

MDHS

Methods for the Determination of Hazardous Substances

Occupational Medicine and Hygiene Laboratory



59

Man-made mineral fibre

Airborne number concentration by
phase-contrast light microscopy

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INTRODUCTION

Nomenclature, nature and health effects

1 A variety of inorganic materials are made into fine fibres and used for structural strengthening or insulation; they are known as man-made mineral fibres (MMMF). Types of MMMF have names such as: mineral wool (which includes rock wool, slag-wool and glass wool), continuous filament, superfine and refractory (or ceramic) MMMF. The names of these classes of materials have different origins and are not necessarily mutually exclusive.

2 A review of MMMF by a working party of the Advisory Committee on Toxic Substances (ACTS) was published in 1979,¹ and an associated survey by HM Factories Inspectorate was also published.² Large parts of these reviews remain useful. Reports of two conferences organised by the World Health Organisation^{3,4} also contain much useful information. Possible health effects are discussed in HSE Guidance Note EH 46.⁵ MMMF can in some circumstances cause irritation of the skin and eyes and upper respiratory tract. Some recent studies have reported an association between mineral wool production and lung cancer, but the exact cause and significance of this are controversial. The latest issue of Guidance Note EH 40 *Occupational exposure limits*,⁶ updated by *Toxic Substances Bulletin*,⁷ should be consulted for applicable occupational exposure limits.

Airborne MMMF and its measurement

3 Most MMMF production processes give a wide range of particle sizes, with median diameters of a few micrometres in the bulk material. Because finer fibres stay airborne longer (and perhaps become airborne more readily), most airborne MMMF clouds have number median diameters $<1\mu\text{m}$, and airborne number medians of superfine glasses can range down to $0.25\mu\text{m}$ or less. Coarser fibres in a cloud tend to be longer on average, but there is a wide range of lengths at each diameter. Exceptional types are continuous filament, and some ceramic fibres manufactured to be substantially all of the

same size. In these materials, submicrometre fibres can be rare. Refractive index depends of course on the parent material, but can also show some variation from fibre to fibre.

4 This MDHS describes a method of measuring the airborne number concentration of respirable MMMF. The total inhalable and respirable mass concentrations can be measured by the methods in MDHS 14.⁸

PRINCIPLE

5 The fibres are collected on a membrane filter in a similar way to the European Reference Method for asbestos,⁹ except that a wider range of flowrates is permitted. The refractive index (r.i.) of the fibres is measured. If r.i. >1.51 , the filter is clarified by the acetone-triacetin technique. If r.i. ≤ 1.51 , the filter is collapsed, etched in a plasma asher, and a film of water is sandwiched between the filter and the cover slip. In both cases, the fibres are then counted by phase contrast light microscopy, with equipment to the same specification as that used for asbestos. The counting rules are those agreed by the WHO/Euro Technical Committee.¹⁰

SCOPE

6 The method can be used to measure personal exposure of fixed-point concentration of respirable MMMF, defined as fibres longer than $5\mu\text{m}$, narrower than $3\mu\text{m}$, with a length:width ratio $>3:1$, and visible by phase contrast light microscopy using the method described here. Fibres outside this size range are excluded by definition, but may still be present in the air. Fibres with width less than about $0.2\mu\text{m}$, may be invisible. It is not possible to identify the chemical nature of the fibres with certainty by the method, so all fibres meeting the definitions in the counting rules must be included in the determination. The method can be applied to natural mineral fibres, but the concentration of fibres where the Control of Asbestos at Work Regulations apply should be measured by the methods in MDHS 39/2.⁹

Accuracy

7 It is not possible to discuss accuracy in relation to an external standard, because the microscope specification (para 21) and the counting rules (para 46) determine which fibres are included; but statements can be made about internal consistency. Counters have been shown on average to undercount dense deposits and overcount sparse deposits.¹¹ In terms of the densities of fibres on the filter surface, results >1000 fibres/mm² may be underestimates, and results of a few tens of fibres/mm² may be serious overestimates. Unlike asbestos by the European Reference Method, fibres are counted regardless of particle or fibre superimposition, so chance superimposition is unimportant. Any microscopical counting method is liable to systematic differences amongst operators and laboratories, which must be controlled by quality checks.

Precision

8 Within a laboratory with a satisfactory quality control scheme, the average coefficient of variation (standard deviation/mean) of counts produced on a sample by a team of counters using the same counting rules is given approximately by

$$(N + b^2N^2)^{1/2}/N \quad (1)$$

where N is the mean number of fibres counted per evaluation, and b is positive and usually less than 0.2, better quality control giving lower b. Expression (1) includes an unavoidable minimum coefficient of variation equal to 1/N, which is due to the Poisson distribution of counts, and additionally a superimposed element due to subjective judgement.¹² Calculated values for 0<b<0.2 are given in Table 1. It must be remembered that interlaboratory coefficients of variation can be twice these values, or more, if quality control is poor.

Table 1 Expected average coefficients of variation within a laboratory for a mean number of fibres N counted per analyst on a sample

N	Range of coefficients of variation
5	45-49%
10	32-37%
20	22-30%
50	14-25%
100	10-22%
200	7-21%

Lower concentration

9 Table 1 shows that errors become very large when small numbers of fibres are counted, and decisions taken on air measurements should have regard to this error. It will be seen that an average concentration on a filter of 10 fibres per 100 microscope graticule areas will sometimes give a result of 5 fibres/100 areas by chance variation. This is the blank count allowed by para 47, so an average count of about 10 fibres/100 areas should be regarded as

the lowest measurable. For a sample of 480 litres, this corresponds to about 0.01 fibres/ml in the air. Such a measurement is clearly very rough, with 90% confidence limits of about 0.005 fibres/ml and 0.018 fibres/ml, taking into account within-laboratory precision.¹² Bias will further seriously degrade the reliability of low-concentration results (para 7).

PRECAUTIONS

10 Inhalation of the vapour from the liquids used should be avoided and manufacturers' recommendations followed carefully. Precautions for acetone clearing are noted in para 38.

REAGENTS

11 Acetone, glycerol triacetate (triacetin), and Cargille liquid r.i. = 1.51 or immersion oil, r.i. 1.515 (optional, see para 32). Analytical grade reagents are not essential, although excessive water in the acetone may reduce filter clarity, and the triacetin should be clean, free from dust and moisture, and with no evidence of hydrolysis (possibly indicated by a smell of acetic acid) or other contamination. The supplier's advice should be followed on storage and lifetime of the Cargille liquids and immersion oil.

APPARATUS

Sampling equipment

12 An open-faced filter holder fitted with an electrically-conducting cylindrical cowl extending between 33 mm and 44 mm in front of the filter, and exposing a circular area of filter at least 20 mm in diameter, must be used. This type of holder is intended to protect the filter, whilst still permitting a uniform deposit. In correct use, the cowl will point downwards. A suitable design is shown in Figure 1, but other designs meeting the above specification are permitted. Flexible tubing is required to connect the filter holder to the pump, and a cap or bung for the cowl entrance to protect the filter from contamination during transport. Clean, degreased tins with well-fitting lids, or similar, may be used to transport the filters, if transport in the filter heads is impracticable.

13 The membrane filter must be of mixed esters of cellulose, of pore size 0.8 to 1.2µm, and 25 mm in diameter, with a printed grid. Flat-tipped metal tweezers of good quality are required for handling them.

14 The pump must give a smooth flow and be capable of having its flow set to within 5%, and of maintaining the chosen flowrate (see para 25) through the membrane filter used to within ±10% during the period of sampling. At 1 litre/min, a 0.8µm pore-sized mixed-ester filter typically has a flow resistance of 2.4 kPa (245 mm water gauge), and a 1.2µm pore size filter has a resistance of 1.8 kPa. Pressure drop is proportional to flowrate. For personal sampling, the pump must be light and portable, and a belt may be required if the pump is too large to fit in the worker's pocket. Reference 13 gives useful information on portable pumps.

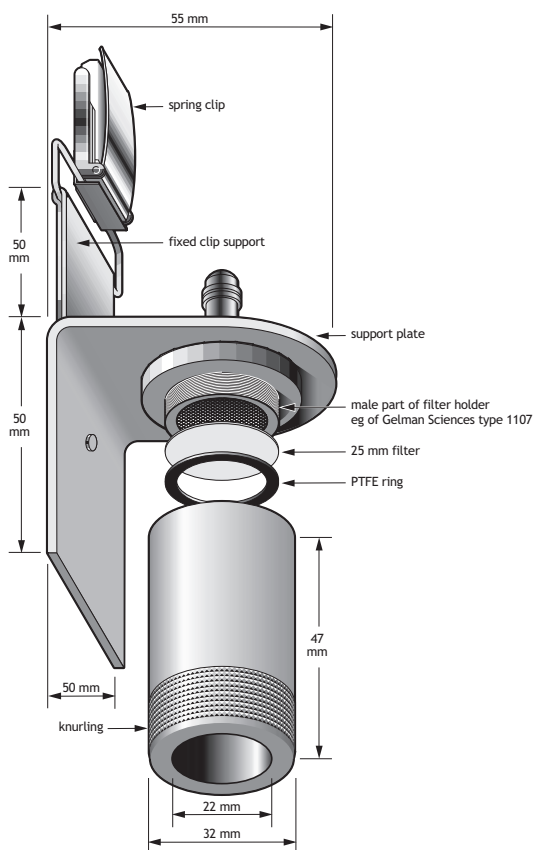


Figure 1 Sketch of a suitable filter holder

Flow measurement

15 A calibrated flowmeter is necessary when the pump is first set up, and when the flow is checked subsequently. This will normally be a portable flowmeter previously calibrated against a primary standard. The primary standard should preferably be a flowmeter whose accuracy is traceable to national standards, used with careful attention to the conditions of the calibration certificate. A bubble flowmeter may be used. This is an arrangement whereby the pump under test draws a soap film up a calibrated tube. The passage of the film is accurately timed between two marks whose separation defines a known volume. A 1-litre burette can form a suitable tube. The volume between the marks can be checked by filling the burette with distilled water, allowing temperatures to stabilise, drawing off the known volume, and weighing the water, making allowance for the dependence of volume on temperature. A suitable bubble solution can be made by mixing one part of concentrated washing-up liquid, two parts of glycerol, and four parts of water. The burette must be thoroughly wetted with the solution, and several attempts at drawing the film up the tube may be necessary before the tube is wet enough for this to be achieved consistently. (Traceability of the calibration will require checking of the clocks, and use of certificated weights.)

16 The portable flowmeter used in the field must have enough sensitivity to permit the flow to be read within $\pm 5\%$ (para 14). If it meets this requirement, the flowmeter incorporated into the pump can be used, but it must be calibrated with a filter holder and filter in line, and read with the flowmeter vertical if of the supported float type. A

flowmeter in the pump or elsewhere in-line can also be misleading if there are leaks in the sampling train between sampling head and flowmeter.

Equipment for filter clearing

17 For acetone clearing, the 'hot block' method¹⁴ is preferred, particularly for field use, but the acetone boiler method may be used in the laboratory. In the 'hot block' method, just enough acetone to clear one filter is injected into a block with an integral heater. The acetone is vaporised and emerges as a vapour jet from an orifice, below which the filter is placed. The acetone boiler is a tall, narrow, flat-bottomed vessel, with a cooling coil near the top (Figure 2). The lid should have a stiff wire cradle attached, to hold a microscope slide securely. The coil carries cold water and confines most of the acetone vapour to the lowest part of the vessel. The water line must include an indicator which shows clearly when water is flowing.

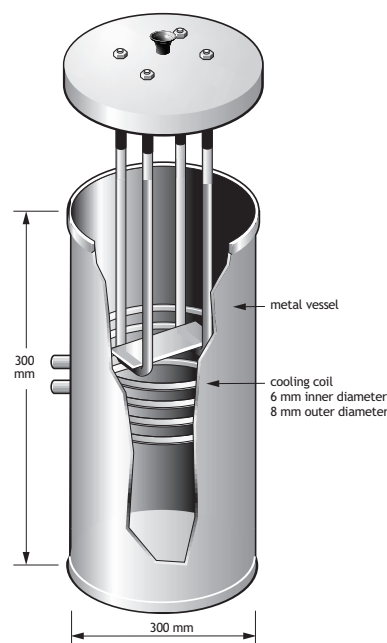


Figure 2 Vessel for acetone clearing

18 If the acetone boiler is used, a source of heat is required which cannot ignite acetone vapour, such as recirculating oil bath. Possible sources of ignition, including electrical switches liable to sparking, must be avoided. The water vapour produced by a water bath may lead to poor clarity of the cleared filters. Use of a fume cupboard is necessary with the boiler, and a lipped surround to contain any spilled acetone is advisable. A fume cupboard is unnecessary with the 'hot block' method, but adequate ventilation is necessary.

19 Fine-tipped pipettes, or other suitable droppers are required, to dispense the clearing liquids.

20 A plasma oven for etching the filter surface is required if the fibre retractive index <1.51. See paragraph 43.

Microscopy

21 The microscope used should be a binocular phase contrast optical instrument with Koehler or Koehler-type illumination. The substage assembly should incorporate an Abbe or achromatic phase contrast condenser in a centring focusing mount, with a phase annulus centring adjustment independent of the condenser centring mechanism. The objective must be 40 times positive phase contrast achromatic, with a numerical aperture of between 0.65 and 0.75 and a phase ring absorption between 65% and 85%, and preferably between 65% and 75%. An optical filter should be used if this improves the performance of the microscope. The eyepiece should be of the wide field type, and should be those supplied by the microscope manufacturer for the instrument in use. The overall magnification should be between 500 and 600 times. It is recommended that the different components of the microscope should all be from a single manufacturer. When properly set up, Block 5 on the HSE/NPL phase contrast test slide must be visible (paragraph 45). At least one of the eyepieces must be of the focusing type and permit the insertion of a graticule. Spectacle wearers will require high eye-point eyepieces, with flexible eye caps. The environment should be vibrationfree, and such that the microscopist can sit in a relaxed and comfortable manner. Any peripheral view beyond the microscope should preferably be an unobstructed distant view in unchanging subdued light, to avoid eyesight fatigue, or alternatively, a matt black background shield can be used.¹⁵

22 The eyepiece graticule must be of the Walton-Beckett type.¹⁶ This graticule must be made for the microscope with which it is to be used, taking into account the true magnification. When ordering the graticule, it is therefore necessary to specify microscope type, the outer diameter of the glass disc of the eyepiece graticule, and also the distance in millimetres on the graticule that corresponds to 100µm in the object plane of the microscope. This can be measured with any other available eyepiece graticule as follows:

- (a) Set up the microscope as if for counting according to this MDHS and the microscope instructions, with the available graticule in place.
- (b) Use a stage micrometer to measure in µm the apparent length 'L' (say) of the scale on the available graticule.
- (c) Remove the available graticule from the eyepiece and measure the scale's true length Y in millimetres, and hence calculate the true length X mm that corresponds to an apparent length of 100µm.

$$X = 100 Y/L \text{ mm}$$

X is the length corresponding to 100µm in the object plane that must be given when ordering. Y must be measured to within ±4%, and within ±2% if possible. If the microscope has a vernier gauge on its stage movement, and if no better method is available, Y can be measured by placing the available graticule on the stage, observing it at low magnification and seeing how many millimetres the stage must be moved to cover the length Y on the graticule. On receipt, the Walton-Beckett graticule should be inserted in the microscope for which it was ordered and its apparent diameter checked with a stage micrometer. It must be between 96 and 104µm.

23 An HSE/INPL phase contrast test slide Mk 2 is required. Microscope slides must be of glass and of conventional type, 76 x 25 mm (approximately) x 0.8 to 1 mm thick. Coverslips should be glass, No 1 1/2 (0.16 to 0.19 mm) thickness, and 25 mm in diameter (or 25 mm side length if square). The microscope slides and coverslips should be clean, and manufactured to BS 3836: Part 1: 1974 (rev 1982) *Microscope cover slips and slides and immersion fluid*.

TAKING THE SAMPLE

Preparation of filters

24 To minimise contamination, the filter holders and cowls must be clean before use, and the filters should be loaded, unloaded and analysed in an area as free from fibre contamination as practicable. Care must be taken to handle the filter at all times only by good quality tweezers, and only by the edge. (It is hard to pick up and hold the filter with poor tweezers.) The entrance to the cowl should be closed with a protective cap or bung during transport through contaminated areas. For every 25 filters (or part of 25) used, at least one filter must be reserved to act as a counting blank. It should be mounted and counted (para 47). It is advisable to do this before sampling, to check that the batch of filters is satisfactory. In addition, as a check on other parts of the procedure, it is good practice to load another blank filter into a sampling head each time filters are prepared for a sampling exercise. This head may be taken to the sampling area, and carried whilst the samplers are placed, and later collected. This sampling blank filter is subsequently mounted and counted (para 47).

Choice of sample volume

25 Sampling flowrate should lie in the range 0.5 to 8 litres/min.¹⁷ Sampling time should have regard to the purpose of measurement. For example, in testing compliance with an exposure limit, an 8-hour time-weighted average concentration may be needed, but this may be derived from two or more consecutive samples.¹⁸ A much shorter sample may be appropriate if the purpose is, for example, to investigate dust production at one particular point in a process. The precision of the evaluation step depends primarily on the number of fibres counted (para 8), so the sampling volume (flowrate x time) should be chosen where possible to keep the fibre density on the filter between 50 (preferably 100) and

1000 fibres/mm². For example, at an airborne concentration *F* of 0.1 fibres/ml, sample volume *V* must exceed about 415 litres for the on-filter concentration *C* to exceed 100 fibres/mm² ($V = 0.415 C/F$ in the units stated; this assumes a deposit diameter of 23 mm). Where low volumes are unavoidable, the number of fibres counted may be increased by increasing the area of filter examined (para 46), but this should not be taken beyond 200 Walton-Beckett graticule areas, because operator fatigue may affect the result. The precision (coefficient of variation) of the sample volume measurement should be within $\pm 10\%$.

Preparation of sampler

26 To allow the pump to warm up, it should be attached to a loaded filter holder using flexible tubing and allowed to run at the chosen flowrate for 15 minutes so that the flowrate can steady (experience may show warm-up to be unnecessary with some types of stabilised flow pumps, although the flow precision in para 14 must be met). The filter is then discarded and a new one fitted for collecting the sample, in such a way that the dust is collected on the gridded side. The flow is then readjusted to the chosen flowrate using the calibrated airflow meter (para 15). A satisfactory procedure is to connect the calibrated meter to the entrance of the cowl by a bung and tube. When flow-setting is complete, the pump should be switched off and a protective cap fitted to the cowl entrance. The pump should not be operated without a filter on the intake, to avoid damage by dust particles.

Background (fixed point) sampling

27 Background samples are generally unacceptable for measuring compliance with occupational exposure limits, but can be useful as a guide to effectiveness of control, or for investigating sources of contamination. The sampling head should be mounted on a stand, with cowl entry facing downwards, allowing free air circulation round the entry. It should be positioned having regard to local sources of dust or clean air. Cross-draughts of more than 1 m/sec may reduce fibre collection.

Personal sampling

28 The filter holder should be fixed to the upper lapel or shoulder of the worker's clothing as close to the mouth and nose as practicable, but in any case within 200 mm of them. Wherever possible, the same sampler position should be adopted for each worker. In some circumstances a higher concentration may be expected on one side of the worker than the other, in such cases the sampler should be positioned on the side expected to give the higher result. At the start of the sampling period, the protective cap must be removed from the filter holder, the pump started, and the time noted. The flowrate should be checked periodically (eg hourly) during sampling, using a calibrated flowmeter, and readjusted to the chosen rate. Operating experience may show this to be unnecessary with some types of pump, but it is still advisable to confirm that the equipment is functioning. At the end of the period the time should be noted, the flowrate checked, the pump

switched off and the protective cap replaced on the filter holder.

End of sampling

29 At the end of the sampling period, the equipment must be removed from the worker, and the cowl closed with a bung or protective cap. The preferred procedure is for the filter to be transported in the filter holder, but if for some reason this is impossible, the filter may be carefully removed in a clean area and placed in a clean tin or similar container. Sprays must not be used to fix the dust to the filter, because they are unnecessary and may cause damage. Adhesive tape can be used to secure the clean unexposed edge of the filter to the tin, if one is used, and the tape can subsequently be cut from the filter with a surgical scalpel. Care must be taken not to contaminate the filter at any stage before evaluation, and the filter holder and cowl must be cleaned before re-use.

30 Wherever possible a bulk sample or samples should be obtained of the MMMF material giving rise to the airborne dust, for determination of refractive index. The samples should be sealed in separate plastic bags and carefully labelled.

DETERMINATION WHETHER R.I. >1.51

31 Three methods are described here. One or two of these methods may be inconclusive or impracticable depending on the fibre being examined. The possibility of mixtures of fibre types must be borne in mind, and the tests must indicate that all the fibres have a refractive index (r.i.) greater than 1.51 for the acetone triacetin mounting method to be used. If any of the fibres have r.i. equal to or less than 1.51, the etching method must be used to make the fibres visible for counting. Most ceramic fibres, including rock wool and slag wool, have r.i. >1.51. Conventional insulation glass wools usually have r.i. >1.51, although for some types r.i. can be around 1.51. Superfine or special purpose glasses or microquartz usually have r.i. close to 1.51 or lower. For these and any other material, the etching method (paras 42 to 44) must be used unless it can be clearly established that the fibre r.i. >1.51. All three r.i. determination methods given here can be used with a microscope as specified in para 21. Use is made of the brightfield condenser position with adjustable aperture iris which is present on all modern phase contrast microscopes. In addition, the removal of any colour filter improves the methods. (If no bulk sample of the fibre is available, the in situ method of determining r.i. may be used.¹⁹) The changes discussed in paras 34 to 36 are summarised in Table 1.

Preparation

32 A small portion of the MMMF bulk material is placed into a drop of liquid of known refractive index on a microscope slide. Immersion oil, which has an r.i. of about 1.515 (this should be specified on the bottle) is non-toxic, and is very suitable. Alternatively a refractive index (Cargille) liquid, of r.i. 1.51 may be used. The MMMF fibres are teased apart with two probes, and a cover slip is

laid on top. When the fibre r.i. is close to 1.51, the fibres can be difficult to find under the microscope. They can be located by placing some easily visible and clearly distinct material, such as a few fibres of tissue paper, in the liquid with the fibres.

33 The microscope should be set up with a total magnification of about $\times 500$ and Koehler or Koehler-type illumination. This is obtained by focusing on a specimen, closing the field iris, and adjusting the condenser position to a point just below the specimen, so that the image of the field iris is brought into focus. After centring the image with the condenser, the field iris is opened to fill the field of view with light. Partially closing the condenser aperture iris will often improve the image of the field iris for these adjustments, although for subsequent use the condenser iris should be set so that it just enters the field of view in the back focal plane. These adjustments may vary with type of microscope and the manufacturer's instructions should be followed.

The principles are:

- (a) the field iris must be in the same plane of focus as the specimen;
- (b) the field iris must be centred in the field of view and opened just beyond it;
- (c) the back focal plane must be fully illuminated;
- (d) the lamp filament (if visible) and the condenser iris must be in focus in the back focal plane.

Method 1: Oblique illumination ^{20,21} (Van de Kolk method)

34 This is suitable for fibres with diameter greater than about μm . It is important that Koehler or Koehler-type illumination is obtained for this method, since the effect will be reversed if the condenser is positioned too low. The fibres are brought into focus using phase contrast, the brightfield condenser position is selected, and the condenser aperture iris and field iris are fully opened. A piece of black paper or card, or even a finger, is inserted under and as close to the condenser aperture iris as possible (this will vary according to the individual microscope). This is inserted from one side until one side of the field of view and one side of the fibre are observed to darken. (The position of the paper can be observed in the back focal plane. It will need to cover the back focal plane almost completely.) The light-dark boundary and the fibre need to be approximately parallel. If the fibre and the field darken on the same side, the fibre refractive index is greater than that of the liquid. If the fibre darkens on the opposite side to the field, the fibre r.i. is less. When the refractive indices match, this effect is not observed, and the fibre may be very difficult to see, or may show a transparent blue colour, possibly with colour fringing. In case of doubt the fibre r.i. should be taken as <1.51 . A further alternative to inserting a piece of paper is to rotate the phase condenser stop carrier slightly from its normal position. This causes one edge to obscure the light path, which produces the required oblique illumination.

Method 2: Becke line and central illumination ^{20,21,22}

35 These are two phenomena which enhance one another. The method is suitable for fibres with diameter great than μm . The fibres are brought into focus using phase contrast, the brightfield condenser position is selected, and the condenser aperture iris is almost closed down. The microscope focus is moved very slightly each way from the position of best focus. If, when the position of focus is raised (ie the stage lowered or objective raised), the fibre becomes darker, with light lines along each side which move away from the fibre as the focus is altered, the fibre r.i. is less than that of the mounting liquid. If the focus is moved in the other direction, the fibre then appears lighter with broad, ill-defined dark lines along each side. These effects are reversed if the fibre r.i. is the greater. Adjustment of the condenser aperture iris may improve visibility of the lines. When the refractive indices match, these effects do not occur, and the fibres may be very difficult to see, or may show a transparent blue colour, possibly with colour fringing. In case of doubt, the fibre r.i. should be taken as <1.51 .

Method 3: Phase contrast ^{20,21}

36 This is suitable for fibres with diameter less than about μm . For positive phase contrast, if a thin fibre is observed to be lighter than the background, the r.i. of the fibre is less than that of the liquid. (Larger diameter fibres can show an inhomogeneous yellow colour, depending on the r.i. difference.) If a thin fibre is observed to be darker than the background, the r.i. of the fibre is greater than that of the liquid. (Note that in this case, phase reversal can occur with larger diameter fibres, and the fibre appears light, with a thin dark outline.) When the refractive indices match, the fibres appear either a transparent blue, sometimes with a red halo, or the same shade as the background, in which case they may be difficult to see. In case of doubt, the fibre r.i. should be taken as <1.51 .

FILTER PREPARATION

37 The principle of the filter clearing method is that the filter is immersed in hot acetone vapour, which condenses on the filter, collapsing its pores and making it transparent, and adhering it to the slide. A liquid must be added to provide optimum contrast. If the fibre r.i. >1.51 , triacetin (glycerol triacetate) is satisfactory, the fibre r.i. ≤ 1.51 , the filter surface is etched to expose the fibres, and water is used as the contrast liquid. As with any method, practice mounting of clean filters is recommended to gain proficiency before a real sample is used. When the sampling filters have been exposed to high humidity, clarity may be improved by drying them before exposing them to the acetone. (The DMF/Euparal method, as described by le Guen and Galvin²³ may be used instead of acetone. It has the advantage of greater permanence of mount and uses simpler equipment. However, if an oven is used, this should be ventilated to remove the solvent vapour, and must be flameproof if more than about 20 slides are cleared at a time.)

38 CAUTION: Acetone vapour is highly flammable and slightly toxic. Less safe methods than the two described here should be avoided. Precautions particular to the acetone boiler method are described in paragraph 40.

Acetone-triacetin: the 'hot block' method

39 The commercial version of the 'hot block' should be used in accordance with the manufacturer's instructions. The filter is placed centrally on a clean microscope slide, sample side up, with the grid lines parallel to the slide edges. The principle is that about 0.25 ml of acetone is injected into the block, so that the vapour emerges in a stream over the filter. The filter clears. The small amount of acetone used minimises the fire risk, although sources of ignition must be kept away, and the acetone bottle must be kept closed when the acetone is not actually being extracted. If another version of the hot block is used,¹⁴ it should be made so as to minimise the amount of acetone, consistent with producing a uniform clear filter, and to keep the acetone completely separate from sources of ignition.

Acetone-triacetin: the boiler method

40 The acetone boiler, described in paragraph 17, creates a region of vapour confined by the cooling coil, which reduces the risk of fire. The quantity of acetone used should be kept to a minimum, it should not be left in the apparatus when this is not in use, and it should not be left boiling when clearing is not actually in progress. The lid should be kept in place whenever possible. The equipment must only be used in a fume cupboard, with a lipped surround to contain any spilled acetone. Sources of ignition must be kept well away from the equipment, and a 'NO SMOKING' rule must be enforced. The cooling water in the clearing vessel coil is turned on, about 30 ml acetone is put into the vessel, the lid is replaced and the vessel placed on a source of heat free from risk of igniting any acetone vapour. A recirculating oil bath is suitable. A water bath may be unsatisfactory because large amounts of water vapour make the filter less clear. When the acetone boils, the lid is removed, a microscope slide is placed in the cradle with the membrane filter placed centrally on the slide, sample side up, with the grid bars parallel to the slide edges. The lid is replaced, thus lowering the slide into the vapour in the vessel; the slide should clear in a few seconds, and it is then removed.

Fibre r.i. >1.51

41 A few minutes are allowed for the acetone to evaporate from the filter, and a micropipette is then used to place a drop (about 10 μ l) of triacetin on the filter or cover slip, enough to cover the whole filter when the cover slip is in place, without excessive overflow round the edges. The cover slip is gently lowered onto the filter at an angle, so that all the air is expelled but should not be pressed onto the filter. The filter becomes less granular in appearance with time for the first few hours after clearing, and the slide should therefore normally be left for 24 hours before counting; if a result is required quickly, the slide may be heated for 15 minutes at about 50°C and then counted. The mounted slide will usually keep for a year or more

without noticeable deterioration, although some small-scale fibre movement can occur; it is desirable to store the slides flat. Long-term keeping qualities can be improved by painting round the edges of the cover slip with a microscopical embedding agent, such as Entellan.

Fibre r.i. \leq 1.51

42 If the fibre r.i. \leq 1.51, or if the r.i. is uncertain, the following method should be used. The acetone clearing procedure (paragraphs 37-40) collapses the filter to about 15% of its original thickness, forming a clear plastic film which has minimal distortion, and which does not deteriorate. The fibres are embedded in the surface, but may be invisible even under phase contrast illumination, if the refractive index of the fibre is close to that of the filter. They must therefore be exposed, by etching away the surface of the filter, and immersing them in a liquid of very different r.i.

43 The slide and filter are placed in a plasma asher, and exposed sufficiently to remove the surface of the filter, leaving the fibres exposed in the air, but still attached to the filter. Le Guen et al²⁴ recommend an oxygen flowrate of 8 cm³/min, with forward and reflected radio frequency power of 100 and 2 W respectively, for about 7 min. They used a Nanotech Plasmaprep P100, but this instrument is no longer available. The same settings should apply on the Biorad PT 7100, 7150 and 7300. Information is available on the calibration of other types of plasma asher.²⁷

44 A drop or two of clean distilled water is placed on the etched filter, and a coverslip placed on top, again taking care not to trap air bubbles. The amount of water should be sufficient to fill the space between filter and coverslip without excessive overflow. Water has a refractive index of 1.33, and provides a good contrast even with low r.i. fibres. It is necessary to mount blank filters to ensure that the water is free of fibrous or bacterial contamination, and it may be necessary to freshly filter the water.

EVALUATION OF SAMPLES

45 The following procedure applies whether the etching method or simple clearing is used. The microscope must be set up according to the manufacturer's instructions (see also paragraph 33), and its performance checked at the beginning of each day or shorter period of use with an HSE/NPL phase contrast test slide Mk 2; the microscopist must be able to see block 5 on the slide when used as specified by the manufacturer. After using the test slide, the fine focus and condenser focus may need readjustment before a sample is counted. The object-plane diameter of the Walton-Beckett graticule should be checked using a stage micrometer. Depending on the microscope, tube length and adjustment of interocular distance can affect the magnification. These should therefore be set correctly before measuring the graticule. The diameter should be between 96 and 104 μ m (see paragraph 22), and the measured diameter must be used in calculations.

46 This slide with the mounted filter is then set up on the microscope, the fibres on the top surface of the filter are brought into focus. The sample may first be examined for uniformity with a low power objective if desired. The X40 objective is then selected, and the fibres are counted according to the following rules, which are based on those agreed for MMMF by a WHO Technical Committee.¹⁰

- (a) Graticule areas for counting are chosen at random within the exposed area of the filter. The fine focus must be adjusted for each new area, and may need to be changed from fibre to fibre.
- (b) A countable fibre is a particle longer than 5µm, with width less than 3µm, and with a length:diameter ratio greater than 3:1. A countable fibre with both ends in the graticule area is counted as one fibre, a countable fibre with only one end in the area counts as half. A fibre completely crossing the graticule, with no ends in, is not counted. (An easy, practical method is to count the number of fibre ends in the graticule area and divide by two, having regard to the proper treatment of split fibres and clumps in (d) below.)
- (c) If the diameter of a fibre varies along its length, a representative average diameter should be used. Bulges in the fibre, such as are sometimes caused by resin, should be ignored. In case of doubt, the diameter should be taken to be <3µm. Fibres which are attached to non-fibrous particles should be assessed as if the particle did not exist.
- (d) Split fibres occur infrequently, but should be treated as single fibres. When several fibres occur together in a group, the fibres should be assessed individually as separate fibres if they can easily be distinguished. Where they form a clump in which the fibres cannot easily be distinguished, the whole clump should be ignored.
- (e) If more than one-eighth of a graticule area is covered by an agglomerate of fibres and/or particles, the graticule area must be rejected and another counted. A graticule area may also be rejected if fibres are so obscured that the microscopist judges that they cannot be counted reliably. The number of rejected areas must be recorded, and if this exceeds ten per cent of the number accepted, then the calculated concentration must be annotated accordingly.
- (f) At least 20 graticule areas must always be examined. If the purpose of measurement is to assess compliance with an exposure limit of L fibres/ml, and the sample volume is V litres, then at least $4295/LV$ graticule areas should be examined.²⁶ (For example, if L = 1 fibre/ml and V = 85 litres, then at least 50 areas need to be examined.) For other purposes, at least 80 fibres should be counted. On sparse samples, counting more than 200 graticule areas may lead to deterioration of counter performance through fatigue. Counting may then be terminated at 200

areas, but if 80 fibres have not been reached, then the counting report should be annotated to the effect that the sample was sparse and the precision is probably reduced.

47 The blank filters should be mounted in the same way as the test filters, and counted. If more than 5 fibres per 100 graticule areas are found on any blank, the airborne measurement should be regarded as a rough estimate only, and the source of the blank count investigated.

48 The diameter of the exposed area of the filter should be measured at least every time a different filter holder or O-ring type is brought into use. The measurement can be made on a filter exposed to workplace dust in the normal course of sampling. The slide is placed on the microscope stage and the filter observed at low magnification while a diameter of the exposed area is traversed by moving the stage. The distance moved can be obtained from the stage vernier scale. Two diameters should be measured, and three filters should be checked in this way. Differences of more than a millimetre may indicate an unsatisfactory clearing technique. The gross appearance of the filter may also show any fault with the filter holder, for example a leak or uneven deposit.

49 The airborne concentration is given by:

$$(1000 ND^2)/(Vnd^2) \text{ fibres/ml}$$

where: N is the number of fibres counted,

n is the number of graticule areas examined,

D(mm) is the diameter of the exposed area of the filter,

d(µm) is the diameter of the Walton-Beckett graticule, as measured with a stage micrometer.

V (litres) is the volume of air sampled.

50 If the sample was mounted by the etching method, the coverslip and Cargille liquid may be removed by dropping 2 to 3 ml of cyclohexane onto the inclined slide from a Pasteur pipette. The sample should be allowed to dry and can then be kept indefinitely.

QUALITY ASSURANCE

51 Because of the large differences in results within and between laboratories obtained with all manual fibre-counting methods, a good quality assurance procedure is essential. Laboratories using the method must participate in checks to assess interlaboratory variation. If none is available for MMMF, participation in the RICE scheme for asbestos may be suitable as a substitute, but the position should be discussed with the RICE organisers. Interlaboratory exchanges should be supplemented by checks of internal consistency using MMMF samples, which should aim to measure the mean and reproducibility of each counter's difference from the average of the laboratory. (It is unsatisfactory for a counter to have an average result equal to the laboratory mean if the average

performance conceals considerable variation from sample to sample.) In a large laboratory, a satisfactory procedure is to have all the counters recount a specified fraction of the routine slides. The fraction should be chosen to provide a quality assurance sample about once a week, and to ensure that these slides are fully representative of the laboratory's routine samples. For example, a laboratory counting 5000 samples a year could select every one hundredth filter (whatever its type) for recounting by all its counters, and could keep a running check of the mean and standard deviation of each individual's difference from the laboratory mean. A laboratory with only one or two counters would have to maintain stock of permanently-mounted and well-characterised slides for periodic check counting, and could again calculate the mean and standard deviation of the counts in relation to those accepted for the stock slides. The reference counts should be obtained by counting the stock slides ten times (say) over a period and taking the mean. The problems of fibre-counting quality assurance and several internal schemes are reviewed in reference 25. Errors in sampling are, of course, additional to those in evaluation. Systematic records of quality assurance results should be kept and regularly examined to assess individual counter and overall laboratory performance. Accreditation by the National Measurement Accreditation Service (NAMAS) provides an independent warranty of a laboratory's acceptability in these and other aspects.

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ADVICE

Advice on this method and the equipment used can be obtained from the Health and Safety Executive, Health and Safety Laboratory, Broad Lane, Sheffield S3 7HQ (telephone 0114 2892000).

The Health and Safety Executive wishes, wherever possible, to improve the methods described in this series. Any comments that might lead to improvements would therefore be welcome and should be sent to the above address.

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18	Health and Safety Executive. <i>Monitoring strategies for toxic substances</i> . HSE Books 1997 HSG173 ISBN 0 7176 1411 5	<i>Oblique illumination</i>	Fibre and field darken on opposite sides	... on same side
19	Vaughan, N.P., Rooker, S.J. and Le Guen, J.M.M. In situ identification of asbestos fibres collected on membrane filters for counting. <i>Ann. Occup. Hyg.</i> , 1981, 24 (3), 281-290.	<i>Becke line/central illumination</i>	Focus raised: fibre darkens; light lines move away	opposite effects
20	Hartshorne, N.H. and Stuart, A. <i>Crystals and the Polarising Microscope</i> , 4th ed. pp 258-264, 422-423. (Edward Arnold, London) ISBN 0 7131 22560.		Focus lowered; fibre brightens; broad, ill-defined dark sides	
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