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Corrections

See [1981;7\(3\):240](#) for a correction.

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Key terms: [biological sample](#); [concentration](#); [human lung tissue](#); [inorganic fiber](#); [inorganic fiber concentration](#); [scanning electron microscopy](#)

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Analysis of inorganic fiber concentrations in biological samples by scanning electron microscopy

by Bjørn Gylseth, MSc, Randi H Baunan, MSc, Rønnaug Bruun, BSc¹

GYLSETH B, BAUNAN RH, BRUUN R. Analysis of inorganic fiber concentrations in biological samples by scanning electron microscopy. *Scand j work environ health* 7 (1981) 101-108. Analyzing fibers with electron microscopic techniques involves several preparation steps, especially during the analysis of fibers in human tissue. The influence of these steps on the analytical result was studied in detail. Fiber number was unaffected by the mild sonication of fiber suspensions analyzed with scanning electron microscopy. Significant fiber losses appeared during the filtration of fiber suspensions through 0.8- μ m pore-size Nuclepore membranes. Shrinkage of the tissue during dry ashing broke long fibers and consequently increased the fiber concentration and affected the fiber length distribution. Dry ashing, however, removed more of the organic debris than wet ashing; thus specimens prepared with dry ashing were more suitable for scanning electron microscopic analysis. A fairly good correlation was demonstrated for the analysis of fibers with scanning and transmission electron microscopy.

Key terms: human lung tissue, inorganic fibers.

The assessment of airborne inorganic fiber concentration has traditionally been performed by optical phase-contrast microscopy. For a better identification and quantification of inorganic fibers, electron microscopy (EM), including scanning (SEM), transmission (TEM), and scanning/transmission (STEM) electron microscopes fitted with X-ray and electron micro-analytical equipment, has recently been introduced.

These techniques are also used for the analysis of inorganic fibers in human autopsy material, especially lung tissue. Different microscopic methods often require different preparation of the tissue samples. In a recent paper by Morgan & Holmes (16) different aspects of the preparation and analysis of lung tissue are discussed. They emphasize that drying,

low-temperature ashing of the tissue and ultrasonic dispersion of the ash are contraindicated. Such techniques have, on the other hand, previously been used by other authors (3, 4, 7). Different preparation techniques have, however, not been thoroughly compared.

In the present investigation we have studied the influence of different preparation steps on inorganic fiber concentration as measured by SEM.

Material and methods

The different preparation steps which may lead to serious fiber losses have been tested on Union Internationale Contre Le Cancer (UICC) standard crocidolite asbestos and on fibers from autopsy material of subjects who died of asbestos-related diseases.

All fibers with an aspect ratio equal to or greater than 3:1 were counted. Fiber numbers were evaluated at a magnification of 4,500 $\times$ in a Jeol JSM-35 SEM, and in each specimen either 100 fibers or the

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fibers in 200 view fields were counted. The calculation of relative standard deviations (RSD) were based on four samples.

All liquids used in the analyses were filtered, and the SEM samples were gold-coated before analysis. The resolution of the SEM was tested on standard UICC chrysotile on a Nuclepore filter with a pore size of 0.8 μm . The TEM analyses were performed in a Philips 301 TEM at a magnification of 5,000 and 20,000 $\times$.

Sample preparation

Stock solutions of the standard UICC samples were prepared by dispersing 1 mg of asbestos in a 100-ml water/ethanol solution in an ultrasonic bath. Ethanol was added to avoid hydrophobic areas on the Nuclepore membranes during the filtration process.

The stock solution was diluted 1:100 with distilled water. Different volumes of this solution were filtered through the membranes by means of a water suction pump. Particle agglomeration and adhesion to the glass walls occurred during storage, and a new stock solution was prepared for each series of analyses.

Tissue samples were cut from lungs preserved in formaldehyde and either dried to constant weight at 80°C or weighed in the wet condition. For the determination of the dry-to-wet weight ratio, adjacent tissue samples were dried as described previously.

Digestion of tissue

The following three procedures were used to remove organic material from the tissue samples:

1. Wet digestion (WD) in 1 N sodium hypochlorite in 0.1 N sodium hydroxide. Sodium hypochlorite (5–10 ml) was added to the tissue in centrifuge glasses that were kept at 60–70°C until a complete reaction was obtained. A mixture of the sodium hypochlorite solution and diethyl ether (5 ml) was thoroughly shaken and thereafter centrifuged at 4,000 r/min for 20 min. Each time the procedure was repeated twice, the ether layer being removed with a pipette. The ether fraction was filtered through Celas silver membranes and analyzed for fibers with SEM. The un-

reacted sodium hypochlorite was neutralized by 1 N hydrochloric acid and sonicated. In one series of analyses the ether extraction was omitted, and the WD samples were filtered directly through the Nuclepore membranes.

2. High-temperature ashing (HTA) in ceramic crucibles in a conventional laboratory oven at 500 or 600°C for 1 h.

3. Low-temperature ashing (LTA) at temperatures below 200°C in a Tracerlab model 505 LTA. Generally 50 W of forward power with an oxygen flow of 225 ml/min was used. Some samples were also ashed at high power LTA, ie, at an energy input greater than 150 W of forward power at an oxygen flow of 150 ml/min. A complete ashing was obtained for 10–40 mg of dry tissue within 2–5 h, depending on the size of the tissue pieces. The ash remaining after LTA was dispersed in 0.5 N hydrochloric acid, a few milliliters of ethanol being added, and kept in the ultrasonic bath for 5 min. The tissue salts were then rapidly dissolved by the hydrochloric acid.

Filtration

Due to their high filtration efficiency, porous membranes are normally used for the air filtration of microfibers. Compared to the structure of plain Nuclepore membranes, the porous structure of the membranes is a poor background for SEM work, but penetration of the Nuclepore may occur depending on the pore size (4, 8).

In this study we have compared 0.2- and 0.8- μm Nuclepore membranes versus 0.8- μm Gelman Metricel polyvinyl chloride (PVC) filters using standard UICC crocidolite prepared from water suspensions. The PVC filters were prepared for SEM according to the method published by LeGuen et al (5). Samples for the TEM analyses were prepared with a modified Jaffe Wick washing technique (2).

Ultrasonic treatment

The samples were treated for 5 min in an ultrasonic bath. Vigorous sonication may split fibers and should be avoided. This possibility was investigated by comparing the direct filtration of standard solutions which had been mildly sonicated in a bath

(less than 0.2 W/ml) to solutions vigorously sonicated with a probe with a power output greater than 3 W/ml.

Results

Large inter- and intralaboratory variations may occur in fiber counting as it is based on subjective observations of the particles with the use of different equipment. In most papers an estimation of the coefficient of variation of the method is not indicated. For the standard UICC samples analyzed in SEM at $4,500\times$ a relative standard deviation (RSD) of 10 % was found for repeated counts on the same sample. Counting different samples prepared from the same stock solution gave an RSD for high fiber loadings (ie, greater than 10,000 fibers/mm²) of 16 % for the Gelman filters and 10 % for the Nuclepore membranes. For lower fiber loadings the precision was even better for the Nuclepore membranes. The RSD for the tissue samples was better than 20 %, including intra- and interlobar fiber density variation (4). A relationship between the magnification employed and fiber numbers has previously been demonstrated by Gylseth et al (4).

Table 1. Comparison of different filter types with regard to filtration efficiency for Union Internationale Contre Le Cancer standard crocidolite asbestos at different fiber densities.

Density	0.2 μ m Nuclepore	0.8 μ m Gelman polyvinyl chloride	0.8 μ m Nuclepore
High			
Fibers/mm ²	12,300	10,473	5,828
SD (%)	10	14	6
Low			
Fibers/mm ²	3,411	2,116	1,754
SD (%)	7	16	8

Fig 1 shows UICC chrysotile fibers at $40,000\times$, indicating the resolution of the SEM employed. In table 1 the results from the comparison of the different filter types are given, and a significant higher filtration efficiency is indicated for Nuclepore 0.2- μ m and PVC 0.8- μ m than for Nuclepore 0.8- μ m membranes.

Tables 2 and 3 show the fiber counts in two different series with different agitation methods. There was no significant in-

Fig 1. Standard Union Internationale Centre Le Cancer chrysotile ($40,000\times$).

Table 2. Comparison of fiber numbers in suspensions treated in an ultrasonic bath and with a probe, respectively.

	Ultrasonic bath < 0.2 W/ml	Ultrasonic probe > 3 W/ml
Fibers/mm ²	3,911	5,250
SD (%)	3	6

Table 3. Comparison of the direct filtration of suspensions to other treatments.

	Direct filtration	Direct filtration + rubber policeman	Direct filtration after 1-d storing	Direct filtration after ultrasonication
Fibers/mm ²	5,894	5,589	3,817	5,966
SD (%)	4	9	6	7

Fig 2. A fiber and asbestos body in the ether extract filtered on a silver membrane (1,500 ×).

Fig 3. Fibers partly covered by organic debris left after the wet digestion of lung tissue (3,000 ×).

Fig 4. Two adjacent fibers from lung tissue clearly affected by the high temperatures (6,000 ×).

Fig 5. Fibers and an asbestos body from a lung sample ashed at the low temperature (3,000 ×).

crease in fiber concentration after the samples had been treated in an ultrasonic bath. Table 3 also shows that the storing of the suspensions causes a reduction in fiber count. These observations agree well with those of Chatfield et al (2) and Spurný et al (9). The storing of the fiber suspensions led to particle adsorption to the beaker walls. This phenomenon can

partly be avoided either by ultrasonication or by the use of a "rubber policeman" before filtration (table 3).

A comparison of the different digestion procedures against direct filtration of standard UICC crocidolite suspensions gave a mean recovery of 85 and 90 % for LTA and WD/ether extraction, respectively. HTA at 600°C for 1 h gave a re-

Fig 6. Unashed tissue residue containing several fibers (3,000 $\times$).

Table 4. Fiber concentrations (million fibers per gram of dried lung tissue) for adjacent tissue samples treated with high-energy plasma ashing and filtered on 0.8- μ m Nuclepore membranes in comparison to low-energy plasma ashing and filtration on 0.2- μ m Nuclepore membranes. (LTA = low-temperature ashing)

Sample	High-energy LTA, 0.8- μ m Nuclepore	Low-energy LTA, 0.2- μ m Nuclepore
1	12	83
2	6.7	41
3	19	74
4	12	61
5	6.4	35
6	77	490
7	40	270

Table 5. Fiber concentrations in adjacent tissue samples ashed by low-temperature ashing (samples 1, 2, 3, 4) and wet digestion (samples 1', 2', 3', 4'). The last column gives the fiber concentration of the wet-digested samples treated with low-temperature ashing after they were filtered and mounted for scanning electron microscopy.

Sample	Fibers per gram of wet tissue $\times 10^3$	Fibers per gram of dried tissue $\times 10^6$	Fibers per gram of dried tissue $\times 10^6$
1	8.2	79	—
2	11.3	123	—
	SD = 13 %	SD = 19 %	
3	10.1	99	—
4	10.2	115	—
1'	6.7	64	63
2'	5.8	63	58
	SD = 12 %	SD = 13 %	SD = 4 %
3'	5.0	51	62
4'	6.3	71	65

covery of less than 20 %. When the temperature was reduced to 500°C, the recovery increased to 55 %.

During tissue analysis both naked fibers and asbestos bodies were found in the ether extract, as shown in fig 2, but in insignificant amounts. Furthermore, the debris (lipids and anthracotic coal pigments) left after WD interfered severely with the SEM analyses, almost covering the fibers (fig 3). High temperatures affected the fibers in tissue seriously. The effect is demonstrated in fig 4, in which two adjacent fibers from tissue which has been high-temperature ashed are shown.

Fig 5 shows fibers from tissue treated with LTA. It is essential that a complete ashing be obtained. Unashed tissue fragments contained several fibers due to the incomplete ashing of big tissue pieces, as shown in fig 6.

Samples ashed at high-power LTA and filtered on 0.8- μ m Nuclepore membranes were compared to adjacent samples ashed at low-power LTA and filtered on 0.2- μ m Nuclepore membranes (table 4). On the average the latter technique gave six times higher fiber concentrations than the former. WD and LTA were compared from adjacent tissue samples (table 5). After

Table 6. Fiber concentrations in adjacent tissue samples after low-temperature ashing (samples 1, 2, 3) and wet digestion (1', 2', 3') followed by two ether extractions.

Sample	Wet weight (mg)	Dry weight (mg)	Fibers per gram of dried tissue $\times 10^6$
1	220	13.69	.. ^a
2	270	24.57	29.1
3	291	22.39	33.3
1'	272	20.94	18.7
2'	283	21.97	20.3
3'	302	23.25	19.4

^a Sample lost.

Fig 7. Diameter and length distribution for 100 random fibers from samples treated by wet digestion (WD) and low-temperature ashing (LTA).

Fig 8. Correlation between the results obtained at 4,500 X in the scanning electron microscope (SEM) and at 20,000 X in the transmission electron microscope (TEM) for five lung samples. (LTA = low-temperature ashing)

Table 7. Fiber concentrations in samples analyzed by scanning electron microscopy (SEM) at a magnification of 4,500 X and transmission electron microscopy (TEM) at magnifications of 5,000 and 20,000 X.

Sample	SEM (4,500 X)	TEM (5,000 X)	TEM (20,000 X)
1	83	67	87
2	41	31	35
3	74	22	68
4	490	380	790
5	270	160	190

complete WD, the solutions were directly filtered onto the membranes. WD gave, on the average, 35 % lower fiber concentrations than LTA. LTA treatment of the wet-digested and SEM-mounted Nuclepore membranes did not improve the results. In fact, the LTA treatment of the Nuclepore membranes gave a filter residue which could be misinterpreted as fibers. In a second series of analyses of adjacent tissue samples from another lung with the ether extraction procedure, the data given in table 6 were obtained; they gave concentrations that averaged 37 % lower than those for LTA. For two corresponding samples of the same lung, the diameter and length of 100 fibers were evaluated in each sample. No difference appeared between the two as regards the diameter distribution, whereas WD, in comparison to LTA gave a significantly larger proportion of longer fibers (fig 7).

A comparison of the LTA samples with SEM and TEM analysis has been performed employing a magnification of 4,500 X for the SEM and 5,000 X and 20,000 X for the TEM (table 7). The results for SEM/4,500 X versus TEM/20,000 X are plotted on a log-log scale in fig 8.

Discussion

As pointed out by Ascroft & Heppleston (1) and Morgan & Holmes (6), fiber losses or even an increase in fiber number may occur during the digestion (WD) of tissue sample for quantitative fiber estimation. Errors may also be introduced by LTA (6). We have employed LTA for some years, and our experience is that, under carefully

controlled conditions, it is an effective and gentle method for the removal of organic material. It entails low fiber losses and reproducible results. High plasma energies (ie, power input greater than 150 W) produce local overheating in the tissue pieces and introduce fiber losses of significant degree. As pointed out, these effects may be avoided with low plasma energies (ie, power input of less than 60 W) at an increased oxygen flow (ie, approx 225 ml/min). The data show that LTA gives a slightly increased fiber count when compared to WD, a finding indicating that fibers break during tissue shrinkage. The difference may, however, partly be caused by the short and thin fibers being covered by the organic debris left after WD. As expected, the diameter distribution is not affected, whereas the WD method, when compared to LTA, gives a length distribution slightly biased towards longer fibers.

The whole ashed sample can be filtered with LTA, and thus any extractions introducing fiber losses can be avoided. For SEM fiber counting it also gives a better background. Furthermore, the most precise tissue mass indicator is dry tissue weight. When the wet tissue is cut, weighing errors are introduced due to liquid loss. Formaldehyde also evaporates fairly rapidly, and therefore contributes to the error. Therefore the samples should be weighed in airtight beakers. Lungs which are intratracheally fixed contain a significant amount of liquid and have a low dry-to-wet weight ratio. Other lungs are infiltrated with tumors or consist of pneumonic areas of highly different density and liquid content, and therefore their weight/weight ratios vary. The dry-to-wet tissue weight ratios varied from 6 to 15 % in our study. If dry weight is used, paraffin-embedded material from retrospective studies may be analyzed; thus a comparison of data from retrospective and prospective studies becomes possible.

With direct filtration of the suspension, fiber losses are reduced when compared to those of the other pretreating steps. It is important to use membranes with a sufficiently small pore size to prevent fiber penetration. Our data show that 0.2- μ m Nuclepore membranes give higher fiber counts than 0.8- μ m porous PVC membranes. The difference demonstrated may

be due to the etching of the PVC membranes necessary for the release of the fibers. Nuclepore membranes also give a better image for counting in SEM than the etched PVC membranes. A comparison of fiber size distributions for 0.2- and 0.8- μ m Nuclepore membranes showed that the 0.2- μ m membranes contained both shorter and thinner fibers than the 0.8- μ m ones. Therefore, the filtration of samples on Nuclepore membranes should only be done with filters with a pore size of 0.2 μ m or less; a compromise between filtration speed and efficiency must be made.

The use of sonication for the homogenization of fiber suspensions has also been discussed by Morgan & Holmes (6). There are two main features, a homogeneous dispersion and a rapid dissolution of the tissue salts. Our data, along with those of Chatfield et al (2) and Spurny et al (9), show that homogenization of fiber suspensions may well be performed by ultrasonics if a sufficiently low power input is used (ie, 0.2 W/ml or less). High-power sonication may significantly increase fiber number.

Before TEM analysis the Nuclepore membranes are cut and transferred to TEM grids; thereafter the membrane is removed by dissolution in chloroform. Fiber losses may occur during these steps. These losses are indicated by the lower counts obtained for the TEM analysis at a magnification of 5,000 $\times$ when it is compared to the SEM analysis at 4,500 $\times$. If the TEM magnification is increased to 20,000 $\times$, the loss is nearly compensated for. At the same time the effects of increased magnification on the fiber number is demonstrated.

The different preparation methods may affect asbestos bodies differently than naked fibers. A study dealing with these problems is at present in progress in our laboratory.

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