Detection and Characterization of Small-Sized Microplastics (≥ 4 µm) in Milk Products

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Abstract

Microplastics (MPs) have gained a high degree of public interest since they are associated with the global release of plastics into the environment. As a result, MPs have also been detected along the food chain. However, information on the ingestion of MPs via the consumption of many commonly consumed foods like dairy products are scarce due to the lack of studies investigating the “contamination” of this food group by MPs. This lack of occurrence data is mainly due to the absence of robust analytical methods capable of reliably quantifying MPs with size < 20 µm in foods.

In this work, we have developed a new methodology to accurately determine and characterize MPs in milk-based products using µRaman technology, entailing combined enzymatic and chemical digestion steps. We demonstrate for the first time the presence of relatively low amounts of small-sized MP (≥ 4 µm) in raw milk collected at farm just after the milking machine and in some processed commercial liquid and powdered cow’s milk products.

Introduction

MPs are generally defined as plastic debris particles less than 5 mm in size (1, 2). Despite the lack of an internationally agreed definition of the lower size limit for MPs, a compromise of 1 µm has been pragmatically accepted in order to be fully consistent with the SI nomenclature for the nanoplastics’ category that ranges from 1 to 1000 nm in size, with a proposed category of sub-microplastics for the range of 100 to 1000 nm (3). MPs are classified by their origin into primary or secondary materials (4). The primary group includes virgin resin pellets and intentionally manufactured MP particles mainly applied in cosmetics, clothing, cleaning and personal care products, whereas secondary MPs are microparticles caused by environmental degradation, e.g. weathering, mechanical tear and abrasion and consequent fragmentation of larger plastic objects or related debris (5).

Thermoplastics encompass a long list of different polymers such as for example polyvinylchloride (PVC), polyethylene terephthalate (PET), polystyrene (PS), acrylonitrile-butadiene-styrene (ABS), polyamide (PA), polycarbonate (PC), polymethylmethacrylate (PMMA) and fluoropolymers such as polytetrafluoroethylene (PTFE). Thermoplastics based on polypropylene (PP) and polyethylene (PE) resins are today the most widely produced, with a world production volume estimated at ca 140 million tons in 2019 (6, 7). The thermally more stable thermoset materials entail, amongst others, polyurethane (PU), unsaturated polyesters (PES), as well as epoxy and acrylic resins.

Large amounts of MPs in the size range from 0.1 to 1 mm are known to accumulate in the environment mainly due to them being directly discharged from land-based sources directly into the marine environment (8), including oceans (9, 10), freshwaters (11) and finally waste waters (12). Concerns about MP pollution of terrestrial ecosystems are also increasing after proof of their presence in soils (13, 14).

The detection of MPs in environment and related biota is however challenging. In the past 10 years, the use of more performant Fourier transform infra-red (15–17) or Raman micro-spectroscopy (18, 19)

compared to pyrolysis-gas chromatography-mass spectrometry (Py-GC-MS) (20, 21) and thermal desorption-gas chromatography-mass spectrometry (TD-GC-MS) (22) have shown the presence of MPs, mainly in the size range from 20 to 250 µm in aquatic fauna, including some common species consumed by humans (23–28). Apart from marine species, recent scientific review papers have highlighted the unequivocal presence of MPs in a wider variety of foods intended for human consumption (29). These foods include poultry meat, snails, edible salts, sugar, honey, and various beverages like drinking and bottled waters, soft drinks, cold tea, beers and liquid milk products (30–40). Reports on the occurrence of MPs have led to increased media interest and concerns about this emerging issue among some international agencies and public health organizations (41–44).

The analytical methods published to date on the analysis of MPs in food show several shortcomings. Many of the methods are either not adequately sensitive to identify MPs with size < 20 µm and/or specific enough to discriminate organic particles from MPs. They are also simply incomplete in terms of performance by lack of information on the MPs size range and on polymer identification.

These difficulties can be explained by the numerous analytical challenges in isolating intact MPs after destruction of the organic food matrix (45, 46). According to these recent critical reviews on sample preparation for MPs analysis, the method of choice for purification of a sample from organic matter that proved to be cost-and time-effective is an alkaline digestion approach using dilute potassium hydroxide solution for 2–3 days with a relative limited degradation of main polymers. Enzymatic methodology also proved to be a promising alternative in degrading the organic matter, especially tissues while not affecting the polymers' integrity. Currently, µRaman amongst all other techniques is considered as the most sensitive technique for MPs analysis after sample digestion, allowing the detection of particle size as small as 1 µm thanks to the high spatial resolution of the laser beam (47–50).

The paucity of data in food commodities in general prompted us to study the occurrence of MPs in selected food materials, starting with cow's milk products. The level of contamination of MPs in milk products remains largely unknown due to the scarcity of published scientific data, and associated difficulty to isolate and quantify MPs in such complex food matrix (39, 40). Along a dairy supply chain, contamination with MPs can occur at various stages. They can be introduced during the milking of the cow at the farm, during downstream processing, or via the final packaging (51).

In this study, a new methodology was developed allowing a fast and efficient extraction of MPs and their identification in samples of cow's milk. This method is based on a two-step preparation that involves multi-enzymatic digestion followed by hot alkaline hydrolysis and a final filtration step through a silicon (Si) filter.

The material retained on the filter was subsequently analyzed using high resolution µRaman equipment. The collected spectra were then processed with an in-house software to reliably characterize for the first time micro-sized MPs (≥ 4 µm) in farm's milk and processed cow's milk products using high resolution µRaman and SEM-EDX technologies.

Results

Mitigating laboratory contamination.

Method blanks performed during method development revealed different sources of MPs contamination. These were mainly the laboratory environment, chemical reagents, containers and laboratory clothing. These sources of contamination were subsequently minimized following the mitigating actions described in the “Materials and Methods” section. After method optimization, Raman analysis of filters retained from procedural blanks showed no significant MP contamination. The total number of MPs found in a set of 10 different daily method blanks used for this study (Figs. 1A and 1B) ranged from 6 to 46 MPs retained on the filter, with an average of 27 ± 12. PE was the most frequently found polymer in method blanks representing ca. 43% of the total amount followed by PES (27%), PP (21%), and by PTFE and PS at lower amounts. More than 80% of MPs found in the set of 10 blanks had a measured surface area of less than 50 μm^2 (ca particle size $\leq$ 7 µm).

Method recovery.

Samples of ultrapure water and pasteurized liquid cow’s milk (Brand B, 3.5% fat) were spiked with commercial standards of five different MPs to determine the recovery rate of the method and to check the potential degradation of MP standards after hot alkaline digestion.

Table 1 shows the recovery of the five standards of MPs spiked in ultrapure water sample without digestion and in a cow’s milk sample following the whole method procedure (i.e. digestion and Raman analysis). Particle recovery for all five different polymer standards (PMMA and PS beads, PE, PP and PA polymers with size ranging from 5 to 40 µm) ranged from 78 to 141% with RSD ranging from 12 to 45%. These results were acceptable given the challenging procedure to prepare repeatable standard polymer solutions with low concentrations of MPs.

Table 1
Recovery rates of five MP standards in ultra-pure water and liquid cow milk (Brand B, 3.5% fat)

Spiked Samples*	PMMA (%)	PS (%)	PP (%)	PE (%)	PA (%)
Ultra-pure Water (N = 7) Without digestion	78 ± 12	97 ± 13	90 ± 18	86 ± 19	94 ± 24
Liquid cow's milk 2 (Brand B) (N = 4) With digestion	97 ± 17	123 ± 45	136 ± 34	119 ± 30	141 ± 12
*After blank subtraction of PP, PE and PS contaminations N represents the number of replicates.					

Figure 2 depicts SEM images of a spiked ultrapure water sample (Fig. 2A, MP standards, no digestion involved) and of a spiked milk sample (Fig. 2C, after digestion), as well as EDX spectra of encountered particles (Fig. 2B and Fig. 2D representing the water solution and cow's milk sample respectively).

The polymers PA, PMMA, PS and polyolefins (PE-PP) were identified in the spiked water sample (Figs. 2A and 2B) by combining the morphology found in the SEM micrographs and the identification of the major chemical elements via EDX detection. These four groups of MP standards (PA, PMMA, PP-PE and PS) were also detected in spiked milk after digestion (Figs. 2C and 2D) since their morphology was well preserved after digestion despite their small sizes.

SEM imaging of the Si filter surface of a spiked milk sample (Brand C, 0.1% fat) provided an overview of the particle structures present after alkaline digestion (Fig. 3). The high-resolution SEM images of the digested sample showed the filter surface at a submicron level and qualitatively distinguishes between the different types of particles (fibers, organic residues, mineral particles and standards of MPs) present after sample digestion. We did not observe significant differences in the MP standards after digestion, neither in morphology (via SEM) nor in chemical composition (via EDX analysis).

For example, in the SEM images of a Si Filter surface, four particle shapes were identified (Fig. 3A). These were assigned to undigested fibrous fragments, digested organic residue, mineral particles, and to the retained MP standards (PA fragments, PS and PMM beads). The MP standards were widely distributed over the entire surface of the filter even if some MPs were agglomerated with some fibers (Fig. 3C).

Mineral based particles (e.g. Mg in Fig. 3D) can be identified through EDX analysis, but fibers, organic residues and MPs show similar EDX spectra for C, O and N elements (Fig. 3E). Therefore, the identification of MPs using EDX information only is very challenging and was only applicable to cases in

which we were aware of their intentional addition and associated well-defined morphology (e.g. beads with single particle size, as observed for the PMMA and PS particles (Figs. 3B and 3F).

Validation of the Raman methodology.

Figure 4 depicts the optical microscope image of surface part of a Si filter for a spiked pasteurized commercial cow's milk sample (Brand C, 0.1% fat) obtained after alkaline digestion and the Raman spectrum of each detected standard MP. This image was obtained using an optical microscope equipped with bright-field illumination (Fig. 4A). The same image was processed using the software of LabSpec 6.5 to highlight the cellulose fiber and the five different types of MPs present in the sample (Fig. 4B). In addition, the Raman spectrum of each MP present is shown in Fig. 4C. The Raman spectra (Fig. 4C) were easily attributed to each polymer using the specific vibrational assignment of the main peaks (Table S1) with a good fit when compared to a reference database (KnowitAll Raman spectral library). Importantly, no significant physico-chemical changes were also identified by µRaman in the polymer structures after alkaline digestion.

Microplastics determination in cow's milk samples.

The Raman method was successfully applied to determine and count the number of MPs in liquid and reconstituted cow's milk samples. The concentration of MPs identified in milk samples ranged from 80 to 968 MPs per 100 mL of sample and between 69 to 99% of the detected MPs in milk samples had a surface area $\leq$ 50 μm^2 (Table 2).

Table 2
Number and type of MPs detected in cow's milk samples

Milk Samples	Total number of MPs per 100 mL**	MP surface < 50µm2 (%)	PE	PES	PP	PTFE	PS	Other polymers
Blank (milking machine) - (2nd collection)	52	92	44	ND	ND	ND	8	ND
Raw milk (1st collection)	96	70	ND	24	44	20	8	ND
Raw milk (2nd collection)	80	69	70	ND	0	10	5	ND
Cow's milk liquid (Brand A)	196	99	ND	4	ND	116	76	ND
Cow's milk liquid 1 (Brand B)	176	93	20	20	8	72	32	4 PU*** 4 PSU*** 16 PVA***
Cow's milk liquid 2 (Brand B)	328	69	136	100	30	24	38	20 PU***
Cow's milk liquid (Brand C)	116	81	16	ND	68	32	ND	ND
Cow's milk powder * (Brand A)	420	81	36	204	80	100	ND	ND
Cow milk powder* (Brand B)	968	86	64	16	12	876	ND	ND

*: reconstituted cow's milk samples (13 g powder in 100 mL water which corresponds to the serving size of liquid and reconstituted cow's milk samples with a standard nutritional energy of ca 67 kcal for milk products).

**: values subtracted from the blank.

***: PU: polyurethane; PSU: polysulfone; PVA : polyvinyl alcohol.

ND: not detected.

The main MPs found in farm's milk, processed liquid milk and reconstituted powdered milk samples were PE, PES, PP, PTFE and PS particles, as depicted in Table 2. The two liquid samples 1 and 2 (brand B) contained additionally PU particles, whilst one also contained small amounts of polysulfone (PSU) and

polyvinyl alcohol (PVA) polymers. The same order of MPs concentration was found in both farm milk and liquid cow's milk samples. Milk powders (Brand A and B) after water reconstitution contained relatively higher levels of MPs compared to farm milk and ready-to-drink milk samples (Brands A, B and C). Based on these data, it could be highlighted that the number of MPs tends to increase from farm's milk to processed milk powders, but the MPs concentrations stay in the same order.

Discussion

The validated digestion procedure used in this study entailed a rapid combined enzymatic and hot alkaline digestion, that effectively removed milk components, hydrolyzing proteins (e.g. whey proteins and caseins), lipids (e.g. triglycerides) and carbohydrates (e.g. lactose) and other organic complex aggregates without degrading the five types of MPs that were tested. The step of chemical soft alkaline microwave hydrolysis based on effective chemical solubilization of the milk sample by a quaternary ammonium salt allows a final rapid filtration in cutting all organic matrix in micrometric fragments without significant sample dilution (52). Another advantage to consider for this rapid digestion procedure is the size of the resulting digested fragments that are as small as possible for an efficient microfiltration of the digested milk sample, otherwise this will lead to clogging of the 5 µm-porosity Si filter (39, 40). It has recently been shown that during the first few minutes of lipase digestion of milk, small fragments of triglycerides (ranging from 1–10 µm) are released and subsequently re-aggregate to a higher size (> 50 µm) if digestion time is prolonged (53). The formation of these so-called "calcium containing soaps" is significantly reduced in this digestion procedure by addition of a calcium chelator, thereby avoiding the rapid clogging of the filter.

Method recovery was acceptable for the spiked cow's milk and water samples. In general, this study showed that the recovery of MPs in replicates of samples of cow's milk after the digestion procedure is generally over 100%. In contrast, the recovery of MPs in undigested water replicates is less than 100%.

The lower recovery obtained for spiked water sample could be linked - due to their hydrophobicity- to the formation of MPs aggregates larger in size than that of added standard MPs particles. Even if MPs in undigested spiked water are evenly distributed on the Si filter, theses aggregates identified as being larger particles than the polymer standard resulted in the total number of particles being finally underestimated after extrapolation of the counting for the total surface.

Higher recovery results found for digested spiked milk samples can eventually be explained by the fact that many MPs particles were stuck to undigested fibers like cellulose, generating "hot spot" regions. The physical phenomenon that leads MPs to aggregate to fibers was not studied in this work. However, we believe that it must be associated with strong electrostatic interactions among the MPs and these organic particles. Since the total number was determined in the central half area analyzed where larger organic fragments are present, MPs particles had an uneven distribution and were consequently slightly overestimated due to presence of these aggregates.

The analysis performed with SEM-EDX corroborate the results obtained with Raman for the cow's milk and water samples spiked with MPs regarding MPs physical integrity. However, the elemental composition analysis by EDX was not sufficiently discriminative to confirm that the microparticle analyzed was a MP or an organic residue from the digestion of cow's milk. Therefore, EDX was undoubtedly a powerful tool to support Raman, but it cannot be used as a confirmatory technique for the correct identification of organic compounds such as MPs.

The Raman method used was performant in detecting and identifying all types of MPs > 4 µm size. The exogenous MPs detected by Raman in samples of cow's milk had a spectral profile very similar to the MP standards but was significantly less intense. This difference may be related to the fact that exogenous MPs are exposed to environmental agents (e.g. sunlight, temperature, humidity, etc.) and various processing steps used to convert farm's milk into milk-based products. Despite this reduction in intensity of the peaks of the spectrum, the Raman method developed was sufficiently sensitive and specific to correctly detect and identify MPs with dimensions ≥ 4 µm.

The lowest number of MPs was found in the two raw milk samples collected at farm level. PE and PS turned out to be the main polymers present in milking machine that are likely contributing to raw milk contamination. The origin of other MPs like PP, PES and PTFE in raw milk can be multiple, e.g. ubiquitous presence in the farm environment and along milking process including storage containers. Depending on the type of packaging, PP particles could likely come from bottle (processed liquid milk - brand C) and PE from multilayer laminated paper (processed liquid milk samples - Brands A and B). Three other types of MP (PU, PSU and PVA) were detected in liquid milk samples (Brand B). The presence of PSU is a potential indication of plastic debris originating from the abrasion of membrane filters used during milk processing (39). PVA can come from the packaging, since PVA is usually used as oxygen and odor barrier polymer in multilayer laminated paper.

Powdered milk samples showed the highest number of MPs (PP, PE, PES and PTFE). These types of MPs are the most ubiquitous in environment and their presence in milk samples may be due to environmental contamination, milking process (i.e. series of macro, micro and ultrafiltration using polymeric membranes and additional drying steps for powder) and packaging conditioning from farms to dairy processing facility. In contrast to the previous works (39, 40) where an average of 6 MPs/L (39) and 40 MPs/L (40) of milk samples (mainly sulfone thermoplastics, PP and PE fragments with size > 10 µm) were identified by µFTIR and µRaman, our study detected a higher average number and types of MPs. The most abundant MPs determined were PP, PE, PES and PTFE with a size < 7 µm (i.e. surface area ≤ 50 μm^2). The higher number of MPs detected in this study may be associated with the fact that a point-by-point Raman approach was able to detect smaller particles in milk products than those detected by the visual detection approach of MPs > 10 µm size adopted in previous studies (39, 40).

If we assume that MPs are considered as migrating polymers and that 100% MPs are spherical particles of 10 µm diameter with a density of ca 1g/cm^3, a number of 1000 MPs found per serving size (i.e. 100 mL) in the milk samples corresponds to a concentration of MPs of ca 5 µg per kg of product. This

concentration is lower than the overall limit of substance migration (60 mg/kg food) and the specific migration of oligomeric fraction (less than 1000 Da) of polymers (50 µg per kg food) specified in the EU N° 10/2011 related to plastic migration (54). However, the size and type of MPs found in foodstuffs have also to be assessed from a toxicological point of view which is the subject of numerous project proposal for funding by different authorities around the world.

Thus, it is urgent to standardize appropriate isolation and identification methods of MPs in food that follow guidelines using reliable protocols with strict quality assurance and quality control measures in order to provide sensitive, accurate and comparable results (55, 56). Due to the different nature and size of MPs in a great range of food matrices, many efficient extraction methods using density separation and chemical or/and enzymatic digestions before filtration steps are available but need to be improved for better MPs recovery without degrading MPs using quicker sample treatments (57). Each analytical method has its strengths and weaknesses for MPs characterization at micrometer level even if µRaman or µFTIR spectroscopy present more advantages in terms of sensitivity and specificity than SEM, flow cytometry, staining techniques or destructive method like Py-GC-MS (58–60).

Conclusion

A new validated methodology for isolation and detection of MPs in milk samples was successfully used for the first time to detect and identify micro-sized MP (≥ 4µm) in raw and processed cow's milk.

This study has shown a slight trend of increase of MPs with the degree of milk processing and packaging conditions. Therefore, this method could be a good analytical tool for route cause analysis of MPs contamination along the dairy chain and therefore to mitigate number and types of MPs in cow's milk products. Additionally, it could be further used in a worldwide survey of dairy products and related ingredients to support the international authorities in performing toxicological risk assessments of human diets.

Finally, with complementary work to optimize µRaman analysis time using a rapid particle counting approach, this could lead to an acquisition time of only 2–3 hours versus ca 20 hours in the current setup. In such case, this could contribute to a mandatory standardization of MPs determination in other foodstuffs including raw materials in order to ensure trust between authorities, food industry and consumers.

Method And Materials

Cow's milk sampling.

Some of the most commonly sold milk products in Switzerland were purchased in stores from three main supermarket chains. Four liquid milk samples and two milk powders were collected and stored in laboratory at 6°C. Raw milk was first directly collected at two different dates into a glass flask (250 mL, particle certified, ThermoFisher Scientific) at a farm located in France (Jura country) using a milking

machine (deLaval), then transported in a cold container to Switzerland and finally stored at 6°C in laboratory before analysis. Samples used for this study are listed in the table S2.

Sample preparation

Milk sample digestion.

Powdered milk samples were initially reconstituted with ultrapure water in a glass flask (1000 mL; particle certified, Thermofisher Scientific) by diluting 25 g test portion in 175 g ultrapure water (Lichrosolv, Merck); 2 mL of multi-enzymatic detergent (Prozyme, Borer Chemie AG) were added in the reconstituted powdered milk and shaken for at least 15 minutes at 40°C into a water bath GFL-1083 Milian.

Processed and raw milk samples were transferred to glass flasks and covered with a glass watch. The samples were digested using a preparation that consists of 2 steps: enzymatic digestion and hot alkaline hydrolysis of milk samples, as described below.

25 mL liquid or reconstituted milk sample and 20 mL ultrapure water (Lichrosolv, Merck) were added into a glass flask (1000 mL; particle certified, ThermoFisher Scientific); 2 mL of multi-enzymatic detergent (Prozyme, Borer Chemie AG) were added and mixed for 2 minutes at 40°C in the container; 10 mL of chelating agent sodium ethylene diamine tetra acetate (EDTA-Na 0.5 M, pH 8, Invitrogen, ThermoFisher Scientific) were added and mixed for 3 minutes at 40°C in the container; 2 mL of alkaline solution tetramethyl ammonium hydroxide (TMAH 25% v/v Sigma-Aldrich) were added into the glass container that was finally immediately put in a microwave (Panasonic NN) at a power of 1000 watts for a maximum of one minute to reach about 80°C. Hot digested milk sample was then immediately submitted to filtration process.

Milk sample filtration.

Hot digested milk sample was directly poured into a glass funnel (100 mL, Sterlitech) mounted onto a filtration unit (glass holder with13 mm frit, stainless steel vacuum manifold, Rocker 400 Vacuum Pump, 220V/50Hz, Sterlitech). Contents of the funnel pass through a Si filter (Silicon filter, 10 x 10 mm size, 500 µm thickness, 5 µm porosity, SmartMembrane) under a vacuum for a maximum of 5 minutes. Home-made filter holder (stainless steel, 10 x 10 mm, 25mm), rubber hole seals (EPDM, 25 mm diameter, 2 mm thickness, 4 and 8 mm diameter holes), seal holder and cover (stainless steel, 25 mm, 8 mm diameter hole) were used for a restricted filtration area of ca 14 mm^2 (i.e. 4 mm diameter area) on silicon filter (Figure S1). Retained digested material was successively flushed with first 5 mL water then 5 mL nitric acid 5% v/v and finally 10 mL water before being stored in a closed glass container before RM analysis.

Microplastics analysis.

Mitigating laboratory contamination.

A major challenge in the measurement of MPs is to avoid contamination of the test sample when handled in the broader laboratory environment, that includes for example particles from the ambient air, clothing, utensils, chemicals, and so forth. Meticulous study of the different possible steps that may introduce “foreign” MPs is a prerequisite to ensure reliable measurement. Therefore, emphasis on lowering the method blanks (i.e. blank following all the method process but omitting the sample matrix) besides the usual method validation parameters is pivotal. Class 2 biosafety cabinet, filtration unit, dedicated glassware and spectroscopy equipment (µRaman and optical microscopy) were installed in clean laboratory rooms with positive air pressure.

Control of laboratory environment was performed measuring MPs retained on some silicon filters put on laboratory benches and in biocabinet for one day before analysis. Grade air purifier (APS-500, Kynio) and particle counter (PC 220 Trotek) were used in the laboratory for mitigating and monitoring particles respectively. Alkaline solution (TMAH) is pre-filtered through 5 µm Ag filter (Sterlitech) whereas all other reagents used for milk preparation (i.e. multi-enzymatic detergent solution, EDTA-Na and ultrapure water) are pre-filtered through 0.65 µm PVDF filter (Merck) using stainless steel filter holders (Merck). Specific 1 L or 250 mL-Particle Free (PF) glass bottles (Thermo) are used for digestion procedure.

All the glass vessels (funnel, beakers, glass bottles), restrictor seals and filter holders were pre-cleaned thoroughly with ethanol 70% v/v and rinsed 3 times with ultrapure water (Merck).

The Si filter was pre-cleaned in a glass container with a pre-filtered aqueous solution of multi-enzymatic detergent (Prozyme, Borer Chemie AG) 10% v/v and finally rinsed with pre-filtered ultrapure water (Merck) in another glass flask. All the reagents used in filtration step (i.e. water, HNO_3 (Merck) were pre-filtered on 0.6 µm PVDF filter (Merck)). Method blanks (i.e. blanks following all the method process but omitting the sample matrix) were systematically analyzed for each series of analysis.

Method recovery.

The two quality criteria used for both efficient digestion and filtration steps were the digestion efficiency and recovery rates of spiked polymers in water and real sample.

Digestion efficiency was verified by counting remaining digested particles and the determination of total related surface covered in the filter by the digested matrix. The particle counting and determination of surface covered was performed using a digital optical microscope VHX-6000 (Keyence). A final maximum decision limit of 300 (with a maximum of 5% surface covered) and 600 retained particles per mm^2 of filtration area (with a maximum of 30% surface covered) were established for procedural blank and digest sample, respectively. This criterion ensures that the sample was digested efficiently and that the filter was not overloaded with organic residues, which could potentially hide MPs or even clog the filter.

Raman method was used to determine the MPs recovery rate that was calculated by spiking one ultrapure water without alkaline digestion step and on one cow’s milk sample (Brand C – 0.1% fat) following the whole procedure of digestion. Different MP standards in solution (PMMA, PS, PA, PE and PP

Sigma-Aldrich) were internally prepared and calibrated by spectral flow cytometry (FC, Cytek – Nestlé Research, Lausanne) in water containing sodium dodecyl sulfate detergent (SDS, Invitrogen) at 5% m/v. Polymer standard solutions used for recovery rates are listed in Table S3.

SEM micrographs of the Si filter surface combined with EDX analysis of the encountered particles were acquired for two spiked samples (ultrapure water and cow's milk (Brand C – 0.1% Fat)) to identify and check the physico-chemical integrity of the MPs without and with alkaline digestion respectively.

SEM imaging - EDX analysis.

Simultaneous particle imaging and elemental chemical composition analysis were performed to check the quality of digestion of one spiked ultrapure water and one spiked cow milk sample (Brand C – 0.1% fat) employing a scanning electron microscope (SEM) "Quattro S" from Thermofischer equipped with an Energy Dispersive X-Ray Spectroscopy (EDX) detector "Xmax 50 mm^2" from Oxford Instruments.

Prior to analysis, the Si filters were coated with either 5 nm of gold (for imaging purposes) or with 5 nm of carbon (for combined imaging and chemical composition).

Imaging of single particles was performed at an accelerating voltage of 5 kV in secondary electron and backscattered electron modes.

EDX spectra were acquired on single particles at 20 kV to confirm the organic or mineral nature of particles and subsequently at 5 kV to improve C, O and N detection. A minimum of 300000 counts were acquired per measurement.

Validation of Raman methodology.

Spiking experiment of one processed cow's milk sample (Brand C – 0.1% fat) using Raman method was performed using different polymer standard solutions (PMMA, PS, PA, PE and PP Sigma –Aldrich) internally prepared and calibrated by flow cytometry (FC) in water containing sodium dodecyl sulfate detergent (SDS, Invitrogen) at 5% m/v with the polymer concentrations displayed in Table S3. Spiking tests were used not only to check the efficiency of digestion and method recovery, as well as possible changes in the Raman fingerprint spectrum. A spectral change could be used as indicator of chemical degradation of the MPs due to the alkaline digestion step.

Raman analysis.

A confocal µRaman Labram HR Evolution (Horiba, SAS France, Villeneuve d'Ascq, France) equipped with an EMCCD detector, 50x magnification long working distance objective (Olympus® ,NA = 0.5), dark field system and 532 nm solid-state laser (power 50 mW), acquisition time of 0.1 second in a spectral range of 550 cm^{-1} to 2000 cm^{-1} (resolution of 4 cm^{-1}) was used to acquire the Raman spectrum. This instrument setting generated a laser beam of ca 1 µm.

The detection and identification of microplastics on the surface of Si filter was performed by the point-by-point mapping approach using steps of 5 µm between two consecutive points. The distances of 5 µm between two consecutive points combined with the laser beam size of ca 1 µm allowed us to confirm that this approach can detect MPs with dimensions equal to or greater than 4 µm. On the other hand, the dimension of ca 1 µm of the laser beam also allowed us to confirm that this approach can potentially detect MPs smaller than 4 µm if the particle was close enough to where the laser beam was irradiated to collect the Raman spectrum.

In this approach, 2 areas representing 50% of total filter surface (i.e. 7 mm^2) were consecutively analyzed (Figure S2). The instrument takes 2 times 10 hours to perform point-by-point mapping with step of 5 µm with a final acquisition of about 250,000 Raman spectra. A maximum limit of 600 retaining particles per mm^2 of filtration area has been established for ensuring both correct µRaman mapping and imaging treatment using Labspec® 6.5 software.

Raman data processing.

After Raman spectra acquisition using LabSpec® 6.5 software, an in-house software was developed for identification and characterization of microparticles. The training data set was constructed using Raman spectra collected from exogenous MPs found in food samples and commercial MPs spike-in milk products. Such diverse set of MPs sources allowed a better representation of the possible range of signals for each given polymer. Furthermore, a wide range of signal quality was selected to better represent the noisier signals that can be found in theses samples.

The spectra database contained few spectra with slightly different spectral range. However, this small variation in the spectral range is not compatible with most machine learning algorithm, including random forest. Therefore, the spectra range and measurement points were refitted (559 cm^{-1} to 1990 cm^{-1}, every 3 cm^{-1}) using R stats splinefun function to allow consistent measurement points of the spectra. Baseline correction was performed using the R package “baseline” to remove the background noise from the spectrum (61). A set of “universal” parameters was chosen to best fit the observed cases. To reduce heavy fluctuation of the signal, the spectra were smoothed using another spline function included in the R package hyperSpec (62). Parameters were chosen so that all data points were used with a reduced smoothing parameter to avoid any degradation of the signal. Finally, the spectra values were scaled over their respective standard deviation.

The Random Forest machine learning algorithm was fed with the preprocessed data. The algorithm was trained with the training set with of 1000 estimators and will classify each spectrum in one of the MP class or in the non-MP (NMP) class (63).

Using the physical position of each measurement, the neighboring signals of the same class of polymer were grouped into particles. Using the R package “lattice” (64), a graph represented the physical connections between spectra. The edges of the graph were then kept if the maximum distance between two spectra of a same polymer class was two. Effectively, this allowed clustering of polymer signals

based on their relative physical location, while jumping over “missed” signals. Missed signal could be caused by a short desynchronization between the computer clock and the speed of acquisition of the spectrum.

Declarations

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Author Contributions

E.P., D.A. and P.A.D.C.F conceived and designed the experiments. D.A. and B.E. conducted the experimental work for sample preparation. P.A.D.C.F., J.B.D. and E.P. conducted the experimental work for Raman analysis. R.P. and B.M.C. developed algorithm for treatment of Raman data. J.B.D. and M.A. conducted the experimental work for SEM-EDX analysis. E.P., P.A.D.C.F., D.A., J.B.D., M.A. B.M.C and R.P wrote the paper. S.D., P.Z. and A.P. provided their expertise and reviewed the manuscript.

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Figures

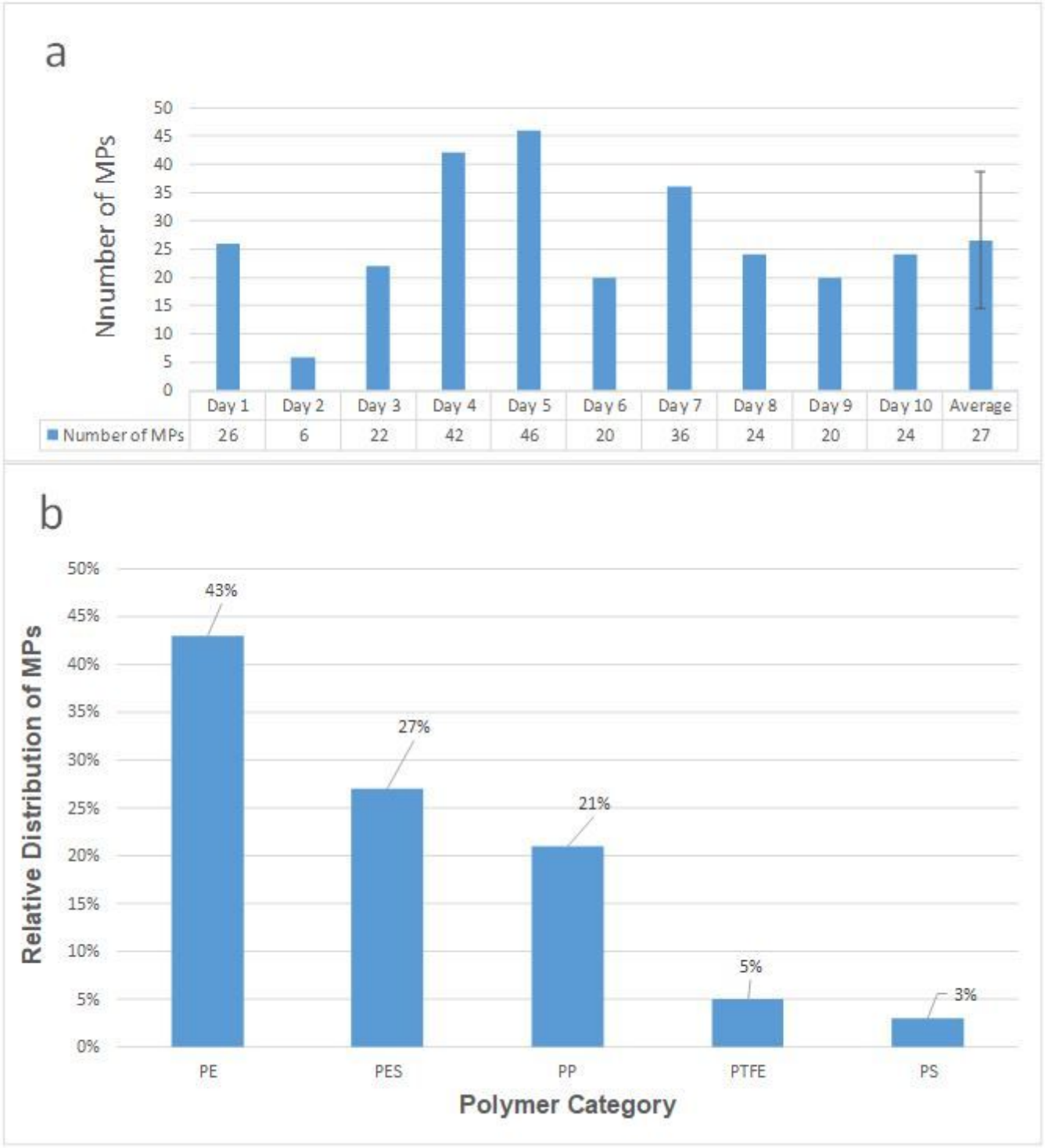

Figure 1

Monitoring of MPs contamination in method blanks. a) Number of MPs in ten procedural blanks; b) relative distribution of MPs in the method blanks based on polymer type

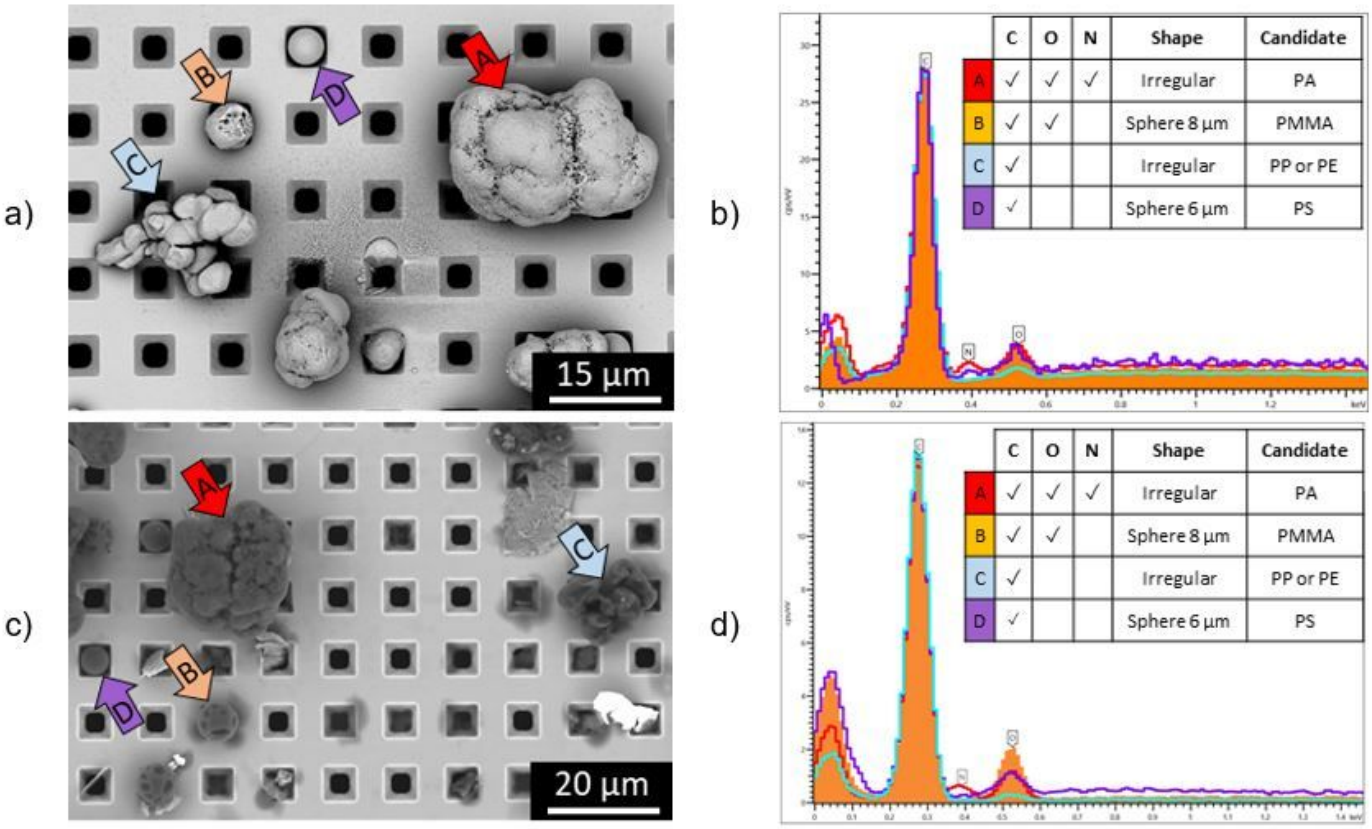

Figure 2

SEM-EDX analysis of MPs in ultrapure water: a) picture showing a SEM micrograph of a spiked water sample with PA polymer (red arrow A), PMMA polymer (orange arrow B), olefins polymers PE-PP (blue arrow C) and PS polymer (purple arrow D) ; b) EDX spectra of the MP standards pointed out in panel 2A; c) SEM micrograph of the milk sample (Brand C, 0.1% fat) spiked with the same polymers; d) EDX spectra of the MP standards selected in panel 2C.

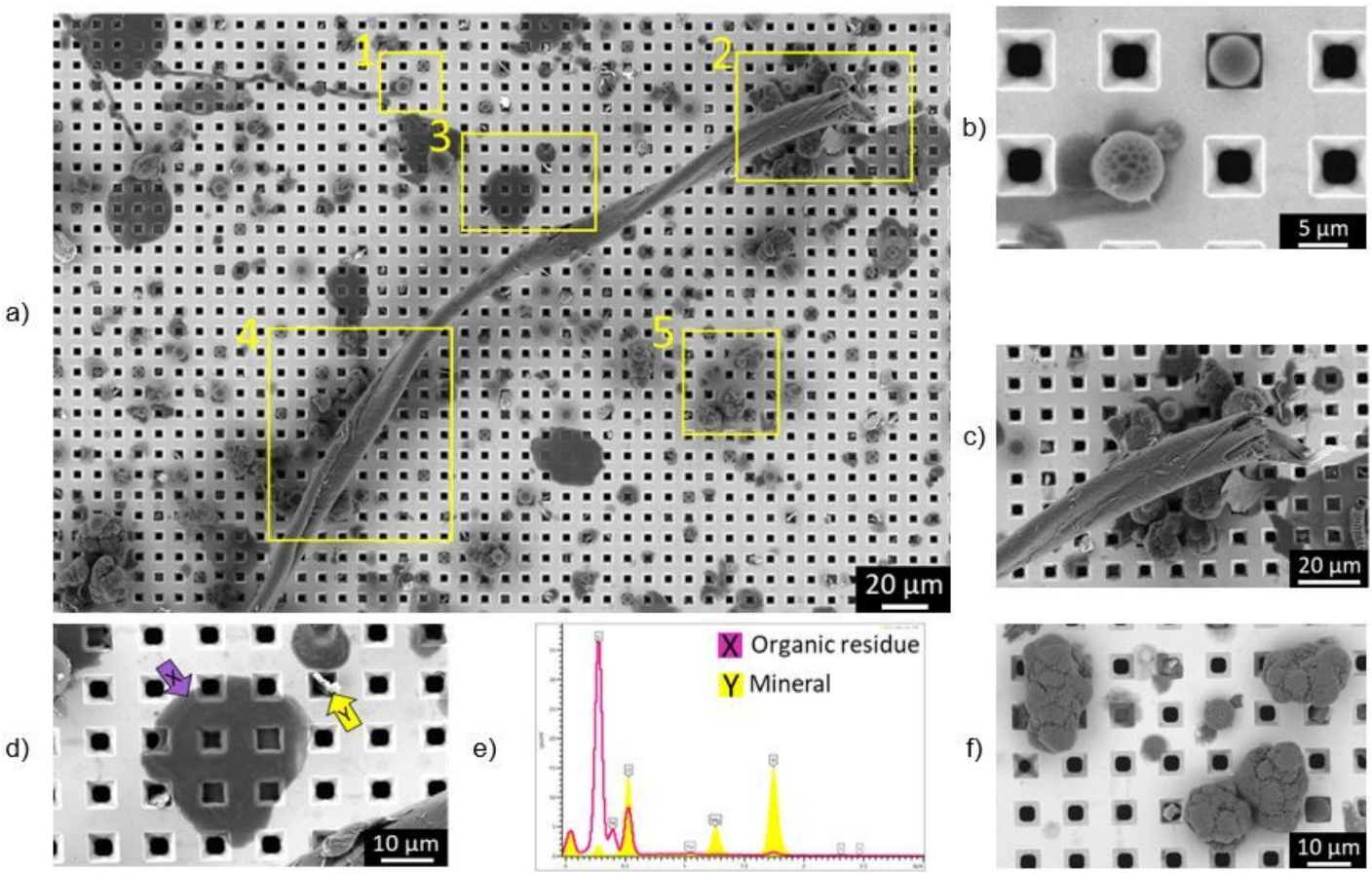

Figure 3

SEM-EDX analysis of a part of the filtered area for a spiked commercial cow's milk sample (Brand C, 0.1% fat) showing: a) different yellow zones (numbered 1 to 5) with different types of particles identified in each zone. Zone 1 = PS and PMMA beads; zone 2 = undigested organic fiber with agglomerates of other particles including polymer standards; zone 3 = organic residue and mineral particle; zone 4 = undigested fiber; zone 5 = polymer beads (PMMA, PS) and PA fragments; b) Magnified zone 1 showing PS and PMMA beads ; c) magnified zone 2 showing agglomerates of PMMA, PS and PA particles stuck to end of a; d) magnified zone 3 showing some organic residue and mineral particles; e) EDX spectrum on the organic residue (X) and mineral particle (Y) present in panels D; f) magnified zone 5 showing PA fragments and PMMA/PS beads.

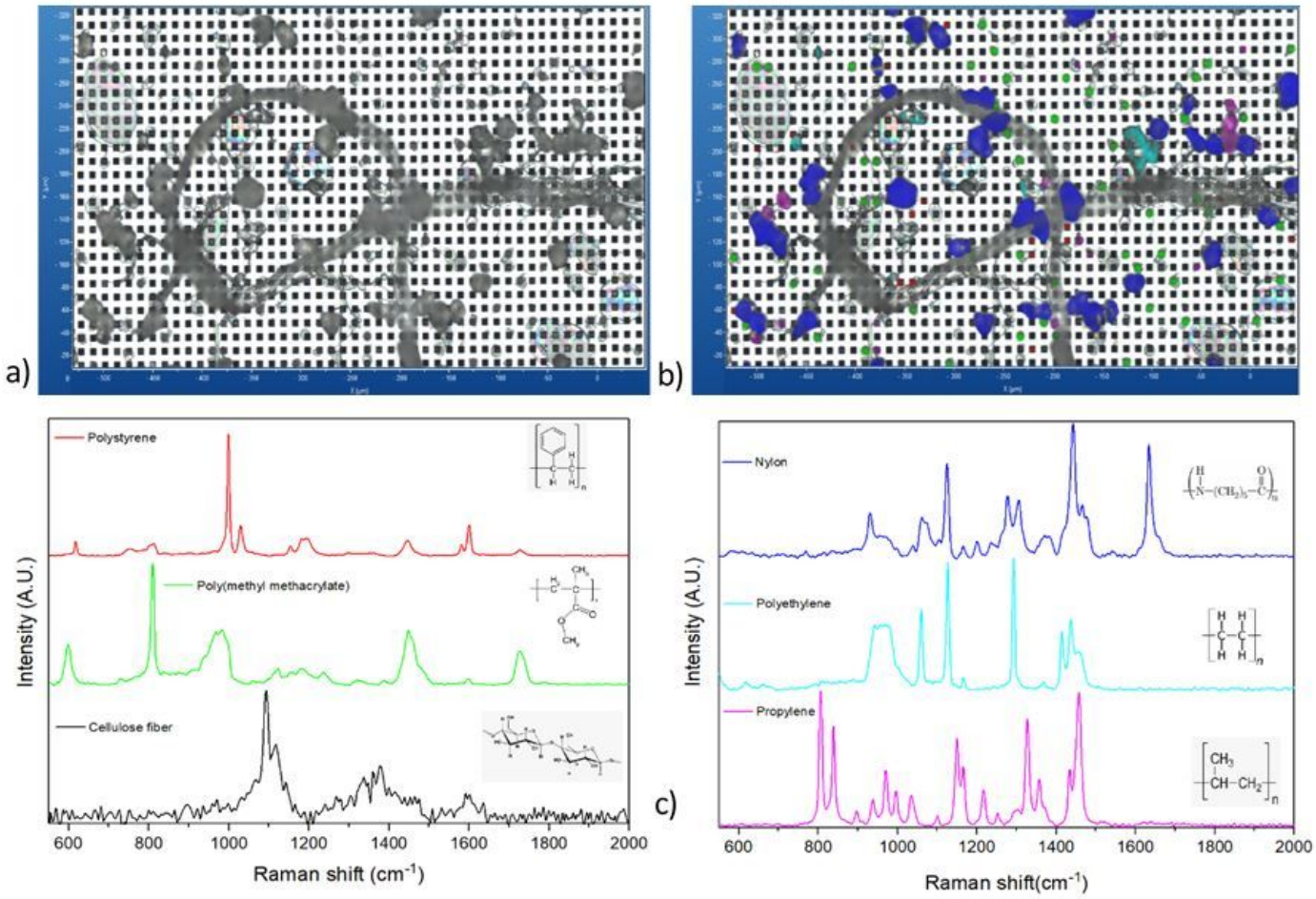

Figure 4

µRaman analysis of a part of the filtered area for a cow's milk (Brand C, 0.1% fat) spiked with five standards of MPs: a) Optical microscope image of digested particles on a part of the Si filter; b) Same image with MPs and cellulose fiber detected and identified (PS colored in red, PMMA in green, cellulose fiber in gray, PA in blue, PE in cyan and PP in pink); c) Related colored Raman spectra collected from five MP standards and cellulose fiber after sample digestion.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Characterizationofsmallmicroplasticsinmilkproductssupplementarymaterial.docx