EVALUATION OF MICROWAVE-ASSISTED ACID/OXIDANT DIGESTION METHOD FOR THE DETECTION OF POLYETHYLENE MICROPLASTICS IN *Merluccius Gayi* FISH BY NILE RED FLUORESCENT STAINING AND IMAGE ANALYSIS

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ABSTRACT

This study proposes a new method for the determination of polyethylene in *Merluccius Gayi* by microwave digestion, Nile Red fluorescent staining, mobile phone camera and image analysis.

We have demonstrated that lyophilization of the sample is an unnecessary step in the process of determining polyethylene microplastics, obtaining recoveries close to 65% and generating sample losses. Furthermore, a method for matrix reduction is validated using direct raw sample digestion with 9/1 HNO_3/H_2O_2 microwave-assisted digestion achieving recoveries of over 98% for microplastics.

Polyethylene fluorescent color obtained was a bright yellow, which allowed it to be distinguished from micro-particles of polystyrene, polypropylene, polyvinyl chloride, and polyethylene terephthalate easily to the naked eye. Additionally, it was possible to train artificial intelligence to perform this selective identification through image analysis.

Keywords: Microplastics, fish, image analysis, microwave digestion, fluorescence.

1. INTRODUCTION

In Chile, the consumption of marine products increased by 6% between 2019 and 2020, rising from 14.9 kg to 15.3 kg per capita, of which 12.25 kg corresponds to fish [1]. The sale of Common Hake (*Merluccius gayi*) surpasses other marine products due to its affordability and traditional consumption. In 2022, it is noted that hake remains one of the most marketed fish, with sales exceeding 25% compared to other ocean-harvested species [2]. Furthermore, it is well known that, historically, *Merluccius gayi* is one of the most abundant carnivorous fish along the coast of Chile [3].

In studies on marine litter, it is suggested that microplastics (MPs) are among the most concerning pollutants for organisms such as fish. Plastic particles can mix with the food sources of marine organisms and may be ingested accidentally or deliberately when mistaken for food [4]. For example, in the review by Azevedo-Santos (2019), it is indicated that of the 400 fish species analyzed, 54.6% where MPs have been reported are marine fish species, and the vast majority are carnivorous fish [5].

In Chile, the work of Pozo and collaborators stands out for determining the presence of MPs in the stomachs of various carnivorous fish, including "Merluccius gayi". Their results highlight the presence of MPs in 10% of the analyzed hake stomachs. These were embedded in the stomach wall in the form of microfibers. For oceanic fish, the types of plastics found were polyethylene terephthalate (PET) and polyethylene (PE), with an abundance percentage of 75% of PET and 25% PE. For coastal fish, MPs of PET and polystyrene (PS) were reported [6].

Despite the existence of reports on the presence of MPs in fish, the reliability of the results has been in question. This is due to the diversity of methods used for treating fish samples and detecting particles, which are rarely subjected to metrological validation studies. Thus, for example, regarding sample pretreatment, studies such as that by Pozo et al. [4] work with the lyophilized sample before digestion, whereas studies like those by Ghosh et al. digest the sample directly without lyophilizing [8]. where the vast majority of studies apply the stages of lyophilization or digestion without validating the necessity or reliability of these treatments.

The review by Dellisanti et al. concludes that the diversity of methods for the determination of MPs in seafood has not only impeded comparison between studies but also reported losses of microplastics when using temperatures above 60°C for digestion steps. It is suggested to use alkaline digestion as a good option to preserve plastic particles, although digestion takes several weeks. This review does not report the use of microwave technology to accelerate the digestion process. Regarding detection, this same review indicates that visual identification has only a 70% accuracy for particles between 50-100 µm, recommending identification methods based on spectroscopic approaches. [9]

Among the spectroscopic detection methods, those based on fluorescence using fluorophores such as Nile red, [10-13] and Rose Bengal which binds to nonpolar particles, are described [14-15]. Although it is true that this fluorophore-based detection method does not provide structural information about the polymer, the identity of a microplastic can be established by comparing the fluorescent color of the native particle with that of pure particles, as different fluorescence colors are obtained depending on the polarity of the monomer[13].

The main objective of this work is to develop and validate a methodology for fish sample treatment based on microwave-assisted acid oxidative digestion, which preserves the integrity of PE plastic particles, shortens digestion times, and is compatible with detection and quantification methods using fluorescent staining and image processing. In summary, a quick, reliable, and inexpensive method for the quantification of PE in fish.

2. EXPERIMENTAL SECTION

2.1 Reagents and standards

All chemicals used in this study were analytical grade. Samples where rinse using an ultrapure water system (Millipore, Bedford, MA, USA). A Nitric Acid 65% and Hydroxigen Peroxide 30% (p.a. Emsure©) was obtained from Merck (Darmstadt, Germany) a Suprapur © Hydrocloric Acid 30% was from Merck (Darmstadt, Germany) Sigma-Aldrich Nile Red for Microscopy was purchased from Merck (Darmstadt, Germany) Acetone HPLC grade (Scharlau ©, Barcelona, Spain) and the standard LDPE PE115 where obtained from Braskem (Sao Paulo, Brasil).

2.2 Instrumentation

A microwave digestion plaform (Ethos Easy ©, Milestone, Sorisole, Italy) a constant temperature incubator (WP-25AB, China), the filters used in both filtrarions where microdisc membrane (Membrane Solutions ©, MCE 47mm, 0.45um, Shanghai, China), for the fluorescence a UV lamp (Spectroline ©, Model ENF-240C/FE, Long Island, United States) a UV cabinet (Spectroline ©, Model CM-10A, Long Island, United States) for the photos where used a Cellphone (Apple Iphone ©, Model 13 Pro Max, Silicon Valley, United States).

2.3 Sample treatment

Merluccius Gayi samples were acquired from local commerce. Sample treatment involves a cleaning process that leaves only skin and flesh, without organs, bones, or scales, thereby retaining only the edible meat fraction. This fraction is then cut into pieces and frozen at -18°C.

For the digestion, 5 grams of the raw fish meat sample is mixed with 18 mL of 65% analytical grade HNO_3 (Emsure™) and 2 mL of 30% analytical grade H_2O_2 (Perhydol®, Emsure™). Subsequently, the microwave digestion technique is

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applied at 1100mW at a temperature of 60°C for 1 hour, with a 10-minute ramp temperature. The sample is then diluted and filtered using a filtration system connected to a vacuum pump (MRC VP-17) with 47mm diameter membrane microdiscs having a pore size of 0.45 µm made of mixed cellulose esters (Membrane Solutions®), carefully transferring the plastic particles adhering to the walls. The filters are placed in 100mm diameter Petri dishes, covered with aluminum foil, and then dried in an incubator (WP-25AB) at 60°C for 2 hours.

2.4 Staining process of polyethylene microparticles with Nile Red

The staining was performed with a 2% solution of Nile Red (Sigma Aldrich, Nile Red for Microscopy, CAS 7385-67-3 in 2% v/v Acetone with water. To each sample, 20 mL of the fluorophore solution was added and kept in an incubator for 30 minutes at 60°C. After 30 minutes of incubation, the sample is removed and frozen at -18°C for 20 minutes and then filtered and placed in a Petri dish, where it is dried at 60°C for 20 minutes again. After this process, it is possible to see the PE MPs with a UV lamp at 254nm.

2.5 Spike and recovery essays

Low density polyethylene was used for the validation process(Braskem Brasil PE115). This plastic was ground by means of a metalic angle grinder, equipped with a diamond cutting disc (Einhell). After this process the particles were sieved with metallic meshes and separated into different diameters, ranging from 1000 µm - 500 µm; 500 µm - 250 µm and < 250 µm. Two hundred microparticles were inserted into the fish flesh at 5 grams of fish flesh by scalpel cuts with the aid of metal tweezers. Each sample was subjected to the same procedure of digestion, staining and subsequent photography to quantify the number of particles recovered.

3. RESULTS AND DISCUSSION

3.1 Acid digestion procedure

Different digestion mixtures were tested by taking 5 g of raw fish with 20 mL of acid mixture. The first mixtures with HNO_3 and HNO_3/HCl did not give the expected results because, as shown in Fig. 1, it left too many solid remnants of the matrix.

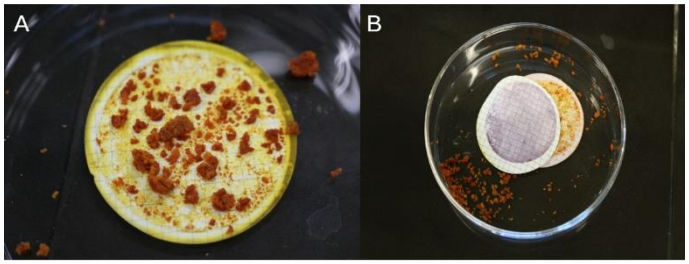

Fig. 1 Remaining from the digestion of 5 grams of fish with: a: 20 mL of HNO_3 and b) 20 mL of HNO_3/HCl 3:1

Considering these digestion remnants, it was decided to increase the degree of oxidation of the mixture by adding H_2O_2 with HNO_3 in different proportions and at 2 sample masses of 10g and 5g of raw fish. The percentages of elimination are given in Table 1.

Table 1 Efficiency of mineralization of fish samples using microwave digestion with different HNO_3/H_2O_2 mixtures, expressed as mass % of the remaining matrix.

Fish mass (g)	20 mL HNO_3/H_2O_2				
	9/1	4/1	7/3	3/2	1/1
	Percentage of remaining fish mass				
5	0.2	1.7	5.1	7.2	7.4
10	1.5	7.6	8.3	10.3	11.4

Table 1 clearly shows us how the 9:1 mixture (18mL HNO_3 and 2 mL H_2O_2) has better digestibility in the chosen matrix for both fish masses. Furthermore, a mass of 5 g of sample is the optimum to achieve maximum mineralization of the sample matrix.

3.2 Recovery studies

To evaluate the reliability of the digestion method, first, the percentage recovery of the polyethylene plastic particles after microwave-assisted digestion with 20 mL of the HNO_3/H_2O_2 9/1 mixture at 60°C for 1 hour was evaluated. Mass recovery was studied at 3 particle diameters. The results are shown in Fig. 2.

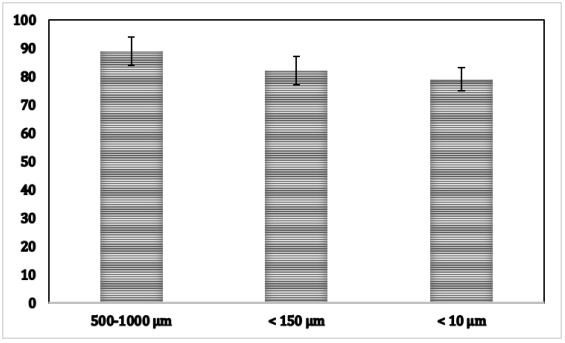

Fig. 2 Mass recovery percentages of polyethylene particles at different particle diameters after microwave-assisted digestion.

We can see that in general the mass recovery percentages are all greater than or equal to 80%, although it is observed that the smaller the particle size, the lower the mass recovery percentage, which may be associated with the larger contact area. On the other hand, recovery percentages were evaluated as a function of particle count, considering the fish matrix and the influence of freeze-drying. The results are presented in Fig. 3.

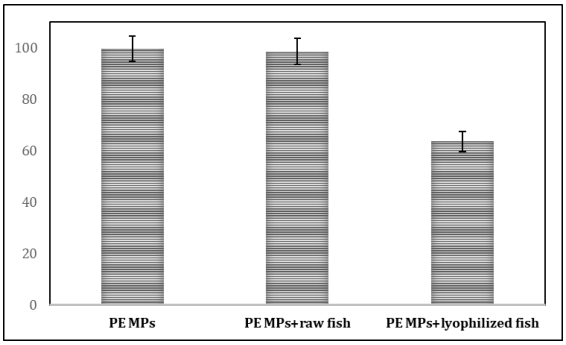

Fig. 3 Recovery percentages in particle count in isolated polyethylene plastic particles, in raw fish matrix medium and in fish medium subjected to freeze-drying.

We can see in Fig. 3 that a good recovery percentage of PE plastic microparticles, both isolated and inserted in the raw fish matrix, is obtained. However, it can be observed that, in the case of the recovery of the PE MPs in the fish matrix subjected to freeze-drying, that the recovery percentage is around 70%. This result can be explained, considering the observable fact that freeze-drying generates an agglomeration of PE microplastics particles generating default biases in the count as can be seen in Fig. 4.

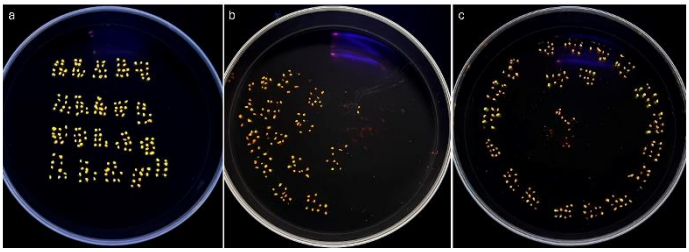

Fig. 4 Visualization of polyethylene microparticles after a recovery study using microwave technology with a 9/1 HNO_3/H_2O_2 mixture: a) without fish matrix; b) with raw fish matrix; c) with fish matrix subjected to prior freeze-drying.

It is important to mention that, in all the analyses, blank samples were taken simultaneously, where no interfering fluorescent particles were detected, except for some light blue fibers which were identified as coming from cellulose fibers.

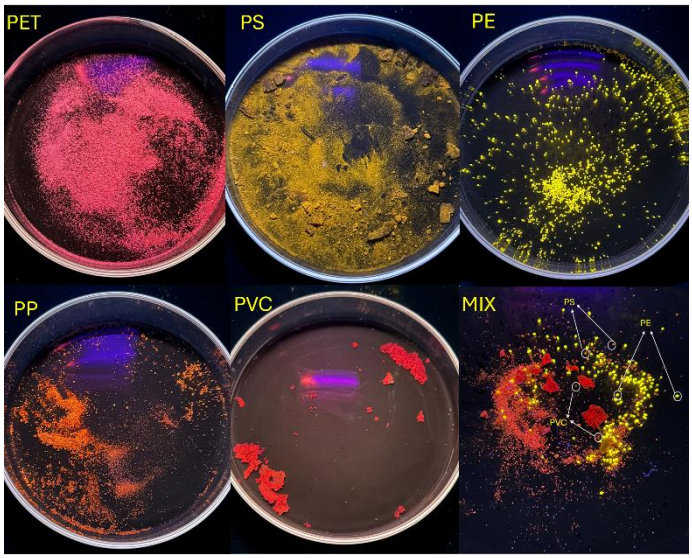

Fig. 5 Fluorescent colors of the five most abundant microplastics in nature after digestion with 9/1 HNO_3/H_2O_2 and subsequent staining with Nile Red.

In Fig. 5, we can observe the different fluorescent colors of the five most abundant microplastics in nature after digestion with 9/1 HNO_3/H_2O_2. It is evident that these microplastics, stained with Nile red, generate distinguishable colors to the naked eye.

As can be seen in Figure 6, the color of the PE particles is a bright yellow, clearly distinguishable in a mixture with the other five polymers, as well as PS and PVC particles, can be differentiated without much difficulty. However, PP and PET particles cannot be distinguished by the naked eye. Therefore, the proposed method is sufficiently selective to differentiate not only PE but also PS and PVC. To automate the process of identifying PE, the artificial intelligence Chat GPT-4 Plus was utilized and tasked with recognizing the color of a photograph of PE. Its response indicated the recognition of a bright yellow color. Subsequently, it was provided with a photograph of mixed plastic micro-particles from the five polymers and instructed to mark them in white. The result of this image processing using artificial intelligence is clearly satisfactory; the AI was able to identify all PE particles in the mixture based on the provided PE standard. This result is significant, considering that identification is an initial step toward achieving automated counting through AI, which was attempted in this case. However, unfortunately, the AI was not able to perform this task with the provided information.

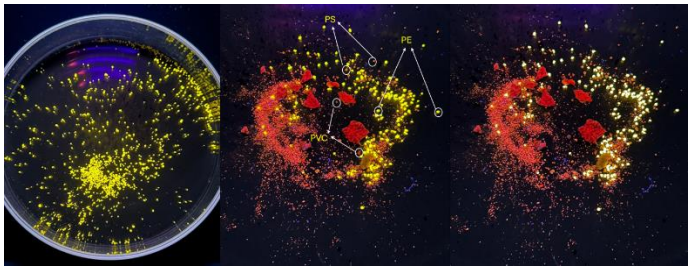

Fig. 6 Recognition of PE particles using artificial intelligence Chat GPT-4 Plus. On the left, we have the photograph of PE particles that was used to train the AI. In the center, the photograph of PE particles mixed with the other four polymers. On the right, the result of the image processing by the AI, which marked the recognized particles in white.

Our method based on microwave-assisted digestion of raw fish takes approximately 80 minutes, compared to most digestion processes that are over 24 hours [16-19]. In Table 2, the digestion times and the one proposed in this work are shown.

Table 2. Comparison of fish sample digestion methods for the determination of plastic microparticles.

Method conditions	Digestion Time	Ref.
Incubator at 60°C	3 hours and 20 minutes	[9]
Incubator at 50°C and then 37°C	72 hours and then 72 hours	[16]
Incubator at 60°C	12 hours	[18]
Incubator at 60°C	12 hours	[21]
Incubator at 50°C	48 hours and then 36 hours	[17]
Incubator at 65°C	72 hours	[19]
Dried at 75°C for 24 h, + 65°C in heating plate for 72 hours.	24 hours drying y 72 hours digestion	[8]
Water bath at 65°C at 50 rpm.	24 hours	[22]
N/A Method just temperature 60°C	48 hours	[23]
Heating plate to boiling.	12 hours and then 30 minutes	[24]
Sealed at room temperature	10 days	[20]
Water bath at 80°C	12 hours and 30 minutes	[25]
Open digestion in heating plate at boiling.	1 day and 10 minutes	[26]
Microwave assisted digestion at 60°C	60 minutes (1 hour)	This work

We can see in Table 2 that the shortest time in references would be from the work of Pozo et al [9] with 3 hours and 20 minutes, while other digestions last up to 10 days as in the case of Gündoğdu et al [20], much longer times compared to this study, in which after 80 minutes, it is already possible to filter the sample. Digesting with microwave assistance crucially decreases the total time of the procedure. Moreover, our digestion method is compatible with the detection method based on fluorophore staining and photography using a cell phone camera, which is much more economically accessible compared to the FT-IR method of the work of Kumar et al. [16] or Leung et al. [23].

CONCLUSIONS

The determination of polyethylene plastic microparticles in fish can be carried out quickly, economically and reliably by means of direct oxidative acid digestion of raw meat, assisted by microwaves, achieving a quantitative mineralization of the fish matrix, without disintegrating the polyethylene plastic particles.

On the other hand, this study demonstrates that it is neither necessary nor advisable to freeze-dry the sample because it agglomerates the polyethylene microparticles, generating default biases in their count.

The bright yellow color obtained through this method offers sufficient selectivity to differentiate micro-particles of PE from those of PVC, PS, PET, and PP. Furthermore, it is possible to train artificial intelligence to perform this recognition, which opens up interesting perspectives for the future in automating the counting of these particles through IA.

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