

II

(Non-legislative acts)

REGULATIONS

COMMISSION REGULATION (EU) 2016/266

of 7 December 2015

amending, for the purpose of its adaptation to technical progress, Regulation (EC) No 440/2008 laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH)

(Text with EEA relevance)

THE EUROPEAN COMMISSION,

Having regard to the Treaty on the Functioning of the European Union,

Having regard to Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No 793/93 and Commission Regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC ⁽¹⁾, and in particular Article 13(2) thereof,

Whereas:

- (1) Commission Regulation (EC) No 440/2008 ⁽²⁾ contains the test methods for the purposes of the determination of the physicochemical properties, toxicity and ecotoxicity of chemicals to be applied for the purposes of Regulation (EC) No 1907/2006.
- (2) It is necessary to update Regulation (EC) No 440/2008 to include new and updated test methods recently adopted by the OECD in order to take into account technical progress, and to ensure the reduction in the number of animals to be used for experimental purposes, in accordance with Directive 2010/63/EU of the European Parliament and of the Council ⁽³⁾. Stakeholders have been consulted on this draft.
- (3) The adaptation contains twenty test methods: one new method for the determination of a physicochemical property, eleven new test methods and three updated test methods for the assessment of ecotoxicity, and five new test methods to assess the environmental fate and behaviour.
- (4) Regulation (EC) No 440/2008 should therefore be amended accordingly.
- (5) The measures provided for in this Regulation are in accordance with the opinion of the Committee established under Article 133 of Regulation (EC) No 1907/2006,

⁽¹⁾ OJ L 396, 30.12.2006, p. 1.

⁽²⁾ Commission Regulation (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) (OJ L 142, 31.5.2008, p. 1).

⁽³⁾ Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes (OJ L 276, 20.10.2010, p. 33).

HAS ADOPTED THIS REGULATION:

Article 1

The Annex to Regulation (EC) No 440/2008 is amended in accordance with the Annex to this Regulation.

Article 2

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

This Regulation shall be binding in its entirety and directly applicable in all Member States.

Done at Brussels, 7 December 2015.

For the Commission
The President
Jean-Claude JUNCKER

ANNEX

The Annex to Regulation (EC) No 440/2008 is amended as follows:

- (1) A note is inserted at the beginning of the Annex, before Part A:

Note:

Before using any of the following test methods to test a multi-constituent substance (MCS), a substance of unknown or variable composition, complex reaction product or biological material (UVCB), or a mixture and where its applicability for the testing of MCS, UVCB, or mixtures is not indicated in the respective test method, it should be considered whether the method is adequate for the intended regulatory purpose.

If the test method is used for the testing of a MCS, UVCB or mixture, sufficient information on its composition should be made available, as far as possible, e.g. by the chemical identity of its constituents, their quantitative occurrence, and relevant properties of the constituents.'

- (2) Chapter A.24 is added:

'A.24. PARTITION COEFFICIENT (N-OCTANOL/WATER), HIGH PERFORMANCELIQUID CHROMATOGRAPHY (HPLC) METHOD

INTRODUCTION

This test method is equivalent to OECD test guideline (TG) 117 (2004)

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

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

The partition coefficient being the quotient of two concentrations, is dimensionless and is usually given in the form of its logarithm to base ten.

2. P_{ow} is a key parameter in studies of the environmental fate of chemical substances. A highly-significant relationship between the P_{ow} of non-ionised form of substances and their bioaccumulation in fish has been shown. It has also been shown that P_{ow} is a useful parameter in the prediction of adsorption on soil and sediments and for establishing quantitative structure-activity relationships for a wide range of biological effects.
3. The original proposal for this test method was based on an article by C.V. Eadsforth and P. Moser (1). The development of the test method and an OECD inter-laboratory comparison test were coordinated by the Umweltbundesamt of the Federal Republic of Germany during 1986 (2).

INITIAL CONSIDERATIONS

4. $\log P_{ow}$ values in the range - 2 to 4 (occasionally up to 5 and more) (1) can be experimentally determined by the Shake-Flask method (Chapter A.8 of this Annex, OECD Test Guideline 107). The HPLC method covers $\log P_{ow}$ in the range of 0 to 6 (1)(2)(3)(4)(5). This method may require an estimation of P_{ow} to assign suitable reference substances and support any conclusions drawn from the data generated by the test. Calculation methods are briefly discussed in the Appendix to this test method. The HPLC operation mode is isocratic.
5. The P_{ow} values depend on the environmental conditions such as temperature, pH, ionic strength etc, and these should be defined in the experiment for the correct interpretation of P_{ow} data. For ionisable substances, another method (e.g. draft OECD guideline on pH metric method for ionised substances (6)) may become available and could be used as an alternative method. Although this draft OECD guideline may appropriate be suitable to determine P_{ow} for those ionisable substances, in some cases it is more appropriate to use the HPLC method at an environmentally relevant pH (see paragraph 9).

(1) An upper limit is given by the necessity to achieve a complete separation phase after adjustments of the partition equilibrium and before samples are taken out for analytical determinations. If proper care is taken, the upper limit can be extended to higher values of P_{ow}

PRINCIPLE OF THE METHOD

6. Reverse phase HPLC is performed on analytical columns packed with a commercially available solid phase containing long hydrocarbon chains (e.g. C₈, C₁₈) chemically bound onto silica.
7. A chemical injected on such a column partitions between the mobile solvent phase and the hydrocarbon stationary phase as it is transported along the column by the mobile phase. The substances are retained in proportion to their hydrocarbon-water partition coefficient, with hydrophilic substances eluted first and lipophilic substances last. The retention time is described by the capacity factor *k* given by the expression:

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

where *t_R* is the retention time of the test substance, and *t₀* is the dead-time, i.e. the average time a solvent molecule needs to pass the column. Quantitative analytical methods are not required and only the determination of retention times is necessary.

8. The octanol/water partition coefficient of a test substance can be computed by experimentally determining its capacity factor *k* and then inputting *k* into the following equation:

$$\log P_{ow} = a + b \times \log k$$

where

a, *b* = linear regression coefficients.

The equation above can be obtained by linearly regressing the log of octanol/water partition coefficients of reference substances against the log of capacity factors of the reference substances.

9. Reverse phase HPLC method enables partition coefficients to be estimated in the log *P_{ow}* range between 0 and 6, but can be expanded to cover the log *P_{ow}* range between 6 and 10 in exceptional cases. This may require that the mobile phase is modified (3). The method is not applicable to strong acids and bases, metal complexes, substances which react with the eluent, or surface-active agents. Measurements can be performed on ionisable substances in their non-ionised form (free acid or free base) only by using an appropriate buffer with a pH below the p*K_a* for a free acid or above the p*K_a* for a free base. Alternatively, the pH-metric method for the testing of ionisable substances (6) may become available and could be used as an alternative method (6). If the log *P_{ow}* value is determined for the use in environmental hazard classification or in environmental risk assessment, the test should be performed in the pH range relevant for the natural environment, i.e. in the pH range of 5,0 - 9.
10. In some cases impurities can make the interpretation of the results difficult due to uncertainty in peak assignments. For mixtures which result in an unresolved band, upper and lower limits of log *P_{ow}* and the area % of each log *P_{ow}* peak should be reported. For mixtures which are a group of homologues, the weighted average log *P_{ow}* should also be stated (7), calculated based on the single *P_{ow}* values and the corresponding area % values (8). All peaks that contribute an area of 5 % or more to the total peak area should be taken into consideration in the calculation (9):

$$\text{weighted average log } P_{ow} = \frac{\sum_i (\log P_{owi})(\text{area } \%)}{\text{total peak area } \%} = \frac{\sum_i (\log P_{owi})(\text{area } \%_i)}{\sum_i \text{area } \%}$$

The weighed average log *P_{ow}* is valid only for substances or mixtures (e.g. tall oils) consisting of homologues (e.g. series of alkanes). Mixtures can be measured with meaningful results, provided that the analytical detector used has the same sensitivity towards all the substances in the mixture and that they can be adequately resolved.

INFORMATION ON THE TEST SUBSTANCE

11. The dissociation constant, structural formula, and solubility in the mobile phase should be known before the method is used. In addition, information on hydrolysis would be helpful.

QUALITY CRITERIA

12. In order to increase the confidence in the measurement, duplicate determinations must be made.
- Repeatability: The value of $\log P_{ow}$ derived from repeated measurements made under identical conditions and using the same set of reference substances should fall within a range of $\pm 0,1$ log units.
 - Reproducibility: If the measurements are repeated with a different set of reference substances, results may differ. Typically, the correlation coefficient R for the relationship between $\log k$ and $\log P_{ow}$ for a set of test substances is around 0,9, corresponding to an octanol/water partition coefficient of $\log P_{ow} \pm 0,5$ log units.
13. The inter-laboratory comparison test has shown that with the HPLC method $\log P_{ow}$ values can be obtained to within $\pm 0,5$ units of the Shake-Flask values (2). Other comparisons can be found in the literature (4)(5)(10)(11)(12). Correlation graphs based on structurally related reference substances give the most accurate results (13).

REFERENCE SUBSTANCES

14. In order to correlate the measured capacity factor k of a substance with its P_{ow} , a calibration graph using at least 6 points has to be established (see paragraph 24). It is up to the user to select the appropriate reference substances. The reference substances should normally have $\log P_{ow}$ values which encompass the $\log P_{ow}$ of the test substance, i.e. at least one reference substance should have a P_{ow} above that of the test substance, and another a P_{ow} below that of the test substance. Extrapolation should only be used in exceptional cases. It is preferable that these reference substances should be structurally related to the test substance. $\log P_{ow}$ values of the reference substances used for the calibration should be based on reliable experimental data. However, for substances with high $\log P_{ow}$ (normally more than 4), calculated values may be used unless reliable experimental data are available. If extrapolated values are used a limit value should be quoted.
15. Extensive lists of $\log P_{ow}$ values for many groups of chemicals are available (14)(15). If data on the partition coefficients of structurally related substances are not available, a more general calibration, established with other reference substances, may be used. Recommended reference substances and their P_{ow} values are listed in Table 1. For ionisable substances the values given apply to the non-ionised form. The values were checked for plausibility and quality during the inter-laboratory comparison test.

Table 1

Recommended reference substances

	CAS Number	Reference substance	$\log P_{ow}$	pKa
1	78-93-3	2-Butanone (Methylethylketone)	0,3	
2	1122-54-9	4-Acetylpyridine	0,5	
3	62-53-3	Aniline	0,9	
4	103-84-4	Acetanilide	1,0	
5	100-51-6	Benzyl alcohol	1,1	
6	150-76-5	4-Methoxyphenol	1,3	pKa = 10,26
7	122-59-8	Phenoxyacetic acid	1,4	pKa = 3,12

	CAS Number	Reference substance	log P _{ow}	pKa
8	108-95-2	Phenol	1,5	pKa = 9,92
9	51-28-5	2,4-Dinitrophenol	1,5	pKa = 3,96
10	100-47-0	Benzonitrile	1,6	
11	140-29-4	Phenylacetonitrile	1,6	
12	589-18-4	4-Methylbenzyl alcohol	1,6	
13	98-86-2	Acetophenone	1,7	
14	88-75-5	2-Nitrophenol	1,8	pKa = 7,17
15	121-92-6	3-Nitrobenzoic acid	1,8	pKa = 3,47
16	106-47-8	4-Chloroaniline	1,8	pKa = 4,15
17	98-95-3	Nitrobenzene	1,9	
18	104-54-1	Cinnamyl alcohol (Cinnamic alcohol)	1,9	
19	65-85-0	Benzoic acid	1,9	pKa = 4,19
20	106-44-5	p-Cresol	1,9	pKa = 10,17
21	140-10-3 (trans)	Cinnamic acid	2,1	pKa = 3,89 (cis) 4,44 (trans)
22	100-66-3	Anisole	2,1	
23	93-58-3	Methyl benzoate	2,1	
24	71-43-2	Benzene	2,1	
25	99-04-7	3-Methylbenzoic acid	2,4	pKa = 4,27
26	106-48-9	4-Chlorophenol	2,4	pKa = 9,1
27	79-01-6	Trichloroethylene	2,4	
28	1912-24-9	Atrazine	2,6	
29	93-89-0	Ethyl benzoate	2,6	
30	1194-65-6	2,6-Dichlorobenzonitrile	2,6	
31	535-80-8	3-Chlorobenzoic acid	2,7	pKa = 3,82

	CAS Number	Reference substance	log P _{ow}	pKa
32	108-88-3	Toluene	2,7	
33	90-15-3	1-Naphthol	2,7	pKa = 9,34
34	608-27-5	2,3-Dichloroaniline	2,8	
35	108-90-7	Chlorobenzene	2,8	
36	1746-13-0	Allyl phenyl ether	2,9	
37	108-86-1	Bromobenzene	3,0	
38	100-41-4	Ethylbenzene	3,2	
39	119-61-9	Benzophenone	3,2	
40	92-69-3	4-Phenylphenol	3,2	pKa = 9,54
41	89-83-8	Thymol	3,3	
42	106-46-7	1,4-Dichlorobenzene	3,4	
43	122-39-4	Diphenylamine	3,4	pKa = 0,79
44	91-20-3	Naphthalene	3,6	
45	93-99-2	Phenyl benzoate	3,6	
46	98-82-8	Isopropylbenzene	3,7	
47	88-06-2	2,4,6-Trichlorophenol	3,7	pKa = 6
48	92-52-4	Biphenyl	4,0	
49	120-51-4	Benzyl benzoate	4,0	
50	88-85-7	2,4-Dinitro-6-sec-butylphenol	4,1	
51	120-82-1	1,2,4-Trichlorobenzene	4,2	
52	143-07-7	Dodecanoic acid	4,2	pKa = 5,3
53	101-84-8	Diphenyl ether	4,2	
54	85-01-8	Phenanthrene	4,5	
55	104-51-8	n-Butylbenzene	4,6	

	CAS Number	Reference substance	log P _{ow}	pKa
56	103-29-7	Dibenzyl	4,8	
57	3558-69-8	2,6-Diphenylpyridine	4,9	
58	206-44-0	Fluoranthene	5,1	
59	603-34-9	Triphenylamine	5,7	
60	50-29-3	DDT	6,5	

DESCRIPTION OF THE METHOD

Preliminary estimate of the partition coefficient

16. If it is necessary, the partition coefficient of the test substance may be estimated preferably by using a calculation method (see Appendix, or where appropriate, by using the ratio of the solubility of the test substance in the pure solvents.

Apparatus

17. A liquid-phase chromatograph fitted with a low-pulse pump and a suitable detection system is required. A UV detector, using a wavelength of 210 nm, or an RI detector is applicable to the wide variety of chemical groups. The presence of polar groups in the stationary phase may seriously impair the performance of the HPLC column. Therefore, stationary phases should have a minimal percentage of polar groups (16). Commercial microparticulate reverse-phase packing or ready-packed columns can be used. A guard column may be positioned between the injection system and the analytical column.

Mobile phase

18. HPLC-grade methanol and distilled or de-ionised water are used to prepare the eluting solvent, which is degassed before use. Isocratic elution should be employed. Methanol/water ratios with minimum water content of 25 % should be used. Typically a 3:1 (v/v) methanol-water mixture is satisfactory for eluting substances with a log P of 6 within an hour, at a flow rate of 1 ml/min. For substances with a log P above 6 it may be necessary to shorten the elution time (and those of the reference substances) by decreasing the polarity of the mobile phase or the column length.
19. The test substance and the reference substances must be soluble in the mobile phase in sufficient concentration to allow their detection. Additives may be used with the methanol-water mixture in exceptional cases only, since they will change the properties of the column. In these cases it must be confirmed that the retention time of the test and reference substances are not influenced. If methanol-water is not appropriate, other organic solvent-water mixtures can be used, e.g. ethanol-water, acetonitrile-water or isopropyl alcohol (2-propanol)-water.
20. The pH of the eluent is critical for ionisable substances. It should be within the operating pH range of the column, usually between 2 and 8. Buffering is recommended. Care must be taken to avoid salt precipitation and column deterioration which occur with some organic phase/buffer mixtures. HPLC measurements with silica-based stationary phases above pH 8 are not normally advisable since the use of an alkaline mobile phase may cause rapid deterioration in the performance of the column.

Solutes

21. The test and reference substances must be sufficiently pure in order to assign the peaks in the chromatograms to the respective substances. Substances to be used for test or calibration purposes are dissolved in the mobile phase if possible. If a solvent other than the mobile phase is used to dissolve the test and reference substances, the mobile phase should be used for the final dilution prior to injection.

Test conditions

22. The temperature during the measurement should not vary by more than ± 1 °C.

Determination of dead time t_0

23. The dead time t_0 can be measured by using unretained organic substances (e.g. thiourea or formamide). A more precise dead time can be derived from the retention times measured or a set of approximately seven members of a homologous series (e.g. n-alkyl methyl ketones) (17). The retention times $t_R(n_C + 1)$ are plotted against $t_R(n_C)$, where n_C is the number of carbon atoms. A straight line, $t_R(n_C + 1) = A t_R(n_C) + (1 - A)t_0$, is obtained, where A, representing $k(n_C + 1)/k(n_C)$, is constant. The dead time t_0 is obtained from the intercept $(1 - A)t_0$ and the slope A.

Regression Equation

24. The next step is to plot a correlation $\log k$ versus $\log P$ for appropriate reference substances with $\log P$ values near the value expected for the test substance. In practice, from 6 to 10 reference substances are injected simultaneously. The retention times are determined, preferably on a recording integrator linked to the detection system. The corresponding logarithms of the capacity factors, $\log k$, are plotted as a function of $\log P$. The regression equation is performed at regular intervals, at least once daily, so that account can be taken of possible changes in column performance.

DETERMINATION OF THE P_{ow} OF THE TEST SUBSTANCE

25. The test substance is injected in the smallest detectable quantities. The retention time is determined in duplicate. The partition coefficient of the test substance is obtained by interpolation of the calculated capacity factor on the calibration graph. For very low and very high partition coefficients extrapolation is necessary. Especially in these cases attention must be given to the confidence limits of the regression line. If the retention time of sample is outside the range of retention times obtained for the standards, a limit value should be quoted.

DATA AND REPORTING

Test report

26. The following must be included in the report:
- if determined the preliminary estimate of the partition coefficient, the estimated values and the method used; and if a calculation method was used, its full description including identification of the data base and detailed information on the choice of fragments;
 - test and reference substances: purity, structural formula and CAS number,
 - description of equipment and operating conditions: analytical column, guard column,
 - mobile phase, means of detection, temperature range, pH;
 - elution profiles (chromatograms);
 - deadtime and how it was measured;
 - retention data and literature $\log P_{ow}$ values for reference substances used in calibration;
 - details on fitted regression line ($\log k$ versus $\log P_{ow}$) and the correlation coefficient of the line including confidence intervals;
 - average retention data and interpolated $\log P_{ow}$ value for the test substance;
 - in case of a mixture: elution profile chromatogram with indicated cut-offs;

- $\log P_{ow}$ values relative to area % of the $\log P_{ow}$ peak;
- calculation using a regression line;
- calculated weighted average $\log P_{ow}$ values, when appropriate.

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*Appendix***P_{ow} calculation methods**

INTRODUCTION

1. This appendix provides a short introduction to the calculation of P_{ow}. For further information the reader is referred to textbooks (1)(2).
2. Calculated values of P_{ow} are used for:
 - deciding which experimental method to use: Shake Flask method for log P_{ow} between – 2 and 4 and HPLC method for log P_{ow} between 0 and 6;
 - selecting conditions to be used in HPLC (reference substances, methanol/water ratio);
 - checking the plausibility of values obtained through experimental methods;
 - providing an estimate when experimental methods cannot be applied.

Principle of calculation methods

3. The calculation methods suggested here are based on the theoretical fragmentation of the molecule into suitable substructures for which reliable log P_{ow} increments are known. The log P_{ow} is obtained by summing the fragment values and the correction terms for intramolecular interactions. Lists of fragment constants and correction terms are available (1)(2)(3)(4)(5)(6). Some are regularly updated (3).

Reliability of calculated values

4. In general, the reliability of calculation methods decreases as the complexity of the substance under study increases. In the case of simple molecules of low molecular weight and with one or two functional groups, a deviation of 0,1 to 0,3 log P_{ow} units between the results of the different fragmentation methods and the measured values can be expected. The margin of error will depend on the reliability of the fragment constants used, the ability to recognise intramolecular interactions (e.g. hydrogen bonds) and the correct use of correction terms. In the case of ionising substances the charge and degree of ionisation must be taken into consideration (10).

Fujita-Hansch π -method

5. The hydrophobic substituent constant, π , originally introduced by Fujita et al. (7) is defined as:

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

where PhX is an aromatic derivative and PhH the parent substance.

$$\begin{aligned} \text{e.g. } \pi_{\text{Cl}} &= \log P_{ow}(\text{C}_6\text{H}_5\text{Cl}) - \log P_{ow}(\text{C}_6\text{H}_6) \\ &= 2,84 - 2,13 \\ &= 0,71 \end{aligned}$$

The π -method is primarily of interest for aromatic substances. π -values for a large number of substituents are available (4)(5).

Rekker method

6. Using the Rekker method (8) the log P_{ow} value is calculated as:

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

where a_i is the number of times a given fragment occurs in the molecule and f_i is the $\log P_{ow}$ increment of the fragment. The interaction terms can be expressed as an integral multiple of one single constant C_m (so-called "magic constant"). The fragment constants f_i and C_m have been determined from a list of 1 054 experimental P_{ow} values of 825 substances using multiple regression analysis (6)(8). The determination of the interaction terms is carried out according to set rules (6)(8)(9).

Hansch-Leo method

7. Using the Hansch and Leo method (4), the $\log P_{ow}$ value is calculated as:

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

where f_i is a fragment constant, F_j a correction term (factor), a_i and b_j the corresponding frequency of occurrence. Lists of atomic and group fragmental values and of correction terms F_j were derived by trial and error from experimental P_{ow} values. The correction terms have been divided into several different classes (1)(4). Software packages have been developed to take into account all the rules and correction terms (3).

COMBINED METHOD

8. The calculation of $\log P_{ow}$ of complex molecules can be considerably improved, if the molecule is dissected into larger substructures for which reliable $\log P_{ow}$ values are available, either from tables (3)(4) or by existing measurements. Such fragments (e.g. heterocycles, anthraquinone, azobenzene) can then be combined with the Hansch- π values or with Rekker or Leo fragment constants.

Remarks:

- (i) The calculation methods are only applicable to partly or fully ionised substances when the necessary correction factors are taken into account.
- (ii) If the existence of intramolecular hydrogen bonds can be assumed, the corresponding correction terms (approx. + 0,6 to + 1,0 $\log P_{ow}$ units) must be added (1). Indications on the presence of such bonds can be obtained from stereo models or spectroscopic data.
- (iii) If several tautomeric forms are possible, the most likely form should be used as the basis of the calculation.
- (iv) The revisions of lists of fragment constants should be followed carefully.

LITERATURE ON CALCULATION METHODS

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- (4) C. Hansch and A.J. Leo. Substituent Constants for Correlation Analysis in Chemistry and Biology, John Wiley, New York (1979).
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- (8) R.F. Rekker. The Hydrophobic Fragmental Constant, *Pharmacology Library*, Vol. 1, Elsevier, New York (1977).
- (9) C.V. Eadsforth and P. Moser. (1983). Assessment of Reverse Phase Chromatographic Methods for Determining Partition Coefficients. *Chemosphere*. 12, 1459.
- (10) R.A. Scherrer. ACS — Symposium Series 255, p. 225, American Chemical Society, Washington, D.C. (1984).'

(3) Chapter C.3 is replaced by the following:

'C.3. FRESHWATER ALGA AND CYANOBACTERIA, GROWTH INHIBITION TEST

INTRODUCTION

1. This test method is equivalent to OECD test guideline (TG) 201 (2006, annex corrected in 2011). The need to extend the test method to include additional species and update it to meet the requirements for hazard assessment and classification of chemicals has been identified. This revision has been completed on the basis of extensive practical experience, scientific progress in the field of algal toxicity studies, and extensive regulatory use, which has occurred since the original adoption.
2. Definitions used are given in Appendix 1.

PRINCIPLE OF THE TEST

3. The purpose of this test is to determine the effects of a chemical on the growth of freshwater microalgae and/or cyanobacteria. Exponentially growing test organisms are exposed to the test chemical in batch cultures over a period of normally 72 hours. In spite of the relatively brief test duration, effects over several generations can be assessed.
4. The system response is the reduction of growth in a series of algal cultures (test units) exposed to various concentrations of a test chemical. The response is evaluated as a function of the exposure concentration in comparison with the average growth of replicate, unexposed control cultures. For full expression of the system response to toxic effects (optimal sensitivity), the cultures are allowed unrestricted exponential growth under nutrient sufficient conditions and continuous light for a sufficient period of time to measure reduction of the specific growth rate.
5. Growth and growth inhibition are quantified from measurements of the algal biomass as a function of time. Algal biomass is defined as the dry weight per volume, e.g. mg algae/litre test solution. However, dry weight is difficult to measure and therefore surrogate parameters are used. Of these surrogates, cell counts are most often used. Other surrogate parameters include cell volume, fluorescence, optical density, etc. A conversion factor between the measured surrogate parameter and biomass should be known.
6. The test endpoint is inhibition of growth, expressed as the logarithmic increase in biomass (average specific growth rate) during the exposure period. From the average specific growth rates recorded in a series of test solutions, the concentration bringing about a specified x % inhibition of growth rate (e.g. 50 %) is determined and expressed as the E_rC_x (e.g. E_rC_{50}).
7. An additional response variable used in this test method is yield, which may be needed to fulfil specific regulatory requirements in some countries. It is defined as the biomass at the end of the exposure period minus the biomass at the start of the exposure period. From the yield recorded in a series of test solutions, the concentration bringing about a specified x % inhibition of yield (e.g., 50 %) is calculated and expressed as the E_yC_x (e.g. E_yC_{50}).