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Assessment of Inorganic Fibre Burden in Biological Samples by Scanning Electron Microscopy – Energy Dispersive Spectroscopy

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Abstract. A protocol to detect inorganic fibres in samples of biological tissues by SEM–EDS is proposed. The sample (500 mg in the case of lung tissue) is digested by NaClO, filtered using a sample holder and fixed onto a SEM stub by clarification. A total of 800 microscopic fields (MF) at 2000× are scanned along 5 parallel strips of the filter preparation at regular intervals for a total area of 1.85 mm^2, representing 0.7% of the total accessible area. In order to test the method and to show that the investigation of animals (sentinel animals) instead of human tissues can provide information on the lung burden of inorganic fibres, the data obtained from a control group I (animals which lived in an environment free of fibre-bearing rocks) consisting of 12 cattle and a test group II (animals which lived in alpine valleys with serpentine outcrops) consisting of 6 cattle and 6 wild animals are compared. As expected, group I shows by far a lesser burden than group II. The proposed SEM–EDS method is a first attempt to standardize SEM–EDS investigations of inorganic particles in biological tissues and is shown to provide results able to significantly discriminate the lung burden between populations even when subjected to non natural environmental exposure alone.

Key words: Burden of inorganic fibres; inorganic fibres in biological samples; protocol for SEM–EDS investigation; sentinel animals.

The hazard for human health caused by occupational exposure to airborne inorganic particles, such as asbestos, quartz, cristobalite and some sheet silicates, is definitively established [1]. In the case of asbestos, fibres breathed in some amounts and over a long period of time can provoke respiratory system diseases such as asbestosis, mesothelioma and lung cancer (this last one also in connection with cigarette smoke), as pointed out in several studies on occupational and para-occupational exposures [2]. The degree of pathological interaction of the asbestos fibres with other apparatuses, like those of the gastrointestinal and kidney systems, is also being investigated [3]. In fact there are no doubts that organs and apparatuses other than the respiratory system can be reached by fibres and particles [4].

The asbestos-correlated pathologies will appear along many decades also owing to their long medical latent period. Note that in many countries the mining and the utilization of asbestos is still allowed (e.g., Canada,

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China and Russia [5]) and big quantities of products containing asbestos are used in the world, fated to release fibres in time causing pollution in the environment. Fibres dispersion, moreover, happens also where regulations of asbestos ban exist and reclamations works are imposed.

Other fibrous minerals have shown pathological effects, e.g. erionite, a fibrous zeolite, abundant in some friable recent volcanic ash deposits (tuffs) and soils of central Turkey, used to made local whitewash and plaster [6]. Recently health effects are also attributed to breathing of some synthetic fibres and investigations are in progress [7].

The threshold, if any [8], of any breathed inorganic species (asbestos fibres, metallic particles and so on) and the duration of exposure necessary to trigger the pathological process is not yet clear. In the last years, also the environmental exposure (i.e., low dose exposure) to several other kinds of inorganic particles seems to be dangerous for the respiratory apparatus and therefore some investigations have been undertaken [9]. At present, the question whether the environmental exposure to airborne inorganic particles causes carcinogenic mechanisms is still open [6]. For instance, it seems that also living near asbestos outcrops may provoke pathological effects, but investigations are still in progress [9].

Therefore, it is important to investigate biological samples (tissues and fluids) in order to: i) establish which inorganic fibrous dusts of environmental background are breathable and persistent in the organism; ii) determine the environmental exposure to harmful inorganic fibres and to get epidemiological data.

Under appropriate conditions, the use of animals can help to reveal the presence of unknown chemical contaminants in the environment or help identify kind and amounts of exposure to contaminants [10]. Investigations about environmental exposure can be carried out on animal populations, ''animal sentinel systems'', living in different environments in order to evaluate the possible health effects. De Nardo [11] has presented a case of mesothelioma diagnosed in a dog and certainly correlated to asbestos domestic exposure. The animals are not subject to professional exposure and their tissues are easier to obtain than human ones. For all these reasons, it seems a good choice to investigate animal tissues, when appropriate.

As concerns the identification and quantification of asbestos fibres in airborne and bulk samples, four techniques (PCM, XRPD, FTIR, SEM–EDS: for acronyms see Appendix 1) are regulated by laws in many countries. About the asbestos fibres in biological materials, there are many researches related to human lungs and few concerning pleura, liver, spleen, kidney, urine and placenta. Unfortunately, for biological samples a standard approach to the sample preparation and examination and for particles identification and quantification does not exist. This leads to a great discrepancy among different laboratories worldwide and it would be useful to standardise an universally accepted technique.

Even if not normally required by regulations in many countries, univocal identification of mineral and crystalline synthetic fibrous species present in any kind of sample can be accomplished only by TEM, using the technique of SAED combined with AEM [12]. With such technique, in fact, it is possible to examine very small particles (e.g. fibrils) and intergrown fibrous phases having sub-micrometer width [13].

To overcome the above mentioned difficulties in comparing results obtained on biological samples in different laboratories, in this work, we propose a standard procedure based on SEM–EDS to analyse inorganic particles (not only fibres) present in biological samples.

According to our experience, the proposed protocol is rather efficient and potentially alternative to the TEM–EDS method for the analysis of particles incorporated in biological materials. Worth noting is that, whereas TEM–EDS instrument is onerous in terms of acquisition, maintenance and personnel training, the SEM–EDS has a better cost/benefit ratio; besides, this instrument is more common and adaptable to routine analyses on more easily prepared samples. A drawback of SEM compared with TEM could be that in the latter case smaller fibres are detectable: with our SEM instrument (SEM/S-360 Cambridge equipped with an EDS Link-Oxford Pentafet ATW2, Si(Li) detector) the minimum dimensions are 0.3 μm. To note, however, that with a 120 kV TEM the same minimum dimension limit is practically valid if chemical information by EDS is necessary.

However, more than absolute values of the burden, often relative burdens alone are required in order to compare, e.g., either environmental with professional cases or the presence of fibres in different environments. Besides, it must be noted that both in epidemiological and clinical studies, it is necessary to examine a large number of samples in a relatively brief period of time; at the same time, the larger amount of sample which can be examined by SEM,

in comparison with TEM, allows a better statistical evaluation. For instance, in the case of biological tissue, normally 500 mg of material is used to prepare the sample to be observed by SEM–EDS, whereas by TEM–EDS the examined sample represents only about 1.5% of the one used in the SEM.

Experimental

As an example of application of the protocol illustrated below, the results obtained from the investigation of 24 lung samples (18 from cows; 6 from wild animals) are here presented.

The selected samples are representative of different geological areas of the Piedmont Region (North-Western Italy). The Lanzo Valley and the Varaita Valley are areas with outcropping rocks bearing tremolite and chrysotile asbestos (serpentinites) [14]; the Asti area is geologically free of asbestos and has been chosen as the control case.

Sample Preparation

The sample preparation essentially consists of three steps: i) digestion of the biological material; ii) filtration of the suspension through a membrane; iii) filter preparation for SEM–EDS analysis. The method, tested for different kinds of biological materials, is the same for all types of sample, but the digested quantity depends on the type of material. For instance, a minimum quantity of 10 ml is needed for urine and bronchoalveolar lavage fluid (and for blood: the test for this material is in progress); for solid tissues (such as lung, bladder, kidney, heart, liver, placenta) 500 mg is a suitable quantity [15].

The whole procedure is detailed in the following steps.

Digestion

A chemical digestion (instead of ashing) is used in order to disregard organic materials [16]. This procedure allows a more "direct method" in comparison to the ashing procedure in which the sample is manipulated via extraction, centrifugation, etc. This is important for instance in presence of ferruginous bodies as well as chrysotile fibres since they are susceptible to damage by manipulation.

A mass of 500 mg of tissue, previously preserved in formalin to 10% (Siac S.r.l., Italy) is digested in 30 ml of NaClO (Merck, Germany), in order to produce a suspension (the NaClO quantity has been optimized after different tests). The necessary time to complete the digestion strongly depends on the freshness of the sample. In the reported examples, the tissues were preserved in formalin from less than six months. They have been digested in NaClO for seventy-two hours at 60 °C in order to accelerate the chemical reaction. A mass of 5 g of the tissue is dehydrated in order to measure its dry weight, a quantity which is used to evaluate the concentration of fibres expressed in number of fibres per gram of dry lung tissue.

Filtering

The obtained suspension is filtered on mixed cellulose esters membrane (Millipore, Italy) with a diameter of 25 mm (the same of the SEM pin stubs used), and with a pore size of 0.45 µm. A core surface of 19 mm in diameter of the filtering surface for a total of 284 mm^2 (hereafter called "exposed area") is accessible to the microscopic observation.

Washing

During the filtering step, it is necessary to wash the membrane thoroughly with warm distilled water to accelerate the dissolution of the micrometric crystals of NaCl, grown during the chemical digestion. In fact, the NaCl precipitated on the membrane can both hide the inorganic particles and be included in the analyzed volume disturbing the chemical analyses.

Dehydration of the Filter

The filter is dried at about 50 °C for at least 12 hours. This temperature is enough to dry the membrane without burning it and without provoking chemical-physical alteration of inorganic particles.

Clarification

Clarification by acetone method (Merck Eurolab, France) glues the membrane to the SEM aluminium pin stub. Moreover, this method avoids two disadvantages in comparison with the most common use of double-side adhesive tape [17]: a) the combustion of the membrane during the carbon sputter coating; b) the displacement on the filter of inorganic particles that are fixed by the formed gel.

Sample Conductivity

Before the SEM–EDS study, the sample is made conductive by carbon sputter coating. If an E-SEM is used, this step is not necessary.

Observation by SEM

To save both time and costs, the SEM observation is performed on a number of selected microscopic fields (MF) which is large enough to obtain a statistic sampling. Taking into account that official methods (ISO 14966, AIA-RTM 2, DM 6/9/94) in order to analyze airborne particles, suggest examining 1 mm^2 of filter at 2000 M, we observe 800 MF's to cover an area (s) of 1.85 mm^2 of the filter, i.e., 0.7% of the total area (S) of the filter. The observation is performed at 2000 M and the MF's are distributed along 5 parallel strips as follows (for details, see Appendix 2). Each strip is 16 mm long and is sampled at steps of 100 µm, thus obtaining 160 MF's per strip. The MF's along the same strip are spaced of 40 µm. The length of the steps (100 µm) and the separation of 2.5 mm between two adjacent strips are such that overlapping between different MF's cannot occur, being the dimension of each MF 40.1×57.8 µm.

Identification of Inorganic Fibres by SEM–EDS

The filter observation is carried out using backscattered electrons in order to detect only the inorganic particles and to disregard the organic ones. According to fibre definition, only particles with length-to-width ratio ≥ 3 are considered and then chemically analyzed by EDS (Figs. 1, 2). The fibres falling across the border of a MF contribute to 100% to the counting because the MF's are not contiguous. Chemical analyses are only qualitative because the nature of the sample does not allow the preparation required to obtain quantitative analyses.

The revealed chemical elements, the relative peak intensities and the morphology of the particles are normally sufficient features for identification. Comparison with spectra of an EDS/SEM database obtained either from samples very well characterized by other techniques (TEM, SAED, TEM–EDS, XRPD, micro-Raman) or simulated by DTSA [18] program reveals very useful.

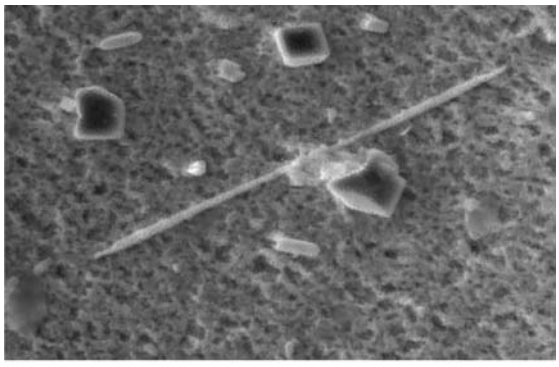

Fig. 1. Secondary electron SEM image of a tremolite fibre detected in sample

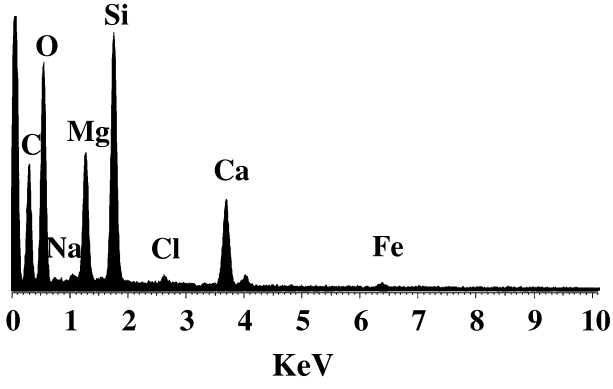

Fig. 2. EDS–SEM elemental spectrum of a tremolite fibre

Quantification of Inorganic Fibres

The number of observed fibres for any species is then standardised to the number of fibres per gram of dry weight lung tissue [fg(LTdw)$^{-1}$]. The observed number of fibres (n) is multiplied by the ratio (X) between the exposed area (S) and the observed area (s) to get the estimated number (N) of fibres deposited on the exposed area. N represents the quantity of fibrous material content in 500 mg of wet lung tissues. By knowing the dry weight (dw) of 5 g of wet tissue (see above), the fg(LTdw)$^{-1}$, indicated as Y, is given by:

$$Y = \frac{n \times X \times 10}{\text{dw}} = \frac{N \times 10}{\text{dw}}$$

10 is the ratio between the mass used to determine the dry weight (5.0 g) and the mass digested (500 mg).

Results and Discussion

Table 1 shows the inorganic fibres that have been identified in the observed samples; also some synthetic fibres (TiO_2 and aluminium silicates) have been found but they are here disregarded. On the whole, fourteen different mineral fibrous species have been identified. Both the number and the variety of fibres in lungs of the control group from the Asti area (group I) is lower than in the group II of animals that lived in alpine areas close to outcrops of serpentinite. In particular, the presence of chrysotile and tremolite asbestos in lungs of group II animals is clearly correlated with the mineralogical content of outcropped rocks in the Lanzo and Varaita valleys [14].

Table 1. Content of inorganic fibrous species in the investigated animal lungs

Animals	Groups	
	I	*II*
Cattle	12	6
Wild animals	0	6
Inorganic fibrous species	*Total fibres [fg(LTdw)$^{-1}$]*	*Total fibres [fg(LTdw)$^{-1}$]*
Silica	3704 (1)	5897 (2)
Tremolite	2857 (1)	14838 (3)
Muscovite	0	5320 (2)
Paragonite	0	3194 (1)
Pyrophyllite	0	3333 (1)
Talc	0	4000 (1)
Chlorite	8978 (3)	2703 (1)
Chrysotile	0	12482 (3)
Antigorite	0	2948 (1)
Halloysite	2667 (1)	2703 (1)
Illite	0	13405 (2)
Smectite/illite	0	7315 (2)
Smectite	0	10754 (4)
Vermiculite	0	1533 (1)
Synthetic fibres	7738 (2)	50672 (5)

Group I: animals from the control area (Asti). *Group II*: animals from test areas (Lanzo Valley and Varaita Valley).

Number of fibres detected on selected filter area is in parentheses. Asbestos minerals are in italics. Synthetic fibres group includes TiO_2 and aluminium silicates.

By using the procedure described in this paper, which involves the counting of at least one fibre over a scanned area of 1.85 mm^2, the minimum lung burdens for animals living in non-urban areas is in the range of 1533–4000 fg(LTdw)$^{-1}$ (Table 1). In the literature, no data obtained on animals are available; for a human population used as control group (i.e., not professionally exposed) a lung burden of 31000 fg(LTdw)$^{-1}$ is reported [19].

The large discrepancy of burden between the two sets of results may be due to at least three reasons: (1) the use of different methods; (2) a background value of fibres higher in a urban than in a country environment; (3) the low statistical significance of investigating samples representing very small volumes of a biological organ. Reason (1) supports the necessity of establishing standard methods of investigation of the burden in biological samples; reason (2) shows how different can be the background in environments where

non-professionally exposed populations live. The reason (3) clearly indicates that the low number of cases so far examined in our research (12 samples each for the control and the case study) puts limits to a statistical discussion of our results on an absolute basis. Also the small quantity of lung explored for each sample involves the same limits, nevertheless the quantity reported in this paper is more than that indicated in the pertinent literature [e.g. 20, 21]. Anyway, the comparison between (a) the significantly high number of animals of the Asti control group for which no fibres have been detected and (b) the constant occurrence of fibres in the samples from the test alpine animals (group II), leads to the following conclusion: the proposed method can definitely discriminate between the lower lung burden expected for a population living in an environmentally safe area (Asti) and the higher lung burden expected for a population living in an environmentally risky area where fibre-bearing rocks occur (Western Alps).

Our proposed method and the case studies presented allow one to draw the following further conclusions.

We have proposed a complete and effective technique to identify and quantify the inorganic particles by SEM–EDS in any kind of biological sample such as lung, bladder, kidney, heart, liver, urine, bronchoalveolar lavage fluid, sputum (perhaps blood).

The presented standardisation of a procedure to obtain the number of fibres per filter by SEM–EDS (Appendix 2) i) avoids the subjectivity of the operator in the choice of the MF's; ii) reduces the risk of mistaking the counting fields; iii) shortens the time necessary to scan a sample.

Because of the impossibility to obtain quantitative chemical analyses for the investigated kind of samples, it is useful to compare the EDS spectra of the detected particles with those accumulated in a database consisting of both experimental and simulated spectra of well characterized samples. We confirm that the use of SEM–EDS technique is particularly suitable to determine inorganic fibres and particles, in biological material, also in low concentration as in the case of natural environmental exposure.

We have proved that the use of a non-experimental animal model is a valid procedure (i) to investigate the type and the environmental diffusion of mineral fibres (e.g. asbestos); (ii) to obtain data useful to study their bio-persistence and the health effects of the exposure to a low fibre concentration such as that occurring in a natural environment.

The illustrated technique can be optimized by a computer-based comparison between the computerized data-base of known EDS–SEM spectra with experimental ones to identify the inorganic phases.

Besides, it would be possible to speed up the positioning of MF's using a computer-controlled sample stage, in the SEM.

Appendix 1

AEM	Analytical Electron Microscopy
AIA-RTM	Asbestos International Association, Recommended Technical Method
DM	Decreto Ministeriale
DTSA	Desktop Spectrum Analyzer
EDS	Energy Dispersive Spectroscopy
E-SEM	Environmental SEM
FTIR	Absorption Infrared Spectroscopy with Fourier Transform
ISO	International Organization for Standardization
M	Magnification
MF	Microscopic Field
PCM	Phase-Contrast Microscopy
SAED	Selected Area Electron Diffraction
SEM	Scanning Electron Microscopy
TEM	Transmission Electron Microscopy
U.S.G.S.	United State Geological Surveys
XRPD	X-Ray Powder Diffractometry

Appendix 2

Procedure Used to Obtain the Number of Fibres Per Filter

- The filter has an observable diameter of 19 mm with an area $S = 284\,mm^2$; its border is marked by a reference mark in order to reposition the sample during different runs.
- The coordinates X_0 and Y_0 (Fig. 3) of the centre of the filter are determined.
- The observations are carried out along five strips which are parallel to the X axis and are located at Y_0, Y_1, Y_2, Y_3, and Y_4; the separation between two contiguous strips is 2.5 mm.

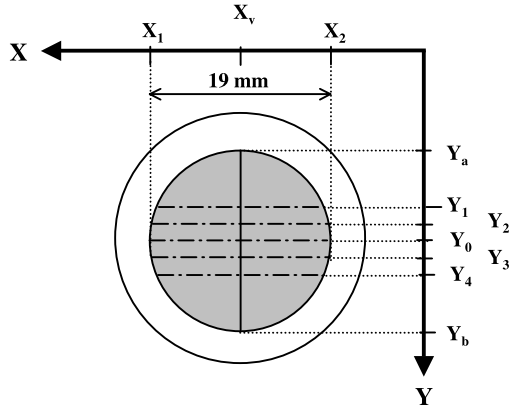

Fig. 3. Position of the scanned strips on the observed filter

- The strips are 16 mm long and their extremities (X_1 and X_2) are symmetric with respect to the vertical diameter containing the centre at X_0, Y_0.
- Along each strip, 160 MF's at steps of 100 μm are examined to search fibres. Note that the step is longer than the base of MF (57.8 μm) to avoid overlapping of MF's.
- The total of measured MF's is $160 \times 5 = 800$; the total of observed area s is $800 \times (40.1 \times 57.8\,\mu\text{m}) = 1.85\,\text{mm}^2$. This area correspond to 0.7% of the total area S of the filter.

The procedure described in this paper has been positively evaluated from the Laboratorio di Ultrastrutture – Istituto Superiore di Sanità – National Health Institute – (a reference agency in Italy) within the quality system control of the laboratories working on asbestos analyses by SEM–EDS.

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