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A Robust Multistep Digestion Method for Microplastics Detection in Human Tissue by MicroRaman Analysis

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ABSTRACT

The presence of microplastics (MPs) in human tissues has raised growing concerns, necessitating robust protocols for their reliable extraction and analysis. This study systematically evaluated and optimized digestion protocols to efficiently process a variety of human tissues—placenta, lung, kidney, adipose tissue, muscle, spleen, liver, thyroid, and brain—while preserving the integrity of MP particles. Initial assessments employing single-reagent protocols such as nitric acid (HNO_3), proteinase K enzymatic digestion, and Fenton oxidative digestion demonstrated limited effectiveness, due to incomplete tissue breakdown or formation of turbid digestates that hindered filtration. Building upon these results, combined digestion approaches were investigated to improve organic matter removal and facilitate filtration through fine pore-size filters (0.2 μm). The optimized 3-day protocol included an initial oxidative Fenton digestion followed by enzymatic digestion (proteinase K). The final step involved lipid removal through ethanol addition and sonication, resulting in clear digestates amenable to filtration. This protocol efficiently digested complex tissue matrices, reducing filter clogging at 1-μm size pore and preserving various common MP polymers, including low-density polyethylene (LDPE), polyethylene terephthalate (PET), polytetrafluoroethylene (PTFE), and polyamides (PA6 and PA12). Application of the optimized digestion allowed successful isolation and characterization of MPs using optical microscopy and Raman spectroscopy. The method showed improved reproducibility and reliability over single-reagent protocols, making it suitable for comprehensive MP analysis in human tissues. The application of an efficient and robust protocol for tissue digestion may contribute to advance human exposure assessment and toxicological studies related to MP contamination.

1 | Introduction

Microplastics (MPs), defined as plastic particles smaller than 5 mm [1–3], have become a pervasive environmental contaminant, raising concerns about their presence in biological tissues and potential health impacts. Despite the extensive body of literature documenting the presence of these contaminants in human tissues [4, 5], there are currently no official protocols or standardized guidelines detailing how to process and analyze the diverse types of biological samples. As a result, many researchers develop and employ their own customized methods, which can vary widely in terms of sample preparation, digestion techniques, and analytical approaches. This lack of standardization complicates comparisons between studies and highlights the urgent need for validated universally accepted protocols to ensure consistency, reliability, and reproducibility in MPs detection and characterization within human tissues. Moreover, accurate identification and isolation of MPs from human tissues require effective digestion protocols that can break down organic material without degrading the plastic particles.

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In this study, an efficient and reproducible protocol for digesting human placenta, lung, kidney, adipose and muscle tissue, spleen, liver, thyroid and brain to be submitted to MPs spectroscopic analysis has been proposed. We have compared six digestion protocols based on acidic (HNO_3), basic (KOH), enzymatic (proteinase K), Fenton protocol, and a combination of them to treat human tissues. Combined digestion (proteinase K + Fenton) and KOH demonstrated suitable for filtration on a 0.2-μm pore size filter. We applied the optimized protocol to digest samples and to isolate and identify the MP debris present by optical microscopy and Raman spectroscopy.

2 | Material and Methods

2.1 | Chemicals

Chemicals used for tissue digestion were Tris-EDTA buffer solution, sodium dodecyl sulfate salt, calcium chloride 1 M, hydrogen peroxide 35%, sodium hydroxide 0.5 M, and proteinase K and were all purchased from Sigma-Aldrich – Merck (Darmstadt, Germany). Iron (III) sulfate pentahydrate was purchased from Acros Organics – Fisher Scientific (Hampton, New Hampshire, USA) and prepared in a 2.5-mM solution. Ethanol was provided by Honeywell Fluka – Fisher Scientific (Hampton, New Hampshire, USA). Nitric acid 65% v/v was purchased from J.T.Baker – Fisher Scientific (Hampton, New Hampshire, USA). Potassium hydroxide was purchased from Carlo Erba Reagents (Milan, Italy). All aqueous solutions were prepared with ultrapure water produced by ELGA PURELAB chorus 2 and filtered before use. Filters used for tissue filtration were Cytiva Whatman alumina anodisc filter discs 0.2-μm pore size, and Cytiva Whatman glass microfibre filter discs 1-μm pore size were purchased from Sigma-Aldrich – Merck KGaA (Darmstadt, Germany). Cellulose nitrate filters 0.45-μm pore size were from Sartorius Biolab Products (Goettingen, Germany). MPs used for recovery experiments were prepared in the Nanostructures & Optics Laboratory of the Department of Chemical Sciences at the University of Padova. The five types of MPs tested were low-density polyethylene (LDPE), polyethylene terephthalate (PET), polytetrafluoroethylene (PTFE), polyamide 12 (PA12), and polyamide 6 (PA6).

2.2 | Tested Digestion Protocols

The following six digestion protocols were tested: HNO_3, KOH, proteinase K, Fenton, and a combination of proteinase K + Fenton, Fenton + HNO_3. Adaptations in volumes and reagents amounts were tested to digest 1 and 3 g of material. Additionally, the required time and the obtained results of each method were compared to establish the most suitable digestion technique. Five types of MPs were tested for qualitative recovery efficiency testing by adding 2 mg of each polymer (LDPE, PET, PTFE, PA12, and PA6) to tissue samples before digestion. The tested parameters were number, size, and shape verified by optical microscopy, Raman spectroscopy, and SEM of the particles left on the filter after the digestion protocol. Biological tissue was included to avoid the precipitation of unreacted reagents during filtration.

Further details regarding filtration and instrumentation are provided in the Supplementary Material (Supplementary Text S1).

2.2.1 | Sampling and Optimized Sample Preparation

Samples of placenta, lung, kidney, adipose tissue, muscle, spleen, liver, thyroid, and brain were collected after approval of Bioethical Committee of University of Bologna (Prot.n. 0060792 04/03/2024) by using a plastic-free protocol to minimize contamination and stored at −20°C in aluminum foils until further processing. For analysis, defrosted material was sampled from the inner region of the organ. To optimize digestion conditions, both 1 and 3 g of tissue samples were tested. The optimized protocol for digestion was carried out over three consecutive days on 1 g of material, as follows: Day 1 (initial digestion): 1 ± 0.2 g of defrosted tissue was added of 20 mL of hydrogen peroxide 35%, sonicated for 10 min and incubated at room temperature for 3 h; at the end of the first incubation period, 10 mL of hydrogen peroxide 35%, 10 mL of NaOH, and 1.5 mL Fe^{2+} 2.5 mM were added. The day 2 (lysis and enzymatic digestion) of the protocol included the addition of 20 mL of lysis buffer (prepared with 0.2-mL EDTA and 0.2-g SDS dissolved in water) and 4 cycles of sonication (5 min) and incubation at 60°C (25 min). Two hundred microliters of 10 mM $CaCl_2$ and 7.5 mg of proteinase K were added before bath incubation at 60°C for 3 h. Another 2.5 mg of proteinase K was added to the solution and then the mixture was left at 60°C in a bath overnight. The last day (final digestion and lipid removal) included the addition of 20 mL of hydrogen peroxide and 10 min of sonication, followed by the addition of 10 mL of ethanol and 10 min of sonication. Before filtration, through 0.2-μm alumina Anodisc filter, the mixture was left to stand for at least 30 min (Figure S1 in SM).

2.2.2 | HNO_3 Protocol

Liu et al. [6] treated placentas with HNO_3 filtering the lysate on a 13-μm stain steel filter. Following their procedure, tissues (1 ± 0.5 g) were added to 15 mL of 11.5% (v/v) HNO_3 and incubated 48 h at room temperature. Final digestion occurred in a bath at 95°C for 3 h. Digestate filtration was not possible.

2.2.3 | KOH Protocol

KOH digestion was based on Ragusa et al. [7] protocol with modification of the solution volumes. Tissues (1 ± 0.5 g) were added to 15 mL of 10% KOH and incubated 1 h at room temperature and left to stand 48 h in a bath at 40°C. Filtration through a 0.2-μm alumina Anodisc filter was successful.

2.2.4 | Proteinase K Protocol

Enzymatic digestion protocol was adapted from the method developed by Cole and colleagues [8] for analyzing biota; it was further optimized for analysis of MPs in human tissues as follows: Tissues (1 ± 0.5 g) were added to 20 mL of lysis buffer (see optimized protocol) and 4 cycles of sonication (5 min) and incubation at 60°C (25 min). Two hundred microliters of 10 mM

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$CaCl_2$ and 7.5 mg of proteinase K were added before bath incubation at 60°C for 3 h. Another 2.5 mg of proteinase K was added to the solution and then the mixture was left at 60°C in a bath overnight. The digestate was filtered through 1-μm glass microfiber.

2.2.5 | Fenton Protocol

Tissues (1 ± 0.5 g) were added to 20 mL of 40% (w/v) H_2O_2 and incubated 1 h at room temperature. Ten milliliters of 0.5 M NaOH and 1.5 mL of 2.5 mM Fe^{2+} were added, and the sample was left to stand overnight at room temperature. Additional 20 mL of H_2O_2 were added before incubation for 3 days at room temperature. Digestate filtration was not possible.

2.2.6 | Combined Protocol Proteinase K + Fenton

This combined protocol was applied to 1 ± 0.5 g of tissue and followed the proteinase K digestion followed by Fenton digestion. Day 1 included lysis and enzymatic digestion by the addition of 20 mL of lysis buffer (prepared with 0.2 mL EDTA and 0.2 g SDS dissolved in water) and 4 cycles of sonication (5 min) and incubation at 60°C (25 min). Two hundred microliters of 10 mM $CaCl_2$ and 7.5 mg of proteinase K were added before bath incubation at 60°C for 3 h. Another 2.5 mg of proteinase K was added to the solution and then the mixture was left at 60°C in a bath overnight. On the second day of the protocol, 20 mL of hydrogen peroxide 35% were added and sonicated for 10 min before incubation at room temperature for 3 h; at the end of the first incubation period, 10 mL of NaOH and 1 mL of 2.5 mM Fe^{2+} were added. The last day (final digestion and lipid removal) included the addition of 20 mL of hydrogen peroxide and 10 min of sonication, followed by the addition of 10 mL of ethanol and 10 min of sonication. Before filtration through a 1-μm glass microfiber filter, the mixture was left to stand for at least 30 min.

2.2.7 | Combined Protocol Fenton + HNO_3

Tissues (1 ± 0.5 g) were added to 20 mL of 40% (w/v) H_2O_2 and incubated 1 h at room temperature. Ten milliliters of 0.5 M NaOH and 1.5 mL of 2.5 mM Fe^{2+} were added and the sample was left to stand 36 h at room temperature. Additional 40 mL of 11.5% HNO_3 were added before incubation for 6 h at room temperature. Digestate filtration was not possible.

3 | Results and Discussion

3.1 | Digestion Protocol Efficacy and Filtration

A comparison of six digestion protocols demonstrated significant differences in their ability to effectively digest human tissue and facilitate subsequent filtration for MP analysis. The digestion process was optimized on a 1-g sample. This optimization reduced reagent consumption and allowed for the use of more compact laboratory equipment. The tissues differed in several aspects, including their consistency, the presence of blood, and their aqueous and lipid fractions. The primary challenge was the complete removal of biological material, which often led to turbid solutions and filter clogging. The protocols led to different results in terms of digestion and filtration efficiency and interpretation of the Raman analysis that will be further discussed (also see Table S1 in SM). The nitric acid (HNO_3) and Fenton protocols proved unsuitable, as the resulting digestates were either particulate-laden or turbid, making filtration through a 1-μm filter impossible. Similarly, the combined Fenton + HNO_3 protocol yielded a turbid and chalky solution that was not amenable to filtration. While the proteinase K protocol successfully produced a brown solution, residual material remained on the filter surface, indicating incomplete digestion.

The most successful protocols for both tissue digestion and filtration were the potassium hydroxide (KOH) and the combined proteinase K + Fenton methods. The KOH protocol yielded a brown, filtrable solution (Figure S2 in SM), while the combined proteinase K + Fenton protocol produced a turbid and colored digestate. Although this digestate formed a yellow precipitate on the filter, likely from unreacted Fe^{2+} catalyst, it was successfully filtered (Figure S3 in SM). Because the suboptimal nature of colored filters for subsequent optical analysis, the optimized protocol's digestion sequence was reversed. Fenton digestion was performed prior to the proteinase K protocol to facilitate a more complete reaction and yield a colorless final product on the filter. The process successfully broke down biological tissues, including lipids, resulting in a solution that could be filtered cleanly. The digestion efficacy of both KOH and the combination of Fenton and proteinase K methods allowed for the use of both 1-μm glass microfiber filters and smaller 0.2-μm alumina Anodisc filters. Replicate digestions were performed to assess the reproducibility of the protocols. The combined protocol showed higher reproducibility than the KOH method. Despite this difference, samples from both digestion techniques were subjected to spectroscopic analysis to ensure a comprehensive evaluation.

3.2 | MP Recovery and Characterization

Qualitative recovery experiments were performed to verify that the optimized multistep protocol successfully broke down the complex tissue matrix and ensured the preservation of the physical and chemical integrity of the tested polymer types, including sensitive polyamides (PA6 and PA12). This successful preservation is crucial, as μRaman spectroscopy, the method used for MP characterization, is inherently a qualitative identification method and relies on the structural integrity of the recovered particles. Qualitative recovery was assessed using the following five common MP polymers: LDPE (low-density polyethylene), PET (polyethylene terephthalate), PTFE (polytetrafluoroethylene), PA12, and PA6 (polyamides). The use of different polymers is well-documented in existing literature. This is because the different behavior of MPs in biological digestion solutions varies depending on their chemical composition and resistance to the reagents used in the protocol. For example, LDPE is chemically stable and resistant to many chemical agents; PET has a higher density, and in the case of peroxide and alkaline solutions, it may undergo some superficial alteration; PTFE is highly chemically and thermally resistant under digestion conditions; PA12 and PA6 can be partially or completely dissolved by strong acid mixtures, while during enzymatic and alkaline digestion, polyamides may show degradation or loss

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of integrity. The optimized protocol and the KOH digestion, which both resulted in a filtrable digestate, were applied to a biological test tissue (placenta) added with a mixture of MP and submitted to optical and Raman detection. The particles and fibers were successfully identified on the filter surface. Optical microscopy at 4× and 10× magnification was used for morphological analysis, while Raman spectroscopy, with a 633 nm excitation and 10× or 20× magnification, allowed for the characterization of the recovered MPs (Figures 1–3 and Figure S4 in SM). Using the optimized protocol, 40 particles were inspected, revealing PAs in five particles and PET in five others. More detailed identification would have been possible at higher magnification (Figures 1 and 2). Using the KOH protocol, 40 particles were inspected, with 23 confirmed as PET, 1 as PTFE, and no PA detected (Figure 3). The MPs quali-quantitative recovery test proved successful for both protocols tested. However, digestion across all tested matrices was more reproducible using the combined protocol, likely due to variations in tissue composition. Moreover, the presence of PAs further confirmed the effectiveness of the optimized digestion protocol. These results demonstrate the suitability of the optimized protocol for isolating MPs from complex biological matrices, ensuring reliable and consistent recovery across diverse sample types.

The protocol proved effective for filtering digestates using filters as small as 1 μm, which are not typically used for tissue samples. The physicochemical characteristics of the tested MP were preserved, allowing for subsequent Raman analysis and identification. Establishing a single, standardized protocol for digesting various biological tissues is crucial for ensuring comparable and reliable MP research. By using the same method across different tissue types, we can reduce methodological variability and produce more consistent results. This approach helps to overcome challenges like differing tissue compositions and the presence of lipids and blood, which can hinder complete digestion and filtration. A unified protocol ensures that variations in MP presence are due to actual differences in the samples, not merely to the applied digestion process. This is essential for a homogeneous comparison of MP loads across different organs and sample types, ultimately improving the quality and integrity of scientific data in this field.

4 | Conclusions

In real-world biological tissues, digestion is tricky, and sometimes unreproducible results are obtained. Several methods are

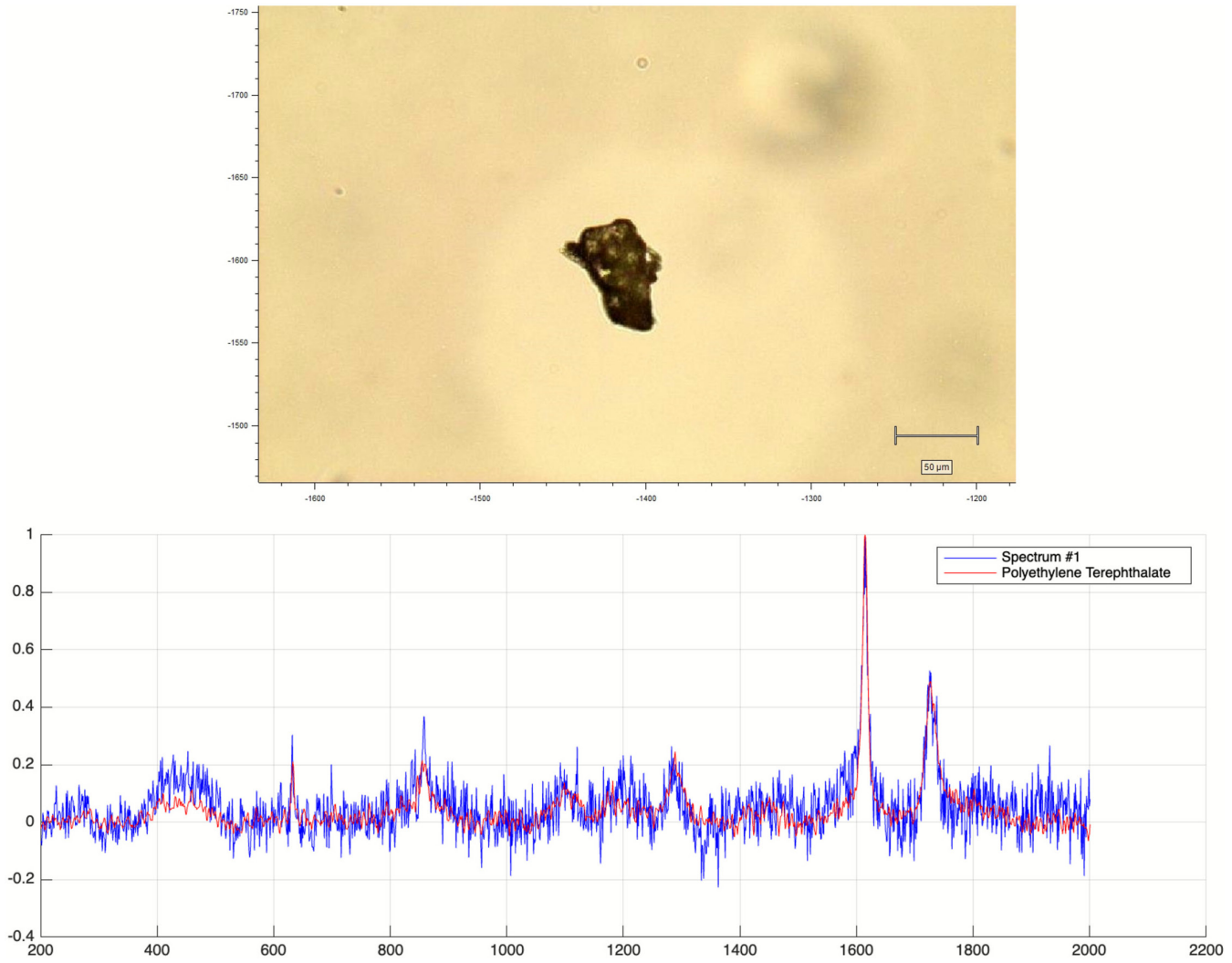

FIGURE 1 | A PET particle identified at 20× magnification by Raman spectroscopy in placenta tissues added of MP for recovery study after digestion with optimized protocol (Fenton + proteinase K).

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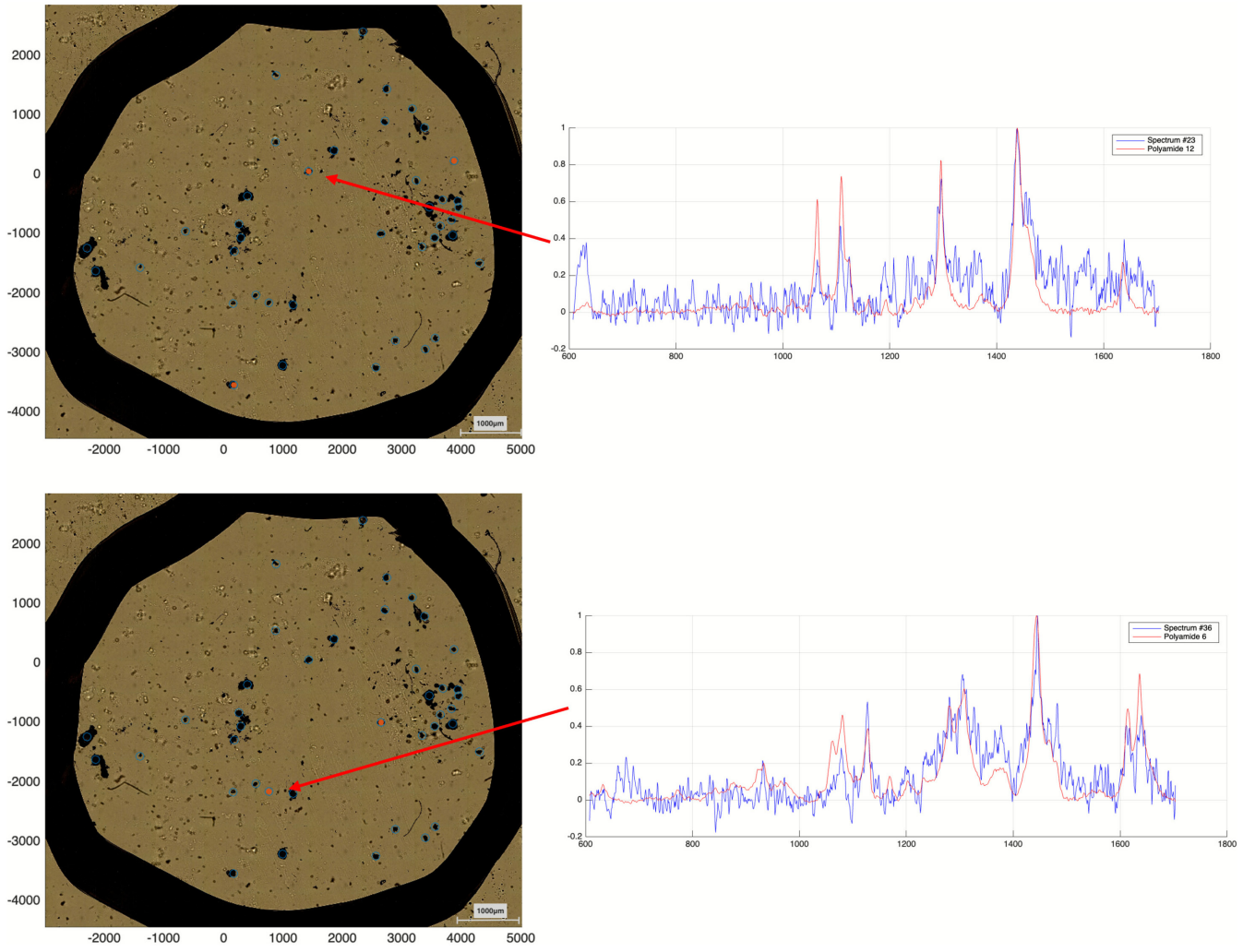

FIGURE 2 | PA12 and PA6 particles identified at 10× magnification by Raman spectroscopy in placenta tissues added of MP for recovery study after digestion with optimized protocol (Fenton + proteinase K).

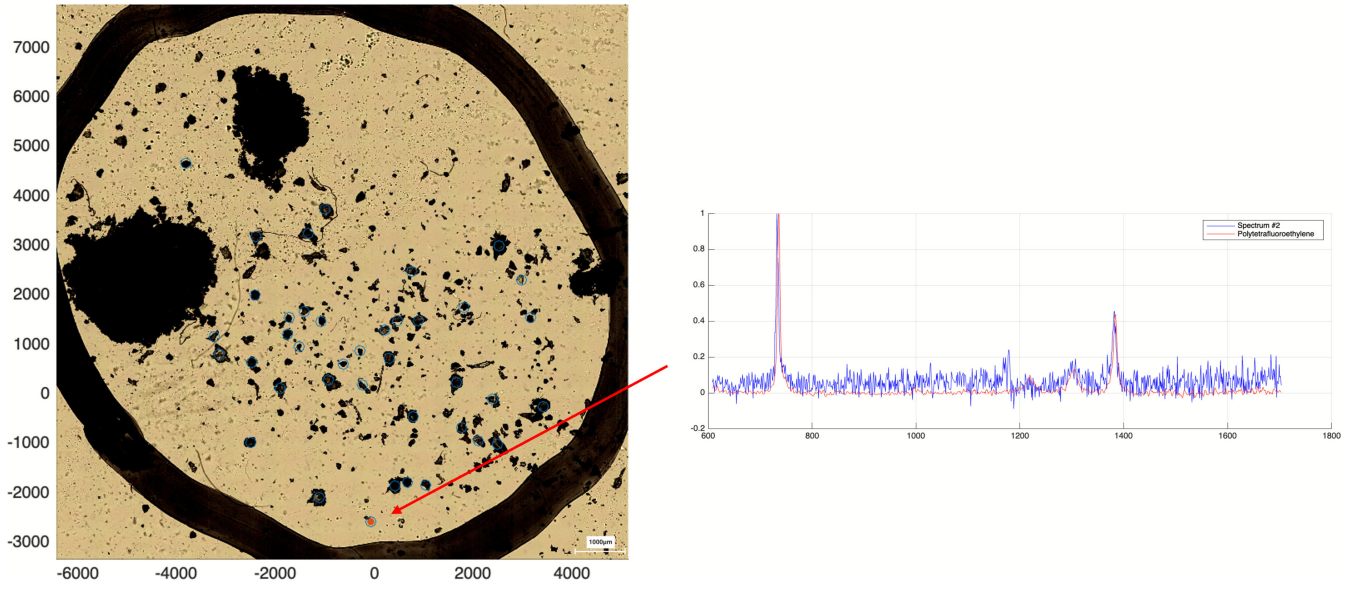

FIGURE 3 | A PFTE particle identified at 10× magnification by Raman analysis in placenta tissues added of MP for recovery study after digestion with KOH protocol.

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currently being developed for the efficient solubilization of solid materials. A major challenge is creating an extraction protocol that can effectively digest biological tissue without affecting the integrity of plastic polymers. In this paper, we addressed the need for a specific digestive protocol by using a combination of Fenton and proteinase K. Although time-consuming, the reproducibility of digestion was good for all the tested material, preserving physical-chemistry properties of the MPs, allowing for the detection of PAs, PET, and PFTE in different biological tissue.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Table S1:** Digestion protocols times and results. **Figure S1:** On the left the digested solution obtained from the optimized protocol Fenton + proteinase K before filtration; on the right, the 0.2-μm alumina Anodisc filter obtained by filtrating the solution of the optimized protocol Fenton + proteinase K. **Figure S2:** On the left, the digested solution obtained from the KOH before filtration; on the right the 0.2-μm alumina Anodisc filter obtained by filtrating the solution of the KOH protocol. **Figure S3:** On the left, the digested solution obtained from the protocol proteinase K + Fenton before filtration; on the right, the 1-μm glass microfiber filter obtained by filtrating the solution of the protocol proteinase K + Fenton. **Figure S4:** Optical microscopic analysis of placenta tissues added of MP for recovery study after digestion with KOH protocol. Left 4×, right 20×.

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