five male animals per experimental and control group are employed. Thus, 10

animals would be sacrificed per time period per group if several test times after treatment are included in the experimental schedule.

1.6.2.3. Route of administration

Test compounds should generally be administered only once. Based on toxicological information, a repeated treatment schedule can be employed. However, the repeated treatment schedule can only be applied if the test compound does not exhibit cytotoxic effects in bone-marrow. The usual routes of administration are oral and intraperitoneal injection. Other routes of administration may be appropriate.

1.6.2.4. Use of negative and positive controls

Both positive and negative (solvent) controls are to be used in each experiment.

1.6.2.5. Dose level

For the base set one dose of the test compound is used, the dose being the maximum tolerated dose or that producing some indication of cytotoxicity, e.g. by a change in the ratio of polychromatic to normochromatic erythrocytes.

Additional dose levels may be used where these are indicated by scientific reasons.

If the test is being used as a method for verification at least two additional dose levels should be used.

1.6.3. Procedure

The test may be performed in two ways:

(i) Animals are treated with the test compound once, at the highest tolerated dose. Sampling times should coincide with the maximum response of the assay, which varies with the test compound. Therefore, using the highest dose, samples of bone-marrow are taken at least three times starting not earlier than 12 hours after treatment, with appropriate intervals following the first sample, but not extending beyond 72 hours.

When additional dose levels are used, samples should be taken at the maximum sensitive period, or, if that is not known, 24 hours after treatment.

(ii) If pharmacokinetic and metabolic information indicate a repeated treatment schedule, repeated dosage can be employed and samples should be taken at least three times starting not earlier than 12 hours after the last treatment and at appropriate intervals following the first sample but not extending beyond 72 hours.

Bone-marrow preparation:

Bone-marrow is obtained from both femora of freshly killed animals by rinsing with fetal calf serum. The cells are sedimented by centrifugation and the supernatant is discarded. Drops of the homogeneous cell suspension are put on slides and spread as a smear. After air-drying the slides are stained.

Analysis:

Slides are coded before microscopic analysis. At least 1 000 polychromatic erythrocytes per animal are scored for the incidence of micronuclei.

The ratio of normochromatic to polychromatic erythrocytes is determined for each animal by counting a total of 1 000 erythrocytes.

2. DATA ,

Data are presented in a tabular form. Thus the number of polychromatic erythrocytes scored, the number of polychromatic erythrocytes with micronuclei, and the percent micronucleated cells are listed separately for each experimental and control animal, as well as the ratio of normochromatic to polychromatic erythrocytes. Mean numbers and standard deviations for each experimental and control group are also listed. The listed data are evaluated by appropriate statistical methods.

3. REPORTING**3.1. Test report**

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

- species, strain and age of animals used,
- number of animals of each sex in experimental and control groups,
- test conditions: detailed description of treatment and sampling schedule, dose levels, toxicity data, negative and positive controls,
- criteria for scoring micronuclei,
- dose/effect relationship when possible,
- statistical evaluation,
- discussion of the results,
- interpretation of the results.

3.2. Evaluation and interpretation

See General Introduction Part B (C).

4. REFERENCES

See General Introduction Part B (D).

B. 13. OTHER EFFECTS — MUTAGENICITY***ESCHERICHIA COLI* — REVERSE MUTATION ASSAY****1. METHOD****1.1. Introduction**

See General Introduction Part B (A).

1.2. Definition

See General Introduction Part B (B).

1.3. References substances

None.

1.4. Principles of the test method

The *Escherichia coli* tryptophan (*trp*) reversion system is a microbial assay which measures *trp*⁻ → *trp*⁺ reversion by chemicals which cause base changes in the genome of the organism.

Bacteria are exposed to test chemicals with and without metabolic activation. After a suitable period of incubation on minimal medium, revertant colonies are counted and compared to the number of spontaneous revertants in an untreated and/or solvent control culture.

1.5. Quality Criteria

None.

1.6. Description of the test method

The following methods may be used to perform the assay: (1) the preincubation method; and (2) the direct incorporation method in which bacteria and test agent are mixed in overlay agar and poured over the surface of a selective agar plate.

1.6.1. Preparation**1.6.1.1. Bacteria**

Bacteria are grown at 37 °C up to late exponential or early stationary phase of growth. Approximate cell density should be 10⁸ to 10⁹ cells per millilitre.

1.6.1.2. Metabolic activation

Bacteria should be exposed to the test substance both in the presence and absence of an appropriate mammalian liver enzyme activation mixture (a cofactor supplemented post-mitochondrial fraction) prepared from mice or rats pretreated with enzyme inducing agents.

1.6.2. *Test condition*

1.6.2.1. *Tester strains*

Three strains, WP2, WP2 uvr A and WP2 uvr A pKM 101 should be used. Recognized methods of stock culture preparations and storage are to be used. The growth requirements and the genetic identity of the strains, their sensitivity to UV radiation or mitomycin C and the resistance to ampicillin in strain WP2 uvr A pKM 101 has to be checked. The strains should also yield spontaneous revertants within the frequency ranges expected.

1.6.2.2. *Media*

An appropriate medium for the expression and selection of mutants is used with an adequate overlay agar.

1.6.2.3. *Use of negative and positive controls*

Concurrent untreated and solvent controls have to be performed. Positive controls have to be conducted also for two purposes:

(i) To confirm the sensitivity of bacterial strains.

Methyl methane sulphonate, 4-nitroquinoline oxide or ethylnitrosourea may be used as positive controls for tests without metabolic activation.

(ii) To ensure the activity of the appropriate metabolizing systems.

A positive control for the activity of one (the) metabolizing system for all strains is 2-aminoanthracene. When available, a positive control of the same chemical class as the chemical under test should be used.

1.6.2.4. *Amount of test substance per plate*

At least five different amounts of test chemical are tested, with half-log intervals between plates. Substances are tested up to the limit of solubility or toxicity. Toxicity is evidenced by a reduction in the number of spontaneous revertants, a clearing of the background lawn, or by degree of survival of treated cultures. Non-toxic chemicals should be tested to 5 mg per plate before considering the test substance negative.

1.6.2.5. *Incubation conditions*

Plates are incubated for 48 up to 72 hours at 37 °C.

1.6.3. *Procedure*

For the direct plate incorporation method without enzyme activation, the chemical and 0,1 ml of a fresh bacterial culture are added to 2,0 ml of overlay agar. For tests with metabolic activation, 0,5 ml of liver enzyme activation mixture containing an adequate amount of post-mitochondrial fraction is added to the agar overlay after the addition of test chemical and bacteria. The contents of each tube are mixed and poured over the surface of a selective agar plate. Overlay agar is allowed to solidify and plates are incubated at 37 °C for 48 up to 72 hours. At the end of the incubation period, revertant colonies per plate are counted.

For the preincubation method, a mixture of test chemical, 0,1 ml of a fresh bacterial culture and an adequate amount of liver enzyme activation mixture or the same amount of buffer is preincubated before adding 2,0 ml of overlay agar. All other procedures are the same as for the incorporation method.

All plating for both methods is done at least in triplicate.

2. DATA

The numbers of revertant colonies per plate are reported for both control and treated series. Individual plate counts, the mean number of revertant colonies per plate and standard deviations should be presented for the tested chemical and the controls. All results are confirmed in an independent experiment. Data should be evaluated using appropriate statistical methods.

3. REPORTING

3.1. Test report

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

- bacteria, strain used,
- test conditions: dose levels, toxicity, composition of media; treatment procedures (preincubation incubation); liver enzyme activation mixture; reference substances, negative controls,
- individual plate count, the mean number of revertant colonies per plate, standard deviation, dose/effect relationship when possible,
- discussion of the results,
- interpretation of the results.

3.2. Evaluation and interpretation

See General Introduction Part B (C).

4. REFERENCES

See General Introduction Part B (D).

B. 14. OTHER EFFECTS — MUTAGENICITY

SALMONELLA TYPHIMURIUM — REVERSE MUTATION ASSAY

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 *Salmonella typhimurium* histidine (his) reversion system is a microbial assay which measures *his*⁻ → *his*⁺ reversion by chemicals which cause base substitutions or frameshift mutations in the genome of this organism.

Bacteria are exposed to test chemicals with and without metabolic activation and plated on minimal medium. After a suitable period of incubation, revertant colonies are counted and compared to the number of spontaneous revertants in an untreated and/or solvent control culture.

1.5. Quality Criteria

None

1.6. Description of the test method

1.6.1. Preparations

1.6.1.1. Bacteria

Fresh cultures of bacteria are grown at 37 °C until late exponential or early stationary phase of growth. Approximate cell density should be 10⁸ to 10⁹ cells per millilitre.

1.6.1.2. Metabolic activation

Bacteria should be exposed to the test substance both in the presence and absence of an appropriate mammalian liver enzyme activation mixture (a cofactor supplemented post-mitochondrial fraction) prepared from mice or rats pretreated with enzyme inducing agents.

1.6.2. Test conditions

1.6.2.1. Tester strains

At least four strains TA 1535, TA 1537, TA 98 and TA 100 are to be used; other strains, such as TA 1538 may be used in addition. Recognized methods of stock culture preparation and storage are to be

used. The growth requirements and the genetic identity of the strains, their sensitivity to UV radiation and crystal violet, and their resistance to ampicillin must be checked. The strains should also yield spontaneous revertants within the frequency ranges expected.

1.6.2.2. Media

An appropriate selective medium is used with an adequate overlay agar.

1.6.2.3. Use of negative and positive controls

Concurrent untreated and solvent controls have to be performed. Positive controls have to be conducted also for two purposes:

- (i) To confirm the sensitivity of the bacterial strains.

The following compounds may be used for tests without metabolic activations:

<i>Strains</i>	<i>Reverts with</i>
TA 1535, TA 100	Sodium azide
TA 1538, TA 98	2-nitrofluorene
TA 1537	9-aminoacridine

- (ii) To ensure the activity of the appropriate metabolizing system.

A positive control for the activity of one metabolizing system for all strains is 2-aminoanthracene. When available a positive control of the same chemical class as the chemical under test should be used.

1.6.2.4. Amount of test substance per plate

At least five different amounts of test chemical are tested, with half-log intervals between plates. Substances are tested up to the limit of solubility or toxicity. Toxicity is evidenced by a reduction in the number of spontaneous revertants, a clearing of the background lawn, or by degree of survival of treated cultures. Non-toxic chemicals should be tested to 5 mg per plate before considering the test substance negative.

1.6.2.5. Incubation conditions

Plates are incubated for 48 to 72 hours at 37 °C.

1.6.3. Procedure

For the direct plate incorporation method without enzyme activation, the test chemical and 0,1 ml of fresh bacterial culture are added to 2,0 ml of overlay agar. For tests with metabolic activation, 0,5 ml of liver enzyme activation mixture containing an adequate amount of post-mitochondrial fraction is added to the agar overlay after the addition of the test chemical and bacteria. The contents of each tube are mixed and poured over the surface of a selective agar plate. Overlay agar is allowed to solidify and plates are incubated at 37 °C for 48 to 72 hours. At the end of the incubation period, revertant colonies per plate are counted. For the preincubation method, a mixture of the test chemical, 0,1 ml of fresh bacterial culture and an adequate amount of liver enzyme activation mixture or the same amount of buffer is preincubated before adding 2,0 ml of overlay agar. All other procedures are the same as for the direct plate incorporation method.

All plating for both methods is done at least in triplicate.

2. DATA

The number of revertant colonies per plate are reported for both control and treated series.

Individual plate counts, the mean number of revertant colonies per plate and standard deviation should be presented for the tested chemical and the controls.

All results are confirmed in an independent experiment.

Data should be evaluated using appropriate statistical methods.

3. REPORTING**3.1. Test report**

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

- bacteria, strain used,
- test conditions: dose levels, toxicity, composition of media, treatment procedures (preincubation, incubation) liver enzyme activation mixture, reference substances, negative, controls,
- individual plate count, the mean number of revertant colonies per plate, standard deviation, dose/effect relationship when possible;
- discussion of the results,
- interpretation of the results.

3.2. Evaluation and interpretation

See General Introduction Part B (C).

4. REFERENCES

See General Introduction Part B (D).

PART C: METHODS FOR THE DETERMINATION OF ECOTOXICITY**C. 1. ACUTE TOXICITY FOR FISH****1. METHOD****1.1. Introduction**

It is desirable to have, as far as possible, information on the water solubility, vapour pressure, chemical stability, dissociation constants and biodegradability of the test substance to help in the selection of the most appropriate test method (static, semi-static or flow-through) for ensuring satisfactorily constant concentrations of the test substance over the period of the test.

Other information required, e.g. structural formula, degree of purity, nature and percentage of significant impurities, presence and amounts of additives, and n-octanol/water partition coefficient should be taken into consideration in both the planning and interpretation of the results of the test.

1.2. Definitions and units

Acute toxicity is the discernible adverse effect induced in an organism within a short time (days) of exposure to a substance. In the present test, acute toxicity is expressed as the median lethal concentration (LC_{50}), that is the concentration in water which kills 50 % of a test batch of fish within a continuous period of exposure which must be stated.

All concentrations of the test substance are given in weight by volume (milligrams per litre) and also expressed as weight by weight (parts per million).

1.3. Reference substances

A reference substance may be tested, to demonstrate that under the laboratory test conditions the response of tested species have not changed significantly.

No reference substances are specified for this test.

1.4. Principle of the test method

The fish are exposed to the test substance(s) added to water at a range of concentrations for a minimum period of 48 hours, but preferably for 96 hours. Mortalities are recorded at least at 24-hour intervals, and the concentrations killing 50 % of the fish (LC_{50}) at each time of observation are calculated where possible.

1.5. Quality criteria

The mortality in the controls must not exceed 10 % at the end of the test.

The oxygen concentration must have been more than 60 % of air-saturation value throughout.

There should be evidence, either from analysis or from chemical properties or from the test system used, that the concentration of the substance being tested has been satisfactorily maintained (e.g. within 80 % of the initial concentration over the test period).

1.6. Description of the test method

Three types of procedure can be used:

Static test:

Toxicity test with aquatic organisms in which no flow of test solution occurs. (Solutions remain unchanged throughout the duration of the test.)

Semi-static test:

Test without flow of solution, but with regular batchwise renewal of test solutions after prolonged periods (e.g. 24 hours).

Flow-through test:

Toxicity test in which the water is renewed constantly in the test chambers, the chemical under test being transported with the water used to renew the test medium.

1.6.1. Reagents

1.6.1.1. Solutions of test substances

Stock solutions of the required strength are prepared by dissolving the substance in deionized water or water according to 1.6.1.2.

Stock solutions of substances of low aqueous solubility may be prepared by ultrasonic dispersion or, if necessary by use of organic solvents, emulsifiers or dispersants. When such auxiliary substances are used the control fish should be exposed to the same concentration of the auxiliary substance as that used in the highest concentration of the test substance. The concentration of such auxiliaries should not exceed 0,1 g per litre.

The chosen test concentrations are prepared by dilution of the stock solution. If high concentrations are tested, the substance may be dissolved in the dilution water directly.

The test should be carried out without adjustment of the pH. If there is evidence of marked change in the pH, it is advised that the test should be repeated with pH adjustment and the results reported. In that case, the pH value of the stock solution should be adjusted to the pH value of the dilution water unless there are specific reasons not to do so. HCl and NaOH are preferred for this purpose. This pH adjustment should be made in such a way that the concentration of test substance in the stock solution is not changed to any significant extent. Should any chemical reaction or physical precipitation of the test compound be caused by the adjustment, this should be reported.

1.6.1.2. Holding and dilution water

Drinking-water supply (uncontaminated by dangerous concentrations of chlorine, heavy metals or other substances), good-quality natural water or reconstituted water (see Appendix 1) may be used. Waters with a total hardness of between 50 and 250 mg per litre (as CaCO_3) and with a pH of 6,0 to 8,5 are to be preferred.

1.6.2. Apparatus

All apparatus must be made of chemically inert material:

- automatic dilution system (for flow-through test),
- oxygen meter,
- equipment for determination of hardness of water,
- adequate apparatus for temperature control,
- pH meter.

1.6.3. *Test fish*

One or more species may be used, the selection being at the discretion of the testing laboratory.

It is suggested that the species used be selected on the basis of important practical criteria, such as their ready availability throughout the year, their ease of maintenance, their convenience for testing, their relative sensitivity and any economic, biological or ecological factors which have any bearing. The fish should be in good health and free from any apparent malformation.

Fish species recommended for testing are given in Appendix 2.

1.6.3.1. *Holding*

Test fish should preferably come from a single stock of similar length and age. The fish must be held for at least 12 days, in the following conditions:

tanks:

at least 300 litres for cold-water fish, at least 100 litres for warm-water fish are recommended,

loading:

appropriate to the system (recirculation or flow-through) and the fish species,

water:

see point 1.6.1.2,

light:

12 to 16 hours photoperiod daily,

dissolved oxygen concentration:

at least 80 % of air-saturation value,

feeding:

three times per week or daily, ceasing 24 hours before the test is started.

1.6.3.2. *Mortality*

Following a 48-hour settling-in period, mortalities are recorded and the following criteria applied:

- greater than 10 % of population in seven days: rejection of entire batch,
- between 5 and 10 % of population: holding period continued for seven additional days. If no further mortalities occur, the batch is acceptable, otherwise it must be rejected,
- less than 5 % of population: acceptance of the batch.

1.6.4. *Adaptation*

All fish must be exposed to water of the quality and the temperature to be used in the test for at least seven days before they are used.

1.6.5. *Test procedure*

A range-finding test can precede a definitive test, in order to obtain information about the range of concentrations to be used in the main test.

One control without the test substance is run, using the auxiliary substance if appropriate, in addition to the test series.

Concentrations should not fall by more than 20 % during the period of the test. Depending on the physical and chemical properties of the test compound, a static, semi-static, or a flow-through test should be selected as appropriate, to meet this requirement.

Fish are exposed to the substance as described below:

- duration:
minimum 48 hours but preferably 96 hours,
- number of animals:
at least 10 per concentration,
- tanks:
of suitable capacity in relation to the recommended loading,
- loading:
maximum loading of 1,0 g per litre for static and semi-static tests is recommended; for flow-through systems, higher loading can be acceptable,
- test concentration:
one control and at least five concentrations differing by a constant factor not exceeding 1,8 and spanning the range of 0 to 100 % mortality,
- water:
see 1.6.1.2,
- light:
12 to 16 hours photoperiod daily,
- temperature:
appropriate to the species (Appendix 2), but within ± 1 °C within any particular test.
- dissolved oxygen concentration:
not less than 60 % of the air-saturation value at the selected temperature,
- feeding:
none.

The fish are inspected after the first 2 to 4 hours and at least at 24-hour intervals. Fish are considered dead if touching of the caudal peduncle produces no reaction and no breathing movements are visible. Dead fish are removed when observed and mortalities are recorded.

Records are kept of visible abnormalities (e.g. loss of equilibrium, changes in swimming behaviour, respiratory function, pigmentation, etc.).

Measurements of pH, dissolved oxygen and temperature must be carried out daily.

2. DATA AND EVALUATION

Plot percentage mortality for each recommended exposure period against concentration on logarithmic-probability paper. Fit a line, by eye, to the points and read off the concentration corresponding to the 50 % response (see Appendix 3).

This is an estimate of the LC_{50} for the appropriate exposure period.

When the data are adequate, the median concentration LC_{50} and its confidence limits ($p = 0,05$) can be estimated using standard procedures.

The LC_{50} value should be rounded off to one (or at most two) significant figures.

In those cases where the slope of the concentration/percentage response curve is too steep to permit calculation of the LC_{50} , a graphical estimate of this value is sufficient.

When two consecutive concentrations, at a ratio of 1,8 give only 0 and 100 % mortality, these two values are sufficient to indicate the range within which the LC_{50} falls.

If it should be observed that the stability or homogeneity of the test substance cannot be maintained this should be reported and care should be taken in the interpretation of the results.

3. REPORTING

The test report shall, if possible, contain:

- information about test organism (scientific name, strain, supplier, any pretreatment, size and number used in each test concentration),
- list of the concentrations used and any available information on the stability at the concentrations of the tested chemical in the test solution,
- description of test equipment,
- if chemical analyses are performed, methods used and results,
- dilution-water source and major chemical characteristics (pH, hardness, temperature),
- concentration of any auxiliary substances,
- in the case of substance of low aqueous solubility, the method of preparation of stock and test solution.
- reasons for the choice and details of the test procedure used (e.g. test duration, static, semi-static, dosing rate, flow-through rate, whether aerated, fish loading, etc.),
- light regime,
- highest test concentration causing no mortality within the period of the test,
- lowest test concentration causing 100 % mortality within the period of the test,
- cumulative mortality at each concentration and the control (or control with the auxiliary substance if required) to the recommended observation times,
- LC_{50} values at each of the recommended observation times (with 95 % confidence limits, if possible),
- statistical procedures used for determining the LC_{50} values,
- graph of this concentration/percentage response curve at the end of the test,
- if possible the slope of the concentration/percentage response curve at the end of the test and its 95 % confidence limits,
- dissolved oxygen concentration, pH values and temperature of the test solutions every 24 hours,
- if a reference substance is used, the results obtained,
- evidence that quality criteria have been fulfilled.

4. REFERENCES

- (1) OECD, Paris, 1981, *Test Guideline 203*, Decision of the Council C(81) 30 final.

*Appendix 1***Reconstituted water***Example of a suitable dilution water*

All chemicals must be of analytical grade.

The water should be good-quality distilled water, or deionized water with a conductivity less than $5 \mu\text{Scm}^{-1}$

Stock solutions

CaCl ₂ ·2H ₂ O (calcium chloride dihydrate):	11,76 g
Dissolve in, and make up to 1 litre with water.	
MgSO ₄ ·7H ₂ O (magnesium sulphate heptahydrate):	4,93 g
Dissolve in, and make up to 1 litre with water.	
NaHCO ₃ (sodium hydrogen carbonate):	2,59 g
Dissolve in, and make up to 1 litre with water.	
KCl (potassium chloride):	0,23 g
Dissolve in, and make up to 1 litre with water.	

Reconstituted dilution water

Mix 25 ml of each of the four stock solutions and make up to 1 litre with water.

Aerate until the dissolved oxygen concentration equals the air-saturation value.

The pH should be $7,9 \pm 0,3$.

If necessary adjust the pH with NaOH (sodium hydroxide) or HCl (hydrochloric acid).

The dilution water so prepared is set aside for about 12 hours and must not be further aerated.

The sum of the Ca and Mg ions in this solution is 2,5 mmol per litre. The ratio of Ca:Mg ions is 4:1 and of Na:K ions is 10:1. The total alkalinity of this solution is 0,8 mmol per litre.

Any deviation in the preparation of the dilution water must not change the composition or properties of the water.

Appendix 2

Fish species recommended for testing

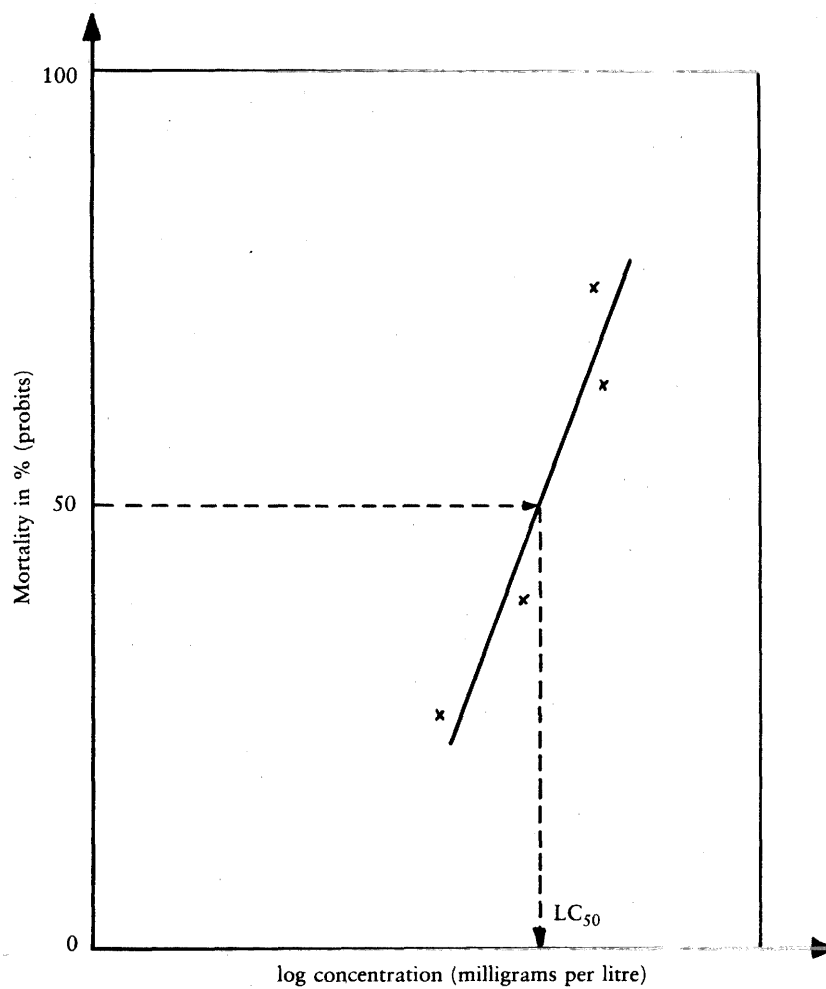
Recommended species	Recommended range of test temperature (°C)	Recommended total length of test animal (cm)
<i>Brachydanio rerio</i> (Teleostei, Cyprinidae) (Hamilton-Buchanan) Zebra-fish	20 to 24	3,0 ± 0,5
<i>Pimephales promelas</i> (Teleostei, Cyprinidae) Fathead minnow	20 to 24	5,0 ± 2,0
<i>Cyprinus carpio</i> (Teleostei, Cyprinidae) (Linnaeus 1758) Common carp	20 to 24	6,0 ± 2,0
<i>Oryzias latipes</i> (Teleostei, Poeciliidae) (Schlegel 1850) Red killifish	20 to 24	3,0 ± 1,0
<i>Poecilia reticulata</i> (Teleostei, Poeciliidae) (Peters 1859) Guppy	20 to 24	3,0 ± 1,0
<i>Lepomis macrochirus</i> (Teleostei, Centrarchidae) (Linnaeus 1758) Bluegill	20 to 24	5,0 ± 2,0
<i>Salmo gairdneri</i> (Teleostei, Salmonidae) (Richardson 1836) Rainbow trout	13 to 17	6,0 ± 2,0
<i>Leuciscus idus</i> (Teleostei, Cyprinidae) (Linnaeus 1758) Golden orfe	20 to 24	6,0 ± 2,0

Collection

The fish listed above are easy to rear and/or are widely available throughout the year. They are capable of being bred and cultivated either in fish farms or in the laboratory, under disease- and parasite-controlled conditions, so that the test animal will be healthy and of known parentage. These fish are available in many parts of the world.

Appendix 3

Example of concentration: percentage mortality

Example of determination of LC_{50} using log-probit paper

Appendix 4

List of examples of standard procedure

- (1) OECD, Paris, 1981, *Test Guideline 203*, Decision of the Council, C(81) 30 final.
 - (2) ISO/TC/147/SC 5/WG/3.- *Draft proposal for screening chemicals and products for acute toxicity to fish using a static, semi-static or flow-through method*, Document 7346/I, II, III, 1980/06/15 ISO/DP.
 - (3) Eidgenössisches Department des Innern, Schweiz: *Richtlinien für Probenahme und Normung von Wasseruntersuchungsmethoden — Part II* 1974.
 - (4) DIN *Testverfahren mit Wasserorganismen*, 38 412 (L1) und L (15).
 - (5) AFNOR, *Determination de la toxicité aigue d'une substance vis-à-vis de Brachydanio rerio*, T90—303.
 - (6) JIS K 0102, *Acute toxicity test for fish*.
 - (7) *Degradability, ecotoxicity and bioaccumulation. The determination of the possible effects of chemicals and wastes on the aquatic environment*, volumes I and II, Government Publishing Office, The Hague, The Netherlands 1980.
 - (8) Environmental Protection Agency, 1975, *Methods for the acute toxicity tests with fish, macroinvertebrates and amphibians*, The Committee on Methods for Toxicity Tests with Aquatic Organisms, Ecological Research Series EPA-660-75-009.
 - (9) Environmental Protection Agency, January 1978, *Environmental monitoring and support laboratory*, Office of Research and Development, EPA-600/4-78-012.
 - (10) Environmental Protection Agency, *Toxic Substance Control*, 16 March 1979, part IV.
 - (11) *Standard methods for the examination of water and wastewater*, 14th edition, 1975, APHA-AWWA-WPCF.
 - (12) Commission of the European Communities, *Inter-laboratory test programme concerning the study of the ecotoxicity of a chemical substance with respect to the fish*, EEC Study D.8368, 22 March 1979.
 - (13) Litchfield, J. T. and Wilcoxon, F., *A simplified method for evaluating dose effects experiments*, *J. Pharm. Exp. Therap.*, vol. 96, 1949, p. 99.
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C. 2. ACUTE TOXICITY FOR *DAPHNIA*

1. METHOD

1.1. Introduction

It is desirable to have, as far as possible, information on the water solubility, vapour pressure, chemical stability, dissociation constants and biodegradability of the substance before starting the test.

Additional information (for instance structural formula, degree of purity, nature and percentage of significant impurities, presence and amount of additives, and n-octanol/water partition coefficient) should be taken into consideration in both the planning and interpretation of the results of the test.

1.2. Definitions and units

The Directive requirement for the LC_{50} for *Daphnia* is considered to be fulfilled by the determination of the EC_{50} as described in this test method.

Acute toxicity is expressed in this test as the median effective concentration (EC_{50}) for immobilization. This is the concentration (in terms of initial values) which immobilizes 50 % of the *Daphnia* in a test batch within a 24-hour exposure period. The 48-hour EC_{50} for immobilization can also be determined when practicable.

Immobilization:

Those animals which are not able to swim for 15 seconds after gentle agitation of the test container are considered to be immobile.

All concentrations of the test substance are expressed as weight by weight (parts per million).

1.3. Reference substances

A reference substance may be tested as a means of demonstration that under the laboratory test conditions the sensitivity of the test species has not changed significantly.

No reference substances are specified for this test.

1.4. Principle of the test method

The *Daphnia* are exposed to the test substance added to water at a range of concentrations for 24 hours; when necessary this duration can be prolonged to 48 hours.

Under otherwise identical test conditions, and an adequate range of test substance concentrations, different concentrations of a test substance exert different average degrees of effects on the swimming ability of *Daphnia*. Different concentrations result in different percentages of *Daphnia* being no longer capable of swimming at the end of the test. The concentrations causing zero or 100 % immobilization are derived directly from the test observations whereas 24-hour EC_{50} (or the 48-hour EC_{50}) is determined by calculation if possible.

A static system is used for this method, hence test solutions are not renewed during the exposure period.

1.5. Quality criteria

Immobilization in the control must not exceed 10 % at the end of the test.

The oxygen concentration must not be less than 2 mg per litre at the end of the test.

Test *Daphnia* should not have been trapped at the surface of the water at least in the control group.

1.6. Description of test method

1.6.1. Reagents

1.6.1.1. Solutions of test substances

Stock solutions of the required strength are prepared by dissolving the substance in deionized water or water according to 1.6.1.2.

Stock solutions of substances of low aqueous solubility may be prepared by ultrasonic dispersion or, if necessary, by use of organic solvents, emulsifiers or dispersants. When such auxiliary substances are used, the control *Daphnia* should be exposed to the same concentration of the auxiliary substance as that used in the highest concentration of the test substance. The concentration of such auxiliaries should not exceed 0,1 g per litre.

The chosen test concentrations are prepared by dilution of the stock solution. If high concentrations are tested, the substance may be dissolved in the dilution water directly.

The test should be carried out without adjustment of the pH. If there is evidence of marked change in the pH, it is advised that the test should be repeated with pH adjustment and the results reported. In that case, the pH value of the stock solution should be adjusted to the pH value of the solution water unless there are specific reasons not to do so. HCl and NaOH are preferred for this purpose. This pH adjustment should be made in such a way that the concentration of test substance in the stock solution is not changed to any significant extent. Should any chemical reaction or physical precipitation of the test compound be caused by the adjustment, this should be reported.

1.6.1.2. Culture and dilution water

Any water suitable for culturing *Daphnia*, either natural or reconstituted water (see Appendix) can be used in this test. To avoid the necessity for acclimation prior to the test, it is recommended that the culture water should be of the same quality as the culture water used for the test.

1.6.2. Apparatus

Normal laboratory apparatus and equipment should be used. Equipment which will come into contact with the test solutions should preferably be made entirely of glass:

- Oxygen meter (with microelectrode or other suitable equipment for measuring dissolved oxygen in low-volumes samples),
- adequate apparatus of temperature control,
- pH meter,
- equipment for the determination of hardness of water.

1.6.3. Test organism

Daphnia magna or *Daphnia pulex*, more than six hours and less than 24 hours old, at the beginning of the test, laboratory bred, free from overt disease and with a known history (e.g. breeding — any pretreatments, etc.).

1.6.4. *Test procedure*

A range-finding test can precede the definitive test, in order to obtain information about the range of concentrations to be used in the main test. One control test, with any auxiliary substance used but without the test substance, should be carried out in addition to the test series.

Daphnia are exposed to the substance as described below:

- duration:
at least 24 hours,
- number of animals:
at least 20 animals at each test concentration preferably divided into four batches of five animals each or two batches of 10,
- loading:
at least 2 ml of test solutions should be provided for each animal,
- test concentration:
the test solution should be prepared immediately before introduction of the *Daphnia*, preferably without using any solvent other than water. The concentrations are made up in a geometric series, at a concentration ratio of 1,8. Concentrations sufficient to give 0 and 100 % immobilization after 24 hours and a range of intermediate degrees of immobilizations permitting calculation of the 24 hours EC₅₀ should be tested together with control,
- water:
see 1.6.1.2,
- light:
a light-dark cycle is optional, complete darkness is acceptable,
- temperature:
the test temperature should be between 18 and 22 °C, but for each single test it should be constant within $\pm 1,0$ °C,
- aeration:
the test solutions must not be bubble-aerated,
- feeding:
none.

The pH and the oxygen concentration of the controls and of all the test concentrations should be measured at the end of the test; the pH of the test solutions should not be modified.

Volatile compounds should be tested in completely filled closed containers, large enough to prevent lack of oxygen.

Daphnia are inspected at least after 24 hours exposure and again after 48 hours if the test has been extended.

2. DATA AND EVALUATION

Plot cumulative percentage immobilization in each concentration after at least 24 hours exposure against concentration on logarithmic-probability paper. Fit a line to the points and read off the concentration corresponding to the 50 % response.

Where the data are adequate, the median concentration and its confidence limits ($p = 0,05$) can be estimated using standard procedures.

The EC_{50} value should be rounded off to one (or at most two) significant figures.

In those cases where the slope of the concentration / percentage response curve is too steep to permit calculation of the EC_{50} , a graphical estimate of this value is sufficient.

When two immediately consecutive concentrations at a ratio of 1,8 give only 0 and 100 % immobilization these two values are sufficient to indicate the range within which the EC_{50} falls.

If it should be observed that the stability or homogeneity of the test substance cannot be maintained then care should be taken in the interpretation of the results and it should be reported.

3. REPORTING

The test report shall, if possible, contain:

- information about the test organism (scientific name, strain; supplier or source, any pretreatment, breeding method — including source, kind and amount of food, feeding frequency),
- number of *Daphnia* used in each test concentration,
- list of the concentrations used and any available information on stability at the concentrations of the test chemical in the test solution,
- description of test vessels, volumes of solution in each, number of animals per vessel,
- if chemical analyses are performed, methods used and the results,
- dilution water source and major chemical characteristics,
- the method of preparation of stock and test solutions,
- concentrations of any auxiliary used (organic solvents, dispersants, etc.),
- light regime,
- highest tested concentration causing no immobilization within the period of the test,
- lowest tested concentration causing 100 % immobilization within the period of the test,
- cumulative immobilization in the blank, in the control with the auxiliary substance and in each tested concentration, at the recommended observation times (24 hours or 24 and 48 hours),
- EC_{50} values at each of the recommended observation times (with 95 % confidence limits, if possible),
- graph of the concentration/percentage response curve at the end of the test,
- statistical procedures used for determining the EC_{50} values,
- if possible, the slope of the concentration/percentage response curve at 24 hours and its 95 % confidence limits,
- dissolved oxygen concentration, pH values, temperature, of the test solutions,
- if a reference substance is used, its name and results obtained must be reported,
- evidence that the quality criteria have been fulfilled.

4. REFERENCES

- (1) OECD, Paris, 1981, *Test Guidelines 202*, Decision of the Council C(81) 30 final.
- (2) ISO *Inhibition of mobility of Daphnia magna Straus (Cladocera — crustacea)* ISO/6341.
- (3) AFNOR *Inhibition of mobility of Daphnia magna Straus (Cladocera — crustacea)* NFT 90 301 (April 1974).
- (4) DIN *Testverfahren mit Wasserorganismen 38412 (L1) und (L11)*.

*Appendix***Reconstituted water***Example of a suitable dilution water*

All chemicals must be of analytical grade.

The water should be good-quality distilled water, or deionized water with a conductivity less than $5 \mu\text{Scm}^{-1}$.

Stock solutions

CaCl ₂ ·2H ₂ O (calcium chloride dihydrate):	11,76 g
dissolve in, and make up to 1 litre with water	
MgSO ₄ ·7H ₂ O (magnesium sulphate heptahydrate):	4,93 g
dissolve in, and make up to 1 litre with water	
NaHCO ₃ (sodium hydrogen carbonate):	2,59 g
dissolve in, and make up to 1 litre with water	
KCl (potassium chloride):	0,23 g
dissolve in, and make up to 1 litre with water	

Reconstituted dilution water

Mix 25 ml of each of the four stock solutions and make up to 1 litre with water.

Aerate until the dissolved oxygen concentration equals the air-saturation value.

The pH should be $7,9 \pm 0,3$. If necessary adjust the pH with NaOH (sodium hydroxide) or HCl (hydrochloric acid).

The dilution water so prepared is set aside for about 12 hours and need not be further aerated.

The sum of the Ca and Mg ions in this solution is 2,5 mmol per litre. The ratio of Ca : Mg ions is 4 : 1 and of Na : K ions is 10 : 1. The total alkalinity of this solution is 0,8 mmol per litre.

Any deviation in the preparation of the dilution water must not change the composition or properties of the water.

C. 3. DEGRADATION

BIOTIC DEGRADATION: MODIFIED OECD SCREENING TEST

1. METHOD

1.1. Introduction

The purpose of the method is the measurement of the biodegradability of water-soluble, non-volatile organic compounds in an aerobic, aqueous medium at a starting test concentration corresponding to 5 to 40 mg DOC (dissolved organic carbon) per litre. If the detection limits of organic carbon analyzers are improved, the use of lower test concentrations may be advantageous, particularly for toxic compounds. The organic carbon content of the test material must be established.

The method is applicable only to those organic test materials which, at the concentration used in the test:

- are at least water-soluble at the concentration to be tested (5 to 40 mg DOC per litre),
- have negligible vapour pressure,
- are not inhibitory to bacteria,
- do not significantly adsorb on glass surfaces.

Information on the relative proportions of the major components of the test material will be useful in interpreting the results obtained, particularly in those cases where the results are low or marginal.

Information on the toxicity to micro-organisms of the chemical may be useful for the interpretation of low results and in the selection of appropriate test concentrations.

1.2. Definitions and units

The degradation is defined as the percentage DOC removal with respect to the test material:

$$D_t = \left[1 - \frac{C_t - C_{bl(t)}}{C_0 - C_{bl(0)}} \right] \times 100$$

where:

- D_t = degradation in percent DOC-removal at time t ,
- C_0 = starting DOC concentration of the culture medium (mg DOC per litre),
- C_t = DOC concentration of the culture medium at time t (mg DOC per litre),
- $C_{bl(0)}$ = starting DOC concentration of the blank (mg DOC per litre),
- $C_{bl(t)}$ = DOC concentration of the blank at time t (mg DOC per litre).

1.3. Reference substances

The use of suitable control chemicals to check the activity of the inoculum is desirable.

Aniline, sodium acetate or sodium benzoate (for example) may be used for this purpose and they have to exhibit DOC-removals of $\geq 70\%$ within 10 days, counting from the day that the observed level of biodegradation first exceeds 10 %. These results must be obtained within the test duration of 28 days, otherwise the test is regarded as invalid, and should be repeated using an inoculum from a different source.

1.4. Principle of the test method

A predetermined amount of the compound is dissolved in an inorganic medium (mineral nutrient solution, fortified with a trace element and essential vitamin solution), providing a concentration corresponding to 5 to 40 mg DOC per litre. The solution is inoculated with a small number of micro-organisms from a mixed population and aerated at 20 to 25 °C in the dark or at least in diffused light only.

The degradation is followed by DOC analyses over a 28-day period.

The procedure is checked by means of a control substance.

A DOC blank must be determined in a parallel test containing neither test nor control material.

1.5. Quality criteria

The reproducibility of the method has been established in the OECD and EEC ring-tests.

The lowest concentration of the test compound for which this method can be used is largely determined by the detection limit of the organic carbon analysis (0,5 mg carbon per litre at the present state of the art) and the concentration of dissolved organic carbon in the nutrient solution.

1.6. Description of the test method

1.6.1. Reagents

1.6.1.1. Water

Deionized or distilled water free of toxic substances (copper in particular), for general use as a solvent. Water which has been deionized by distillation or ion exchange is suitable.

The distilled water must not contain more than 10 % of the organic carbon introduced by the test material.

High-purity water is necessary in view of the DOC analyses in the concentration range of 0 to 40 mg per litre. The contaminations result from inherent impurities but also from the ion-exchange resins and microbial developments (bacteria, algae under the influence of light, etc.). Only one water batch must be used for one test series which is to be monitored beforehand by DOC analysis. If necessary, suitable water may be obtained by UV irradiation or other means.

1.6.1.2. Nutrient solution

The nutrient solution contains per litre, 1 ml of each of the following solutions (a) to (f) in water (1.6.1.1.) (AR means analytical reagent):

- | | |
|--|------------|
| (a) KH_2PO_4 (potassium dihydrogen phosphate): | 8,50 g AR |
| K_2HPO_4 (dipotassium hydrogen phosphate): | 21,75 g AR |
| $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ (disodium hydrogen phosphate dihydrate): | 33,40 g AR |
| NH_4Cl (ammonium chloride): | 20,00 g AR |
| Dissolve in and make up to 1 000 ml with water (1.6.1.1). The pH value should be pH: 7,2 | |
| (b) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (magnesium sulphate heptahydrate): | 22,50 g AR |
| (c) CaCl_2 (calcium chloride): | 27,50 g AR |
| Dissolve in and make up to 1 000 ml with water (1.6.1.1) | |

- (d) $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (iron (III) chloride hexahydrate) 0,25 g AR
 Dissolve in and make up to 1 000 ml with water (1.6.1.1).
 This solution is freshly prepared immediately before use.
- (e) Trace element solution
 $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ (manganese (II) sulphate tetrahydrate) (= 30,23 mg $\text{MnSO}_4 \cdot \text{H}_2\text{O}$): 39,9 mg AR
 H_3BO_3 (boric acid): 57,2 mg AR
 $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (zinc sulphate heptahydrate): 42,8 mg AR
 $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$ (ammonium heptamolybdate (VI)) (= 36,85 mg $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$): 34,7 mg AR
 Fe-chelate ($\text{FeCl}_3 \cdot \text{EDTA}$): 100,0 mg AR
 Dissolve in and make up to 1 000 ml with water (1.6.1.1).
 Sterilization of the trace element stock solution at 120 °C, 2 atm 20 minutes.
- (f) Vitamin solution
 Biotin 0,2 mg AR
 Nicotinic acid 2,0 mg AR
 Thiamine 1,0 mg AR
 p-Aminobenzoic acid 1,0 mg AR
 Pantothenic acid 1,0 mg AR
 Pyridoxamine 5,0 mg AR
 Cyanocobalamin 2,0 mg AR
 Folic acid 5,0 mg AR
 dissolve in and make up to 100 ml with water (1.6.1.1).
 The solution is filtered sterile through 0,2 µm membrane filters. Instead of solution 1.6.1.2 (f), 15 mg of yeast extract may be used per 100 ml of water (1.6.1.1).
- 1.6.1.3. Control substances
 Aniline (freshly distilled), sodium acetate, sodium benzoate.
- 1.6.1.4. Mercuric chloride solution
 1 % of HgCl_2 in water (1.6.1.1).
- 1.6.2. Apparatus
- 1.6.2.1. Shaking machine accommodating 2-litre Erlenmeyer flasks either with automatic temperature controls or used in a constant temperature room at 20 to 25 °C.
- 1.6.2.2. Narrow neck 2-litre Erlenmeyer flasks (creased or fluted flasks are recommended). The flasks must be carefully cleaned with, e.g., alcoholic hydrochloric acid before use, rinsed and dried in order to avoid contamination with residues from previous tests. The flasks also have to be cleaned before their first use since they may be contaminated.
- 1.6.2.3. Membrane filtration apparatus.
- 1.6.2.4. Membrane filters 0,2 µm.
- 1.6.2.5. Carbon analyzer.
- 1.6.3. Preparation of the inoculum
 Any one of the following four sources may be used as inoculum provided that its viability is checked by means of a control substance (1.6.1.3).

1.6.3.1. Inoculum from a secondary effluent

The inoculum is obtained preferably from a secondary effluent of good quality collected from a treatment plant dealing with a predominantly domestic sewage. The effluent must be kept under aerobic conditions in the period between sampling and use. To prepare the inoculum, the sample is filtered through a coarse filter, the first 200 ml being discarded. The filtrate is kept aerobic until used. The inoculum must be used on the day of collection.

1.6.3.2. Inoculum from soil

100 g of soil (fertile, not sterile) are suspended in 1 000 ml of chlorine-free drinking water (soils with an extremely large content of clay, sand or organic carbon are unsuited). After stirring the suspension is allowed to settle for 30 minutes.

The supernatant is filtered through a coarse filter paper, the first 200 ml being discarded. The filtrate is aerated immediately, and aeration is continued until use. The inoculum must be used on the day of collection.

1.6.3.3. Inoculum from a surface water

An inoculum is drawn from a suitable surface water. The sample is filtered through a coarse filter paper, the first 200 ml being discarded. The filtrate is kept aerobic until used. The inoculum must be used on the day of collection.

1.6.3.4. Composite inoculum

Equal volumes of the three inoculum samples are mixed well, and the final inoculum drawn from this mixture.

The suitability of the inoculum is checked by means of a control substance (1.6.1.3).

1.6.4. *Test procedure*

The test materials are evaluated simultaneously in duplicate together with the control substance (1.6.1.3). A blank test with inoculum but without either test or control material for the determination of DOC blanks is also carried out.

A stock solution of the test material in water (1.6.1.1) is prepared. A volume of this stock solution is added to the nutrient solution (1.6.1.2) so that a carbon concentration of 5 to 40 mg DOC per litre is attained. The control material (1.6.1.3) is tested at a starting concentration corresponding to 20 mg DOC per litre.

900 ml of the nutrient solution are placed into two reaction vessels (1.6.2.2) and inoculated with 0,5 ml per litre portions of the inoculum (1.6.3). The opening of the vessel is covered with, e.g., aluminium foil in such a way that the exchange of air between the flask and the surrounding atmosphere is not unduly impeded (cotton wool is unsuitable because of the DOC analysis). The vessels are then inserted in the shaking machine. The temperature of 20 to 25 °C must be maintained unchanged during the test, and the vessels should be shielded from light. The air should be free of pollutants and toxic materials (chlorinated solvents, etc.).

In the course of the biodegradation test the DOC concentrations are determined in duplicate on the first day and on days 27 and 28. At least three additional analyses (around the seventh, 14th and 21st days) have to be performed to follow the course of the degradation.

Only the necessary volumes of culture medium may be drawn for each determination. The centrifugation or membrane filtration preceding the actual carbon determination requires differing volumes for the different instruments. Evaporation losses of the culture medium are to be made up by adding water (1.6.1.1) in the required amounts. The culture medium is to be mixed well before withdrawing a sample. Material adhering to the wall of the vessel has to be dissolved or suspended before sampling. The membrane filtration or centrifugation has to be done immediately. The filtered or

centrifuged samples have to be analyzed on the same day, otherwise they must be preserved with 0,05 ml of the HgCl_2 solution (1.6.1.4) for each 10 ml of nutrient medium or by storing them at 2 to 4 °C for up to 24 hours, or below -18 °C for longer periods.

If a plateau is observed before the 28th day, the test can be finished. If degradation has obviously started by day 28, but has not reached a plateau, it is considered good practice to extend the experiment for one to two weeks longer.

All steps require great care and cleanliness of the vessels, pipettes, etc. must be assured (but not sterility).

1.6.5. *DOC determinations*

Membrane filters are suitable if it is assured that they neither release carbon nor adsorb the substance in the filtration step.

If the samples are centrifuged, this has to be done at $40\,000\text{ ms}^{-2}$ ($\sim 4\,000\text{ g}$) for 15 minutes, preferably in a refrigerated centrifuge, in any case less than 40 °C.

Note

The differentiation TOC/DOC by centrifugation at very low concentrations does not seem to work well since either, not all bacteria are removed, or carbon as part of the bacterial plasma is redissolved. At higher test concentrations ($\geq 10\text{ mg C per litre}$) and the same small inoculation the centrifugation error seems to be comparatively small.

The sample withdrawn for the culture medium (about 30 ml) is centrifuged or membrane filtered immediately in the filtration apparatus (1.6.2.3) using the membrane filters according to 1.6.2.4. The first 20 ml of the filtrate are discarded.

The DOC concentration is determined twice in the remaining filtrate (about 10 ml) by means of the TOC/DOC instrument (1.6.2.5). If the filtrate cannot be analyzed on the same day it has to be conserved according to point 1.6.4.

2. DATA AND EVALUATION

The analytical results are registered on the attached form sheet (Appendix 1) and the biodegradation values are calculated according to 1.2.

The DOC concentrations are calculated to the nearest 0,1 mg per litre. The means of the D_t values are rounded up to the nearest whole percent.

The course of the degradation test is followed graphically in a diagram as shown in the attached example (Appendix 2).

The results of the degradation test are valid, if the following condition is met: that in the same test series the control material yields DOC removals of $\geq 70\%$ within 10 days, counting from the day that the observed level of biodegradation first exceeds 10 %. This result must be obtained within the test duration of 28 days, otherwise the whole series has to be discarded.

3. REPORTING

3.1. Test reports

The test report shall, if possible, contain the following:

- data should be reported according to the form sheet (Appendix 1),

- the course of the degradation test is represented graphically in a diagram showing the lag phase, degradation phase, slope and time window ('time window' means here a 10-day period, starting from the day that the observed level of biodegradation first exceeds 10 %),
- proof of validity of the test (control substance ≥ 70 % DOC-removal in 10 days, counting from the day that the degradation exceeds 10 %, this result obtained within the test duration of 28 days).

3.2. Interpretation of results

Owing to the stringency of this test, a low result does not necessarily mean that the test compound is not biodegradable under environmental conditions, but indicates that more work will be necessary to establish this.

Test chemicals showing a high DOC loss in this test should be regarded as easily biodegradable provided that this level is reached within the time window of 10 days, counting from the day that the observed level of biodegradation first exceeds 10 %.

4. REFERENCES

- (1) OECD, Paris, 1981, *Test Guideline 301E*, Decision of the Council C(81) 30 final.
- (2) Gerike, P., Fischer, W. K., A correlation study of biodegradability determinations with various chemicals in various tests, *Ecotoxicology and Environmental Safety*, vol. 3, No 2, 1979, pp. 159 to 173.
- (3) Gerike, P., Fischer, W. K., A correlation study of biodegradability determinations with various chemicals in various tests II. Additional results and conclusions, *Ecotoxicology and Environmental Safety*, vol. 5, No 1, 1981, pp. 45 to 55.

Appendix 1

Biotic degradation: modified OECD screening test

Testing institute

Test material

Experiment No

Test data

Theoretical concentration mg DOC per litre

Carbon determinations

	Flask No		DOC concentrations after x days (milligrams per litre)						
			0 (C ₀)						
<i>Blank</i> Mineral nutrient solution without test material but with inoculum	1	a ₁							
		a ₂							
		$C_{a(t)} = \frac{a_1 + a_2}{2}$							
	2	b ₁							
		b ₂							
		$C_{b(t)} = \frac{b_1 + b_2}{2}$							
<i>Test</i> Mineral nutrient solution with test material and with inoculum	3	bl ₁							
		bl ₂							
		$C_{bl(t)} = \frac{bl_1 + bl_2}{2}$							

Evaluation of raw data

Flask No	Calculation of results	% DOC removal after x days						
		0						
1	$D_1 = \left(1 - \frac{C_{a(t)} - C_{bl(t)}}{C_0 - C_{bl(0)}}\right) \times 100$	0						
2	$D_2 = \left(1 - \frac{C_{b(t)} - C_{bl(t)}}{C_0 - C_{bl(0)}}\right) \times 100$	0						
Mean (*)	$D_t = \frac{D_1 + D_2}{2}$	0						

* D₁ and D₂ should not be averaged if there is a considerable difference.

Biotic degradation: modified OECD screening test (form sheet)

Testing institute

Study director

Date of start of test Experiment No

Test material

Chemical structure

Stock solution:

	milligrams per litre	milligrams per litre TOC (*)	milligrams per litre DOC (**)
Test material concentrate			

* Disagreement between DOC and TOC values points towards insufficient solubility of the test material.

** All DOC values determined after membrane filtration or centrifugation.

Carbon analyzer

Inoculum:

*Test result*D_t = % DOC removal after days,*Validation of result*

Control chemical

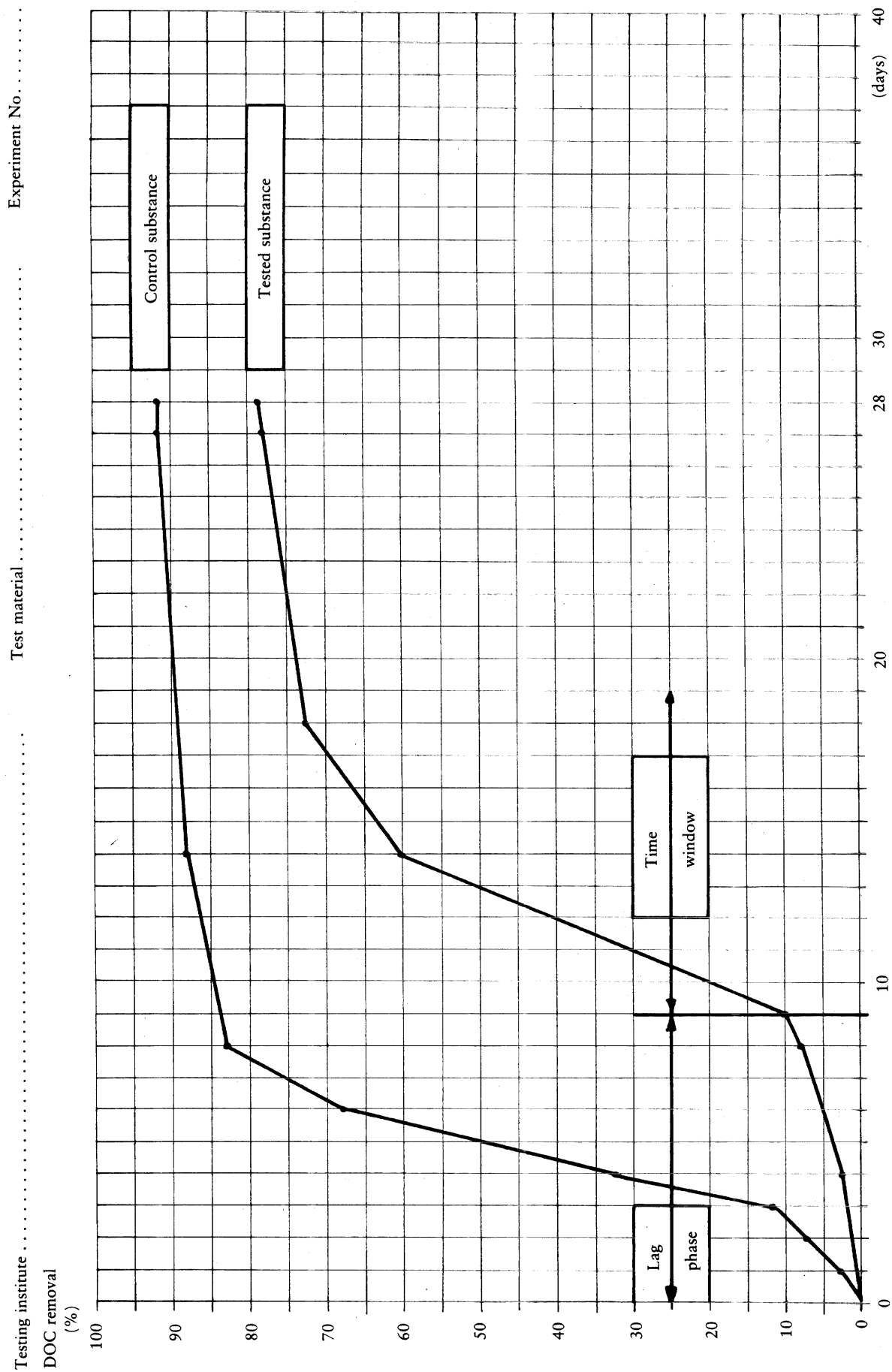
Result % DOC removal after days.

Reference experiment No

Remarks:.....
(Date).....
(Signature)

Appendix 2

Modified OECD screening test

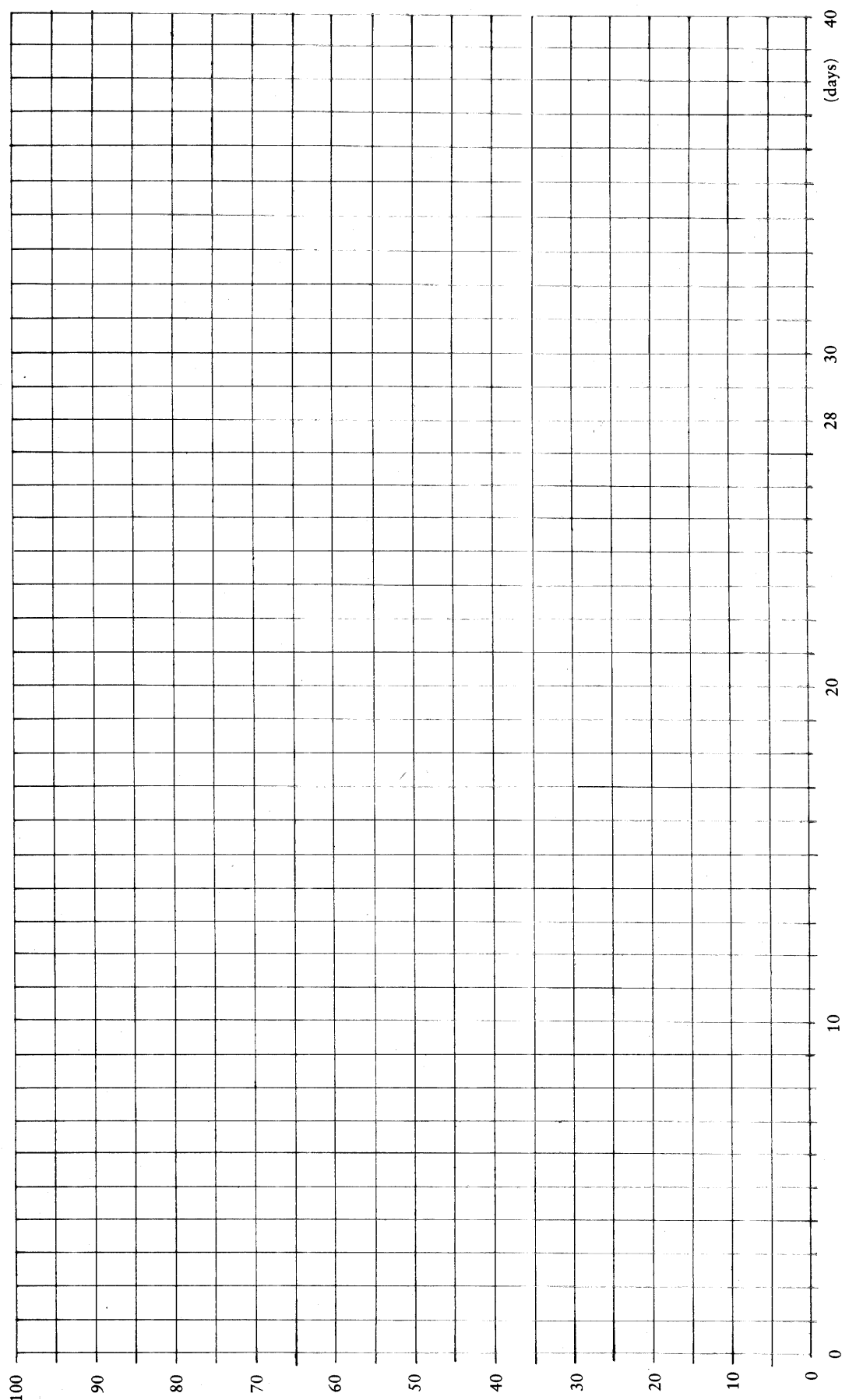


Modified OECD screening test

Experiment No.

Test material

Testing institute

DOC removal
(%)

C. 4. DEGRADATION

BIOTIC DEGRADATION: MODIFIED AFNOR TEST NF T 90/302

1. METHOD

1.1. Introduction

The purpose of the method is the measurement of the biodegradability of water-soluble, non-volatile organic compounds in an aerobic, aqueous medium at a starting test concentration corresponding to 40 mg DOC (dissolved organic carbon) per litre. If the sensitivity limits of organic carbon analyzers are improved, the use of lower test concentrations may be advantageous, particularly for toxic compounds.

The organic carbon content of the test material must be established.

The method is applicable only for those organic test materials which, at the concentration used in the test:

- are at least water-soluble at the concentration to be tested (40 mg DOC per litre),
- have a negligible vapour-pressure,
- are not inhibitory to bacteria,
- do not significantly adsorb on glass surfaces.

Information on the relative proportions of the major components of the test material will be useful in interpreting the results obtained, particularly in those cases where the results are low.

Information on the toxicity to micro-organisms of the chemical may be useful for the interpretation of low results.

1.2. Definitions and units

The degradation is defined as the percentage DOC removal with respect to the test material:

$$D_t = \left[1 - \frac{C_t - C_{bl(t)}}{C_0 - C_{bl(0)}} \right] \times 100$$

where:

- D_t = degradation in percent DOC removal at time t,
- C_0 = starting DOC concentration of the culture medium (mg DOC per litre),
- C_t = DOC concentration of the culture medium at time t (mg DOC per litre),
- $C_{bl(0)}$ = starting DOC concentration of the blank (mg DOC per litre),
- $C_{bl(t)}$ = DOC concentration of the blank at time t (mg DOC per litre).

1.3. Reference substances

The use of suitable control chemicals to check the activity of the inoculum is desirable.

Aniline, sodium acetate or sodium benzoate (for example) may be used for this purpose and they have to exhibit DOC removals of $\geq 70\%$ within 28 days, otherwise the test is regarded as invalid and should be repeated using an inoculum from a different source. In this specific test method, glucose is used

especially for the inhibitory test and to check the activity of the inoculum which can also be checked by aniline, sodium acetate and sodium benzoate.

1.4. Principle of the test method

Organic substances dissolved in water are biodegraded by chemi-organotrophic micro-organisms using them as the sole source of carbon and energy. These products are studied at a concentration such that the initial content of organic carbon is 40 mg per litre. Organic carbon remaining in solution is measured at least after three, seven, 14 and 28 days. Simultaneously, the test substance is checked for possible inhibitory effects on the inoculum.

The procedure is checked by means of a control substance.

1.5. Quality criteria

The reproducibility of the method has been established in the OECD and EEC ring-tests.

The lowest concentration of the test compound for which this test method can be used is largely determined by the sensitivity limit of the organic carbon analysis (0,5 mg carbon per litre at the present state of the art) and the concentration of dissolved organic carbon in the nutrient solution.

1.6. Description of the test method

1.6.1. Reagents

The chemicals used must be of analytical purity.

1.6.1.1. Distilled water

Distilled water must not contain more than 10 % of the organic carbon introduced by the test material.

1.6.1.2. Nutrient solution

Prepare the test medium as indicated below, using sterile materials. For one litre of solution, dissolve the following in distilled water (AR means analytical reagent):

$(\text{NH}_4)_2\text{SO}_4$ (ammonium sulphate):	0,300 g AR
NH_4NO_3 (ammonium nitrate):	0,150 g AR
KH_2PO_4 (potassium dihydrogen phosphate):	0,300 g AR
$\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ (disodium hydrogen phosphate dodecahydrate):	2,000 g AR
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (magnesium sulphate heptahydrate):	0,050 g AR
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (calcium chloride dihydrate):	0,050 g AR
Yeast extract:	0,005 g AR

The pH is $7,5 \pm 0,1$.

Add 1 ml of a trace element solution of the following composition:

$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (iron (II) sulphate heptahydrate):	0,100 g (AR)
$\text{MnSO}_4 \cdot \text{H}_2\text{O}$ (manganese (II) sulphate monohydrate):	0,100 g (AR)
K_2MoO_4 (potassium molybdate):	0,025 g (AR)
$\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ (disodium tetraborate decahydrate):	0,025 g (AR)

Co(NO ₃) ₂ · 6H ₂ O (cobalt (II) nitrate hexahydrate):	0,025 g (AR)
CuCl ₂ · 2H ₂ O (copper (II) chloride dihydrate):	0,025 g (AR)
ZnCl ₂ (zinc chloride):	0,025 g (AR)
NH ₄ VO ₃ (ammonium vanadate):	0,010 g (AR)
Distilled water to 100 ml	

(The trace element solution can be kept for one month at a temperature between +1 and +4 °C.)

Make up to volume (1 litre), and mix. The medium must be used within 12 hours.

1.6.1.3. Control substances

Aniline (freshly distilled), sodium acetate, sodium benzoate, glucose.

1.6.2. Apparatus

Usual laboratory equipment and:

- apparatus for the assay of organic carbon,
- spectrophotometer,
- centrifuge 4 000 g,
- shaker allowing adequate aeration and shaking,
- apparatus for the assay of dissolved oxygen, pH-meter, 500 ml sterile, wide-neck conical flasks,
- apparatus for sterile filtration (membrane filters of 0,22 µm porosity).

The glassware must be thoroughly cleaned and completely free from all traces of organic or toxic matter.

1.6.3. Preparation of the inoculum

Take an adequate volume of a mixture of three samples from polluted surface waters and effluents from municipal sewage treatment plants free from major specific pollutants. The bacterial count for each sample must be at least 10⁵ bacteria/ml.

The samples must be used for inoculation within a period of 12 hours, including transportation, and must not remain for more than six hours without aeration.

Filter through paper to eliminate the larger insoluble particles, collect the filtrate and pass through a membrane filter of pore size 0,22 µm.

Wash with any suitable isotonic solution. Take up the bacteria deposited on the membrane filter in a small volume of any isotonic solution. Mix well. Measure the absorbance at 620 nm and from this deduce the concentration of bacteria in relation to a standard curve obtained previously by means of solid medium counts of *Pseudomonas fluorescens* strain ATCC 15453. Add the volume of solution required to adjust the concentration of bacteria to $5 \pm 3 \times 10^7$ /ml. Use the inoculum within the next hour.

1.6.4. Test procedure

The incubation must be carried out in the absence of any intense lighting, in an incubator maintained at 20 to 25 °C and free from toxic vapours.

Prepare the following solutions:

1. Solution of test substance in the test medium in such a way as to obtain a concentration of 40 mg per litre of organic carbon.
2. Solution of glucose in the test medium in such a way as to obtain a concentration of 40 mg per litre of organic carbon.
3. Solution containing in the test medium the concentrations of test substance and glucose used.
4. Also have available an adequate volume of test medium.

Mix the four solutions individually and sterilize by filtration through a membrane filter.

Membrane filters are suitable if it is assured that they neither release carbon nor adsorb the substance in the filtration step.

All the necessary manipulations must be carried out by sterile methods. Divide solutions into the test flasks (previously sterilized) according to the following scheme:

flask 1 (test):	150 ml of solution 1
flask 2 (test):	150 ml of solution 1
flask 3 (test):	150 ml of solution 1
flask 4 (sterile control):	150 ml of solution 1
flask 5 (glucose control):	150 ml of solution 2
flask 6 (monitoring of inhibitory action):	150 ml of solution 3
flask 7 (blank):	150 ml of solution 4

Seed flasks 1, 2, 3, 5, 6 and 7 with 1,5 ml of inoculum and mix well by manual shaking.

Take an aliquot of 3 to 5 ml from each flask.

Centrifuge the aliquots at 4 000 g for 15 minutes, keeping the temperature below 26 °C.

Collect the supernatants for assay of organic carbon at time 0.

Place the flasks on the shaker and leave them there throughout the test period: the concentration of dissolved oxygen on day 3 in flask 5 must be at least 5 mg per litre.

In the same way as for the assay of organic carbon at time 0, carry out this assay on flasks 1, 2, 3, 5, 6 and 7 after at least three, seven, 14, 28 days of incubation. However, if the reduction in carbon content reaches 95 % of the initial content in flasks 1, 2 and 3, consider the test as finished.

The test can be finished before the 28th day if a plateau is observed sooner.

If a degradation has obviously started by day 28, but did not reach a plateau, it is considered good practice to extend the experiment for one to two weeks longer.

At the end of the test carry out an assay of organic carbon in flask 4 in the same manner as at time 0, and check for sterility by seeding into a tube of liquid culture medium and incubating at 25 °C for five days.

Culture medium:

dehydrated yeast extract	3 g
pancreatic casein peptone	6 g
water	1 000 ml

Dissolve the components of the dehydrated complete medium in boiling water. If necessary adjust the pH in such a way that after sterilization it is $7,2 \pm 0,2$ at 20 °C.

If the assays of organic carbon content have to be deferred, keep the supernatant at 4 °C in the dark in hermetically sealed glass flasks; the maximum acceptable duration of preservation is 24 hours. If the analysis cannot be carried out within 24 hours, then freeze at a temperature below -18 °C.

To compensate for loss of water due to evaporation, before each sampling check the volume of medium in the flask and, if necessary, make up to volume with distilled water sterilized by filtration through a membrane filter of 0,22 µm pore size to restore the volume measured after the previous sampling.

2. DATA AND EVALUATION

The analytical results are recorded on the attached form sheet (Appendix 1) and the biodegradation values are calculated according to 1.2.

The results of the degradation test are valid, if the conditions are met:

- that the level of the glucose degradation in flask 5 must be at least 80 % by day 7,
- that at the end of the test, flask 4 must still be sterile,
- that the concentration of dissolved oxygen on day 3 in flask 5 must be at least 5 mg per litre.

The level of glucose biodegradation in flask 6 must by day 7 be at least 75 % of that observed in flask 5. If this limit is not reached, it may be assumed that the test substance subjected to the test displays an inhibitory effect towards the bacteria present and that the method is therefore not applicable at the concentration specified.

Notes

The comparison of the percentage eliminations of carbon in flasks 1, 2 and 3, on the one hand, and in flask 4, on the other hand, allows the causes of the degradation observed to be differentiated:

- the physico-chemical mechanism in flask 4,
- the physico-chemical plus biological mechanisms in flasks 1, 2 and 3.

3. REPORTING

3.1. Test report

All the experimental results concerning the tested substance, the reference substance and the blanks should be reported.

Mention the following points in particular:

- extent of the disappearance of the product in flask 4 at the end of the test,
- any inhibition phenomena observed,
- proof of validity,
- the course of the degradation test is represented graphically in a diagram showing the lag phase, degradation phase, slope and time window ('time window' means here a 10-day period, starting from the day that the observed level of biodegradation first exceeds 10 %).

3.2. Interpretation of results

Owing to the stringency of this test, a low result does not necessarily mean that the test compound is not biodegradable under environmental conditions, but indicates that more work will be necessary to establish this.

Test chemicals giving a high result of DOC loss in this test should be regarded as easily biodegradable providing that this level is reached within the time window of 10 days, counting from the day that the observed level of biodegradation first exceeds 10 %.

4.

REFERENCES

- (1) OECD, Paris, 1981, *Test Guideline 301A*, Decision of the Council C(81) 30 final.
 - (2) Gerike, P., Fischer, W. K., A correlation study of biodegradability determinations with various chemicals in various tests, *Ecotoxicology and Environmental Safety*, vol. 3, No 2, 1979, pp. 159 to 173.
 - (3) Gerike, P., Fischer, W. K., A correlation study of biodegradability determinations with various chemicals in various tests. II. Additional results and conclusions, *Ecotoxicology and Environmental Safety*, vol. 5, No 1, 1981, pp. 45 to 55.
 - (4) Afnor, *Method for the evaluation in aqueous medium of the biodegradability of so-called 'total' of organic products*, T 90-302.
-

Appendix 1

Form sheet for the modified Afnor test

Experiment No
 Date of start of test
 Test/control material
 Theoretical test concentration
 Carbon analysis

DOC determinations

Culture medium	Flask No	DOC concentration (mg per litre) after				
		t = 0	3	7	14	28 (days)
Test	1	1 _{C₀}	1 _{C₃}	1 _{C₇}	1 _{C₁₄}	1 _{C₂₈}
Test	2	2 _{C₀}	2 _{C₃}	2 _{C₇}	2 _{C₁₄}	2 _{C₂₈}
Test	3	3 _{C₀}	3 _{C₃}	3 _{C₇}	3 _{C₁₄}	3 _{C₂₈}
Test mean	1 - 3	$\bar{C}_0$	$\bar{C}_3$	$\bar{C}_7$	$\bar{C}_{14}$	$\bar{C}_{28}$
Sterile control	4	4 _{C₀}				4 _{C₂₈}
Glucose control	5	5 _{C₀}	5 _{C₃}	5 _{C₇}	5 _{C₁₄}	5 _{C₂₈}
Inhibitory control	6	6 _{C₀}	6 _{C₃}	6 _{C₇}	6 _{C₁₄}	6 _{C₂₈}
Blank	7	C _{bl(0)}	C _{bl(3)}	C _{bl(7)}	C _{bl(14)}	C _{bl(28)}

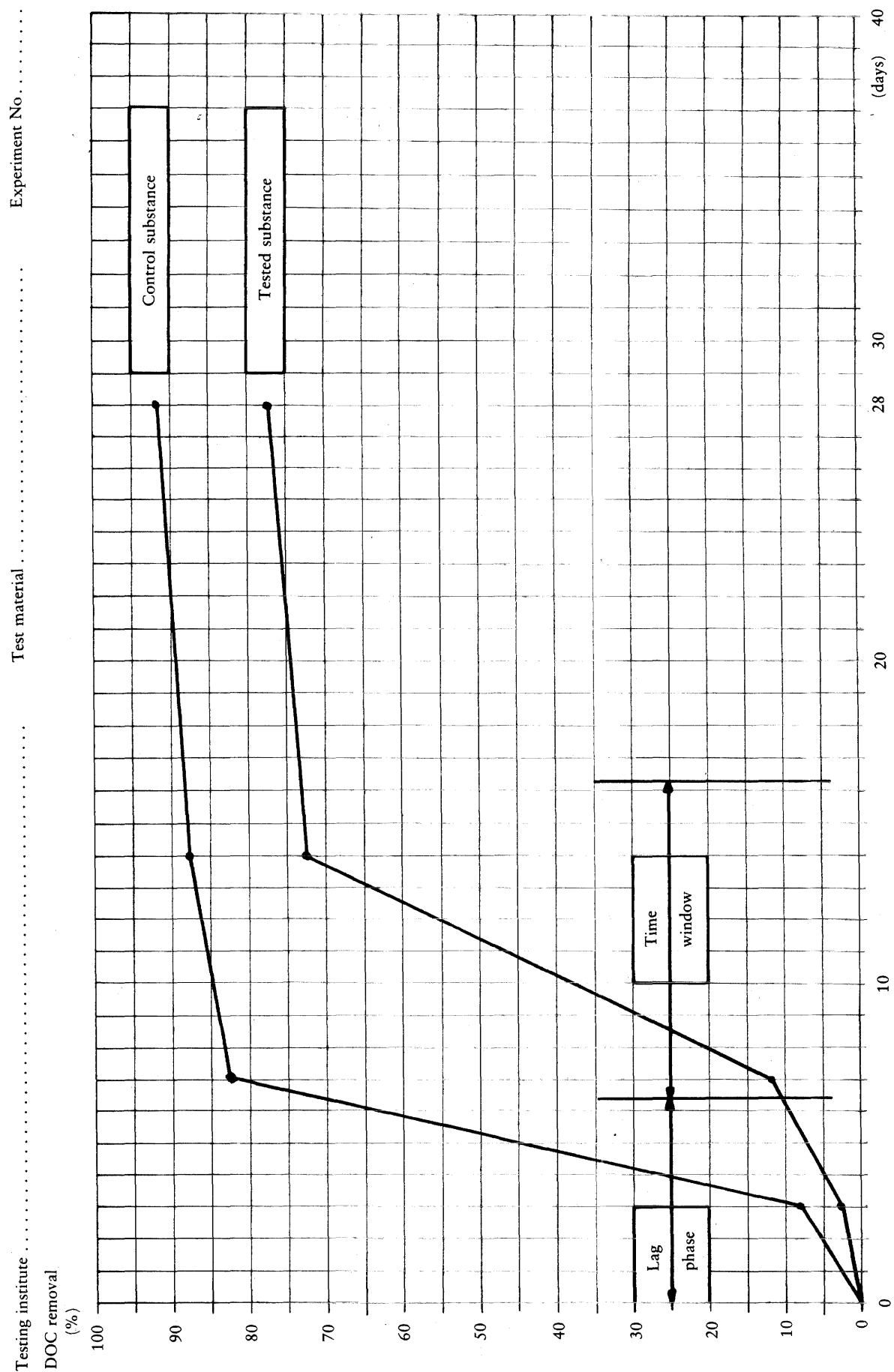
Evaluations of the results

	t = 0	3	7	24	28 (days)
Test $\left[1 - \frac{C_t - C_{bl(t)}}{C_0 - C_{bl(0)}} \right] \times 100$	0				
Glucose control $\left[1 - \frac{5_{Ct} - C_{bl(t)}}{5_{C_0} - C_{bl(0)}} \right] \times 100$	0				
Inhibitory control $\left[1 - \frac{6_{Ct} - C_{bl(t)}}{6_{C_0} - C_{bl(0)}} \right] \times 100$	0				

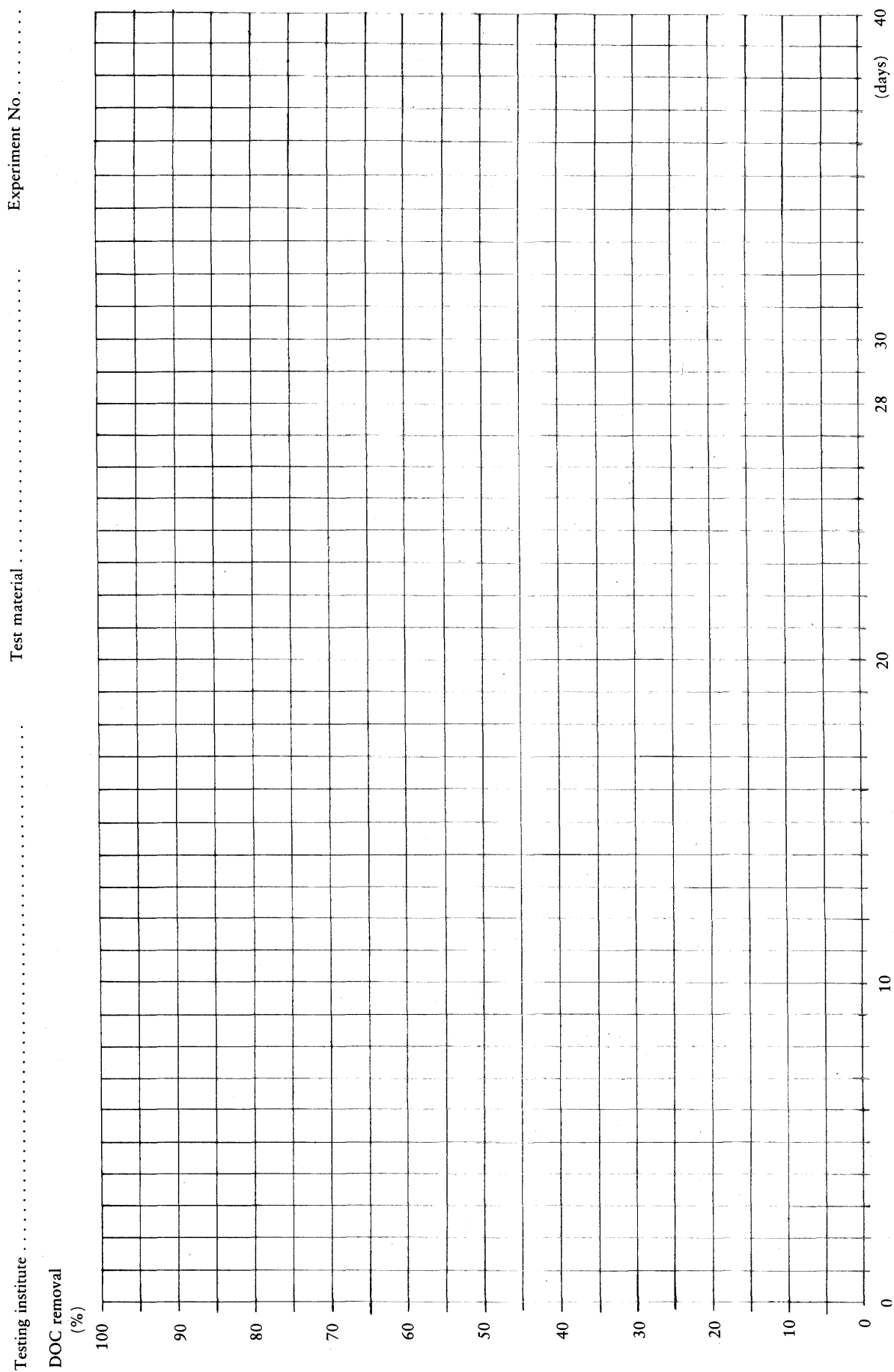
Validity:

- Dissolved oxygen flask 5, day 3: mg per litre
- % biodegradation flask 5, day 7: %
- % biodegradation flask 6, day 7: %
- Sterile flask 4:

Appendix 2
Modified Afnor test NF T 90/302



Modified Afnor test NF T 90/302



C. 5. DEGRADATION

BIOTIC DEGRADATION — MODIFIED STURM TEST

1. METHOD

1.1. Introduction

The purpose of the method is the measurement of the biodegradability of non-volatile organic substances in an aerobic, aqueous medium, at two starting test concentrations of 10 and 20 mg per litre (standard concentrations).

The organic carbon content of the test material must be available (TOC analysis or estimate using the empirical formula to allow the calculation of the CO₂ theoretical yield).

The method is applicable only to those organic test materials which, at the concentration used in the test:

- have negligible vapour pressure,
- are not inhibitory to bacteria.

This method may, at least in principle, be applied to substances which are insoluble at the test concentrations.

Information on the relative proportions of the major components of the test material will be useful in interpreting the results obtained, particularly in those cases where the results are low.

Information on the toxicity to micro-organisms of the chemical may be useful for the interpretation of low results and in the selection of appropriate test concentrations.

1.2. Definitions and units

The degradation is defined as the CO₂ produced by the substance, as a percentage of the theoretical CO₂ it should have produced (ThCO₂), calculated from the organic carbon content of the substance.

1.3. Reference substances

The use of a suitable control chemical to check the activity of the inoculum is desirable.

Aniline, sodium acetate or sodium benzoate, for example, may be used for the purpose and they have to exhibit a CO₂ production $\geq 60\%$ within 28 days otherwise the test is regarded as invalid and should be repeated using an inoculum from a different source.

1.4. Principle of the test method

The test material is added to a chemically defined liquid medium, inoculated with sewage micro-organisms and aerated at 20 to 25 °C. The temperature is recorded during the test period.

The CO₂ released is trapped as BaCO₃ and the degradation is followed by CO₂ analysis over a 28-day period. After reference to suitable blank control(s), the total amount of CO₂ produced by the test

compound is determined for the test period and calculated as a percentage of the total CO₂ that the test material, based on its carbon content, could have theoretically produced.

The procedure is checked by means of an inoculum control substance (see 1.6.1.3).

1.5. Quality criteria

The reproducibility of the method has been established in the OECD and EEC ring-tests.

The endogenous CO₂ production of the inoculum, measured in the blank flask, is the main reason why a test substance concentration lower than 5 mg per litre cannot be used. (When the test is adapted to ¹⁴C labelled test substance, the concentration can be much lower.)

1.6. Description of the test method

1.6.1. Reagents

1.6.1.1. High-quality water (HQW)

Double-distilled water, free of toxic substances (copper in particular), with low carbon content (< 2,0 mg per litre of TOC) and with resistivity ≥ 18 megohms/cm. Distilled water must not contain more than 10 % of organic carbon introduced by the test material.

1.6.1.2. Nutrient solution

(a) Stock solution

FeCl ₃ · 6H ₂ O (iron (III) chloride hexahydrate):	0,25 g
Dissolve in and make up to 1 000 ml with water 1.6.1.1.	
MgSO ₃ · 7H ₂ O (magnesium sulphate heptahydrate):	22,50 g
Dissolve in and make up to 1 000 ml with water 1.6.1.1.	
CaCl ₂ (calcium chloride):	27,50 g
Dissolve in and make up to 1 000 ml with water 1.6.1.1.	
KH ₂ PO ₄ (potassium dihydrogen phosphate)	8,50 g
K ₂ HPO ₄ (dipotassium hydrogen phosphate)	21,75 g
Na ₂ HPO ₄ · 2H ₂ O (disodium hydrogen phosphate dihydrate).	33,40 g
NH ₄ Cl (ammonium chloride):	1,70 g
Dissolve in and make up to 1 000 ml with water 1.6.1.1.	
(NH ₄) ₂ SO ₄ (ammonium sulphate):	40,00 g
Dissolve in and make up to 1 000 ml with water 1.6.1.1.	

(b) Test medium

The test medium will contain per litre of water (1.6.1.1) the following reagents:

- 4 ml of the above ferric chloride solution,
- 1 ml of the above magnesium sulphate solution,
- 1 ml of the above calcium chloride solution,
- 2 ml of above phosphate solution,
- 1 ml of above ammonium sulphate solution.

The pH value should be 7,2 ± 0,2.

1.6.1.3. Control substances

Aniline (freshly distilled), sodium acetate, sodium benzoate.

1.6.1.4. Barium hydroxide 0,025 N (0,0125 M)

Dissolve 4,0 g $\text{Ba}(\text{OH})_2 \cdot 8\text{H}_2\text{O}$ per litre HQW. Filter through filter paper and seal the clear solution to prevent absorption of CO_2 from the air. It is wise to prepare more than 5 litres at a time when running the series.

1.6.2. Apparatus

1.6.2.1. CO_2 scrubbing apparatus

A series of 12 carboys (three test materials). ('Carboy' means here a 4- to 5-litre brown glass bottle. If clear glass containers are used, the test must be conducted in the dark.)

Four 1-litre plastic bottles, containing 700 ml of 10 N (10 M) NaOH.

One 1-litre Erlenmeyer flask containing 700 ml of 0,025 N (0,0125 M) $\text{Ba}(\text{OH})_2$ solution.

One empty 1-litre Erlenmeyer to prevent liquid carry-over.

These bottles are connected in series, using inert tubing, to a pressurized air source and air is sparged through the scrubbing solutions at a constant rate.

For each additional set of four carboys, add one additional 1-litre plastic bottle containing 700 ml 10 N (10 M) NaOH.

1.6.2.2. CO_2 production apparatus

Four 4- to 5-litre brown carboys for each test material. Stoppers, flexible tubing, plastic tubing.

1.6.2.3. CO_2 absorber bottles

100-ml barium hydroxide absorber bottles.

1.6.3. Preparation of the inoculum

The source of test organisms is activated sludge freshly sampled from a well-operated municipal sewage treatment plant. This sewage treatment plant should receive no or minimal effluents from industry.

On arrival at the laboratory, the activated sludge is aerated for four hours. 500 ml of the mixed liquid is sampled and homogenized for two minutes with a mechanical blender. It is then settled for 30 minutes.

If the supernatant still contains high levels of sludge solids at the end of 30 minutes, it may be settled for an additional 30 to 60 minutes or adapted to laboratory conditions to improve its ability to settle.

The supernatant is decanted to provide sufficient volume for a 1 % inoculum for each CO_2 test flask. Avoid carry-over of sludge solids which would interfere with the measurement of CO_2 production.

Although optional, it is useful to perform viable counts on the supernatant fraction to determine microbial numbers. This inoculum should normally contain 10^6 to 20×10^6 colony forming units per millilitre.

It should be used on the day it is prepared.

1.6.4. *Test procedure*

1.6.4.1. Stock solution

An initial stock solution of the substance to be tested is prepared by dissolution in high-quality water to give a concentration of 1 000 mg per litre.

Stock solutions are made up on the basis of the organic carbon content of the test material. If this is unknown, stock solutions are made up to a concentration on a weight basis. To obtain a homogeneous sample, it is necessary to mix well, at the same time avoiding any foaming which may concentrate the test substance. For solid samples it may be necessary to melt and mix the entire contents of the sample bottle before taking an aliquot. This part of the procedure is extremely important because calculations of percent biodegradation depend on having the correct amount of carbon in the test system.

The pH of the stock solution need not be adjusted unless it falls outside the range of 3 to 10 since the phosphate buffer in the test medium will control it. If the pH lies outside this range, adjust an aliquot of the stock solution to pH $7,0 \pm 1,0$ with 1N (1M) HCl or NaOH, making sure that the solution is being vigorously mixed during the addition of acid or base.

To confirm the nominal concentration of organic carbon of the test compound, the stock preparation (or the neutralized aliquot) may be analyzed for the total organic carbon. A TOC analysis is also required for the control stock solution.

If a test material is insoluble in water, add the appropriate amount of test material directly to the carboy on a weight or volume basis.

If the test material is not soluble at the test concentration, special measures, such as the use of ultrasonic dispersion, may have to be employed to achieve a good dispersion of the test material.

1.6.4.2. Conditions

Since a 1 % inoculum is used in the CO₂ test, it is necessary to make dilutions in the CO₂ test medium.

This is most easily achieved as follows:

- (a) to each of the 4- to 5-litre test carboys, add 2 470 ml of high-quality water (HQW see 1.6.1.1);
- (b) to each of the 4- to 5-litre test carboys, add 3 ml each of the ammonium sulphate, magnesium sulphate, and calcium chloride stock solutions, add 6 ml of the phosphate buffer stock solution and 12 ml of the ferric chloride solution;
- (c) to each of the 4- to 5-litre test carboys, add 30 ml of the activated sludge inoculum.

This mixture is aerated with CO₂-free air for 24 hours, to purge the system of carbon dioxide.

After the aeration period, 100 ml 0,025 N (0,0125 M) Ba(OH)₂ is introduced into each of three CO₂ absorber bottles and connected in series to the exit air line of each test carboy.

1.6.4.3. Performance of the test

Test material is added to two of the four carboys to begin the test period. Each material is tested at two concentrations: 10 and 20 mg per litre.

The amount of test material stock solution required in the carboy is calculated as follows:

$$\text{millilitres of stock solution per carboy} = \frac{B \times C}{A}$$

where:

B = test substance concentration in test carboy (milligrams per litre),

A = test substance concentration in stock solution (milligrams per litre),

C = final volume of test medium in test carboy (millilitres).

Sufficient stock solution to reach the desired test concentration, as calculated above, plus enough distilled water to make 473 ml (stock solution + HQW) are added to the appropriate carboys. To the third carboy, used as blank control and containing no test material, 473 ml of HQW are added. The final volume in each carboy is now 3 000 ml.

A control substance at a concentration of 20 mg per litre is added to the last of the four carboys.

The test is started by bubbling CO₂-free air through the solution at a rate of 50 to 100 ml per minute per carboy (= one to two bubbles per second).

Water-insoluble test materials, may be added dry to the CO₂ test carboy, and agitated with a magnetic stirrer. For foaming chemicals, CO₂-free air bubbling can be replaced by overhead aeration and magnetic stirring.

The CO₂ produced in each carboy reacts with the barium hydroxide and is precipitated as barium carbonate; the amount of CO₂ produced is determined by titrating the remaining Ba(OH)₂ with 0,05 N (0,05 M) standardized HCl. Periodically (every two or three days), the CO₂ absorber nearest the carboy is removed for titration. The remaining two absorbers are each moved one place closer to the carboy, and a new absorber containing 100 ml of fresh 0,025 N (0,0125 M) Ba(OH)₂ is placed at the far end of the series.

Titration are made as needed (before any BaCO₃ precipitate is evident in the second trap), approximately every other day for the first 10 days, and on every fifth day until the 28th day.

On the 27th day, the pH of the carboy contents is measured again, and then 1 ml of concentrated HCl is added to each of the test carboys to drive off inorganic carbonate. The carboys are aerated overnight, and samples are removed from each carboy for DOC analysis. The final titration is made on day 28.

Titration on the 100 ml Ba(OH)₂ solution are made after removing the bottles closest to the carboys. The Ba(OH)₂ is titrated with 0,05 N (0,05 M) HCl, using phenolphthalein as an indicator.

The test is run at room temperature 20 to 25 °C and temperature is recorded during the test period.

If a plateau is observed before the 28th day, the test can be terminated.

If degradation has obviously started by day 28, but has not reached a plateau on day 28, it is considered to be good practice to extend the experiment for one to two weeks longer.

1.6.5. CO₂ determination

Ways of measuring CO₂ evolution other than by back titration of Ba(OH)₂ traps could be considered. This does not change the principle of this test and could possibly lead to a continuous reading of the biodegradation as it progresses.

The first step in calculating the amount of CO₂ produced is to correct the test material carboys for endogenous CO₂ production. The control carboy serves as a 'seed blank' to correct for CO₂ which may be produced through endogenous respiration of bacteria. The amount of CO₂ produced by a test material is determined by the difference (in millilitres of titrant) between the experimental and blank Ba(OH)₂ traps.

When using 0,05 N (0,05 M) HCl to titrate the absorber bottle, each millilitre of HCl titrated corresponds to 1,1 mg of CO₂ produced.

2. DATA AND EVALUATION

The analytical results are recorded on the attached form sheet (see Appendix 1) and the biodegradation values are calculated according to point 1.2.

The CO₂ concentrations are calculated to the nearest 0,1 mg per litre. The biodegradation values are rounded up to the nearest whole percent.

The course of the degradation test is followed graphically in a diagram as shown in the attached example (see Appendix 2).

The results of the degradation test are valid, if the following conditions are met:

- that in the same test series the control material yields ≥ 60 % biodegradation within 28 days (if this is not the case, the whole series has to be discarded and repeated with an inoculum from another source),
- that no significant amount of CO₂ is evolved from the blank flask during the test (contamination of the medium, glassware and air supply). Total CO₂ evolution at the end of the test may not exceed 50 mg CO₂ per 3 litres of medium.

3. REPORTING

3.1. Test report

The test report shall, if possible, contain the following:

- data should be reported according to the form sheet (see Appendix 2),
- the course of the degradation test is represented graphically in a diagram showing the lag phase, degradation phase, slope and time window ('time window' means here a 10-day period, starting from the day that the observed level of biodegradation first exceeds 10 %),
- dispersion procedure for substances which are not soluble under test condition,
- indicate data and location where test organisms were sampled and how they were handled prior to inoculation,
- temperature range recorded during the test period must be noted,
- if measured as suggested under point 1.6.2 (inoculation), report the number of micro-organisms per millilitre (colony forming units (CFU) per millilitre),
- proof of validity of the test (control substance ≥ 60 % degradation in 28 days).

3.2. Interpretation of results

Owing to the stringency of this test, a low result does not necessarily mean that the test compound is not biodegradable under environmental conditions, but indicates that more work will be necessary to establish this.

Test chemicals giving a high result of biodegradation in this test should be regarded as easily biodegradable provided that this level is reached within the time window of 10 days, counting from the day that the observed level of biodegradation first exceeds 10 %.

4. REFERENCES

- (1) OECD, Paris, 1981, *Test Guideline 301B*, Decision of the Council C(81) 30, final.
- (2) Gerike, P., Fisher, W. K., A correlation study of biodegradability determinations with various chemicals in various tests, *Ecotoxicology and Environmental Safety*, vol. 3, No 2, 1979, pp. 159 to 173.
- (3) Gerike, P., Fisher, W. K., A correlation study of biodegradability determinations with various chemicals in various tests II. Additional results and conclusions, *Ecotoxicology and Environmental Safety*, vol. 5, No 1, 1981, pp. 45 to 55.
- (4) Larson, R. J., Estimation of biodegradation potential of xenobiotic organic chemicals, *Applied and Environmental Microbiology*, vol. 38, 1979, pp. 1153 to 1161.

Appendix 1

Form sheet for the modified Sturm test

Experiment No

Date of start of test

Test/Control material

Theoretical test concentrate

Carbon analysis

Theoretical ThCO_2

Temperature range during the test

 CO_2 production

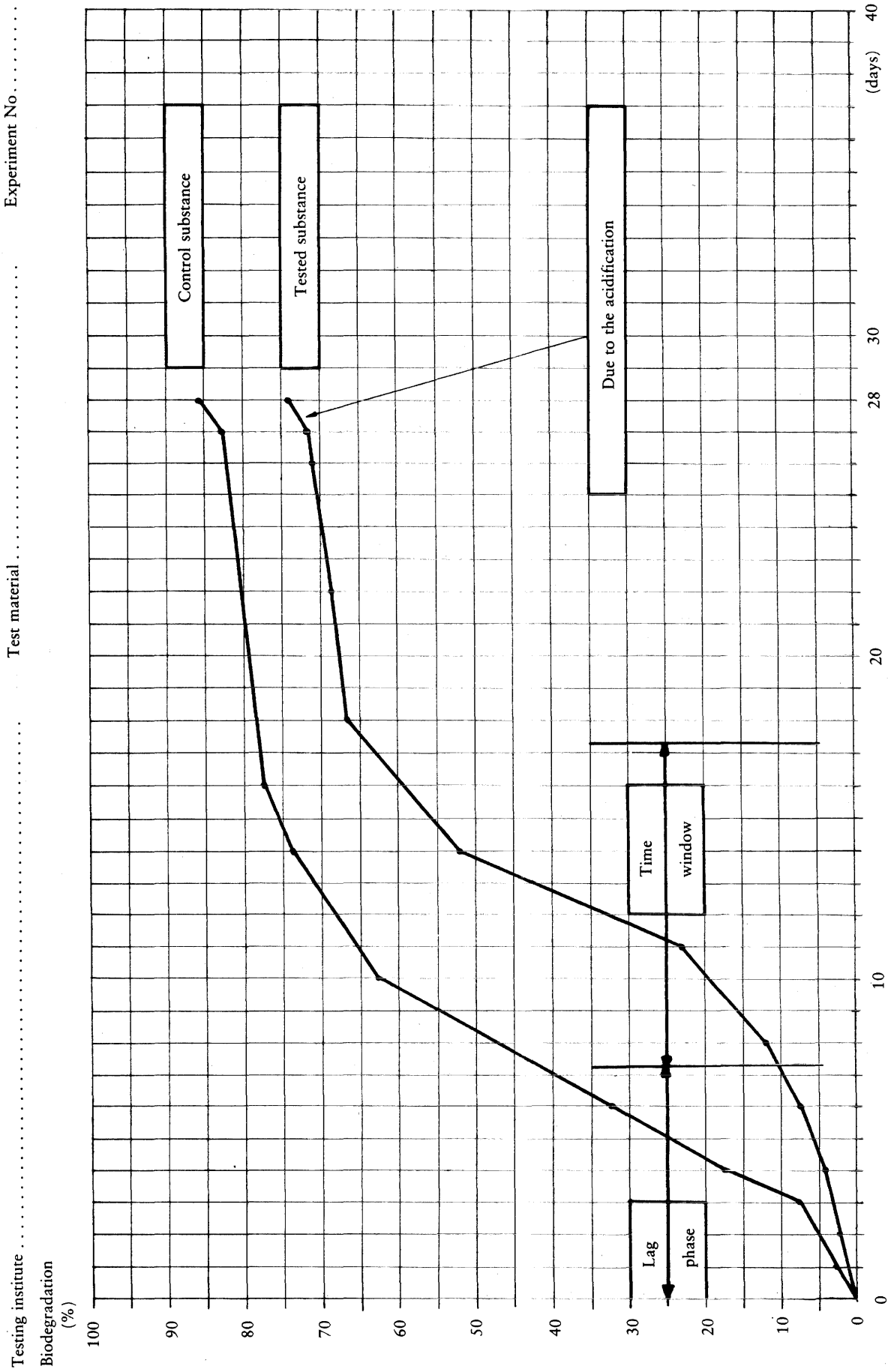
Days	CO_2 found (mg)	CO_2 cumulative (mg)	% of ThCO_2
28			

Validity:

— % biodegradation control material

— CO_2 total evolution of blank flask.

Appendix 2
Modified Sturm test



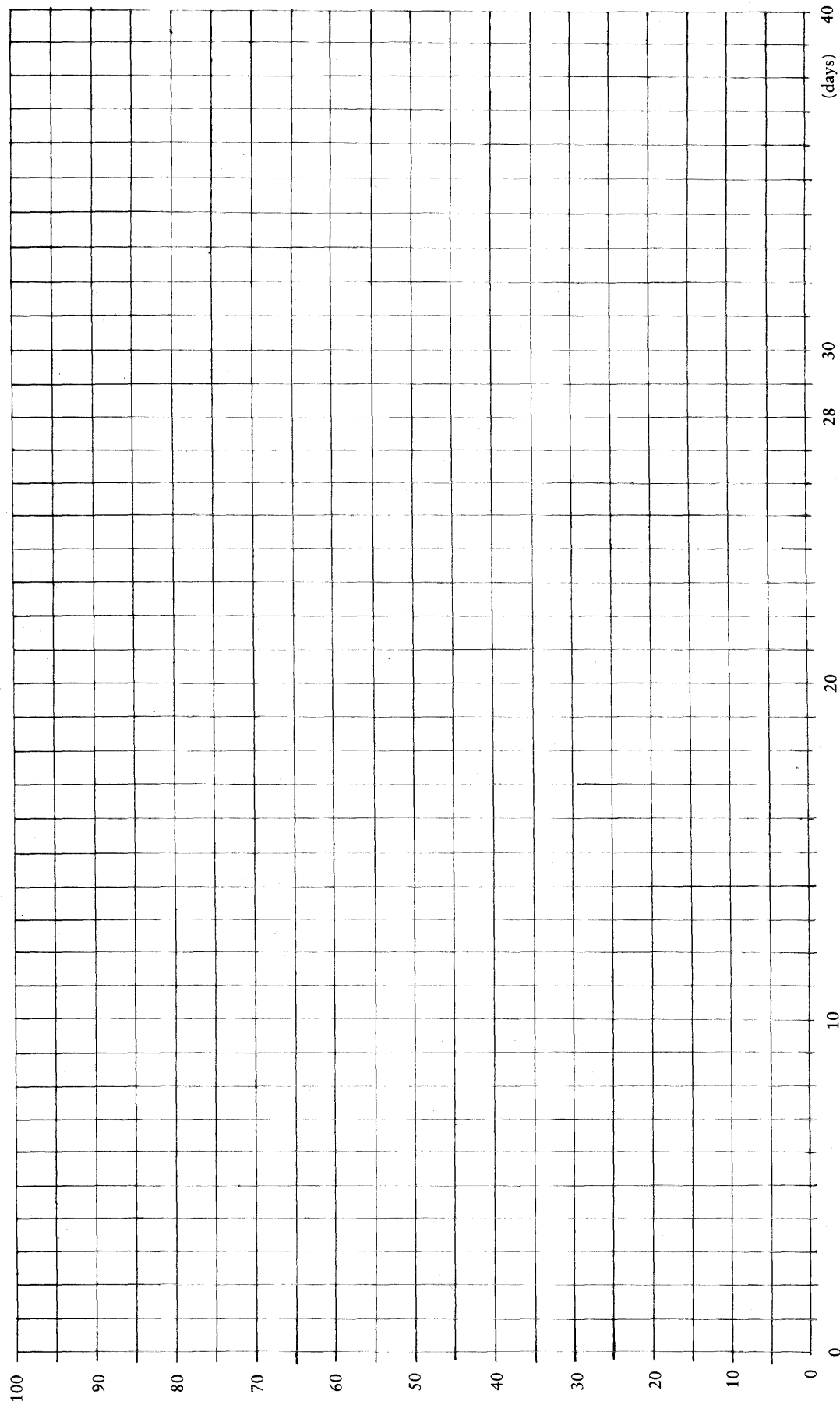
Modified Sturm test

Experiment No.

Test material

Testing institute

Biodegradation
(%)



C. 6. DEGRADATION

BIOTIC DEGRADATION: CLOSED-BOTTLE TEST

1. METHOD

1.1. Introduction

The purpose of the method is the measurement of the biodegradability of organic compounds in an aerobic, aqueous medium at a test concentration of 2 (standard concentration) to 10 mg per litre of test substance.

At the present state of development, the test is especially suitable for the biodegradability evaluation of water-soluble compounds. However, volatile compounds as well as poorly soluble compounds may also, at least in principle, be tested.

The empirical formula of the test material is required so that the theoretical oxygen demand (ThOD) may be calculated; if it is not known the chemical oxygen demand (COD) of the test material may serve as the reference value (see Appendix 1).

The method is applicable only to those organic test materials which at the concentration used in the test are not inhibitory to bacteria. If the test material is not soluble at the test concentration, special measures, such as the use of ultrasonic dispersion, may have to be employed to achieve a good dispersion of test material.

Information on the relative proportions of the major components of the test material will be useful in interpreting the results obtained, particularly in those cases where the results are low.

Information on the toxicity to micro-organisms of the chemical may be useful for the interpretation of low results and in the selection of appropriate test concentrations.

This method can be used for determining the BOD.

1.2. Definitions and units

The biochemical oxygen demand (BOD) is calculated as the difference of the oxygen depletion between a blank and a solution of test material under the conditions of the test. After division by the concentration (w/v) of the substance, the net oxygen depletion is obtained in milligrams of BOD per milligram of substance.

The degradation is defined as the ratio of the biochemical oxygen demand to either the theoretical oxygen demand (ThOD) or the chemical oxygen demand (COD) and expressed in percent.

Note

Sometimes the two ways of calculation (percentage of the ThOD or percentage of the COD) do not give the same results.

$$\% \text{ biodegradation (of ThOD)} = \frac{\text{mg O}_2/\text{mg tested substance}}{\text{ThOD}} \times 100$$

or

$$\% \text{ biodegradation (of COD)} = \frac{\text{mg O}_2/\text{mg tested substance}}{\text{mg COD/mg tested substance}} \times 100$$

where:

ThOD = theoretical oxygen demand (calculation see Appendix 1),

COD = chemical oxygen demand, determined experimentally.

1.3. Reference substances

The use of suitable control chemicals to check the activity of the inoculum is desirable.

Aniline, sodium acetate or sodium benzoate (for example) may be used for this purpose and they have to exhibit a degradation of $\geq 60\%$ within 28 days, otherwise the test is regarded as invalid and should be repeated using an inoculum from a different source.

1.4. Principle of the test method

A predetermined amount of the compound is dissolved in an inorganic medium (mineral nutrient solution) providing a concentration of usually 2 mg of test substance per litre. The solution is inoculated with a small number of micro-organisms from a mixed population and kept in closed bottles in the dark in a constant temperature bath or enclosure at 20 to 21 °C.

The degradation is followed by oxygen analyses over a 28-day period. The procedure is checked by means of an inoculum control substance.

An oxygen blank must be determined in a parallel test containing neither test nor control material.

Simultaneously, the test substance may be checked for possible inhibitory effects on the inoculum.

1.5. Quality criteria

The reproducibility of the method has been established in the OECD and EEC ring-tests.

1.6. Description of the test method

1.6.1. Reagents

1.6.1.1. Distilled or deionized water

Distilled or deionized water containing not more than 0,01 mg Cu per litre, air saturated. The volume according to the need of the day (e.g. 50 litres), is kept at room temperature, as close as possible to 20 °C and aerated strongly for 20 minutes with clean compressed air. Generally, the water is ready for use after standing for 20 hours at 20 °C. Oxygen is determined for control purposes. The concentration at 20 °C should be 9,09 mg O₂ per litre. All transfer and filling operations of the air-saturated water have to be conducted, bubble-free, by siphon.

1.6.1.2. Nutrient solution

(a) Stock solutions:

KH ₂ PO ₄ (potassium dihydrogen phosphate):	8,50 g
K ₂ HPO ₄ (dipotassium hydrogen phosphate):	21,75 g
Na ₂ HPO ₄ · 2H ₂ O (disodium monohydrogen phosphate dihydrate):	33,30 g
NH ₄ Cl (ammonium chloride):	1,70 g

Dissolve in and make up to 1 000 ml with distilled water.

The pH value should be 7,2

MgSO ₄ · 7H ₂ O (magnesium sulphate heptahydrate):	22,50 g
--	---------

Dissolve in and make up to 1 000 ml with distilled water.

CaCl ₂ (calcium chloride):	27,50 g
---------------------------------------	---------

Dissolve in and make up to 1 000 ml with distilled water.

FeCl ₃ · 6H ₂ O (iron(III) chloride hexahydrate):	0,25 g
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Dissolve in and make up to 1 000 ml with distilled water.

(b) Test medium:

The test medium will contain, per litre of water (1.6.1.1) 1 ml of each of the above stock solutions.

The pH value should be $7,2 \pm 0,2$.

1.6.1.3. Control substances

Aniline (freshly distilled), sodium acetate, sodium benzoate.

1.6.2. *Apparatus*

1.6.2.1. Calibrated 250- to 300-ml BOD bottles with glass stoppers or uncalibrated narrow neck 250-ml bottles with glass stoppers whose volumes have to be determined, may be used.

1.6.2.2. Several 2-, 3- and 5-litre bottles with litre marks for the preparation of the experiment and for the filling of the BOD bottles.

1.6.2.3. Pipettes of 1 to 10 ml volume; funnels and coarse filter paper; bottles for the preparation of the inoculum.

1.6.2.4. Waterbath for keeping the bottles at constant temperature with the exclusion of light.

1.6.3. *Preparation of the inoculum*

Any one of the following four sources may be used as inoculum provided that its viability is checked by means of a control substance (1.6.1.3).

1.6.3.1. Inoculum from soil

An aqueous suspension of unfertilized garden soil. 100 g of garden soil which has not recently been fertilized (soil from a greenhouse which is at constant temperature throughout the year is especially advantageous) are dispersed in 1 litre of chlorine-free tap-water. After 30 minutes the suspension is filtered through a coarse filter paper and the first 200 ml of the filtrate are discarded. The following main part of the filtrate serves for the inoculation (one drop out of a pointed pipette per litre of final volume). The inoculum is prepared immediately before the experiment. It has to be aerated if it is to be kept for several hours. The number of bacteria may be determined by plate count or with nutrient pad sets. There should be no more than 10^3 to 10^5 bacteria per millilitre of final volume.

1.6.3.2. Inoculum from secondary effluent

The inoculum should preferably be prepared by using a secondary plant (activated sludge plant or trickling filter dealing with a predominantly domestic sewage). The effluent must be kept under aerobic conditions in the period between sampling and application. To prepare the inoculum, the sample is filtered through a coarse filter paper, the first 200 ml being discarded. The rest of the filtrate is kept aerobic until used. The inoculum must be used on the day of collection.

1.6.3.3. Inoculum from a laboratory activated sludge

The effluent from a strongly aerated laboratory activated sludge unit is used. The inoculum is prepared as described under 1.6.3.2.

1.6.3.4. Composite inoculum

Equal volumes of the three inoculum samples (1.6.3.1 to 1.6.3.3) are mixed well, and the final inoculum drawn from this mixture.

1.6.4. Test procedure

All necessary manipulations before incubation have to be performed at about 20 °C.

Groups of bottles (1.6.2.1) are prepared for the determination of the BOD of the test and control substances in simultaneous experimental series (see Appendix 2). If chemical analyses are performed simultaneously, a sufficient number of bottles — including the controls for the inoculum and the blank — have to be prepared, e.g. either seven or 15 parallel bottles are prepared for one test material for the 0, 5, 15 and 28 day tests after a sufficient volume of water has been prepared in large bottles (1.6.2.2).

These large bottles are first filled to about one-third of their volume with distilled water (1.6.1.1) by siphon. Then the individual salt stock solutions (1.6.1.2) are pipetted into these bottles according to the final volume, and the respective test or control materials are added in such amounts that the final concentrations of 2 and sometimes 5 or 10 mg per litre are attained.

The concentration of about 9 mg of dissolved oxygen per litre of dilution water at 20 °C limits the possible starting concentration of the test material to about 2 mg per litre in order to guarantee a significant oxygen concentration remaining after the oxidation of the test substrate.

Poorly degradable substances or substances with a low ThOD are advantageously tested in parallel at higher concentrations. Subsequently the solutions are inoculated with one drop from a pipette per litre of final volume, and likewise the blank.

Finally the solution is made up to volume with a siphon which reaches down to the bottom of the flask. This ensures sufficient mixing. Subsequently, each prepared solution is siphoned immediately to fill the respective group of bottles by siphon from the lower quarter (not from the bottom) of the bottle.

Furthermore, the zero controls are analyzed or preserved for later analysis (for the O₂ determination by precipitation with MnCl₂ (manganese(II) chloride) and NaOH (sodium hydroxide)).

The remaining parallels are placed in a water bath at 20 °C, kept in the dark, and removed from the bath or enclosure after five, 15 and 28 days, respectively, and analyzed.

Each series is accompanied by a complete parallel series for the determination of the blank, the oxygen depletion without inoculation, and the control substance.

Inhibition test:

Substances may easily and simply be tested for inhibitory effects in the closed bottle test:

series 1: 2 mg per litre of a well-degradable compound, e.g. fatty alcohol condensed with ethylene oxide, in a molecular ratio of 1/10 or any of the control chemicals,

series 2: x mg per litre of test material (x is usually 2),

series 3: 2 mg per litre of the well-degradable compounds plus x mg per litre of test material.

If the BOD values of series 3 is less than the sum of those of series 1 and 2, the test material can be considered to be inhibitory to bacteria at this concentration. This control experiment is always necessary if a negative or poor degradability result seems illogical in view of the structure of the test material, i.e. if there are indications that it may be caused by inhibition.

1.6.5. Dissolved oxygen determination

The dissolved oxygen is determined according to a chemical or electrochemical method of international or national recognized standard.

2. DATA AND EVALUATION

The analytical results are recorded on the attached form sheet (see Appendix 3).

The course of the degradation test is followed graphically in a diagram as shown in the attached example (see Appendix 4).

The results of the degradation test are valid, if the following conditions are met:

- that, in the same test series, the control material yields ≥ 60 % biodegradation within 28 days. If this is not the case, the whole series has to be discarded,
- that the oxygen depletion in the bottle without inoculation should not exceed 0,3 mg O₂ per litre after five days and 0,4 mg O₂ per litre after 28 days respectively; the blank with inoculation should not exceed 0,5 mg O₂ per litre after five days and 0,6 mg O₂ per litre after 15 and 28 days.

3. REPORTING

3.1. Test reports

The test report shall, if possible, contain the following:

- data should be reported according to the form sheet (see Appendix 3),
- the course of the degradation test is represented graphically in a diagram showing the lag phase, degradation phase, slope and time window ('time window' means here a 10-day period, starting from the day that the observed level of biodegradation first exceeds 10 %),
- method used for the COD determination,
- method used for the oxygen measurements,
- dispersion procedure for substances which are poorly soluble at the test conditions,
- proof of validity of the test.

3.2. Interpretation of results

The possibility that nitrogen-containing compounds may affect the results should be considered.

Because of the stringency of this test, a low result does not necessarily mean that the test compound is not biodegradable under environmental conditions, but indicates that more work will be necessary to establish this.

Test chemicals showing a high oxygen-uptake result in this test should be regarded as easily biodegradable provided that this level is reached within the time window of 10 days, counting from the day that the observed level of biodegradation first exceeds 10 %.

4. REFERENCES

- (1) OECD, Paris, 1981, *Test Guideline 301D*, Decision of the Council C(81) 30 final.
- (2) Gerike, P., Fischer, W. K., A correlation study of biodegradability determinations with various chemicals in various tests, *Ecotoxicology and Environmental Safety*, vol. 3, No 2, 1979, pp. 159 to 173.
- (3) Gerike, P., Fischer, W. K., A correlation study of biodegradability determinations with various chemicals in various tests. II. Additional results and conclusions, *Ecotoxicology and Environmental Safety*, vol. 5, No 1, 1981, pp. 45 to 55.

Appendix 1

Calculation of the theoretical biochemical oxygen demand

The ThOD of the substance $C_cH_hCl_{cl}N_nNa_{na}O_oP_pS_s$ of the molecular weight MW is calculated according to:

$$\text{ThOD}_{\text{NH}_3} = \frac{16 \left[2c + \frac{1}{2} (h - cl - 3n) + 3s + \frac{5}{2}p + \frac{1}{2}na - o \right]}{\text{MW}}$$

This calculation implies that C is mineralized to CO_2 , H to H_2O , P to P_2O_5 and Na to Na_2O . Halogen is eliminated as hydrogen halide and nitrogen as ammonia.

Example:

Glucose $\text{C}_6\text{H}_{12}\text{O}_6$, MW = 180.

$$\text{ThOD} = \frac{16 \left(2 \times 6 + \frac{1}{2} \times 12 - 6 \right)}{180} = 1,07 \text{ mg O}_2/\text{mg glucose}.$$

Molecular weights of salts other than those of the alkali metals are calculated on the assumption that the salts have been hydrolyzed.

Sulphur is assumed to be oxidized to the state of +6.

Example:

Sodium n-alkylbenzenesulphonate $\text{C}_{18}\text{H}_{29}\text{SO}_3\text{Na}$, MW = 348

$$\text{ThOD} = \frac{16 \left(36 + \frac{29}{2} + 3 + \frac{1}{2} - 3 \right)}{348} = 2,34 \text{ mg O}_2/\text{mg substance}.$$

In the case of nitrogen-containing substance the nitrogen may be eliminated as ammonia, nitrite, or nitrate corresponding to different theoretical biochemical oxygen demand.

$$\text{ThOD}_{\text{NO}_2^-} = \frac{16 \left[2c + \frac{1}{2} (h - cl) + 3s + \frac{3}{2}n + \frac{5}{2}p + \frac{1}{2}na - o \right]}{\text{MW}}$$

$$\text{ThOD}_{\text{NO}_3^-} = \frac{16 \left[2c + \frac{1}{2} (h - cl) + 3s + \frac{5}{2}n + \frac{5}{2}p + \frac{1}{2}na - o \right]}{\text{MW}}$$

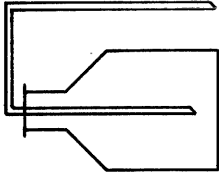
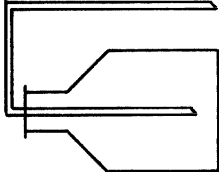
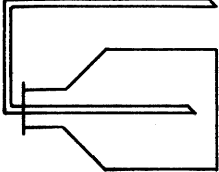
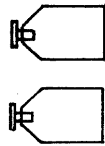
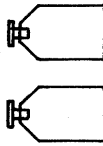
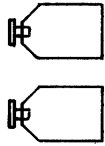
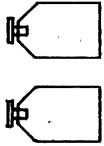
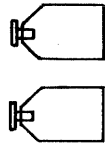
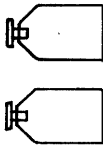
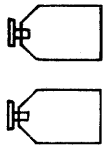
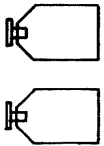
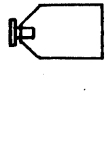
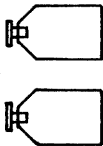
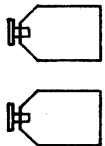
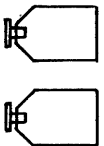
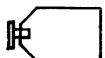
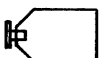
Suppose full nitrate formation had been observed by analysis in case of a secondary amine: $(\text{C}_{12}\text{H}_{25})_2\text{NH}$, MW: 353

$$\text{ThOD}_{\text{NO}_3^-} = \frac{16 \left(48 + \frac{51}{2} + \frac{5}{2} \right)}{353} = 3,44 \text{ mg O}_2/\text{mg substance}.$$

Appendix 2

Scheme for the bottle arrangement for the closed-bottle test

(* = Specific analysis if available)

Controls		Determinations	
Analyses immediately 5 days 15 days 28 days	distilled water salt solutions ↓ mineral nutrient solution (control of the oxygen blank)	distilled water salt solutions inoculation ↓ inoculation blank	distilled water salt solutions inoculation test material ↓ test materials
			
	O ₂ -det.    	O ₂ -det.    	O ₂ -det.    
	*-det. 	*-det. 	*-det.    

Appendix 3

Biotic degradation: closed bottle test (form sheet)

Testing institute
Study director
Date of start of test Experiment No
Test material
Chemical structure

Analysis (Winkler method or oxygen electrode)
ThOD or COD of test material mg O₂/milligram
Temperature of the dilution water after aeration
O₂-concentration of the water after aeration and standing before start of test mg O₂ per litre
Inoculum:

Test results

D_t = BOD expressed in % ThOD after 28 days or
 D_t = BOD expressed in % COD after 28 days

Validation of results

Control chemical
Result BOD expressed in % THOD after 28 days
Experiment Reference No

Remarks:

Testing institute

Test material

Experiment No

A: O₂ determinations:

	Flask No		mg O ₂ per litre after x days			
			0	5	15	28
Mineral nutrient solution without test material and without inoculum	O ₂ - control	c ₁		—	—	—
		c ₂		—	—	—
	Mean	$m_o = \frac{c_1 + c_2}{2}$				
Mineral nutrient solution without test material but with inoculum	1	c ₃				
	2	c ₄				
	Mean blank	$m_b = \frac{c_3 + c_4}{2}$				
Mineral nutrient solution with test material and with inoculum	1	a ₁				
	2	a ₂				
	Mean test material	$m_t = \frac{a_1 + a_2}{2}$				

B: O₂ depletions (mg BOD per litre) after x days

$$BOD_x = (m_o - m_{t_x}) - m_o - m_{b_x} (*)$$

(*) This difference is important as a check for the validity of the test.

mg BOD per litre after x days		
5	15	28

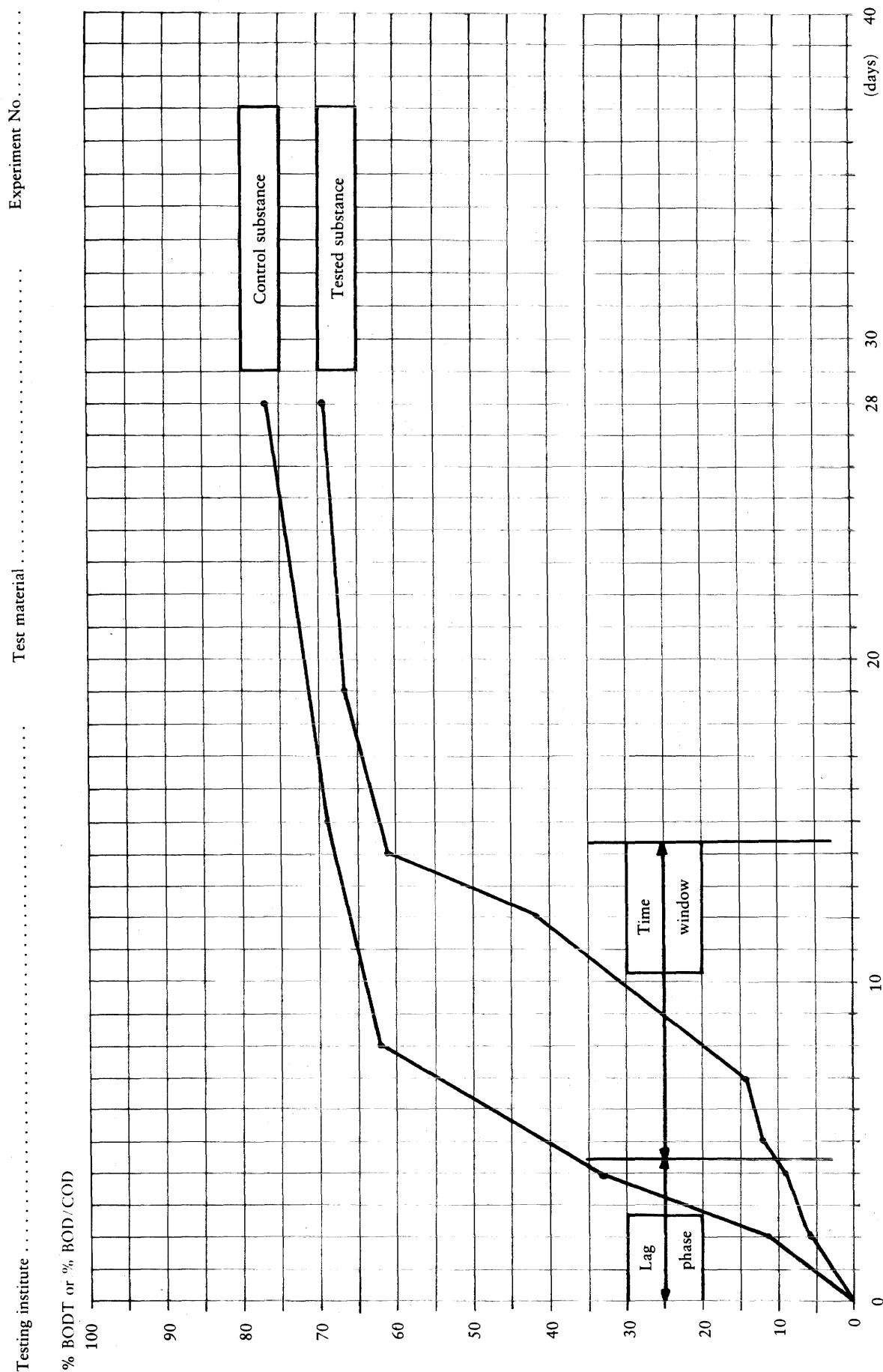
C: Evaluation

$$D_t = \frac{\text{mg BOD}_x \text{ per litre}}{\text{mg}_{\text{subs}} \text{ per litre} \cdot \text{ThOD}} \cdot 100 \quad \text{or} \quad \% \text{ BOD}_x / \text{COD} = \frac{\text{mg BOD}_x \text{ per litre}}{\text{mg}_{\text{subs}} \text{ per litre} \cdot \text{COD}} \cdot 100$$

	After x days		
	5	15	28
% BOD/ThOD			
% BOD _x /COD			

Appendix 4

Closed-bottle test

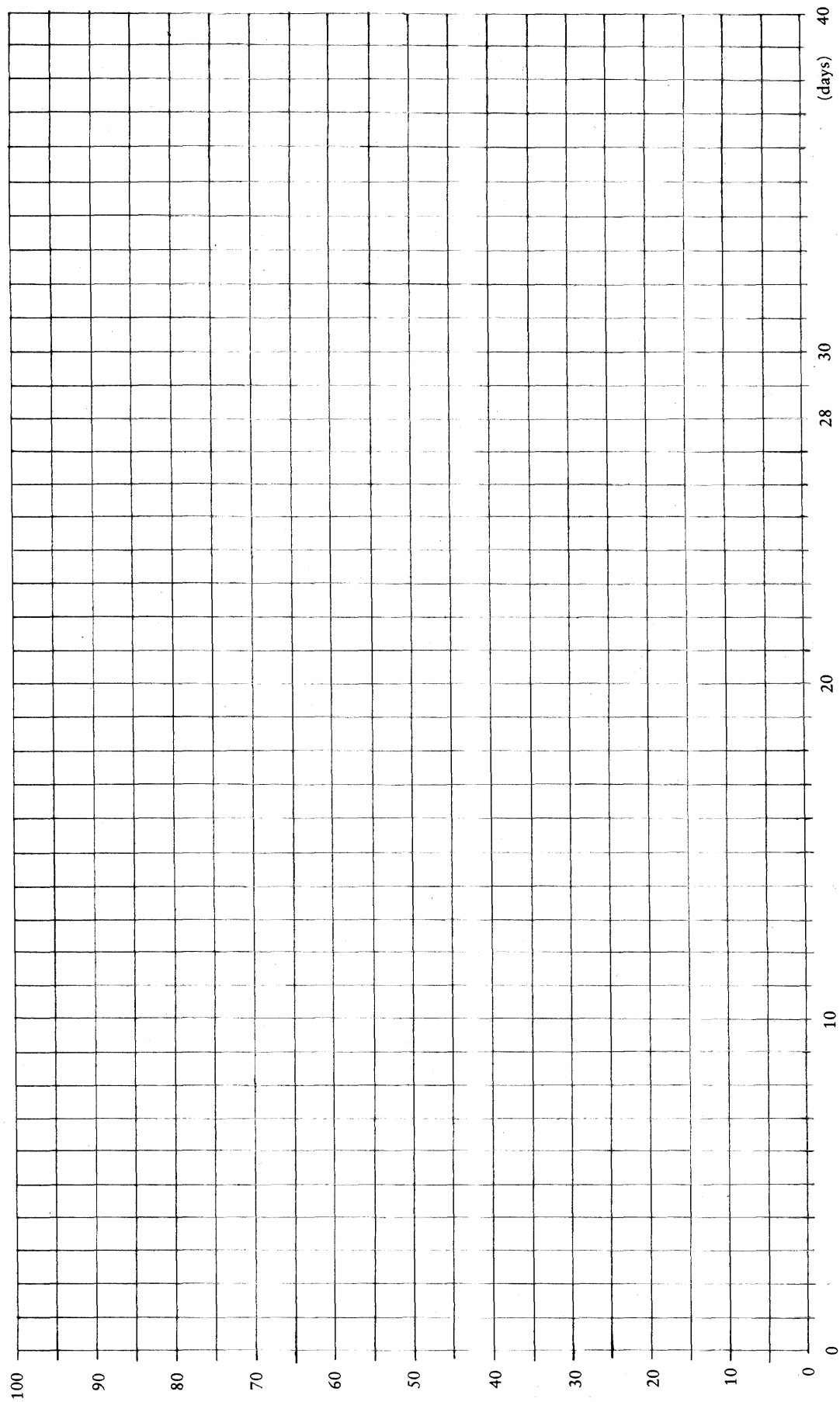


Closed-bottle test

Testing institute Experiment No.

Test material

% BODT or % BOD/COD



C. 7. DEGRADATION

BIOTIC DEGRADATION: MODIFIED MITI TEST

1. METHOD

1.1. Introduction

The purpose of this test method is to measure the biodegradability of organic substances in an aqueous medium with an enclosed respirometer giving readings of the biochemical oxygen demand.

The empirical formula of the test material is required so that the theoretical oxygen demand (ThOD) may be calculated, otherwise COD may be used.

The method is applicable only to those organic test substances which, at the concentration used in the test:

- have negligible vapour pressure,
- are not inhibitory to bacteria,
- do not reach and react with the CO₂ absorbent.

If the test material is not soluble at the test concentration, special measures, such as the use of ultrasonic dispersion, may have to be employed to achieve a good dispersion of the test substance.

Information on the toxicity to micro-organisms of the chemical may be useful for the interpretation of low results and in the selection of appropriate test concentrations.

Information on the relative proportions of the major components of the test substance will be useful in interpreting the results obtained.

1.2. Definitions and units

$$\text{Percentage degradation} = \frac{(\text{BOD} - \text{B})}{\text{ThOD (or COD)}} \times 100 \%$$

or

$$\text{Percentage degradation} = \frac{(\text{Sb} - \text{Sa})}{\text{Sb}} \times 100 \%$$

where:

BOD = biochemical oxygen demand of the test compound (experimental) (in milligrams) measured on the BOD curve,

B = oxygen consumption of basal culture medium to which the inoculum is added (experimental) (in milligrams) measured on the BOD curve,

ThOD = theoretical oxygen demand required when the test compound is completely oxidized (theoretical) (in milligrams),

Sa = residual amount of the test compound after completion of the biodegradability test (experimental) (in milligrams),

Sb = residual amount of the test compound in the blank test with water to which only the test compound has been added (experimental) (in milligrams).

1.3. Reference substances

In order to check the activity of the inoculum, the use of control substances is desirable. Aniline, sodium acetate or sodium benzoate may be used for this purpose. If the percentage of degradation of aniline

calculated from the oxygen-consumption does not exceed 40 % after seven days and 65 % after 14 days the test is regarded as invalid. If the recovery rate of Sb is found to be rather low, the test is also regarded as invalid.

1.4. Principle of the test method

The test chemicals are sole organic carbon sources, and there is no prior adaptation of micro-organisms to test chemicals.

An automated closed system oxygen consumption measuring apparatus (BOD meter) is used. Chemicals to be tested are inoculated with micro-organisms in the testing vessels. During the test period, biochemical oxygen demand is measured continuously by the BOD meter. Biodegradability is calculated on the basis of BOD, and supplementary chemical analysis is undertaken, such as measuring dissolved organic carbon concentration, concentration of residual chemicals, etc.

1.5. Quality criteria

1.5.1. Reproducibility:

Generally good, especially so for chemicals of water solubility greater than 0,1 g per litre.

1.5.2. Sensitivity:

- (A) Oxygen consumption: detection limit = 1 mg (oxygen consumption by micro-organisms)
- (B) Chemical analysis: depends on the sensitivity of analytical methods.

1.5.3. Specificity:

Applicable to every kind of chemical, for which $(C)_{\text{water}}/(C)_{\text{air}} \geq 1$. For volatile chemicals a 'modified BOD meter', composed of capillary tubing and normal BOD meter, should be used (see Appendix 1).

1.6. Description of the test method

1.6.1. Reagents

1.6.1.1. The distilled water must not contain more than 10 % carbon introduced by the test substance.

1.6.1.2. Basal culture medium

To each 3 ml of solution A, solution B, solution C and solution D, water is added to make up to 1 000 ml (deionized water is used throughout).

(A) K_2HPO_4 (dipotassium hydrogen phosphate):	21,75 g
KH_2PO_4 (potassium dihydrogen phosphate):	8,50 g
$Na_2HPO_4 \cdot 12H_2O$ (disodium hydrogen phosphate dodecahydrate):	44,60 g

NH ₄ Cl (ammonium chloride):	1,70 g
Dissolve in and make up to 1 000 ml with water (1.6.1.1).	
The pH value should be 7,2.	
(B) MgSO ₄ ·7H ₂ O (magnesium sulphate heptahydrate):	22,50 g
Dissolve in and make up to 1 000 ml with water (1.6.1.1).	
(C) CaCl ₂ (calcium chloride):	27,50 g
Dissolve in and make up to 1 000 ml with water (1.6.1.1).	
(D) FeCl ₃ ·6H ₂ O (iron(III) chloride hexahydrate):	0,25 g
Dissolve in and make up to 1 000 ml with water (1.6.1.1).	

1.6.2. Apparatus

BOD meter equipped with six bottles (300 ml each):

bottles 1 and 2:

deionized water, 300 ml + test chemical, 30 mg

bottles 3 and 4:

basal culture medium, 300 ml + activated sludge, 9 mg (dry base) + test chemical, 30 mg

bottle 5:

basal culture medium, 300 ml + activated sludge, 9 mg (dry base) + aniline or other reference substance, 30 mg

bottle 6:

basal culture medium, 300 ml + activated sludge, 9 mg (dry base)

1.6.3. Preparations of the inoculum

1.6.3.1. Activated sludge

Sludge sampling sites: Sludge sampling is made, in principle, at not less than 10 places throughout the country, chiefly in those areas where a variety of chemical substances may be considered to be consumed and discarded.

For example, for Japan, standard activated sludge of the Japanese Chemical Biotesting Centre is obtained from the following places, and mixed:

- City sewage plant: three plants located in the northern, central and southern parts of Japan,
- Industrial sewage plant: one plant used for the waste-water treatment of chemical industries,
- River: three rivers located in the northern, central and southern parts of Japan,
- Lake: one lake located in the middle of Japan,
- Sea: two inland seas of Japan.

Frequency of sludge sampling: Sludge sampling should be made, in principle, four times a year (March, June, September and December).

Sludge sampling methods:

- City sewage: one litre of recycled sludge at a sewage treatment plant,
- Rivers, lakes and marshes or sea: one litre of surface water and one litre of surface soil on the beach which is in contact with atmosphere,

Preparation:

The sludge samples collected from the sampling sites are mixed by stirring in a single container, and the mixture is allowed to stand. The floating foreign matter is removed and the supernatant is filtered with No 2 filter paper. The filtrate is adjusted to pH $7,0 \pm 1,0$ with sodium hydroxide or phosphoric acid, transferred into a culture tank and aerated.

Culture:

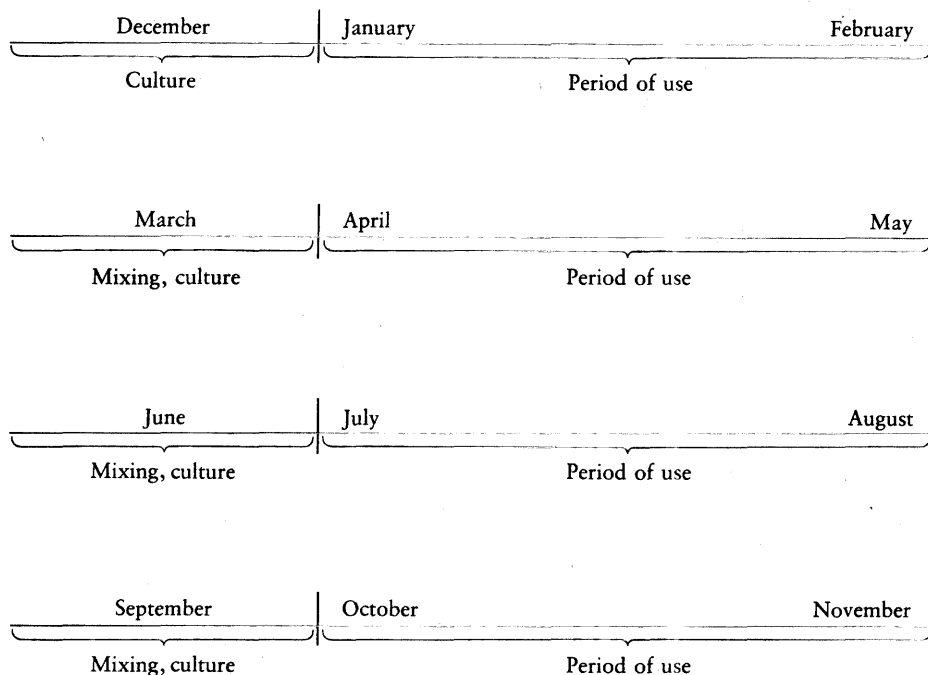
After ceasing the aeration of the solution obtained above for about 30 minutes, approximately one-third of the whole volume of the supernatant is removed. An equal volume of 0,1 % synthetic sewage is added to the remaining portion of the supernatant and the mixture is aerated again (0,1 % synthetic sewage: 1 g of glucose, 1 g of peptone and 1 g of monopotassium phosphate are dissolved in 1 litre of water and the solution is adjusted to pH $7,0 \pm 1,0$ with sodium hydroxide). This procedure is repeated once every day. The culturing is carried out at 25 ± 2 °C.

Control:

For the control of the culturing step, the following items are checked and necessary adjustments are made:

- Appearance of supernatant: the supernatant of activated sludge should be clear.
- Settling properties of activated sludge: the activated sludge in large flocs, must have good settling properties.
- State of formation of activated sludge: in the case where growth of flocs is not observed, either the volume of 0,1 % synthetic sewage to be added or the frequency of addition of synthetic sewage is increased.
- The pH of the supernatant is $7,0 \pm 1,0$.
- Temperature: the temperature for cultivation of activated sludge is 25 ± 2 °C.
- Amount of aeration: in replacing the supernatant with the synthetic sewage, the suspension in the culturing tank must be sufficiently aerated to maintain the dissolved oxygen concentration of the solution above 5 mg per litre.
- Microflora of activated sludge: when the activated sludge is microscopically observed (at $\times 100$ to 400 magnification), a number of protozoa of different species together with cloudy flocs must be seen.
- Mixing of fresh and old activated sludge: in order to maintain fresh and old activated sludges at the same activity, the filtrate of the supernatant of an activated sludge in use in the test is mixed with an equal volume of the filtrate of the supernatant of an activated sludge freshly collected and the mixture is cultured.
- Checking the activity of activated sludge: activity of activated sludge should be checked periodically, at least once every three months, with standard substances applying the test method provided below. Especially in the case where fresh and old activated sludge samples are mixed, careful checking must be done in relation to the old activated sludge.

Example of preparation of activated sludge samples and period of use:



(The same pattern of preparation and use follows.)

1.6.4. *Pretreatment of test chemical*

If the test compound is not soluble in water up to the test concentration, it should be pulverized as finely as possible.

1.6.5. *Addition of test compound and preparation for test*

The following test vessels (see 1.6.2) are required and adjusted to the test temperature:

- (1) Two test vessels containing water to which is added 100 mg per litre of the test compound (vessels 1 and 2);
- (2) Two test vessels containing the basal culture medium, to which is added 100 mg per litre of test compound: the pH of this solution is adjusted to 7 before the inoculation of activated sludge, if necessary (vessels 3 and 4);
- (3) A test vessel containing basal culture medium to which is added 100 mg per litre of aniline or any other reference substance (vessel 5);
- (4) A test vessel for the control blank test, containing only the basal culture medium (vessel 6).

1.6.5.1. *Inoculation with activated sludge*

Inoculum is added to the test vessels 3, 4, 5 and 6 above so that the suspended matter defined in the Japanese Industrial Standards (3), for example, is contained at a concentration of 30 mg per litre.

1.6.5.2. *Test conditions*

Concentration of test chemicals: 100 mg per litre.

Concentration of activated sludge: 30 mg per litre.

Test temperature: 20 to 25 °C,

Period: 28 days.

Perform in darkness. Every day, the temperature, the change in colour of the content of the culturing vessel should be checked. Stir vigorously with mechanical stirrer.

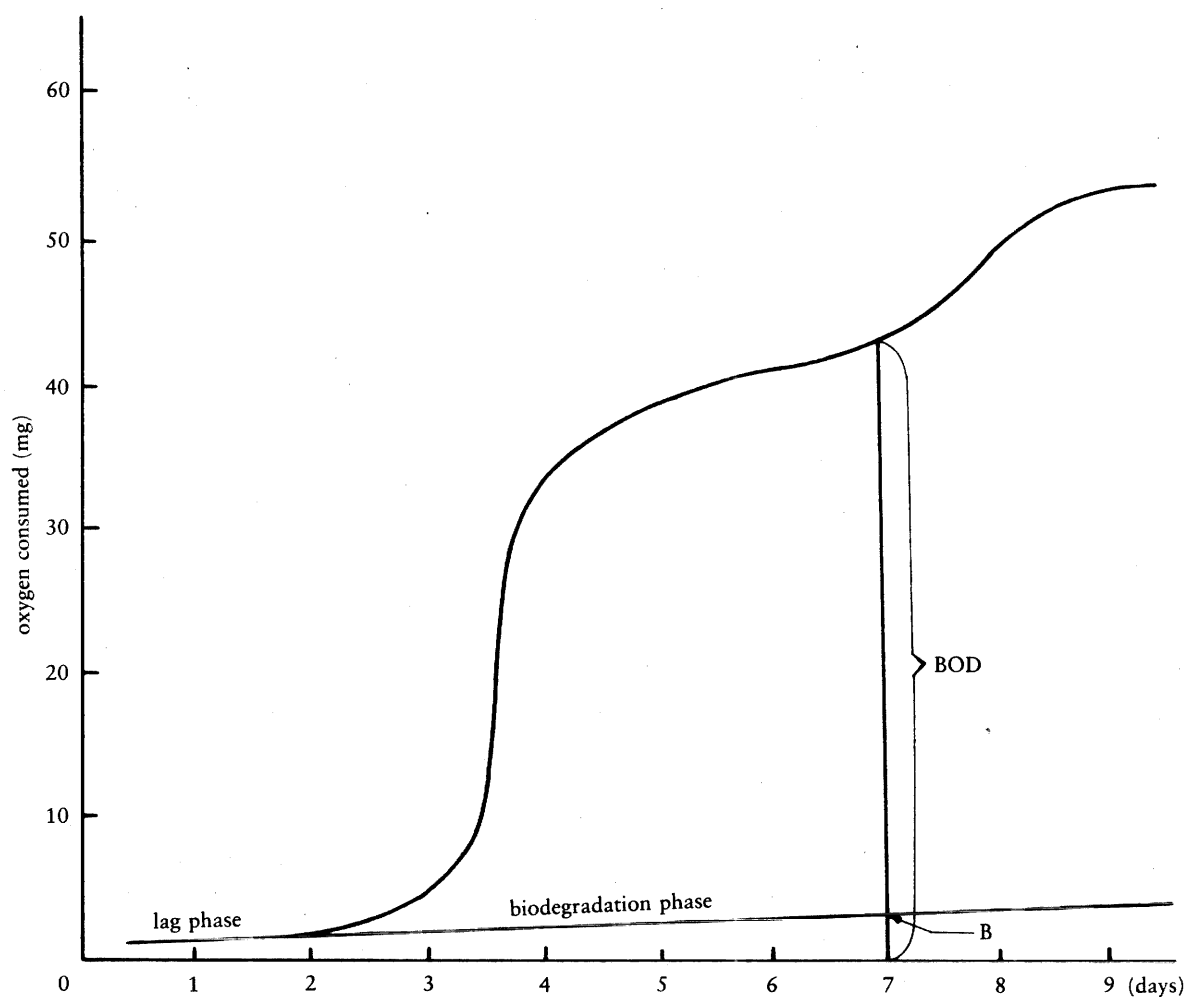
1.6.6. *Performance of the test*

The BOD curve is recorded as a continuous line for 28 days (see figure).

After the 28-day testing period, the pH and concentration of residual chemicals and intermediates in the testing vessels are determined.

Figure

BOD curve of aniline



The test chemicals in the testing vessel without activated sludge are also analyzed in order to confirm whether there is any change in the test chemical during the testing period or any loss of the original test chemical by evaporation or adsorption by the walls of the test vessels, etc.

1.6.7. *Analytical equipment*

If the test compound is soluble in water, the residual amount of total organic carbon is also determined at the end of the test.

(a) When a total organic carbon analyzer is used:

10 ml of the tested solution is sampled from the test vessel and centrifuged at 3 000 g for five minutes. The residual amount of the total organic carbon in the supernatant is determined on a total organic carbon analyzer.

(b) When other analyzers are used:

The total content of a test vessel is extracted with a suitable solvent for the test compound and, after proper pretreatment, such as concentration, the residual amount of the test compound is determined on an analyzing instrument (gas chromatograph, mass spectrometer, spectrophotometer, etc.).

For volatile chemicals, the temperature control bath of the BOD meter should be cooled to 10 °C and this temperature held for at least 30 minutes, in order to prevent evaporation. The analytical procedures (a) and (b) should then be started.

2. DATA AND EVALUATION

2.1. Treatment of results

The method for calculating the percentage degradation from the oxygen consumption and from the result of direct analysis are defined in 1.2.

2.2. Evaluation of results

The theoretical oxygen demand may be calculated either as shown in Appendix 2 or using the original MITI procedure:

<i>Element</i>	<i>Oxidized form</i>
C	CO ₂
H	H ₂ O
N	NO ₂
S	SO ₂
X (halogen)	X

3. REPORTING

3.1. Test report

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

- information on the test chemicals (name, structural formula, molecular weight, purity, nature of impurities, physical chemical properties of test chemical, identification data of test chemical),
- test conditions,
- Activated sludge: sludge sampling sites and concentration,
- test chemical: concentration,
- test period,
- test temperature,
- analytical procedure:
 - pretreatment,
 - analytical conditions of instrument,
 - recovery rate of analysis,
 - identification of intermediate,

— results:

biodegradation curves (check of the inoculum activity + curve of the substance)

BOD (mg)

B (mg)

Sa_a (mg)

Sb_b (mg)

ThOD (mg)

Percentage of degradation by BOD

percentage of degradation by chemical analysis,

chromatograms or spectra of test chemicals obtained and used for the purpose of analysis,

proof of validity (see point 1.3).

3.2. Interpretation of results

The possibility that nitrogen-containing compounds may affect the results should be considered.

If the recovery rate of Sb_b is found to be in the order of 10 % or less, this would indicate analytical problems or, for instance, hydrolysis; in such a case, special care should be taken with the interpretation.

Owing to the stringency of this test, a low result does not necessarily mean that the test compound is not biodegradable under environmental conditions, but indicates that more work will be necessary to establish this.

Test chemicals showing a high result of oxygen uptake in this test should be regarded as easily biodegradable providing that this level is reached within the time window of 10 days, counting from the day that the observed level of biodegradation first exceeds 10 %.

4. REFERENCES

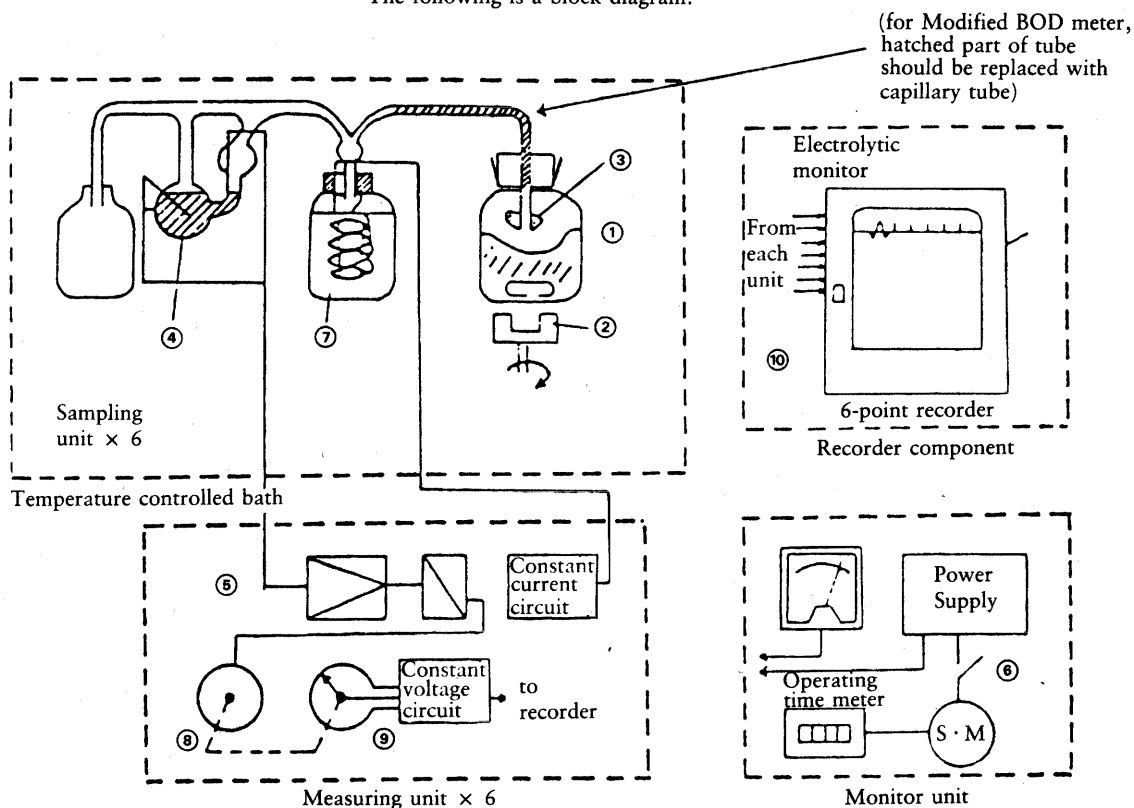
- (1) OECD, Paris, 1981, *Test Guideline 301 C*, Decision of the Council C(81) 30 final.
 - (2) *Biodegradability and bioaccumulation test of chemical substances* (C-5/98/JAP), 1978.
 - (3) *The chemical substances control law in Japan* (Chemical Products Safety Division, Basic Industries Bureau, MITI) (C-2/78/JAP), 1978.
 - (4) *The biodegradability and bioaccumulation of new and existing chemical substances*, 5,8 (C-3/78/JAP), 1978.
-

Appendix 1

Principle of closed-system oxygen consumption measuring apparatus

The oxygen consumption of micro-organisms can be determined by using an electro-chemical analysis process (e.g. coulometry).

The following is a block diagram:



The specimen contained in cultivating bottle (1) is stirred by means of a magnetic stirrer (2). When the reaction progresses, the dissolved oxygen in liquid will be consumed. Oxygen (O_2) in the space in the cultivating bottle is dissolved in liquid, resulting in generation of CO_2 in its place.

As this CO_2 is absorbed by soda lime (3), the partial pressure of oxygen in the space and the total pressure decrease.

The drop in pressure is detected and converted into an electric signal by means of an electrode type manometer (4) and is amplified by an amplifier (5) for operating a relay circuit (6), resulting in operation of a synchronous motor (8). Simultaneously, by constant current, electrolytic oxygen is generated from sulphuric acid copper solution contained in an electrolytic bottle (7).

This oxygen is supplied to the cultivating bottle and restoration of pressure is detected by means of the manometer, resulting in switching off of the relay circuit and stopping the electrolytic and synchronous motor.

The upper space in the cultivating bottle is always kept under a constant pressure of oxygen and the quantity of oxygen consumed in the cultivating bottle is proportional to the quantity of electrolytic oxygen. As this quantity of electrolytic oxygen is proportional to electrolytic time, there is a constant electrolysis current. Accordingly, the revolution angle of a synchronous motor (9) is converted to a mV signal by means of the interlocking potentiometer, resulting in an indicator quantity of consumed oxygen at the recorder (10).

Appendix 2

Calculation of the theoretical biochemical oxygen demand

The ThOD of the substance $C_cH_hCl_{cl}N_nNa_{na}O_oP_pS_s$ of the molecular weight MW is calculated according to:

$$\text{ThOD}_{\text{NH}_3} = \frac{16 \left[2c + \frac{1}{2}(h - cl - 3n) + 3s + \frac{5}{2}p + \frac{1}{2}na - o \right]}{\text{MW}}$$

This calculation implies that C is mineralized to CO_2 , H to H_2O , P to P_2O_5 and Na to Na_2O . Halogen is eliminated as hydrogen halide and nitrogen as ammonia.

Example:

Glucose $\text{C}_6\text{H}_{12}\text{O}_6$, MW = 180

$$\text{ThOD} = \frac{16 \left(2 \times 6 + \frac{1}{2} \times 12 - 6 \right)}{180} = 1,07 \text{ mg O}_2/\text{mg glucose}$$

Molecular weights of salts other than those of the alkali metals are calculated on the assumption that the salts have been hydrolyzed.

Sulphur is assumed to be oxidized to the state of +6.

Example:

Sodium n-alkylbenzenesulphonate $\text{C}_{18}\text{H}_{29}\text{SO}_3\text{Na}$, MW = 348

$$\text{ThOD} = \frac{16 \left(36 + \frac{29}{2} + 3 + \frac{1}{2} - 3 \right)}{348} = 2,34 \text{ mg O}_2/\text{mg substance}$$

In the case of nitrogen-containing substance the nitrogen may be eliminated as ammonia, nitrite, or nitrate corresponding to different theoretical biochemical oxygen demand.

$$\text{ThOD}_{\text{NO}_2^-} = \frac{16 \left[2c + \frac{1}{2}(h - cl) + 3s + \frac{3}{2}n + \frac{5}{2}p + \frac{1}{2}na - o \right]}{\text{MW}}$$

$$\text{ThOD}_{\text{NO}_3^-} = \frac{16 \left[2c + \frac{1}{2}(h - cl) + 3s + \frac{5}{2}n + \frac{5}{2}p + \frac{1}{2}na - o \right]}{\text{MW}}$$

Suppose full nitrate formation had been observed by analysis in case of a secondary amine:

$(\text{C}_{12}\text{H}_{25})_2\text{NH}$, MW: 353.

$$\text{ThOD}_{\text{NO}_3^-} = \frac{16 \left(48 + \frac{51}{2} + \frac{5}{2} \right)}{353} = 3,44 \text{ mg O}_2/\text{mg substance.}$$

Appendix 3

Biotic degradation: modified MITI test

Testing institute
 Study director
 Date of start of the test
 Test material Experiment No
 Chemical structure

 Analytical procedure
 ThOD or COD of test material
 Inoculum
 Sampling site
 Concentration

Test results

..... % degradation = $\frac{\text{BOD} - \text{B}}{\text{ThOD}} \times 100 \%$ after 28 days

or

..... % degradation = $\frac{\text{BOD} - \text{B}}{\text{COD}} \times 100 \%$ after 28 days

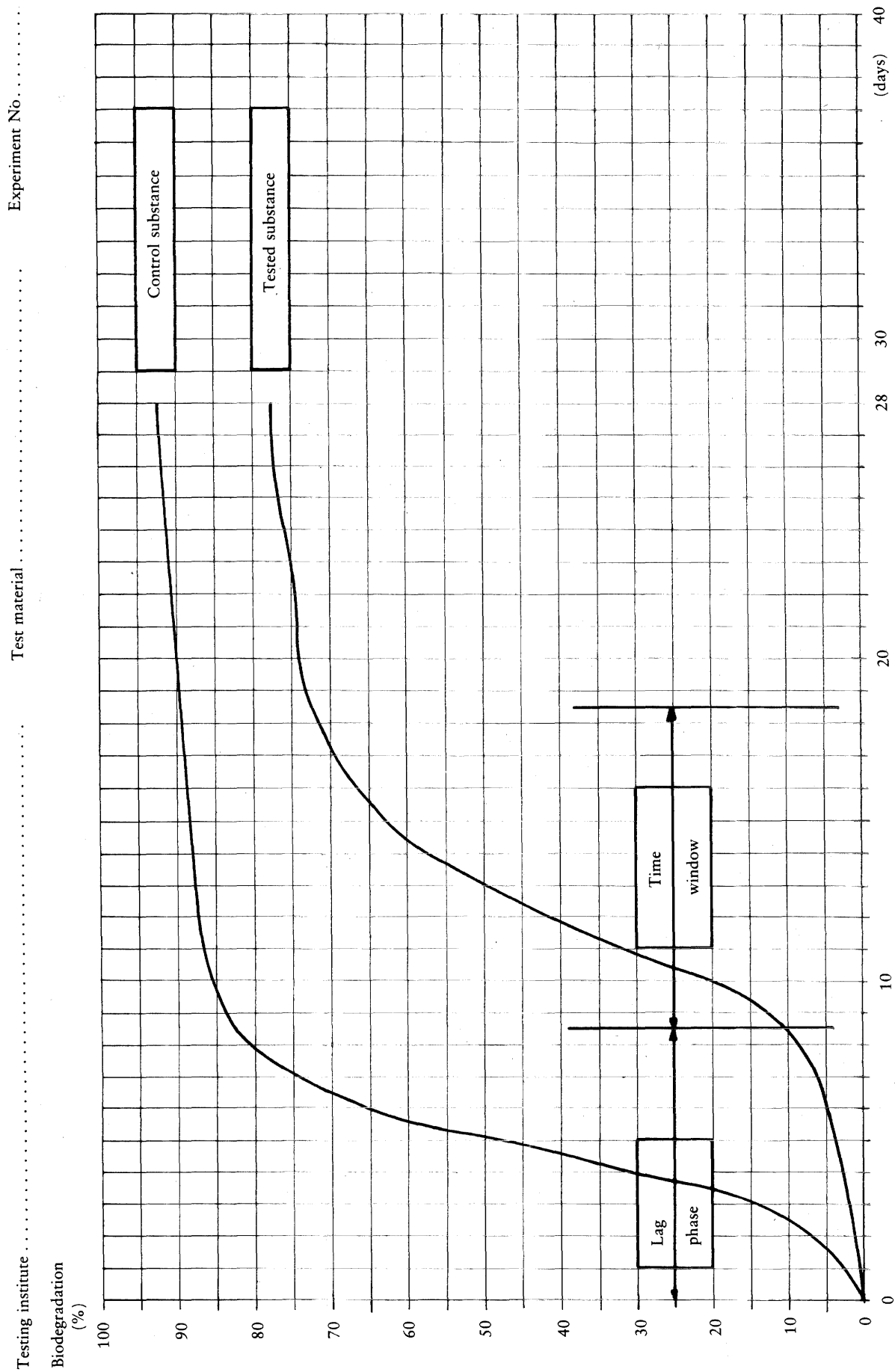
..... % degradation = $\frac{\text{Sb} - \text{Sa}}{\text{Sb}} \times 100 \%$ after 28 days

Validation of the results

Control chemical
 Result: % degradation after 28 days
 Experiment reference No

Remarks:

Appendix 4
Modified MITI test

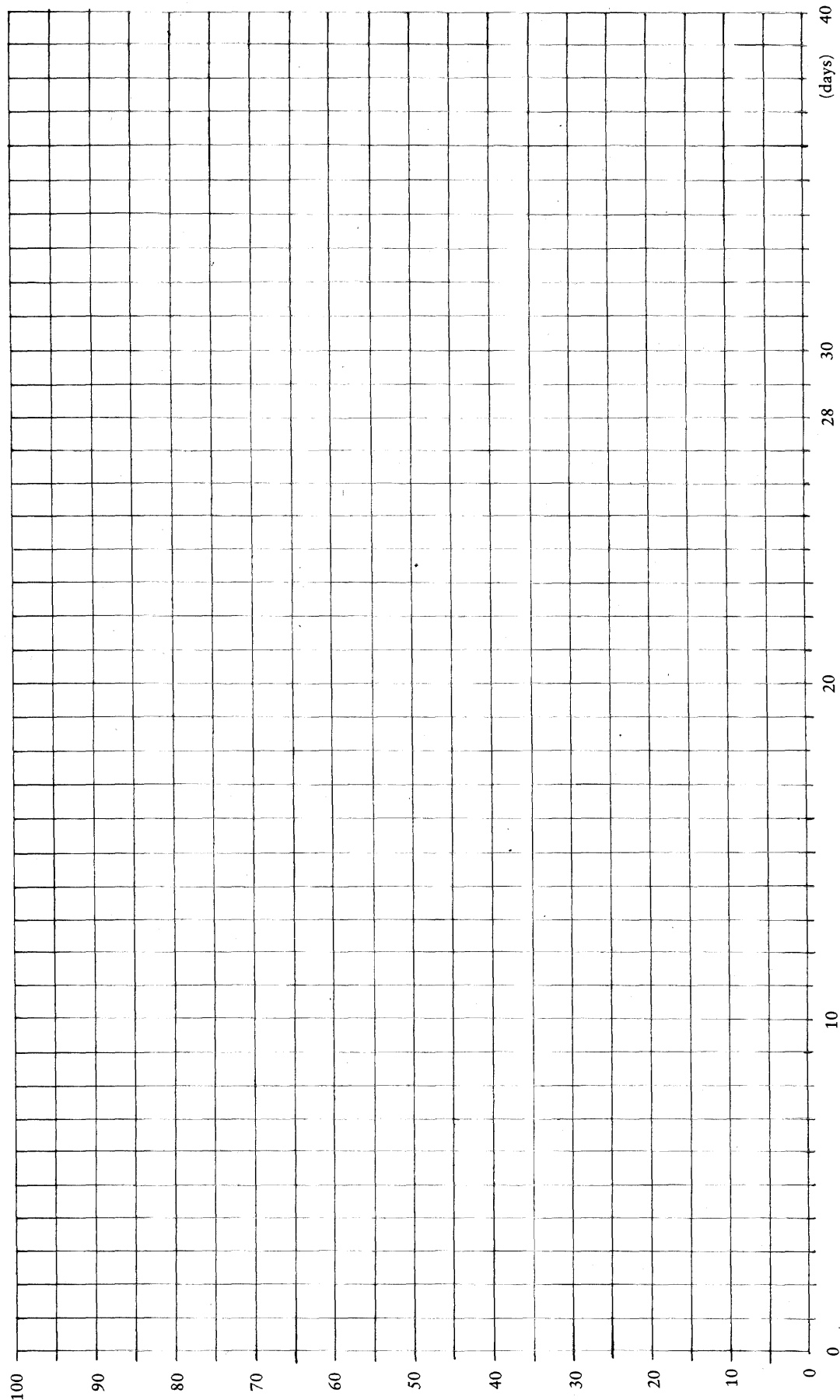


Modified MITI test

Testing institute Experiment No.

Test material

Biodegradation
(%)



C. 8. DEGRADATION

BIOCHEMICAL OXYGEN DEMAND

1. METHOD

1.1. Introduction

The purpose of the method is the measurement of the biochemical oxygen demand (BOD) of solid or liquid organic substances.

Data elaborated with this test pertain to water-soluble compounds; however, volatile compounds and those of low water solubility may also, at least in principle, be tested.

The method is applicable only to those organic test materials which are not inhibitory to bacteria at the concentration used in the test. If the test material is not soluble at the test concentration, special measures, such as the use of ultrasonic dispersion, may have to be employed to achieve good dispersion of test material.

Information on the toxicity of the chemical may be useful to the interpretation of low results and in the selection of appropriate test concentrations.

1.2. Definition and units

The BOD is defined as the mass of dissolved oxygen required by a specified volume of solution of the substance for the process of biochemical oxidation under prescribed conditions.

The results are expressed as grams of BOD per gram of tested substance.

1.3. Reference substances

Reference substances for calibration cannot yet be recommended. The use of a suitable control chemical to check the activity of the inoculum is desirable.

1.4. Principle of the test method

A predetermined amount of the substance, dissolved or dispersed in a well-aerated suitable medium, is inoculated with micro-organisms and incubated at a constant defined ambient temperature in the dark.

The BOD is determined by the difference in dissolved oxygen content at the beginning and at the end of the test. The duration of the test must be at least five days and not more than 28 days.

A blank must be determined in a parallel assay containing no test substance.

1.5. Quality criteria

The BOD determination cannot be considered as a valid determination of the biodegradability of a substance. This test may only be regarded as a screening test.

1.6. Description of the test method

A preliminary solution or dispersion of the substance is prepared to obtain a BOD concentration compatible with the method used. The BOD is then determined following any suitable national standardized method. An international one, yet to be agreed, would be preferred.

2. DATA AND EVALUATION

The BOD contained in the preliminary solution is calculated according to the selected normalized method, and converted into grams of BOD per gram of tested substance.

3. REPORTING

The method used shall be stated.

The biochemical oxygen demand should be a mean of at least three valid measurements.

All information and remarks relevant for the interpretation of results have to be reported, especially with regard to impurities, physical state, toxic effects and inherent composition of the substance which would affect the results.

The use of an additive to inhibit biological nitrification must be reported.

4. REFERENCES

List of standardized methods, for example:

NF T 90 — 103: Determination of the biochemical oxygen demand.

NBN 407: Biochemical oxygen demand.

NEN 3235 5.4: Bepaling van het biochemisch zuurstofverbruik (BZV).

The determination of biochemical oxygen demand, *Methods for the examination of water and associated materials*, HMSO, London.

C. 9. DEGRADATION

CHEMICAL OXYGEN DEMAND

1. METHOD

1.1. Introduction

The purpose of the method is the measurement of the chemical oxygen demand (COD) of solid or liquid organic substances in a standard, arbitrary manner, under fixed laboratory conditions.

Information on the formula of the substance will be useful to conduct this test and interpret the result obtained (e.g halogen salts, ferrous salts of organic compounds, organochlorine compounds).

1.2. Definitions and units

The chemical oxygen demand is a measure of the oxidizability of a substance, expressed as the equivalent amount in oxygen of an oxidizing reagent consumed by the substance under fixed laboratory conditions.

The result is expressed in grams of COD per gram of tested substance.

1.3. Reference substances

Reference substances do not need to be employed in all cases when investigating a new substance. They should serve primarily to calibrate the method from time to time and to allow comparison of results when another method is applied.

1.4. Principle of the test method

A predetermined amount of the substance, dissolved or dispersed in water, is oxidized by potassium dichromate in a strong sulphuric acid medium with silver sulphate as a catalyst, under reflux for two hours. The residual dichromate is determined by titration with standardized ferrous ammonium sulphate.

In case of chlorine-containing substances, mercuric sulphate is added to reduce chloride interference.

1.5. Quality criteria

Because of the arbitrary manner of determination, COD must be considered more as an 'oxidizability indicator' than as a measure of organic matter.

Chloride can interfere in this test; inorganic reducing or oxidizing agent may also interfere with the COD determination.

Some cyclic compounds are not fully oxidized by this test.

1.6. Description of the test method

A preliminary solution or dispersion of the substance is prepared to obtain a COD between 250 and 600 mg per litre.

Remarks:

In the case of poorly soluble and non-dispersible substances, an amount of finely powdered substance or liquid substance corresponding to about 5 mg of COD can be weighed and put in the experimental apparatus with water.

The COD is then determined following any suitable national standardized method, pending the publication of an internationally standardized method, which would be preferred.

2. **DATA AND EVALUTION**

The COD contained in the experimental flask is calculated following the selected normalized method, and converted to grams of COD per gram of tested substance.

3. **REPORTING**

The reference method used should be stated.

The chemical oxygen demand should be a mean of at least three measurements. All information and remarks relevant to the interpretation of the results have to be reported, especially with regard to impurities, physical state and inherent properties of the substance (if known) which would affect the results.

The use of mercuric sulphate to minimize the chloride interference must be reported.

4. **REFERENCES**

List of standardized methods, for example:

NBN T 91 — 201	Determination of the chemical oxygen demand.
ISBN 0 11 7512494	Chemical oxygen demand (dichromate value) of polluted and waste waters.
NF T 90 — 101	Determination of the chemical oxygen demand.
DS 217 = water analysis	Determination of the chemical oxygen demand.
DIN 38409 — H — 41	Determination of the chemical oxygen demand (COD) within the range above 15 mg per litre.
NEN 3235 5.3	Bepaling van het chemisch zuurstofverbruik.
ISO DP 6060	Water quality: chemical oxygen demand dichromate methods.

C. 10. DEGRADATION

ABIOTIC DEGRADATION: HYDROLYSIS AS A FUNCTION OF pH

1. METHOD

This method is based on the OECD Test Guideline (1).

1.1. Introduction

Hydrolysis is an important reaction controlling abiotic degradation. This reaction is particularly relevant for substances with low biodegradability; and it can influence the persistence of a substance in the environment.

Most hydrolysis reactions are of *pseudo* first-order and, therefore, half-life times are independent of concentration. This usually allows the extrapolation of results found at laboratory concentration to environmental conditions.

Furthermore, several examples have been reported (2), showing a satisfactory agreement between the results found in pure and natural waters for several types of chemicals.

It is useful to have preliminary information on the vapour pressure of the substance to perform this test.

This method is only applicable to water-soluble substances. Impurities will usually affect the results.

Hydrolytic behaviour of chemicals should be examined at pH values more commonly found in the environment (pH 4 to 9).

1.2. Definitions and units

Hydrolysis refers to a reaction of a chemical RX with water. This reaction may be represented by the net exchange of the group X with OH:



The rate at which the concentration of RX decreases is given by:

$$\text{rate} = k [\text{H}_2\text{O}] [\text{RX}] \quad [2]$$

Because water is present in great excess compared to the chemical, this type of reaction is usually described as a *pseudo*-first order reaction in which the observed rate constant is given by the relationship:

$$k_{\text{obs}} = k \times [\text{H}_2\text{O}] \quad [3]$$

This constant can be determined for one pH value and one temperature, T, using the expression:

$$k_{\text{obs}} = \frac{2,303}{t} \times \log \frac{C_0}{C_t} \quad [4]$$

where:

t = time,

C₀ = the concentration of the substance at time 0,

C_t = the concentration of the substance at time t, and

2,303 = the conversion factor between natural and base 10 logarithms.

The concentrations are expressed in grams per litre or moles per litre.

The dimension of this constant k_{obs} is $(\text{time})^{-1}$.

'The half-life period' $t_{1/2}$ is defined as the time required to reduce the concentration of the test substance by 50 %, that is:

$$C_t = \frac{1}{2} C_0 \quad [5]$$

From the expressions (4) and (5) one can demonstrate that:

$$t_{1/2} = 0,693/k_{\text{obs}} \quad [6]$$

1.3. Reference substances

It is not necessary to use reference substances in all cases when investigating a new substance. They should serve primarily to check the performance of the method from time to time and to allow the comparison of results when another method is applied.

Acetylsalicylic acid (aspirin)

Phosphorothioic acid 0,0-diethyl 0-(6-methyl-2-(1-methylethyl)4-pyrimidinyl ester. (Dimpylate, Diazinon), have been used as reference substances (1).

1.4. Principle of the test method

The substance is dissolved in water at a low concentration, the pH and the temperature are controlled.

The decrease of the concentration of the substance with time is followed by any suitable analytical procedure.

The logarithm of the concentration is plotted against time and, if the plot is a straight line, the first-order rate constant may be obtained from its slope (see point 2).

When it is not practical to determine a rate constant directly for a particular temperature, it is usually possible to estimate the constant through the use of the Arrhenius relationship, which gives the temperature dependence of the rate constant. From the linear plot of the logarithm of the rate constant as determined at appropriate temperature as a function of the reciprocal of the absolute temperature, K, it is possible to extrapolate the rate constant value which was not directly obtainable.

1.5. Quality criteria

It is reported in reference (2) that measurements of hydrolysis rate-constants on 13 classes of organic structures can be of high precision.

The repeatability depends in particular on the control of the pH value, on dissolved oxygen concentration and might be affected by the presence of micro-organisms.

1.6. Description of the test method

1.6.1. *Reagents*

1.6.1.1. Buffer solutions

The test is carried out at three pH values: 4,0, 7,0 and 9,0.

For this purpose, buffer solutions should be prepared using reagent grade chemicals and distilled or deionized, sterile water. Some examples of buffer systems are presented in the Appendix.

The buffer system used may influence the rate of hydrolysis; if there is evidence of this, an alternative buffer system should be employed. The use of borate or acetate buffers is recommended in reference (2) instead of phosphate.

If the pH value of the buffer solutions is not known at the temperature used during the test, this can be determined with a calibrated pH meter at the selected temperature with a precision of $\pm 0,1$ pH units.

1.6.1.2. Test solutions

The test substance should be dissolved in the selected buffer and the concentration should not exceed 0,01 M or half the saturation concentration, whichever is the lower.

The use of water-miscible organic solvents is recommended only for substances of low water solubility.

The amount of solvent should be less than 1 %, and should not interfere with the hydrolytic process.

1.6.2. *Apparatus*

Stoppered glass flasks should be used, but grease must be avoided on the ground joint.

If the chemical or the buffer system is volatile, or if the test is being conducted at elevated temperatures, sealed or septum-closed tubes are preferred and head space should be avoided.

1.6.3. *Analytical method*

The analytical method used will depend on the nature of the substance and must be sufficiently precise and sensitive to detect a reduction of 10 % of the initial concentration.

The method must be specific to allow determination of the test substance at the test solution concentrations and may well consist of some combination of suitable analytical techniques.

1.6.4. *Test conditions*

The tests will be carried out using a thermostatically controlled enclosure or a constant-temperature bath set at $\pm 0,5$ °C of the chosen temperature. The temperature will be kept and measured to within $\pm 0,1$ °C. Photolytic interference should be avoided by appropriate means.

All suitable precautions should be taken to exclude dissolved oxygen (e.g. by bubbling with nitrogen or argon for five minutes before preparation of the solution).

1.6.5. *Test procedure*

1.6.5.1. Preliminary test

For all substances a preliminary test should be performed at $50 \pm 0,5$ °C at three pH values: 4,0, 7,0 and 9,0. A sufficient number of measurements are made, in order to be able to estimate whether, for each pH value and at 50 °C, the half-life time ($t_{1/2}$) is lower than 2,4 hours or less than 10 % of hydrolysis is observed after five days. (One can estimate that these values correspond respectively to half-life times lower than one day or higher than one year under conditions more representative of those of the environment (25 °C)).

If the preliminary test indicates that 50 % or more of the test substance has been hydrolyzed in 2,4 hours at 50 °C, or less than 10 % has been hydrolyzed after five days at each of the three pH values (4, 7 and 9), no further testing is necessary.

In other cases, and for individual pH values for which this condition has not been fulfilled, test 1 is carried out.

1.6.5.2. Test 1

Test 1 is carried out at one temperature; preferably at $50 \pm 0,5$ °C, if possible, under sterile conditions at those pH values for which the preliminary test has shown the necessity for further testing.

A sufficient number of samples (not less than four) should be chosen to cover the range 20 to 70 % of hydrolysis to test for *pseudo*-first order behaviour at the specified pH values.

For each pH value at which test 1 is performed the order of reaction is determined.

Estimation of rate constant at 25 °C:

The decision on how to proceed experimentally depends on whether it may be concluded from test 1 that the reaction is *pseudo*-first order or not.

If it cannot be concluded with certainty from test 1 that the reaction is *pseudo*-first order, further experiments must be carried out as described in test 2.

If it can be safely concluded from test 1 that the reaction is *pseudo*-first order, further experiments should be carried out as described in test 3. (Alternatively, it may, under special circumstances, be possible to calculate the rate constants at 25 °C from constants at 50 °C, calculated using results from test 1, (see 3.2)).

1.6.5.3. Test 2

This test is performed, at each pH value for which the results of test 1 have shown the necessity to do so:

- either at one temperature lower than 40 °C,
- or at two temperatures above 50 °C differing from each other by at least 10 °C.

For each pH value and temperature where test 2 is carried out at least six adequately spaced data points should be taken so that the degrees of hydrolysis are in the range 20 to 70 %.

For one pH value and one temperature, a determination is carried out in duplicate. When test 2 is done at two temperatures above 50 °C, the duplicate is preferably performed at the lower of these two temperatures.

For each pH value and temperature where test 2 is carried out, a graphical estimation of the half-life time ($t_{1/2}$) will be given when possible.

1.6.5.4. Test 3

This test is carried out, at each pH value for which the results of test 1 have shown the necessity to do so:

- either at one temperature lower than 40 °C,
- or at two temperatures above 50 °C differing from each other by at least 10 °C.

For each pH and temperature where test 3 is performed, three data points are chosen, the first at time 0 and the second and third when the degree of hydrolysis is greater than 30 %; the constant k_{obs} and $t_{1/2}$ should be calculated.

2. DATA

For *pseudo*-first order behaviour the values of k_{obs} for each pH value and each temperature of the tests can be obtained from the plots of the logarithms of the concentration versus time using the expression:

$$k_{\text{obs}} = - \text{slope} \times 2,303$$

Furthermore $t_{1/2}$ can be calculated according to equation [6].

Evaluate $k_{25\text{ °C}}$ by applying the Arrhenius equation where appropriate.

For non-*pseudo*-first order behaviour see 3.1.

3. REPORT**3.1. Reporting**

The test report shall if possible include the following information:

- specification of the substance,
- any results obtained with reference substances,
- the principle and details of the analytical method used,
- for each test: the temperature, pH value, buffer composition and a table of all concentration-time data points,
- for *pseudo*-first order reaction, the values of k_{obs} of $t_{1/2}$ and its calculation procedure,
- for non-*pseudo*-first order reaction, plot results as logarithm of concentration versus time,
- all information and observations necessary for the interpretation of the results.

3.2. Interpretation of results

It may be possible to calculate acceptable values of the rate constant (at 25 °C) of the test substances, provided that experimental values of the activation energy already exist for homologues of the test substance and provided that it can be reasonably assumed that the activation energy of the test substance is of the same order of magnitude.

4. REFERENCES

- (1) OECD, Paris, 1981, *Test Guideline 111*, Decision of the Council C(81) 30 final.
 - (2) OECD, Paris, 1981, *Test Guideline 111*, Decision of the Council C(81) 30 final, reference 2.
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Appendix

BUFFER MIXTURES

A. CLARK AND LUBS

The pH values reported in these tables have been calculated from the potential measurements using Sørensen standard equations (1909). The actual pH value are 0,04 unit higher than the tabulated values.

Composition	pH
0,1 M potassium hydrogen phthalate + 0,1 N HCl at 20 °C	
2,63 ml 0,1 N HCl + 50 ml phthalate to 100 ml	3,8
0,1 M potassium hydrogen phthalate + 0,1 N NaOH at 20 °C	
0,40 ml 0,1 N NaOH + 50 ml phthalate to 100 ml	4,0
3,70 ml 0,1 N NaOH + 50 ml phthalate to 100 ml	4,2
0,1 M monopotassium phosphate + 0,1 N NaOH at 20 °C	
23,45 ml 0,1 N NaOH + 50 ml phosphate to 100 ml	6,8
29,63 ml 0,1 N NaOH + 50 ml phosphate to 100 ml	7,0
35,00 ml 0,1 N NaOH + 50 ml phosphate to 100 ml	7,2
0,1 M H ₃ BO ₃ in 0,1 M KCl + 0,1 N NaOH at 20 °C	
16,30 ml 0,1 N NaOH + 50 ml boric acid to 100 ml	8,8
21,30 ml 0,1 N NaOH + 50 ml boric acid to 100 ml	9,0
26,70 ml 0,1 N NaOH + 50 ml boric acid to 100 ml	9,2

B. KOLTHOFF AND VLEESHOUWER

Composition	pH
0,1 M monopotassium citrate and 0,1 N NaOH at 18 °C (add tiny crystal of thymol or a few milligrams of mercuric iodide to prevent growth of moulds)	
2,0 ml 0,1 N NaOH + 50 ml citrate to 100 ml	3,8
9,0 ml 0,1 N NaOH + 50 ml citrate to 100 ml	4,0
16,3 ml 0,1 N NaOH + 50 ml citrate to 100 ml	4,2

C. SÖRENSEN

0,05 M borax + 0,1 N HCl

Composition		pH			
Borax (ml)	HCl (ml)	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 borax + 0,1 N NaOH

Composition		pH			
Borax (ml)	NaOH (ml)	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