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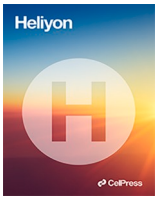

Research article

Raman microspectroscopy and laser-induced breakdown spectroscopy for the analysis of polyethylene microplastics in human soft tissues

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ABSTRACT

People are exposed to microplastics (MPs) on a large scale in everyday life. However, it is not clear whether MPs can also be distributed and retained in certain tissues. Therefore, the development of analytical methods capable of detecting MPs in specific human organs/tissues is of utmost importance. In this study, the use and combination of spectroscopic techniques, namely Raman microspectroscopy and laser-induced breakdown spectroscopy (LIBS), was tested for the detection of polyethylene (PE) MPs in human tonsils. Preliminary results showed that Raman microspectroscopy was able to detect MPs down to 1 μm in size and LIBS down to 10 μm. In the next step, human tonsils were spiked with PE MPs, and digested. The filtered particles were analyzed using Raman microspectroscopy and LIBS, and complemented by X-ray fluorescence (XRF). The results showed that Raman microspectroscopy could reliably detect PE MPs in spiked human tonsils, while LIBS and XRF served as a reference analytical method to characterize particles that could not be classified by Raman microspectroscopy for their non-organic origin. The results of this study, supported by a current feasibility study conducted on clinical samples, demonstrated the reliability and feasibility of this approach for monitoring MPs in biotic samples.

1. Introduction

Microplastic pollution is an important global issue, as microplastics (MPs) have been found in many natural ecosystems around the globe and also in various organisms [1]. The latter has attracted the interest of researchers and society, as questions concerning a potential threat to human health have arisen [2]. Therefore, intensive research has been conducted to detect MPs in human tissues and

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organs in order to understand the human exposure [3].

Research has revealed that MPs may enter the human body through inhalation and ingestion. In fact, humans are continuously exposed to atmospheric MPs when inhaling, estimations range from 6 to 272 MPs/day [4]. After ingestion or inhalation, small particles (<2.5 μm) can even penetrate cell membranes. Consequently, MPs were detected in the human placenta [2], human lung tissue [5], nasal mucosa [6], blood [7], human stools [8], semen [9] and follicular fluid [10], implying that MPs may be widespread in the human body. MPs in the human body may generate oxidative stress and inflammation which are associated with an increased risk of cardiovascular and respiratory disorders and lung cancer [11]. According to experiments on laboratory animals, presence of MPs is linked to immunological response, endocrine disruption, lipid and energy metabolism alteration, and other disorders [12].

To understand the health risk, it is essential to reliably detect MPs in tissues and organs. However, detecting MPs within a human tissue can be challenging because of their non-homogeneous distribution, low concentrations, and broad range of sizes and shapes [13]. Until now, many analytical approaches have been adopted but all of them require a preliminary step – digestion of the investigated samples. So far multiple tissue digestion methods have been proposed, and their selection is based on the interaction of the used solution with the analyzed material. Some of the methods are either not very efficient in tissue digestion, or there is a risk that they will degrade MPs along biological matter [14]. Next step includes a detection of recovered MPs. The most common techniques for detecting MPs are optical microscopy, vibrational spectroscopy, and tandem mass spectrometry, each offering distinct advantages and limitations [11].

Optical microscopy encompasses stereomicroscopy, allowing the morphological and color determination and type identification. However, it has limitations when recognizing small MPs mixed with other materials. Fluorescence staining enhances MPs visibility but lacks specificity in identifying MPs types and may alter their natural characteristics. Vibrational spectroscopy, such as Fourier Transform Infrared Spectroscopy (FTIR), can detect MPs down to 20 μm [15]. Next, Laser Direct Infrared (LDIR) imaging can detect MPs also down to 20 μm [16], but it suffers from a narrower infrared band (ca. 1800–900 cm^{-1}) which might result in misidentification [17]. It also demands an extensive sample pretreatment. The last-mentioned spectroscopic method is Raman microspectroscopy which can detect MPs as small as 1 μm. Tandem mass spectrometry, specifically Pyrolysis-Gas Chromatography-Mass Spectrometry (Pyr-GC-MS), eliminates the need for sample pretreatment and analyzes additives. However, it is less effective for a small amount of MPs, non-homogeneous materials, and fibers in the sample. When selecting a method for MPs research, researchers should carefully evaluate these advantages and limitations in order to align the chosen technique with their study objectives and sample characteristics.

In this work, we aimed to develop an optimized protocol for a reliable identification of a specific type of MPs in human samples. For this experiment, polyethylene (PE) MPs were chosen as PE represents one of the most extensively utilized polymer across various industries worldwide [18] and PE has been previously identified as one of the most commonly detected polymer types in human tissue [5]. As human samples, neck tonsils were selected as a representative soft tissue that is easy to obtain in sufficient quantity and comparable quality. Tonsils are also important in MPs research as they can capture exogenic material and activate immunologic reactions. Previously, researchers investigated the possibility to detect polystyrene (PS) nanoparticles in human tonsils and emphasized the necessity for an improved sample preparation protocol due to the high likelihood of particle degradation during the standard tissue digestion protocols [19].

In the proposed procedure, we mainly focused on i) evaluating the effects of the digestion procedure on PE MPs, ii) determining the detection limits of selected methods and iii) using the optimized protocol to investigate the presence of MPs in model tissue (human tonsils). In the first part, alkaline digestion was chosen as it has been shown to be suitable for the digestion of soft tissue previously [20,21]. However, we focused on a comprehensive evaluation of the size change and morphological changes of PE particles due to the digestion process to assess a possible destruction of the particles. In the second step, Raman microspectroscopy and laser-induced breakdown spectroscopy (LIBS) together with X-ray fluorescence (XRF) were selected for the detection and characterization of PE MPs. LIBS and XRF were primarily selected for analyzing some exogenous (potentially non-polymeric) materials that cannot be classified with the available Raman libraries. Size limitations were identified for Raman microspectroscopy and LIBS. Addressing these limitations was crucial for improving the accuracy and reliability of our protocol for the identification of PE MPs in human tissue. Finally, the optimized protocol was applied to detect PE MPs in native and spiked (i.e., PE MPs added) human tonsils.

2. Materials and methods

2.1. Human tonsils

Human tonsils used in the experiment as model human tissue were provided by multiple departments at University Hospital Brno, Czech Republic (UHB), including the Department of Paediatric Otorhinolaryngology, Department of Paediatrics, Department of Otorhinolaryngology and Head and Neck Surgery, and Department of Internal Gastroenterology. Tonsils were used as model tissue solely for optimization purposes, not for verifying the presence of MPs. Consequently, samples were randomly selected and obtained from 4 underage patients (age range 8–12 years, two males and two females). To ensure compliance with ethical standards, all participating patients were required to provide informed consent by signing a consent form by their guardian. To prevent the sample contamination, no plastic materials were used during biopsies and sample transport. The samples were stored in glass jars filled with saline solution and sealed with glass caps. The sample preparation procedures were conducted at CEITEC Brno University of Technology (Czech Republic). The project workload (sample collection and handling) was approved by the Ethic Committee of the Faculty Hospital Brno on June 21, 2023. The approval is listed under number 03–210623/EK.

2.2. Microplastics

MPs used for the assessment of the digestion procedure effect (Section 2.3) were PE MPs in the form of fragments prepared by grinding of low-density polyethylene (LDPE) pellets (Sigma-Aldrich, USA) as they represent the most common MPs found in the environment [22]. Their particle size distribution was determined as described in Section 2.3. The chemical composition was initially confirmed by FTIR (Spectrum Two FT-IR spectrometer, PerkinElmer, USA) and it was confirmed that they were made of PE (data not shown). To determine the detection limit for Raman microspectroscopy and LIBS, PE MPs with a specific size range, namely 1–4 μm and 3–16 μm, were used. They were spheres obtained from Cospheric LLC (USA).

2.3. Digestion procedure

Based on the literature review, alkaline digestion with 10 % KOH solution was selected, as it offers an effective digestion of soft tissues [23]. Human tonsils were individually placed in separate glass jars and subjected to a 16-h digestion process using 9 % (w/v) KOH at a temperature of 50 °C. Following digestion, the digestate was filtered through a 0.45 μm cellulose membrane filter (Whatman, Merck KGaA, Darmstadt, Germany) using a vacuum pump connected to a filter funnel. The pressure applied during filtration was adjusted to prevent undesired filter permeation. Lastly, the filters were left to dry in a sterile fume hood overnight.

In the next step the effect of the digestion on MPs was evaluated. PE MPs (fragments, Section 2.2) were weighed to the nearest 50.0 mg in ten replicates, and the digestion procedure followed the same methodology as for tonsil digestion (described above). To evaluate the effects of the digestion procedure on MPs, we examined the changes in mass, size, and morphology of MPs before and after treatment. Changes in mass were determined in ten replicates based on the weight of the MPs before and after treatment. Particle size distributions were determined with a laser diffraction analyser (S3500 Bluewave, Microtrac, Germany) using the dry unit. The measurement was repeated ten times for each sample, and each repetition included the measurement of approximately 0.5 g of the sample. The results were expressed as mean number size distribution with standard deviations (which can reveal creation of new small particles due to fragmentation during the digestion procedure) and mean volume size distribution with standard deviations (which can reveal changes on the surface of particles and thus affect their overall volume). The statistical difference between the frequency of MP sizes before and after the digestion procedure for each size group was tested using the Mann-Whitney *U* test (OriginPro 2022b, OriginLab Corp., USA), and differences were considered significant if $p < 0.01$. Morphological changes were studied using a field emission scanning electron microscope (FE-SEM, Ultra Plus, Zeiss, Germany) at an accelerating voltage of 2 kV and an aperture size of 30 μm. Prior to analysis, samples were coated with a thin Au/Pd layer. The morphology of at least 30 particles was observed at 500x and 2000x magnification.

2.4. Detection of microplastics in tonsils

Each tonsil was divided into two halves; one served as a test sample with manually added PE MPs, and the other as a control sample. PE MP fragments, as described in Section 2.2, were introduced into a glass jar containing the test sample, and then the digestion process was carried out. This approach ensured that the microplastics and the sample underwent digestion simultaneously, allowing for an integrated analysis of the microplastics within the test sample matrix. Thus, both test and control samples were individually placed in separate jars and subjected to a 16-h digestion process using 9 % (w/v) KOH at a temperature of 50 °C as described in Section 2.3 and depicted in Fig. 1. After digestion and filtration, dried filters with MPs were placed on a glass Petri dish and carefully transported for further analysis using Raman microspectroscopy (Section 2.5), LIBS with XRF as its reference measurement (Section 2.6).

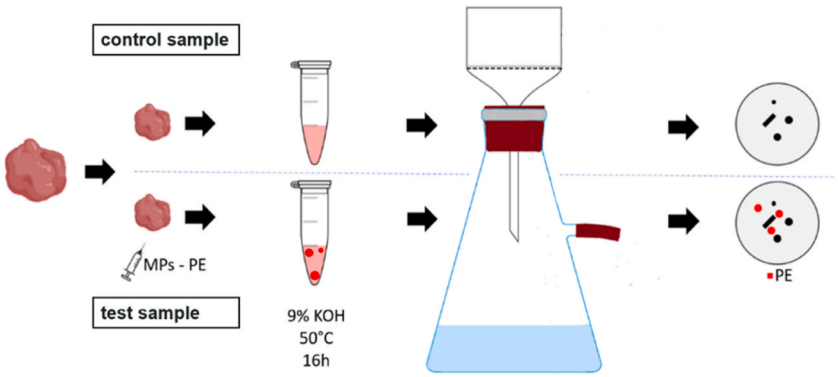

Fig. 1. Sample preparation protocol for subsequent analysis by spectroscopic methods. The workflow is optimized for PE particles. Control and test samples undergo the initial digestion in a 9 % KOH solution, followed by filtration using a vacuum filter. The analysis focuses on the residue collected on the filter to detect particle presence.

2.5. Raman microspectroscopy

Initially, the detection limit of Raman microspectrometer for the identification of MPs in terms of their size was determined by investigating the specified ranges of PE MPs (spheres, Section 2.2). The measurements were conducted using the RENISHAW inVia Raman microspectrometer (Renishaw, Wotton-under-Edge, UK). The setup consisted of Leica N PLAN EPI objective with 100x magnification (Leica, Wetzlar, Germany), a laser with a wavelength of 785 nm, laser power was set to 80 mW. Moreover, we opted for a CaF_2 slide as the substrate to mitigate potential issues related to glass fluorescence. The central Raman shift was fixed at 1200 cm^{-1}, and we collected 20 spectra per batch. To ensure the acquisition of precise and dependable Raman spectra, we judiciously selected an integration time of 1 s. This choice prevents the detector oversaturation, and the subsequent accumulations enable an extended overall acquisition time, ultimately resulting in an improved signal-to-noise ratio. A total of 10 accumulations were performed. During data processing, we applied rolling circle filtering with a radius of approximately 500 cm^{-1} and 20 passes. The spectra were smoothed utilizing Savitzky-Golay algorithm with the order set to 2 and a frame length 7. The spectra depicted in Fig. 3 were normalized at their maximum and stacked for better clarity. For Fig. 4, intensities of the PE peaks without normalization were plotted against the size of the particle. Intensity of the noise to calculate the signal-to-noise ratio was defined as standard deviation from the 1600–1700 cm^{-1} range as in this range no significant PE peaks were present. The samples under investigation were PE spherical MPs with two size ranges: 1–4 μm and 3–16 μm. A small amount of PE spheres was transferred onto the CaF_2 slide for measurement, ensuring that the laser was continuously refocused on the surface of each bead. Finally, the spheres were selected randomly, with a higher emphasis on the smaller ones. The size of the particles was determined with the utilization of a reference 2, 7, and 10 μm PS beads. The pictures of the PE MPs from the microscope were compared with the reference PS MPs. Utilizing the reference beads, the size was converted from pixels to μm using the "Measurement" function in the software ImageJ (ImageJ, Bethesda, USA). A line was drawn from one edge of the particle to the other (within the photo) and its length in pixels was correlated to its size declared by the manufacturer. This procedure was performed for 3 different beads for each reference size.

Further, the same instrument was used to investigate MPs recovered from tonsils after digestion and filtration. However, as the surface of the filter is uneven and the filter itself was not perfectly flat, Leica objectives with 20x (spiked and native samples) and 50x (only native samples) magnification were used to mitigate this negative effect of the surface on the analyses. The power of 14 mW (only control samples) and 140 mW (test and control samples) were used depending on the varying amount of fluorescence to avoid its overwhelming background signal, enabling flexibility in the experimental conditions. Other settings remained unchanged from the previous measurements. Meticulous attention was taken to ensure a precise laser focus on the surface of the particles. Particles of varying sizes were localized and measured while also being documented through photography. In some cases, photographs were taken using different magnifications to capture the particles in detail, ensuring proper scaling for accurate representation. 5 spectra were collected from spiked samples, 5–35 spectra collected from native samples. Data processing was the same as with the limit of detection of the PE MPs analysis.

2.6. Laser induced breakdown spectroscopy and X-ray fluorescence

For LIBS analysis a LIBS system (FireFly, Lightigo, CZ) was equipped with a 532 nm laser operated at 40 mJ. The pulse duration was 7 ns and the spot size was approximately 80 μm resulting in a fluence of approximately 230 GW/cm^2. The plasma emission was collected with an optical system and transported with an optical fibre to a Czerny-Turner type spectrometer (0.05 nm resolution) with a range of 240–410 nm equipped with a CMOS camera that was set to a gate delay of 500 ns and a gate width of 50 μs The interaction area was purged by air during the experiment.

For the analysis of clinical samples, obtained filters after digestion and filtration were placed on a glass slide and investigated using LIBS. Two filters were measured – with a test sample and a control sample.

However, for the estimation of the detection limit, a different sample preparation was needed to fixate the MP particles specified in Section 2.2. An epoxy embedment was selected as the sample preparation method as it also simulates a carbon-rich background for other parts of the experiment and also prevents particles from being scattered around when shot by laser. The area where MPs were embedded was scanned with 100 μm resolution on an 86 × 36 shot area. The resulting data set was analyzed using a custom python script with the employment of Principal Component Analysis (PCA). PCA is a dimension reduction method used widely in the LIBS community, it is useful especially in highlighting differences between measured spectra [24]. By transforming the data to low-dimensional space, additional methods like k-means clustering can be used to determine and characterize sample types in the data set.

Furthermore, XRF served as a reference to the LIBS and was used for MPs recovered from tonsils. The measurement was conducted using the Elvax Pro instrument from Elvatech on a 1.5 mm spot in diameter. A calibration-free method based on the fundamental parameter algorithm was used to calculate the elemental content.

3. Results

3.1. Digestion of tonsils and effect on microplastics

The selected digestion procedure was successful for digestion of human tonsils. There were not any larger pieces of the soft tissue that could cover MPs on the filter and potentially affect the detection.

In the next step, the effect of the selected digestion procedure on PE MPs (fragments, Section 2.2) was tested. The mass of PE MPs

did not change after treatment (0.0 %, n = 10), so no degradation occurred as a result of KOH digestion. The particle size distribution by number (Fig. 2A) and volume (Fig. 2B) showed no change in the size of the MPs either due to the digestion process. Statistical tests revealed no significant changes in the size of the various MP size groups, with the exception of a significantly increased frequency of size 352 μm (size distribution by volume) and sizes 88, 74, and 62 μm (size distribution by number) after treatment. In the absence of a trend or pattern showing, for example, a decrease in the frequency of larger particles and an increase in smaller particles, the differences were considered negligible.

FE-SEM images showed no significant changes in the morphology of the PE MPs. Both the MPs before treatment (Fig. 2C and D) and the MPs after treatment (Fig. 2E and F) had an irregular surface, but no cracks or other morphological changes were detected on the surface after digestion. From the results, we concluded that the digestion method had no effect on the physical properties of PE MPs and that it was suitable for use in MP research to digest soft tissue containing a wide range of MPs.

3.2. Detection limits of Raman microspectroscopy

The resulting spectra clearly displayed the main peaks of PE MPs, even for the smallest particle (1.57 μm) analyzed. As the size of the MPs increased, the intensity of these peaks and the signal-to-noise ratio also improved. Three examples of particles with different sizes (1.57, 3.46, and 15.84 μm) were selected to illustrate the spectra. The PE vibrations are clearly visible, with the smallest particle showing significantly more noise compared to the larger ones. Additionally, a noticeable peak around 1350 cm^{-1} is attributed to residual fluorescence from the glass in the objective lenses. This fluorescence occurs because the laser, which is line-shaped, likely has a larger focal plane than the particle. Consequently, some of the laser light is refracted back by the CaF_2 slide, passing through the glass lenses with higher intensity and causing the fluorescence. Before the normalization, the intensities of the highest PE peak (1296 cm^{-1}) were examined for all particles and signal/noise ratios were calculated (Fig. 4).

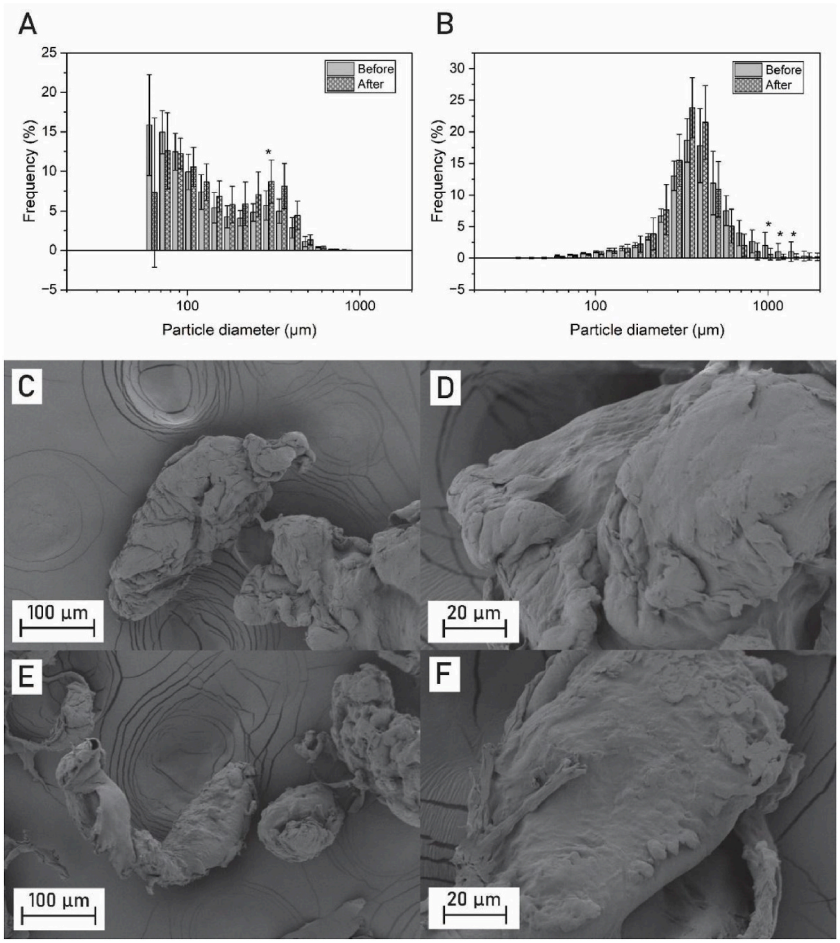

Fig. 2. Mean particle size distribution by number (A) and volume (B) of the microplastics before and after digestion (mean ± SD, n = 10), FE-SEM images of the MPs before digestion at the 500x magnification (C) and 2000x magnification (D), and after digestion at the 500x (E) and 2000x magnification (F). A statistically significant difference between the frequency of MP sizes before and after the digestion at a certain size group ($p < 0.01$) is denote by asterisks (*).

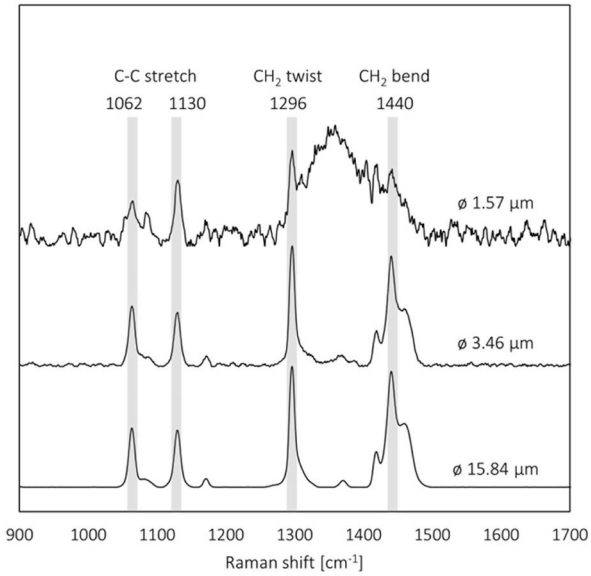

Fig. 3. Selected spectra of PE MPs (spheres, Section 2.2) with different dimensions. The main PE peaks are addressed. Spectra stacked for better clarity.

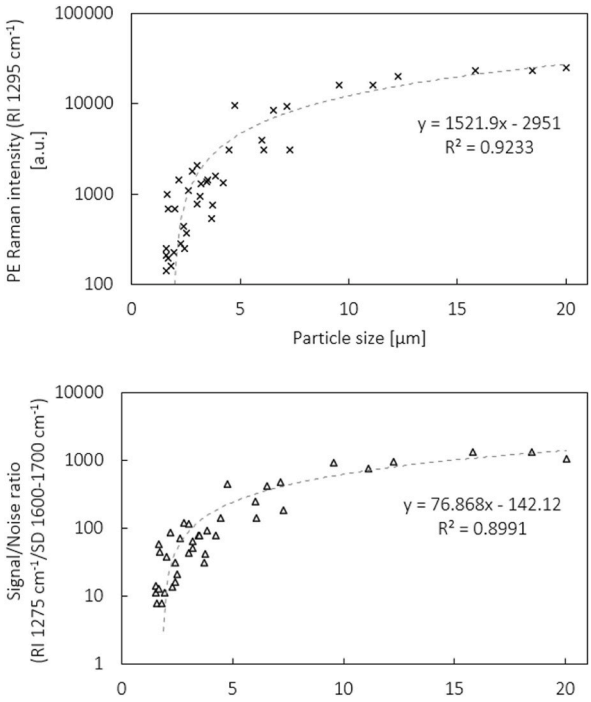

Fig. 4. Intensities of the main PE peak (top), Signal/noise ratios (bottom). The Y-axis in logarithmic scale for a better clarity.

Both the intensity of the main peak and the signal-to-noise ratio (S/N) increased linearly with the size of the particles, with coefficients of determination (R^2) of 0.92 and 0.90, respectively. Although the line shape of the laser likely contributed to the increased fluorescence background for smaller particles, the linear relationship between peak intensity and particle size remains evident. Thus, the intensity of the Raman signal can be used to estimate the particle size, even though an analysis of particles smaller than 2 µm is primarily qualitative.

3.3. Detection limits of laser-induced breakdown spectroscopy

The detection limit for LIBS was obtained by measurement of PE MPs (spheres, Section 2.2) embedded in epoxy. The reference PE signal was gathered by measuring the bulk PE sample (40 × 60 × 10 mm) beforehand. The Principal Component Analysis (PCA) was used to cluster acquired data and explore the mean spectra of found clusters. Three possible clusters could appear in the Principal Component (PC) space, where one would be clear epoxy, one PE MPs signal and the last one a mixture of signals of epoxy and PE MPs. A signal that can be assigned to MP particles was detected down to the group of 3–16 μm-sized MPs. Hence, the detection of MPs down to 10 μm by LIBS was successful. However, the signal of these smaller particles is typically a mixture of PE MPs and epoxy, caused by the larger spot size of the utilized system. On the other hand, a sample group of 1–4 μm-sized MPs was not detected by LIBS. In Fig. 5, 3–16 μm PE MPs with already clustered and labelled data in the PC space are depicted. The k-means algorithm used for separation of different matrices was set to three clusters. In the case of small particles, no clear MP signal could be acquired, that is why only two matrices are present. The boundary between gray and blue points in Fig. 5 is an arbitrary remainder from the k-means clustering set to three clusters and does not represent the change in the matrix.

The comparison of the mean spectrum of the smallest cluster with the bulk PE signal (Fig. 6) showed that the signals correlated to each other well. However, it has to be noted that the spot size of approximately 80 μm in the LIBS analysis prevents the ablation of only the MP particles of sizes 3–16 μm. Thus, there will always be an interference of the epoxy. Moreover, the clear PE MPs cluster cannot be found in the PC space for such small MPs. This is also visible in the spectra, as the PE bulk and PE MPs cluster from the 3–16 μm size group spectra have some differences. Nonetheless, the presented results have shown that the detection in epoxy by LIBS of PE MPs with an approximate diameter of 10 μm is possible with adequate data processing. Thus, this approach may be consequently applied to detection of PE MPs within biological samples.

3.4. Detection of microplastics in tonsils

The proposed methodology for detecting PE MPs in human soft tissues was further tested on several clinical samples. A test sample with added PE MPs along the control samples (native tissue with MPs spiking MPs) were subjected to digestion and filtration. The Raman microspectroscopy confirmed the positive presence of PE particles in test samples, while no MPs were detected in control tissue (Fig. 7). The Raman spectra from control tissues were compared with the available library of polymers spectra, and multiple mismatched spectra from particles localized on the filter were found. These spectra seem to have characteristic lignin peaks at 1600 and 1660 cm^{-1} throughout all control samples, which suggests the presence of particles from an organic material of non-clinical origin. Consequently, these regions were further analyzed using LIBS and XRF.

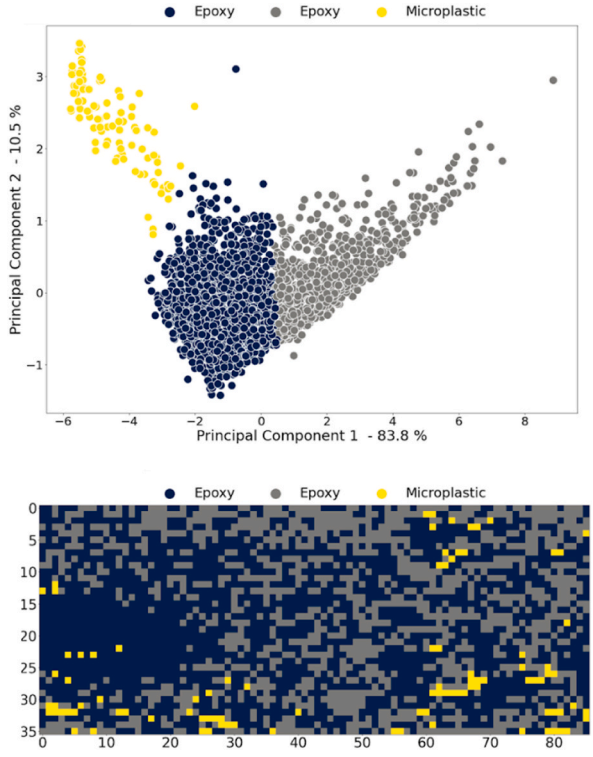

Fig. 5. PCA score plot for first two PCs of LIBS data from the 3–16 μm MP in epoxy. The yellow cluster contains spectra, where an MP signal was detected. Two groups for epoxy were selected for automatic clustering as it enables a better separation of MPs apart the epoxy.

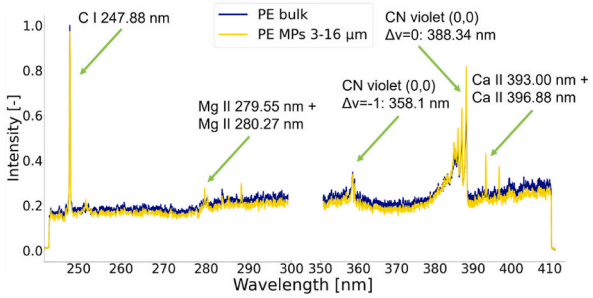

Fig. 6. Comparison of the mean spectrum of the MP cluster from PCA score plot and mean spectrum of bulk PE sample. The most important spectral lines are labelled.

LIBS did not show significant differences when comparing the control and test samples (Fig. 8) and thus could not detect PE MPs. However, the control sample exhibited higher Mg, Mn, and Si signals (Fig. 8B). XRF as a complementary measurement include chemical elements which most likely come from the digestion solution (K) or used filter (S, Mg, Al, Si). Moreover, Ba was detected in two control samples with concentrations of 0.07 % and 0.14 %, and P was measured in all control and test samples, with concentrations below 0.04 % and 0.01 %, respectively.

4. Discussion

The issue of plastic pollution, including the presence of MPs, has recently attracted considerable attention as these particles have been detected in various human body tissues. However, the development of reliable methods for the robust determination of MPs in human tissue is still ongoing. In this context, we introduce an alternative approach for the detection of PE MPs in human soft tissue that combines Raman microspectroscopy, LIBS and XRF. The first step in the successful recovery of MPs from human tissue is digestion. In the last decades, numerous digestion procedures have been proposed [20,25–27]. Recently, however, it has been found that not all of them are suitable, as some MPs may be affected by the procedure [26,28], so that their size, shape, and morphology may be altered. Often, studies investigating the detection of MPs in biological tissues do not include validation of their digestion methods. For example, a study on MPs in tonsils pointed out that the absence of particles could be due to degradation during sample preparation [19]. This oversight raises concerns about the accuracy of such studies, as unverified digestion methods may compromise the reliability of the results.

In this study we carefully reviewed available reports on digestion of human as well as animal tissues (e.g. fish, mussels) [2,29–31] and selected alkali digestion. Mass, particle size and morphology analysis, performed before and after the digestion step, confirmed that the PE MPs were not lost and remained unaltered, approving the suitability of the method. However, this method was only tested on MPs made from PE, another research showed that it had no degradation effect on PA, PC, PET, PMMA, PP, PTFE, PS, PSXL, PUR, uPVC, except for CA [20]. In any case, the effect of tissue digestion procedures on MPs is crucial to ensure that the integrity and properties of the particles are maintained during sample preparation. Failure to do so could lead to misinterpretation of results due to a possible degradation or loss of microplastics during the digestion process.

The next step in the analysis of MPs in human tissue is the detection of the recovered particles. The testing of detection limits showed that Raman microspectroscopy can detect PE particles as small as 1 µm. The reliable detection of PE MPs was consequently confirmed also for spiked tonsils. In addition, the analysis revealed some lignin material which could be fragments from wood spatula,

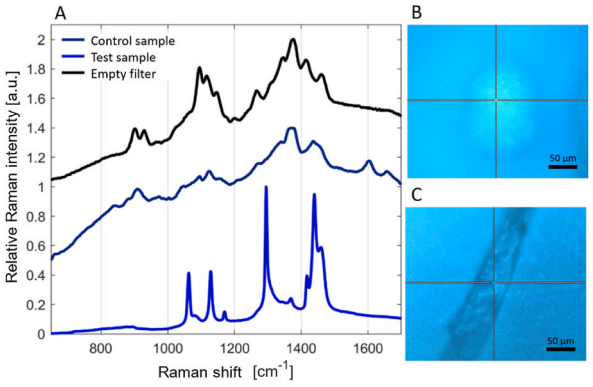

Fig. 7. The spectra of analyzed filters are the control sample, test sample, and empty filter (A). The spectrum of the test sample was obtained from the PE MPs shown in (B), and the spectrum of the control sample from particle (C) which was not classified during this measurement.

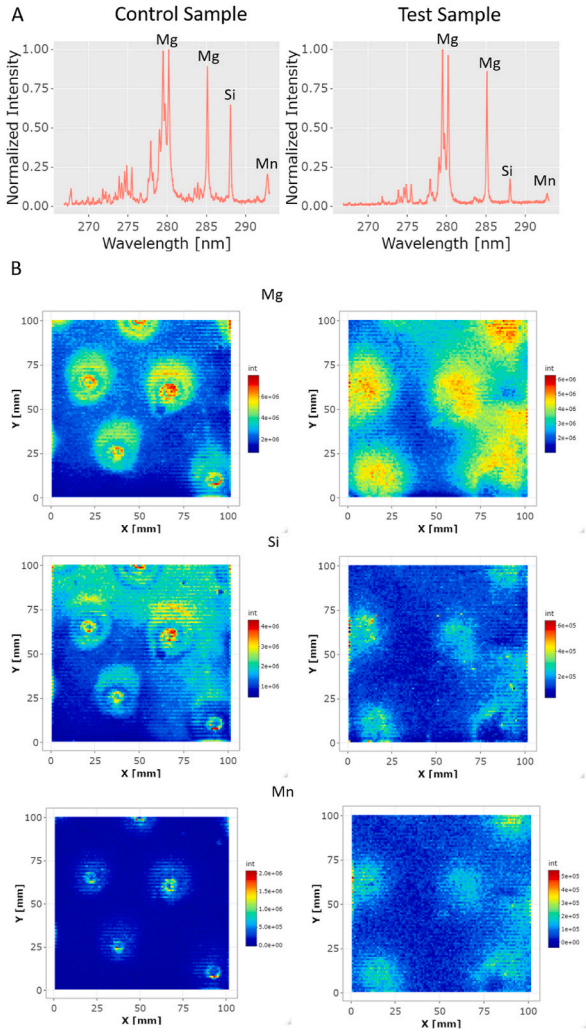

Fig. 8. LIBS measurement of the test and the control sample, (A) spectra and (B) elemental maps.

which are often used for tonsil investigation in human patients. Previously, Raman microspectroscopy was also successfully used for analysis of MPs in human lungs [5]. This confirmed the suitability of Raman microspectroscopy for analysis of human tissues. For detection of MPs, also FTIR can be used, but commonly sizes larger than 20 μm can be analyzed [15]. On the other hand, Py-GC-MS can be used to obtain data on the presence of MPs expressed as mass of MPs in investigated tissue but it cannot indicate the number of MPs present [32]. Therefore, Raman microspectroscopy seems to be a superior method for the detecting MPs distributed in human tissues, as particles between 4 and 20 μm are most likely to be accumulated in tissue [33]. Moreover, with these limitations it is possible to detect particles which can penetrate the cell membrane (<2.5 μm) [12]. Obtaining information about particles closer to the nanoscale dimensions might require some additional steps, such as mapping or collecting multiple spectra to be analyzed.

The use of Raman tweezers could also be valuable for the detection of nanoplastics, as successfully tested for the MPs detection in seawater [34]. Another possibility for the detection of nanoplastics could be the application of Surface-Enhanced Raman spectroscopy that was successfully employed for spiked water samples [35]. However, the detection of such small particles in tissues brings many challenges, especially in the sample digestion and filtration before the analysis. The risk of destruction or loss of particles is very high. In addition, measurements on the filter could be problematic due to the background signal and the risk of the filter being destroyed (burned) by the laser.

In the case of LIBS, the detection limit is higher, namely it reliably detects particles down to 10 μm. However, it has to be noted that LIBS measurement was efficient only when MPs were embedded in epoxy. Consequently, the sample preparation requires some additional effort. Therefore, the use of LIBS can be beneficial in the analysis of environmental MPs as previously demonstrated [36,37]. When MPs were retained on the filter after the digestion of tonsils with added PE MPs, particles jumped out upon impact of the laser pulse, indicating that the direct detection of MPs on a filter is unsuitable for LIBS. LIBS was successful for analysis of elemental composition of various particles that could not be classified using Raman microspectroscopy. Both LIBS and XRF confirmed the presence of Mg, Mn and Si across the samples. The majority of elements detected on control samples using XRF were also found on

empty filters, with the exception of two elements: P, likely originating from the tonsil tissue, and Ba, likely due to the sample contamination, accounting for the low concentration observed in the results. The detected elements are either also present in the filter or can be found in biological samples, contributing to the human body (except for lignin). Therefore, based on the results of Raman microspectroscopy, LIBS and XRF analysis, it was concluded that the native tonsils did not contain PE MPs. This confirmed that the tonsils were not contaminated during the procedure. This is important as airborne contamination is a serious problem in microplastic research and including proper controls is crucial to ensure the accuracy and credibility of the findings.

5. Conclusions

Analysis of MPs in human samples is challenging as there are not established procedures that could be routinely used. We have introduced an optimized protocol for detecting PE MPs in human soft tissue. The selected digestion procedure did not alter the size, shape, or morphology of the PE MPs. The size detection limits for PE MPs were determined to be 1 μm for Raman microspectroscopy, measured directly on filter paper, and 10 μm for LIBS, when embedded in epoxy. Raman microspectroscopy effectively identified PE MPs on filters obtained from test samples. LIBS and XRF supported conclusions about the non-polymeric origin of some particles found after digestion. The proposed protocol holds a significant potential for application to various plastic types. However, a successful implementation relies on optimizing the approach based on different MP types and sizes.

Data availability

All raw data used to create the presented figures and tables is available on Zenodo under the following link: https://doi.org/10.5281/zenodo.10794133.

CRediT authorship contribution statement

Viktória Parobková: Writing – original draft, Formal analysis, Data curation. **Daniel Holub:** Writing – original draft, Formal analysis, Data curation. **Martin Kizovský:** Formal analysis, Data curation. **Gabriela Kalčíková:** Writing – review & editing, Writing – original draft, Project administration, Methodology. **Ula Rozman:** Validation, Formal analysis, Data curation. **Milan Urík:** Project administration, Methodology, Investigation, Conceptualization. **Karel Novotný:** Formal analysis, Data curation. **Ota Samek:** Resources, Methodology, Funding acquisition. **Tomáš Zikmund:** Writing – review & editing, Visualization, Supervision, Methodology. **Pavel Pořízka:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Jozef Kaiser:** Writing – review & editing, Resources, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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