Method Article

Dimensional distribution control of elongate mineral particles for their use in biological assays

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A B S T R A C T

The aim of the present method is to reduce the dimensional variability of asbestos, elongate mineral particles, and other asbestiform minerals for use in biological assays. Here, the pristine mineral sample is filtered through two nylon meshes of different sizes to obtain a narrower dimensional distribution following a power law. Furthermore, we show that anoxic preparation, autoclaving and storage of the mineral prior to addition into biological cultures did not affect the mineral's chemical properties. This approach avoids the use of highly reactive chemicals modifying mineralogical characteristics and surface properties, which can affect to a major extent mineral toxicity as well as interactions between minerals and biological matter or biofluids. The method can be combined with additional selective approaches to further refine the dimensional range of the minerals. The advantages of this protocol over previous methods are:

- Exclusive use of distilled water and 2-propanol, thus eliminating chemicals that can modify bulk or surface properties of the studied minerals.
- Successful sterilization of the resulting mineral particles for use in biological assays without compromising mineralogical characteristics.
- Applicability of this method across various types of asbestos, elongate mineral particles and, potentially, other hazardous minerals.

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Specifications table

Subject area	Earth and Planetary Sciences (Direct submission)
More specific subject area:	Mineral processing
Method name:	Elongate mineral particle preparation
Name and reference of original method	S.J. Chipera, G.D. Guthrie Jr., D.L. Bish, Preparation and purification of mineral dusts, in Health Effects of Mineral Dusts, Rev. Mineral. 28 (1993), 235–249
Resource availability	Not applicable

Method details

Asbestos is an industrial term that defines a group of regulated, naturally-occurring silicate minerals which, when inhaled, ultimately lead to diseases such as asbestosis, lung cancer, and mesothelioma [1–4]. Understanding how asbestos minerals or elongate mineral particles interact with living organisms requires experimental control over their dimensional properties as a way for generating reproducible results [5]. Several methods are available to shape and partially control the dimensional distribution of elongate mineral particles [5], including: sieving (*e.g.*, [6]), magnetic separation (*e.g.*, [7]), single-fluid density separation [8], field-flow fractionation [9], and separation based on settling velocity (*e.g.*, [10]). Here, we present a simplified sieving method based on filtration through two different nylon mesh sizes, which aims at narrowing the width (*w* or diameter, *d*) and length (*L*) distributions of mineral particles while maintaining a nearly constant aspect ratio (*L/w*) among the two dimensions in the filtered product. This selection method can be applied to any elongate mineral particles (*e.g.*, asbestos and asbestiform fibers) because it is (i) exclusively based on the dimensionality of the subject particle, and (ii) independent of other physical properties (*e.g.*, paramagnetism, density) varying between mineral species, for which some of the above-listed methods are based upon.

Experimental setup and standard operating procedures

Before working with any powdered materials, all equipment and utensils must be cleaned using 2-propanol or acetone (both water-miscible solvents). Additionally, all personnel must wear personal protective gear (*i.e.*, laboratory coats, gloves, disposable respirators) and must work inside a chemical fume hood to prevent contamination by and exposure to asbestos or elongate mineral particles. The hood surfaces used for the described work were also protected by covering them with disposable mats/diaper pads or paper towels. Clean-up was completed by thoroughly wiping equipment, utensils and lab benches with 2-propanol, followed by multiple water rinses and a final rinse with 2-propanol.

Naturally occurring samples and initial purification step

Chrysotile asbestos was obtained from the Balangero mine in Piedmont, Italy after industrial processing/purification. Tremolite-actinolite was obtained from Passo di Caldenno, in the Province of Sondrio, Italy as a rock with a weight of approximately 200 g. Both minerals were selected for this method because their mineralogical features have been previously characterized [11,12]. The method also considered minerals of different starting purities, as described next in the initial purification step for the tremolite-actinolite sample.

The 200 g rock containing elongate mineral particles of tremolite-actinolite was first broken into smaller pieces no larger than a couple of cm. This can be done with a hammer after the rock has been wrapped in at least three layers of wet paper towels, followed by at least three layers of plastic bags. Each rock piece was either selected for further manipulation or discarded after a preliminary naked-eye identification to confirm the presence of different phases of interest and their habit. Subsequently, the mineral fragments were visually separated with a 10X lens under a stereomicroscope (Leica M165 C stereomicroscope equipped with a Leica IC80 D camera and an LED illumination system) to remove visible impurities, other accompanying phases, and/or organic material/soil particles. Complex, heterogeneous, cm-wide fragments were further broken up using an agate mortar and pestle with

2-propanol until the pieces were mm-sized. The smaller fragments were inspected again under the stereomicroscope to repeat the purification step until all the mineral fragments appeared as a single phase under the stereomicroscope. At this stage, we observed that the broken fragments consisted of mostly bladed, fibrous (possibly asbestiform) and/or prismatic crystals.

To ensure that only tremolite-actinolite particles were present, the pieces were transferred to a large beaker (600 mL) filled with distilled water and left to settle for 3 h at room temperature, as calculated after Pollastri et al. [10]. This last step aims to remove any remaining soil particles and/or other lighter mineral phases present in the sample by concentrating the target mineral in the bottom of the beaker. After 3 h, the overlaying distilled water was discarded, and the purified mineral transferred to a clean container while covered with 2-propanol.

Grinding and filtration of the mineral particles

A small portion (about 4 mm^3) of both purified mineral samples were transferred separately into an agate mortar (50 mm diameter) containing 2-propanol and ground with an agate pestle for 10 min. Occasionally, additional 2-propanol was poured into the mortar in order to prevent the purified mineral from drying completely. Wet grinding for 10 min was sufficient for making both chrysotile and tremolite-actinolite samples appear as homogenous powders. Once pulverized, the entire suspension was transferred to a clean beaker (600 mL) and the mortar rinsed with 2-propanol to collect all of the remaining mineral particles into the beaker. The grinding step was repeated until all the initially purified mineral was used up. Below, this gently ground mineral will be called "pristine" and represents our control as opposed to the "filtered" mineral (undergoing the filtration procedure documented here). The ground-mineral suspension was left overnight at room temperature for the particles to settle, after which the overlaying 2-propanol was carefully removed with a single-use pipette without disturbing the bottom mineral layer. Any remaining 2-propanol was evaporated by placing the beaker in nearly boiling water (80–90 °C). If necessary, the evaporation can be done overnight at room temperature instead.

The dried ground mineral was resuspended in 500 mL of distilled water in glass bottles, capped, and shaken to separate the particles. The suspension was then transferred to a vacuum filtration system (MilliporeSigma, see Fig. 1) [e.g. 13] and filtered twice to decrease the size fraction in terms of width (*w* or diameter, *d*) and length (*L*). We specifically used 5 μm and 20 μm nylon meshes (Spectrum Labs) because we wanted to use the filtered particles for microbiological applications. Furthermore, we used nylon mesh filters because nylon is suitable for all aqueous solutions, "washable", and eventually autoclavable.

The entire suspension was initially allowed to filter through the 5 μm mesh to collect any particles larger than 5 μm. The use of a smaller mesh size (5 μm) before the larger mesh size (20 μm) turned out to be more effective than the other way around in reducing agglomeration of and clogging by massive bundles since smaller particles act as a binding material between larger particles and bundles. To facilitate the filtration process, we attached the system to a vacuum pump and constantly stirred the mineral suspension in a 1000 mL glass Büchner funnel while under vacuum to avoid fiber/particle agglomeration and laminar flow reduction through the mesh holes, which may promote alignment of elongated particles with the mesh holes and thus selection by particle width only. Two batches of 500 mL ground-mineral suspensions (about 10 mm^3 of powdered mineral) were filtered through for a total of 1000 mL volume. The fragments smaller than 5 μm were conserved in the water suspension for subsequent waste disposal. The 5 μm mesh was then removed, placed upside-down in a clean 600 mL beaker containing 300 mL of distilled water, and subsequently sonicated in a sonic bath (60 Hz) for 1–5 s. Use of higher-power sonication and longer time should be avoided since the dimensional distribution can be modified at higher frequencies and extended time [14]. The mesh was then held with clean forceps and washed with additional distilled water to remove any remaining fibers. The clean mesh can then be reused for no more than 7 additional times as a precaution to avoid possible damages to the filter. These steps were repeated until the entire ground-mineral suspension was passed through the 5 μm mesh.

Afterwards, the entire filter flask was thoroughly cleaned initially by wiping with 2-propanol soaked paper-towels, then by wiping and rinsing with distilled water before placing the 20 μm mesh

Fig. 1. Vacuum-filtration flask used for particle preparation. This system included a 1 L glass Büchner flask (bottom), a 90 mm diameter stainless-steel filter support housed in a glass base, and a 1 L glass Büchner funnel (top).

on the filtration system. Distilled water was first filtered while placing a finger over the hose barb (where the vacuum pump would be connected during vacuum filtration) to prevent the water from just passing through the mesh due to the larger pore size. The previously obtained suspension of >5 μm fibers in the 600 mL beaker was poured into the water-containing Büchner funnel and the final volume was brought up to 1000 mL, using the extra volume to wash the 600 mL beaker. At this point, the finger slowly uncovered the hose barb, allowing the mineral suspension to filter through very quickly (though the process can be facilitated with a vacuum pump, if necessary). The suspension should be constantly stirred as it passes through the 20 μm mesh. Once this step is completed, the particles of interest are contained within the filtrate, whereas the mesh should hold all particles larger than 20 μm. The particles on the mesh can be saved, re-ground, and re-filtered. The "5–20" μm particle suspension in the filter flask was transferred to a large beaker and placed on a hot plate under low heat to slowly evaporate the distilled water. The beaker was removed when the water level reached approximately 50–100 mL and left to evaporate in a fume hood at room temperature. Subsequently, the beaker containing the "5–20" μm dry powder was kept covered with parafilm^{TM}. The dry powder can be resuspended in water or 2-propanol to prepare SEM stubs or TEM grids for further analyses.

Sterilization and preparation/storage under anoxic conditions

For use in biological assays, the filtered "5–20" μm particles were sterilized via autoclave in order to rule out potential contamination in downstream experiments. Dried particles were first carefully placed in glass containers and closed shut to prevent airborne particles from escaping (*i.e.*, Balch tubes sealed with butyl rubber stoppers and crimped with aluminum seals). Preparation of mineral particles under anoxic conditions included flushing sealed tubes with O_2-free gas (*e.g.*, N_2) using two

22-gauge (or above) needles, one as an inlet needle inserted vertically and the other as the outlet needle pointing towards the wall of the glass tube, for a few minutes at a low gas-flow rate in order to minimize airborne particles from escaping through the needles. The mineral samples in the sealed tubes were then autoclaved at 121 °C and 110 kPa for 20 min using a dry cycle. Afterwards, these sealed particles can be suspended in any pre-sterilized solution (*e.g.*, water or culture media under either oxic or anoxic conditions) and subsequently added to biological assays using a 16-gauge needle and syringe. For procedures requiring the handling of open tubes, we recommend using a disposable glove bag for ensuring airborne particle containment.

Measurements, data treatment, and elaboration

Samples of both chrysotile and tremolite-actinolite before (pristine) and after (filtered) the protocol were transferred onto 12.7 mm metal stubs with conductive carbon tabs for analysis by Scanning Electron Microscopy (SEM). Specifically, the mineral-particle suspension was initially vigorously shaken, and four droplets were deposited directly on the carbon tape avoiding contact between the pipette tip and carbon tape. The initial shaking and the fast evaporation of the 2-propanol in the fume hood helped in avoiding particle agglomeration. A FEI Quanta 600 FEG Mark II Field SEM was set to environmental mode with a voltage of 30 kV and a chamber pressure of 0.38 torr to image samples. Images were obtained in Secondary Electron (SE) mode at a magnification of 10^2X and 10^3X for each. Since changing the magnification changes the particle distribution range and shape [12], we decided to test the effectiveness of our method at two different scales. For each magnification (10^2X and 10^3X), 25 particles in each of 6 fields of view were counted, yielding a total of 150 particles for each mineral sample and magnification. Additionally, regulations on asbestos and other elongated mineral particles vary between countries, so it is useful to provide data over multiple magnification ranges [see 12].

The SEM images were used to measure diameter (*d*) for chrysotile, and both length (*L*) and width (*w*) for tremolite-actinolite by applying the software *ImageJ* [15]. For the tremolite-actinolite samples, the aspect ratio (*L/w*) was also calculated. The chrysotile fiber curvature and uninterrupted bundles did not allow us to obtain clear results in terms of length, and therefore, we decided to avoid the use of this potentially biased data set. Furthermore, the transfer to the carbon tape on the SEM stubs allows for the sample to dry, thus generating further fiber agglomeration and leading to an artefact fiber/bundle length. In case the investigator is solely interested in the dimensional transformation of samples and not the chemistry, it would be useful to add a dispersion agent before collecting the powder suspension on a mixed cellulose ester filter to be attached to the SEM stub.

Since we expected right-skewed distributions for our data, we first normalized it using the natural logarithm to obtain a bell-shaped Gaussian distribution. A double Grubb's test was subsequently applied to the normalized data distributions to remove outliers at both of its extremes, and the detected outliers were then removed from the original dataset. Treated data were distributed into a number of bins that were calculated using Scott's method [16]. The number of bins depended on the data parameter (*bins_n*) according to:

$$bins_n = \frac{max - min}{3.5 \cdot \frac{St.\ Dev.}{3\sqrt{n}}},$$

where *max* and *min* are the highest and lowest values in the data, respectively, and *n* is the total number of values. Next, data discretization was applied by using a K-means algorithm within Addinsoft [17] for 10 repetitions, 500 iterations, and a convergence of 1×10^{-5}, to generate centroids for each bin. The obtained discretized distribution was fitted using a power law. The data treatment was performed separately for each parameter (*d, w, L,* and *L/w*) and mineral.

Method validation

The distribution calculated for the filtered mineral-particle populations showed a good rearrangement to generally lower values of *L, w,* and *d,* and a steeper curve than that of the pristine, unfiltered samples (Figs. 2A–D and 3). Most of the particle lengths from filtered samples are lower than 20 μm, as expected. In the tremolite-actinolite samples, the rearranged aspect ratio (*L/w*) was
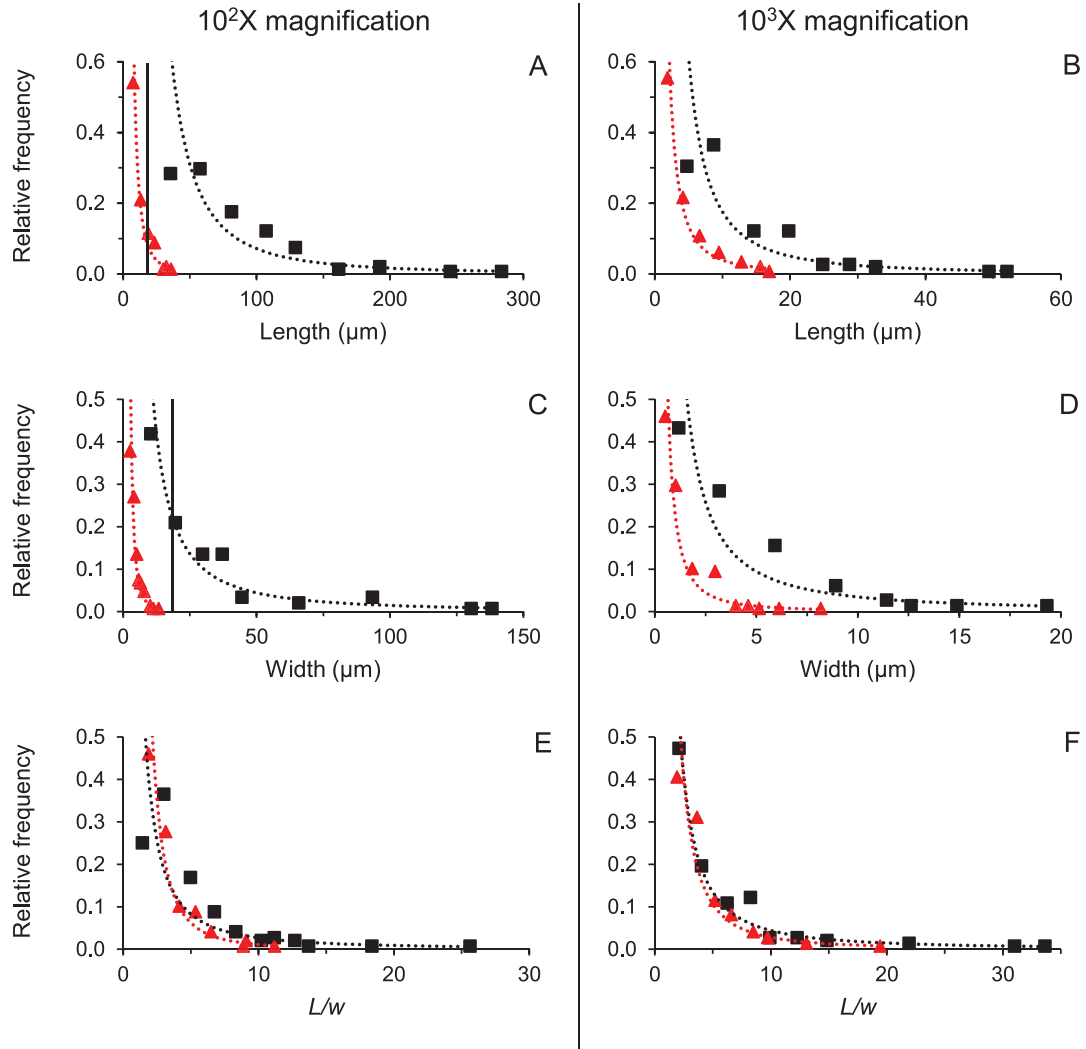
Fig. 2. Dimensional distribution of length (*L*, in A and B), width (*w*, in C and D), and aspect ratio (*L/w*, in E and F) for filtered tremolite-actinolite particles (red triangles) versus pristine samples (black squares) at 10^2X and 10^3X. The continuous vertical black line in A and C represents the 20 μm mesh upper limit.
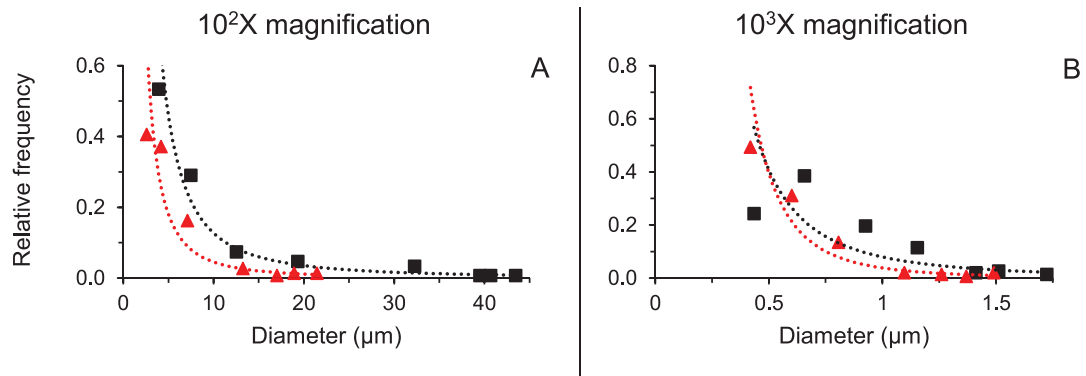
Fig. 3. Dimensional distribution, using the diameter (d), for filtered chrysotile elongate particles (red triangles) versus pristine samples (black squares) at (A) 10^2X, and (B) 10^3X.

Table 1

Statistical parameters for tremolite-actinolite samples.

	Length 10^2X			Length 10^3X		
	Pristine (µm)	Filtered (µm)	% Difference	Pristine (µm)	Filtered (µm)	% Difference
Mean	73.66	12.48	−83.06	11.58	4.09	−64.68
Median	61.56	9.99	−83.77	8.52	2.67	−68.66
Highest frequency centroid	57.54	7.44	−87.07	8.66	1.87	−78.41
Standard deviation	42.51	7.12		8.45	3.46	
Relative error	0.58	0.57		0.73	0.85	
Max.	283.55	36.23		52.03	16.93	
Min.	19.48	3.75		2.37	0.67	
	Width 10^2X			Width 10^3X		
	Pristine (µm)	Filtered (µm)	% Difference	Pristine (µm)	Filtered (µm)	% Difference
Mean	25.24	4.24	−83.20	3.81	1.25	−67.19
Median	17.75	3.80	−78.59	2.80	0.84	−70.00
Highest frequency centroid	10.31	2.53	−75.46	1.18	0.51	−56.78
Standard deviation	22.36	1.95		3.65	1.21	
Relative error	0.89	0.46		0.96	0.96	
Max.	138.21	13.25		19.82	8.17	
Min.	3.33	1.25		0.27	0.24	
	L/w 10^2X			*L/w* 10^3X		
	Pristine (µm)	Filtered (µm)	% Difference	Pristine (µm)	Filtered (µm)	% Difference
Mean	4.37	3.19	−27.00	5.08	3.95	−22.24
Median	3.18	2.75	−13.52	3.47	3.28	−5.48
Highest frequency centroid	2.99	1.86	−37.79	2.09	1.92	−8.13
Standard deviation	3.55	1.80		4.91	2.68	
Relative error	0.81	0.56		0.97	0.68	
Max.	25.62	11.18		33.63	19.41	
Min.	0.78	0.92		0.83	1.05	

Table 2

Statistical parameters for the chrysotile samples.

	Diameter 10^2X			Diameter 10^3X		
	Pristine (µm)	Filtered (µm)	% Difference	Pristine (µm)	Filtered (µm)	% Difference
Mean	8.04	4.77	−40.67	0.76	0.58	−23.68
Median	5.51	3.73	−32.31	0.68	0.52	−23.53
Highest frequency centroid	3.92	2.59	−33.93	0.66	0.42	−36.36
Standard deviation	7.78	3.59		0.31	0.24	
Relative error	0.97	0.75		0.40	0.41	
Max.	43.45	21.88		1.75	1.50	
Min.	1.86	1.18		0.25	0.25	

similar before and after the filtering procedure (Fig. 2E and F). These observations are valid in spite of magnification differences. Our data reveal a successful dimensional restriction of the mineral particles (Fig. 2 and 3) and, in the case of the tremolite-actinolite sample, a conservation, or only small modification, of the original aspect ratios (Fig. 2).

Our statistical parameters (Tables 1 and 2) show a consistent reduction of the particle dimensions and variability, confirming what is displayed in Figs. 2 and 3.

According to the World Health Organization (WHO) [18], fibers are defined as those particles with $L \geq 5$ µm, w (or d) ≤ 3 µm, and an aspect ratio (L/w or L/d) ≥ 3. We have therefore also used our method to determine how it affects the number of fibers, as defined by the World Health Organization (WHO) [18], that are present before and after the proposed sample preparation (see raw dataset in Supplementary Materials). In the tremolite-actinolite particle population, the percentage of

WHO fibers observed at 10^2X was 0.67% and 19.33% before and after the application of the sample preparation protocol, respectively. At a magnification of 10^3X, the number of WHO fibers in the same sample was 35.33% and 16.00% before and after the treatment, respectively. In the case of chrysotile, the percentage of WHO fibers at 10^2X was 12.00% before the treatment and 28.67% after the treatment. At a magnification of 10^3X, however, all the fibers can be considered WHO fibers both before and after the treatment. These considerations were made just taking into account the chrysotile diameter for the reasons previously explained, but from a qualitative point of view, we can cautiously assume that the chrysotile count would not change due to the short length or small aspect ratio of the observed fibers and bundles. The disparities among the number of fibers observed at different magnifications are due to the fact that the "WHO fiber" is not a definition that applies coherently at all scales. The different microscope resolution capabilities and counting rules do not produce results that are directly comparable to each other. Furthermore, the data collection at different magnifications commonly generates different dimensional distributions [19], which do not necessarily reflect the real (overall) dimensional distribution of the sample.

The method described here has several advantages over previous methods. First, this method replaces the use of heavy liquids with distilled water, avoiding mineral-surface modifications and preparing selected fibers for direct transfer into biological experiments [20]. Second, it avoids milling and ultrasonic treatment [21], which can induce surface modification [22] and partial collapse of the crystal structure, respectively, thus maintaining mineralogical characteristics. Third, the use of a large Büchner funnel during filtration helps avoid particle clogging and agglomeration through vigorous stirring, which is not applicable during commonly used sieving/filtration methods. Fourth, our approach successfully adjusts the distribution of the lengths, widths (or diameters), and aspect ratios of the filtered particles compared to the pristine particles. Whereas gravimetric methods could be used in combination with the presented approach, on their own they may fail to correctly separate particles by dimensional category since masses can be the same for a short, wide particle and a long, thin fiber. Lastly, the obtained power-law distribution is mathematically easy to describe and can be used to document the rearrangement of the dimensional distribution of elongated particles after comminution or other mechanical stressors (e.g., [23]).

Conflict of Interest

The authors certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.mex.2020.100937.

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