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Optimization of a new multi-reagent procedure for quantitative mussel digestion in microplastic analysis

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ABSTRACT

Over the last few years, different digestion protocols have been proposed to extract microplastics from mussels, an important product from aquaculture and a relevant economic resource, always scrutinized as a potential pollutant concentrator. In this study, a full factorial experimental design technique has been employed to achieve efficiency in removing biological materials while maximizing the recoveries of five common microplastics (polyethylene, polystyrene, polyethylene terephthalate, polypropylene and polyamide). A robust setpoint was calculated, 2.5% potassium hydroxide at 60 °C for 3 h with 5% hydrogen peroxide and 2.7% of methanol, permitting the quantitative digestion of mussel tissues and recovery of microplastics. These experimental conditions were successfully used to digest whole mussels bought from a local market, which possess high levels of microplastic contamination (41 items/g dry weight). The results highlight the importance of optimizing protocols to develop robust, easy to use and cheap quantitative approaches for analysing microplastic accumulation in edible organisms.

1. Introduction

The word "plastic" includes a huge variety of polymer types, with each type having a different chemical composition and varying properties, used to meet the end-product demand in the best way. A report published by Jambeck et al. reported that in 2010, 275 million metric tons (MT) of plastic waste was produced in 192 coastal urban centres distributed worldwide, with an input from land to the sea of about 4.8 to 12.7 million MT (Jambeck et al., 2015). The most common polymers dispersed in the marine environment are polyethylene (PE), polypropylene (PP), polystyrene (PS), nylon (PA), polyethylene terephthalate (PET), poly (vinyl chloride) (PVC) and cellulose acetate (CA) (Andrady, 2011). According to the data since 2006, there has been a positive trend in recycling (PlasticsEurope, 2020); however, there is still great concern about plastics that enter the marine environment, calculated to be ca. 10% of the total plastics produced (da Costa et al., 2017). The dangerous interactions of microplastics with the marine ecosystems and biota have been widely reported (Andrady, 2011; da Costa et al., 2017; de Sá et al., 2018; Franzellitti et al., 2019; Hidalgo-Ruz et al., 2012; Soares et al., 2020; Troost et al., 2018; Zhang et al., 2019) and also the possible threat posed by this pollutant to humans by the ingestion of seafood (Dehaut et al., 2016).

Several analytical methodologies have been proposed to detect and quantitate microplastics in marine samples, for example tagging with fluorescent Nile Red (Maes et al., 2017), density separation for sediment samples (Graca et al., 2017) and Fenton's reagent to digest complex organic environmental samples (Hurley et al., 2018). However, the scientific community has a common concern about the absence of standardized operating protocols for their analysis (Dehaut et al., 2016; Hurley et al., 2018; la Nasa et al., 2021; Silva et al., 2018; Vandermeersch et al., 2015; von Friesen et al., 2019). In the fisheries and aquaculture industries, the pollutant concentration has been always a concern due to its repercussions in human health (FAO, 2020). Although since 2012, several studies have focused on critical issues associated with the analysis of microplastics, especially the extraction of these materials from their matrices (Lusher et al., 2017; Shim et al., 2017; Silva et al., 2018), a consensus method has not been established yet. This aspect is particularly important for biota.

Mussels are one of the first animals used to assess the environmental quality of seawater (Goldberg, 1975); they meet almost all the required

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criteria for a useful bioindicator species, for example widespread distribution, sedentary, accessibility and high tolerance to pollutants (Goldberg, 1986); in addition, they are active filter feeders able to uptake microplastics. Their importance is also highlighted in their role as seafood consumed by humans (Dehaut et al., 2016). They are considered ideal indicators for microplastic pollution monitoring since they accumulate these items in different parts of their anatomy (Li et al., 2019). The request of some international bodies such as OSPAR Commission or the Marine Strategy Framework Directive is to implement standard, effective and cost-efficient analytical methods to study microplastics in the marine environment, biota and seafood (Guidance, 2013; ICES, 2015). In fact, risk assessment on ecosystem health (Compa et al., 2019) and, consequently, the effects of microplastic ingestion by humans are important topics to be considered (Lim, 2021.) Although there are few studies on the effects of microplastics on humans, microplastic contamination could be a threat for the human population (Campanale et al., 2020; FAO, 2020). Thus, there is an increasingly urgent need to develop protocols that can perform microplastics analysis as a routine process to monitor contaminants as a food quality control measure, like those normally applied for heavy metals and pesticides, or that can reduce the time (and money) needed to process samples providing rapid results, making the monitoring of contaminants in the marine biota easier. Numerous methods have been developed to extract microplastics from biological tissues; they can be classified as acidic, alkaline, oxidizing and enzymatic methods (Dehaut et al., 2016). Acid digestion with simple and/or mixtures of strong acids (nitric, hydrofluoric, perchloric and sulphuric acid) is reported to achieve high digestion efficacies but also to destroy some of the polymers tested. In addition, damages, such as yellowing or melting, are reported for polyethylene terephthalate (PET) and high-density polyethylene (HDPE) (Catarino et al., 2017; Lusher et al., 2017) whereas dilute nitric acid affects low density polyethylene (LDPE) (Bitencourt et al., 2020). Some authors employing enzymes such as proteinase K, protease and trypsin report a high percentage of digested tissues and good recoveries of plastic particles (Catarino et al., 2017; Cole et al., 2011; Courtene-Jones et al., 2017). Although it's an effective approach, enzymatic digestion is very expensive and difficult to be routinely used in microplastic digestion protocols, due to the long time required for the complete destruction of tissues. To address this drawback, some procedures take advantages of oxidizing reagents that, in combination with the selectivity of the enzyme, can improve the organic matter degradation (Burek et al., 2019; da Costa et al., 2017; Dehaut et al., 2016; Löder et al., 2017; Prata et al., 2019; Thiele et al., 2019). As far as the oxidizing digestion is concerned, one of the problems reported is foam production when using hydrogen peroxide (H_2O_2) (Thiele et al., 2019) At present, alkaline digestion seems to be the most promising technique (Hurley et al., 2018) for microplastics analysis in seafood tissues (Dehaut et al., 2016): potassium hydroxide (KOH) is not a chemical of major public health concern; it is cheap (Thiele et al., 2019) and affords good recoveries for different microplastics (Ding et al., 2018). Microwave-assisted extraction (MAE) allows faster sample pre-treatment steps: in conventional heating, in fact, a defined time is needed to heat the vessel before to transfer heat to the solution, whereas microwaves heat the solution directly (Sanchez-Prado et al., 2010). The MAE method has been applied to extract microplastics from sand and sediment samples (la Nasa et al., 2021), and from tissues of various seafood (shark species, acoupa weakfish, tuna fish, trahira, and pink shrimp) with acid digestion solution (Bitencourt et al., 2020). MAE can be regarded as a green chemistry method as it promotes significant reduction of the solvent amount (reducing waste generation), minimizes extraction time, allows the simultaneous treatment of many samples and reduces contamination risks by using a closed vessel system (Beser et al., 2014, 2011; Carro et al., 2017; Król et al., 2012; Sanchez-Prado et al., 2010).

Traditionally, procedure optimization has been achieved changing one-parameter-at-time, monitoring the effect of that single factor on an experimental output (Bezerra et al., 2008). Unfortunately, it does not include the effect of the interactions between the studied variables. As a result, a higher number of experiments are performed, the result often being not the best one, increasing time and cost (Bezerra et al., 2008; Leardi, 2009). The design of experiments (DOE) allows the control of the operational time and costs and, using a programmed number of experiments, it is possible to determine a set of optimum conditions for the investigated task along with the knowledge of the factors' contribution and the degree of interaction between the variables (Costa et al., 2010; Miller and Miller, 2018). The importance of this approach has been shown in clam tissue digestion (Zhang et al., 2020): temperature, solution volume, incubation time and shaking speed were optimized but keeping KOH concentration as high as 10%.

Aiming at developing a faster, cheaper, and more effective procedure both in term of mussel digestion efficiency and microplastic recoveries, the present study proposes a multi-reagent method: using a KOH concentration lower than those reported generally, we explored the possibility to enhance the digestion efficiency, by the concurrent use of methanol (CH_3OH) and H_2O_2. The former was used to take advantage of its ability to enhance the proteolytic activity of KOH by dissolving fats, and the latter was used to accelerate organic tissue degradation, at a low concentration to reduce the risk of damaging microplastics. Microwave-assisted extraction in a closed vessel system reduced problems related to foaming. Time and temperature were the other experimental variables considered. The DOE on tissue and most common polymers allowed the identification of the experimental regions where complete digestion of mussel tissue and quantitative recoveries of microplastics in a short time are achieved. This new procedure, apart from making the analysis faster and cheaper, will allow routine monitoring in aquaculture and fisheries.

2. Materials and methods

KOH pellets, H_2O_2 (30%) solution and LCMS-grade CH_3OH were purchased from Sigma-Aldrich (Milano, Italy). All the solutions were prepared with ultrapure water. Glass fibre filters (Whatman 47 mm, 1.2 μm pore size) were supplied by Sigma-Aldrich.

Microwave-assisted digestion was performed with the use of an Ethos system (Milestone, Sorisole, Italy) described elsewhere (Faraco et al., 2016) and operated in the closed-vessel mode to avoid excessive foaming and subsequent sample loss. The internal vial temperature was controlled during all the experiments by a temperature sensor probe inserted in one of the vessels. *Mytilus galloprovincialis* tissues were dried at 50 °C in an oven for 48 h, cut in pieces before weighing (dry weight 0.2–0.5 g), and then introduced in glass vials. Five different polymers were selected: bottle taps for HDPE, mussel nest for polypropylene (PP), plastic bottles for PET, cable ties for polyamide (PA) and plastic containers for polystyrene (PS). The chemical properties of the polymers were determined by Raman microspectroscopy (see Figs. 1S–5S). These polymers (HDPE, PP, PET, PA and PS) were frozen separately, ground mechanically with a crusher (Wu et al., 2021) and then sieved to remove fragments greater than 1 mm. About 100 mg of each microplastic polymer were weighed accurately and transferred into glass vials. Reagents at the concentration reported in Table 1S in 2.5% KOH for a final volume of 10 times the dry weight were then added. Each vial was vortexed for 10 s and then heated in the microwave closed vessel system (temperature and time as in Table 1S). Irradiation power reached its maximum of 500 W at the beginning of digestion during the heating of the mixture and then decreased to lower and almost steady values. After the digestion step, the solutions were cooled to room temperature and neutralized with a few drops of citric acid (10%) before filtration through glass fibre membranes using Buchner apparatus. The vials were rinsed with ultrapure water three times to recover all the digestion residues and the rinsing water was filtered. After that, the filters were washed with a few millilitres of ethanol and dried in an oven at 30 °C for 30 min and then in a vacuum desiccator until constant weight was achieved.

Table 1
Experimental conditions and relative response values expressed in terms of mussel digestion efficiency (DE%) and plastic recoveries (PR %).

Exp no	Run order	Temp	Time	H_2O_2	CH_3OH	Mussel (DE%)	HDPE (PR%)	PP (PR%)	PA (PR%)	PS (PR%)	PET (PR%)
1	25	60	1	0	0	88.4	100	99.1	100.4	100	99
2	9	90	1	0	0	95	100.3	100	102.4	100	98
3	1	60	3	0	0	97.6	100	100	102.6	99.3	98.8
4	37	90	3	0	0	98.9	100	99.1	102.3	100.4	102.5
5	23	60	1	5	0	96	100	101.2	99.7	99.7	99.5
6	38	90	1	5	0	96.7	99.5	100	100.7	99.5	98.9
7	36	60	3	5	0	98.9	99.5	99.5	99.7	100	100
8	28	90	3	5	0	99.6	98.5	100	101.1	99.5	103
9	30	60	1	0	20	92.2	100.4	100.5	100.9	100	98.7
10	8	90	1	0	20	98.5	100.6	98.6	99.4	99.4	98
11	6	60	3	0	20	98.1	100.4	99.2	102.1	100	99.2
12	31	90	3	0	20	99.4	98.9	99.6	102.7	100	98.1
13	7	60	1	5	20	93.5	99.4	100	99.6	99.3	100
14	14	90	1	5	20	98.9	99.7	100	98.9	98.5	102.3
15	24	60	3	5	20	99	99.2	101	102.9	100.7	98.8
16	29	90	3	5	20	97.1	97.9	98.5	102.8	99.6	102
17	12	75	2	2.5	10	96.9	99.4	99.5	104	101.2	99.2
18	20	75	2	2.5	10	97.1	99.9	99.1	104.6	100.8	101.3
19	4	75	2	2.5	10	95.7	100.7	100	103.9	101.3	100.6
20	19	60	1	0	0	91.6	100	100	100.8	100	100
21	15	90	1	0	0	94.9	100.2	99.6	101.2	100.1	100
22	5	60	3	0	0	97.1	100	98.6	101.9	100	99.7
23	33	90	3	0	0	97.7	101	100	103.6	99.6	102.9
24	11	60	1	5	0	95.2	99.6	100	100.2	100	99.9
25	3	90	1	5	0	97	99.5	100.6	100.9	98.9	100.3
26	10	60	3	5	0	99.2	99.2	99	100	100.4	101.1
27	18	90	3	5	0	98.3	99.3	100	101.8	99.6	104
28	21	60	1	0	20	91	100	99	100.5	100	99.1
29	13	90	1	0	20	96.2	100.1	99.4	100.6	100	99
30	2	60	3	0	20	98.5	99.5	99.9	102.8	99.6	98.1
31	27	90	3	0	20	98.4	99.5	100	103.8	100	97.3
32	22	60	1	5	20	96.2	100.1	100	100	100	98.4
33	34	90	1	5	20	98.5	100	99.5	99.7	99.3	101
34	17	60	3	5	20	99.1	99.4	100.6	102.5	100	100.6
35	35	90	3	5	20	99.3	97.4	99.5	102.3	100	103.3
36	26	75	2	2.5	10	98.6	100	100	103.5	101.2	99.7
37	32	75	2	2.5	10	94.4	99.5	99	103.8	100.7	100.3
38	16	75	2	2.5	10	96.3	99.7	100	104.4	100.9	99

2.1. Optimization of microwave-assisted digestion of mussel tissues

Microwave-assisted digestion parameters were selected by applying a full factorial experimental design with three runs of the central points (2^k, k = 4; central points: 3, Table 1S). All the runs were performed randomly to avoid the occurrence of unwanted systematic effects. The experimental design was created using MODDE 12.1 software (Sartorius). All responses were centred and scaled to unit variance and the models were developed using the same software, which also provides a framework for optimization and for finding robust set points.

The polynomial equation for the 2^k factors design is the following:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \sum_{j(\neq i)}^{k} \beta_{ij} X_i X_j + \varepsilon, \quad (1)$$

where Y denotes the digestion efficiency (%DE) and polymer recovery (%PR), variables used to evaluate mussel tissues and plastic stability, respectively, under the experimental conditions (vide infra). X_i represents the independent variables (time, temperature, H_2O_2 and methanol), and β_0, β_i and β_{ij} refer to the regression coefficients for intercept, linear and interaction terms, respectively, and ε represents the unexplained error.

2.2. Assessment of digestion efficiency

The digestion efficiency (%DE), i.e. the percentage of the digested tissue, was calculated by the following formula (Karami et al., 2017):

$$\%DE = \left[1 - \frac{(\mathrm{Wfa} - \mathrm{Wfb})}{\mathrm{Wm}}\right] 100, \quad (2)$$

where Wm corresponds to the sample weight and Wfa and Wfb are the dry weights of the filter after and before filtration of the digested tissue, respectively.

2.3. Assessment of plastic's integrity

To evaluate the effects of the treatments on the polymer integrity, all plastic samples were weighed before and after the digestion treatments. The relevant recovery (PR) was calculated as percentage using the following equation:

$$\%\mathrm{PR} = \left[1 - \frac{\mathrm{Wpa} - \mathrm{Wpb}}{Wp}\right] 100 \quad (3)$$

where Wp is the plastic weight and Wpa and Wpb correspond to the weight of the filter containing plastic after treatment and the dry filter, respectively.

2.4. Raman microspectroscopy

The microplastics were also subjected to Raman analysis to check the polymer chemical stability. Raman microspectroscopy was performed using a Renishaw InVia instrument with a Leica microscope with 50×/20×/5× magnification lens and a 785 nm diode laser: dark particles spectra in the 500–1800 cm^{-1} region were recorded using a laser power of 1% (1–2 mW on sample) to avoid sample overheating or burning, while spectra of white and transparent particles were recorded using a 5–10% laser power (5–10 mW on sample), 10 s exposure time and three accumulations. WiRE 3.4 software was used to process the Raman data.

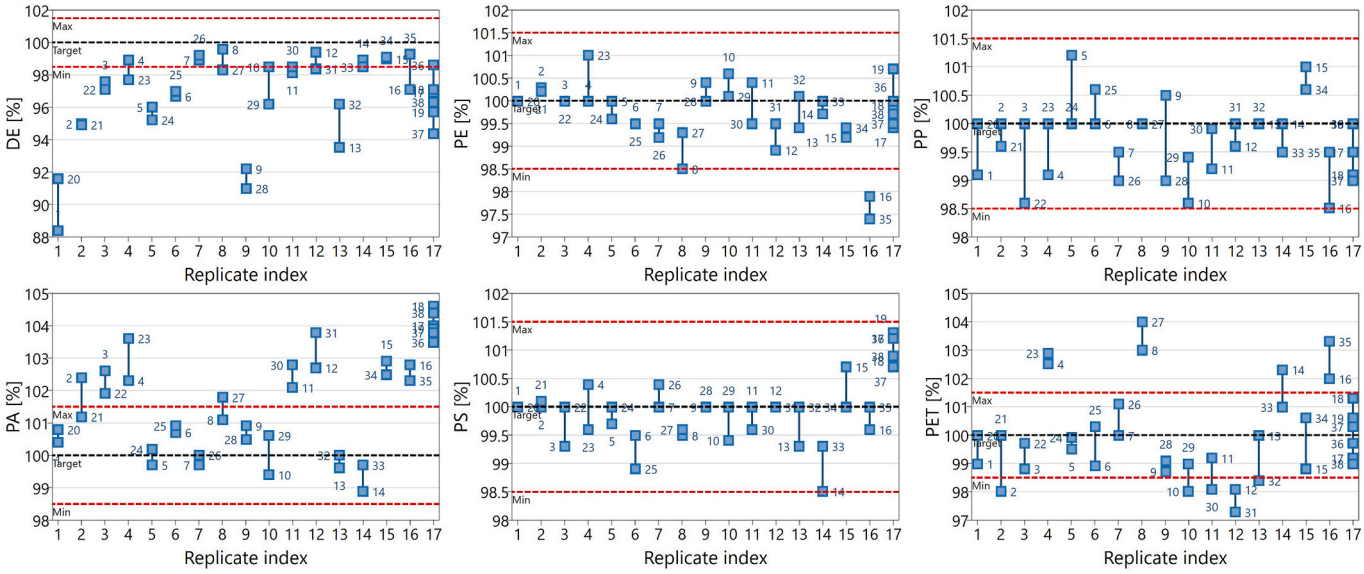

Fig. 1. Plot of the experimental results obtained in all the 228 runs in terms of mussel digestion efficiency (DE%) and plastic recoveries (polymer %).

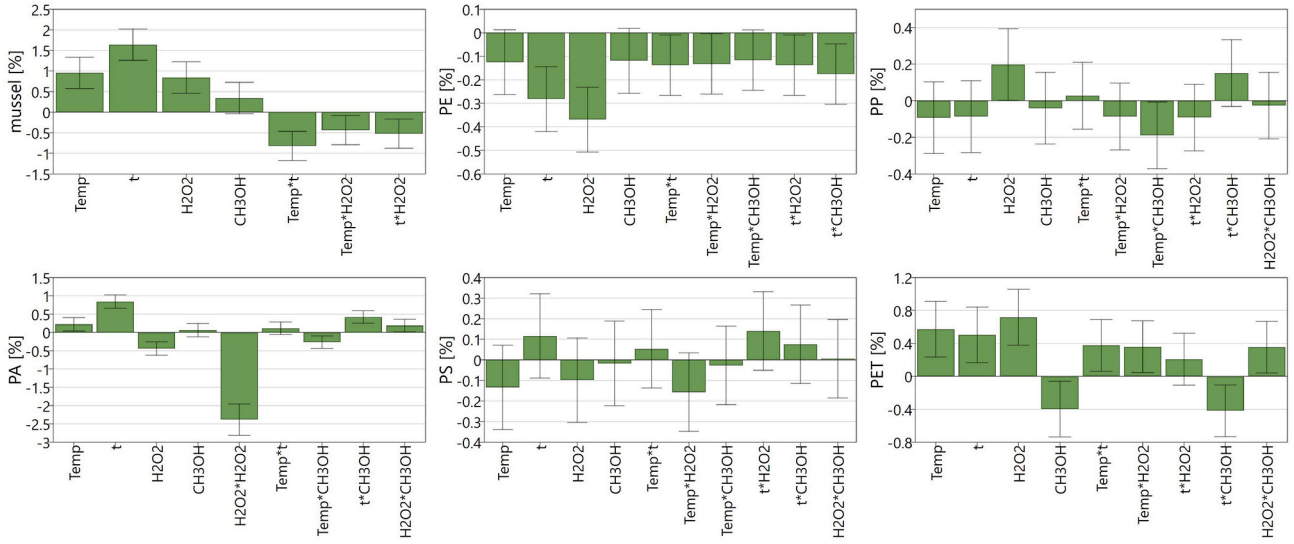

Fig. 2. Scaled and centred coefficients of the factors affecting digestion efficiency and plastic recoveries.

Table 2
Factor values and the relevant contribution at the robust set point identified using MODDE software around the optimal set point.

Factor	Role	Value	Factor contribution
Temperature	Free	60	17.2626
Time	Free	3	22.0331
H_2O_2	Free	5	46.3731
CH_3OH	Free	2.66667	14.3312

Table 3
The predicted results for each response along with the selected criterion from the robust set point (value column), the normalized distance to the target (Log(D)), the probability of failure and the capability estimate (Cpk).

Response	Criterion	Value	log(D)	Prob. of failure	Cpk
Mussel	Maximize	98.9339	−0.546426	5%	0.552624
HDPE	Target	99.424	−0.831297	0.01%	1.4134
PP	Target	99.6738	−1.32523	0.04%	1.22788
PA	Target	100.545	−0.880061	0.1%	1.02921
PS	Target	100.353	−1.25747	0.08%	1.14856
PET	Target	100.499	−0.956724	3.2%	0.623866

2.5. Application of the optimized protocol

The optimized robust conditions, i.e. KOH (2.5%) 60 °C, 3 h, H_2O_2 (5%) and CH_3OH (2.7%), were used to digest the whole mussels soft tissue (*Mytilus galloprovincialis*), bought from a local market. Microplastics on filters were characterized by optical microscopy, using a Nikon Eclipse 80i microscope equipped with a Nikon camera at two different magnifications (5× and 20×) and the relevant photographs were collected using ACT-2U acquisition software. The microplastics on each filter were counted and classified according to their shape, dimension and colour. They were then analysed by Raman spectroscopy to identify the polymer.

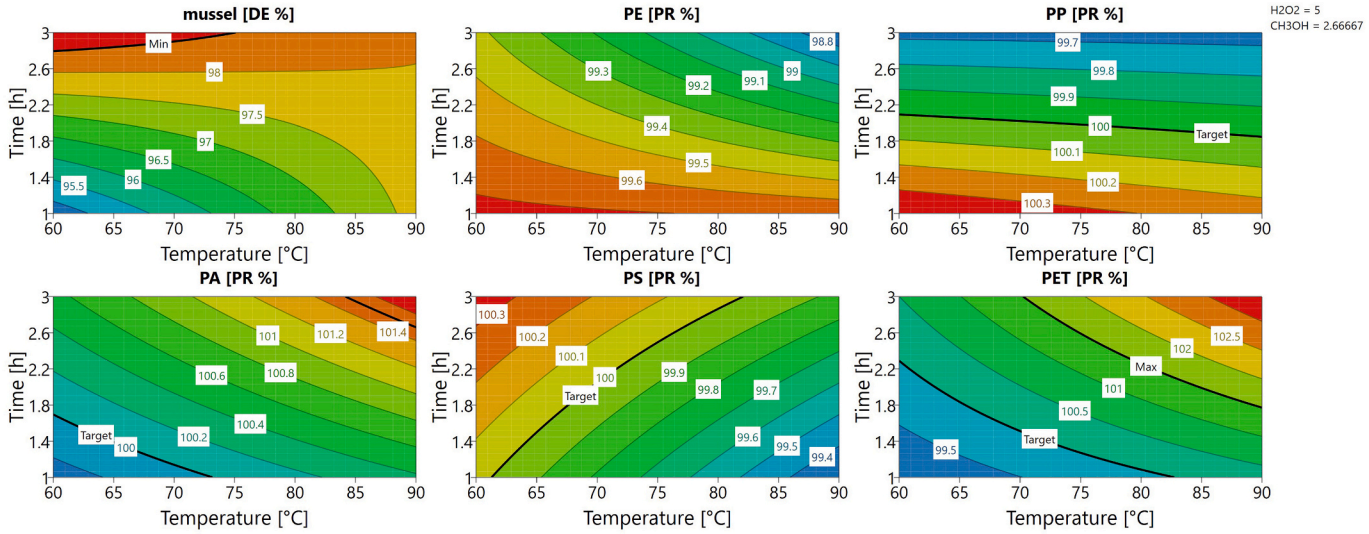

Fig. 3. Response contour plots showing the digestion efficiency and plastic recoveries as a factor of run time vs. temperature at 5% H_2O_2 and 2.7% CH_3OH, i.e. in the experimental condition of the robust set point.

2.6. Contamination control

All tests were conducted in glass vials. During operation, all the staff was wearing cotton laboratory coats and nitrile gloves to prevent contamination. Whenever possible, all the samples were kept in closed containers to prevent airborne contamination. These actions were effective: method blanks, in fact, carried out twice during the DOE development showed 5 and 3 transparent fibres on the whole filter, so the relevant mean, 4 transparent fibres, was subtracted from each sample.

3. Results and discussion

In this study, multivariate analysis was performed to evaluate the effect of the selected factors on the digestion efficiency without damaging the potential ingested plastics, covering all possible interactions between the parameters, and different responses were recorded simultaneously: 1) the digestion efficiency (%DE) of the organic matter and 2) the plastic recoveries (%PR). Several prior research studies have shown the ability of bases such as NaOH or KOH to remove organic compounds. Although 10 M KOH or 10 M NaOH solution allowed the complete destruction of the organic matter in a couple of weeks (Foekema et al., 2013; Cole et al., 2011), several polymers such as PET were degraded using 10 M NaOH (Dehaut et al., 2016; Hurley et al., 2018). H_2O_2 is an oxidizer that is often used to remove organic materials but 30% H_2O_2 caused sample loss because of foaming and polymer modifications (Nuelle et al., 2014). CH_3OH is an organic solvent that is often used in the extraction mixtures of lipids; its use can facilitate tissue digestion by lipid dissolution. The presence of H_2O_2, thanks to its oxidizing effect on organic tissues combined with the effect of methanol, could allow the use of a lower KOH concentration. During laboratory screening experiments, KOH solutions at three different concentrations, 1%, 2.5%, and 5%, were tested. 1% KOH solution (60 °C, 12 h) was not able to digest mussel tissue, conversely 2.5% and 5% KOH solutions could. As a result, 2.5% KOH was selected as the digestion medium: in terms of molarity, it corresponds to about 0.44 M, lower than that used generally. The H_2O_2 concentration was kept low (0–5%) due to the damage usually observed in microplastics at higher concentrations, whereas the methanol (0–20%) was used to take advantage of the ability of this solvent to enhance the proteolytic activity of KOH. It is important to develop a procedure to optimize tissue digestion while preserving microplastics from degradation. Till date, few developed protocols have taken into consideration the interaction between the factors involved in the digestion of biota organic matrices and their synergic effect on tissues and plastics. Temperature, time, H_2O_2 and methanol concentration were evaluated using a full factorial design, providing a unique statistical approach for the optimization of time, cost and effectiveness of the proposed protocol. It was decided to use the 60–90 °C and 1–3 h as the digestion temperature and time duration range. We selected a digestion time range lower than that reported generally, thanks to the concurrent use of hydrogen peroxide and methanol, to provide a method that could make the sample preparation phase easy and fast to be routinely performed especially in a monitoring framework. In fact, operational simplicity, low-cost reagents and short time of analysis were the followed criteria (Süssmann et al., 2021). The set of 19 experimental conditions, as listed in Table 1S, was used and replicated, resulting in a total of 228 runs, 38 runs for each material (mussel tissue, HDPE, PP, PET, PA, and PS). The experiments were performed in a random order, and the measured responses, reported in Table 1 and showed in Fig. 1, were analysed using MODDE 12.1 software.

3.1. Mussel digestion efficiency

As shown in Fig. 1, the variability of the response at the central point is limited, so a good reproducibility in the whole experimental domain is expected. Moreover, the collected experimental data shows that changes in the factors resulted in changes in the digestion efficiency of the mussel tissues that varied from about 88% to 100%. To find the mathematical relationship among the different factors, i.e. H_2O_2 and CH_3OH concentrations, temperature and time, a PLS model was developed and the following equation, including linear and significant interaction terms for mussel digestion efficiency, was obtained:

$$\%DE = 96.7105 + 0.953231\ X_1 + 1.63909\ X_2 + 0.842794\ X_3 + 0.342931\ X_4 - 0.821621\ X_1\ X_2 - 0.437837\ X_1\ X_3 - 0.524324\ X_2\ X_3 \quad (4)$$

Here %DE is the response variable, and X_1, X_2, X_3 and X_4 represent the factors, as reported in Table 1 (i.e. temperature, time, H_2O_2 and CH_3OH, respectively). All the terms in Eq. (4) have a level of significance better than 0.05 but X_4, i.e., methanol, which is 0.07. The residuals are normally distributed on the normal probability plot as they follow a straight line (Fig. 6S), evidencing the good quality of the model.

Bar charts provide an overview of which factors most influence digestion efficiency. Fig. 2 shows that time was the most influential

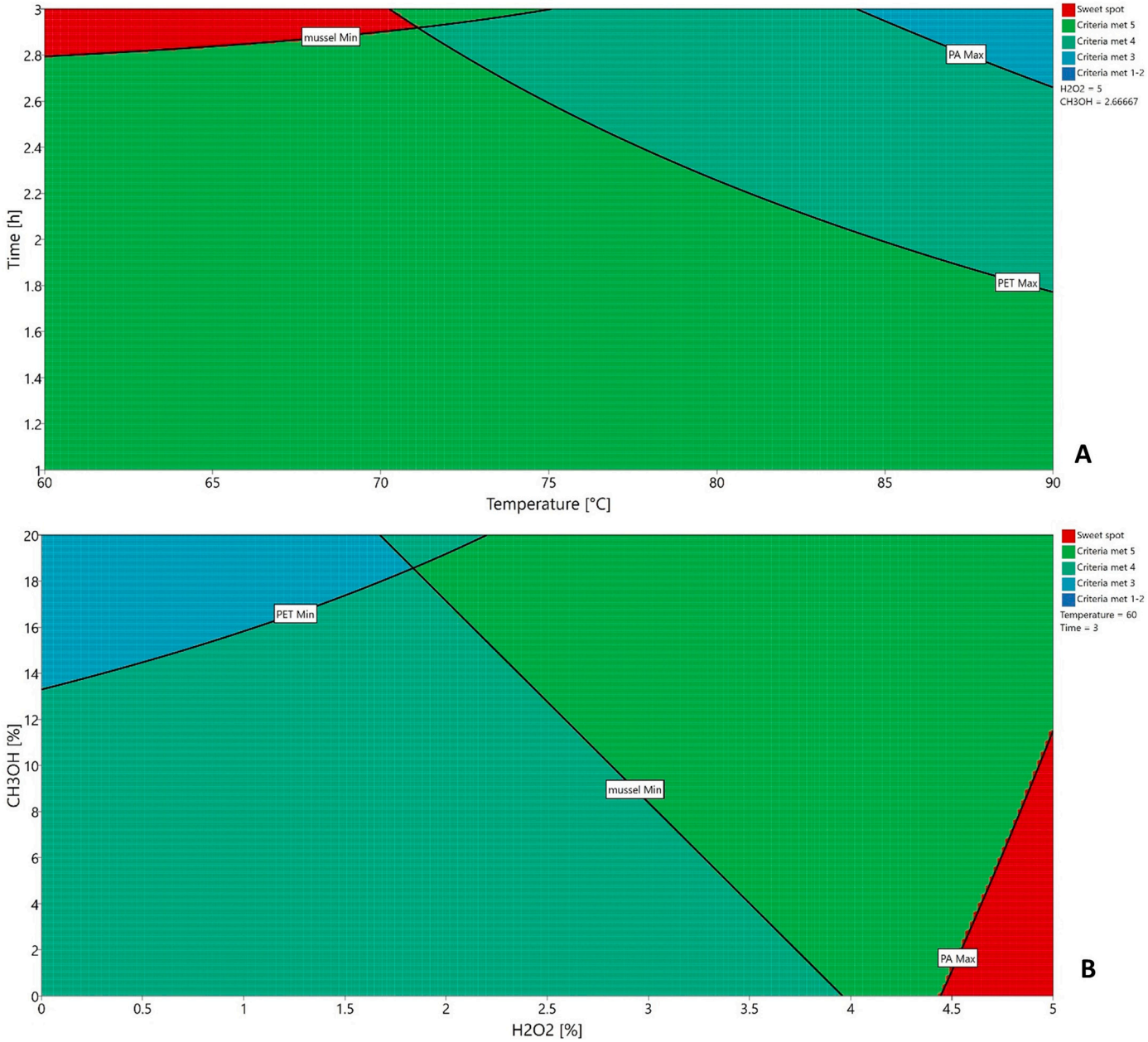

Fig. 4. Sweet spot plots showing the optimal region, where the red region indicates the sweet spot. Time vs. temperature at 5% H_2O_2 and 2.7% methanol (A) and H_2O_2 vs. methanol at 60 °C for 3 h (B). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

factor for DE%, higher than temperature and H_2O_2 concentration. All these factors have positive effects on DE%. There are also small interaction effects between those parameters, but these generally have a negative effect. In contrast, methanol has no influence on the digestion efficiency of mussels nor are there any significant interactions between methanol and the other parameters.

The selected mathematical model can explain the global variability of the experimental conditions chosen, supported by the high correlation between the R2 and Q2 values and the reproducibility. R2 is the goodness of fit value and represents the ability of the model to fit the raw data and to explain data variance; an R2 value close to 1 implies that the model can fit the data very well. Q2 is the goodness of prediction and estimates the model's ability to predict the responses. Model reproducibility is a measure of the variations of the response under the same conditions. A perfect model has a value of 1 for all the three parameters. The model showed a total explained variance R2(Y) equal to 0.85 and a cross-validated predictability Q2(Y) equal to 0.71; hence, both the individual explained variance and the predictability of the responses in the investigated domain were good.

3.2. Plastic recovery

Microplastics behaviour during digestion experiments is somehow dependent on their size. To have a broader distribution, common plastic objects have been ground, and fraction separated through a 1 mm sieve retained for DOE. As a result, the experimental recoveries of these microplastics are more descriptive of the actual resistance of polymers to the selected conditions.

HDPE, PP, PA, PS and PET were treated as reported in Table 1S, and the results are collected in Table 1, whereas Fig. 1 shows the distribution of the experimental points for all these materials.

HDPE, one of the most common microplastic polymer found in the

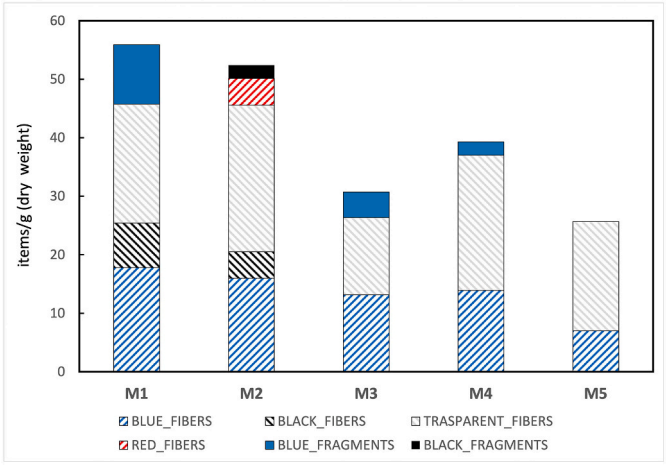

Fig. 5. Number of microplastics per gram of dry tissue counted on the filter for each digested mussel according to their colour and shape. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

marine environment is not stable under all the experimental conditions: in fact, it cannot be exposed to 90 °C for 3 h in the presence of 5% H_2O_2 and 20% methanol because it possibly starts oxidizing and its recovery is not quantitative. Time and H_2O_2 have a notably strong single factor effect; moreover, time has significant interactions with both H_2O_2 and methanol for HDPE recovery. Overall, the conditions comprising high H_2O_2 concentration and temperature and 3 h were found to be not beneficial for HDPE recovery.

PP and PS microplastics showed a greater stability, and in all the experiments performed, the recoveries were in the interval 98.5–101.5%. In contrast, PA was prone to degradation and the recovery was not quantitative in many experiments: it swelled for longer digestion times and at higher temperatures; however, the presence of H_2O_2 seemed to slow this process possibly because the oxidation of the polymeric chains makes them less susceptible to solvent penetration. Time and H_2O_2 are significant factors. Methanol is not a significant factor, but its interaction with time is. The square test showed the presence of non-linear terms and the addition of H_2O_2* H_2O_2 could bring the R2 and Q2 values to over 0.9 and to about 0.8, respectively, indicating a rather good model.

The experiments carried out on PET showed that these microplastics were also sensitive to high temperature (i.e., 90 °C) and long digestion time (3 h) but significantly less than PA: only the experiments at 90 °C lasting 3 h led to recoveries outside the acceptance window (98.5–101.5%). Temperature, time, H_2O_2 and methanol have all significant single factor effects, whereas among the interactions, Temp * t, Temp * H_2O_2, t * CH_3OH and H_2O_2 * CH_3OH are all significant. The model fit R2 is 0.72, whereas the estimate of the prediction precision Q2 is 0.55, indicating that the ability of the model to predict the responses is acceptable.

3.3. Optimization

The aim of the experimental design was to achieve complete mussel digestion along with significant plastic recovery. We found that when mussel digestion is greater than 98%, the filtration step, the slowest step in the procedure, is fast. As a result, the acceptance window for the digestion efficiency was set to 98.5–100%. With respect to polymer recovery, swelling was observed in some of the polymers in different experiments (i.e. a recovery rate of greater than 100% for microplastics), indicating the need to adopt a symmetrical acceptance window that was set to 98.5–101.5%. PP and PS were in the range of 98.5–101.5% in all the experiments, but for the other polymers, i.e. HDPE, PA and PET, different experiments showed a recovery rate outside the 98.5–101.5% window, indicating the need for the optimization step. The criteria, of course, were different for tissue digestion (maximize) and polymers (target 100%). For each response, a desirability function is calculated and the optimizer looks for the combination of factor settings that predicts a result inside the response acceptance windows as close as possible to the targets for all responses. All the factors were set as free in their intervals. The highest level of time and H_2O_2 concentration and the lowest temperature and methanol concentration were the optimal conditions.

Starting from the optimizer set point, it is possible to search for a robust set point through Monte Carlo simulations, which converge if a set point with the ability to predict all responses within its limits can be found. The robust set point was quite similar, but with about 2.7% of methanol and 5% of H_2O_2. Table 2 lists the factor values and contribution at the robust set point, whereas Table 3 collects the predicted robust responses for the mussels and all the polymers. The robustness of the model is qualified by the limited probability of failure.

The contour plots allow a more immediate interpretation of the results (Fig. 3). The mussel digestion efficiency (mussel DE%), plotted in a time vs. temperature graph, shows that increases in time and H_2O_2 concentration are always beneficial to improve digestion efficiency. Temperature at a fixed level of H_2O_2 and methanol does not influence at the same extent the destruction of the organic matrix. Methanol, even if not a significant variable, has an interesting behaviour: the increase in its concentration increases the digestion efficiency only when the H_2O_2 concentration is low.

Mussels could be quantitatively digested at 60 °C in 3 h, using 5% H_2O_2 and 2.7% CH_3OH concentrations without any loss of microplastics. Moreover, these experimental conditions did not cause any discolouration or yellowing of the synthetic items that, as reported in the experimental section, are everyday objects and whose wide granulometric distribution can be considered representative of the actual microplastics present in the environment.

Sweet spot plots obtained by plotting time vs. temperature at 5% H_2O_2 and 2.7% CH_3OH (Fig. 4A) and H_2O_2 vs. CH_3OH at 60 °C for 3 h (Fig. 4B) help visualize the experimental conditions promoting both high mussel digestion efficiency and significant plastic recoveries. Regions where only one criterion is met are coloured in blue, whereas regions where all criteria are met are coloured in red. It is peculiar that the red region is not wide; as a result, the range of the experimental condition that can be used is limited and this must be considered when dealing with microplastics in tissue.

Polymers were analysed by Raman spectroscopy after the selected robust treatment and the relevant spectra were compared to evaluate whether any modifications occurring during the digestion procedure could impede the spectroscopic identification of the polymers. These spectra along with those of the pristine polymers are shown in Figs. 1S–5S, and they are almost superposable. This result confirms that the proposed digestion protocol does not destroy the polymers and has limited impact on their chemistry. This multireagent digestion mixture also allows a complete digestion of the mussel tissue not attainable with the use of the base alone thus making the filtration step fast; moreover, the neutralization step before filtration is faster and more cost effective as requires less citric acid.

3.4. Application of the optimized protocol

The robust conditions were applied to digest mussels from an aquaculture facility with controlled feeding (manuscript in preparation) and mussels' samples from a local market. After digestion, plastic particles were counted and classified based on their colour, shape and size (sample dry weights and items found on each filter are listed in Table 2S). In Fig. 5, the number of microplastics per gram of dry mussel is shown.

Microplastics were detected in each sample; the transparent ones

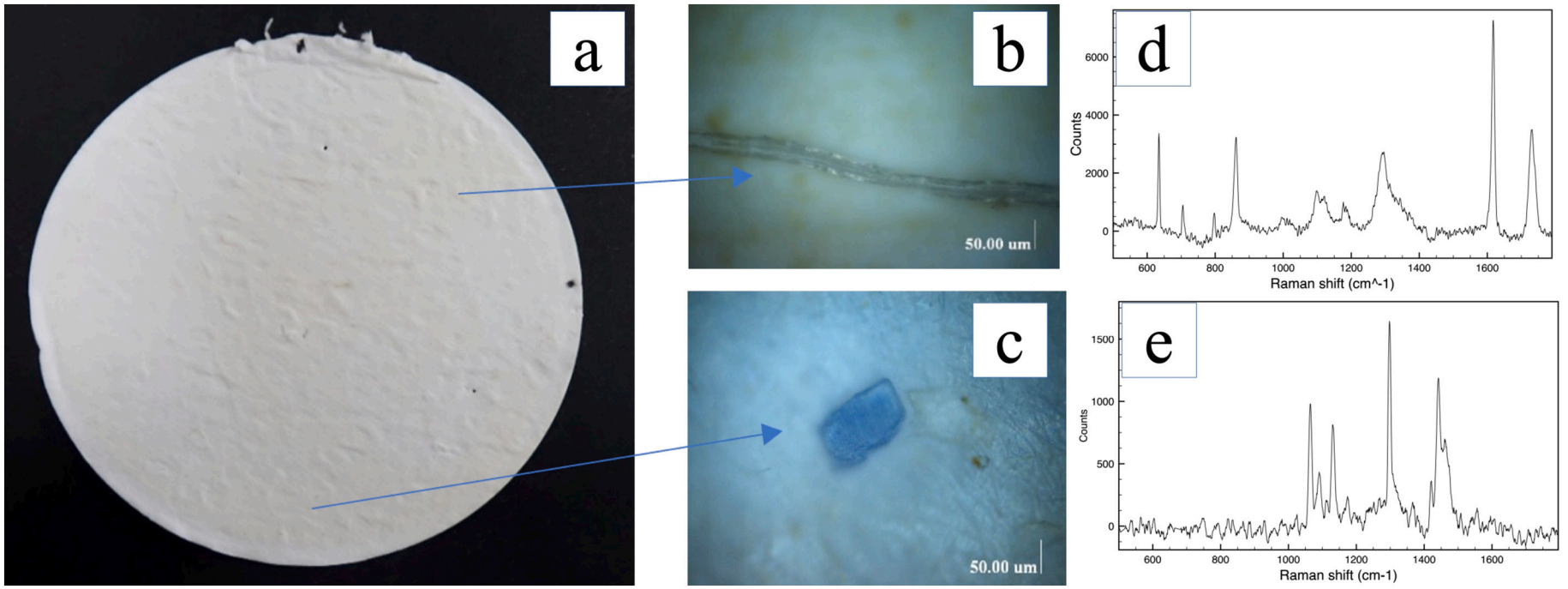

Fig. 6. Photograph of the filter after filtration of the digestate of mussel M3. (a) Photograph at 20× magnification of the transparent fibre (b) and blue fragment (c) identified by Raman spectroscopy as PET (d) and PE (e), respectively. Raman spectra have been background subtracted. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

were more in number than the coloured ones and fibres more in number than fragments, as also reported by other authors (Li et al., 2021; Ugwu et al., 2021). Overall, 41 ± 13 items/g dry weight could be counted. With respect to their size, Fig. 7S shows the mean percentage of the microplastics as a function of the particle size: the monomodal distribution shows a maximum at 80–150 μm.

A set of these microplastics was characterized using Raman spectroscopy. Spectral identification was carried out according to the literature (Araujo et al., 2018; Dehaut et al., 2016; Frère et al., 2016; Käppler et al., 2015) and the Renishaw polymer database. Fig. 6 shows one of the samples: the effective digestion of the mussel tissue is apparent as the filter is almost white and some transparent fibres and blue fragments denoted as PET and PE, respectively, could be identified at the microscope by Raman microscopy.

4. Conclusion

Over the last few years, the presence of microplastics in the marine environment has been reported worldwide, raising concerns about its consequences on wildlife and ecosystems and for their goods and services. Microplastics are one of the most important challenges for the scientific community, and there is a need to develop fast and effective methods for their analysis in samples of animal origin. One of the processes that causes a major concern is the sample preparation process. Several protocols were developed to make this phase a brief step before characterization, but only few studies have been published that use a statistical approach to find extraction protocols that can effectively and quickly eliminate biological matter while ensuring the recovery of the plastic polymers. In this study, we demonstrated that a 2.5% KOH solution at 60 °C containing 5% H_2O_2 and 2.7% methanol allows a 99% digestion in 3 h. This protocol, validated by a statistical model, is robust and reproducible. Because of their synergic effect, KOH and H_2O_2 concentrations lower than those used generally allowed a complete destruction of mussel tissues and the quantitative recovery of microplastics, positively impacting cost-effectiveness and eco-sustainability. Such efficiency and the fact that the procedure is cheap and fast suggest that it could be very useful for the routine detection of microplastics in marine biota tissues. Further studies are still needed to expand our knowledge on this topic and apply it to other animal tissues and polymers for which the present method represents a reliable starting point.

CRediT authorship contribution statement

Silvia Fraissinet: Writing – original draft, Writing – review & editing, Investigation, Data curation, Visualization. **Antonio Pennetta:** Methodology, Writing – original draft, Formal analysis. **Sergio Rossi:** Writing – review & editing, Methodology, Funding acquisition. **Giuseppe E. De Benedetto:** Conceptualization, Formal analysis, Validation, Writing – review & editing, Supervision. **Cosimino Malitesta:** Conceptualization, Supervision, Funding acquisition.

Declaration of competing interest

Silvia Fraissinet, Antonio Pennetta, Sergio Rossi, Giuseppe E. De Benedetto, and Cosimino Malitesta declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marpolbul.2021.112931.

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