Method Article

Validation of an FT-IR microscopy method for the determination of microplastic particles in surface waters

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ABSTRACT

For analysis of microplastic (MP) particles in aquatic or solid compartments, standardized methods are required, yet data obtained by current methods are of limited comparability. Current methods include Fourier-transform Infrared (FT-IR) microscopy, Raman microscopy or thermo-analytical methods and attempts to compare data-sets from these methods have largely failed. Only little quality data based on validated methods and appropriate quality standards is available. Thus, reports of presence and numbers of MP still vary significantly from each other without a reliable indicator which of the reported data fulfils data acceptable quality requirements.

A methodology for the determination of MP via FT-IR microscopy is introduced and critically discussed regarding mandatory validation parameters and applicability. Furthermore, advantages and challenges of this method are put into relation to other spectroscopic and spectrometric techniques.

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Strategy

The lack of harmonised methods, reference material, and thus comparative data is one of the biggest challenges regarding the determination of MP in the environment [1,2]. Thus, efforts have to be taken to evade these challenges. This methodological setup was primarily chosen for aqueous samples, but may also be extended for solid samples such as sediments or soils. The general strategy was to trap the MPs after sample preparation on 25 mm diameter membrane filters with 0.2 μm pore size (AnoDisc™ 0.2 μm pore size and 25 mm diameter, Whatman, GE Healthcare UK

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(http://creativecommons.org/licenses/by-nc-nd/4.0/)
limited, Buckinghamshire United Kingdom) and to determine them via software-based microscopy. The polymer component of the MP was identified using FT-IR spectroscopy. Sample preparation techniques applied during these experiments were described by Klein et al. (2015) [3] and included density separation via saturated NaCl-solution and an oxidative treatment with H$_2$O$_2$ (30%):H$_2$SO$_4$ (96%)(1:3) prior to filtration. All measurements were performed on an FT-IR spectrometer (Spectrum TWO) coupled to a Spotlight200i Microscope both from Perkin Elmer, Waltham, Massachusetts, USA.

Following sample preparation, aqueous samples or standards (reference suspension spiked to 100 mL) were transferred to an AnoDisc® membrane via vacuum enhanced filtration. The used AnoDisc® filter membranes consist of aluminium oxide and are thus transparent for IR radiation with wavenumbers above 1500 cm$^{-1}$ enabling transmission as measurement mode for FT-IR. Transmission was selected as the IR-beam traverses the entire particle determining the bulk properties of the particles. Potential interferences caused by surface modifications as biofouling, loading with natural organic matter (NOM), or weathering are circumvented by this method. As only drawback the identification of non-transparent particles is hampered.

For identification purposes, the generated spectrum of each particle was compared with an IR spectra reference database allowing to characterise the particle. The database was generated by obtaining the IR spectra of the major homo-polymer materials. The reference materials analysed included polyethylene (PE), polypropylene (PP), polycarbonate (PC), polymethyl methacrylate (PMMA), and polyamide (PA) which were provided by BASF (Ludwigshafen Germany); and polyethylene terephthalate (PET), polystyrene (PS), polyvinyl chloride (PVC) which were purchased from PSS were recorded and included (reference spectra with respective measurement parameters can be found in the supporting information (SI)). The advantage of a small and targeted database is the simplification of the crucial decision in MP particle analysis: Is the particle of synthetic polymeric or natural origin? Furthermore, a simple database facilitates the output as the material of the particle is classified into limited few main categories defined by the bulk polymers (i.e. PE based, including HD-PE, LD-PE, and weathered PE etc.). An argument in favour of the more complex databases (with more entries and has a wider, more specific classification scheme including co-polymer types) is the improved tracking of contamination sources if the polymer material is precisely identified [4]. However, since the major portion of MP is of secondary origin the polymers have already undergone weathering and fragmentation, and a biofilm has formed which are strongly hampering an allocation of the emitter [5–7].

A complete transfer of (MP) particles onto the AnoDisc™ membrane via vacuum enhanced filtration is required to ensure robust and reproducible quantification of MP particles. Therefore, it may be necessary to flush the glassware several times with purified water or purified water containing 0.05% of the surfactant SF100 (NovaChem) to ensure a complete transfer of the particles to the filter membrane. This is particularly relevant when working with “fresh” MP particles as these particles tend to agglomerate due to their hydrophobic surface. The authors experienced a less drastic effect for MP found in environmental samples as their surface gained hydrophilicity by coating with NOM or due to weathering. The filter membrane was held on a custom made stainless steel bracket on the microscopy table enabling measurement via FT-IR microscopy. Prior to particle detection, a microscopic image ($A = 4$ mm$^2$) of a part of the AnoDisc™ membrane was generated. Particle detection is automated based on an algorithm that detects particles by contrast (of pixels to filter background), minimal area (number of pixel with certain contrast), and a split factor (distinguishing between single overlapping and large particles) which can be varied for each generated microscopic image. For each particle a respective measurement spot was assigned and IR spectra of the particle and of the background (reference spectrum containing only filter material) were recorded. After automatic background correction the resulting particle spectrum is automatically compared to the spectra in the database. As stated above the created microscopic images possess an area of 4 mm$^2$ which represent only 0.82% of the total filter area. Depending on the number of particles on a filter membrane and as explained below, it was not possible to investigate each particle on the filter due to the limitation of time. Measurement times of a 4 mm$^2$ filter area depending on the number of detected particles range from 30 to 120 min. Measuring the entire filter takes a minimum of 48 h. Thus, a general reduction of covered filter area is required. To obtain representative results, a larger
filter area than 0.82% has to be investigated and resulting in averaging a multitude of images prior to extrapolation to the total filter area.

These facts lead to three main problems for data analysis and interpretation, which are inherent for all spectroscopic methods which use particle detection on different filters. The first problem regards the definition of suitable parameters for the recording of “clean” spectra in an acceptable time frame. The second problem concerns the decision on whether a particle is polymeric or not, which is defined by the comparison with the database and thus, the cut-off to be used for the correlation factor. And the third problem is based on how much of the filter area or number of particles have to be investigated to generate reproducible and representative results for the sample entity on the whole filter area.

Method adaptations/solutions

Parameters for the IR-measurement were selected after several measurements of PS particles ($M_w = 3460$ g mol$^{-1}$, shape: fragments) standard particles on different filter materials such as KBr and AnoDisc membrane. A resolution of 4 cm$^{-1}$ and a scan number of two were found as reasonable compromise between measurement time and data quality, while taking correlation factor into account (detailed data can be found in the SI). A doubling of resolution (i.e. 4 cm$^{-1}$ to 2 cm$^{-1}$) also leads to a doubling of the covered mirror path, and thus the required measurement time. This is also valid for the accumulation of single scans. The selected parameters were taken as basis in further method validation.

Parameters for particle detection (minimum contrast, split factor, threshold, brightness, and contrast) cannot be standardized for the applied software (Spektrum, Perkin Elmer) as these parameters are highly dependent on the nature of the sample. Especially when working with surface water samples or sediment samples, a modification of parameters such as contrast and brightness, are crucial to ensure reproducible results for particle detection. These parameters have to be optimised by the operator for each sample or even for each recorded microscopic image. From the researcher’s experience, high brightness and contrast result in dark spots for particles facilitating the automatic detection of these. This minimises the false detection of shades and stains on the membrane as particles and thus shortens measurement time. Other approaches for particle detection implement modified algorithms and are described elsewhere [8].

Since the classification of the particles is based on an automatic comparison of a recorded spectrum with database spectra and the resulting correlation factor (equation for the calculation of the correlation factor is shown in the SI) a cut-off for the correlation factor was determined and was chosen as a decision criterion in deciding whether the spectrum of a particle can be assigned to a database spectrum and thus be identified as polymer. Therefore, spectra of a PS-particle were recorded in a 10-fold replicate measurement on an AnoDisc™ membrane and a correlation factor for every single measurement was automatically calculated via database comparison. To generate the decision criterion, the mean of the correlation factors was calculated and the triple standard deviation was subtracted from the mean (correlation factor = 0.73; standard deviation = 0.044). This resulted in following decision criterion: A correlation factor of 0.6 or higher is sufficient to successfully assign a recorded spectrum to a database spectrum.

To determine the effect of surface modifications such as coating with NOM (humic substances, proteins, or extracellular polymeric substances (EPS)), embrittlement, and biofouling on the correlation factor and thus on the differentiation whether the particle is originally from synthetic polymer the abovementioned methodology was repeated for the PS-particles that were incubated with humic substances. PS-particles were incubated for 24 h with humic substances (Suwannee River Reference Standard; $\beta = 2$ mg L$^{-1}$). Again a single particle was selected and spectra recorded in a 10-fold replicate measurement. The decision criterion was calculated from the mean of the correlation factors (0.93) and the corresponding standard deviation (0.019) resulting in a value of 0.87. Both correlation factors of the single measurements and the resulting decision criteria are shown in Fig. 1. The correlation factors in this methodological setup were not dependent on surface modifications. Calculated decision criteria were not adversely affected by the presence of humic substances. This is reasonable as with transmission measurement, a spectrum of the entire particle including the internal
Fig. 1. Correlation factors of repeated measurements (n = 10) for a pristine PS particle (orange) and a PS particle coated with humic substances (Suwannee River Reference Material) (blue). Error bars indicate relative standard deviation and the data points at the end the mean with triple standard deviation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

particle material is recorded, which is not affected by surface modifications. It is expected that further surface modifications or additives do not adversely affect the correlation factors significantly, as the polymer is always present in excess rendering transmission measurement a robust technique for MP identification. As result, the lower decision criterion (correlation factor >0.6) was selected for this method which signifies a 95% certainty that MP is not falsely classified as non-polymeric particle.

The methodology proved to be robust as surface modifications of particles did not interfere with qualification as MP particles. On basis of this method, the determination of reproducibility and correctness was performed. Being very robust is the central advantage of FT-IR microscopy compared to other spectroscopic approaches like Raman microscopy. Raman spectroscopy is a surface technique and suffers from interferences due to fluorescence (of i.e. humic substances) as major drawback. The advantage of Raman over FT-IR is the minimal detectable particle size. Raman microscopy is capable of detecting particles down to 1 μm [9]. This advantage of Raman creates also another big challenge. Detectable particle numbers of the sample are for Raman thus much higher than for FT-IR leading to much higher measurement times [9,10].

Scanning of entire filters or measurements of every single particle are neither possible nor feasible for both Raman and FT-IR microscopy. Time required for recording both spectra and images is very time consuming. For Raman, measurement times can even add up to months or years, depending on the one hand on chosen parameters and on the other hand on the total number of particles on the filter. Even with fully automated procedures the measurement times exceed any reasonable time frame for routine analysis. Thus, the measurements of entire filters are not feasible and it is necessary to define particle number or filter area rates which are representative for the sample entity. There are different approaches for the generation of representative data sets including determination of filter area or total particle numbers [8]. Both approaches require a sufficient number of particles or filter area to be representative for the sample entity. Therefore, a template was developed covering between 8 and 20% of the total filter area. The scheme of the template can be seen in Fig. 2. Each microscopic image has a size of 4 mm² (2 × 2 mm). Up to 21 microscopic images can be placed along a helical path across the filter. This positioning of the individual microscopic images was chosen to avoid false quantification due to local particle hotspots as the results of all microscopic images is averaged before extrapolation to the entire filter area.
The limit of detection (LOD) can be calculated via a hypothesis test. Therefore, two assumptions were made: I) homogenous distribution of particles on the filter and II) successful detection of a particle when it is located inside an area being measured. Due to the second assumption, the particle LOD (95% detection probability) is not dependent on particle size, but solely on particle numbers. The LODs were determined as 36 particles per filter (for 8.2% of the filter area) and 13 particles per filter (for 20% of the filter area). The calculation of the hypothesis testing is shown in the SI.

Very high hydrophobicity of MP particles poses a serious problem for a reliable detection. MP, especially pristine particles, show high aggregation potential as the surface is not weathered or coated and thus highly hydrophobic. These particles tend to aggregate or stick to all kinds of surfaces. To overcome this, a surfactant (0.05w% NovaChem SF100) was added for suspension stabilization and to prevent particles from clogging. Stabilization is on the one hand important for a quantitative transfer of all MP particle in the sample and to minimize particle loss. On the other hand, it is required to generate a homogenous distribution of MP particles on the filter. Another advantage of stabilized suspensions is that aliquoting defined volumes with known particle numbers is possible. This is the basis of the following validation. Method development and choice of the detergent is described elsewhere [11].

Fig. 2. Template of helical layout of the single measurement areas. The diameter of the filter is 25 mm and the area of a single measurement area is 4 mm². The total area of the red squares accounts for 8.15% of the entire filter area and 7.33% for the green squares. The numbers code the coordinates of the centre of each measurement area, if the centre of the filter is defined as 0/0. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
A stabilized reference suspension of PS MP-particles was generated by cryo-milling (CryoMill, Retsch, Germany) transparent polystyrene pellets (Polystyrol 158 K, BASF, Germany) resulting in irregularly shaped microplastic particles (<200 μm), which were stabilized with a surfactant. The particle concentration was determined via a particle counter (SVSS, PAMAS, Germany) by light extinction in a laser-diode sensor (type HCB-LD-50/50). Detailed information is given in the SI. This reference suspension was used for all experiments dealing with the determination of reproducibility, accuracy, and correctness of the developed method.

Method validation

As contamination with artefacts is a major issue, [12,13] blanks were measured to determine if and how many particles were introduced during sample preparation and measurement. Therefore, 100 mL of type 1 water with 0.05% surfactant (SF100) were filtered on an AnoDisc™ membrane and ten single microscopic images (red squares in Fig. 2, each image had a size of 4 mm², equalling 8.2% of the total filter area) were examined for MP particles. In an additional experiment blanks for the oxidising solution (10 mL of H₂O₂:H₂SO₄ (1:3, v:v)) were also recorded. In all investigated blank samples, no MP could be detected, although the whole procedure was not carried out under clean bench conditions, but samples were covered with glass lids or aluminium foil when applicable. This shows that airborne contamination with particles >20 μm is very unlikely and does not play a significant role for detection via FT-IR microscopy.

Reproducibility was determined by triplicate measurements of an aliquot of the reference suspension. Precision was determined once with a density separation (sample matrix was sand and MP) and once without. Both relative standard deviations were <20% (16.5 & 19.4%), which were deemed acceptable. Recoveries were determined for a sample (spiked with reference suspension) which was once directly filtered on an AnoDisc™ membrane and once following an oxidative treatment (H₂O₂: H₂SO₄; 1:3 for 1.5 h at room temperature). Both experiments yielded acceptable recoveries 88.4% (540 detected PS-particles, no sample pre-treatment, RSD 16.5%, n = 3) and 101.1% (190 detected PS-particles, oxidative treatment RSD 35%, n = 3). As expected, a lower standard deviation was observed with increasing total particle number. The recoveries for both experiments were within measurement uncertainty and thus the deviations were not caused by bias but by random distributions and particle numbers. A further indication for the confirmation of this hypothesis was obtained during comparative studies. Samples analysed within the comparative studies which used different methodological setups require much higher mass- and thus particle - concentrations to overcome their high limit of detection [14], in particular for spectrometric approaches. This results in samples with very high particle concentrations which due to overlapping particles cannot be correctly determined by single particle techniques (FT-IR and Raman).

Conclusion

FT-IR microscopy is a robust method for low to medium particle numbers (150–1000 particles per sample). Higher particle numbers facilitate clogging and heterogeneous distributions, which hinder correct quantification as clogged particles evade detection. Thus, at high particle concentrations, an underestimation of MP numbers is likely. This can be seen in Fig. 3 as the numbers of small particles are determined one to two orders of magnitude too low, whereas for bigger particles (50–100 μm) the opposite was observable, leading to the conclusion that the particles were not homogenously distributed and clogged during sample preparation albeit surfactant was added.

Sample preparation did not show a significant impact on recoveries or precision of the detection method. As only particles (and fragments) with sizes >20 μm can be detected via FT-IR microscopy, issues with blanks or artefacts were not observed. Small airborne MP particles or microplastic fibres and very small suspended MP were not captured with this FT-IR microscopic method.

In comparison to other spectrometric approaches, this methodology of MP detection via FT-IR microscopy has several benefits, but also some limitations: Detection via Raman microscopy captures particles up to 1 μm in size, but suffers from laborious sample preparation. This sample preparation is required to avoid blanks and fluorescence of the matrix. Additionally, every microscopic method
Fig. 3. Part A: shows the results for the determination of precision and part B the results for determined particle recoveries. Therefore, aliquots of a PS reference suspension (provided by TU Berlin) were used to spike blank samples. The blue bars represent the results obtained by this reference suspension. The purple displays the precision of an unknown sample, which was provided by TU Berlin and underwent a density separation with saturated NaCl solution prior to measurement.

Part C displays the results for correctness, which was determined by comparative samples, which were provided by TU Berlin. Reference particle numbers were unknown at the time of measurement. Yellow, green, and red bars represent a different sample and polymer material.

Yellow and green code samples with PE and red represents a sample containing PP. Striped bars are reference particle numbers, which were provided by TU Berlin. Filled bars represent the measured particle numbers. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

faces issues regarding heterogenic particle distribution on the filter material and clogging due to high particle numbers. The issue with high particle numbers extends with the decreasing size of detectable particles. This can be explained by the fact that particles <20 μm are by one to two orders of magnitude more abundant than particles >20 μm resulting in a higher number of particles which have to be investigated.

Spectrometric approaches as pyrolysis GC–MS or thermal-extraction desorption (TED) GC–MS can be fully automated and have thus a higher sample throughput. Limitations are high Limits of Detection and Quantification (LOD and LOQ). Furthermore, pyrolysis products of the matrix can interfere with quantification and size distributions of MP particles cannot be determined.

This FT-IR methodology provides an easy and robust method which can be applied in all laboratories without restrictions. Sample preparation and measurement are in an acceptable time frame for research purposes (one-day sample preparation, four to eight hours measurement time) and do not suffer from any major limitations. Depending on the analytical question this method can be applied for all kinds of environmental samples and particle size down to 20 μm.

Declaration of Competing Interest

The authors state that there is no conflict of interest regarding this work.
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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.2020.100874.

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