Science of the Total Environment 947 (2024) 174493

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Development and application of a novel extraction protocol for the monitoring of microplastic contamination in widely consumed ruminant feeds

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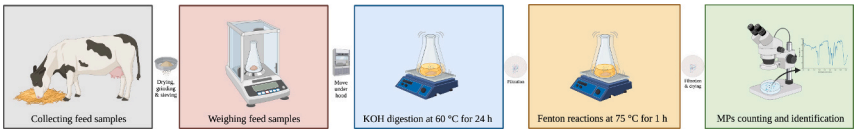
HIGHLIGHTS

- Sources of MPs exposure in ruminants but lack of protocols for feeds monitoring.
- MPs first non-destructive protocol for MPs extraction from 4 most used ruminant feeds.
- Optimal combination of KOH/Fenton oxidation for the removal of interfering matter.
- First visual characterization of MPs in ruminant feeds from N—W Italian farm.
- MPs contamination in feeds from 8.38.3 ± 3.51 to 39.3 ± 7.02 MPs/g.

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Microplastics
Ruminants
Feeds
Extraction protocol optimization
Identification
Pyr-GC/MS

ABSTRACT

Plastics and, in particular, microplastics (MPs) (< 5 mm) are emerging environmental pollutants responsible for interconnected risks to environmental, human, and animal health. The livestock sector is highly affected by these contaminants, with 50–60 % of the foreign bodies found in slaughtered domestic cattle being recognized as plastic-based materials. Additionally, microplastics were recently detected inside ruminant bodies and in their feces. MPs presence in ruminants could be explained by the intensive usage of plastic materials on farms, in particular to store feeds (i.e. to cover horizontal silos and to wrap hay bales). Although feed could be one of the main sources of plastics, especially of microplastics, a specific protocol to detect them in ruminant feeds is not actually present. Hence, the aim of this study was to optimize a specific protocol for the extraction, quantification, and identification of five microplastic polymers (high-density polyethylene, low-density polyethylene, polyamide fibers/particles, polyethylene terephthalate and polystyrene) from feeds typically used in ruminant diets (corn silage, hay, high protein feedstuff and total mixed ration). Several combinations of Fenton reactions and KOH digestion were tested. The final extraction protocol involved a KOH digestion (60 °C for 24 h), followed by two/three cycles of Fenton reactions. The extraction recoveries were of 100 % for high-density, low-density polyethylene, polyamide particles, and polystyrene and higher than 85 % for polyethylene terephthalate and polyamide fibers. Finally, the optimized protocol was successfully applied in the extraction of microplastics from real feed samples. All the feeds contained microplastics, particularly polyethylene, thus confirming the exposure of ruminants to MPs.

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https://doi.org/10.1016/j.scitotenv.2024.174493
Received 6 November 2023; Received in revised form 21 May 2024; Accepted 2 July 2024
Available online 3 July 2024

1. Introduction

Plastic pollution is one of the most pressing and growing problems that humanity should face in the 21st century (Du and Wang, 2021; Golwala et al., 2021), which also affects the livestock sector (Borreani and Tabacco, 2017; Urli et al., 2023). In fact, 50 to 60 % of the foreign bodies found in slaughtered domestic cattle are plastic-based materials (Galyon et al., 2023a, 2023b).

The limited and inappropriate recovery of plastic wastes has led to a visible accumulation of polymer debris in the environment, where they suffer from systematic fragmentation, generating different sizes of plastics: megaplastics, macroplastics, mesoplastics and microplastics (Du and Wang, 2021; Souza Machado et al., 2018; Yang et al., 2021). Among them, microplastics (MPs), classified as emerging ubiquitous pollutants (Ivleva, 2021), are commonly defined as particles smaller than 5 mm (Souza Machado et al., 2018). Indeed, MPs could act as carriers for inorganic and organic contaminants (e.g. heavy metals, persistent organic pollutants, antibiotics, pesticides, and fungicides) (Kinigopoulou et al., 2022; Okeke et al., 2022; Silva et al., 2018), being potentially responsible for interconnected risks to environmental, human, and animal health (Prata et al., 2021).

In the livestock sector, the presence of MPs was recently detected in sheep feces (Beriot et al., 2021), cow follicular fluid (Grechi et al., 2023), cow milk (Da Costa Filho et al., 2021; van der Veen et al., 2022) and cow blood and meat (van der Veen et al., 2022), thus confirming the exposure of ruminants to MPs. Among these sources, ruminant feeds should be also considered. In fact, plastic materials are intensively used as storage medium, for dairy cow, pig, and beef farms, with the formers consuming the largest quantities. As an example, an average of 10.3 kg of plastic/cow is yearly used in the northern part of Italy, 3.9 kg/cow of which were used to cover bunker silages (Borreani and Tabacco, 2015). Plastic products mainly used in dairy farms are silage bags, bale wraps, and plastic bunker silo covers, typically composed by low-density polyethylene (LDPE) and LDPE coextruded with polyamide (LDPE-PA). Moreover, veterinary drugs are typically packed in high-density polyethylene (HDPE) supports (Borreani and Tabacco, 2015, 2017). Finally, atmospheric transport of MPs could also account for MPs contamination in feeds (Evangeliou et al., 2020).

It was estimated that 0.07 % of polyethylene-based plastics is contained in ruminant diets (Pizol et al., 2017). When these materials enter the reticulum, they aggregate to form large spherical masses that press against the rumen epithelium during rumination. With prolonged presence, they could reduce rumen functionality (Akraiem and Abd Al-Galil, 2016), lead to erosion and ulcerations of ruminal papillae (Otsyina et al., 2017), and promote heavy metal leaching (Mahadappa et al., 2020).

Based on these premises, plastic contamination was shown to highly affect ruminants' feedings. Routinary protocols able to quantify and to characterize this plastic contamination (in particular MPs) are, therefore, highly requested, even if highly challenging. Indeed, feeds are complex sample matrices, since they contain a variety of components such as grains, protein sources, minerals, vitamins, and additives (Bueno et al., 2020), which could interfere with MPs identification. In addition, feed composition varies greatly according to animal characteristics such as breed, body size, sex, life stage, and digestive capacity (McGrath et al., 2018; Tedeschi et al., 2019).

Hence, the development of a specific MPs extraction protocol requires a careful optimization of several operational steps to remove the interfering inorganic and organic compounds in the matrix, avoiding, at the same time, degradation, and loss of MPs.

Unlike other environmental and food matrices (He et al., 2021; Hurley et al., 2018; Sridhar et al., 2022), currently specific non-destructive protocol for the extraction, visual evaluation, and quantitation of MPs in ruminant feeds is not available yet. The only work devoted to MP investigation in feeds is a pilot study from van der Veen et al. (2022), in which the presence of MPs was investigated in cow and pig feeds (pellets, fresh and shredded). The target of van der Veen work is the quantitation of several polymers in feeds using pyrolytic GC–MS, without focusing on the development of an effective protocol for their selective extraction and singular characterization. In this regard, MPs were extracted by dissolution in tetrahydrofuran, thus not preserving their physical integrity, which is essential for carrying out further visual, and spectroscopic investigations (e.g. stereomicroscope or μ-FTIR).

Based on the above-mentioned assumptions, the aim of this work was the optimization of an effective protocol for the extraction, quantification, and identification of five types of MPs from feeds typically used in ruminant diets, in particular those of dairy cows. In this regard, it should be mentioned that in 2021 the European Food Safety Authority (EFSA) expressed the urgent need to develop MP detection methods in food and feed (European Food Safety Authority, 2021).

The optimization of extraction conditions was performed directly on real feed samples, previously fortified with MP standards, using extraction recoveries as the analytical response. Finally, the optimized protocol was applied for the screening of selected MPs in real feed samples from local farmers. To the best of our knowledge, this work represents the first study reporting MPs contamination in dairy cow feeds.

2. Materials and methods

2.1. Feed samples

As summarized in Table 1, four dairy cow feeds were investigated, namely: corn silage (CS), hay (H), high protein feedstuff (HPF) and total mixed ration (TMR). All these matrices were sampled from an Italian dairy farm located in Piedmont, avoiding the use of plastic equipment. Each sample was: dried at 60 °C overnight and grinded (5 mm sieved); fortified with MPs to optimize the protocol (section 2.3) and extracted using the optimized protocol (section 2.4).

2.2. MP standards and reagents

2.2.1. MP standards

Five widespread types of MPs (Ajith et al., 2020; Khalid et al., 2021) were used to optimize the protocol, namely: high-density polyethylene (HDPE), low-density polyethylene (LDPE), polyamide (PA), polyethylene terephthalate (PET) and polystyrene (PS). In addition, to differentiate the MP shape, the protocol was customized for fibers (cylindrical shape and length to diameter ratio > 3) and particles (non-cylindrical shape and length to diameter ratio < 3) (ECHA, 2019), choosing PA as model polymer (hereafter called PA fibers and PA part., respectively). The list of each MP, together with its density (Harper,

Table 1
Feed sample, acronym, composition, and picture of chosen dairy cow feeds.

Feed	Acronym	Composition	Picture
Corn Silage	CS	Whole corn plant: stover (stalk, leaves, husk that covers the cob, cob that holds the maize grain) and maize grain.	
Hay	H	Rye grass hay.	
High Protein Feedstuff	HPF	Corn, dehulled soybeans, wheat bran, partially dehulled sunflower, beet pulp, corn flour, soybean husks, sugarcane molasses, inactivated yeast pool, minerals.	
Total Mixed Ration	TMR	Main ingredients: silages (mostly corn silage), rye grass hay, high protein feedstuff.	

2002), origin, length range and picture are reported in Table 2. All the MP standards were produced in the laboratory from real samples (bags, bottles, etc.) using a stainless-steel grinder and were subsequently sieved by 5 mm mesh-sieves to discard MPs larger than 5 mm.

2.2.2. MP reagents

Throughout this work, ultrapure water (18.2 MΩcm at 25 °C) obtained with a Milli-Q Academic system (Millipore, Billerica, MA, USA) was used. In addition, the following reagents were used: 30 % hydrogen peroxide (H_2O_2) (VWR Chemicals, Milan, Italy); iron sulphate heptahydrate ($FeSO_4\ 7H_2O$) (VWR Chemicals, Milan, Italy); 95–97 % sulfuric acid (H_2SO_4) (Honeywell, Charlotte, North Carolina, USA) and potassium hydroxide (KOH) (Honeywell, Charlotte, North Carolina, USA).

2.2.3. MPs background contamination: Quality assurance

Contamination from laboratory equipment or airborne particles during sample transfers should be addressed as the main MP background sources. Currently, in the field of microplastic research, there are no standardized protocols to account for background contamination. Hence, in the context of this study, the following precautions were adopted: i) plastic equipment was completely avoided, using a metal/glass apparatus, specifically developed by our research group; ii) airborne contamination: use of cotton clothes, laboratory coats and nitrile gloves for operators; iii) contamination from equipment: rinsing of all glass equipment and containers with 0.45 μm filtered ultrapure water before use and capping with aluminum foil during each step. The extraction steps were conducted under a shielded hood to minimize unfiltered air flow. Two procedural blanks were processed in parallel to each extraction (environmental MPs were <5 % of the total contamination for each sample and, hence, were always considered negligible).

2.3. Optimization of MPs extraction protocol

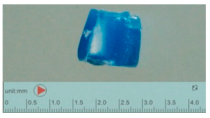
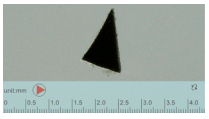
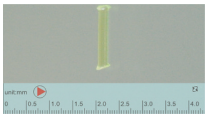
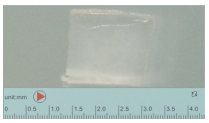
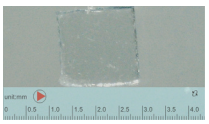
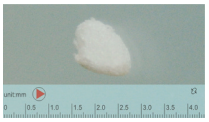
To achieve a quantitative extraction of MPs from feed samples, several oxidation protocols were tested, as hereafter described.

2.3.1. Degradation of organic matter (OM)

2.3.1.1. Fenton reactions. The protocol of Rodrigues et al. (2019), that investigated the Fenton reaction applied for MPs extraction from estuarine waters, was adapted to feed samples. Each feed sample (1 g) was placed in a glass flask with 20 mL 0.05 M Fe^{2+} solution and two subsequent aliquots of 20 mL of 30 % H_2O_2 (added at $t = 0$ min and $t = 30$ min respectively). The reaction was performed on a hot plate at 75 °C under stirring conditions (300 rpm, magnetic stirrer) for 1 h. To obtain the complete removal of organic matter, progressive Fenton reactions were tested (from one to three subsequent reactions), each one followed by a filtration step through a metallic sieve. In detail, flask content was filtered through a metallic filter (200 mesh, pore dimension <0.03 mm, Humboldt). The flask was rinsed three times with 0.22 μm filtered ultrapure water, to remove residual MPs. Subsequently, the solid retained by the filter was also washed with ultrapure water to remove any residual water-soluble contamination. Afterwards, it was transferred using a metallic spatula in the new flask and, to avoid any possible loss, the filter was rinsed directly into the flask, using the reagent for the subsequent reaction. After the oxidation step, the filtrate was dried at 60 °C overnight and transferred into a glass Petri dish to observe it by a stereomicroscope.

2.3.1.2. Alkaline digestion coupled with Fenton reactions. Due to the high amount of interfering organic matter present in the feed matrices

Table 2
List of MPs chosen for the protocol optimization, together with their density, origin, length range (mm) and their visual aspect.

MP	Density (g/cm^3)	Origin	Length range (mm)	Picture
HDPE	0.95	Falcon tube cup	1.147–3.483	
LDPE	0.92	Rubbish bag	0.682–3.436	
PA fibers	1.13	Fishing net	0.541–3.174	
PA part.	1.13	Ties	1.188–2.524	
PET	1.56	Bottle	1.590–3.286	
PS	1.05	Food packaging	0.579–3.396	

investigated, an alkaline digestion pre-treatment was implemented before the Fenton reactions. Tests were performed with 40 mL of KOH (10 %) at 60 °C (Dehaut et al., 2016), studying the effect of different incubation times (namely 6 h, 18 h and 24 h). After the alkaline digestion, one, two or three Fenton reactions (section 2.3.2.1) were performed according to the experimental design of Fig. 1. Each reaction was followed by filtration through a metallic sieve (see paragraph 2.3.1.1). At the end of each trial, the final filtrate was dried at 60 °C and was transferred into a glass Petri dish for the subsequent identification and quantitation procedures.

2.3.2. MP extraction recoveries and polymers integrity check

MP extraction recoveries (R%) were evaluated fortifying each feed sample with 0.01 ± 0.005 g of each of the five laboratory prepared MP standards.

It should be mentioned that the same weight could correspond to different numbers of MP pieces because density, sizes, shapes, and weights of MP pieces can vary from one to another polymer. Hence, the specific MP pieces counted and added to the feed samples are reported in Table S1 of the Supplementary Material Information.

In addition, fortified MPs were properly prepared to be easily distinguishable from native contamination, potentially present in feeds (see section 2.2.1).

After extraction, MPs were again visually counted and observed using the stereomicroscope in order to both assess that no physical degradation occurred (e.g. fragmentation) and to evaluate the extraction recoveries (R %). The extraction recoveries were calculated according to the following equation:

$$R\% = \frac{n^{\circ}\ of\ MPs\ after\ the\ extraction}{n^{\circ}\ of\ MPs\ fortified\ in\ the\ sample} \bullet 100$$

Each protocol was repeated three times for each feed sample, and control blanks were run in parallel (no background contamination was observed). Extraction rates (expressed as percentages) obtained for each MP and each feed after the protocol optimization are reported and commented in the Results and Discussion session (Section 3.1.1.2).

In addition to the physical integrity check, the chemical integrity (potential alteration of polymer chains) of MPs after the sequential application of the oxidation cycles was also performed through FTIR analysis on both virgin and post-extracted MPs. The *spectra* were acquired using a Perkin Elmer Spectrum Two μ-FTIR ATR spectrometer (wavenumber range of 4000–400 cm^{-1}, 32 scans per spectrum, spectral resolution of 4 cm^{-1}).

2.4. Application of the optimized protocol to real samples

The optimized protocol was applied in the determination of the native contamination by MPs (HDPE, LDPE, PA fibers/part., PET, PS) in four real feed samples. For each tested matrix (CS, H, HPF and TMR), the analysis was performed in triplicate and two control blanks were run in parallel. MP concentration results were expressed as: *i*) total number of MPs detected per gram ($\frac{MPs}{g}$); *ii*) total number of MP fibers per gram ($\frac{MP\ fibers}{g}$) and *iii*) total number of MP particles (all MPs not belonging to fibers) per gram ($\frac{MP\ particles}{g}$), where $\frac{MPs}{g} = \frac{MP\ fibers}{g} + \frac{MP\ particles}{g}$.

2.4.1. Characterization of polymers by stereomicroscope and Pyr-GC/MS

After the extraction of MPs from each feed, the residual particles retrieved from the metallic filter were preliminary characterized by visual observation using a Nikon H550S stereomicroscope equipped with Micro Capture Ver6.9.12 software (DIV = 0.01 mm). The flow chart suggested by Lusher et al. (2020) was carefully followed in order to reduce subjectivity and random errors, by classifying suspected fragments into three categories, namely morphology (e.g. size, shape and texture), optical properties (e.g. the color) and behavior (e.g. flexibility and density). Once classified, MPs were further analyzed through pyrolysis-gas chromatography/mass spectrometry (Pyr-GC/MS) to confirm their nature and to identify polymer composition. The use of Pyr-GC/MS as a complementary technique to the visual identification allows to achieve integrated analysis of MPs, for example for source-apportionment and risk assessment studies (Dong et al., 2023; Narloch et al., 2022; Wang and Wang, 2018).

Pyr-GC/MS analysis were performed with a micro-furnace pyrolizer (EGA/Py-3030D, Frontier Laboratories Ltd., Japan) interfaced to a GC/MS system (8890 and 7000D, Agilent Technologies, Inc., USA). The GC was equipped with a fused silica capillary column coated with 0.25 μm

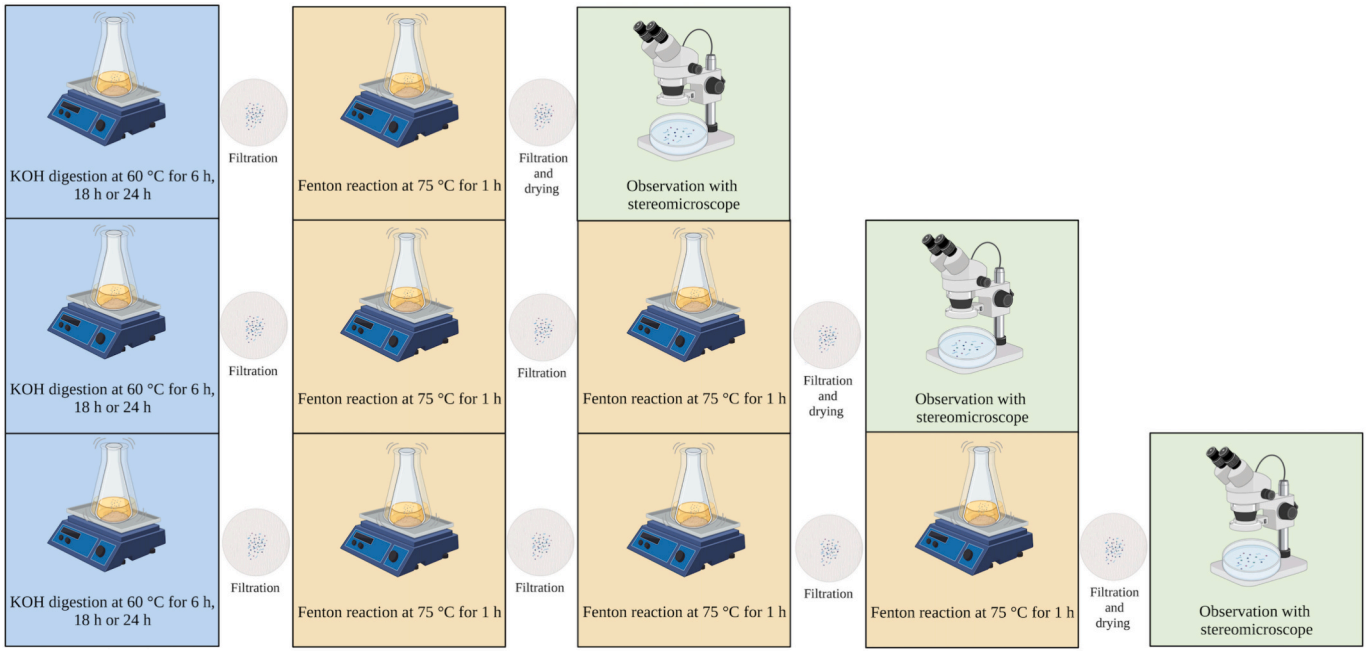


Fig. 1. Degradation of OM. Experimental trials of alkaline digestion coupled with Fenton reactions (created with BioRender.com).

film thicknesses of 5 % diphenyl 95 % dimethylpolysiloxane (HP-5MS, 30 m × 0.25 mm id, Agilent Technologies, Inc., USA). The GC was operated with a constant helium (He) flow as carrier gas of 1 mL/min. To avoid saturation of the mass *spectrum*, a 1:100 split ratio was chosen. GC injector was set at 300 °C.

For the analysis, the MP was directly placed in a sample cup. Then it was inserted into the pyrolizer furnace. The temperature program curves were set according to the work of Anuar et al. (2022). Polymer identification was achieved using total ion pyrograms and recognizing characteristic pyrolysis products. The recognition of pyrograms and pyrolytic products was performed by comparison with the *"Pyrolysis-GC/MS data book of synthetic polymers: pyrograms, thermograms and MS of pyrolyzates"* which includes pyrograms of 163 polymers and MS data for the main pyrolyzates by means of Pyr-GC/MS (Tsuge et al., 2011).

2.5. Statical analysis

Data were analyzed using R statistical software (R 4.2.2) and its extension RStudio (2022.07.2 + 576). The Kruskal-Wallis non-parametric test (significance threshold level 0.05) was used to detect the statistical differences for: MP extraction recoveries, MP concentrations (MPs/g, MP fibers/g and MP particles/g, each variable analyzed separately) and MP range lengths. Kruskal-Wallis non-parametric test was used after the invalidation of the normality variance with Shapiro-Wilk test. Least significant difference Fisher's criterium was used as the post hoc test while the t-student as the adjustment method.

3. Results and discussion

3.1. Optimization of MP extraction protocol

As previously mentioned, the aim of the extraction protocol here optimized is both to remove the interfering matrix, avoiding, at the same time, MP degradation. Feeds are considered complex samples because they have a complex composition. Main feed constituents (such as lignocellulose) are difficult to be removed, similarly to other matrices such as soils, sand, organic-rich sediments, sludge, biota, etc. (Monteiro and Pinto Da Costa, 2022). In this regard, within the protocol, several steps were evaluated.

It should be mentioned that preliminary density separation tests were performed with saturated salt solutions (such as NaCl and $ZnCl_2$), with unsuccessful removal of the organic matter. Hence, density separation step was discarded.

3.1.1. Degradation of organic matter (OM)

Even if it is universally recognized that the organic matter present in samples should be efficiently removed to avoid interferences in MP determination, oxidation techniques can potentially damage MPs themselves. Indeed, depending on the concentration and duration of the oxidation treatment, the oxidizing agents can lead to degradation or alteration of MPs. This can result in changes to their size, shape, and surface characteristics, which may negatively impact the subsequent analysis or characterization (Savino et al., 2022). Based on the above-mentioned premises, the following oxidation treatments were carefully optimized, in order to degrade OM without damaging MPs.

3.1.1.1. Fenton reactions. A single cycle of two Fenton reactions (as described in section 2.3.2.1) was initially tested for all matrices. Visual observation of treated samples showed that for H and TMR, degradation was slightly more efficient than for CS and HPF. However, for all the samples, poor recoveries (below 10 %) were obtained, since MPs were fully hindered by interfering matrix clusters (residual organic matter in H and TMR is reported as an example in Fig. S1 in Supported Information). The higher degradation resistance of CS and HPF could be ascribed to some of their components rich in cellulose and lignin (i.e. corn stalk for CS and seed shell for HPF) (Jahirul et al., 2012; Passoth and Sandgren, 2019). However, it should be mentioned that for none of the tested matrix, a complete removal of OM was achieved. Hence, two and three consecutive Fenton cycles were tested, but they were still not enough to efficiently remove the OM (Fig. S1 in Supported Information). Even if a partial increase in the extraction recoveries was observed, recoveries still remained below 18 % for all the feeds.

3.1.1.2. Alkaline digestion coupled with Fenton reactions. Given that the three consecutive Fenton reaction cycles were not efficient enough for the complete OM removal, the effect of a KOH alkaline digestion before Fenton reactions was studied for all feed samples. Three different KOH digestion times were tested, namely, 6 h, 18 h and 24 h. Visual observation of the post-oxidized samples through the stereomicroscope showed that both 6 h and 18 h reaction times provided higher degradation of interfering organic matter than Fenton reactions only, even if a complete degradation could not be achieved. A similar trend as for Fenton degradation was shown also for KOH digestion, with more efficient OM removal for H and TMR than for CS and HPF.

Finally, 24 h KOH digestion followed by two Fenton reactions (for H and TMT)/ three Fenton reactions (for CS and HPF) allowed the quantitative degradation of OM for H, TMR, CS, and HPF, as shown in Fig. S2 (Supporting Information).

For CS and HPF, a residual fraction of OM was still observed. Nevertheless, this fraction was considered negligible, since MPs were easily discernible from these residual matrix particles and easily classifiable through the Lusher et al. (2020) identification flow-chart (see section 2.4.1).

Based on these results, the optimized protocol for OM degradation is: 24 h KOH digestion followed by Fenton reactions (two cycles for H and TMR and three for CS and HPF, respectively).

3.2. MP extraction recoveries and polymers integrity check

In Fig. 2, the MP extraction recoveries obtained through the optimized protocol are reported. Results show that quantitative extractions were obtained for HDPE, LDPE, PA part. and PS for all the tested matrices, while for PA fibers and PET recoveries were above 85 %. For each MP type, the extraction recoveries did not differ significantly in the four feeds (Kruskal-Wallis test, significance threshold level 0.05, $n = 3$). In addition, repeatability was confirmed with relative standard deviation equal to 0 % for HDPE, LDPE, PA part. and PS, and lower than 12 % and 8 % for PA fibers and PET respectively (n = 3).

These results obtained within our study are in good agreement with the work of Way et al. (2022), in which a meta-analysis study was conducted on the extraction yields of 71 protocols available in the literature for the extraction of MPs from different matrices. According to their research, extraction recovery rates were 84.5 % for microplastics ranging from 1 μm to 1 mm ($n = 60$ works) and 84.8 % for microplastics sized between 1 mm and 5 mm ($n = 30$ works). Additionally, according to the above-mentioned work, extraction yields were higher for plant material, whole organisms, and excreta from swine human, chicken, and zebrafish (> 88 %), and lower for fishmeal, water, and soil (58–71 %); furthermore, high-density MPs (like PA and PET) are characterized by lower extraction recovery yields. However, in the study of Way et al. (2022), no ruminant feed was considered and, to the best of our knowledge, the present work appears to be the first in which MP extraction yields from ruminant feed are evaluated.

To check the integrity of each target MP after the sequential application of the oxidation cycles, μ-FTIR analysis on both virgin and post extracted MPs was performed. The signals typical of each polymer were still clearly distinguishable, thus demonstrating that no alteration of polymer chains occurred (Fig. S3 of the Supporting Information).

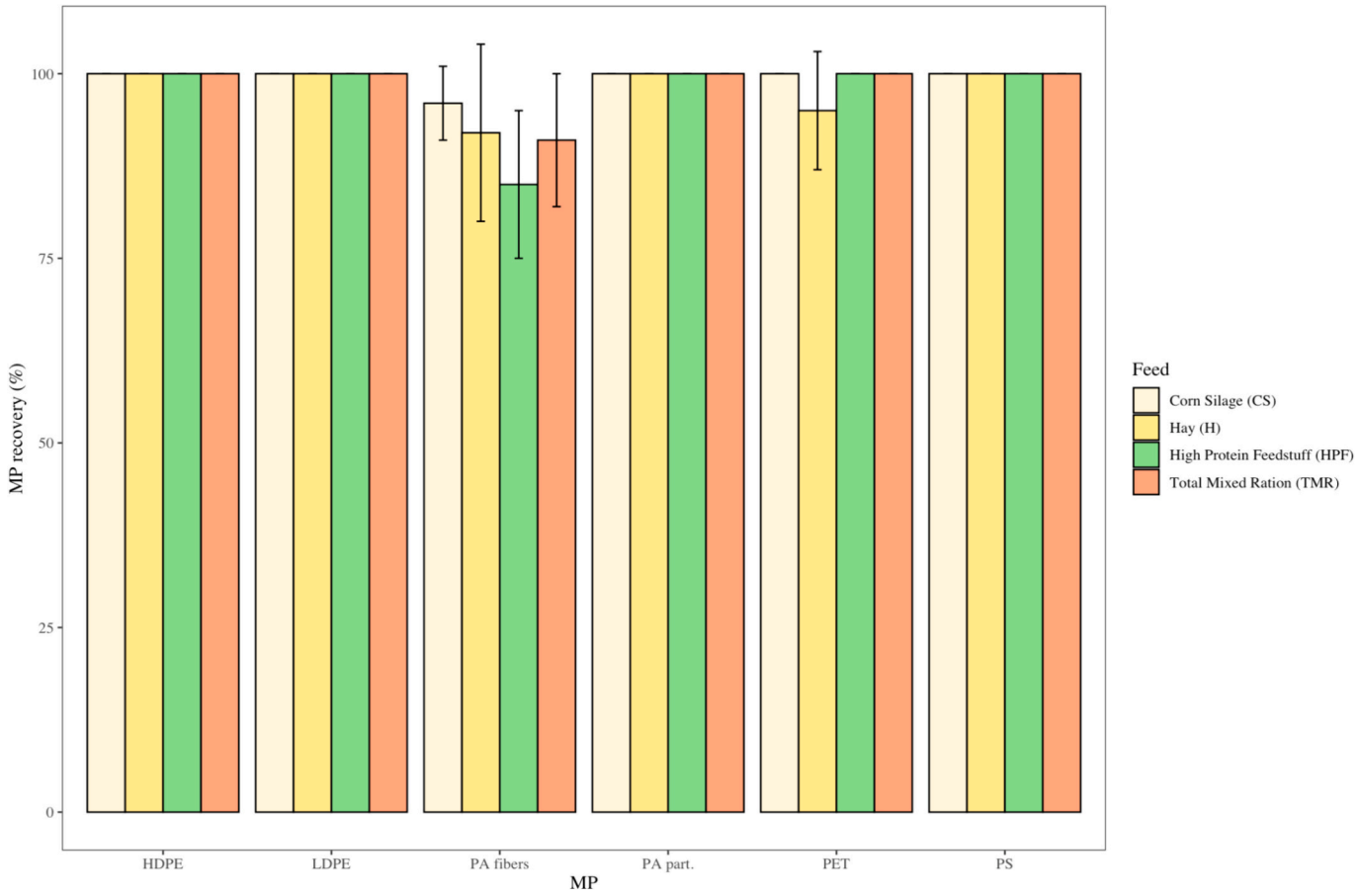


Fig. 2. MP extraction recoveries (%) obtained using the optimized MP extraction protocol on the four fortified feeds. For each MP, the extraction recoveries did not differ significantly in the four feeds (Kruskal-Wallis test, significance threshold level 0.05, n = 3).

3.3. Optimized MP extraction protocol for ruminant feeds

The optimized MP extraction protocol for ruminant feeds is summarized, together with all its operational steps, in Table S2 (Supporting Information).

Considering the studies currently available in the literature regarding the extraction of MPs from complex environmental samples, it can be noted that, due to the differences in density between the MPs and the matrix components, a step based on density separation is generally present. In particular, density separation is exploited for the analysis of MPs in: i) soils (Isari et al., 2021, Li et al., 2019, Liu et al., 2019; ii) marine sediments and oysters (Rivoira et al., 2020); iii) lake sediments (Zobkov et al., 2020); iv) sand (Kedzierski et al., 2017; Maynard et al., 2021); and v) marine biota (Yu et al., 2019). In contrast to other complex matrices, the optimized protocol for the extraction of MPs from ruminant feeds has the advantage of bypassing the density separation step, thus reducing the extraction time and increasing the feasibility.

The first step of the protocol here optimized for the extraction of MPs from ruminant feeds consists of a first KOH (10 %) digestion at 60 °C for 24 h. This alkaline digestion was shown to be one of the most effective digestive treatments in MP extraction protocols (Phuong et al., 2018; Prata et al., 2019). Alkaline digestion was also applied by Thamizhakaran Stanley et al. (2022) for the pretreatment and degradation of lignocellulosic biomass, which has a similar chemical composition to that of ruminant feeds, thus making it a promising approach to remove organic matrix in the tested feeds. Although alkaline digestion could promote discoloration and degradation of PA fibers (Prata et al., 2019), it was here demonstrated that under the optimized extraction conditions, the chemical structure of target MPs is maintained.

The second step of the optimized protocol was the oxidation of OM by Fenton reaction cycles: two reactions for TMR and H and three for CS and HPF. A similar approach based on sequential Fenton reactions was used to extract MPs from the secondary effluent of a wastewater treatment plant (Dyachenko et al., 2017) and from sludge (Cunsolo et al., 2021), a matrix affected by a high organic matter content.

As discussed in section 3.1.2, the use of KOH treatment only or multiple Fenton reactions was not sufficient to efficiently remove the interfering OM. Conversely, the proposed synergic combination of both KOH digestion together with Fenton reactions achieved the goal. A similar approach was followed also for other complex matrices, such as marine biota, as shown by Alfonso et al. (2021) and Avila et al. (2023).

3.4. Application of the optimized protocol to real samples

The optimized protocol previously described was successfully applied in the analysis of target MPs in feed samples (CS, H, HPF and TMR) daily used by local farmers. The results are summarized in Table 3. The highest concentration of MPs/g was detected in H (39.3 ± 7.02 MPs/g); this concentration is more than double that detected in the other matrices. CS, HPF and TMR had MPs/g concentrations not significantly different from each other (12.3 ± 5.76 MPs/g, average concentration). For all four feeds tested, the majority of MPs detected are in fiber form. The MP colors were: colorless, black, blue, green, red, grey, and brown.

The total detected MPs were additionally classified by their dimensions, as reported in Fig. 3 (and further discussed in detail in section S1 of the Supporting Information).

For all four feeds, the box plots were positively skewed having the

Table 3
MP concentrations in the four feeds obtained by applying the optimized MP extraction protocol. Concentrations are expressed in MPs/g, MP fibers/g and MP particles/g, expressed as mean ± SD(Kruskal-Wallis test, significance threshold level 0.05, n = 3).

Feed	MPs/g	MP fibers/g	MP particles*/g
CS	13.3 ± 3.21^{b}	13.0 ± 3.46^{bc}	0.3 ± 0.58^{b}
H	39.3 ± 7.02^{a}	32.0 ± 2.00^{a}	7.3 ± 5.03^{a}
HPF	8.3 ± 3.51^{b}	8.0 ± 3.61^{c}	0.3 ± 0.58^{b}
TMR	15.3 ± 5.77^{b}	15.3 ± 5.77^{b}	0.0 ± 0.00^{b}

Different superscript letters in the same column refer to mean values statistically different according to the Kruskal-Wallis test (significance threshold level 0.05). Conversely, common letters in the same column refer to mean values not statistically different from one to the other. MPs/g = MP fibers/g + MP particles/g; MP fibers = length:diameter > 3; MP particles = length:diameter < 3.
* "MP particles" refers to the overall non-fiber fraction, as detailed in section 2.2.1.

means higher than the median, and the upper quartiles larger than the lower quartiles. All the samples had values outside the quartiles showing wide variability in lengths. Using the Kruskal–Wallis test, at the level of 0.05 of significance, H showed to have MPs of significantly shorter lengths than other feeds.

The application of the optimized MP extraction protocol on real ruminant feed samples demonstrated the presence of MPs in all dairy cow feeds: CS, H, HPF and TMR, as also reported for HPF by the pilot study of van der Veen et al. (2022). Regardless of particle size, these results confirm the existence of an "environmental MP cycle" that involves ruminants, as hereafter phase-by-phase reported and commented. Phase 1: agricultural soils, including those of livestock farms, are contaminated with MPs (from 7.1 to 42.96 MPs/g of agriculture soils; 9.56 MPs/g of pasture soils) (Álvarez-Lopeztello et al., 2021; Zhang and Liu, 2018). Contamination of soils by MP arises from plastic equipment (e.g. mulching), substances already contaminated with plastics (e.g. wastewater sludges used as an amendment) or atmospheric deposition (Chang et al., 2022). MPs (especially nano-sized microplastics) could move from soil to plants through root uptake (Zhang et al., 2022). Phase 2: from soils and plants they could pass to animal feeds and this contamination could be enhanced by plastic material used for storing ruminant feeds (Borreani and Tabacco, 2017) (19.05 MPs/g of ruminant feeds in our study). Phase 3: ruminants eat contaminated feed and MPs enter their bodies (i.e. 38.6 MPs/mL of bovine follicular fluid), interfering with their physiologic functionalities (e.g. reproductive system) (Grechi et al., 2023), until they return to the soil via feces (from 0 to 5 MPs/g of sheep feces) (Beriot et al., 2021), becoming a new source of pollution.

3.4.1. *Characterization of polymers by stereomicroscope and Pyr-GC/MS*

In Fig. 4 selected subset of MPs, including three micropolymers for each feed (ten fibers and two particles) are reported. It is possible to observe the variety of colors (green, colorless, red, and blue) shapes (fibers/particles) and lengths.

After stereomicroscopic inspection, MPs were analyzed by Pyr-GC/MS. The pyrograms are reported in Fig. S4-S7 (Supporting Information).

The majority of the fragments in CS, H, HPF and TMR were correctly recognized as polyethylene, typically employed for feed wrapping and storage (Borreani and Tabacco, 2015, 2017). Similarly to our study, polyethylene was detected in cow HPF by the only preliminary study by van der Veen et al. (2022). Furthermore, the presence of polyethylene has been detected in blood, milk, meat, and follicular fluid of cows (Grechi et al., 2023). This result confirms the widespread diffusion of polyethylene and the tendency of objects made with polyethylene to degrade and form microplastics (Da Costa Filho et al., 2021; Grechi et al., 2023; van der Veen et al., 2022).

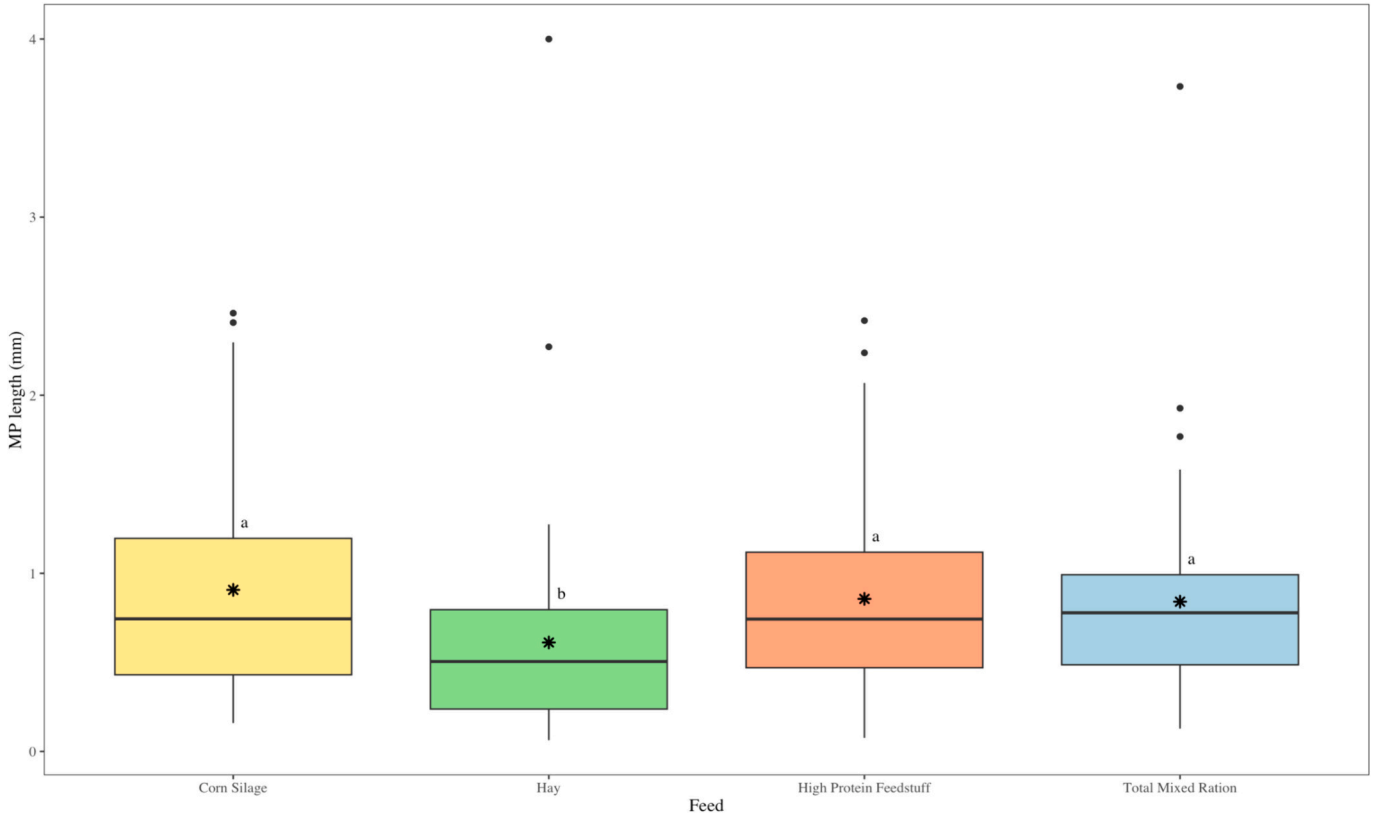


Fig. 3. Range length of MPs found in feed samples expressed in mm. The horizontal line represents the median value of data (Q50), the edges of the boxes are the quartiles (Q25 and Q75), the asterisk is the mean (Kruskal-Wallis test, significance threshold level 0.05, different superscripts (a–b) within feeds indicate significant differences).

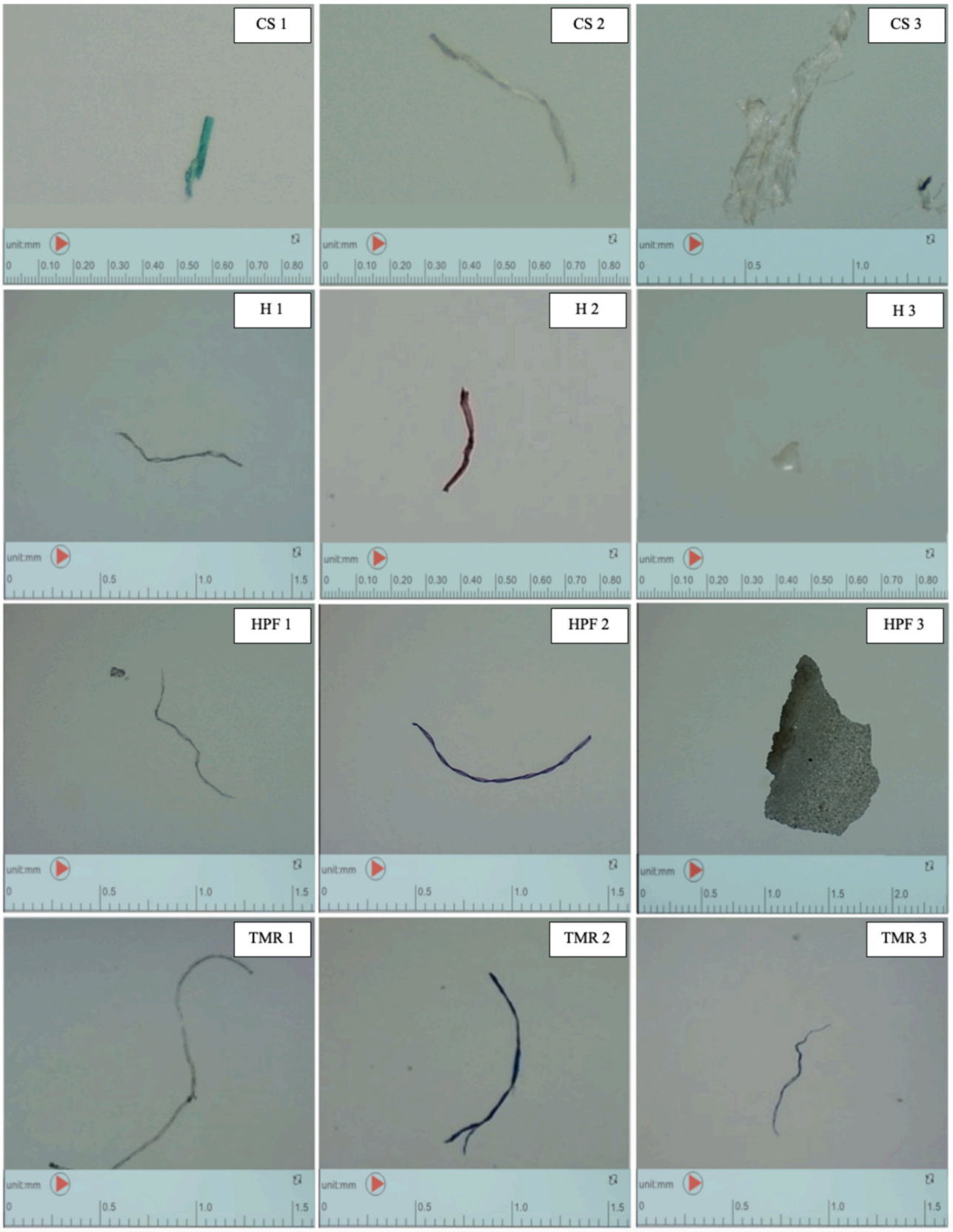


Fig. 4. Pictures captured under stereomicroscope of the three MPs for each feed sample that were selected and identified by Pyr-GC/MS.

It should be mentioned that, among the MPs detected in HPF (see Table 3), one fragment (classified as HPF 3 in Fig. 4) was not ascribable to any of the polymers considered within this study (hence suggesting that the proposed protocol could be also effective for additional polymers than those here studied).

4. Conclusions

For the first time, a non-destructive extraction protocol dedicated to the analysis of five types of MPs from ruminant feed is proposed. High consumption feeds for dairy cows were investigated (corn silage, hay, high protein feedstuff and total mixed ration). The developed protocol, which represents a novelty in the scientific literature, was intentionally developed with the final aim of both removing interfering species and not altering the structure of microplastics, to make possible reliable qualitative and quantitative analysis. In this regard, different combinations of procedural steps based on Fenton reactions and KOH digestion were tested and discussed. The final protocol, which leads to recoveries as high as 85 % for all the MPs and feeds, requires a KOH digestion at medium temperature (60 °C for 24 h) followed by two/three cycles of Fenton reaction. The effectiveness of the proposed procedure avoids the use of additional density separation steps, thus reducing the operating times compared to already existing procedures developed for other complex matrices (e.g. soil, biota).

The optimized procedure was successfully applied for the extraction, visual quantitation, and mass spectrometric identification of MP contamination in the four tested animal feeds. The detection of polyethylene in all the matrices confirms the exposure of ruminants to MPs.

The effectiveness, ease, and robustness of the proposed protocol suggest its full compatibility with future monitoring campaigns aimed at evaluating MP contamination in ruminant feed and crucial for understanding the real impact of these pollutants in animal farming.

Funding

SGP was funded by Ph.D. PROGRAMS, 37th CYCLE (PON - D.M. 1061/21) - GREEN TOPICS. This research has received funding from Project CH4.0 under the MUR program “Dipartimenti di Eccellenza 2023-2027” (CUP: D13C22003520001) and from “Fondazione Cassa di Risparmio di Torino” under the grant PLASTISOIL, RF 2021.1938.

CRediT authorship contribution statement

Sara Glorio Patrucco: Conceptualization, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Luca Rivoira:** Conceptualization, Funding acquisition, Methodology, Supervision, Validation, Writing – review & editing. **Maria Concetta Bruzzoniti:** Conceptualization, Validation, Writing – review & editing. **Salvatore Barbera:** Writing – review & editing, Funding acquisition. **Sonia Tassone:** Conceptualization, Supervision, Writing – review & editing.

Declaration of competing interest

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

Data availability

Data will be made available on request.

Acknowledgments

The authors would like to acknowledge the local farm that provided feed samples, Dr. Alessandra Eleonora Carioli and Dr. Mattia Isola for their help during the experiments, and PhD student Matteo Dho for his suggestions in the use of RStudio.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2024.174493.

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