Technical Report

EURIMA test guideline: In-vitro acellular dissolution of man-made vitreous silicate fibres

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1. Introduction

The potential pathogenicity of a specific fibre type mainly depends upon the extent to which the fibres can be inhaled and can persist in the lung [1]. The pathogenicity of a fibre is determined by animal studies. However, the comments to the Commission Directive 97/69/EC, 1997 (Classification of man-made vitreous fibres with respect to carcinogenicity) express the firm EU intention, that, to the greatest possible extent, in-vitro methods should replace in-vivo studies.

The last few years have seen the development of fibre types with increased bio-solubility, that still assure the relevant product qualities for the various application areas. For this work, two pH environments, pH 7.4 and pH 4.5, found in the lung environment [2] were simulated in in-vitro dissolution measurements.

Earlier publications [3 and 4] have reported results from in-vitro measurements obtained on different fibres and by different laboratories.

The present test guideline was worked out by two consecutive working groups, established by EURIMA (The European Insulation Manufacturers Association) and with participants both from industry and from independent research laboratories. The working groups first developed a draft guideline, which was used in a Round Robin (parallel test) that involved laboratories from most of the participants. Based on the findings in the Round Robin, the guideline was slightly revised and tightened, and then followed by a second Round Robin.

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Based on evaluations of the Round Robin results and on comparisons between in-vitro dissolution rates and in-vivo biopersistence results, we conclude:

a) The guideline provides a state-of-the-art method for measuring in-vitro dissolution rates relevant for evaluating the bio-persistence of a fibre.
b) Although the Round Robin tests show a correlation between in-vitro tests and in-vivo biopersistence, the in-vitro method as described in the guideline does not allow direct comparison between actual values obtained by any laboratory with values obtained by another laboratory. The values obtained cannot yet be used directly for regulatory purposes.
c) In-vitro measurements performed according to the guideline may be used for internal control and documentation provided reference values for known fibres are measured and given, as stated in the guideline.

A future paper will publish the results of the Round Robins and discuss the influence of the various test parameters.

2. EURIMA test guideline

2.1 Introductory information

2.1.1 Objective and rationale

To provide guidelines for assessing in-vitro acellular dissolution of man-made vitreous silicate fibres (MMVFs) at pH 4.5 and 7.4.

In order to minimize the future use of experimental animals, it is desirable to develop in-vitro methods that

may be used to estimate the biopersistence of fibres with different chemical compositions. Dissolution of fibres in-vivo is believed to take place in a near neutral pH of 7.4 (the extra-cellular environment) and an acidic pH of 4.5 to 5 (the environment found within the phagolysosomes of the macrophages and in the microenvironment at the surface of activated macrophages) [2, 5 to 7]. Solubility at pH 7.4 has been shown for a number of fibres to be a good predictor of the outcome of chronic inhalation and injection tests [8]. Evolution of the chemistry of these fibres has been shown to be similar in vivo and in vitro [9]. For some fibres, however, the biopersistence can only be predicted by considering the dissolution rates at both pH 4.5 and pH 7.4 [10]. Measurements at both pH values have been reported [3, 4, 11 to 17].

2.1.2 Scope and mechanisms

The test method described covers silicate-based MMVFs and may be used for all such commercial and experimental inorganic synthetic vitreous fibre materials composed of individual fibres whose geometry can be adequately described as that of a circular cylinder.

The major processes that occur when an amorphous fibre is in contact with an aqueous solution are network dissolution and ion exchange. The dissolution of silicon represents the network dissolution, and the dissolution of calcium and magnesium represents the additional ion exchange (leaching) processes.

$$\begin{aligned}
&(\mathrm{Si{-}O{-}R})_{\mathrm{glass}} + \mathrm{H^+} \quad \Leftrightarrow \quad (\mathrm{Si{-}OH})_{\mathrm{glass}} + \mathrm{R^+} \\
&\qquad\qquad\qquad\qquad\qquad\quad \text{(ion exchange)}, \\
&(\mathrm{Si{-}O{-}R})_{\mathrm{glass}} + \mathrm{H_2O} \quad \Leftrightarrow \quad (\mathrm{Si{-}OH})_{\mathrm{glass}} + \mathrm{R^+} + \mathrm{OH^-}, \\
&(\mathrm{Si{-}O{-}Si})_{\mathrm{glass}} + \mathrm{OH^-} \quad \Leftrightarrow \quad (\mathrm{Si{-}OH})_{\mathrm{glass}} + (\mathrm{Si{-}O{-}})_{\mathrm{glass}} \\
&\qquad\qquad\qquad\qquad\qquad\quad \text{(network dissolution)}, \\
&(\mathrm{Si{-}O{-}Si})_{\mathrm{glass}} + \mathrm{OH^-} \quad \Leftrightarrow \quad (\mathrm{Si{-}OH})_{\mathrm{diss}} + (\mathrm{Si{-}O{-}})_{\mathrm{glass}},
\end{aligned}$$

where R represents the network modifiers, such as earth alkaline and alkaline elements.

2.1.3 Definition and units

The dissolution rate constant (constant decrease of mass per unit surface area) is calculated assuming a surface reaction controlled dissolution independent of time. The in vitro dissolution coefficient (K_{dis}) is expressed in ng $\mathrm{cm^{-2}\,h^{-1}}$.

Dissolution has often been published as a dissolution rate (v) in nm $\mathrm{d^{-1}}$ (for example, in [11]). The relation between K_{dis} and v is: $K_{\mathrm{dis}} = v \cdot \varrho$, where ϱ is the density of the glass.

2.1.4 Reference materials

Investigation of a new material should always include reference materials covering the relevant range of dissolution rates. They should serve to check the performance of the method and allow comparison with results from other methods. These reference materials may be textile-drawn or product samples, especially prepared for test purposes. MMVF 11, MMVF 21, Fibre C and MMVF 34 [18 and 19] are suitable reference materials[1)].

2.1.5 Principle of the test method

Replicate fibre samples are subjected to a continuous constant flow of buffer solution in a measurement apparatus for determining the dissolution coefficient (figure 1).

The dissolved amounts of silicon and other main components present in the glass composition in sufficient amounts (mainly aluminium, calcium, boron) are determined at regular intervals by a suitably validated analytical procedure. From these data and the fibre diameter distribution of the fibres in the samples, the dissolution coefficients (in ng $\mathrm{cm^{-2}\,h^{-1}}$) of the test substance and reference materials are determined.

The coefficients (K_{dis}) shall be expressed as K_{dis}(Si), which is an estimate of network dissolution, and as K_{dis}(Al, Ca, B) (and others if measured), which has a higher value, taking into account the possible leaching/dissolution of more soluble components of the fibre.

2.1.6 Quality control, precision criteria, and standard documents

The appropriate quality control and precision criteria should be established using formally conducted validation procedures.

3. Description of the test method

3.1 Characterization of materials

The following information should be available for both test and reference fibres:

- description (or reference) of process for producing the fibres;

[1)] MMVF 11 and MMVF 21 are available from NAIMA, 44 Canal Center Plaza, Suite 310, Alexandria, VA 22314 (USA). MMVF 34 is available from Rockwool International A/S, Hovedgaden 584, DK-2640 Hedehusene (Denmark). Fibre C is available from Isover St Gobain, Les Miroirs, F-92096 Paris La Défense (France).

- description (or reference) of process for producing the sample to be tested – including storage conditions;
- content and type of binder or coating, if any;
- chemical composition in weight percentage of oxides of the fibres using a suitable measurement method (defining at least 99 % of the composition); all elements present in amounts greater than 0.1 % should be stated;
- density of fibres (calculated from content of oxides or determined using a suitable method, such as the pycnometric method);
- length-weighted fibre diameter distribution data of fibres using a suitable method [20 and 21] and based on at least 500 measured fibres.

From the density and the length-weighted diameter distribution, the specific surface is calculated assuming cylindrical geometry of the fibres (see section 5.1)

The following optional information may also be provided:

- BET measurements may be used to assess the initial surface area.
- FeO and Fe_2O_3 may be recorded separately.

3.2 Determination of fibre diameter distribution

The length-weighted fibre diameter distribution is determined using SEM (or OM) and measuring at least 500, preferably 800 fibres. The length-weighted fibre diameter distribution is determined either by bivariate measurements of the length and the diameter of each individual fibre or by using the intercept method (probabilistic method), where all fibres crossing a line in the microscope are measured (the probability of crossing the line being proportional to the length) [20 and 21]. A robust determination of the length-weighted diameter distribution is crucial, and both methods should be properly validated.

3.3 Buffer solutions

The test is carried out using a modified low calcium Gamble's solution. For this purpose, buffer solutions should be prepared using reagent-grade chemicals and de-ionized water, see table 1. The buffer solutions can be prepared from various reagents. However, the buffer system used influences the rate of dissolution. If an alternative liquid is used, reference materials should be run for comparison.

Table 1. Composition of the pH 4.5 and pH 7.4 buffer solutions[4] in mg l^{-1}

pH value 4.5[5]		pH value 7.4[6]	
NaCl	7120	NaCl	6600
$NaHCO_3$	1950	$NaHCO_3$	2703
$CaCl_2 \cdot 2H_2O$	29	$CaCl_2$	22
Na_2HPO_4	148	$Na_2HPO_4 \cdot 12H_2O$	358
Na_2SO_4	79	Na_2SO_4	79
$MgCl_2 \cdot 6H_2O$	212	$MgCl_2 \cdot 6H_2O$	212
$H_2NCH_2CO_2H$ (glycine)	118	$H_2NCH_2CO_2H$ (glycine)	118
Na_3-citrate$\cdot 2H_2O$	152	Na_3-citrate$\cdot 2H_2O$	153
Na_2-tartrate$\cdot 2H_2O$	180	Na_2-tartrate$\cdot 2H_2O$	180
Na-pyruvate	172	Na-pyruvate	172
90 % lactic acid	156	Na-lactate	175
HCl[7] (1:1) addition in ml	4 to 5	HCl[7] (1:1) addition in ml	1 to 1.5

[4] The components should be added to a large quantity of water (minimum 75 % of final volume). Gentle heating is sometimes required to dissolve all components.
[5] On mixing, the solution is adjusted to approx. pH 4.5 and re-adjusted after 24 h. Formaldehyde is added at 3 to 4 ml l^{-1} to prevent growth of algae.
[6] pH is adjusted to approximately 7.4. Formaldehyde is added at 3 to 4 ml l^{-1} to prevent growth of algae. The solution is bubbled with N_2/CO_2 at between (95/5) % and (90/10) %.
[7] Approximate amount for pH adjustment.

3.4 Test conditions

3.4.1 Temperature and pH of buffer solution

The temperature should be maintained at (37 ± 1)°C. The flow cells may be placed in thermostatic cupboards or water baths. The pH of the buffer solutions should be kept at within ± 0.1 of the target pH, on entering the flow cells. The exit pH should be measured and reported.

3.4.2 Ratio of flow rate to surface area (*F/A*)

The ratio of the flow rate to the initial surface area (*F/A*) should be (0.030 ± 0.005) µm sec^{-1}. The flow rate should be chosen to be within 120 to 150 ml d^{-1} and should be kept constant (within ± 5 % of target at all time points) during the test. The amount of fibres in a flow cell should be between 50 and 300 mg. The exact amount of fibres to be put into the cell should be determined based on the flow rate and specific surface area of the sample.

The flow rate (*F*), the surface area (*A*), the mass (*M*) and the fibre geometry are linked. As an example, for an *F/A*-ratio of 0.030 µm sec^{-1}, the settings shown in table 2 are reasonable. If an alternative flow rate is required, reference materials should also be tested at the alternative flow rate.

3.5 Apparatus

3.5.1 Dissolution apparatus

Schematic arrangements of a typical system are presented in figures 1 and 2.

Table 2. Examples of settings for an F/A-ratio of 0.03 μm s^{-1}

sample type	typical diameter in μm	flow (F) in ml d^{-1}	surface area (A) in cm^2	mass (M) in mg
fine fibres	± 1	150	575	75
industrial fibres	± 3	130	500	175
textile fibres	±10	120	465	300

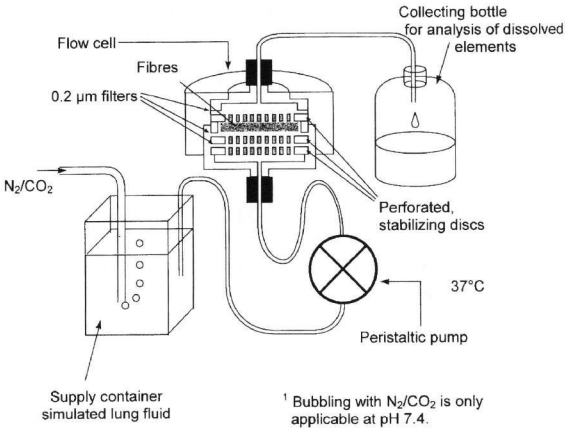

Figure 1. Apparatus and set-up for flow-through in in-vitro measurements.

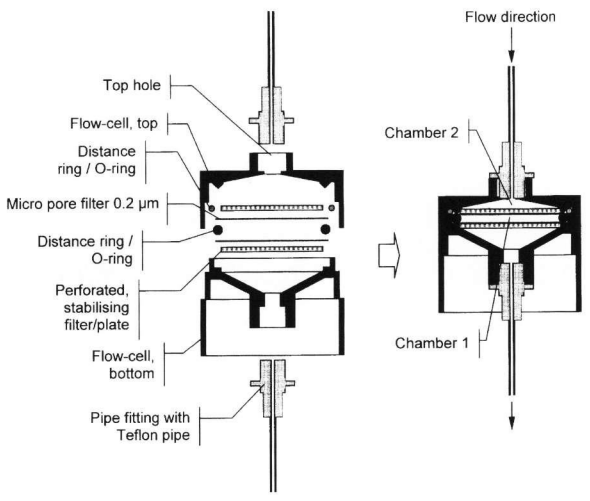

Figure 2. Illustration of flow cell.

The following parts are involved:

- thermostatic chamber or bath;
- pump capable of delivering a constant flow;
- pipes causing no contamination of the buffer solution[2)];
- "flow cells" of defined dimensions[3)], with a diameter of 37 to 50 mm (figures 1 and 2);
- micropore filters at the inlet to and outlet from the flow cells; they should remain stable during the test and have a pore size of 0.2 μm.

3.5.2 Analytical apparatus

A specific analytical method should be used to determine the concentrations of the dissolved elements. Examples of such methods are Atomic Absorption Spectrometry (AAS), Graphite Furnace Atomic Absorption Spectrometry (GF-AAS), Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES). For certain elements such as boron, wet-chemical methods may be relevant. The method used should be properly validated.

The pH in the fluid is measured using a pH meter with a glass electrode, both when entering the flow cells, at the outlet, and in collected sample liquids (>5 ml). If possible, the use of indicator paper (pH 4.5: pH range 4 to 7, Merck, Darmstadt (Germany), pH 7.4: pH range 6.5 to 10, Merck) should be avoided or limited.

4. Test procedure

4.1 Preparation

The buffer solution is prepared by weighing the amount of ingredients as stated in section 2.3. pH is adjusted by adding HCl. The pH 4.5 buffer solution is left for degassing and readjusted after 24 h. The composition is controlled by analysing the elements calcium and magnesium and measuring the pH.

Dependent on the type of the fibre sample, some preparation of the sample is needed: continuous fibres should be shortened; for industrial fibres, any shots (non-fibrous particles) should be removed by sieving, and the fraction that has passed through a 63 μm sieve should be used as the sample. In general, no preparation is necessary for specially prepared fine fibre samples.

From the calculated specific surface area and the predetermined flow rate (120 to 150 ml d^{-1}), the amount of test material required to obtain the required F/A ratio (0.03 μm s^{-1}) is calculated (table 2).

For each test material, at least three cells and one blank cell are mounted. To allow for discard of one cell,

[2)] High-quality Teflon is preferable. Some tubes contain polyethylene that might affect the pH on decomposing. The use of silicones should be avoided completely.

[3)] Suitable flow cells are available from Sartorius AG, D-3400 Göttingen (Germany).

it is advisable to use four flow cells and minimum two blank cells. The dispersion of the fibres in the cells should be controlled. Initially the fibres should cover the entire micropore area of the flow cell.

4.2 Observation period

Where the buffer fluid enters the cell and also at the outlet, the pH is measured twice a day (up to 48 h) and later in the period once a day. Samples are taken at regular intervals to determine the concentrations of the dissolved elements, however, most frequently in the beginning of the test. The total volume of effluent is measured, and the time for sample taking registered. Typically, samples are taken after 1, 4, 7, 11, 14 and 21 d. The appropriate sample intervals and duration of the total test should be determined based on the results of a pre-test. The dissolution data from at least three intervals should be used for calculation of the dissolution rate.

The calculation of the dissolution coefficient is mostly based on the results up to 14 d. However, the dissolution coefficient of highly soluble fibres is made up to the maximum time when less than 50 % of the SiO_2 in the fibre sample has dissolved. A fibre is defined as highly soluble if more than 50 % of its mass has dissolved within 14 d.

4.3 Optional post observation procedures

After the test has been stopped, the fibre samples may be rinsed in de-ionized water and dried to constant weight in a desiccator. The remaining weight of the sample may then be determined and the weight loss compared to the weight loss determined from the elements dissolved. The fibres may be investigated with respect to the integrity of the fibre structure using scanning electron microscopy (SEM).

5. Evaluation of data

Data should be given in tabular form, showing individual data as well as summarized data (table 3). Where possible, data should be supported by graphical presentation.

5.1 General

The dissolution data from 1 to 14 d (or up to the maximum observation time when less than 50 % of the SiO_2 in the fibre sample has dissolved, if this occurs before 14 d) together with the length-weighted fibre diameter distribution data are used to calculate an average dissolution rate.

Table 3. Test parameters

material parameters
- chemical composition
- density
- length-weighted fibre diameter distribution data
- amount of fibres
- surface area

buffer solutions
- amounts of ingredients
- concentration of Na (theoretical), K (theoretical), Ca, Mg
- pH-value

test conditions
- temperature
- flow rate
- *F*/*A* ratio
- pH value (inlet/outlet)
- gas flow (pH 7.4)
- descriptive quality control (deviations)

dissolution parameters
- ppm contents of Si and Ca (and other elements) measured in the effluent from the flow cells with test materials, reference materials and blank cells
- volume of effluent (from each cell)
- time for sample taking

after the test (optional)
- amount of remaining fibres
- SEM photos

The calculation is based on the assumption that the dissolution is surface reaction controlled, i.e. that the dissolution in ng per unit surface area (cm^2) of each fibre remains constant. The calculation is made separately for each element analysed.

$$D = D_0 - 2\frac{K_{dis} \cdot t}{\varrho}, \tag{1}$$

$$D = D_0 - 2\upsilon \cdot t, \tag{2}$$

$$K_{dis} = \upsilon \cdot \varrho, \tag{3}$$

where D_0 is the diameter at time zero, D is the diameter at time t and ϱ is the density of the fibres. With the units generally used for υ ($nm\,d^{-1}$) and K_{dis} ($ng\,cm^{-2}\,h^{-1}$), and with the density expressed in $g\,cm^{-3}$, the following conversion formula applies:

$$K_{dis} = \frac{\upsilon \cdot \varrho \cdot 100}{24}. \tag{4}$$

The calculation may be done in two ways, depending on the type of sample investigated. Section 5.2 describes a single step method, which is normally applicable but subject to certain conditions with respect to the fibre

diameter distribution and the percentage dissolved. Section 5.3 describes the general method, which is always applicable.

D_{0m}, the mean length-weighted diameter, S_0, the standard deviation of the length weighted fibre diameter distribution, and β, the relation between S_0 and D_{0m} are defined as follows:

$$D_{0m} = \frac{\Sigma D_i \cdot L_i}{\Sigma L_i}, \tag{5}$$

$$S_0^2 = \frac{\Sigma D_i^2 \cdot L_i}{\Sigma L_i} - D_{0m}^2, \tag{6}$$

$$\beta \equiv \frac{S_0}{D_{0m}}, \tag{7}$$

where D_i = measured diameter of fibre i, and L_i = measured length of fibre i.

The surface area SA is calculated from:

$$SA = \frac{4 M_0}{\varrho} \frac{D_{0m}}{D_{0m}^2 + S_0^2}. \tag{8}$$

5.2 Calculation using a single step formula

This method may be used if $\beta < 1$, and if the total mass loss in the calculation period is less than 50 % of the total mass.

If M_0 is the mass of the sample at time zero and ΔM is the mass dissolved at time t, the equation below should be used for the calculation of v:

The dissolution rate v is calculated from:

$$\frac{\Delta M}{M_0} = 4v \cdot t \frac{D_{0m} - v \cdot t}{D_{0m}^2 + S_0^2} \tag{9}$$

which (together with the basic relation between v and K_{dis}) may be rearranged to:

$$K_{dis} = \frac{D_{0m} \cdot \varrho}{2t} \left[1 - \sqrt{\left(1 - \left(1 + \frac{S_0^2}{D_{0m}^2}\right) \frac{\Delta M}{M_0}\right)}\right]. \tag{10}$$

5.3 Calculation using the general formula

This method is the general method. It can be used for any fibre diameter distribution (any β-value) and allows for some fibres to dissolve fully during the experiment – i.e. the percentage dissolved may be higher than 50 %. It may also be used for fibres fulfilling the criteria for calculation according to the method in section 5.2.

If M_0 is the mass of the sample at time of zero and ΔM is the mass dissolved at time t, the equation below should be used for the calculation of v:

$$M_0 - \Delta M = \frac{\varrho \cdot L_0 \cdot \pi}{4} \int_{2v \cdot t}^{\infty} (D - 2v \cdot t)^2 \, \mathrm{d}L(D). \tag{11}$$

In this case, L_0 is the total fibre length at time zero, and $L(D)$ is the length-weighted diameter distribution function of the original sample.

In practice, converting the integral to the following sum formula may solve the integral:

$$1 - \frac{\Delta M}{M_0} = \frac{\sum_{i=1}^{i=n} \left[(D_i - 2v \cdot t)^2 \cdot L_i\right]}{\sum_{i=1}^{i=n} D_i^2 \cdot L_i} \tag{12}$$

$$\text{for } (D_i - 2v \cdot t) > 0,$$

where n is the number of fibres measured for the determination of the fibre diameter distribution.

The formula calculates the dissolution of each individual fibre, and the equation may be solved by the use of suitable mathematical tools. The integral may also be solved iteratively as indicated in figure 3.

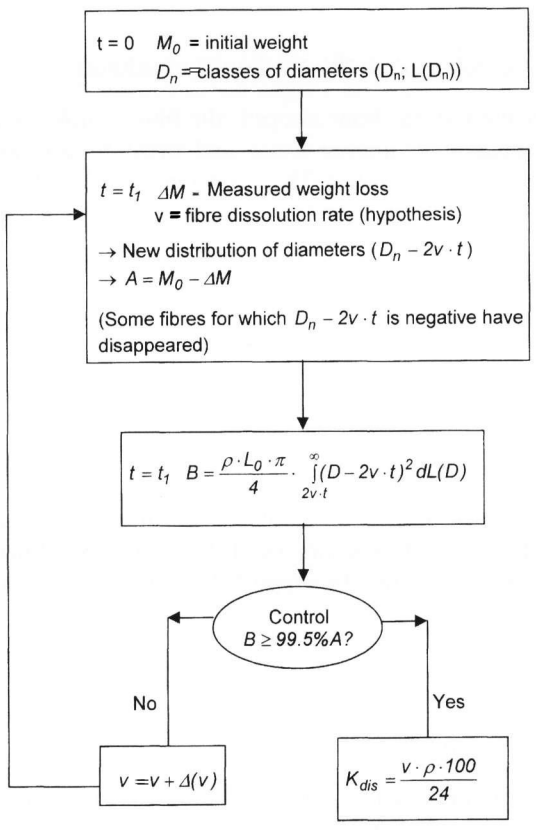

Figure 3. Flowchart for iterative calculation procedure.

5.4 Criteria for acceptance

The test run should fulfil the following criteria:

- flow: 120 to 150 ml/d (as specified in section 2.4);
- variations in flow: within ±5 % of target at all time points (as specified in section 2.4);
- initial F/A ratio: (0.030 ± 0.005) µm/s (as specified in section 2.4);
- low inter-cell variation on mg silicon in solution at all time points (within ± 20 % on average);
- consistent development in K_{dis} over time (excluding day 1), until 50 % SiO_2 has dissolved;
- acceptable results (fulfilling the above criteria) from minimum three cells.

The results from one cell can be discarded if the flow was out of range (provided that more than three cells were used). In-vitro tests failing to fully comply with these criteria should be re-done with minimum three new cells.

5.5 Confidence intervals

The result of the measurement, the K_{dis} value, should be given with its 95 % confidence interval.

The (potential) major contribution to the imprecision of the dissolution coefficients originates from the determination of the length-weighted fibre diameter distribution and the calculated specific surface area. Special efforts should be made to determine these as precisely as possible.

When repeat measurements of a specific dissolution coefficient have been carried out, the confidence interval may be determined directly. It is recognised that repeat measurements of a specific dissolution coefficient may not always be carried out, and in that case, an approximate confidence interval may be provided as described in the next paragraph. However, routinely, sufficient repeat measurements should be carried out for the laboratory to be sure that the experimental variation is so small that the use of approximate limits is satisfactory.

In the absence of repeat results in a particular measurement, the K_{dis} value may be given with an approximate confidence interval of $K_{dis} \pm 0.3 \cdot K_{dis}$ for samples of product man-made vitreous silicate fibres or size-selected fine fibres. For continuous filaments, the corresponding approximate interval is $K_{dis} \pm 0.2 \cdot K_{dis}$.

Examples of the dissolution coefficients at pH 7.4. and 4.5, respectively, for the test materials (section 1.4) are given in table 4.

Table 4. Examples of the dissolution coefficients for the test materials (section 2.1.4)

sample	pH value	K_{dis} in ng cm^{-2} h^{-1}	95 % confidence interval
MMVF 11	7.4	85	60 to 110
MMVF 21		20	14 to 26
Fibre C		1000	700 to 1300
MMVF 11	4.5	1	0.7 to 1.3
MMVF 21		53	37 to 69
MMVF 34		618	433 to 803

6. Test report

6.1 Required report items

The test report should include the following information or specify that it conforms to the standard procedures of this guideline:

- the principle of the method used;
- test material characterization;
- the composition of the buffer solution;
- descriptions of dissolution apparatus including design, type, and dimensions; the equipment and methods for monitoring pH, temperature, and flow rate and the method to determine the dissolved components should also be described;
- for each test, the test conditions, including volume and time of sample taking;
- data on concentrations measured in effluents;
- the F/A ratio;
- the mass loss at each time point;
- listings of individual sample data should be included as an appendix: data report;
- a statement as to whether the requirements for acceptance are fulfilled (section 5.4).

The mathematical and statistical treatment of the results should give:

- the dissolution coefficient (K_{dis} for silicon and calcium) in ng cm^{-2} h^{-1} for test and reference materials;
- the overall calculated, or estimated, confidence interval for each value of K_{dis};
- the average dissolution coefficients with the (weighted) standard deviation between the dissolution coefficients determined for the various sampling periods in the test;
- the test period, the number of samples, and the number of cells (repeat samples) used in the calculations.

6.2 Optional report items

The test report may also include the following:

- the analysis may additionally be presented in terms of the dissolution rate (v) in nm d^{-1}, with the same detail as for K_{dis};
- discussion of the results;
- interpretation of results.

7. References

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