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A reliable method for the isolation and characterization of microplastics in fish gastrointestinal tracts using an infrared tunable quantum cascade laser system

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ABSTRACT

Societal and environmental concern due to frequent reports of microplastics in fish stomachs raised as they may accumulate along the trophic chain. The request for analysing microplastics in fish stresses two major analytical issues: sample treatment and final characterization. The, so far, workhorse for chemical characterization is infrared spectroscopy which is time-consuming. Here, a quantum cascade laser-based device is used to accelerate the characterization stage. Its novelty poses new challenges for sample processing and particle handling because the unknown particles must be transferred to a reflective slide. In this study, three sample digestion protocols (alkaline-oxidative with H_2O_2, and alkaline-oxidative with NaClO and enzymatic-oxidative) and three different procedures to transfer the filter cake to reflective slides are compared. A simplified enzymatic-oxidative digestion (validated through an interlaboratory exercise) combined with a Syncore® automatic evaporation system and a Laser Direct Infrared Imaging (LDIR) device is proposed first time as a reliable and relatively fast method to treat gastrointestinal tracts of fish. Analytical recoveries were studied using samples of *Scomber scombrus* and they were ca. 100% for big –i.e., >500 μm- and ca. 90% for medium –i.e., 200–300 μm- particles and ca. 75% for 10 μm thick fibres.

1. Introduction

Current research presents clear evidences of pelagic and benthonic fish (Ugwu et al., 2021; Xu et al., 2020), as well as marine mammalians (Hernandez-Gonzalez et al., 2018; Novillo et al., 2020), ingesting microplastics (MP) worldwide, most of them accumulating in their stomachs. A major concern here relates to tiniest particles that might translocate and, so, bioaccumulate through the trophic chain and ultimately affect highest predators, including humans (Kwon et al., 2020; Zhang et al., 2021).

The raising demand for reliable, validated and fast analytical protocols to determine MPs in biota stresses two major analytical aspects: sample treatment and particle characterization. The current workhorse for the latter (identification plus characterization) is infrared (IR) spectroscopy and/or microspectroscopy, either in transmittance or reflectance modes (Cowger et al., 2020; Lusher et al., 2020), although it can take many hours of data collection alone. When dealing with sample preparation, a main pitfall of almost all protocols is that they are very time-consuming and, so, they are not efficient for current environmental monitoring where tens or hundreds of samples are taken (Cowger et al., 2020). Hence, more and more alternatives are being sought for to measure samples as fast as possible, as a matter of example, using subsampling method (Brandt et al., 2021; Huppertsberg and Knepper, 2020), focal plane array (Primpke et al., 2020) or open system models (Chen et al., 2022).

Recently, a major instrumental player presented a breakthrough concept: the tunable IR quantum cascade laser (QCL) device developed by Agilent and commercialized as 'Laser Direct Infrared Imaging' (LDIR). It can obtain a reasonably good reflectance (or transflectance, whenever the particles are very thin) spectrum in just 1 s (Hildebrandt et al., 2020). Nevertheless, it is not possible to use it to measure directly commonly-used 47 mm diameter filters, although a possibility might be to use small (13 mm diameter) gold-coated polycarbonate (PC) filters. However, these are very expensive, not reusable and suffer deformation

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during vacuum filtration (Agilent, 2019). Following, in most applications the contents of typical filters used to retain the MPs after sample treatment have to be transferred to a reflective surface (whose shape is like typical microscope slides). This challenge needs to be addressed because literature on this issue is still very scarce due to the novelty of the LDIR system.

Agilent itself recommended filter sonication for 20 min, <35 °C, to resuspend the particles in a 99% ethanol phase and withdraw 20 μL aliquots (Agilent, 2019). Nevertheless, the use of such small aliquots can compromise representativeness and reproducibility because it is not easy to pipet suspensions of particles, as they have a random Brownian motion, can agglomerate, stick to the walls or clog the pipette tips. Hildebrandt et al. (2020) suspended the filter in 50% water: ethanol and deposited the suspension on a reflective slide. Unfortunately, it was not specified how to perform the transference, nor whether it used aliquots or the entire suspension volume.

Previous applications to real samples were not too conclusive. For example, Bringer et al. (2021) did not specify how to transport the particles to the reflective slide. Li et al. (2021) washed a membrane filter with ca. 25 mL of ethanol and concentrated them to 1 mL blowing N_2 (which is time consuming). No details about volume, required time or validation were disclosed. Tian et al. (2021) transferred the particles from a stainless steel filter (10 μm mesh size) to 25 mL of ethanol, sonicated for 1.5 h and transferred a fifth of the total volume to a glass tube, where it was concentrated to 1 mL in a N_2 flow, which was then poured on a reflective slide. Pfohl et al. (2021) sonicated a metallic mesh filter (25 μm pore size) in 50% ethanol in a glass vial for 2 min. It was not detailed how to transfer the solution to the slide. As a general conclusion it can be said that there is a lack of detailed information in literature on how to perform this critical step and, therefore, there is room for the study presented here.

Despite the transfer procedure being an important step, it is undeniable that the previous sample treatment is of utmost importance as well. Different options can be found in literature to get rid of the biological material to isolate the suspicious particles. But the compromise between organic matter destruction and preservation of integrity of plastics yields controversy.

The main objectives of this paper are twofold: to establish a protocol for a microplastic-friendly digestion of gastrointestinal tracts of fish (comparing three alternative protocols) and to lay down an effective and simple protocol to transfer the filter contents to the reflective slides required for the QCL-based measurement device. Therefore, the article is organized according to two topics: i) sample digestion, and ii) sample transfer to reflective slides. In the former an enzymatic-oxidative method and an alkaline-oxidative one are studied. Three modes of transference were considered: collection of 10% of the volume of an ethanol suspension, evaporation of the whole suspension using an automatic Syncore® system, and the traditional use of microtweezers plus a stereomicroscope.

2. Experimental part

2.1. Samples, reagents, and materials

To optimize the methodology under development samples of *Scomber Scombrus* were obtained from local markets and their gastrointestinal tracts were extracted and spiked with 20 MPs particles of each studied polymer (PS, PP, PVC, PET, PE and PA6.6) plus 20 PET fibres (Korntex X217O). The polymers used throughout this work, but the PET fibres, were provided by the Universität of Bayreuth (Germany), within the framework of the JPI-Oceans-funded Baseman project. They were prepared in the form of powders (pure substance, only with the mimimum amount of additives required to manufacture them). The spiked MPs are around 200–300 μm whereas PVC particles are around 70 μm. The PET fibres are 1–5 mm length and ca. 10 μm diameter. The polymers (PS, PP, PVC, PET, PE and PA6.6) were chosen because they represent ca. 74,1% of the global polymer demand (Plastics Europe, 2021) and, so, they appear more frequently in environmental studies (see e.g., (de Haan et al., 2019) (Wang et al., 2022) (Ugwu et al., 2021) (Xu et al., 2020)). On the other hand, PET is the commonest microfiber in synthetic garments (Corami et al., 2020). PP (polypropylene) was from Borealis (commercial name HL508FB); PS was from INEOS Styrolution (commercial name, Styrolution PS 158 N/L); PA6.6 was provided by BASF (commercial name, 'Ultramid'); PET (Polyethylene Terephthalate) was purchased from Neogroup, commercial name Neopet 80; LDPE (low density polyethylene) was from LyondellBasell (commercial name Lupolen 1800P); finally, PVC (Polyvinyl chloride) was fabricated by Vinnolit Gmbh (Germany, product Vinnolit S3268).

Once spiked, the samples were frozen at −20 °C until further treatment, as it is usually done in monitoring programs while sampling at sea.

The reagents for the alkaline treatment were KOH (100% purity, Emsure), and Triton X-100 (Sigma-Aldrich); the enzymatic treatment required sodium dodecyl sulfate (SDS ≥ 98.5% purity), Tris (tris (hydroxymethyl)aminomethane), protease from *Streptomyces griseus* (Type XIV activity ≥3.5 units/mg), lipase (from *Thermomyces lanuginosus* with activity ≥100.000 U/g), all them from Sigma-Aldrich. From these, working solutions of SDS (2% *w*/w) and Tris (1 M) were prepared. To adjust the pH of Tris, HCl (37% w/w, PA-ACS-ISO, Panreac) was used. For the oxidative treatment H_2O_2 (≥ 30%) was from Sigma-Aldrich.

Ultrapure MilliQ-type water (18 MΩ•cm-1 resistivity) was from a Direct-Q 3-V Millipore (Molsheim, France) device, collected and used daily. The 20 μm mesh size (open bore, square weave mesh type) metallic filters were from Bopp & Co. A.G: (Switzerland) and the 1000 μL pipette tips were from Eppendorf (Hamburg, Germany).

2.2. Quality control

All laboratory material and glassware were washed with alkaline soap (Extran® MA01) for 48 h and rinsed thoroughly with tap and Milli-Q water before and during all working steps. All materials and recipients were covered with aluminium foil during storage and use (Enders et al., 2020). All the solutions were made with fresh ultrapure Milli-Q water. Dedicated air flow cabinets were not used although all works were done inside fume hoods. Operators always wore cotton clothing to avoid cross-contamination by microplastic fibres. Stainless steel filters were washed following the protocol described by Enders et al. (2020) in Module 0, although with Triton-X100 instead of Tween80; in addition, an oven treatment at 450 °C for 3 h after washing was done following Prata et al. (2021).

Negative controls as procedural blanks were made for each experiment using all reagents and filtration instruments, according to quality criteria proposed by Hermsen et al. (2018). The presence of cellulose and *co*-polymer fibres from the laboratory environment was detected. Contaminant particles of the studied polymers were absent from all blank filters ($n = 9$) and the fibres identified as *co*-polymers were of different colour as the spiked PET ones. Recoveries were calculated using the expression: R (%) = 100 * *Number of particles recovered/Number of particles spiked* (=20).

2.3. Apparatus

A quantum cascade laser-based system (8700 LDIR, Laser Direct Infrared, from Agilent Technologies, USA) working in the 1800–600 cm^{-1} mid-IR region and using flat reflective slides (MiRR, Kevley Technologies, Chesterland, USA), was used.

An automatic evaporation system composed of a V-800/805 vacuum controller, Vacuum line and R-12 analyst Syncore-Plus® Line plus dedicated glass containers (residual volume 1.0 mL) (Büchi, Switzerland); a Rotabit P incubation system (Selecta, Spain), with temperature and agitation controls; a Pobel vacuum filtration system combined with a Millipore vacuum pump (Millipore, Ballerica, MA,

model WP6122050); a 3,000,867 Selecta ultrasonic bath (Barcelona, Spain); and a 2001 pH-meter from Crison (Barcelona, Spain), were employed throughout. Further, a Leitz Wetzlar stereomicroscope (10× ocular and manual adjustment of the objective zoom up to 5×, total magnification 50×) was employed for the 'manual method'.

3. Results and discussion

In the following, a preliminary section reflects briefly on how a correct polymer assignment to a particle can be made from a spectrum using the QCL-LDIR system. Then, two major sections present the digestion protocols and the transference of the filter cake to the reflective slides. The working temperatures never exceeded 40 °C, as Lusher et al. (2020) recommended.

3.1. Polymer identification

From an operational viewpoint, although the QCL-LDIR system detects particles and fibres automatically, the spectra of the fibres were assessed further with 20-point-series measurements along their main length. In some cases, the autoscan mode was disable to identify some small particles more adequately with the high-magnification view. The measurement time for the overall Kevley slide takes ca. 5 h/sample, which is significantly less than that required by a single-point μ-FTIR system to scan a selected suite of particles in a dedicated holder (López-Rosales et al., 2021).

Once a spectrum with a good signal-to-noise ratio is obtained a comparison with a spectral library is needed. The spectrum is related to one or several candidates of the library and, usually (although not necessarily correct) the user selects that with the upper match index (or other denominations). The way in which the correlation (match) is calculated becomes predetermined by the software and very few times can be modified by the user (which is a serious drawback common to most instrumental software).

To consider that a particle was identified positively as a polymer in the database a match coefficient >90% is recommended. However, unfortunately, for real samples that may contain highly degraded particles it is not possible to set a definitive, unique criterion. The best recommendation is to enlarge the database; for example customising it with the most common polymers (pristine and weathered). As an indication, Tian et al. (2021) accepted matches up to 65%. Nevertheless, using that value we experienced many wrong polymer identifications (see Fig. 1 for an example) because a visual study of the spectrum revealed quite clear differences between the two spectra (unknown and candidate). The polyurethane (PU) suggestion is clearly not reasonable once the two spectra are visualized, regardless of the correlation index. Therefore, in our view, it is mandatory to study every 'high' match for a sound decision-making. One should be very cautious when concluding from automatic identification criteria.

3.2. Sample digestion

As sample matrices may hide suspicious particles it is required to destroy them although without affecting microplastics. There are contradictory reports about the use of acid media (Miller et al., 2017). It was seen that acids can dissolve some nylon and PS, PP, PE and PA fibres (Avio et al., 2015; Catarino et al., 2017; Claessens et al., 2013; Dehaut et al., 2016; Naidoo et al., 2017). LDPE, HDPE and PP were degraded in an experiment made by Karami et al. (2017). Besides, PA, PET and PS damage was found also by Pfeiffer and Fischer (2020). Despite the general trend is to avoid acids (Dehaut et al., 2016; Enders et al., 2017; Karami et al., 2017; Thiele et al., 2019), nitric acid was proposed for a rapid dissolution of the biological material (Lusher et al., 2017; Naidoo et al., 2017) and, even, an initial report of the International Council for

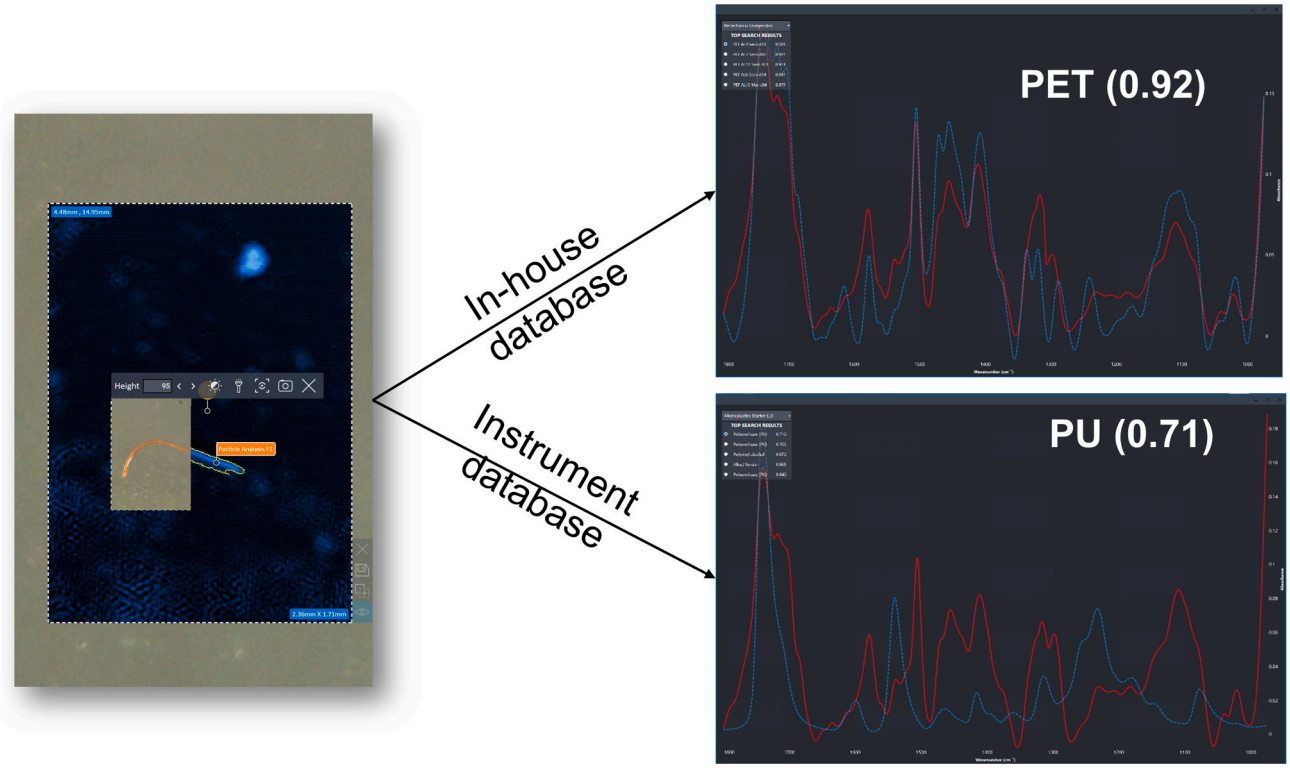

Fig. 1. Example of two correlation indexes derived for the same fibre using the customized (in-house) database and the common available one included by default in the instrument controlling software. In the first case, the spectral match is very good whereas in in the second case, polyurethane is a wrong identification. Red spectra corresponds to the fibre spectra and blue corresponds to databases. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the Exploration of the Sea (ICES) recommended a mixture of 65% nitric acid and 68% perchloric acid, in a 4:1 ratio (De Witte et al., 2014).

Alkaline digestion is considered a more efficient way to get rid of the organic matter (Kühn et al., 2017) as it respects more the integrity of the polymers. Nevertheless, NaOH yielded unsatisfactory results as it degraded polymeric fibres, damaged PET, PA, PVC, PC and PE (Cole et al., 2014; Dehaut et al., 2016; Hurley et al., 2018) and damaged biopolymers like PLA (Pfohl et al., 2021). KOH became the preferred option to treat biological tissues, like stomachs and intestines (Karami et al., 2017; Lusher and Hernandez-Milian, 2018; Phuong et al., 2018; Prata et al., 2019; Thiele et al., 2019). Besides, Karami et al. (2017) concluded that 10% KOH for 48 h could represent a good tradeoff to destroy the organic matter efficiently while preserving the MPs. Dehaut et al. (2016) suggested 60 °C to treat gastrointestinal tracts, but changes in the shape and degradation of PET were reported (Hurley et al., 2018; Karami et al., 2017). Further, other problems were found when KOH was used with PET fibres and some polymers: Hurley et al. (2018) observed a 16% reduction in the weight of PC, and low recoveries were observed for PVC and Nylon 66 (PA 6.6), plus changes in colour (Karami et al., 2017).

Oxidizing agents, mostly H_2O_2, were proposed either alone (Hurley et al., 2018; Prata et al., 2019; Tagg et al., 2015) or combined with Fe(II) as catalyst to yield the Fenton's reaction and accelerate the final oxidation process (Hurley et al., 2018; Prata et al., 2019; Tagg et al., 2016; Treilles et al., 2020). The most remarkable problem when using H_2O_2 is the excessive foam formation that can yield poor recoveries (Avio et al., 2015). Furthermore, the Fenton's reaction can involve high temperatures, much foam (Treilles et al., 2020) and Fe^{+2} residues (Babuponnusami and Muthukumar, 2014). Besides, concentrated H_2O_2 (ca. 30%) may also damage some synthetic polymers (Crawford and Quinn, 2017; Lusher et al., 2017; Nuelle et al., 2014). An experiment found total PA6.6 degradation, PP size reduction and superficial surface degradation ('crazing') in PS when 30% H_2O_2 at 70 °C was employed (Hurley et al., 2018). That concentration was also found to affect PET and PA fibres (Treilles et al., 2020), even at 50 °C (Karami et al., 2017). Recently, it was found that PLA and tire rubber changed their surface after Fenton's reaction (Pfohl et al., 2021) and also that it can change PVC Raman peaks (Lenz et al., 2021). Tsangaris et al. (2021) established that a good trade-off between organic matter destruction and polymer preservation is 15% H_2O_2.

An alkaline-oxidative treatment proposed by Enders et al. (2017, 2020) including 17% KOH and 15% NaClO (*v*/v) (active chloride 14%) was reported to be efficient and preserve plastics below 100 μm size (Lenz et al., 2021). However, such combination can damage strongly semi-synthetic fibres (Cai et al., 2019) and change the shape of PS particles (López-Rosales et al., 2021).

The so far most conservative treatments employ enzymes, like proteinase-K (Cole et al., 2014), trypsin (Courtene-Jones et al., 2017), corolase 7089 (Catarino et al., 2017), alcalase (Rist et al., 2019) and pancreatic enzymes (von Friesen et al., 2019). Proteinase-K reported very good results (Hildebrandt et al., 2020) but it is very expensive. Railo et al. (2018) used a one-step digestion based on a mixture of lipase, protease, amylase and SDS.

Löder et al. (2017) presented a Universal Enzymatic Digestion Protocol (UEPP) that can be applied to a wide range of biological matrices although it needs a preliminary evaluation of the matrix composition to select the proper digestion steps (Löder et al., 2017). The problem when so many enzymatic steps are required is that various losses of MPs can occur and the lengthy, time-consuming workload, up to 15 days.

3.3. Digestion protocols studied in the present work

Three treatments were tested here: two alkaline-oxidative ones, based in Enders et al. (2020) and López-Rosales et al. (2021); and an enzymatic-oxidative one, adapted from the UEPP protocol (Löder et al., 2017).

For the alkaline-oxidative treatment 250 mL of 10% KOH and 0.1 mL of Triton X-100 were used (López-Rosales et al., 2021). The surfactant improves the digestion of fatty tissues (Bessa et al., 2019) and facilitates the MP recovery. After 48 h of incubation 30% H_2O_2 is added gradually in 10 mL increments, until obtaining 15% H_2O_2 in the total volume. In some cases agitation must be interrupted due to foams (Tsangaris et al., 2021). This process lasted for 72 h and incubation extended for another 48 h (sometimes this is not required, depending on the sample; see Fig. 2).

For the enzymatic-oxidative treatment the enzymatic phase involved a pretreatment with 100 mL of 2% SDS for 24 h. SDS performed a first maceration of the gastrointestinal tract (Löder et al., 2017). Subsequently, 200 mL of 1 M TRIS (pH = 9) and 300 mg of protease were added and incubated for 48 h. Then, 10 mL of lipase were added and incubated for 24 h. The oxidizing phase implied adding 10 mL increments of 30% H_2O_2 until its concentration reaches 15% (referred to total volume). This can take 48 h and incubation is extended for another 48 h. Incubation conditions were always 130 U/min and 40 °C. The overall procedure is depicted in Fig. 2.

The enzymatic-oxidative protocol depicted above had been validated previously in an inter-laboratory exercise within the framework of the JPI-Oceans BASEMAN project where gastrointestinal tracts of salmo had been used, its preliminary results were presented elsewhere (López-Rosales et al., 2020). There, recoveries ca. 98% were obtained for the polymers included in the study (PS, PE, PA6.6 and PET), which outperformed other approaches, see Table SM1 (Supplementary Material).

Fig. 2 reveals that both protocols destroyed the organic matter. However, the combination of KOH and NaClO (*v*/v) (active chloride 14%) proposed by Enders et al. (2020) was not able to sufficiently eliminate the organic matter, despite its working time was extended until 10 days. Indeed the 3 filters that were used got totally clogged (Fig. 2). In our opinion, the procedure seems too mild to digest the kind of stomachs we had here and eventually needs further adjustments that will not be considered in this paper. Additional digestion of the filters would be necessary.

The oxidizing stage is important not only to complete the digestion but to bleach the final solutions and make them almost transparent (this is the reason for the last 48 h). This stage was slower for the alkaline treatment, as the foam formation was more aggressive and, therefore, the 30% H_2O_2 was added at a slower pace; for example, 10 mL/40 min (approx.). The enzymatic treatment required initial intervals of about 25 min that after 6 additions were reduced to ca. 10 min. The alkaline-oxidative treatment resulted in more viscous solutions, whose filtrations were slower than those from the enzymatic-oxidative treatment, and whose filter cakes contained more residues.

The recoveries of both procedures were compared using one of the transference methods validated in next section (in particular, the Syncore method, see there for more details). Table 1 revealed that there were not statistical differences (Student's *t*-test, 95% confidence level) between the recoveries obtained for the PP, PS, PE, PET, PA6.6 and PVC particles in the two digestion methods and that all the confidence intervals overlap. The overall recovery is quite good, ca. 90% for the enzymatic digestion and slightly lower for the alkaline treatment (although statistically they are coincident). Results for the PET fibres were not so good as their recovery by the alkaline treatment was low (52%). This was attributed to the potential damage of alkaline treatments to PET fibres, as reported by Treilles et al. (2020), Hurley et al. (2018) and Karami et al. (2017); however, by the enzymatic treatment higher recovery was achieved (76%). Some problem could also be related to the filters mesh size, not being able to retain 100% of the fibres (Cai et al., 2020). Despite the recovery for fibres is a little lower than that obtained for particles, it is comparable to recent studies (Yuan et al., 2022) whose figures were around 78 ± 5%, 68 ± 10% and 78 ± 6% for PET fragments, PET fibres and Nylon fibres, respectively (in-line filtration) and around 55 ± 17%, 61 ± 12% and 56 ± 9% for PET particles, PET fibres and Nylon fibres, respectively (laboratory filtration). There, the fibres were 15 μm diameter and the filter was of PC, 10 μm pore

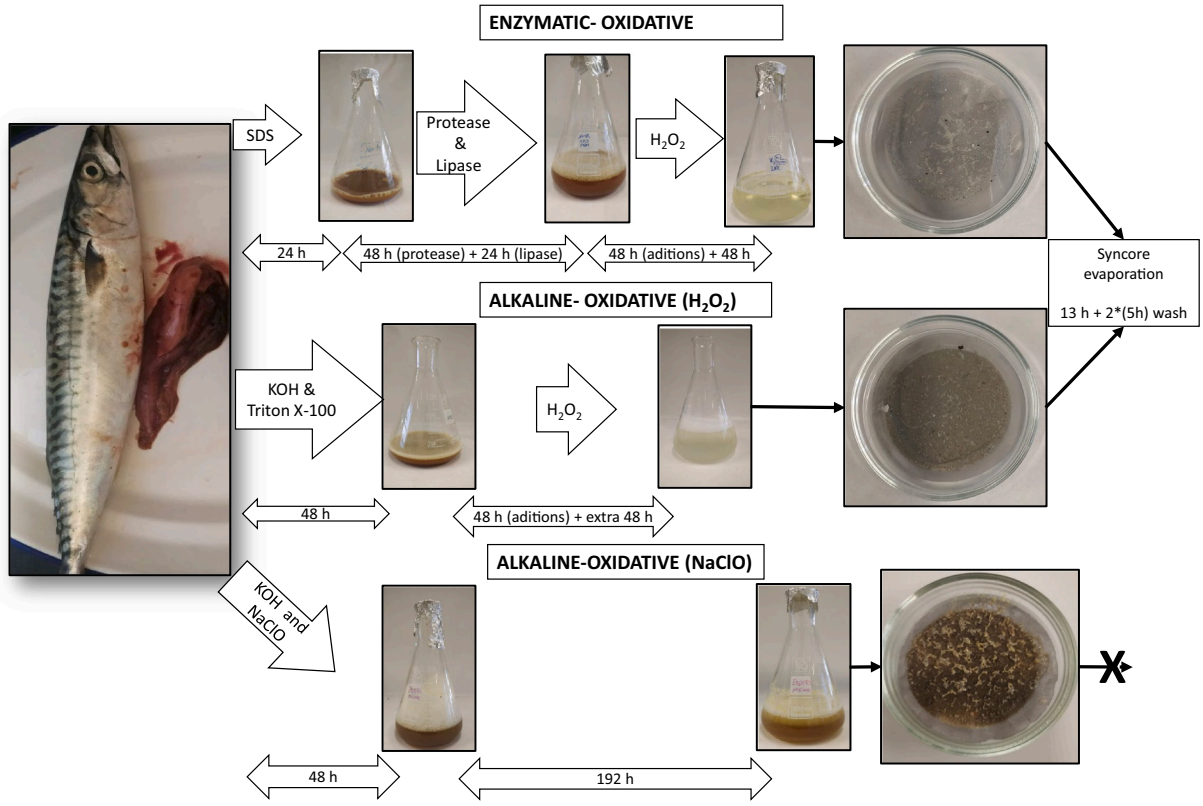

Fig. 2. General scheme of the digestion protocols (see text for details).

Table 1
Recoveries (%) obtained for the two different digestion protocols (enzymatic and alkaline) as a function of the polymer particles and fibres considered in this study (average ± standard deviation, n = number of replicated studies).

	PP (n = 3)	PS (*n* = 3)	PE (n = 3)	PET (n = 3)	PA (n = 3)	PVC (n = 3)	All particles (*n* = 18)	PET Fibres (*n* = 9)
Enzymatic	87 ± 3	90 ± 5	87 ± 3	85 ± 5	88 ± 2	92 ± 3	88 ± 4	76 ± 7
Alkaline	87 ± 6	82 ± 8	85 ± 5	78 ± 3	88 ± 6	83 ± 3	84 ± 5	52 ± 14

diameter.

Considering all the issues raised above, it was concluded that the enzymatic-oxidative digestion is the most adequate one to digest gastrointestinal tracts, although it is also more time consuming (8 days vs 7 days of the alkaline one) and costly (roughly, 41 €/sample vs. 8.5 €/sample of the alkaline option). Nevertheless, the alkaline-oxidizing alternative can be a good option, mainly for monitoring studies, providing fibres are not the main scope of the campaign, as it is faster and much cheaper, with statistical equivalent recoveries. Somehow, the selection depends on the trade-off one can accept between analyte preservation, time, number of samples and cost.

3.4. Transference protocols

After digesting the gastrointestinal tracts, the resulting solutions were vacuum filtrated through the metallic filters; the material thus retained was washed with abundant MilliQ water and dried. The challenge now is to quantitatively transfer the filter cake to a reflective slide, distribute the particles homogeneously over its surface and evaporate the solvent.

A resuspension of the filter contents in a 50% water:ethanol mixture and withdrawal of an aliquot of the total volume to the slide was recommended (Agilent, 2019; Tian et al., 2021). The problem here is to assure that a representative aliquot was collected due to the Brownian motion of the particles and their trends to agglomerate and stick to the walls or the pipette tips. In the present work that procedure was applied although considering an aliquot that represented 10% of the total volume (let us denote this option as the 'aliquot method'). A second approach consisted of using microtweezers and a stereomicroscope to pick up the particles to the reflective slide for further measurement (this will be called the 'manual method'). A third option consisted of avoiding the use of aliquots by evaporating the whole solvent where the filter contents were resuspended. This can be done with a Syncore® evaporation system (let us term this option as 'Syncore method').

The performance of the three approaches was compared using the enzymatic-oxidative digestion method to ensure maximum preservation of the MPs. In the *aliquot method* the filter is washed with up to 50 mL of 96% ethanol in a Büchi glass tube and sonicated for 30 min, $\leq$ 40 °C. Then, the filter was washed with another 10 mL (5 mL/side) and removed. 10% of the total volume of the final suspension (i.e. 6 mL was collected by means of repeated 0.5 mL aliquots and poured on the reflective slide waiting for solvent evaporation between the withdrawals.

The *manual method* consists of manually collecting the particles and fibres from the filter cake with microtweezers and a stereomicroscope to the reflective slide. It is acknowledged that this method has a practical size limitation to handle the particles that, in our experience, is ca. 70 μm diameter for particles and ca. 2.5 mm length for fibres (10 μm diameter). Despite some authors suggested that even particles ca. 30 μm could be handled manually (Cai et al., 2020) we could not reproduce

that.

The *Syncore method* followed the same initial steps as the former two, although considering the whole solvent volume (to transfer the entire filter content) using dedicated Büchi glass tubes with a 1.0 mL bottom deposit. The temperature and agitation were 40 °C and 160 rpm. A pressure gradient was programmed starting at 250 mbar and decreasing to 150 mbar for 10 min, then, became reduced to 80 mbar for 20 min, and kept for 800 min. The small remnant volume at the bottom of the Syncore flask is sonicated for 10 s and a volume between 0.3 and 0.7 mL was quickly collected, carefully poured on the reflective slide and the solvent allowed to evaporate (Fig. 3). The remains at the bottom of the flask are suspended on 0.5 mL ethanol and transferred to the slide. The Syncore tubes were washed twice with 20 mL of ethanol, repeating the evaporation and transfer processes (ca. 5 h/each). The enormous advantage of this procedure is that the entire filter contents are transferred to the slide so that representativeness (of potential aliquots) is not an issue.

A final note is in order here to explain the lower, complementary route in Fig. 3. Big particles (≥1 mm) that can appear in some fish samples cannot be aspirated throughout the very tip of the pipette, or they can clog it. For that reason, when such particles are visible at the filter cake, we recommend picking-up them (before sonicating the filter), with microtweezers and a stereomicroscope. The particles are easily deployed onto the reflective slide.

The transfer protocol including this optional step was validated using 3 new samples, spiked with 20 PE particles (200–250 μm), 20 PE pellets (1–2 mm) and 20 PET fibres (2.5 mm, 10 μm diameter). The results were excellent as recoveries were 100% for major particles, 92% (±3%) for the medium ones and 78% (±4%) for the fibres, where the ± refers to standard deviation ($n = 3$). Note that the recoveries in this study are consistent with those of the previous ones.

Table 2 compares the recoveries obtained for the three transfer procedures. The Syncore and manual methods were highly satisfactory, with no significant differences between them (Student's *t*-test, 95% confidence level). But it should be noted that the manual method has a detection limit of about 70 μm, being the LDIR system one around 10 μm in routine, automatic particle detection. Fig. 4 compares the overall recoveries for particles and fibres using the well-known Box-and-Whisker test. The aliquot method revealed as clearly problematic and seems not a good option. It tended to mostly overestimate the true value of the number of particles, and to underestimate the number of fibres (note the very high standard deviations). First, the fact that any error occurring in the counting of the aliquot is magnified when extrapolating to total volume (in our case, this is ×10). Another situation might be that when pipetting some particles (likely, the smallest ones) may be aspirated with preference to others, but this is something we could not verify.

With regard to the fibres, the Syncore method offered much better recoveries (and consistent with those in Table 1) than the manual option because fibres smaller than 2.5 mm length (10 μm diameter) were difficult to pick up manually from the stainless-steel filter and this explains the so poor recoveries of the manual approach. On the other hand, the aliquot method showed a high variability (as somehow expected), Fig. 4, and accordingly cannot be recommended for routine use, likely due to the difficulty in assuring the homogeneity and representativeness of the aliquots.

4. Conclusions

Out of the three digestion methods studied in this work (alkaline oxidative with H_2O_2, alkaline oxidative with NaClO, and enzymatic oxidative with H_2O_2), the enzymatic oxidative one can be established as a safe and reliable method to digest the gastrointestinal tracts of *Scomber Scombrus*, with good particle recoveries. Nevertheless, the alkaline-oxidative (H_2O_2) option yielded statistically equivalent results to the enzymatic one and can also be a good option for monitoring studies as it is a bit faster than the alkaline-oxidative way that uses NaClO as a source of active oxygen (which performed poorly for the studied gastrointestinal tracts).

With regards to the transfer of the filter cake to reflective slides to be measured by the QCL-LDIR system, the automatic evaporation of the suspension resulting from the filter sonication was the best option among the studied ones. The manual way, consisting of picking-up particules manually was good but time-consuming, tiresome and with worst limits of detection (ca. 70 μm particle diameter and 2.5 mm fibre length) due to visual and handling constraints. The option of withdrawing partial aliquots of the final working suspension yielded quite irreproducible results and it is not recommended.

Therefore, the enzymatic-oxidative digestion, coupled to a Syncore automatic evaporation of the extract, and final measurement with an

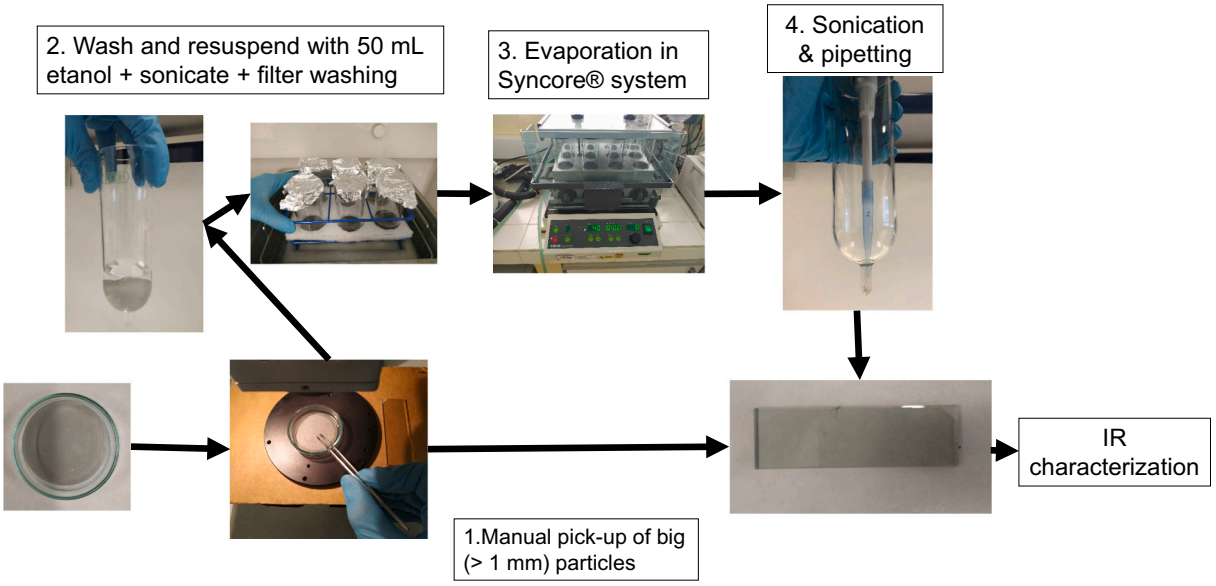

Fig. 3. Scheme of the final Syncore procedure to transfer the filter cake contents to reflective slides. The step depicted at the bottom is optional to collect big particles that might be present on the filter cake.

Table 2
Recoveries (%) calculated for the three transfer procedures (average value ± standard deviation).

	PP ($n = 3$)	PS (n = 3)	PE (n = 3)	PET (n = 3)	PA (n = 3)	PVC (n = 3)	All particles ($n = 18$)	PET Fibres ($n = 9$)
Syncore	87 ± 3	90 ± 5	87 ± 3	85 ± 5	88 ± 3	92 ± 3	88 ± 4	76 ± 7
Manual	92 ± 3	92 ± 3	92 ± 3	95 ± 3	90 ± 5	93 ± 3	92 ± 4	50 ± 4
Aliquot	183 ± 126	270 ± 184	137 ± 71	187 ± 148	200 ± 132	250 ± 100	204 ± 120	50 ± 17

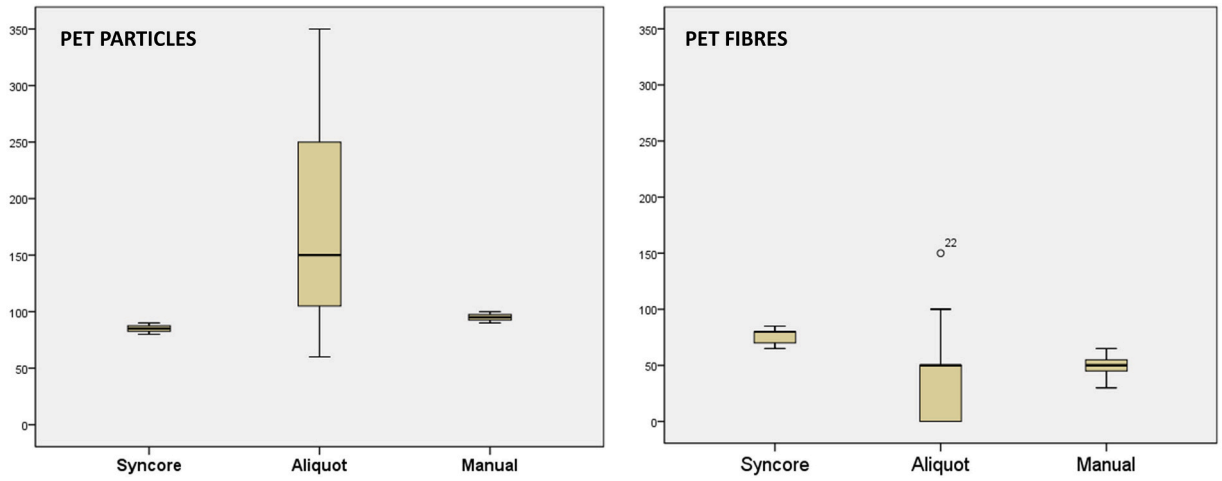

Fig. 4. Traditional Box-and-Whisker plots to compare the recoveries (%) of PET particles and fibres. Note the high variability of the 'aliquot method' and the bad performance of the 'manual procedure' to recover fibres (see text for details).

LDIR system revealed as a very reliable methodology to measure microplastics up to 10 μm that may be present in the fish stomachs, with excellent recoveries, ca. 90% for the commonest polymers, PS, PP, PVC, PET, PE and PA6.6. Recoveries for PET fibres were satisfactory, ca. 75%, similar to those reported in literature.

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CRediT authorship contribution statement

ALR performed the experiments and FTIR determinations, discussed the data and wrote the first draft of the manuscript. VFG collaborated in sample treatment experiments and microplastic identification by FTIR microscopy, discussed and interpreted the data. SML, JMA, PLM designed the study, discussed the data, review & editing manuscript and acquisition of funding. All authors were involved in writing and revision of the manuscript.

Declaration of competing interest

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

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