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InPlasTwin

Increasing expertise in micro- and nanoplastics analysis through twinning action

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D4.2 Knowledge exchange on MNPs extraction protocols: Developed working sample preparation procedure

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Abstract:	Deliverable 4.2 summarizes the activities carried out within WP4, focusing on knowledge exchange among all InPlasTwin project partners with the aim of developing working procedures for extracting MNPs from soil and strawberry samples. Three extraction procedures for soil were developed: a conventional approach using density separation and Fenton digestion for polyethylene MPs, and a novel two-step protocol combining TSPP extraction with gentle hydrogen peroxide digestion for conventional and biodegradable NPs, followed by an additional density separation step for biodegradable MPs. For strawberries, an alkaline digestion combined with enzymatic digestion, filtration and hydrogen peroxide digestion was adapted for biodegradable MNPs. Assessment of extraction efficiency using spiked samples confirmed the robustness of the proposed procedures, although challenges remain in addressing matrix interferences and improving specificity. This work represents an important step toward making the developed extraction protocols applicable for analysing MNPs in soil and strawberry samples in subsequent activities of the InPlasTwin project.

Document Revision History

Date	Version	Author/Contributor/ Reviewer	Summary of main changes
06-11-2025	1	Janja Vidmar, Katrin Loeschner	First draft
26-11-2025	2	Majda Nikezić	Description of the sample preparation procedure development conducted at DTU Food
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9-12-2025	5	Pia Leban	Description of the Procedure 2: Extraction of MNPs from soil, spICP-MS analysis
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List of Abbreviations and Acronyms	
MNPs	Micro- and nanoplastics
MPs	Microplastics
NPs	Nanoplastics
PE	Polyethylene
PP	Polypropylene
PMMA	Polymethyl methacrylate
PVC	Polyvinyl chloride
PS	Polystyrene
TSPP	Tetrasodium pyrophosphate
PBAT	Polybutylene adipate terephthalate
PLA	Polylactic acid
PTFE	Polytetrafluoroethylene
PET	Polyethylene terephthalate
SDS	Sodium dodecyl sulphate
DLS	Dynamic light scattering
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
spICP-MS	Single Particle Inductively Coupled Plasma Mass Spectrometry
IR microscopy	Infrared microscopy
QCL	Quantum Cascade Laser
pyGC-MS	Pyrolysis Gas Chromatography Mass Spectrometry
EtOH	Ethanol
KOH	Potassium hydroxide
HCl	Hydrochloric acid
HNO_3	Nitric acid
H_2O_2	Hydrogen peroxide
CH_3COOH	Acetic acid
Eu-PS NPs	Europium-doped polystyrene nanoplastics
NaBr	Sodium bromide
$CaCl_2$	Calcium chloride
$FeSO_4$	Iron sulphate
AF4	Asymmetrical Flow Field-Flow Fractionation
AF4-LS	AF4 hyphenated to light scattering detection
WP	Work Package
PES	Polyether sulfone
H_2SO_4	Sulfuric acid
GFF	Glass fibre filter
FTIR	Fourier Transform Infrared Spectroscopy

1. Introduction

Microplastics (MPs) and nanoplastics (NPs) have emerged as contaminants of global concern, not only in aquatic systems but increasingly in terrestrial environments and food commodities. Their presence has prompted extensive research into analytical methodologies for their quantification, characterization, and identification, processes that first require the effective isolation of micro- and nanoplastics (MNPs) from complex sample matrices. However, the diversity of polymer types, particle sizes, and the complexity of environmental and food matrices make the development of reliable extraction protocols highly challenging.

Several sample preparation procedures have been proposed for extracting MNPs from soils, typically combining density separation with chemical or enzymatic digestion to remove organic matter. Common approaches include the use of high-density solutions such as zinc chloride, sodium bromide, or sodium iodide for density separation, followed by oxidative digestion using hydrogen peroxide or Fenton's reagent (Radford et al., 2021; Schütze et al., 2022). Recovery rates of these sample preparation procedures vary widely depending on soil type, organic matter content, and polymer size and composition (Gündoğdu et al., 2025). For example, advanced workflows such as sequential enzymatic-oxidative digestion combined with freeze-drying and sieving have demonstrated recovery of particles down to 10 µm without significant damage to conventional polymers, yet biodegradable polymers like polylactic acid (PLA) degrade under protease treatment (Möller et al., 2022).

For food matrices, protocols often rely on enzymatic digestion (with the use of, e.g., pectinase, cellulase, or proteolytic enzymes) combined with oxidative or alkaline treatments to remove complex organic matter while preserving particle integrity. Studies have shown that enzymatic digestion can achieve high recovery rates (>90%) for various polymers when optimized for specific matrices (Courtene-Jones et al., 2017; Kwon et al., 2020). However, challenges remain in completely removing matrix components for spectroscopic analysis (e.g., micro-Fourier Transform Infrared Spectroscopy (FTIR) or Raman) (Aloia et al., 2025).

The absence of harmonized, polymer-specific protocols for MNP extraction from soils and food samples remains a major barrier to reliable and reproducible quantification, limiting comparability across studies. Existing methods often compromise particle integrity, particularly for smaller-sized MPs and NPs, and biodegradable, aged, or weathered plastics, which are highly susceptible to chemical degradation during oxidative digestion steps (Kubowicz & Booth, 2017). This issue is critical because biodegradable polymers such as PLA and polybutylene adipate terephthalate (PBAT) are increasingly used in agricultural applications (e.g., mulching films), yet the extraction of MNPs composed of these materials remains poorly standardized (Mhaddolkar et al., 2025).

Together, these challenges highlight the urgent need for improved, polymer-specific extraction methods that effectively isolate MNPs from complex environmental and food matrices while preserving particle integrity. Developing such protocols is essential for accurate risk assessment, environmental monitoring, and food safety evaluations.

The activities carried out within work package 4 (WP4) focus on knowledge exchange among all InPlasTwin project partners, aiming to develop robust working sample preparation procedures for extracting MNPs from soil and strawberry samples. This collaborative effort builds upon the experience gained in extracting MNPs from environmental and food samples during the hands-on trainings conducted in Tasks 4.1 and 4.2, as described in Deliverable 4.1. It also builds on online trainings and consultations conducted under Task 4.3 (Virtual Training: Enhancing MNP Extraction Techniques Knowledge Exchange), where researchers from VITO, DTU, and IMR shared their expertise

with JSI and AUA. During these efforts, extraction procedures were refined, particularly for MNPs generated from the degradation of mulching films, and adapted to ensure compatibility with specific analytical methods. This work represents an important step toward enabling the developed extraction procedures to be applied to the analysis of MNPs expected in soil and strawberry samples arising from mulching film degradation during strawberry cultivation. These applications will be addressed in subsequent phases of the InPlasTwin project through hydroponic (WP7) and field-scale (WP8) experiments.

2. Sample preparation procedures for extracting MNPs from soil

2.1. Selection of the sample preparation approaches

A series of sample preparation procedures for extracting MNPs from agricultural soil samples was tested during a one-month stay of JSI and AUA researchers at VITO (as described in Deliverable 4.1).

The first procedure (Procedure 1), suitable for extracting MPs composed of conventional polymer types, such as PE, consists of a density separation step, utilizing sodium bromide (NaBr) solution, and an organic digestion step with the use of Fenton reagent. This conventional approach was specifically tested on agricultural soil spiked with PE MPs (28-63 µm) derived from both pristine and naturally weathered PE mulching films, supplied by a Greek company, PLASTIKA KRITIS. This enables an assessment of the method's applicability under realistic agricultural conditions, particularly for strawberry cultivation. In the Western Peloponnese—and more broadly in Greece and other Mediterranean regions—strawberry growers predominantly use PE mulching films because of their durability and reusability over 4–5 consecutive years in the same greenhouse. Consequently, developing soil extraction protocols targeting MPs derived from PE films provides a realistic representation of the MPs expected in agricultural soils and ensures consistency with the strawberry experiment planned in WP8.

In the second procedure (Procedure 2), an approach to isolate NPs from soil matrices was tested and developed. For NPs, we hypothesized that their significantly altered physicochemical properties (e.g., higher surface area-to-mass ratio and surface charge) would result in distinct behaviour within the soil compared to their micro-size counterparts, necessitating a novel extraction technique. This new protocol utilized a dispersing agent, tetrasodium pyrophosphate (TSPP), to effectively disperse and stabilize the NPs in suspension. The resulting suspension was then subjected to mild organic digestion using hydrogen peroxide, which is expected to be a more subtle method than the Fenton reaction, thus reducing potential damage to the NPs. This extraction procedure was developed and tested first with NPs standards composed of conventional polymers, including metal-doped polystyrene (PS) NPs, which enabled the use of the spICP-MS method to evaluate the extraction efficiency during various steps of the protocol. At the same time, we also tested this protocol on a series of NPs that do not contain any metal tracers, to assess extraction efficiency using pyGC-MS, which is intended for NP detection in real samples.

Once the nanoplastics-specific workflow was established, it was expanded into Procedure 3, a unified method capable of isolating both biodegradable NPs and MPs from the same soil sample. Procedure 3 was further adapted for biodegradable MPs by incorporating an additional density separation step, followed by a second mild organic matter digestion with hydrogen peroxide. The proposed approach is also suitable for extracting biodegradable MNPs from soil samples, which are more susceptible to chemical degradation by aggressive reagents that can partially or completely degrade biodegradable polymers, leading to underestimation or loss of MNPs. This final approach was tested on MNPs

fraction <63 µm, produced from the cryo-milled biodegradable mulching film in WP3, in order to demonstrate the method's applicability for extracting biodegradable MNPs expected to be present in soil following strawberry cultivation with biodegradable mulching films in WP8. The two-phase approach, therefore, enabled comprehensive particle size fractionation from a single sample. Details of each extraction step of this procedure are discussed below.

2.2. Quality assurance and quality control

2.2.1. Contamination prevention

For all sample preparation procedures, the same measures were applied to prevent secondary contamination of MNPs. Before any extraction, all non-volumetric glassware and the stainless-steel meshes (28 µm and 500 µm) were combusted in a furnace at 480 °C for 6 h and subsequently covered with pre-burned aluminum foil. Plastic materials were avoided wherever possible. Tweezers and volumetric flasks were rinsed with ultrapure water, followed by rinsing with ethanol prior to use. All reagents, including NaBr, hydrogen peroxide (30% H_2O_2), and iron sulphate ($FeSO_4$), were filtered through 0.7 µm glass fibre membranes before use. For the extraction of NPs, the TSPP solution and 30% H_2O_2 were filtered through a 100 nm filter (NC10 Cellulose Nitrate Membrane filter, Schleicher& Schuell) or through a 100 nm syringe filter (polyether sulfone (PES) filter media, Minisart, Sartorius). The calcium chloride (CaCl2) solution was filtered through a 1 µm syringe filter (PES filter media, Puradisc, Whatman). Procedural blanks (x2 per sample treatment batch) were applied along with each batch and were treated in the same way as the soil samples.

2.2.2. Assessment of method recovery

To evaluate the extraction efficiency of each developed sample preparation procedure by pyrolysis gas chromatography mass spectrometry (pyGC-MS) and single particle inductively coupled plasma mass spectrometry (spICP-MS), various plastic materials were spiked into test soil samples prepared in triplicate (or in additional replicates when required). These spiked samples were processed alongside background soil samples that contained no added plastics.

The concentration of plastics recovered from the spiked samples was corrected by subtracting the amount detected in the background (non-spiked) soil. This correction ensures that the measured recovery reflects only the contribution from the intentionally added plastics rather than naturally occurring contaminants.

Method performance was assessed by calculating recovery rates using the following equation:

$$Recovery\ (\%) = \left(\frac{C_{spiked} - C_{background}}{C_{added}}\right) \times\ 100;$$

where C_{spiked} is the amount of plastic measured in the spiked soil sample,
$C_{background}$ is the amount of plastic detected in the non-spiked soil sample, and
C_{added} is the known amount of plastic introduced into the sample.

Additional performance indicators, such as false positive results, limits of detection/quantification, may also be evaluated depending on the analytical requirements of the study.

2.3. Procedure 1: Sample preparation procedure for extracting PE MPs from soil

A sample preparation procedure (Procedure 1) was developed for the extraction of MPs composed of conventional polymer types, such as polyethylene (PE; density 0.92 g cm^{-3}) or polymers with similar densities, from soil. All reagents were prepared prior to extraction. A saturated aqueous NaBr solution (400 mL; density 1.41 g cm^{-3}) was prepared by dissolving 259.55 g of NaBr in ultrapure water. A 0.05 M $FeSO_4$ solution (500 mL) was prepared by dissolving 7.5 g of $FeSO_4 \cdot 7H_2O$ in water; to ensure complete reduction of Fe^{3+} to Fe^{2+}, which serves as the active oxidizing species, 3 mL of sulfuric acid (H_2SO_4) was added. Before extraction, all reagents (NaBr, $FeSO_4$, and 30% H_2O_2) were filtered through a glass fiber filter, and all glassware and stainless-steel filters were rinsed and baked, as described in Section 2.2.1, to minimize secondary contamination with MNPs.

15 g of soil was weighed into a 50 mL polypropylene centrifuge tube. A volume of 40 mL of NaBr solution (density 1.4 g cm^{-3}) was added to the tube to perform density separation. The soil–salt mixture was vigorously shaken (200 rpm, 2 h) and then left to settle overnight to allow mineral particles to sink and lighter microplastics to remain in suspension.

After settling, the tube was flash-frozen using liquid nitrogen to immobilize the settled fraction, facilitating the clean decanting of the supernatant. The tube walls and settled material were then thoroughly rinsed with water (x3) to ensure maximal recovery of floating particles. The collected supernatant was poured through a 28 µm stainless-steel sieve under vacuum, which was subsequently rinsed with deionized water and ethanol to rinse off NaBr and remove smaller particles sticking onto the filter surface.

The material retained on the sieve was transferred into a beaker containing 20 mL of 0.05 M $FeSO_4$, followed by 10 minutes of sonication to release any particles adhering to the sieve. The stainless-steel filter was then rinsed with 3–5 mL of water into the sample, and the filter was placed in a Petri dish for subsequent use. To initiate Fenton oxidation, 20 mL of 30% H_2O_2 was added to the beaker. The reaction mixture was left overnight to ensure complete digestion of organic matter.

Following digestion, the suspension was filtered again through the same 28 µm stainless-steel mesh, and then sequentially through a 500 µm stainless-steel sieve to remove coarse particles that were not fully digested, along with any larger plastic fragments unintentionally present. The resulting filtrate was collected in a 100 mL or 200 mL volumetric flask and brought to volume with ultrapure water.

From the homogenized filtrate, an aliquot of 3 mL or 5 mL (subsample) was taken and filtered onto a glass fibre filter with a pore size of 1 µm (13 mm in diameter). The filter was carefully folded and placed into a pyrolysis cup (Frontier Laboratories, Japan) for subsequent pyGC-MS analysis to quantify and characterize MNPs mass content.

2.3.1. Assessment of the extraction efficiency

2.3.1.1. Spiking experiment

To evaluate the extraction efficiency of Procedure 1, a composite sample of control agricultural soil from Western Peloponnese was spiked with a known amount of PE MPs generated from conventional black PE mulching films (PLASTIKA KRITIS) using a cryogenic mill. Both pristine films and naturally weathered films (used for four years in strawberry field cultivation) were included. The resulting material was sieved to obtain the 28–63 µm size fraction. An amount of 3 mg of each type of PE MPs

was weighed into 15 g of soil using a microbalance, corresponding to an MNP concentration of 0.02% (w/w) in soil.

Agricultural soil (control and from the exposed site) was collected from strawberry fields in Western Peloponnese, Greece, in four replicates. Control soil, used for the spiking experiment, was collected from a field with no documented use of mulching films, while exposed soil was obtained from a field with extensive (8 years) use of PE mulching films in strawberry cultivation. Soil samples were dried at 40 °C over the weekend and subsequently sieved using 1 mm stainless steel sieves to remove plant debris and larger stones, as well as to identify any visible microplastics larger than 1 mm. Soil texture (sand, silt, and clay content) was determined, and carbon analysis was performed for each of the collected soil samples (Table 1).

Table 1. Properties of agricultural soil collected from Western Peloponnese, Greece.

		Soil texture			Carbon analysis (m/m % C ds)		
Description	**Sample ID**	% sand	% loam	% clay	Total carbon	Total inorganic carbon	Total organic carbon
Exposed soil- sample 1	C60	60.8	16.3	22.9	0.62	0.22	0.40
Exposed soil- sample 2	C61	57.5	17.2	25.3	0.48	0.04	0.45
Exposed soil- sample 3	C62	60.3	19.3	20.4	0.40	<0,05	0.40
Exposed soil- sample 4	C63	52.0	25.2	22.8	0.38	<0,05	0.38
Control soil - sample 1	S64	40.28	32.3	27.4	4.99	3.12	1.87
Control soil - sample 2	S65	54.6	23.6	21.8	4.84	2.75	2.09
Control soil - sample 3	S66	51.4	24.6	24.0	5.18	2.68	2.50
Control soil - sample 4	S67	52.51	27.5	20.0	6.06	2.59	3.46

2.3.1.2. PyGC-MS measurements

PyGC-MS was used to identify and characterize the polymer composition as well as the mass of the MPs extracted from the soil, thereby assessing the method recovery based on polymer mass.

The pyrolysis cups containing folded filters with soil extracts were loaded into the auto-shot sampler (AS-2020E, Frontier Laboratories Ltd.). While the pyrolysis cups were used without quartz wool for sample measurements, quartz wool (Frontier Laboratories Ltd.) was used for calibration standards measurement to prevent their loss from the cups. Pyrolysis was performed in flash mode at 590 °C for 0.3 min using the multi-shot pyrolizer (EGA/PY-3030D) under a helium atmosphere. The resulting pyrolysis products were transferred to a GC-MS system (Agilent 5977B MSD) equipped with a capillary column (DB-5Q, 30 m × 0.25 mm × 0.25 µm). The GC oven program started at 40 °C (held for 2 min), followed by a ramp of 10 °C/min to 320 °C with a final hold of 10 min. Helium was used as the carrier gas at a flow rate of 1 mL/min, and injections were performed in split mode with either a 50:1 or 100:1 split ratio. The MS was operated in electron ionization (EI) mode at 70 eV, with a scan range of m/z 35–500, and ion source and quadrupole temperatures set to 230 °C and 150 °C, respectively.

PyGC-MS data analysis was performed with polymer identification based on reference pyrograms and characteristic marker ions. Library matching was first conducted in F-Search (Frontier Laboratories Ltd.), where mass spectra of detected pyrolysis products were compared against reference libraries. Batch identification and quantification were carried out using the MassHunter Workstation software (Quantitative Analysis for GC-MS, version B.08.00/Build 8.0.598.0, Agilent Technologies) together with the NIST 23 mass spectral library. For each polymer marker, 3–5 characteristic ions were monitored simultaneously, and a single designated quantifier ion with a minimum signal-to-noise ratio of 10 was

used to integrate peak areas and determine polymer mass using calibration curves obtained from the calibration standard.

Calibration was carried out using external polymer standards (e.g., PE from Lab 261, CA, USA, for improved homogeneity), which were later compared to standards prepared from cryo-milled PE mulching film. All standards were analysed under identical conditions as the soil extracts, and sample concentrations were corrected for instrument response and sample mass.

For PE, the selection of appropriate marker compounds is essential because pyrolysis produces a broad series of oligomers (typically C_5–C_{30}), each appearing as characteristic triplets consisting of an alkane, alkene, and alkadiene (Figure 1). Since multiple oligomers can be used for quantification, a simultaneous batch evaluation was conducted to identify the most reliable markers. Oligomers in the range of C_{10}–C_{25} were selected for assessment, as this range is widely recognized to provide stable, high-intensity signals with minimal chromatographic interference. Within each triplet, the alkene peak was chosen as the primary marker because it consistently exhibited the highest peak intensity and the best signal-to-noise ratio, making it more robust for quantification across different sample matrices. In addition, the relative peak ratios among these oligomers were evaluated to detect potential false positives and to characterize polymer-specific patterns, which is particularly important for PE because various environmental or matrix-derived compounds can produce pyrolysis fragments that resemble PE signatures.

To further assess marker stability and ensure method reproducibility, five characteristic ion fragments were simultaneously monitored for each oligomer (m/z 57, 70, 83, 97, and 111). Monitoring multiple ions enabled verification that their relative ion ratios remained consistent across standards and samples, a critical criterion for confident polymer identification using mass spectrometry. Stable ion ratios indicate that pyrolysis and ionization conditions are well-controlled and that no significant matrix effects are altering fragmentation behaviour. This combined approach ensured that the selected markers were chemically reliable, analytically reproducible, and suitable for accurate and confident PE quantification.

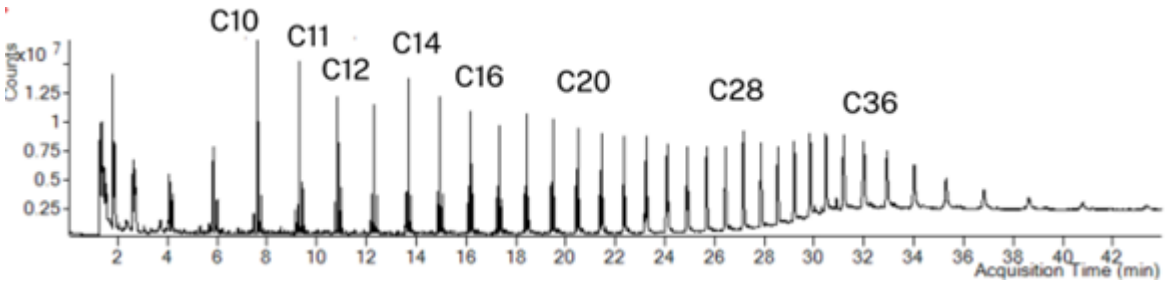

Figure 1: Pyrolysis GC-MS–derived total ion chromatogram (TIC) of polyethylene, illustrating its characteristic thermal degradation products.

Results from pyGC-MS confirm that PE can be detected in all analysed samples, i.e., spiked soil as well as control and exposed soil samples collected from strawberry fields in Western Peloponnese. Recoveries were calculated using control agricultural soil spiked with PE MPs (within the 28–63 µm size range), with corrections applied for the background soil signal to eliminate false-positive contributions from residual organic matter. The recovery was determined to be 31.5 ± 9.1% for PE MPs derived from pristine black PE mulching film and decreased to 11.5 ± 5.6% for those generated from field-weathered black PE mulching film.

Assessment of soils from strawberry fields in Western Peloponnese showed PE concentrations of 4.8 ± 3.6 µg/g in exposed soil from mulching film–applied fields and 8.9 ± 1.1 µg/g in control (non-exposed) soil samples. The control soil contained approximately six times more organic carbon (2.5% vs. 0.45% total organic carbon, Table 1), which likely accounts for the higher apparent PE signal due to stronger false-positive interference compared with the soil from the exposed strawberry field.

Based on the results obtained, the method currently demonstrates low analytical specificity. The pyrolysis of PE generates a range of oligomers, but these same compounds are also produced from the soil matrix, even after applying chemical digestion to remove organic matter. Consequently, distinguishing polyethylene-derived pyrolysis products from soil-derived ones remains challenging. Additional tests were performed to evaluate the influence of both the polymer and the soil matrix on the pyGC-MS signal. Preliminary results show that at present, the soil still contributes to nearly all components of the PE pyrolysis product profile, making it difficult to reduce background soil signals. Ongoing work will focus on introducing additional digestion steps or, more preferably, exploring alternative pyrolysis modes (e.g., double-shot or heart-cut) to improve specificity.

2.4. Procedure 2: Sample preparation procedure for extracting NPs from soil

2.4.1. Procedure development

Procedure 2 was developed for extracting NPs of various polymer compositions and sizes from the soil matrix and was first tested on NPs standards with different sizes and polymer compositions. The information on the NP standards used is gathered in Table 2.

Table 2. Nanoplastics standard used for the development of the NPs extraction protocol.

Polymer	**Polystyrene (PS) (Europium Chelate)**	**Polymethyl methacrylate (PMMA)**	**Polyvinyl chloride (PVC)**	**Polypropylene (PP)**	**Polyethylene (PE)**
Manufacturer	Bangs Laboratories, Inc. (9025 Technology Drive Fishers, IN 46038)	Lab 261 (265 Cambridge Avenue 60601 Palo Alto, CA 94306)			
Particle size	200 nm and 300 nm	219 nm	404 nm	56 nm	68 nm
Content of solids	1%	1%	1%	1%	1%

For the extraction of NPs, standard soil samples, including loamy sand, clay loam, and silt clay, purchased by LUFA Speyer, were used (Table 3). These soils were selected to expand the applicability of the extraction method to soils with varying physical and chemical properties (organic content, pH, particle size distribution, etc.) and to test the influence of different soil characteristics on the efficiency of NPs extraction.

Table 3. Chemical and physical characteristics of standard soils (LUFA Speyer, 2025).

LUFA Speyer soil type	2.1	2.4N	6S
	Loamy sand	**Clay loam**	**Silt clay**
Organic carbon [% C]	0.94 ± 0.15	2.36 ± 0.25	1.78 ± 0.21
Microbial carbon / total organic carbon in %	3.8 ± 0.4	4.1 ± 0.7	4.0 ± 0.8
pH value	5.00 ± 0.09	7.45 ± 0.08	7.32 ± 0.05
Particle size distribution (%)			
< 2 µm	3.1 ± 1.1	33.1 ± 5.2	40.3 ± 1.4
2 µm – 50 µm	11.1 ± 2.1	34.4 ± 1.9	36.3 ± 2.1
50 µm – 2 mm	85.8 ± 1.8	32.6 ± 5.8	23.4 ± 1.6

Tetrasodium pyrophosphate (TSPP) was selected as the extraction solution to disaggregate soil and release NPs, followed by organic matter digestion with hydrogen peroxide and subsequent nanofiltration. TSPP was chosen because it has been reported in the literature as an efficient reagent for extracting inorganic nanoparticles from soil samples (Yi et al., 2020).

Initially, various extraction parameters were tested and optimized, including approaches commonly employed to separate NPs from larger colloids (i.e., filtration and mild centrifugation), methods for pre-concentrating the samples, the volume of H_2O_2 used for organic matter digestion, and procedures for sample loading to pyrolysis cups.

2.4.1.1. Procedure 2a

In the initial test, the following protocol was applied: 2 g of soil was weighed into 60 mL glass vials. The samples were spiked with 100 µg each of PE, PP, PMMS, PVC, and Eu-PS NPs standards. Subsequently, 40 mL of 2.5 mM TSPP solution was added to each vial. The mixtures were shaken horizontally on an orbital shaker at 200 rpm for 2 h, followed by 5 min bath sonication. After sonication, the inner walls of the vials were rinsed with an additional 5 mL of TSPP solution, and the suspensions were left to settle overnight (Figure 2). The following day, 5 mL of the supernatant was filtered through a 5 µm syringe filter. An additional 3 mL of ethanol was filtered to rinse the filter and recover any NPs that may have adhered to it. The vials containing the samples were then covered with aluminum foil pierced with holes and placed in an oven at 60 °C for 24 h to evaporate the solvent and maximize NPs concentration (Figure 3). After 24 h, 10 mL of 30% H_2O_2, pre-filtered through a 200 nm filter, was added to the samples, which were then sonicated for 10 min. The samples were subsequently placed on a shaker for 24 h to digest the organic matter present (Figure 4, Figure 5).

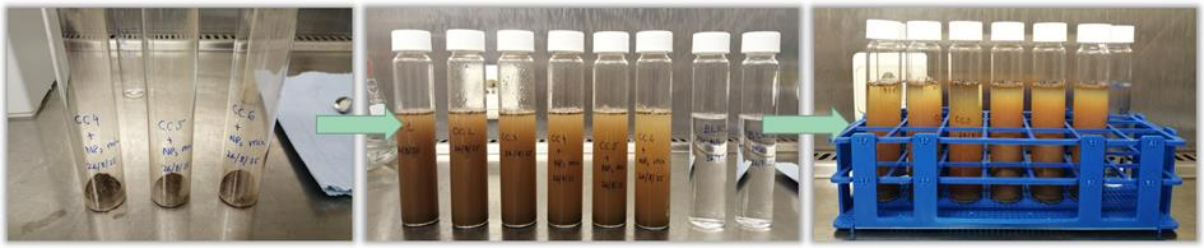

Figure 2. Soil samples (left), after the TSPP solution addition (middle), and after overnight settling (right).

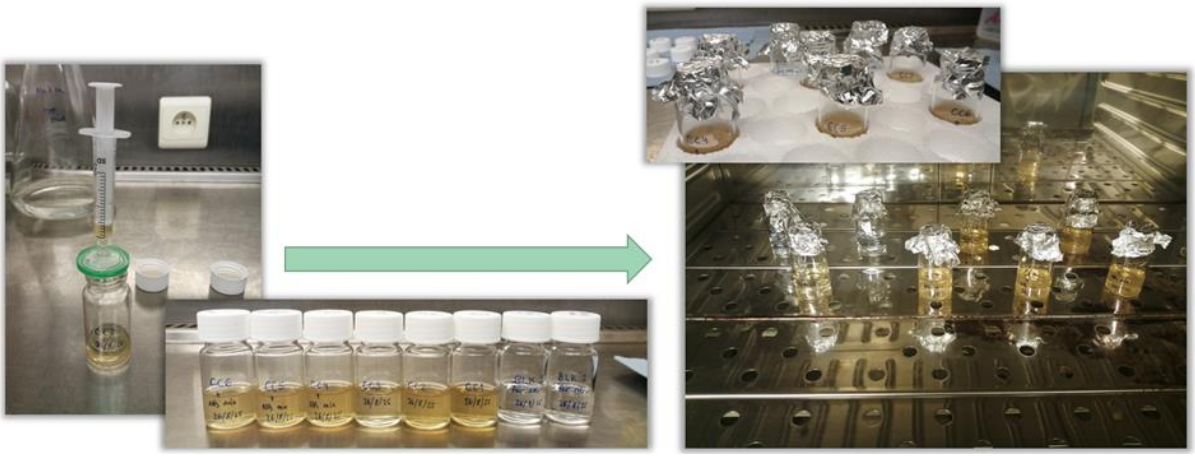

Figure 3. Filtration of the supernatant and sample drying in the oven at 60 °C for 24 h.

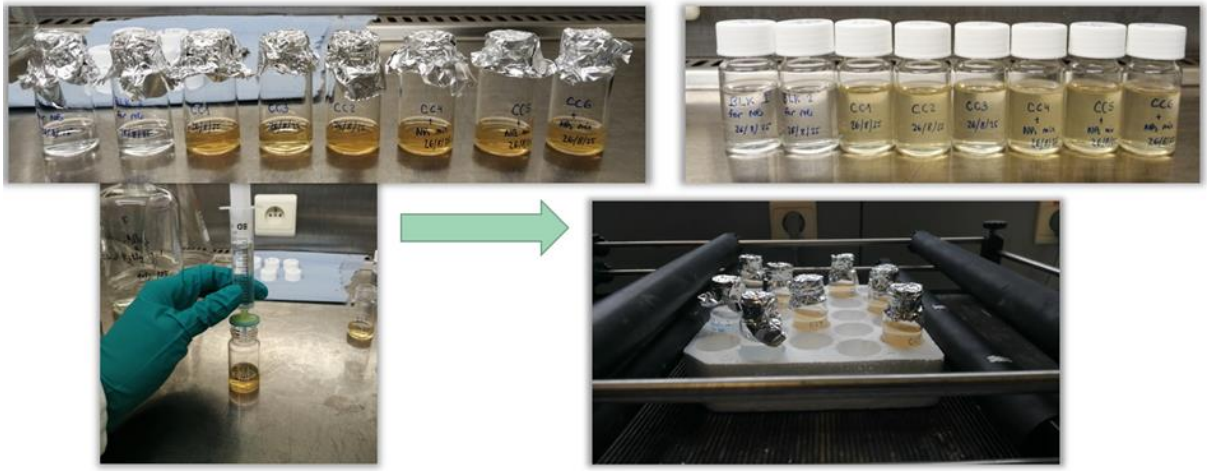

Figure 4. Digestion of organic matter with the addition of H_2O_2.

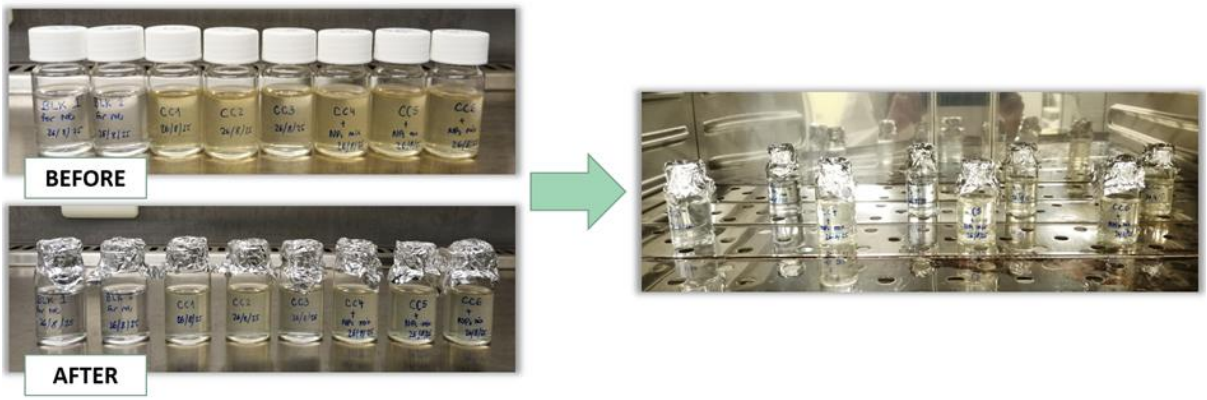

Figure 5. Samples before and after the organic matter digestion with H_2O_2 (left) and sample evaporation in the oven (right).

Prior to pyGC-MS analysis, which requires a limited sample volume, a pre-concentration step was performed to increase the detectable mass of polymer markers. The initial liquid samples (10 mL H_2O_2) were slowly evaporated in a laboratory oven maintained at 60 °C. This temperature was chosen to promote gradual solvent evaporation while minimizing thermal degradation of polymer constituents.

Due to the oven's low ventilation, the evaporation rate was slow, and reducing the sample volume from 10 mL to approximately 0.5 mL required more than a week.

Following pre-concentration, 50 µL aliquots were pipetted into an 80 µL stainless-steel pyrolysis cup (Frontier Lab, Japan). After each addition, the cup was returned to the 60 °C oven until the sample was fully dried to ensure complete removal of residual moisture prior to pyrolysis. This loading and drying procedure was repeated five times, yielding a total of 250 µL of concentrated extract in the pyrolysis cup. This stepwise approach prevents overflow and maximizes the amount of material available for pyrolysis and subsequent GC-MS analysis.

2.4.1.2. Procedure 2b

In the second test, an alternative approach was evaluated for separating NPs from soil residues by applying mild centrifugation instead of filtration. After overnight settling of the soil samples in the TSPP solution, 10 mL of the supernatant was transferred to 15 mL centrifuge tubes and centrifuged at 2000 rpm for 3 min (497 × g) to remove particles larger than 1 µm with densities exceeding 2.6 g cm^{-3}. From the resulting supernatant, a 5 mL aliquot was collected, to which 30% H_2O_2 (filtered through a 0.1 µm syringe filter) was added. In this approach, the volume of H_2O_2 used for organic matter digestion was also reduced from 10 mL to 5 mL (Figure 6).

In this second procedure, pre-concentration of the sample extract for pyGC-MS analysis was achieved using a filtration-based approach. The 2 mL sample aliquot was filtered through a 20 nm Anodisc membrane, allowing for the collection of NPs larger than 20 nm. This method was developed as an improvement over the first protocol, which required more than a week of oven drying and resulted in substantial particle loss due to thermal degradation and particle adhesion along the edges of the glass vials.

Following filtration, the Anodisc membranes were carefully fractured into small pieces using a sharp stainless-steel tweezer. To prevent the fragments from dispersing during this process, each membrane was placed on a pre-wetted glass fibre filter, which helped secure the Anodisc fragments in place. The glass fibre filter containing the fractured membrane pieces was then folded and transferred into a pre-burned stainless-steel pyrolysis cup for pyGC-MS analysis, following the same sample-loading procedure as described in the first protocol.

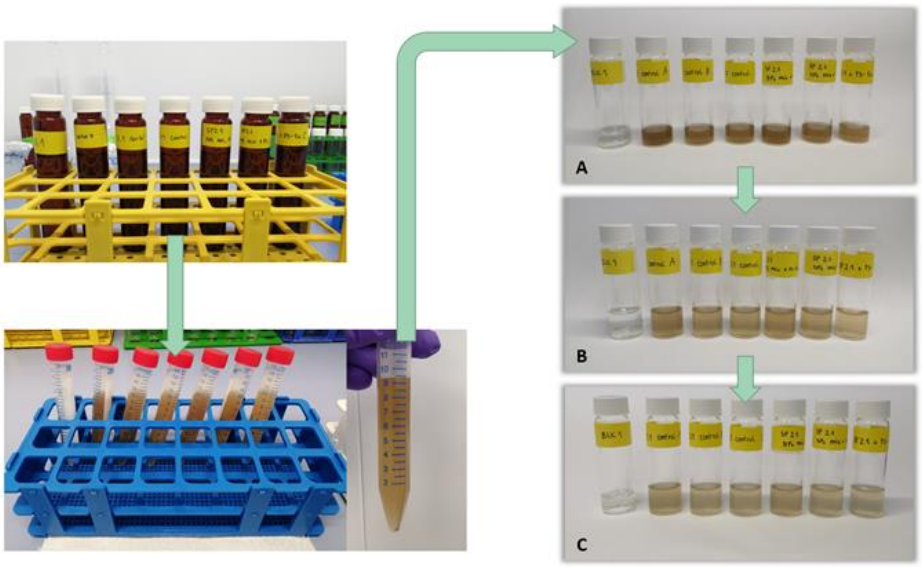

Figure 6. Second extraction procedure using TSPP, applying mild centrifugation to separate NPs from soil residues (left), followed by a comparison of the sample before H_2O_2 addition (A), after H_2O_2 addition but before 24 h digestion (B), and after 24 h digestion with H_2O_2 (C).

2.4.2. Assessment of the extraction efficiency

The extraction efficiency of the sample preparation Procedure 2 was evaluated using two analytical methods.

SpICP-MS, which enables the analysis of individual NPs based on their elemental composition, was applied to assess extraction efficiency in soil samples spiked with Eu-doped PS NPs. The spICP-MS technique provides information on particle number concentration, mass concentration, and particle size based on monitoring the europium isotope ^{153}Eu. The ^{153}Eu signal was recorded in fast time-resolved mode with 0.1 ms dwell time. Signal spikes from individual particles were converted to PS particle mass using ionic Eu standards and the known Eu content (1.67%) in Eu-PS NPs. Assuming spherical shape and PS density (1.06 g/cm³), particle diameters were calculated from the particle mass. The particle mass concentration was obtained by multiplying the particle number concentration by the mean particle mass. Data was analysed with MassHunter software (Version 5.3). During method development (Procedures 2a and 2b), subsamples were collected at different extraction stages to determine extraction efficiency by spICP-MS. In Procedure 2a, aliquots were taken before and after H_2O_2 treatment, while in Procedure 2b, aliquots were collected before and after mild centrifugation and after H_2O_2 treatment.

The second analytical method used to evaluate extraction efficiency was pyGC-MS, which is suitable for environmental samples containing non–metal-doped plastic materials. Owing to the high complexity of the samples and the method's sensitivity to matrix interferences, only samples from the final stage of the extraction procedure were analysed by pyGC-MS. In addition, the analysis of NPs in soil requires extensive sample pre-concentration to achieve sufficient sensitivity, given the low sample intake of the technique. PyGC-MS measurements were performed as described in Section 2.3.1.2, with the exception that a split ratio of 1:5 was applied instead of 1:50 or 1:100, in order to reach the required limit of detection. For the identification of 5 polymers, including PE, PP, PMMA, PVC, and PS, the identification and quantification ion markers are described in Table 4.

Table 4: The identification and quantification ion markers for pyGC-MS analysis of PE, PP, PS, PVC and PMMA.

Polymer	Pyrolysis products	Characteristic ions for identification m/z	Indicator ion for quantification (m/z)
PE	α,ω-Alkenes: n-C16-26-Alkadienes (calculated as mean from at least 5 up to 11 signals)	55, 97, [226 (M) – 366 (M)]	83
	1,20-Heneicosadiene	41, 55, 97, 296 (M)	83
PP	2,4-Dimethylhept-1-ene	43, 55, 70, 83, 126 (M)	126
	2,4,6,8-Tetramethyl-1-undecenes (3 isomers)	69, 83, 111, 210 (M)	
PS	Styrene	104 (M)	
	2,4-Diphenyl-1-butene	91, 208 (M)	
	2,4,6-Triphenyl-1-hexene	91, 117, 207, 312(M)	91
PVC	Benzene	78 (M)	
	Chlorobenzene	112 (M)	
	Napthalene	(64), 102, 128 (M)	128
PMMA	Methyl acrylate	55, 86 (M)	
	Methyl methacrylate	41, 69, 100 (M)	100

2.4.2.1. Procedure 2a

The extraction efficiency of Procedure 2a was evaluated before and after H_2O_2 digestion by analysing 300 nm Eu-PS NPs using spICP-MS. The results showed recoveries of approximately 46% for particle number concentration and 94% for particle mass concentration prior to H_2O_2 digestion. After digestion, these values decreased to about 39% and 49%, respectively. The digestion step also induced Eu leaching from PS NPs, as evidenced by an increase in the ionic Eu fraction from 4% to 30% and a decrease in median particle size from 435 nm before digestion to 405 nm after digestion. Double-peak size distributions for the 300 nm Eu-PS NPs, with two peaks appearing at approximately 440 nm and 600 nm, were observed both in the standard particle suspensions used for soil spiking and in the soil extracts. This indicates pronounced particle agglomeration already present in the stock suspension, which could not be mitigated by extensive sonication or shaking. Owing to this persistent agglomeration, the assessment of the extraction efficiency for Procedure 2b was conducted using 200 nm Eu-PS NPs.

The extraction efficiency of Procedure 2a at its final stage was also evaluated by pyGC-MS measurements of all five types of polymers spiked to soil samples. Across all spiked soil samples, PP, PS, and PMMA showed detectable pyrolysis markers in every replicate. PVC was detected in 4 out of 5 samples. PE was detected only through a limited set of markers (e.g., decene, tetradecene, pentadecene, hexadecene) with low signal intensity, but the characteristic triplicate pattern of alkane, alkene, and alkadiene series was not observed, and higher-chain hydrocarbons (e.g., heneicosene) were absent. Due to the incomplete marker pattern and insufficient diagnostic signals, PE was excluded from further quantification and assessment of method recovery (Figure 7).

Concentrations measured in the spiked samples were corrected for background by subtracting the levels detected in the corresponding unspiked soil. When no polymer was detected in the unspiked soil, the correction was instead based on the procedural blank. Although each sample was spiked with 100 µg of each NPs type (PE, PP, PVC, PS, PMMA), the recovered masses were consistently below 1 µg

across all polymers. PMMA showed a mean recovery of 0.85 ± 0.27% (n = 5), while PP exhibited a recovery of 0.48 ± 0.17% (n = 5). PVC recovery was 0.52 ± 0.36% (n = 5), and PS yielded the lowest recovery at 0.17 ± 0.11% (n = 5).

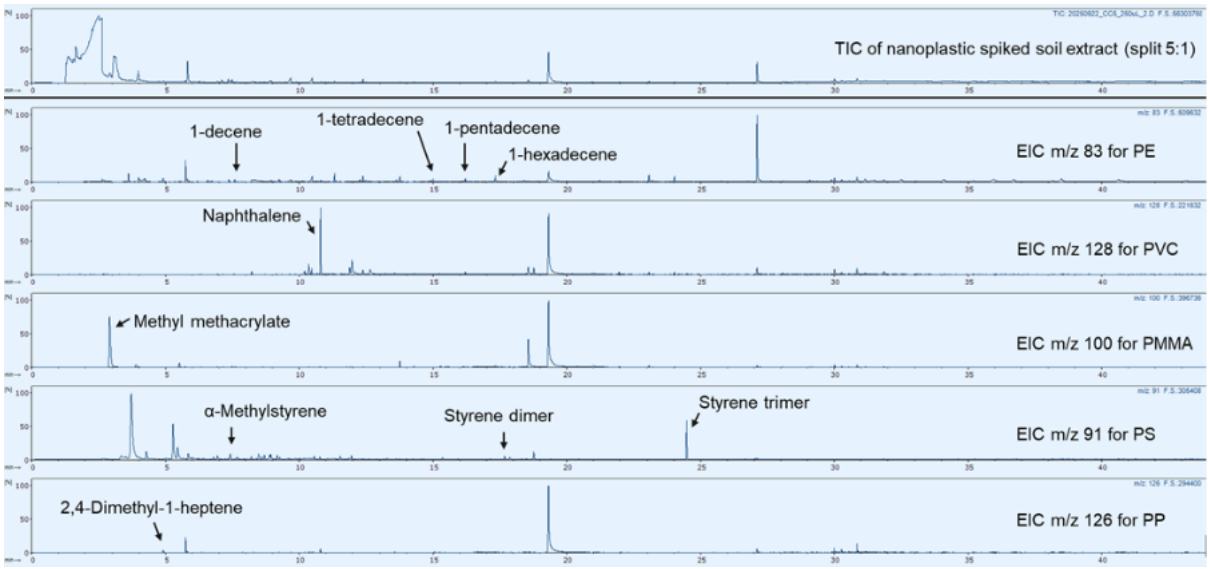

Figure 7: Top panel: Total ion chromatogram (TIC) of an extract from a soil sample spiked with a mixture of five nanoplastics. Major pyrolysis products corresponding to each polymer are visible across the chromatographic run. Lower panels: Extracted ion chromatograms (EICs) for the diagnostic ions used to confirm polymer identity, including m/z 83 for PE, m/z 128 for PVC, m/z 100 for PMMA, m/z 91 for PS, and m/z 126 for PP.

2.4.2.2. Procedure 2b

The extraction efficiency of Procedure 2b was evaluated before and after mild centrifugation and after H_2O_2 digestion by analysing 200 nm Eu-PS NPs using spICP-MS. The results demonstrated that extraction recoveries were influenced by soil type. Loamy sand exhibited the highest recoveries for both particle number (86–95%) and particle mass concentrations (84–95%), followed by clay loam and silt clay (Table 5). spICP-MS analysis showed that the mean particle mass remained consistent across the different extraction steps, indicating no significant leaching of the metal tracer or NPs aggregation. Particle size distributions were similar among soil types and across sample aliquots collected at different stages of the extraction procedure, and were comparable to those of the reference Eu-PS NP standard (Figure 8). These findings indicate that the mild centrifugation used to separate extracted NPs from soil residues, together with the reduced H_2O_2 volume (5 mL) for organic matter digestion applied in Procedure 2b, did not compromise particle integrity, in contrast to Procedure 2a, which employed filtration and a higher volume (10 mL) of 30% H_2O_2.

Table 5: Recoveries of particle number concentration, particle mass concentration, and mean particle mass for Eu-PS NPs extracted from different soil types and analysed by spICP-MS. Extraction recoveries were determined from sample aliquots collected at various stages of the extraction protocol and are reported as mean ± standard deviation (n = 3).

	Particle number concentration recovery (%)			**Particle mass concentration recovery (%)**			**Mean particle mass recovery (%)**		
Soil type	Supernatant before centrifugation	Supernatant after centrifugation	Supernatant after H_2O_2 treatment	Supernatant before centrifugation	Supernatant after centrifugation	Supernatant after H_2O_2 treatment	Supernatant before centrifugation	Supernatant after centrifugation	Supernatant after H_2O_2 treatment
Loamy sand	86 ± 5	86 ± 6	95 ± 9	89 ± 9	84 ± 4	95 ± 8	99 ± 3	99 ± 3	99 ± 3
Clay loam	66 ± 6	65 ± 8	71 ± 10	70 ± 5	70 ± 5	81 ± 8	107 ± 4	106 ± 4	99 ± 3
Silt clay	58 ± 2	58 ± 5	59 ± 3	65 ± 2	61 ± 5	64 ± 2	106 ± 2	106 ± 0	99 ± 3

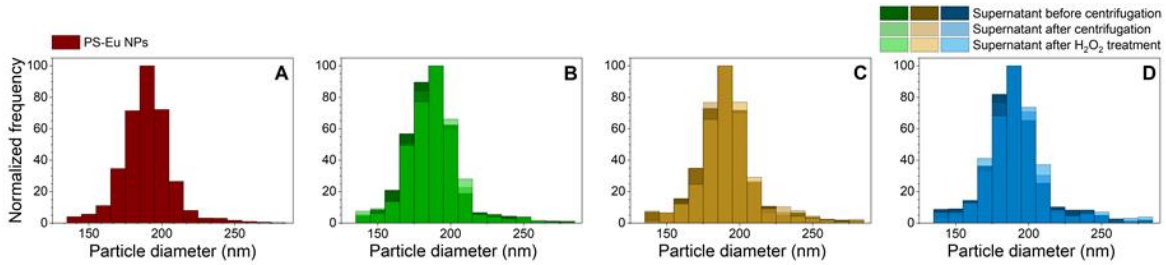

Figure 8: Particle size distribution histograms of Eu-doped PS NPs (bin size 10 nm). (A) Reference standard in aqueous solution, and Eu-PS NPs collected at various stages of the extraction procedure from spiked (B) loamy sands, (C) clay loam, and (D) silt clay.

Preliminary pyGC-MS results for soil extracts obtained using Procedure 2b indicate a substantially improved qualitative recovery of the spiked NPs, including PE, PP, PVC, PMMA, and PS (Figure 9), compared with Procedure 2a, when using a split ratio of 10:1. Characteristic pyrolysis markers were identified for each polymer: PE showed triple-peak patterns of alkenes and alkadienes in the C10–C20 range; PVC was indicated by naphthalene; PS by styrene monomers and trimers; PP by 2,4-dimethyl-1-heptene; and PMMA by methyl methacrylate. The preliminary data also suggest differences in the types of plastics recovered across soil types, likely attributable to electrostatic interactions between NPs and soil matrices that affect recovery efficiency. A fully quantitative evaluation is currently in progress to determine the recovery of each NP type in different soil types and to assess method reproducibility and repeatability.

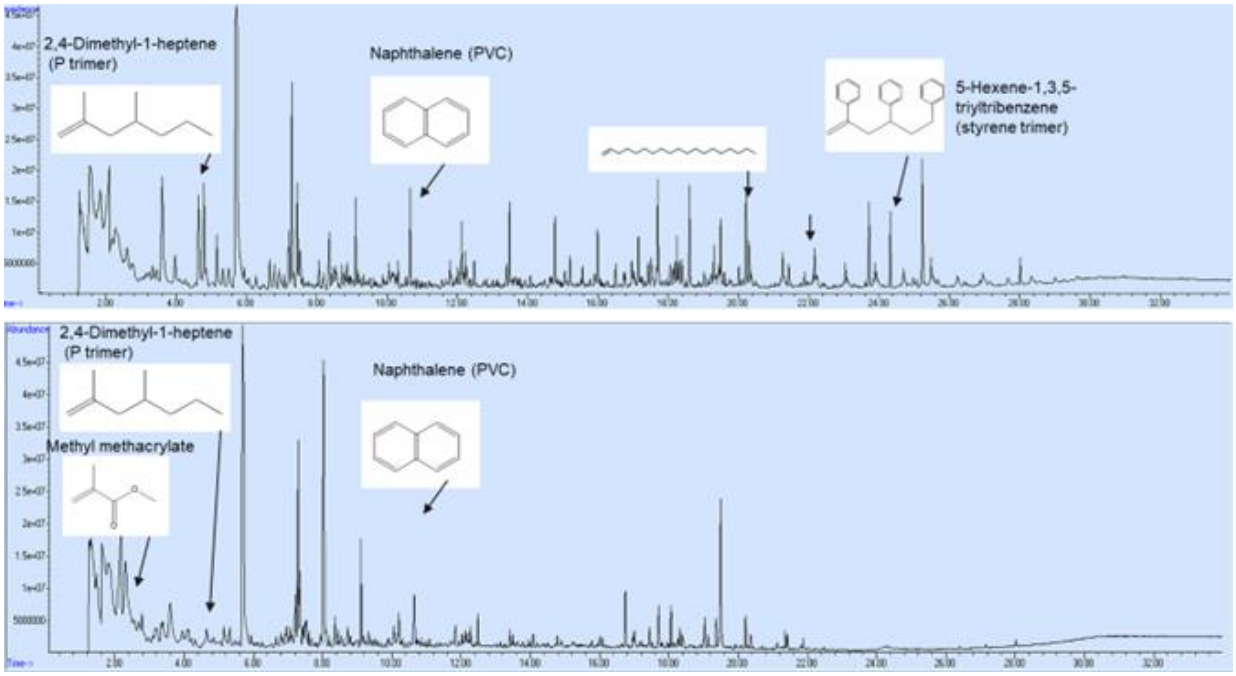

Figure 9: Extracted ion chromatograms (EICs) of an extract from a soil sample spiked with a mixture of five nanoplastics. Major pyrolysis products corresponding to each polymer are visible across the chromatographic run.

2.4.3. Final sample preparation procedure for extracting NPs from soil

Prior to extraction, the reagents were prepared as follows: 2.5 mM TSPP was prepared by weighing 0.5576 g of tetrasodium diphosphate decahydrate (Merck) into a 500 mL glass measuring flask and dissolved in ultrapure water. To ensure complete dissolution, the solution was shaken and vortexed. The TSPP solution was then filtered through a 100 nm filter (NC10 Cellulose Nitrate Membrane filter, Schleicher & Schuell) or through a 100 nm syringe filter (PES filter media, Minisart, Sartorius).

Approximately 2 g of soil was weighed into clean, prebaked 60 mL glass vials. Subsequently, 40 mL of 2.5 mM TSPP solution was added to each vial. The mixtures were shaken horizontally on an orbital shaker at 200 rpm for 2 h, followed by sonication for 5 min. After sonication, the inner walls of the vials were rinsed with an additional 5 mL of TSPP solution, and the suspensions were left to settle overnight. After settling, 10 mL of the supernatant was transferred into 15 mL centrifuge tubes. The samples were centrifuged at 2000 rpm for 3 min (497 × g) to remove particles larger than 1 µm with a density exceeding 2.6 g cm^{-3}. From the resulting supernatant, a 5 mL aliquot was taken, to which 5 mL of 30% H_2O_2 (filtered through a 0.1 µm syringe filter) was added. The mixtures were sonicated for 10 min and shaken for 24 h at 125 rpm with open vials to digest organic matter. Vials with samples were weighed during the extraction procedure to accurately determine the final dilution of the sample.

For pyGC-MS measurements, a 2 mL sample aliquot was filtered through a 20 nm Anodisc membrane, allowing for the collection of NPs larger than 20 nm. Following filtration, the Anodisc membranes were carefully fractured into small pieces using a sharp stainless-steel tweezer. To prevent the fragments from dispersing during this process, each membrane was placed on a pre-wetted glass fibre filter, which helped secure the Anodisc fragments in place. The glass fibre filter containing the fractured membrane pieces was then folded and transferred into a pre-burned stainless-steel pyrolysis cup for pyGC-MS analysis.

2.5. Procedure 3: Sample preparation procedure for extracting biodegradable MNPs from soil

Procedure 2 was further extended to enable the extraction of both biodegradable NPs and MPs from the same soil sample. Procedure 3 builds upon Procedure 2, which targets NP extraction as described in Section 2.4.3, and continues with the removal of most of the TSPP solution by pipetting, leaving 5 mL in each vial. Subsequently, 35 mL of calcium chloride ($CaCl_2$) was added. The 100 mL of $CaCl_2$ solution with a density of 1.32 g cm^{-3} was prepared by weighing approximately 49 g of anhydrous $CaCl_2$, to which 91 g of ultrapure water was slowly added, with continuous stirring due to the exothermic dissolution. After the salt had fully dissolved and the solution cooled to room temperature, the final volume was carefully adjusted to 100 mL with additional ultrapure water. The solution was then filtered through a 1 µm syringe filter (PES filter media, Puradisc, Whatman). The density was determined gravimetrically by weighing a known volume of the solution and dividing the mass by its volume. After adding $CaCl_2$, solution, the mixtures were shaken on an orbital shaker at 200 rpm for 2 h, followed by 5 min of sonication. After sonication, the vial walls were rinsed with an additional 5 mL of $CaCl_2$ solution, and the suspensions were left to settle overnight. On the following day, 10 mL of the supernatant was collected. 5 mL of 30% H_2O_2 (filtered through a 0.1 µm syringe filter) was then added, followed by 10 min of sonication, and the samples were shaken for 24 h at 125 rpm with open vials to digest organic matter.

The described protocol is schematically presented in Figure 10.

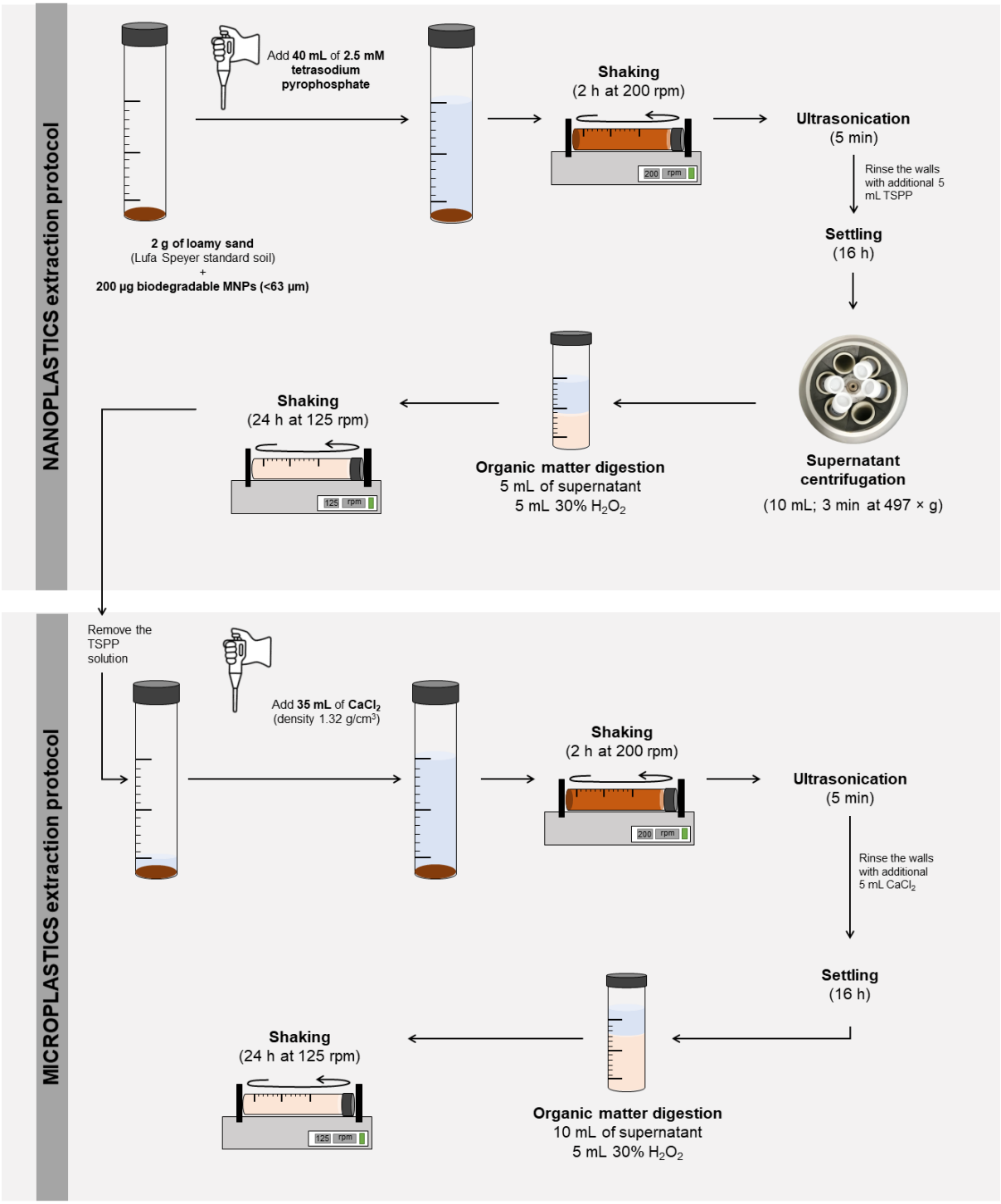

Figure 10. The scheme of the final two-phase protocol for extracting biodegradable nanoplastics and microplastics from the same soil sample.

Subsamples of the H_2O_2 digests described above were taken for filtration prior to pyGC-MS measurements. To avoid instrument overloading, only 1 mL or 2 mL of the extract was used. The subsamples were filtered through Anodisc filters (13 mm diameter, 0.02 µm pore size) using a Merck stainless-steel filtration setup. The filters were subsequently rinsed with ultrapure water, followed by rinsing with acetone. The Anodisc filters were then placed upside down onto a pre-wetted glass fibre filter (GFF) to ensure transfer of the retained particles. The Anodisc filters were carefully cracked into

small pieces (<1 mm) on the glass fibre substrate. The GFF containing pieces of Anodisc was then folded and placed into a pre-burned pyrolysis cup, which was loaded into the pyGC-MS system. pyGC-MS analysis was performed following the measurement parameters described previously.

2.5.1. Assessment of the extraction recovery

To evaluate the extraction efficiency of Procedure 3, two grams of a standard soil sample from LUFA, characterized as loamy sand, was spiked with 200 µg of MNPs generated from the cryo-milled biodegradable mulching film (fraction < 63 um) at DTU Sustain during WP3. According to the FTIR analysis, these biodegradable MNPs were composed of polybutylene adipate terephthalate (PBAT) and corn starch. Loamy sand soil was selected due to its properties being similar to those of the agricultural soil collected from the Western Peloponnese (Table 1), which had been long-term exposed to mulching films. Biodegradable MNPs fraction < 63 µm was spiked into blank and soil matrices, and both the NPs fraction (if present) and the remaining MPs fraction were separated according to the protocol described above. Background (non-spiked) soil samples were processed in parallel to assess potential contamination and baseline signals.

Figure 11 illustrates the pyGC-MS results of the biodegradable plastics extracted from the spiked soil samples. Distinct pyrolysis products derived from biodegradable plastic spikes were identified, including bis(but-3-enyl) terephthalate, but-3-enyl adipate, and cyclobutanone. Compounds characteristic of PLA degradation, such as benzoic acid and 3,6-dimethyl-1,4-dioxane-2,5-dione, were also observed. In addition, biphenyl and benzophenone were detected in the pyrogram.

When the biodegradable NPs were spiked into a blank matrix and subjected to the extraction procedure (thereby avoiding soil interference), characteristic PBAT pyrolysis products, particularly ester-derived compounds such as but-3-enyl terephthalate, but-3-enyl adipate, and benzoate derivatives, were not observed in the pyGC-MS chromatograms. This absence may be attributed to several factors. First, prolonged exposure of the materials to hydrogen peroxide (approximately one month), applied during a period of laboratory inactivity associated with facility relocation at VITO, may have induced oxidative degradation of the biodegradable polymers, resulting in the loss of ester functionalities. Second, the concentration of NPs in the spiked material may have been below the detection limit of the analytical method. Third, incomplete recovery during sample handling and preparation cannot be excluded. Collectively, these factors likely contributed to the absence of characteristic PBAT ester signals.

Nevertheless, the presence of compounds such as benzophenone, cyclopentanone, and acetophenone in the blank samples suggests a high likelihood that biodegradable plastic-derived material was present, though this still requires further testing and validation. Interestingly, most compounds detected in the spiked blank samples were also observed in the spiked soil samples, indicating that the soil matrix did not adversely affect plastic recovery using the tested extraction procedure. Further experiments are planned to systematically assess the effects of prolonged hydrogen peroxide exposure on biodegradable plastics and possibly to repeat the spiking experiments under more controlled conditions.

For biodegradable MPs, a similar observation was made; however, the detected signals were even less pronounced than those observed for NPs. This may be explained by the use of $CaCl_2$ with insufficient density to effectively recover biodegradable plastics (with a density of around 1.4 g cm^{-3}), potentially resulting in incomplete separation and reduced recovery. Further experiments are planned to evaluate the effects of other saturated salt solutions with higher densities (e.g., sodium iodide, 1.7–1.8 g cm^{-3}) on the extraction efficiency of biodegradable MPs from soil.

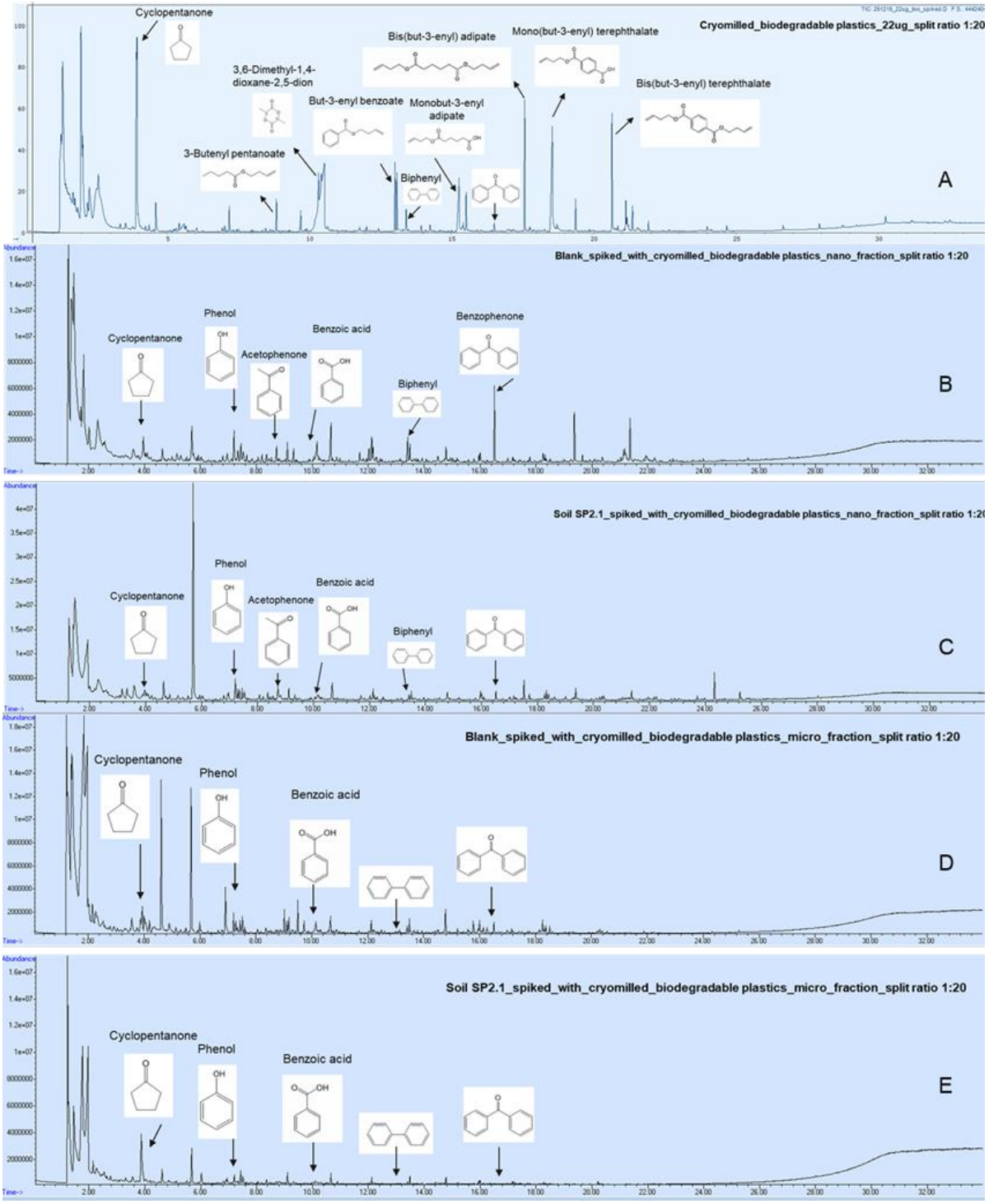

Figure 11: Total ion chromatograms (TICs) are shown for: (A) cryomilled biodegradable MNPs test material (<63 µm); (B) nanoplastic fraction extracted from a blank; (C) nanoplastic fraction extracted from a soil sample; (D) microplastic fraction extracted from a blank; and (E) microplastic fraction extracted from a soil sample (SP2.1). All samples were analysed using the pyGC-MS parameters described previously, with a split ratio of 1:20. Major pyrolysis products for each sample are indicated in the figure.

3. Sample preparation procedures for extracting MNPs from strawberries

3.1. Procedure development

3.1.1. Selection of the sample preparation approach

The assessment of the fresh strawberry composition was essential to identify the most appropriate enzyme or enzyme combination capable of efficiently degrading the organic matrix, without compromising the integrity of plastic particles (Table 6). Methodologically, strawberries represent a complex soft-fruit matrix, characterized by a high-water content, abundant sugars, and relatively low levels of proteins and lipids.

Table 6. Chemical composition of strawberries (Source: https://frida.fooddata.dk/food/1?lang=en)

Component	Content/100g	Additional information
Protein	0.7 g	
Carbohydrate	8.3 g	
Available carbohydrates	6.9 g	Mainly fructose and glucose, little sucrose
Dietary fibre	1.5 g	Mainly pectin and cellulose, hemicellulose, lignin
Fat	0.6 g	
Ash	0.4 g	
Water	90.0 g	

The selection of the enzyme was guided by the prior experience of the DTU Food, hosting the one-month training for two researchers from JSI and AUA on sample extraction protocols for MNPs from food samples, and by existing literature addressing similar analytical challenges (Loeschner et al., 2023; López-Mayán et al., 2022). The Macerozyme R-10 was identified as an appropriate choice for disrupting plant cell walls prior to MNPs determination. This enzyme mixture, derived from *Rhizopus* sp., contains cellulase (0.1 U mg^{-1}), hemicellulose (0.25 U mg^{-1}), and pectinase (0.5 U mg^{-1}), which together facilitate the degradation of polysaccharide components within plant tissues. The digestion procedure involved adding the enzyme solution to the strawberry matrix suspended in a citrate buffer, followed by incubation under controlled conditions.

3.1.2. Test of different digestion parameters

A protocol previously developed at JSI for extracting europium-chelated PS NPs from tomato fruit samples was applied (Sahai et al., manuscript under revision). Briefly, in this protocol, 0.025 g of dried plant material was mixed with 8 mL of citrate buffer (pH 6.5) in a 15 mL tube, briefly shaking the mixture, and adding 2 mL of an aqueous solution containing 50 mg of Macerozyme R-10. Samples were incubated at 37 °C for 24 h, then left to settle for 30 min. The supernatant was collected and diluted with water for spICP-MS analysis. Extraction conditions (buffer pH, digestion temperature, digestion time, and enzyme concentration) were optimized prior to final protocol selection.

In our experiment, the enzymatic extraction procedure was adapted to strawberry matrices, with modifications introduced to sample mass, mixing steps, and digestion conditions to ensure efficient disruption of the fruit tissue.

Both freeze-dried and fresh strawberries were included in protocol development to assess potential differences in digestion efficiency. For the digestion protocol, 0.025 g of freeze-dried strawberry was weighed into a 15 mL PTFE tube. To obtain an equivalent amount of fresh material, the required mass

was calculated based on the dry-matter content of fresh strawberries (Table 6). Accordingly, 0.25 g of fresh strawberry was used. Freeze-dried strawberry powder was purchased from RawFoodShop, while fresh strawberries were obtained from a grocery store in Denmark. Fresh strawberries were homogenized using a food blender before taking a sample.

The enzymatic procedure (Figure 12) included adding 8 mL of 0.002 mol L^{-1} citrate buffer (pH 6.5) to the sample, after which it was vortexed for 4 min to ensure thorough mixing. Subsequently, 2 mL of an aqueous solution containing 50 mg of Macerozyme R-10 enzyme was added. This mixture was then incubated in a water bath with a magnetic stirrer at 37°C for the specified digestion time. After incubation, the samples were allowed to settle for 30 min, after which the supernatant was collected and diluted with ultrapure water for spICP-MS analysis, or 0.05% sodium dodecyl sulphate (SDS) for analysis by asymmetric flow-field flow fractionation hyphenated to light scattering detection (AF4-LS).

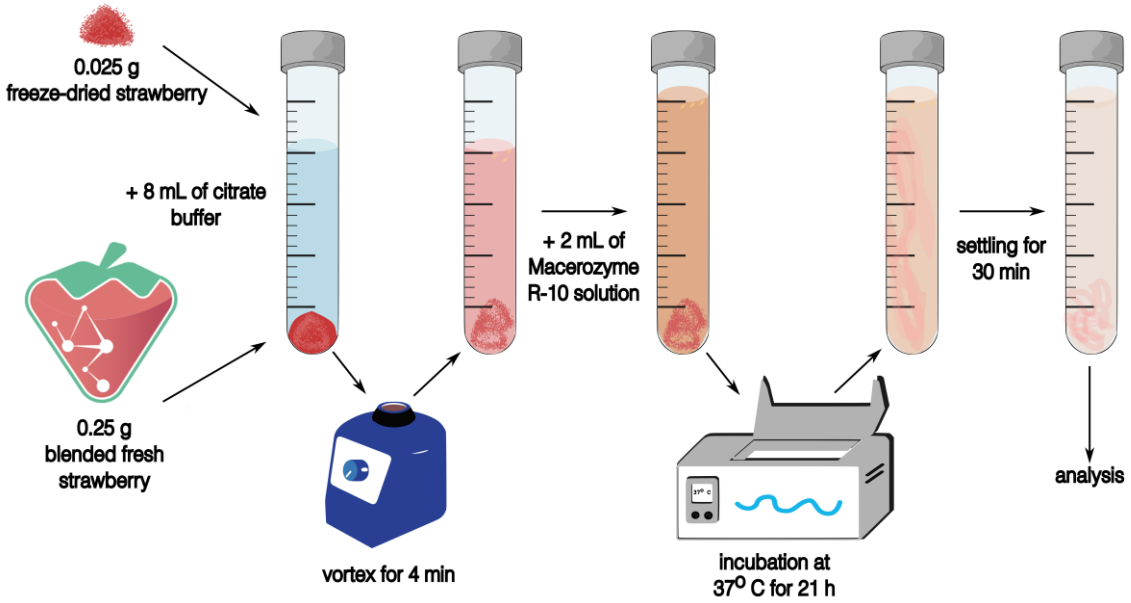

Figure 12. Graphical scheme of the protocol for extracting NPs from the strawberry matrix.

Initial visual inspection during the sample preparation

After mixing the strawberries with citrate buffer, the seeds (achenes) floated to the surface. Due to their relatively large size, they do not interfere with the digestion process or affect the efficiency of the protocol.

Increase in the digestion time

Two digestion times were evaluated: 21 h and 48 h. After 21 h, aside from the seeds that remained floating on the surface, the samples still contained visible undigested or partially digested organic material. These residues likely represent lignified cell-wall components that are not susceptible to Macerozyme R-10 activity. The remaining material appeared as diffuse, cloudy structures without evidence of aggregation or clump formation (Figure 13). To assess whether extended incubation would enhance matrix breakdown, the digestion was prolonged to 48 h. However, no visual differences were observed compared to the 21 h samples, indicating that additional incubation did not result in further digestion.

Comparison of freeze-dried and fresh material

The pink-red coloration of the digested samples originates primarily from strawberry anthocyanins, mainly pelargonidin derivatives. Freeze-dried samples exhibited a slightly more intense colour, but no other notable visual differences were observed compared to the fresh samples (Figure 13).

Settling prior to supernatant collection

Following digestion, a settling period was required to allow remaining insoluble residues to separate from the liquid phase before supernatant collection. A settling time of 30 min was considered sufficient, as the supernatant was clear enough to be used for subsequent analyses after appropriate dilution. Extending the settling period did not result in further noticeable improvement. The samples shown in Figure 14, which were left to settle for 48 h, were kept exclusively for visual inspection and were not subjected to further analysis.

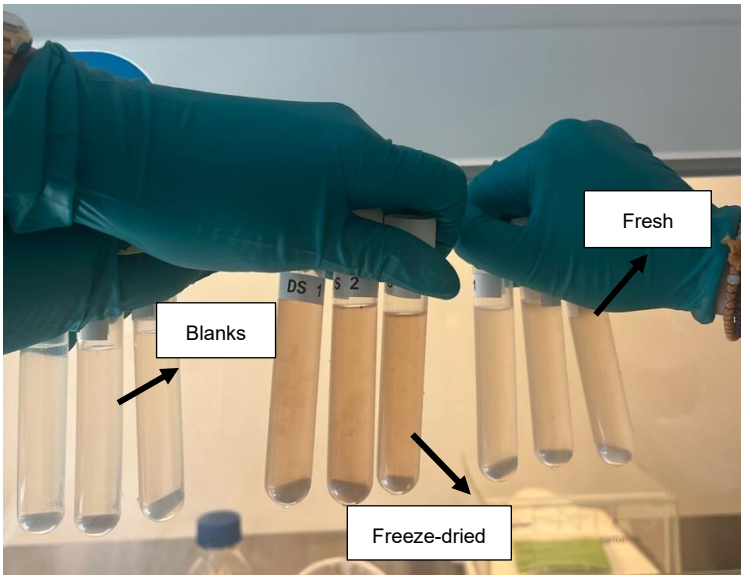

Figure 13. Visual appearance of strawberries following the 21 h incubation. The white items at the bottom of the tubes are the magnetic stir bars used during incubation.

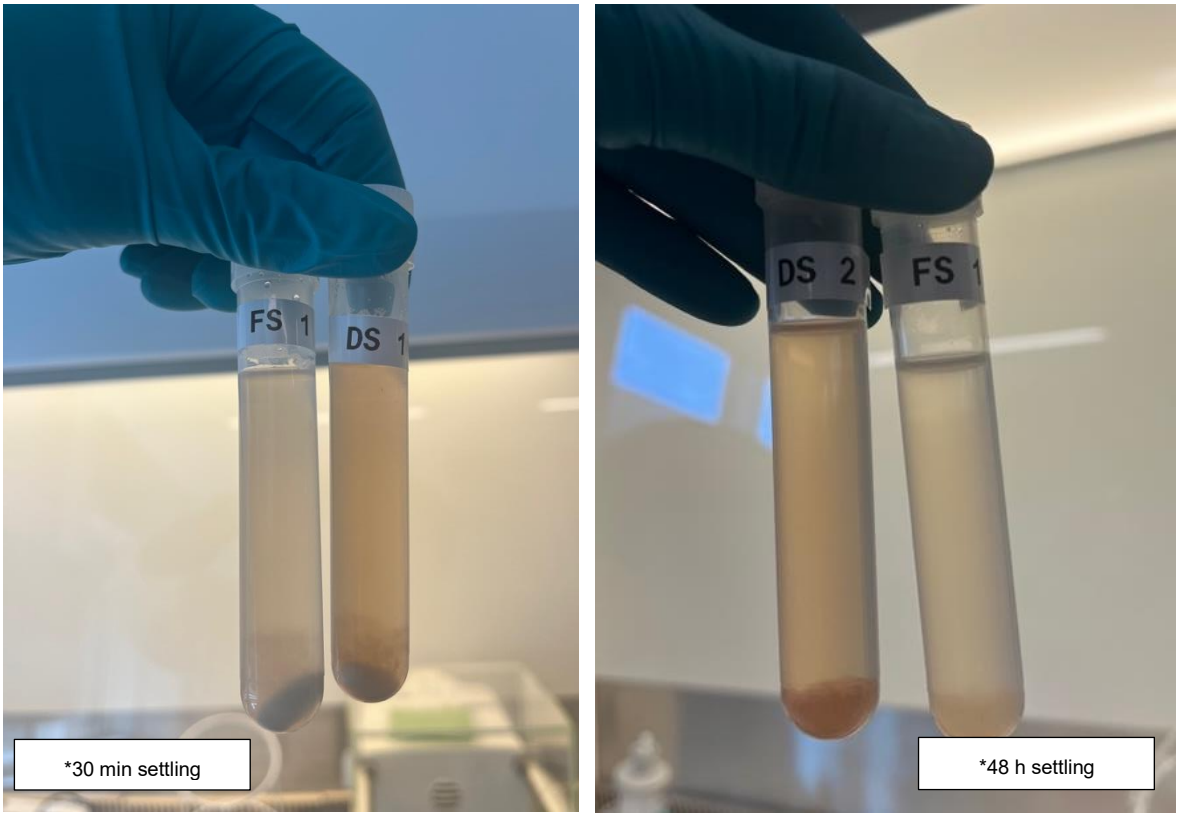

Figure 14. Settling of digested strawberry samples (FS – Fresh strawberry sample, DS – Freeze-dried strawberry sample).

3.1.3. Assessment of residual material

Dynamic light scattering (DLS) analysis was applied primarily to evaluate the presence of residual material remaining after enzymatic digestion of the strawberry samples. The derived count rate served as an indicator of the sample's optical quality and particle concentration, providing a rapid assessment of how efficiently the digestion removed matrix components (Figure 15).

Prior to analysis, all samples were diluted tenfold with ultrapure water to minimize multiple scattering effects. To test whether the surfactant used for AF4-LS (see section 3.1.5) affected the measurement, the same samples were also diluted in a 0.05% SDS solution. The comparable scattering signals obtained under both conditions confirmed that SDS did not influence the DLS results.

To further confirm the impact of filtration, both fresh and freeze-dried samples were analysed before and after filtration through a 0.45 µm filter. The filtered samples exhibited substantially lower derived count rates, indicating effective removal of larger matrix residues and confirming that filtration improves sample clarity.

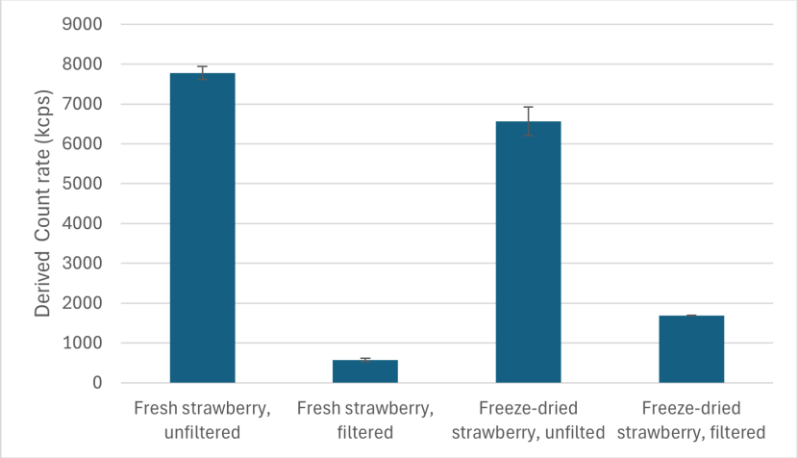

Figure 15: Derived count rates obtained by DLS analysis after the enzymatically digested strawberry matrix (fresh and freeze-dried; unfiltered and after filtration using a 0.45 µm filter)

3.1.4. Assessment of the extraction efficiency

Metal-doped NPs were chosen to allow precise quantification of the extraction efficiency by spICP-MS. Considering the expected size range relevant for uptake by strawberries, particles of 200 nm diameter were selected. A suspension of 200 nm Eu-doped PS particles was obtained from Bangs Laboratories, Inc. (Hirschberg an der Bergstrasse, Germany) and stored at 4 °C. The beads characterized by high stability under storage conditions were supplied as ~1 % (w/v) aqueous suspensions.

Spiking solutions were prepared to achieve a final spike level of 10 µg Eu per kg of freeze-dried strawberry sample. For this, the Eu-PS suspension was first diluted 66800-fold, and then a volume of 100 µL was added to 25 mg of strawberry powder. Eu recovery was subsequently assessed based on total Eu concentrations determined by inductively coupled plasma mass spectrometry (ICP-MS) on the same samples on the following day. Total Eu concentrations were measured in both spiked and unspiked strawberry digests to evaluate potential background Eu contributions from the strawberry matrix or enzyme solutions. Samples were diluted twofold and analysed without filtration using an iCAP-TQ ICP-MS (Thermo Fisher Scientific) equipped with an ASX-560 autosampler (Teledyne CETAC Technologies).

Results revealed that:

- Strawberries themselves contained 4 ng/g Eu
- Relative recoveries (NPs spiked to strawberry vs. pure NPs) were approximately 91%.

Single particle ICP-MS was used to evaluate the recovery of Eu-PS NPs based on the number of detected particles, providing also information on particle number concentration and size distribution, and allowing the assessment of potential particle losses during extraction and digestion. The samples were analysed using a triple quadrupole ICP-MS (ICP-QQQ, Agilent 8900, Santa Clara, CA, USA). The Eu-PS NP suspension was diluted 40 million-fold with ultrapure water prior to analysis. Spiked strawberry samples were analysed directly after the settling step.

The acquired data were processed using the open-source software SPcal (Lockwood et al., 2025). Measurements of the Eu-PS NPs standard were included to verify agreement with the expected values (Table 7).

Table 7. Results of spICP-MS analysis of 200 nm Eu-PS NPs (considering particle density of 1.06 g cm^{-3} and Eu mass content per particle of 1.67 wt%; N=2)

Eu-PS NPs standard 200 nm	PS Mass concentration	Mean size	Median size
	mg/L	nm	nm
Average	9543	178	177
St. dev.	542	0.2	/
Expected value	10000	200	200
Recovery (%)	**95.4**	**89.2**	**88.5**

The number of detected particles was 1152 ± 50 (N=2) in the Eu-PS NPs suspension prepared in ultrapure water, and 1669 ± 35 (N=3) in Eu-PS NPs that underwent enzymatic digestion. This might indicate a stabilizing effect of the enzyme solution on the particles. Analysis of the spiked strawberry samples showed that 1790 ± 69 particles (N=3) were detected by spICP-MS. To determine recoveries, spiked strawberry samples and Eu-PS NP suspension (after enzymatic digestion) were compared. Recovery for particle number concentration was 104%, for particle mass concentration 92%, and for particle size 95%. In conclusion, the extraction efficiency of the proposed procedure was considered satisfactory.

3.1.5. Test of AF4 as an additional sample preparation step

The digests of freeze-dried and fresh samples were spiked with Eu-PS NPs at an approximate concentration of 1 g per kg of strawberries (dry weight). The sample set included:

- Enzymatically digested blanks,
- Enzymatically digested suspensions of Eu-PS NPs,
- Enzymatically digested strawberry matrix without spiking, and
- Enzymatically digested spiked strawberry matrix prepared under identical conditions.

The first AF4-LS measurements revealed a high peak in the unspiked strawberry samples (blue curve, Figure 16). The strong scattering signal from the residual organic matrix overlapped with the expected NPs peak (pink curve). This highlighted one of the main challenges in analysing food matrices by AF4-LS – matrix residues can mask or distort nanoparticle signals.

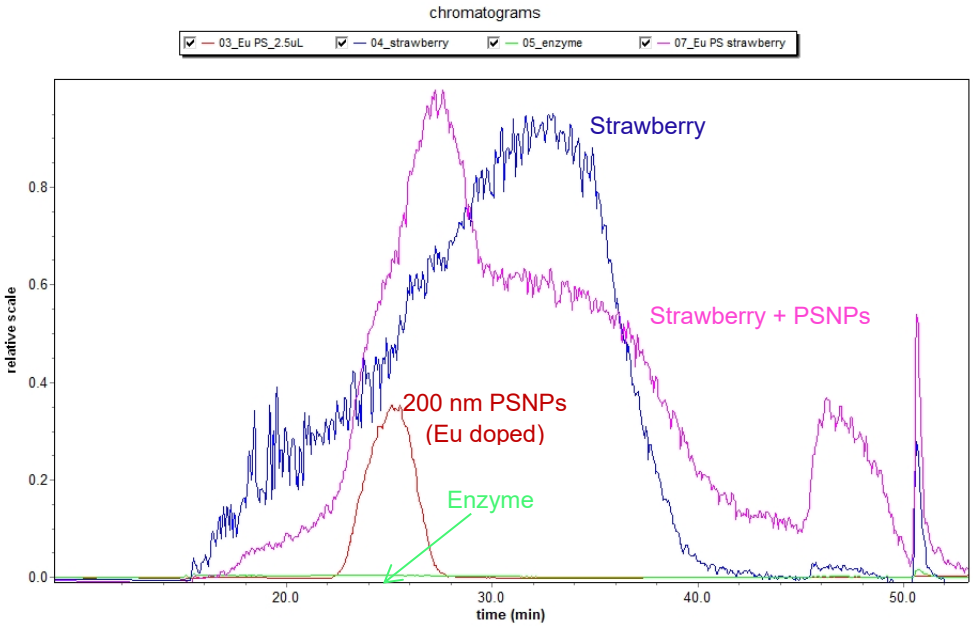

Figure 16. AF4-LS chromatograms of analysed samples (10 kDa regenerated cellulose membrane, 0.05 (v/v) % SDS, detector flow 1 mL/min, cross flow 0.3; 0.1 mL/min in 30 min, injection volume 10 µL (50 ng – 100 ng Eu-PSNPs)).

To address this issue, several approaches were tested:

- Comparison of freeze-dried and fresh samples, which yielded similar interference patterns.
- Introduction of a filtration step prior to AF4 analysis, using 0.45 µm filters. This step effectively removed a large portion of the matrix, reducing background scattering and improving the clarity of the NP signal, even though some loss of Eu-PS NPs occurred (Figure 17).

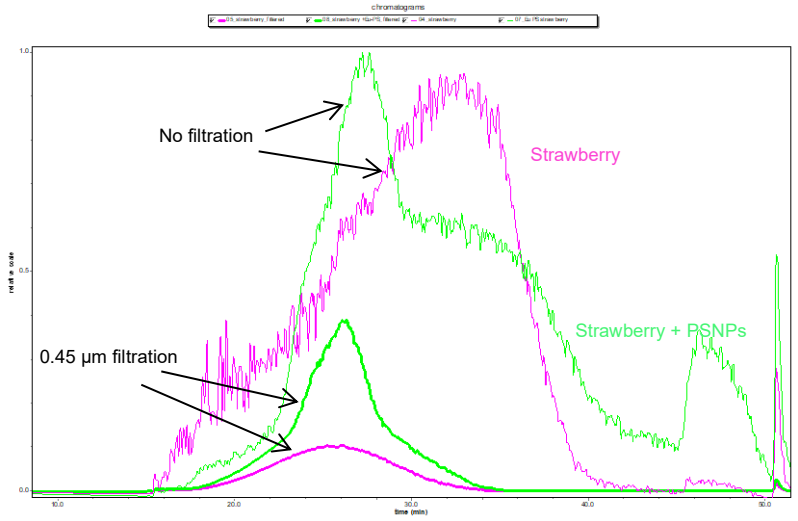

Figure 17. AF4-LS chromatograms of filtered VS. non-filtered samples

In addition, two fractions were collected based on the obtained AF4-LS chromatograms: one corresponding to the elution window of the Eu-PS NP peak and one collected from a baseline region without detectable signals, serving as a blank (Figure 18). The ability to selectively collect the NP-containing fraction highlights the potential of AF4 as a preparative and clean-up step prior to downstream analyses, as it can reduce the contribution of the original sample matrix. However, it remains to be assessed whether SDS present in the carrier solution interferes with downstream analytical methods, such as pyGC.MS. Furthermore, given the mass requirements and sensitivity of pyGC-MS, an additional up-concentration step of the collected fractions may be necessary.

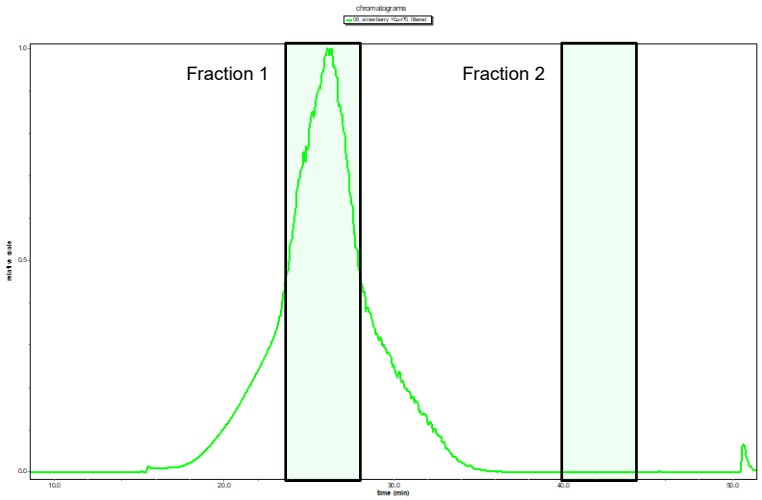

Figure 18. Fractions collected during the AF4-LS analysis

3.1.6. Adjustment of the extraction procedure for IR microscopy

Following the completion of the hands-on training at DTU Food, where the primary focus was on developing a protocol suitable for extracting NPs from the strawberry matrix, method development was continued in close collaboration with IMR to establish an extraction procedure compatible with infrared (IR) microscopy. At this stage, DTU Sustain provided <63 µm plastic MNPs generated by cryomilling of UV-weathered biodegradable mulching film, intended also for use in WP7. Consequently, our efforts shifted toward optimizing and evaluating the extraction protocol for these particles.

The Daylight Solutions Spero-QT IR microscope was selected as the analytical instrument for particle detection due to its advanced mid-IR chemical imaging capabilities. The Spero-QT employs a broadly tuneable mid-IR quantum cascade laser (QCL) light source, enabling high-throughput, label-free spectroscopic imaging across the molecular fingerprint region with rapid hyperspectral data acquisition and live imaging modalities.

For IR microscopy using the Spero-QT, sample preparation aims to obtain a clean, particle-containing suspension that can be evenly deposited onto an IR-transparent filter. Because matrix components strongly interfere with infrared spectra, the sample must be digested or otherwise treated to produce a clear solution that can pass easily through the analysis filter. After filtration, the filter is dried and subsequently analysed on the Spero-QT microscope to acquire chemical images and spectra of the retained particles.

3.1.6.1. Adjustment of enzymatic digestion

For comparability purposes, freeze-dried strawberries from the same producer as those obtained at DTU Food were purchased and used as the matrix for protocol optimization. Initially, we applied the protocol previously used at DTU Food, as detailed in Figure 12, which is based on enzymatic digestion with Macerozyme R-10. The samples were incubated for 21 h at 37 °C with continuous shaking at 120 rpm.

To improve the applicability of the method for realistic sample conditions, we increased the mass of freeze-dried strawberries used in the digestion. We ultimately worked with 125 mg of freeze-dried strawberries, five times the mass of the initial 25 mg, which, although still relatively small, provides a sample size more closely aligned with the fresh-weight equivalent of a whole strawberry and yields

better estimates of MPs occurrence. The volume of citrate buffer added was consequently increased to 40 mL, followed by the addition of 10 mL of enzyme solution containing 100 mg of Macerozyme R-10 (Figure 19).

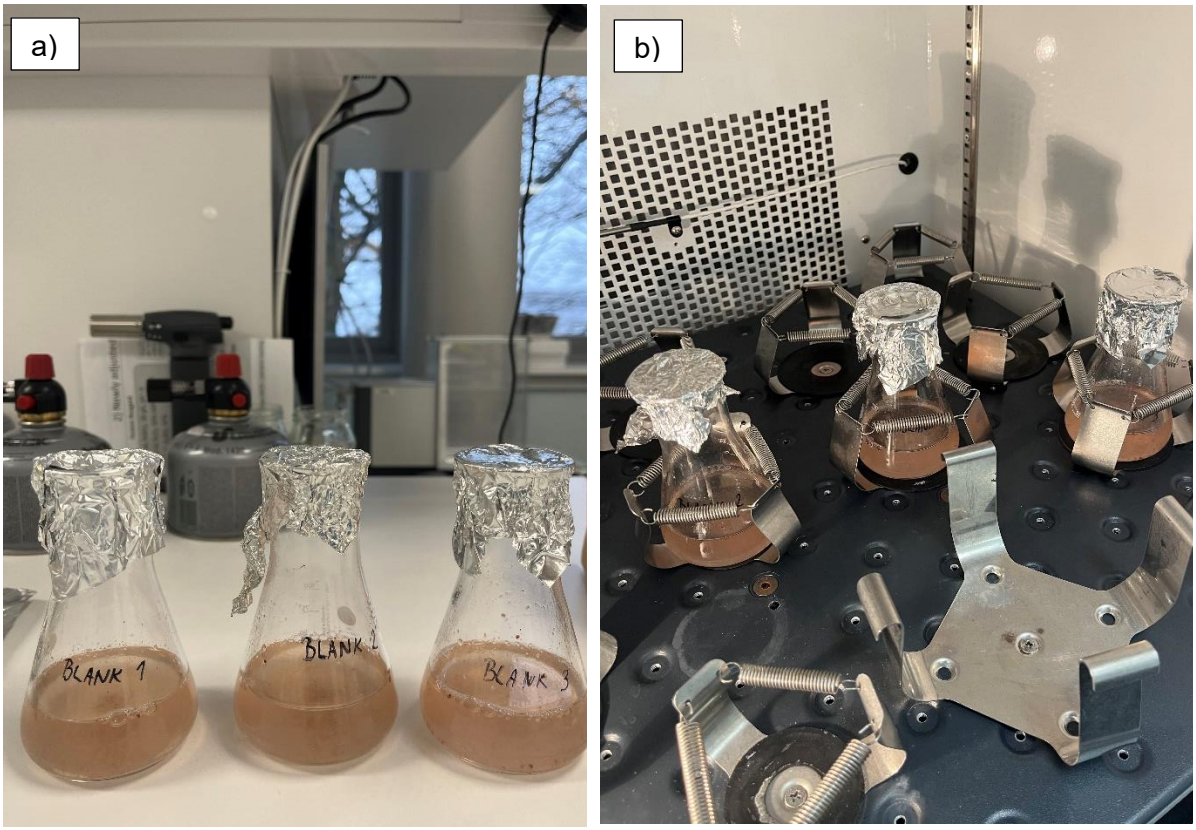

Figure 19. Initial upscaled sample preparation: a) samples after addition of citrate buffer and enzyme solution; b) samples placed in the incubator.

Because the protocol developed at DTU Food targeted smaller particles (200 nm Eu-PS NPs), adaptations were necessary when working with the <63 µm biodegradable MNPs (PBAT/corn-starch based) used in this study. In this size range, a 30 min settling step was not appropriate, as the target particles would settle together with the remaining undigested strawberry matrix. To avoid particle loss, the entire sample therefore had to be filtered prior to IR microscope analysis. Filtration prior to analysis was performed using a 3.0 µm PTFE filter with a polymethyl pentene support ring (25 mm diameter; Environmental Express, FL, USA).

However, the applied digestion protocol did not achieve complete degradation of the strawberry matrix, and substantial undigested material remained. As shown in Figure 20, alongside the strawberry seeds, additional particulate residues were still present, posing a risk of clogging the filter and compromising particle recovery.

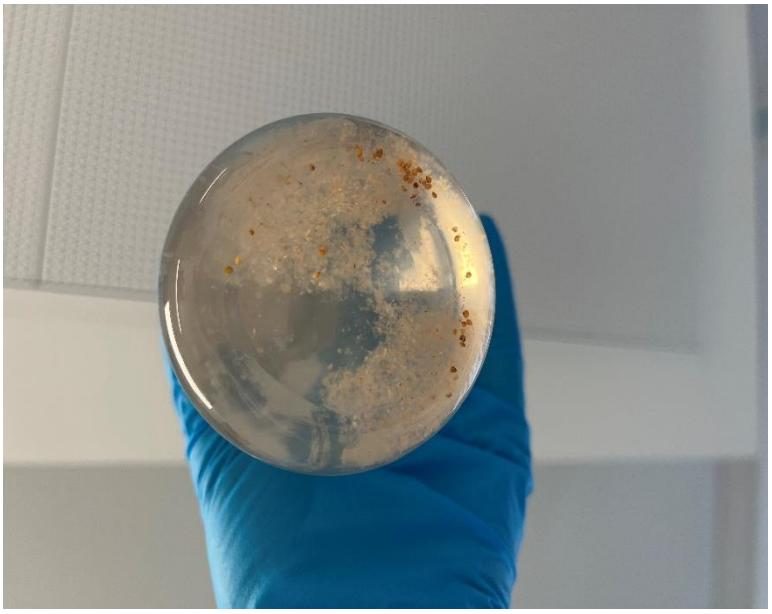

Figure 20. Bottom of the Erlenmeyer flask containing the sample after 21 h of enzymatic digestion.

3.1.6.2. Additional sample purification

Adjustments to the procedure based on enzymatic digestion focused primarily on improving the filtration strategy to obtain cleaner filters for subsequent IR microscope analysis. Following incubation, the sample was first passed through stainless steel mesh filters with pore sizes of 315 µm and 180 µm to remove strawberry seeds and larger undigested matrix fragments (Figure 21). To minimize particle loss, the filters were thoroughly rinsed with a large volume of ethanol (EtOH) and water (1:1, v/v). This step improved the visual quality of the remaining sample, as both the seeds and a portion of the jelly-like strawberry residues were effectively removed.

Figure 21. Stainless steel mesh filters following filtration of the strawberry digests, showing the 180 µm mesh (left) and 315 µm (right).

Following this pre-filtration step, the sample was passed through the analysis filter (results shown in Figure 22). Filtration at this stage was quite time-consuming, and the filter surface remained heavily covered with strawberry matrix, indicating the need for further refinement of the protocol. Notably, no substantial differences were observed between filters originating from samples pre-filtered with the 315 µm and 180 µm meshes. To assess the extent of matrix interference, the analysis filter was subsequently examined using the Spero-QT instrument to characterize the spectral signature of the

remaining residues and evaluate whether a usable background signal could be obtained. As anticipated, the resulting IR image was not satisfactory, as also illustrated in Figure 22.

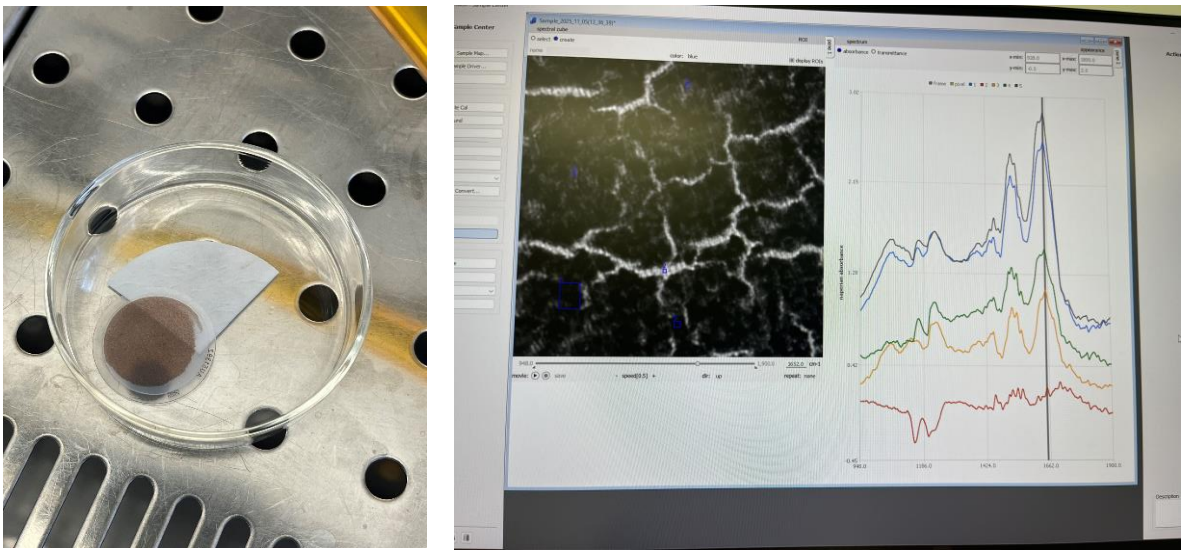

Figure 22. The analysis (3 µm) filter and the corresponding live view on the Spero-QT IR microscope.

In addition, an extra filtration step was introduced prior to filtration on the 3 µm analysis filter. This intermediate filtration was performed using a 5 µm PTFE filter (47 mm diameter). The resulting filter is shown in Figure 23. The collected material on the 5 µm filter was resuspended in an EtOH: water (1:1, v/v) solution and incubated for 30 minutes, followed by 1 minute of sonication.

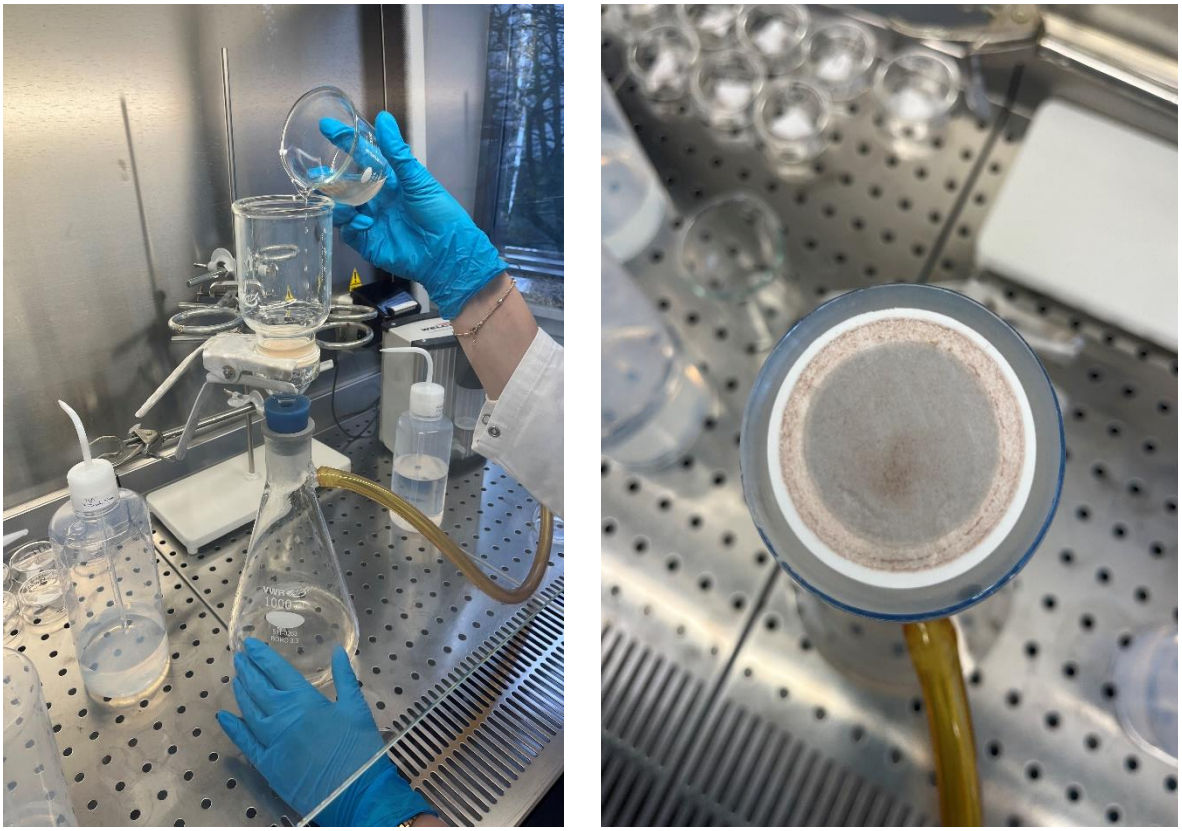

Figure 23. Filtration apparatus and resulting material collected on the 5 µm filter

The filter was then additionally rinsed with the EtOH: water (1:1, v/v) solution, and the resulting suspension was then filtered again using the 3 µm analysis filter. However, the appearance of the resulting analysis filter did not show any improvement compared with the previous attempt. Given the lack of improvement and persistent matrix interference, we discontinued further testing of this protocol beyond the blank samples, as it was evident that the approach would not yield usable filters for IR microscope analysis. In the following phase, we explored combining alternative approaches while maintaining mild conditions to avoid damaging the biodegradable MNPs and introduced spiked samples to systematically evaluate particle recovery under the new conditions.

3.1.6.3. Alternative approaches

At this stage, in addition to blank samples, we also introduced strawberry samples spiked with <63 µm biodegradable MNPs. Incorporating spiked samples enabled us to monitor how different chemical treatments affected both the strawberry matrix and the particles, thereby providing a clearer assessment of the suitability and robustness of the tested procedure.

The spiking suspension was prepared by weighing 0.001 g of MNPs (<63 µm biodegradable mulching film particles) and dispersing them in 25 mL of an EtOH: water (1:1, v/v) mixture.

To rapidly assess the impact of various chemicals, we performed a series of preliminary tests in which 125 mg of freeze-dried strawberry powder was combined with different chemical solutions. In parallel, the particles were also exposed directly to the chemicals in the absence of the strawberry matrix, allowing us to more clearly observe their stability. The chemicals tested included 10% potassium hydroxide (KOH), 10% hydrochloric acid (HCl), 32.5% nitric acid (HNO_3), 30% hydrogen peroxide (H_2O_2), and 100% acetic acid (CH_3COOH).

Nitric acid led to the complete degradation of the biodegradable MNPs. In contrast, acetic and hydrochloric acid did not visibly affect either the particles or the strawberry matrix. KOH, however, partially degraded the strawberry material while preserving the integrity of the particles, making it a promising candidate for incorporation into the sample preparation procedure. Examples from these tests are shown in Figure 24.

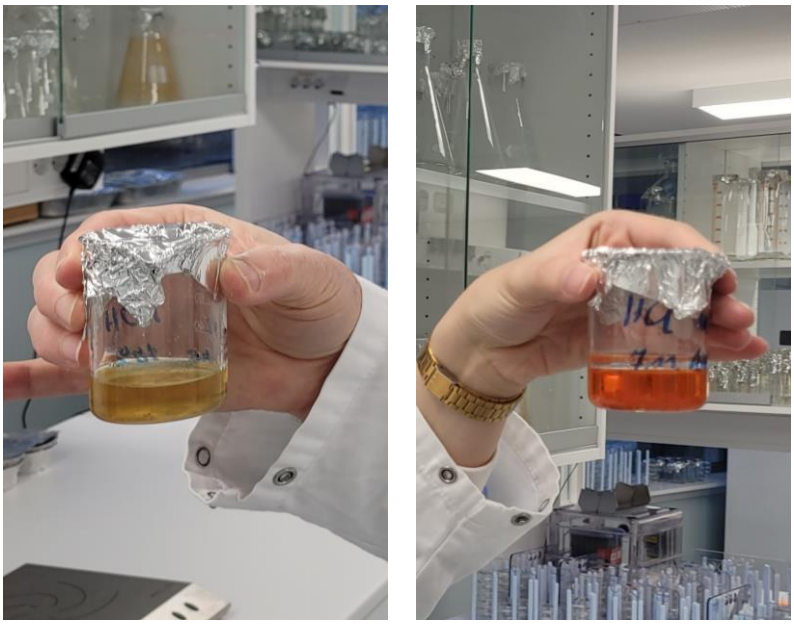

Figure 24. Strawberry powder digested in 10% KOH (left) and 10% HCl (right).

After identifying KOH as a potential agent for degrading the strawberry matrix, we proceeded to test a mild alkaline protocol while maintaining the same sample mass. To 125 mg of freeze-dried strawberry powder, we first added 1 mL of the prepared spiking solution and then brought the volume to 25 mL with 10% KOH, followed by overnight incubation (21 h) at 37 °C. Although some improvement in matrix degradation was observed the following day, residual strawberry material was still present. Consequently, we first filtered the mixture through a 315 µm mesh and then through a 5 µm filter, after which we continued with enzymatic digestion of the material retained on the 5 µm filter, as previously tested. For this purpose, the 5 µm filter was submerged in 40 mL of citrate buffer and supplemented with 10 mL of enzymatic solution containing 100 mg of Macerozyme R-10, followed by incubation under the same conditions. Despite the visually clearer appearance of the resulting suspension, the outcome remained suboptimal (Figure 25). Therefore, we investigated whether altering the sequence of the two protocol phases would yield improvement. In this subsequent approach, enzymatic digestion was performed first under identical conditions, followed by filtration through a 5 µm filter and then alkaline digestion using 25 mL 10% KOH. During these trials, we also evaluated the effect of extending the alkaline incubation time to 48 h and observed enhanced matrix degradation. On this basis, the duration of the alkaline digestion phase was extended to 48 h in the next tests.

Figure 25. Step 1: Enzymatically digested sample (left) and Step 2: The 5 µm filter after filtration of the digest, suspended in 10% KOH (right).

Based on these tests, it appeared more effective to perform the alkaline digestion first. Initiating the protocol with the alkaline step resulted in greater reduction of the strawberry matrix early in the process, which facilitated subsequent filtration through the 5 µm filter and reduced the burden on the enzymatic digestion phase. Therefore, the workflow in later tests was adjusted to begin with 48 h alkaline digestion followed by enzymatic treatment.

To further improve matrix degradation, we also evaluated an alternative enzymatic treatment. Viscozyme L (≥100 FBGU g^{-1}), a cellulolytic enzyme mixture from *Aspergillus* sp. (Merck/formerly Sigma-Aldrich, Darmstadt, Germany), was selected for testing alongside the existing protocol (Toto et al., 2023; Steiner et al., 2024; Liu et al., 2024). All enzymatic digestion trials were conducted under the same conditions as before, using citrate buffer at pH 6.5, to ensure comparability and because Viscozyme L is active within this pH range. Preliminary experiments showed that Viscozyme L produced a stronger degradation effect on the strawberry matrix, resulting in clearer suspensions and cleaner analysis filters (Figure 26). These observations indicated that Viscozyme L was a more suitable enzyme for this application, and it was therefore incorporated into the subsequent sample preparation procedure in combination with the alkaline digestion step, followed by additional digestion with hydrogen peroxide.

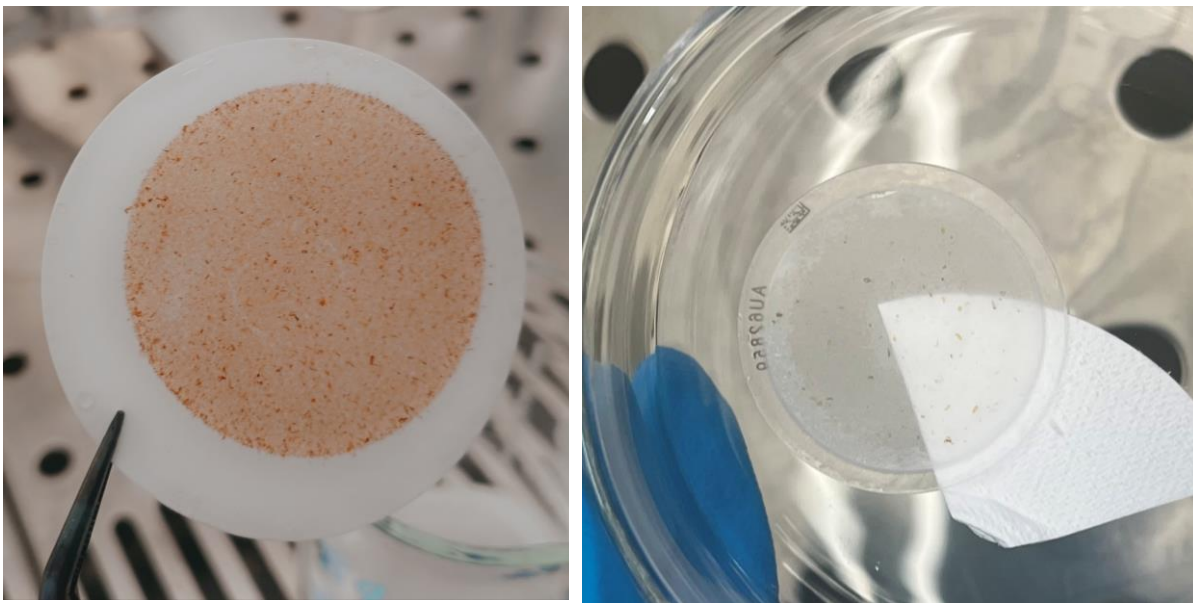

Figure 26. The 5 µm filter obtained after the alkaline digestion step (left) and the 3 µm analysis filter obtained after the subsequent enzymatic digestion step (right).

3.2. Final sample preparation procedure for extracting MNPs from strawberries

All glassware used in the sample preparation procedure was pre-burnt to remove potential organic contaminants, and all solutions employed during the extraction were pre-filtered through 0.7 µm glass fibre filters to prevent contamination by foreign particulate matter.

For the extraction of MNPs from freeze-dried strawberries, 125 mg of strawberry powder was weighed into clean glass beakers. The samples were spiked with 1 mL of the prepared <63 µm biodegradable MNPs suspension, after which the volume was brought to 25 mL with 10% KOH. The mixtures were incubated at 40 °C and 120 rpm for 48 h to achieve alkaline degradation of the organic matrix. Following incubation, the suspensions were first filtered through a 5 µm filter (PTFE, 47 mm), and the filter was washed with water and an EtOH: water (1:1, v/v) mixture to remove all remaining potassium hydroxide. The 5 µm filters containing the retained material were then transferred into clean beakers and submerged in 40 mL of 0.002 mol L^{-1} citrate buffer (pH 6.5). Subsequently, 200 µL of Viscozyme L (≥100 FBGU g^{-1}; Merck/Sigma-Aldrich, Darmstadt, Germany) was added to each beaker. The samples were incubated at 40 °C and 120 rpm for 48 h to facilitate enzymatic digestion of the remaining matrix

components. After digestion, the resulting suspensions were first passed through a 315 µm stainless steel mesh to remove the seeds and thereafter filtered onto another 5 µm filter (PTFE, 47 mm). This filter was placed into a beaker, to which 30 mL of hydrogen peroxide solution (30%) was added. To enhance the reaction, small amounts of a 10% KOH solution were added until a gentle bubbling was observed. Finally, the sample was filtered onto a 3 µm PTFE filter (analysis filter), which served as substrates for IR microscopy.
The analysis filters were dried overnight at room temperature to remove residual moisture. Once dried, the filters were analysed using the Spero-QT IR microscope to obtain chemical images and spectra of the retained MNPs.

3.2.1. Assessment of the extraction efficiency

To assess the suitability of the newly developed sample preparation procedure for extracting MNPs from strawberry samples for IR microscopy, analysis filters loaded with spiked strawberry extract were imaged using the Spero-QT 340. The implementation of the spectra, generated by the biodegradable MPs, into the machine learning model as an individual class is still ongoing. Therefore, the sample was analysed "manually" by inspecting IR-spectra generated by particles identified in the images and matching them to previously recorded ones (Figure 27). The spectrum could be identified as one belonging to the biodegradable mulching film/PBAT (Figure 28), thereby indicating that the protocol developed is capable of identifying target MPs in the presence of the strawberry matrix using IR microscopic analysis.

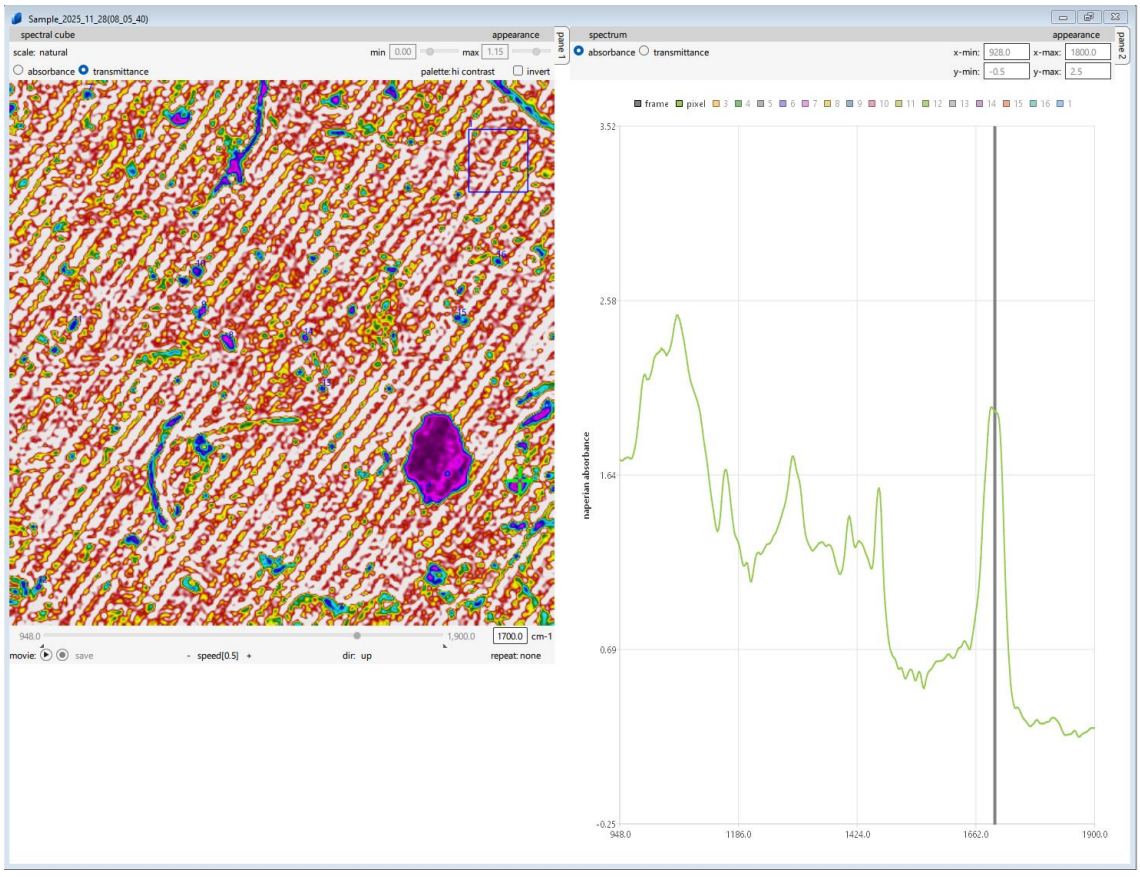

Figure 27: Screenshot of a "manual" analysis of a filter loaded with spiked strawberry extract according to the newly developed protocol. To the left, an intensity-based image of an approx. 2x2 mm portion of the filter surface is shown with a green crosshair indicating the currently observed pixel. To the right, the infrared spectrum recorded for the chosen pixel is presented.

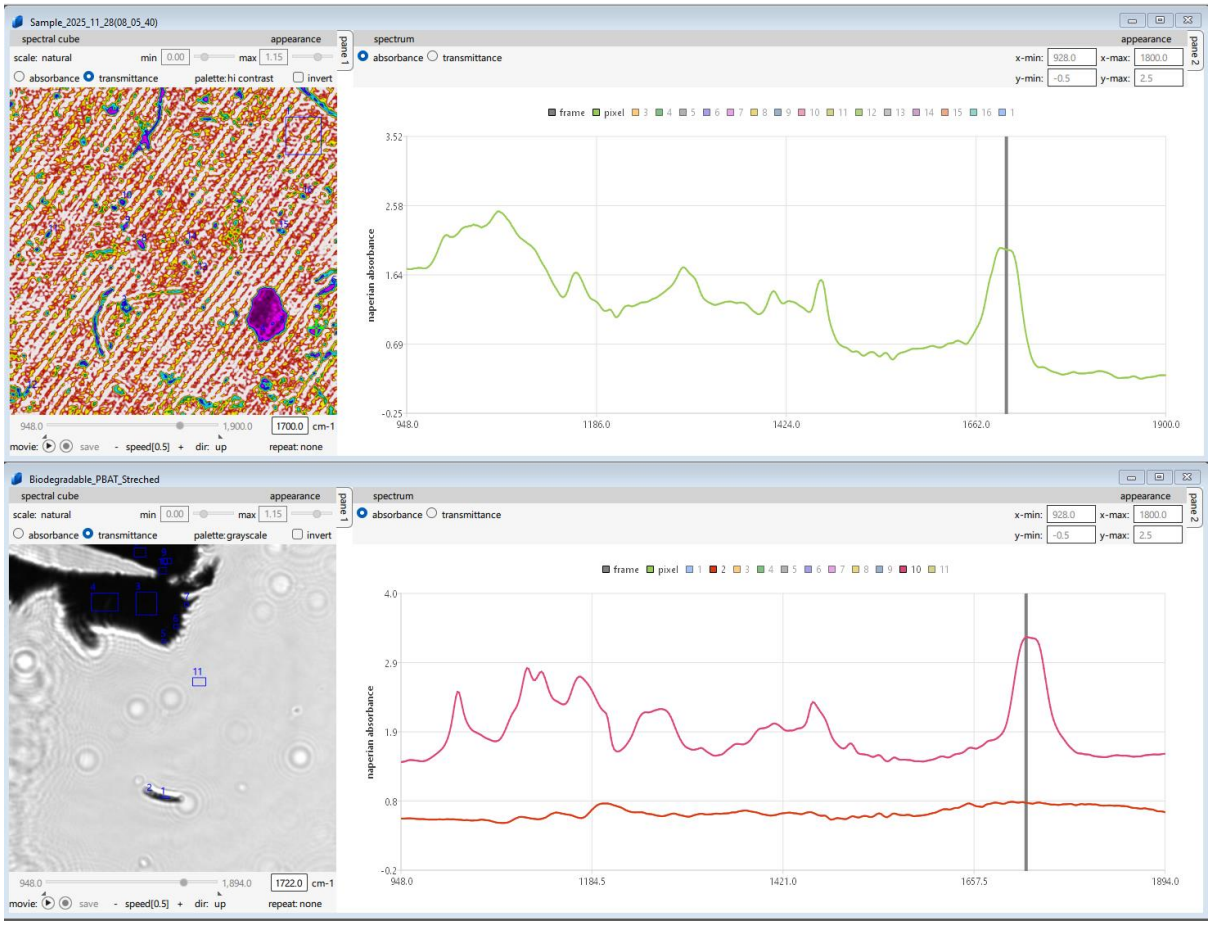

Figure 28: Screenshot of the comparison between the infrared spectrum of a particle found in strawberry extract (up) and the infrared spectrum of the biodegradable mulching film (bottom).

The applicability of the newly developed sample preparation procedure was further assessed with regard to its ability to sufficiently remove the strawberry matrix and enable the detection of MPs by Spero-QT analysis. In both unspiked and spiked strawberry extracts, MPs were detected, and were most frequently classified as PS, although in low numbers (Figure 29). The probability threshold for PS identification was increased to 0.6 during data reprocessing, which substantially reduced the number of misclassified PS particles, likely arising from the spectral interference from residual strawberry matrix components, and improved classification reliability. In the spiked sample, PBAT, the main constituent of the biodegradable MPs, could not be identified because this polymer is not yet included in the current spectral classification model. Importantly, PBAT was not misclassified as polyethylene terephthalate (PET), despite its chemical similarity, indicating adequate specificity of the model under the applied analytical conditions.

Overall, the results demonstrate that the strawberry matrix was digested sufficiently to allow reliable IR microscope analysis using the Spero system. The detection of low background levels of various polymer types is consistent with expected environmental or handling-related contamination and provides important context for interpreting results from spiked samples.

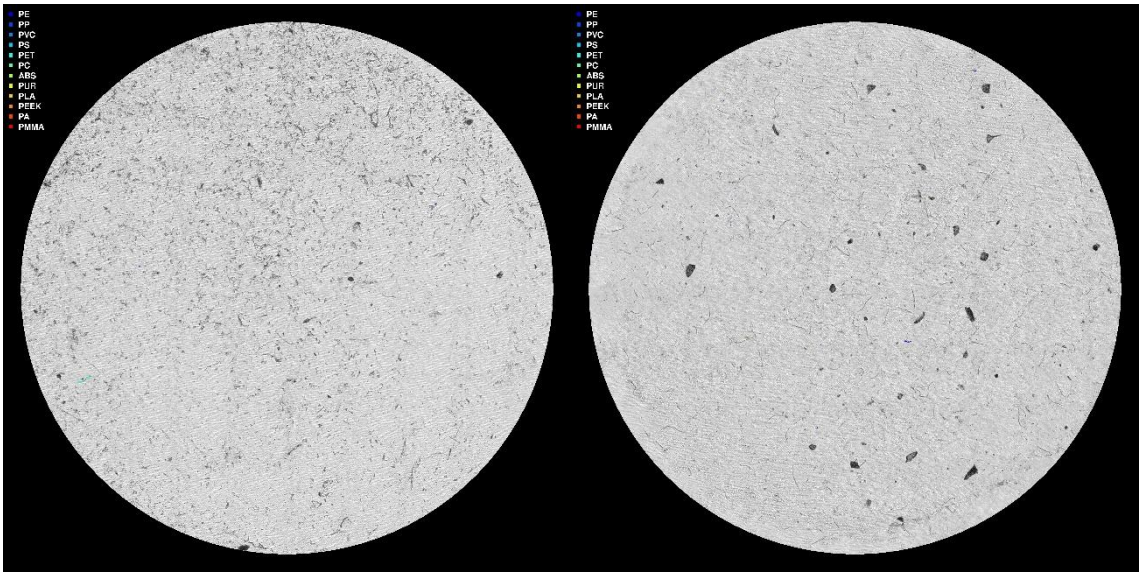

Figure 29: IR microscopy images of filter loaded with unspiked (left) and spiked (right) strawberry extracts processed using the newly developed sample preparation procedure. Polymer types were identified by comparing the measured spectra with polymer classes implemented in a machine-learning classification model.

4. Related virtual trainings

In addition to hands-on training provided to researchers from JSI and AUA on sample preparation procedures for extracting MNPs from soil (at VITO) and strawberry samples (at DTU Food), WP4 activities also included regular virtual trainings and consultations. These online interactions, conducted under Task 4.3 (Virtual training: Enhancing MNP extraction techniques knowledge exchange), facilitated knowledge exchange among InPlasTwin partners by addressing methodological challenges and supporting discussions aimed at refining and optimizing MNP extraction procedures. A key focus of these activities was the adaptation of extraction methods initially developed using MNP standards composed of conventional polymers to enable the analysis of MNPs generated from the degradation of mulching films in WP3.

Prior to the hands-on trainings, several online planning meetings were organized. During these sessions, hosting institutions provided overviews of extraction and analytical methods available for MNPs analysis in soil (VITO) and strawberries (DTU Food). Different sample preparation procedures were discussed in terms of their suitability for soil and food matrices, including advantages, limitations, and laboratory feasibility. These discussions formed the basis for the experimental work conducted during the hands-on trainings.

- For Task 4.1, the online planning meeting organized on the 27th of February 2025 via Teams defined the strategy for testing and developing soil extraction methods. This included selecting suitable MNPs for spiking and identifying agricultural soil samples from strawberry fields in Western Peloponnese for sample preparation development. Through online consultations and email exchanges, partners compared different extraction protocols, including the marine sediment protocol at IMR (density separation in a custom separation and sedimentation chamber, followed by filtration and chemical digestion with HCl, urea/NaOH, or H_2O_2), VITO's soil protocol (density separation, followed by flash-freezing, Fenton digestion, filtration), and DTU Sustain's oil-based extraction of bioplastics from compost (Figure 30).

- For Task 4.2, the online planning meeting on 6th of June 2025 focused on determining whether to target MPs, NPs, or both when developing extraction procedures from strawberries (considering MPs expected on the surface versus NPs inside strawberries); selecting suitable test materials (mulching-film–derived particles or metal-doped standard plastic materials such as Eu-doped PS NPs); evaluating sample preparation strategies, including acidic, alkaline, H_2O_2-based, and enzymatic digestion (e.g., R-10 Macerozyme), and identifying analytical techniques to evaluate protocol efficiency.

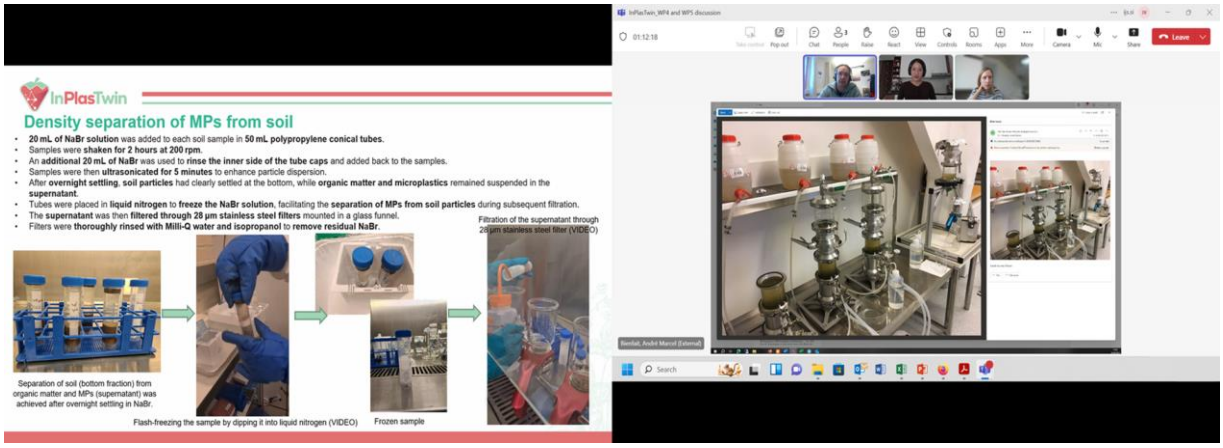

Figure 30: Online meeting of researchers from VITO, JSI, and IMR discussing the extraction protocols for MNPs from soil samples available at VITO and IMR.

Following the hands-on training, the main learning outcomes were presented in an online session on the 25th of September 2025 (Figure 31). Janja Vidmar (JSI) shared her training experience at VITO, highlighting tested and developed sample preparation steps and pyrolysis GC-MS analysis of MNPs in soil. Nhu Phan (VITO) discussed challenges in analysing PE MPs by pyGC-MS, including low recovery rates and interference from soil organic matter, and low recovery rates of polymer markers from NPs, suggesting sample pre-concentration for improved detection. Majda Nikezić (JSI) presented the outcomes of the DTU Food training on NPs extraction from strawberries, reporting good recovery rates based on (sp)ICP-MS analysis of metal-doped NPs. Discussions emphasized limitations in analysing MNPs in real environmental and food samples and highlighted the need to refine protocols to improve recovery rates and analytical accuracy.

Overall, these virtual trainings and consultations successfully facilitated knowledge exchange among partners, contributing to the development and refinement of robust and effective sample preparation procedures for extracting MNPs from both soil and food matrices within the InPlasTwin project.

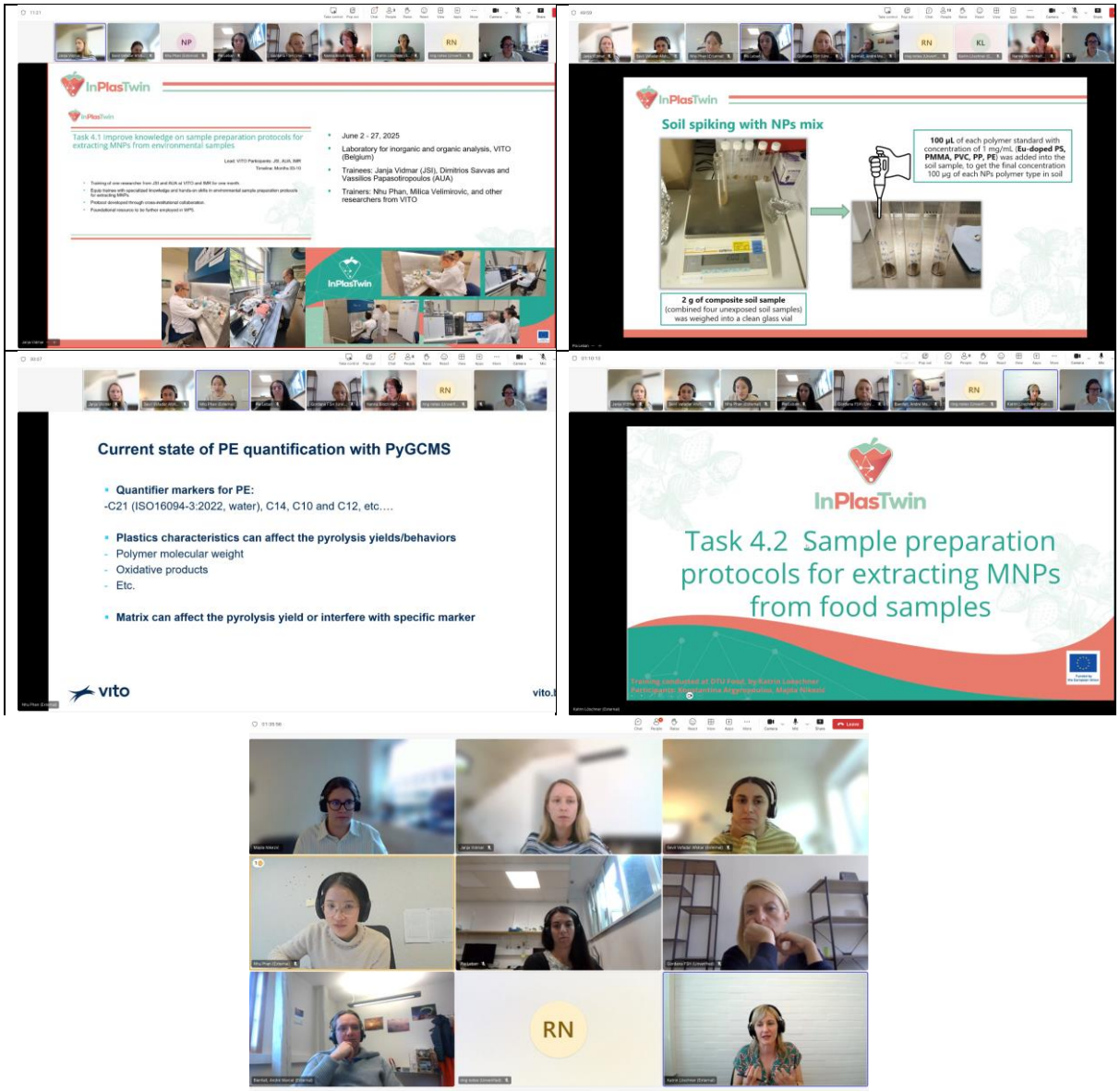

Figure 31: Online presentation and discussion of the key outcomes from the hands-on trainings conducted under Tasks 4.1 and 4.2.

5. Conclusions

The work reported in Deliverable 4.2 provides an overview of the InPlasTwin activities carried out within WP4 (Knowledge exchange in the MNPs extraction from complex samples), building upon the hands-on trainings conducted at the experts' institutions in Tasks 4.1 and 4.2 and using virtual knowledge exchange activities in Task 4.3, all aimed at developing working sample preparation procedure for efficient and reliable extraction of MNPs from agricultural soil and strawberry samples. Additional experiments were undertaken to refine extraction procedures, particularly for MNPs released from the degradation of mulching films (a key element of WP3) and to ensure compatibility with specific analytical methods. The optimization aimed to effectively isolate MNPs from complex matrices while preserving particle integrity.

For the extraction of MNPs from agricultural soil samples, three extraction procedures were developed: (i) a conventional approach based on density separation with NaBr and organic matter digestion using Fenton reagent, suitable for extracting PE MPs; (ii) a novel protocol combining TSPP extraction with mild hydrogen peroxide digestion for the recovery of conventional and biodegradable NPs; and (iii) a two-step protocol that integrates NP extraction with a subsequent density separation step, enabling the simultaneous extraction of biodegradable NPs and MPs from the same soil sample.

For strawberry samples, the protocol initially tested on metal-doped PS NPs included sample incubation in Macerozyme R-10 enzyme, followed by short settling and collection of the supernatant for spICP-MS analysis. While this approach showed satisfactory recovery for metal-doped NPs, it was insufficient for removing the strawberry matrix for IR microscope analysis, prompting further refinement of the protocol. This approach involved alkaline digestion with KOH combined with enzymatic digestion using Viscozyme L, followed by filtration and hydrogen peroxide digestion, with all steps adapted to preserve biodegradable MNPs.

Contamination prevention measures, recovery assessment, and evaluation of analytical sensitivity and selectivity across different particle concentrations, polymer types, and sizes were integral to assessing the performance of the developed protocols. Extraction efficiency was evaluated by spiking soil and strawberry samples with metal-doped PS NPs and analysing them using spICP-MS. The efficiency of the protocols was further tested for various plastic materials relevant to InPlasTwin research, including PE and biodegradable MNPs originating from mulching films used in strawberry cultivation.

The conventional protocol applied for the extraction of PE MPs from soil yielded recoveries (as determined by pyGC-MS) between 11.5% (for MPs generated from field-exposed mulching films) and 31.5% (for MPs generated from pristine mulching films), with remaining challenges in distinguishing PE-derived pyrolysis products from soil-derived compounds. The two-step novel protocol for extracting MNPs achieved recoveries for metal-doped PS NPs ranging from 60% to 95% (depending on soil type), as determined by spICP-MS. When applying the two-step sample preparation procedure for extracting biodegradable MNPs for pyGC-MS analysis, the absence of characteristic PBAT pyrolysis markers suggests that the MNPs were likely degraded or incompletely recovered during extraction, whereas similar signals in spiked blank and soil samples indicate that the soil matrix did not negatively impact recovery.

Enzymatic digestion of strawberries using Macerozyme R-10 achieved near-complete recovery (approximately 100%) for metal-doped PS NPs, as determined by spICP-MS, while the protocol developed for extracting biodegradable MNPs for IR microscopy was qualitatively shown to effectively digest the strawberry matrix and enable the identification of target MPs with sufficient specificity in the presence of the matrix.

Overall, the proposed extraction procedures demonstrated acceptable recoveries and robustness, while highlighting remaining challenges such as matrix interferences and analytical specificity. This work represents an important step toward making the developed extraction procedures applicable for analysing MNPs expected in soil and strawberry samples obtained from hydroponic experiments (WP7) and field-scale experiments (WP8), which will be analysed in subsequent activities of the InPlasTwin project.

6. References

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