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Textiles and textile products — Microplastics from textile sources —

Part 2:
Qualitative and quantitative analysis of microplastics

Textiles et produits textiles — Microplastiques d'origines textiles —

Partie 2: Analyse qualitative et quantitative des microplastiques

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Foreword

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This document was prepared by Technical Committee ISO/TC 38, *Textiles*.

A list of all parts in the ISO 4484 series can be found on the ISO website.

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Introduction

There is significant evidence that the textile sector releases microplastics (MPs) into the environment.

These particles, when present in the environment, can affect the biota, and so, their number, shape and size can be relevant parameters for the assessment of their potential impact and, consequently, the development of a counting technique can be a helpful approach.

Moreover, many of the microparticles analysed are not of synthetic origin and therefore it is necessary to identify and distinguish them from microplastics (MPs).

This document is designed to provide the nature, numerical concentration, surface area and (estimated) mass of the microplastics produced or released by the textile sector and collected in a solid, aqueous or aeriform matrices.

Depending on the matrice, pre-treatment of the sample is necessary to concentrate the microplastics and eliminate inorganic and organic (for example biological) components that can interfere with their identification. This document involves a preliminary observation of the sample by an optical microscope (OM) and then identification of the microplastics (MPs) by molecular spectroscopy. This document provides the possibility of using two different techniques of molecular spectroscopy, Micro-FTIR and Micro-Raman to identify and count plastic particles down to submicron size.

This document is designed to allow the re-evaluation of microplastic counting data when toxicological and environmental impact indications become available.

This document describes the method of analysis for a single filter. However, errors in the qualitative and quantitative determination of microplastics that can result from the variability between different filters imply that replicates should be performed to establish precision.

This document provides useful information (e.g. dimensional classes, shape, composition, etc.) that can be taken into account for a possible eco-toxicological assessment of health and environmental impacts. It is well known that some microplastics (MPs) are lipophile and can be vehicles for toxic compounds (e.g. PCBs, PAHs, dioxins) or vehicles of pathogenic microorganisms adhered to their surface and can be assimilated (with their dose of toxicity) and permeate into organisms and cells.

The sources of microplastics are numerous. Their shapes and sizes are also variable. In the case of those released by textiles, the typical (but not the only) morphology is fibrous and their diameter and length can vary depending on the construction parameters of yarns and fabrics or cleaning conditions.

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Textiles and textile products — Microplastics from textile sources —

Part 2:
Qualitative and quantitative analysis of microplastics

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1 Scope

This document establishes a qualitative-quantitative analytical evaluation (i.e. determination) of microplastics to be able to define their:

- particle number;
- morphology (morphological characteristics);
- dimensional distribution;
- the type, chemical origin or nature of polymers and their colour, if present.

This document is applicable to the determination of microplastics (from the textile sector) collected in various matrices (for example textile process wastewater, clothes washing water, textile process air emissions, textile process solid waste).

This document specifies expression of results in terms of estimated surface area and mass of microplastics (MPs) per unit sample. It enables the expression of the results of the quantification of microplastics (MPs) from various sources, including samples related to the production, processing, treatment and use of textiles (raw material, manufacturing process, sample like wastewater from washing clothes, air, and industrial process water).

This document applies to textile sector samples of matrices of different physical states (solid, liquid or aeriform), for example:

- solid samples from textile production processes;
- water samples from the textile production process and/or from the washing of clothing (e.g. garments or other textiles, ISO 4484-1 or ISO 4484-3 can be applied in order to prepare a liquid to be tested);
- air samples to test the air quality in the workplace of textile companies.

This document, being able to provide information such as size, shape, surface and mass (estimated), enables the transfer of useful information for ecotoxicological assessments to specialists.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 1833-4, *Textiles — Quantitative chemical analysis — Part 4: Mixtures of certain protein fibres with certain other fibres (method using hypochlorite)*

ISO 3696, *Water for analytical laboratory use — Specification and test methods*

EN 481, *Workplace atmospheres — Size fraction definitions for measurement of airborne particles*

EN 13284-1, *Stationary source emission — Determination of low range mass concentration of dust- Part 1: Manual gravimetric method*

EN 13284-2, *Stationary source emissions — Determination of low range mass concentration of dust — Part 2: Quality assurance of automated measuring systems*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminology databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at https://www.electropedia.org/

3.1
microplastic
MP
material consisting of a solid polymer containing particles, to which additives or other substances may have been added, and where a weight fraction of ≥1% particles have:

a) all sizes 100 nm ≤ × ≤ 5 mm,

b) for fibres, a length of 300 nm ≤ × ≤ 15 mm and a length/diameter ratio >3

Note 1 to entry: Polymers that occur in nature that have not been chemically modified (other than by hydrolysis) are excluded, as are polymers that are (bio) degradable.

[SOURCE: ECHA, ANNEX XV Restriction Report - Microplastics, 22 August 2019, par 1.2.2.1, modified on lower size recommended dimensions, by Commission Recommendation C/2022/3689 of 10 June 2022 on the definition of nanomaterial (OJ C 229, 14.6.2022, p. 1), modified — "≥1% w/w" was changed to "a weight fraction of ≥1 %"; additional information has been given as a note to entry.]

3.2
significant sample volume
amount of filtered volume to be analysed considering the source of the sample and the values of: total suspended solid (TSS) and chemical oxygen demand (COD)

Note 1 to entry: See Table 1 and Table 2.

3.3
subsample
aliquot (fraction) of the primary sample diluted (as known) with water

3.4
washing solution
solution used to wash equipment to recover any MP which can be left on the equipment

3.5
image analysis
identification and classification of particles/fibres according to their morphology (shape) and size, providing additional sample information.

Note 1 to entry: The sample information are, for example, distribution percentage, number and size of microparticles and microparticles with fibre shape.

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3.6

molecular micro-spectroscopy

analysis (FTIR or Raman) coupled to optical microscope (OM)

Note 1 to entry: The equipment is used to identify the polymer that composes the particle and to classify the particles recognized by image analysis, providing information such as distribution, percentage, number and size of MPs identified.

4 Principle

In order to be able to determine the MPs in a matrice, it is first necessary to transfer a significant portion of them to a suitable filter (6.3) to allow subsequent microscopic analysis with regards to quantity (molecular spectroscopy).

Different approaches shall be followed depending on the physical state of the starting matrice:

— powdery solid, or a mixture of solid materials;

— aqueous (liquid) suspension;

— aeriform.

In the case of solid powdery samples, the preliminary dispersion of a representative aliquot of the samples is carried out in a known volume of water (5.1), or in a dispersing solution consisting of a non-ionic surfactant (5.4.2) in filtered water (5.1). If the sample is solid (non-powdery) an appropriate disintegration treatment (e.g. ultrasonic treatment) shall be carried out.

For the analysis of liquid suspensions, the sample shall be filtered through a filter (6.3) of suitable material, pore size and shape (depending on the spectroscopic technique used). Analysis of the material on the filter (6.3) according to the following description:

a) identification of the composition of microparticles present by Micro-FTIR (6.1) /Micro-Raman (6.2) spectroscopy in order to identify any plastic microparticles and related measurement of their dimensions by image analysis;

b) counting of the number and identification of the size class of particles and fibres observed, and possible calculation of the total masses of MP present on the filter (6.3) in accordance with Formula (6).

Aeriform samples shall be considered in accordance with EN 13284-1, and for dust collection in the air (air emissions, air working environment) shall be considered in accordance with EN 13284-2.

All MP assessments, regardless of the different matrices analysed, shall be compared. The data of the produced waste, the discharged water, the air of the workplace or the emissions into the atmosphere can be analysed and compared to obtain a balance of the MPs of:

— a specific textile production process;

— textile products during their life cycle as garments;

— any other textile processes/semi-finished/finished products.

According to the analytical method used, the different sampling, preparation and purification procedures shall be considered. They shall be chosen according to the characteristics of the sample to be tested.

The analytical method shall be adequately applicable to all samples prepared in advance.

Preliminary analysis should be performed in accordance with the different sample preparation and purification procedures and the analytical techniques subsequently applied.

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In particular, in the case of textile sector source samples with potential presence of salts and organic substances, investigative analyses shall first be carried out, for example in the case of liquid samples of aqueous matrices:

- determination of conductivity;
- determination of COD;
- determination of TSS, with membrane filtration technique;
- pre-screening with OM for estimating microscopic image quality vision.

5 Reagents

All reagents [analytical reagent grade (AR)] or their solutions and the demineralised water (5.1), shall be filtered through filters (6.3) with a pore size of at least 0,45 µm.

5.1 **Demineralised water**, Grade 3 quality as specified in ISO 3696.

5.2 **Hydrogen peroxide**, with a volume fraction of 15 % in demineralised water (5.1).

For the oxidation of organic matter prior to filtration, hydrogen peroxide AR may be used.

5.3 **Sodium hypochlorite (NaClO)**, 1 M or 1 mol/l.

Freshly prepared sodium hypochlorite solution containing (35 ± 2) g/l active chlorine (±1) mol/l, in accordance with ISO 1833-4 is used for the dissolution of wool fibre during standards preparation.

5.4 Washing solutions

5.4.1 **Sodium chloride**, with a mass per volume of 1 %.

Dilute 10 g of pure NaCl AR in 1 000 ml with demineralised water (5.1)

The solution shall be prepared by diluting pure NaCl AR salt in demineralised water (5.1).

A sample of this solution shall be analysed to determine the MPs content of the salt to be taken into account in the results of the analyses.

5.4.2 **Non-ionic surfactant,** with a mass per volume of 1 %.

Dilute 10 g of pure non-ionic surfactant in 1 000 ml of demineralised water (5.1).

The solution shall be prepared by diluting the surfactant, preferably, not ionic, for example Triton X (whose composition and/or IR/Raman spectrum shall be known in order to subtract it during analysis), in demineralised water (5.1).

A sample of this solution shall also be analysed in order to determine the MP content to be taken into account in the results of the analyses.

5.5 Ethanol solution

Ethanol with a volume fraction of 95 % mixed 1:1 with demineralised water (5.1)

It is possible to use the ethanol solution previously filtered with a filter (6.3) made of mixed esters of cellulose or cellulose nitrate.

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5.6 Acetic acid solution 0,1 mol/l, with a volume fraction of 60 %.

Remove salt or organic material by dosing a solution obtained by diluting 5,7 ml of acetic acid (a volume fraction of 60 %) in 1 000 ml of demineralised water (5.1).

If necessary, the molarity of the solution can be increased to make the removal of salt or organic material more efficient.

A sample of this solution shall also be analysed in order to determine the MP content so that it can be taken into account in the results of the analyses.

6 Apparatus

6.1 Micro-FTIR, for particles greater than 10 µm with the following requirements:

a) transmission/transflectance or reflectance;

b) spectral resolution: minimum 4 cm^{-1};

c) Spectra format: absorbance;

d) Depending on the system an aperture of 150 µm × 150 µm can be used;

e) Detector spectra range 4 000 cm^{-1} to 675 cm^{-1};

f) Collection time and scans depend on the system and used sources.

6.2 Micro-Raman, for particles greater then 0,2 µm to 0,5 µm with the following requirements:

a) lasers can have a wavelength of 457 nm, 532 nm, 633 nm, and 785 nm;

b) several objectives with different magnifications and numerical apertures;

c) spectroscopic systems;

d) Ultra-High-Throughput spectrometer (UHTS300) in the VIS range Grating: minimum 600 g/mm;

e) Ultra-High-Throughput spectrometer (UHTS400) in the NIR range Grating: minimum 300 g/mm.

6.2.1 CCD camera with one of the following specifications:

a) black illuminated camera for operation in VIS range;

b) low dark current CCD camera in NIR range;

c) dark field microscopy; particle identification and measurement software

6.3 Filters, according to the type of spectroscopy chosen and the size of the particles to be determined.

The pore size that can usually be used are: 0,45 µm, 0,8 µm, 1 µm, 5 µm.

All the filters shall have a suitable shape and fit the instrument of molecular micro-spectroscopy. Circular or square shapes are preferred.

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6.3.1 Micro-FTIR Filters

6.3.1.1 In the case of Micro-FTIR (reflection and transmission) analysis, filters (6.3) with a pore size area of at least 5 µm shall be used.

The filters shall be one of the following.

a) Aluminium oxide filter, spectral range between 4 000 cm^{-1} and 1 250 cm^{-1}, in transmission mode (pore diameter 0,02 µm to 0,2 µm).

b) Silicon filter, spectral range between 4 000 cm^{-1} to 600 cm^{-1} in transmission or reflection mode. Pore diameter 5 µm, pitch 12 µm, thickness 500 µm.

c) Gold polycarbonate filter, spectral range 4 000 cm^{-1} to 400 cm^{-1} in reflection mode (1 µm, 25 mm)

d) Cellulose acetate nitrate filter, spectral range between 4 000 cm^{-1} to 400 cm^{-1} in reflection mode or micro-ATR.

6.3.1.2 In the case of attenuated total reflectance (ATR) analysis, the possible filters (6.3) that can be used have a pore size area of at least 5 µm, in detail:

a) cellulose acetate nitrate;

b) pore diameter 0,45 µm and/or 0,8 µm; filter diameter 47 mm and/or 25 mm;

c) PVDF (polyvinylidene fluoride);

d) pore diameter 5 µm filter diameter 47 mm.

6.3.2 Micro-Raman filters

In the case of Micro-Raman (6.2), the possible filters (6.3) that may be used are:

a) cellulose acetate nitrate, gold coated polycarbonate membrane, silicon filter;

b) 13 mm diameter, 25 mm diameter, 47 mm diameter, 10 mm side.

All materials used shall be washed with demineralised water (5.1) and subsequently with ethanol (5.5) beforehand to remove any residual MP, and immediately after washing shall be left to dry in the air covered with aluminium foil or watch glass.

6.3.3 Reading the filter

To reduce bend upon drying after the filtration process, mount them as flat as possible for measurement.

Silicon filters have two faces (one mirror face and the other darker). Use the filter with the mirror face facing upwards (face to be used for filtering), in this way the micropores facilitate the adhesion of the MPs and their maintenance, even when the filter is dry, during the following counting and identification steps with Micro-FTIR (6.1) or Micro-Raman (6.2).

Silicon filters show strong Raman peaks but no fluorescence. Silicon peaks may be ignored in the particle identification.

6.4 **Light microscope,** suitable for fibre identification, involves the use of projection microscopes and visual microscopic image analysers. Transmitted-light microscopes with direct graduated scale equipped with an optical lens are also applicable.

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6.5 Filtration system, made of steel or glass (see Figure 1), with funnel of 100 ml, 500 ml, 1 000 ml, 2 000 ml complete with sintered septum, clamp and coded Erlenmeyer flask.

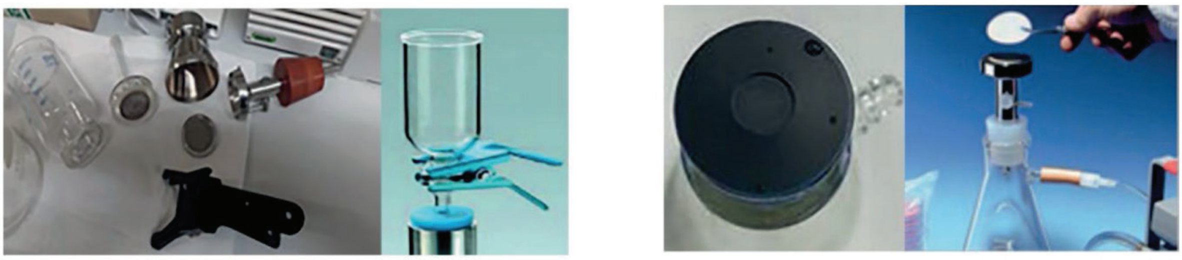

Figure 1 — Examples of filtration system, filter holder and filters

6.6 Flasks and bottles, made of glass, with glass cap.

6.7 Tweezers, made of steel.

6.8 Petri dishes, made of glass.

6.9 Microslides, made of glass.

6.10 Filter holder, (see Figure 1).

6.11 Mechanical stirrer.

6.12 Conductivity meter, accurate to a minimum of 5 microSiemens/cm (µS/cm).

6.13 Equipment for COD determination, rapid kits are also allowed.

6.14 Analytical balance, accurate to a minimum of 0,1 mg.

6.15 Ultrasonic bath, (see 7.3).

6.16 Vacuum filtration system.

6.17 Software, for automatic image analysis, morphological identification, dimensional classification, mapping.

7 Cleaning procedure

7.1 Cleaning of the materials and the test environment

Airborne fibre contamination is possible and using procedures to reduce it is recommended.

The following is a list of mandatory actions to be taken to reduce contamination and keep equipment clean:

a) All glassware shall be previously washed with demineralised water (5.1) filtered through filters (6.3) with a pore size of at least 0,45 µm (nitrate, acetate, mixed cellulose esters) and with washing solution, then rinsed before each use; then washed with ethanol solution (5.5) filtered through filters (6.3) with a pore size of 0,45 µm (nitrate, acetate, mixed cellulose esters) and with washing

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solution, then rinsed before each use with the same ethanol solution (5.5). Final rinse before each use shall be done 3 times.

The same actions shall be applied to sample and container for which glass and metals should be preferred.

Avoid any plastic apparatus or components for filtration and sample conservation as they can release MPs to the sample.

After washing, the glassware shall be stored and protected with the aid of suitable barriers (for example aluminium foil closing the inlets) to reduce the possible deposition of MP present in the air.

b) It is recommended to wipe down all surface using paper saturated with ethanol, or acetone before measuring/testing each sample.

c) Rinse all tweezers (6.7), probes and hands before each filtration procedure.

d) Garments (included white laboratory coat) worn by analysts during sample handling should preferably be made of natural fibres instead of synthetic ones such as synthetic fleece or similar materials.

e) Minimize movement in the lab or working space. It is suggested to conduct sensitive work inside a fume hood or laminar flow cabinet.

7.2 Blank test to evaluate environmental contamination

Environmental contamination of MPs can be considered, by carrying out in parallel, for each lot of analysis, a blank test where a sample of water (5.1) and any other washing solution used on the test is subjected to the analysis procedure. The MP value detected shall be defined as MPs environmental contamination and shall be considered in the various analytical assessments carried out on the various samples subjected to analysis.

The determination of the blank test shall be carried out for each lot of analysis.

7.3 Cleaning procedure for filters

Store filter in glass petri dishes (6.8) in order to reduce contamination from the dish itself. Keep your filter (6.3) covered whenever possible before the observation.

All filters (6.3) shall be new or cleaned before using.

Before filtration, observe the whole surface of the filter (6.3) on OM to check that they do not have any interfering particles on their surface which may have come from the packaging, its handling or the production process itself. Cleaning depends on the kind of filter (6.3).

It is possible to use either a physical treatment which requires an ultrasonic procedure or a chemical one (e.g. simple immersion in pure ethanol AR for 10 min), for all types of filters.

However, for silicon filters in order to reduce the overall cost of analysis, the possibility of reusing the same filter for several analyses has been foreseen with following washing and quality control procedure:

- minimum of 3 repeated sonications in an ultrasonic bath, each of 10 min in demineralised water (5.1) (to be replaced at each repetition);
- before using immerse the silicon filter in 10 ml pure ethanol AR.

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8 Sample preparation

8.1 Solid matrices (fibre, textile or any derivates)

In the case of solid powdery samples, the preliminary dispersion of a representative aliquot of the samples is carried out in a known volume of water (5.1), or in a dispersing solution consisting of a non-ionic surfactant (5.4.2) in filtered water (5.1). If the sample is solid (non-powdery) an appropriate disintegration treatment (e.g. ultrasonic treatment,) shall be carried out.

8.2 Liquid matrices (aqueous)

8.2.1 Choice of sample volume/mass to be filtered and number of filters (6.3).

Filter through one or more filters (6.3) of different dimensions and pore size depending on sample volume.

Table 1 shows recommended sample volumes for filtration through a 13 mm filter with 1 µm pore size.

Table 2 shows recommended sample quantity for filtration through a 47 mm filter with 0,45 µm pore size.

Table 1 — Recommended sample quantity for filtration through a 13 mm filter with 1 µm pore size

Sample quality - Source (like a) - TSS content	**TSS** mg/l	**Unpretreated sample** **(minimum filterable volume)** ml	**Pretreated sample** **(minimum filterable volume)** ml
Wastewater - wwtp Out - low content	<100	10	100
Wastewater - wwtp In - medium content	<2 500	2	20
Activated sludge - Suspended biomass - high content	<10 000	1	5

Table 2 — Recommended sample volumes for filtration through a 47 mm filter with 0,45 µm pore size

Sample quality - Source (like a) - TSS content	**TSS** (mg /l)	**Unpretreated sample** **(minimum volume to be filtered)** ml	**Pretreated sample** **(minimum volume to be filtered)** ml
Wastewater - wwtp Out - low content	<100	50	200
Wastewater - wwtp In - medium content	<2 500	10	100
Activated sludge - Suspended biomass - high content	<10 000	5	20

8.3 Air matrices

Use the following techniques and sampling volumes.

- The determination of airborne particles shall be in accordance with EN 13284-1.
- The determination of dust shall be in accordance with EN 481.

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8.4 Preliminary checking of sample and pre-treatment

In order to identify the possible needs for pre-treatment of the liquid sample with unknown origin, the values of conductivity and the COD and TSS of the sample to be analysed shall be measured and, in addition, an observation in OM at 10 x and/or 50 x magnification shall be carried out.

The liquid samples treated are textile process wastewater, laundry washing effluent, industrial process water.

8.4.1 Liquid sample property checking and requirements

8.4.1.1 Conductivity

Check the conductivity of liquid sample. If the conductivity is higher than 3 000 µS/cm, it shall be necessary to wash the filter (6.3) with a solution of acetic acid 0,1 mol/l (5.6) to eliminate any traces of inorganic substances.

8.4.1.2 Chemical oxygen demand (COD)

Check the COD of liquid sample. If the COD is higher than 100 mg O_2/l, perform a pre-oxidation with hydrogen peroxide (5.2) 15 % (for a duration of not less than 7 days to 30 days depending on the complexity of the matrice) where the volume of hydrogen peroxide (5.2) to be added is calculated using Formula (1), with the aim of eliminating any trace of non-plastic organic material.

$$V = \frac{(COD \times S_{\mathrm{v}})}{70{,}06} \tag{1}$$

where

V is the volume of hydrogen peroxide (5.2, a volume fraction of 15 %) to dose in l;

COD is the chemical demand of oxygen expressed in mg O_2/l;

S_{v} is the sample volume to be treated in l.

8.4.1.3 OM observation

Depending on the TSS and the COD ratio as well as the OM observation, the recommended minimum significant sample volume should be defined in order to have acceptable filtration times.

In addition, during the OM pre-screening, if the presence of overlapping microparticles is high, pre-dilute (8.4.4) the sample with water (5.1), to obtain an optimal microparticle concentration lower than 200 (see Annex B). If the concentration is higher, it may be necessary to use different filters to avoid overlapping.

8.4.2 Pre-treatment if required

8.4.2.1 General

The acidic and/or oxidant pre-treatment may be carried out in accordance with 5.6 and 5.2, during one of the following analysis steps in which the sample may be:

a) an untreated raw sample;

b) a subsample before proceeding with the filtration procedure;

c) the filter (6.3) used after the microparticle collection (see Figure 2).

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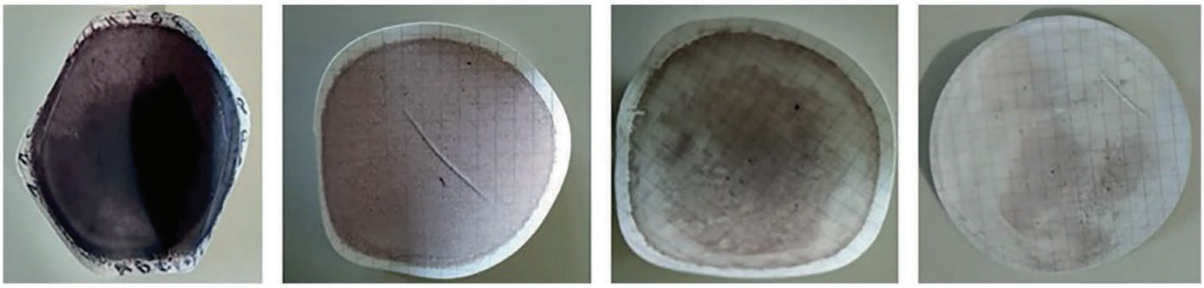

Figure 2 — Examples of nitrate/acetate filters after different pre-treatments with hydrogen peroxide

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8.4.2.2 Salt removal

The acetic acid (5.6) treatment may be carried out in two different ways:

a) on the untreated raw sample by dosing acetic acid (5.6) to attain pH 5 in the sample suspension and in the case of high conductivity or presence of precipitated inorganic salts;

b) by washing the filter (6.3), after completion of the filtration steps, in case of evidence of precipitation of salts, with a direct dosing of 1 ml acetic acid (5.6). If necessary, this action can be repeated.

8.4.2.3 Organic removal

8.4.2.3.1 General

To be carried out on the sample prior to filtration.

The oxidation process takes place over time and as a result of the dosage of hydrogen peroxide (5.2).

8.4.2.3.2 Hydrogen peroxide treatment

The volume fraction of 15 % hydrogen peroxide (5.2) to be added to the sample is proportional to the COD of the sample to be treated. Dose 1,4 ml of 15 % hydrogen peroxide (5.2) volume fraction for each 100 mg O_2/l (COD) referred to 100 ml of sample. To calculate the dosage for a real sample, refer to Formula (1).

Satisfactory results are obtained after 7 days, if they are still not adequate (see Figure 3) extend the reaction time.

Figure 3 — Water samples treated with different hydrogen peroxide volume fractions of 15 %

8.4.2.3.3 Reaction time

Depending on the matrice, different reaction times can occur.

The minimum oxidation time depends on the quality of the non-plastic organic matrice to be oxidized. The time can vary between 7 days and 30 days. Verify the oxidation effect visually by checking the clarity of the sample and the reduction of the colour/turbidity.

Consequently, a production of sediment can occur.

Figure 4 highlights this effect.

a) 1 day b) 3 days c) 7 days

Figure 4 — Water sample treated with hydrogen peroxide (5.2) volume fractions of 15 % at different times

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8.4.3 Test sample homogenization/hydration and/or sonication

The sample should be stirred vigorously or alternatively sonicated to produce the subsample; these operations can be carried out at room temperature or higher, up to a maximum of 50 °C.

In order to minimize the ability of the microparticles to adhere to the walls due to their high surface tension and hydrophobicity, all samples are subjected, before being handled and filtrated to a process of ultrasonication designed to significantly increase the hydrophilicity of MPs and consequently reduce/minimize the wall effect and agglomeration, as that would result in loss of significance and representativeness of the sample analysed.

The procedure involves treatment with ultrasonic bath (6.15) 20 kHz to 40 kHz at different treatment times. The wetting effect of the MPs shall make them perfectly dispersible in aqueous solution.

The subsample shall then be diluted (see 8.4.4) by a factor to perform an adequate particle counting (without particle overlapping) on the final filter (6.3).

In the calculation of the total volume, the value of the volume used for the washing of the equipment (for example pipettes, cylinders, filtration container) shall be included.

8.4.4 Pre-dilution

In order to produce a subsample to be tested, pre-dilution shall be performed immediately after the preliminary checking phase (8.4).

In the pre-dilution phase of the sample, its homogenization shall be prepared according to 8.4.3.

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9 Preparation procedure

9.1 General

This protocol provides a consistent method for preparing standardised suspensions containing microfilaments/microplastic with fibre shape[4] for:

- standard or reference materials currently unavailable for purchase[1][2];
- evaluation and validation of all approaches from sampling to identification of microplastic.

9.2 Preparation of micro-sized fibres sample

This procedure describes how to cut synthetic threads in order to obtain solutions with a number of filaments per litre between 70 filaments per litre and 850 filaments per litre. Table 3 provides a series of types of threads used for the preparation of standard samples and their relative concentrations.

Table 3 — Examples of preparation of standard samples (example)

Sample name (polymer, colour, number of filaments)	Water (5.1) collection volume (ml)	Theoretical concentration (Number of filaments/litre)
Polyamide 6,6 blue (68 filaments)	300	227
	500	136
	900	76
Polyamide 6 multicolour (128 filaments)	300	427
	500	256
	900	142
Polyamide 6 orange (180 filaments)	300	600
	500	360
	900	200
Polyester beige (256 filaments)	300	833
	500	512
	900	284
Polypropylene orange (72 filaments)	300	240
	500	144
	900	80

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In order to cut sample from 200 µm to 800 µm a manual microtome (see Figure A.1) can be used.

It is necessary to use a yarn of commercial material with a known number of filaments for the preparation of the standards. Before cutting, check that the number of filaments is equal to the one declared by the producer by using an OM (see Figure 5 and Figure 6).

Figure 5 — Example of polyamide thread

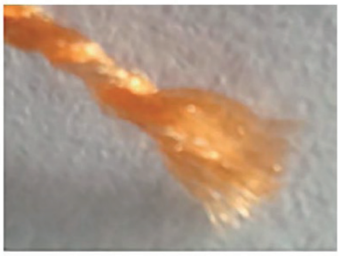

Figure 6 — Example of polyamide thread with 180 filaments

Proceed with the cutting using a microtome (only one cut for sample), see Annex A. In order to fill the microtome slot completely wool fibre shall be used (see Figures 7, 8, 9, and 10) All the cut fibres are collected in a recipient and the wool fibres are identified by using an OM as they have an irregular diameter of around 16 µm to 20 µm and scales on the surface. Moreover, they may be eliminated by an oxidative treatment (see Figure 11) as described in 7.2.

Figure 7 — Polyamide and wool fibres inside the microtome

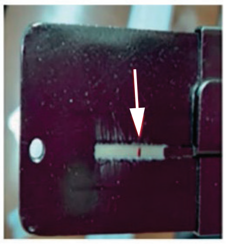

Figure 8 — Cut Polyamide thread inside the wool fibres

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Figure 9 — Fibres lifting (0,2 mm)

Figure 10 — Cutting the fibres with a blade

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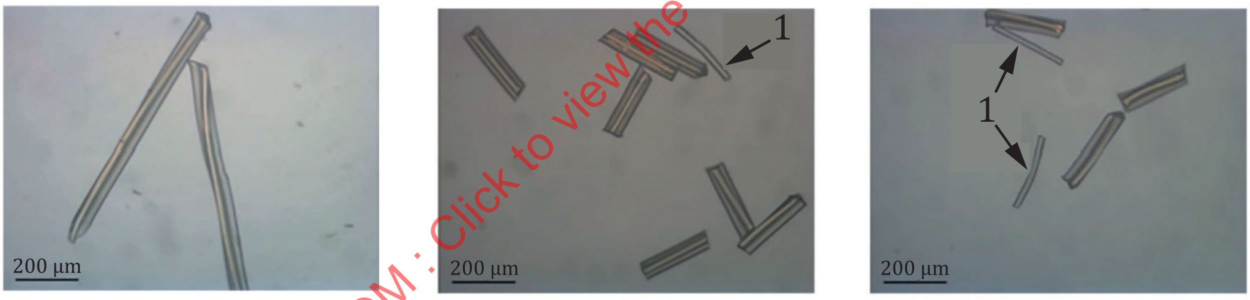
After cutting determine the fibre diameter and their lengths by using an OM as shown in Figure 11.



a) L: 829 µm d: 55 µm

b) L:216 to 235 µm d:55 to 60 µm

c) Example of wool fibre (1)

Key

1 wool fibre

Figure 11 — Optical microscope images of polyamide fibres cut with a microtome at 800 µm and 200 µm (mixed with wool fibres)

9.3 Standard fibre sample preparation (Water based)

After cutting with the microtome (see Annex A), the synthetic filaments are wrapped with wool fibres. All the fibres are collected with the cutting blade and dispersed in 10 ml of water (5.1) and 7 ml of sodium hypochlorite (5.3) in a 50 ml flask (6.6).

A Sodium hypochlorite solution NaClO (5.3) is used to dissolve wool fibres in accordance with ISO 1833-4 making it easier to count synthetic fibres under a microscope. The suspension is stirred in a 50 ml flask (6.6) with a mechanical stirrer (6.11) at 130 r/min for 40 min without heating.

After the treatment the synthetic fibre suspension is spilt into a large Erlenmeyer flask (6.6). The flask (6.6) is washed with 50 ml aliquots of water (5.1) to reach the stated volumes (300 ml, 500 ml or 900 ml).

Before reaching the stated volumes, all the fibres on the flask walls are recovered and transferred to the Erlenmeyer flask (6.6).

The flask (6.6) is also rinsed with 10 ml of ethanol solution (5.5) to collect all the remaining fibres. Then the final aliquot of water (5.1) is added to reach the stated volume.

9.4 Water based standard fibre sample filtration

9.4.1 General

The collection of fibre-shaped microparticles is performed on one or more filters (6.3) with pore size lower than the minimum diameter of the fibres used for the preparation of the standard suspension.

9.4.2 Filtration procedure

Gradually filter all the suspensions (50 ml at a time).

After filtering the whole suspension, wash the funnel walls with a few ml of ethanol solution (5.5) using a glass Pasteur pipette in order to recover the possible microparticles adhering to the glass.

Carry out the final recovery wash (of the filtering system, the gasket and the flask (6.6) containing the suspension with an ethanol solution (5.5) and filter on Filter 2.

The washing operation shall be repeated at least three times, each time using an aliquot of 30 ml of ethanol (5.5). Use an aliquot from the last wash with a glass Pasteur pipette to wash thoroughly the filter funnel, and the gasket.

Two filters (6.3) are used to collect all the microparticles derived from all the filtering and washing procedures for each standard suspension.

- Filter N°1 is used to collect the microparticles of the standard suspension and the solution of the first rinse of the flask (6.6) and of the filtering system.
- Filter N°2 is used to collect microparticles from the subsequent rinses with the washing ethanol solution (5.5) and from the solution of the subsequent rinses of the Erlenmeyer flask (6.6) and all the components of the filtering system.

9.5 Requirements for standard fibre samples

At least 5 replicates per type of synthetic polymer shall be carried out for each sample. For each yarn and polymer, at least five suspensions at 300 ml, 500 ml and 900 ml shall be prepared to obtain five different solutions with the same number of filaments.

For each replicate, the number of particles (in filament shape) at a given volume results from the sum of the particles collected on Filter 1 (main solution) and Filter 2 (washing water). See Formula (2):

$$\frac{N_{\mathrm{tp}}}{L} = \frac{N_{\mathrm{p}}}{S_{\mathrm{v}}} \tag{2}$$

where

N_{tp} is the number of total particles obtained by automatic or manual counting as sum of particles counted on Filter 1 and Filter 2;

N_{p} is the number of particles;

S_{v} is the solution volume (l).

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Table 4 shows how the data shall be entered and calculated for each sample analysed.

Table 4 — Average of MPs (Number of filament/litre) of 5 replicate compared with theoretical values and % recovery

	replicates	**Number of filaments of the first sample: 300 ml (total solution)**	**Number of filaments (rinse)**	**Total filaments**	**% recovery**
Sample names (polymer, number of filaments) Number of filaments/litre (theoretical)	1°				
	2°				
	3°				
	4°				
	5°				
	Average				
	replicates	**Number of filaments of the first sample: 500 ml (total solution)**	**Number of filaments (rinse)**	**Total filaments**	**% recovery**
Sample names (polymer, number of filaments) Number of filaments/litre (theoretical)	1°				
	2°				
	3°				
	4°				
	5°				
	Average				
	replicates	**Number of filaments of the first sample: 900 ml (total solution)**	**Number of filaments (rinse)**	**Total filaments**	**% recovery**
Sample names (polymer, number of filaments) Number of filaments/litre (theoretical)	1°				
	2°				
	3°				
	4°				
	5°				
	Average				

The minimum acceptable standard recovery rate is 80 %.

The standard recovery rate was observed (see Annex B) to depend on the ratio between the number of particles and the volume (ml) in which they are dispersed. A recovery rate between 80 % and 100 %, was obtained per solution containing MPs with a concentration of not fewer than 200 particles/l.

If the above recovery rate is not reached, check that the washing and filtration procedures have been correctly applied and that the filtration equipment is functioning properly and that it is leak-proof.

10 Test procedure

10.1 Addition of internal standard fibre sample

For a more precise determination of microplastic fibre shape in real samples, an internal standard should be added considering procedure described in 9.2 and 9.3, with a concentration similar to the number of particles counted/estimated in the sample. In this way, it shall be possible to determine the quality of all the operations leading to the identification of microplastic fibre shape. The internal standard should have a different colour from those present in the sample identified during the pre-screening tests (8.4). It should be added with a concentration similar to the number of particles counted/estimated in the sample.

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10.2 Test sample filtration

Any pre-treated sample is vacuum filtered using the vacuum filtration system (6.15).

If the sample shows a high presence of microparticles and suspended solids even after the pre-treatment, the filtration is carried out in two separate steps.

a) Filtration of decanted supernatant.

b) Filtration of the final deposit (precipitated) with recovery by final washing operation.

The microparticles/fibres collected on both filters shall be counted and added to obtain the final result, which is to be reported in the final test report.

10.3 Recovery procedure

Once the filtration phase has been completed, in order to recover any MP deposit on the bottom of the bottle (6.6) and on the walls of the glassware used (both the container and the filtration system, 6.5), it is necessary to proceed to a recovery washing that allows the detachment from the walls and the collection of sample MPs.

After filtering the entire suspension, wash the funnel walls with a few ml of washing solution, using a glass Pasteur pipette in order to recover any possible microparticles adhering to the glass.

Carry out the final recovery washing (of the filtering system, the gasket and the flask (6.6) containing the suspension) with washing solution and filter it through the same filter.

The washing operation shall be repeated at least three times, each time using an aliquot of 30 ml of recovery solution. Use an aliquot of the last washing with a glass Pasteur pipette to wash thoroughly the filter funnel and the gasket.

The washing solution shall be chosen (depending on its availability) from one of the three:

— sodium chloride solution a mass fraction of 1 % (5.4.1);

— non-ionic surfactant solution a mass fraction of 1 % (5.4.2);

— ethanol solution (5.5);

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10.4 Image analysis

The MPs collected on the filter are then subjected to image analysis that is carried out manually or automatically by software (6.17).

The following information shall be collected at this stage:

— optical microparticles and microplastic fibre shape recognition;

— morphological identification (microparticles including fibre shaped);

— dimensions L x L;

— X, Y mapping;

— section area estimate (L x W or with software);

— external surface estimate (as sphere - cylinder (rotation solid) or with automatic 3D modelling system);

— sub classification of colour (if any different), morphological and dimensional classes.

Figure 12 and Table 5 show examples of automatic image analysis (6.17) of particles collected on filters.

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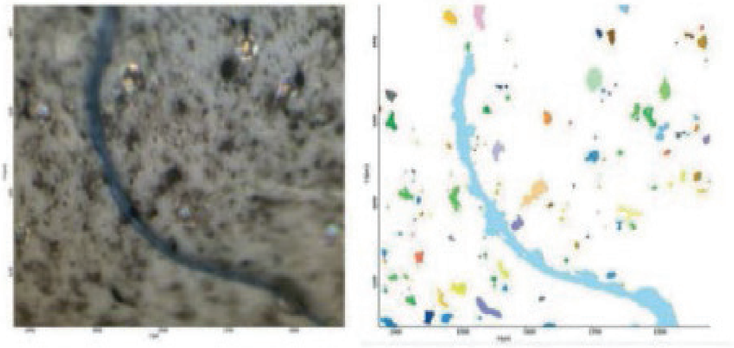

Figure 12 — Automatic image analysis — Example (1)

Table 5 — Automatic image analysis — Example (2) dimensional classification

Length (µm)	Width (µm)	Aspect ratio	Num pixels	Area (µm^2)	Convex area (µm^2)	Perimeter (µm)	Convex perim. (µm)	Feret max (µm)	Feret min (µm)	CE Diam. (µm)	Circularity	Convexity	Solidity	Volume (µm^3)
17,793	13,489	0,759	476,500	179,385	195,196	60,242	54,013	19,524	13,498	15,113	0,788	0,897	0,919	1 807,352
5,640	5,206	0,923	58,500	22,023	22,964	18,135	17,652	6,136	4,909	5,295	0,917	0,973	0,959	77,747

10.5 Identification of MPs

10.5.1 General

For the identification of MPs, molecular spectroscopic techniques may be used:

a) Micro-FTIR (6.1);

b) Micro-Raman (6.2).

10.5.2 Detection limits

Detection limits of methods are dependent on the following factors:

a) Instrumental:

- minimal size detection of the instrument [Micro-FTIR (6.1) or Micro-Raman (6.2)];
- % of matching during chemical mapping;
- spot of analysis dimension;
- X-Y minimal translation steps.

b) Microplastic morphology:

- shape, colour.

c) Solution characteristics:

- concentration of the solution: Number of particles/l.

A high concentration of MPs Number of particles/l determines overlapping of the MPs on the filter, increases counting errors and makes chemical identification difficult.

- Filter size and pore size

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NOTE In the case of automatic MP identification (through specific software), a manual overview might be necessary to avoid any counting errors or any difficulty in chemical identification.

10.5.3 Calculation

Since analysis times shall be kept to a minimum without losing the quality of the data acquired, statistical evaluations shall be carried out to identify the minimum number of particles to be analysed that may be representative of the sample as referred to in the scientific paper[3] and also as described in Annex B.

The calculation of the minimal required number of particles is based on the following assumptions:

- all the particles collected on the filter are divided into two groups: MP and non-MP;
- all the particles on the filter are separated from each other;
- all the particles are randomly distributed on the filter, meaning that the MP to non-MP ratio is constant all over the filter;
- heterogeneous spatial distribution of the particles is allowed;
- all the particles are identified by image processing;
- regardless of their size, all the particles are treated equally as single measurement points;
- flawless identification of particles by analytical method (for example molecular spectroscopy).

Formula (3) gives the algorithm to determine the significant number of particles.

$$\mathrm{n} \geq \frac{P(1-P)}{\dfrac{e^2}{{z_{c;0.9}}^2} + \dfrac{P(1-P)}{N}} \tag{3}$$

Formula (3)defines the minimum number of statistical units to be analysed, *n*, taken from a finite population of size *N*, to estimate the proportion, *P,* with an error inferior to the previously specified one, *e*. Formula (4) is based on the confidence interval constructed by normal approximation of the hypergeometric distribution and it follows by rearranging and solving [see Formula (5)] by *n*:

$$\hat{P} \pm z_{c;0.9} \sqrt{\hat{P}\left(1-\hat{P}\right)\frac{N-n}{N-1}} \tag{4}$$

$$z_{c;0.9} \sqrt{\hat{P}\left(1-\hat{P}\right)\frac{N-n}{N-1}} \leq \mathrm{e} \tag{5}$$

where

z_(c;0,9)	is the standard Gaussian distribution quantile of level 90 %;
N	is the total number of particles found on the filter through image processing;
$\hat{P}$ /decimals	is the estimate of the MP fraction;
e/decimals	is the margin of error;
n	is the sample size/number of particle required.

10.5.4 Spectra comparison and MP identification

In either Micro-FTIR (6.1) or Micro-Raman (6.2), if the spectrum is not recognized with a matching greater than 75 %, the corresponding particle shall be indicated as "unknown composition".

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The operation of spectra comparison with the DB spectra (data base) shall be carried out for all the points mapped or for an, n, statistically representative sample for each morphological dimensional class.

The DB spectra are commercial ones, published in peer-reviewed journals or internally created starting from known MP samples. In the latter case, it is possible to create DB spectra with MP samples from a range of environments.

For automatic Raman identification, good matches shall be higher or equal to 90 %. In case of lower values, particle identification is carried out by using customized databases.

10.5.5 Determination of external surface area and volume

The determination of the surface, volume values and morphology can be carried out automatically if specific software is available.

If not, estimation can be done manually, with this assumption that the particles are spherical and the fibres are cylindrical.

10.5.6 Calculation of total surface and total mass (optional)

For each family of polymers recognized by molecular spectroscopy:

- add the calculated/estimated surface values per polymer family;
- calculate the volumes of the identified particles (as sphere - cylinder (rotation solid) or with an automatic 3D modelling system);
- add the calculated/estimated mass values per polymer family;
- calculate the total mass by type of polymer (starting from the volume and density typical of the polymer being considered) using Formula (6):

$$P = V \ x \ d \tag{6}$$

where

P is the calculated total mass (g);

V is the calculated total volume (cm^3);

d is the specific density for the main polymers (g/cm^3, refer to Table 6 when possible).

NOTE Mass calculation of MPs can be applied to any kind of MPs identified on the filter independently from the source (e.g. fibre, textile product, or any kind of other plastic used in textile processes).

Table 6 — Density values of some synthetic polymers

Chemical name	**Density** (g/cm^3)
Polypropylene, atactic	0,87
Polypropylene, isotactic	0,90
Polyisoprene	0,91
Polyethylene, low density	0,92
Polyethylene, high density	0,95
Polydimethylsiloxane	0,97
Polystyrene	1,05
NOTE In case of no data available for density of the specific polymer in analysis no weight will be calculated and a N.D. will be indicated in the report.	

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Table 6 *(continued)*

Chemical name	**Density** (g/cm^3)
Polyamide 6,6 [Poly(hexamethylene adipamide)]	1,08
Polyamide6 [Poly(caprolactam)]	1,12
Poly(vinyl acetate)	1,19
Polycarbonate	1,20
Poly(vinyl alcohol)	1,29
Polyacrylamide	1,30
Polybutylene terephthalate	1,31
Cellulose acetate	1,31
Poly(ethylene terephthalate)	1,39
Poly(vinyl chloride)	1,40
Cellulose nitrate	1,60
Poly(vinylidene fluoride)	1,76
Poly(tetrafluoroethylene)	2,00
NOTE In case of no data available for density of the specific polymer in analysis no weight will be calculated and a N.D. will be indicated in the report.	

10.5.7 Calculation of recovery rate (R_r) COUNTING

In case of internal standard addition, determine the value of the recovered MPs (MP internal standard) from the sample analysis and calculate the recovery ratio using Formula (7). This rate should be included in the test report, in order to give an indication of the MP recovery level value for the sample tested. A low R_r value shall highlight any erroneous operations during the tests in the event of significant sample losses.

$$R_r = \frac{R_c}{T_h} \tag{7}$$

where

R_r is the recovery rate

R_c is the number of recognized Internal standard MP

T_h is the theoretical number of Internal standard MP added

11 Test report

11.1 The test report shall consist of at least:

a) the sample and the sampling procedure;

b) the International Standard used (including its year of publication);

c) the method used (if the standard includes several);

d) the result(s), including a reference to the clause which explains how the results were calculated;

e) any deviations from the procedure;

f) any unusual features observed;

g) the date of the test;

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h) a summary table indicating:
  - morphological and dimensional classes
  - numbers, types and colours (optional) of polymers found
  - estimated external surface area (expressed in mm^2/unit of sample analysed) per identified polymer family
  - estimated mass (optional, expressed in mg/unit of test sample) per identified polymer family

For the size classes, those defined in ISO/TR 21960, are:

- 5 000 µm to 1 000 µm;
- <1 000 µm to 500 µm;
- <500 µm to 100 µm;
- <100 µm to 50 µm;
- <50 µm to 10 µm;
- <10 µm to 5 µm;
- <5 µm to 1 µm;
- <1 µm to 0,1 µm

The size classes are dependent to the type of instrument and filter (6.3) used.

The example of classification of MPs is shown in Annex C.

11.2 The report may also include the following:

a) recovery rate

b) photographs of samples

c) OM images

d) particle mapping images

e) number of microparticles analysed (N)

f) spectra

g) summaries and graphs of the different classes identified:
  - morphological,
  - dimensions,
  - polymer families

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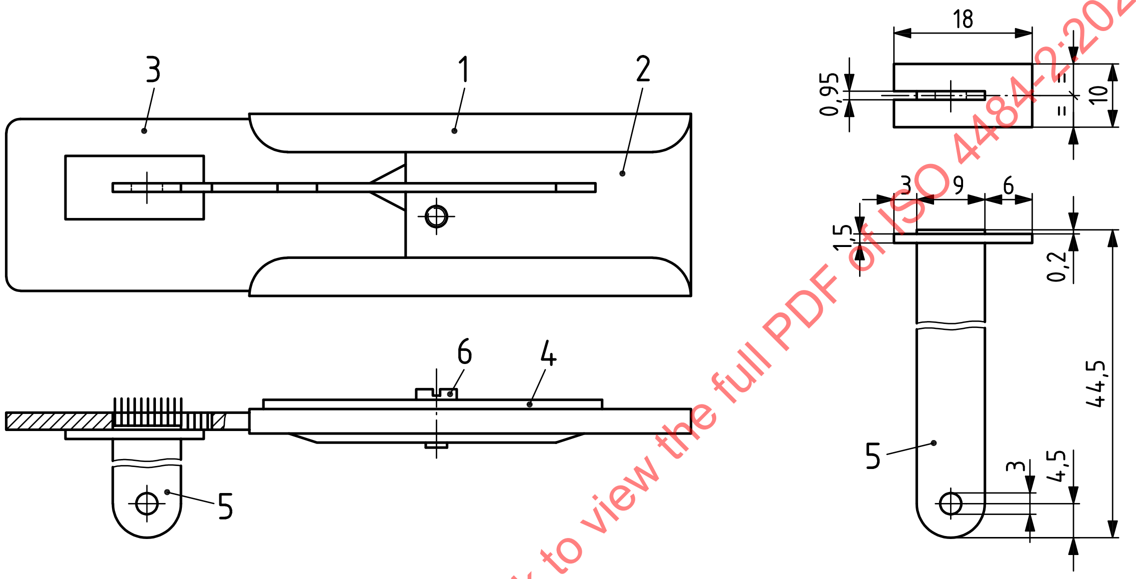
Annex A
(informative)

Microtome for standard preparation

Test specimen preparation using a microtome as described in ISO 137[5] has been found suitable, see Figure A.1.

Dimensions in millimetres



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Key

1 guide
2 fixed plate
3 mobile plate
4 flap
5 punch
6 guide (1) position fixing screw

Figure A.1 — Example of manual microtome drawing

Annex B
(informative)

Examples of statistics elaboration

Logit regression analysis (see Formula B.1) was used to investigate the relationship between concentration and single microfilament detection probability as well as the impact of the type of material used (synthetic polymer). The logit model is widely used to investigate the relationship between a binary response variable and some other explanatory ones. In this study[4], the logit model was chosen due to the binary nature of the data, in which a dependent variable has two possible values expressed as identification or non-identification for each individual microfilament in the suspension. Let Y_{ij}, $i=1,\ldots,n$, $j=1,\ldots m$, denote the response, that is the number of detected microfilaments for the i-th sample and j-th replication. Since for each sample, K is the theoretical number of microfilaments and it represents the number of independent trials that can be performed on it. Y_{ij} is distributed as a binomial random variable of size K and probability p_i. The logit model used explicit the relationship between the probability of detection of the single microfilaments, p_i, and the covariates by modelling:

$$logit(E(Z_{ijk}|X_{1,ij},X_{2,ij})) = log(p_i/(1\text{-}p_i)) = \beta_0 + \beta_1 X_{1,ij} + \beta_{M(i)} \qquad \text{(B.1)}$$

where

$Z_{ijk,}$ k=1,

K is a Bernoulli random variable representing the detection of the K-th microfilament in the i-th sample and j-th replication,

$X_{1,ij}$ is the concentration used;

$\beta_{M(i)}$ is the parameter representing the material's effect for the i-th sample.

To determine the sum of microfilaments collected on the filters for each thread, 5 replicates per type of synthetic polymers were carried out for each sample and the data were statistically analysed. The results are graphically shown in Figures B.1 and B.2.

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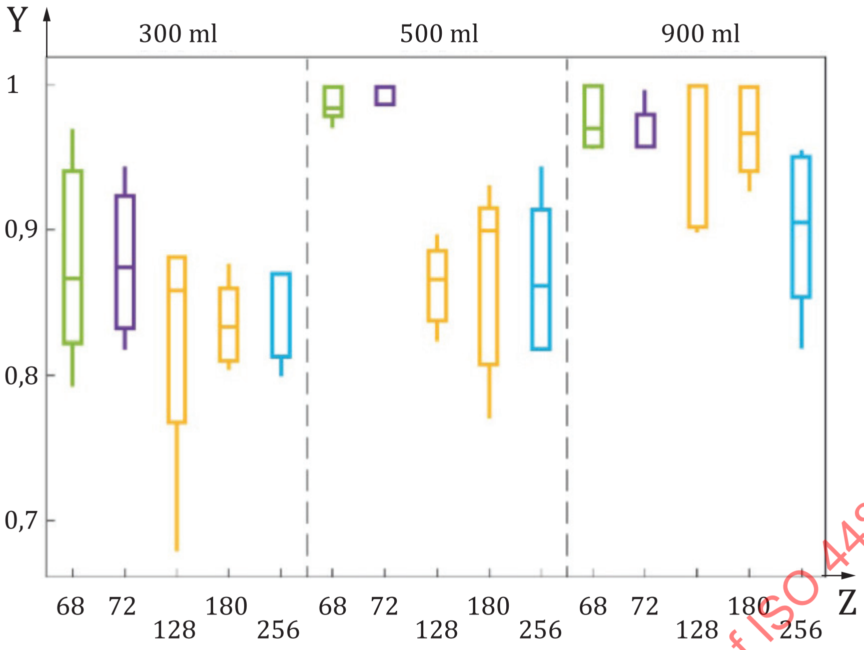

Key

X	concentration (microfilaments/L)
Y	probability
—	polyamide 6,6
—	polypropylene
—	polyamide 6
—	polyester

Figure B.1 — Boxplots showing the relation between concentrations and the probability of detection of the single microfilament

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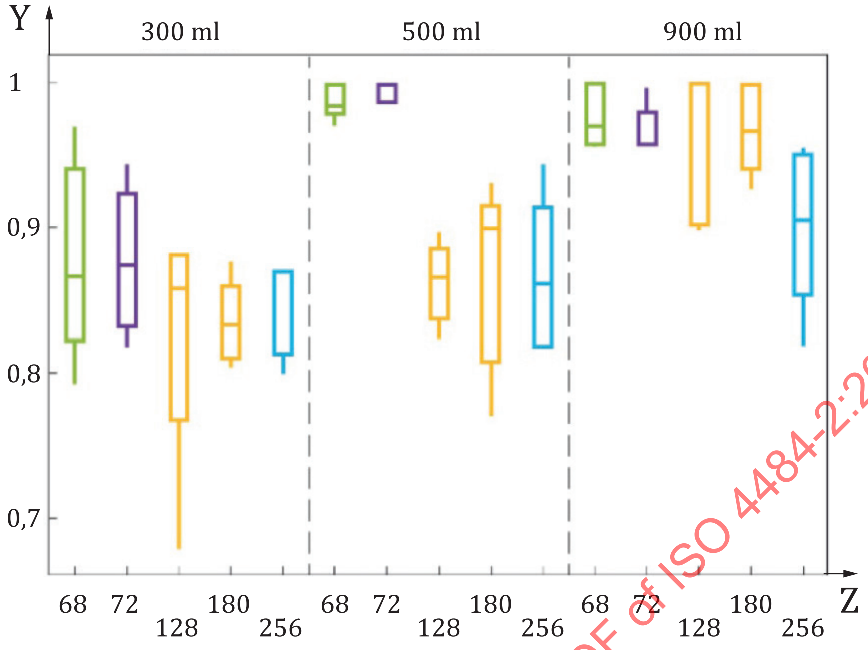

Key

X	concentration (microfilaments/L)
Y	probability
🟩	polyamide 6,6
🟪	polypropylene
🟨	polyamide 6
🟦	polyester

Figure B.2 — Boxplots showing the relation between suspension volumes, theoretical microfilaments and the probability of detection of the single microfilament

Figure B.1 shows the relationship between concentration and the probability of the detection of the single microfilaments. The results show that the probability of detecting the microfilaments is higher than 95 % when the concentration of microfilaments/l is lower than 200 Number of microfilaments/l, whereas, Figure B.2 shows the relationship between the theoretical microfilaments contained in the samples and the detection probability of the single microfilament, for each suspension volume.

Therefore, the higher the suspension volume is, the higher the detection probability is, while the more theoretical microfilaments are present, the lower the detection probability is. These relationships are also related to different kinds of materials represented by different colours.

The estimates of the parameters of the model and inference are obtained with the maximum likelihood method, the standard error and the p-values are reported in the Table B.1[4].

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