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# Focused ultrasound-based extraction for target analysis and suspect screening of organic xenobiotics in fish muscle

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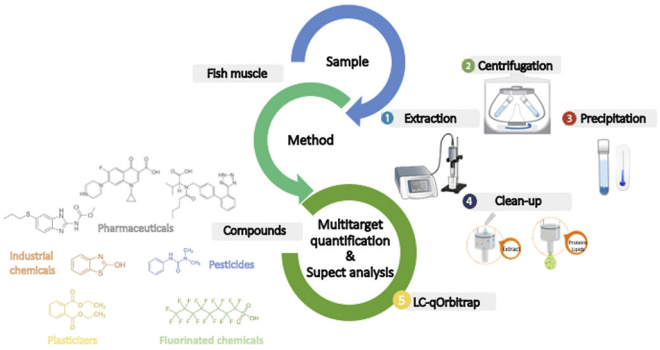
## HIGHLIGHTS

- Multitarget method for the analysis of 180 pollutants in fish muscle
- Cold acetonitrile was the common extraction solvent.
- Filters for protein and lipid elimination rendered the best clean-up conditions.
- Extension of the method to suspect screening of approximately 9000 compounds
- First time focused ultrasounds applied for suspect screening in biota

## GRAPHICAL ABSTRACT

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## ABSTRACT

The development of multitarget and/or suspect screening methods for the analysis of xenobiotics in fish samples is compulsory due to the lack of works in the literature where a deep evaluation of the variables affecting extraction and clean-up steps is performed. The aim of the present work was to optimize and validate a multitarget (180 compounds) method for the analysis of priority and emerging xenobiotics in fish muscle using focused ultrasound-assisted solid-liquid extraction. From the different extraction solvents studied, a single extraction in cold acetonitrile rendered the best consensus results in terms of absolute recoveries and the number of target compounds extracted. Matrix effect was minimized using commercially available Captiva ND-Lipid filters, which provided clean extracts and satisfactory repeatability compared to other approaches. Absolute recoveries were corrected using matrix-matched calibration and apparent recoveries in the 43%-105%, 73%–131% and 78%–128% ranges were obtained at low (20 ng $g^{-1}$), medium (100 ng $g^{-1}$), and high (200 ng $g^{-1}$) spiking levels, respectively. A 60% of the xenobiotics showed limits of identification lower than 20 ng $g^{-1}$. The developed method was successfully applied to the quantification and suspect screening of samples bought in a local market (hake, gilt-head bream, sea bass and prawn) and fished (thicklip grey mullet) at the Urdaibai estuary (north of Spain). Food additives, antiparasitic drugs and PFOS were quantified at ng $g^{-1}$ level. Moreover, the targeted method was extended to the suspect screening, revealing the presence of plastic related products (caprolactam, phthalates, polyethylenglycols), pharmaceutical products (albendazole, mebendazole, valpromide) and pesticides or insect repellents (icaridin, myristyl sulfate, nootkatone). Therefore, FUSLE in cold acetonitrile combined with Captiva ND-Lipid filters and liquid chromatography tandem high-resolution mass spectrometry (LC-q-Orbitrap) were successfully applied to both multitarget quantitative analysis and suspect screening of approx. 17,800 compounds.



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## 1. Introduction

Conventional wastewater treatment plants (WWTPs) are not capable of completely removing the millions of chemical compounds released to the aquatic systems by industry, hospitals and rural/urban daily life (Chiaia-Hernandez et al., 2013; Nürenberg et al., 2015). When the effluents of those WWTPs enter the water bodies, the distribution of xenobiotics in the different compartments of the ecosystems occurs, but little is known about neither their final fate nor the effects they may pose in wild organisms and humans. While regulations are found for classical pollutants such as polychlorinated biphenyls, organochlorine pesticides, polycyclic aromatic hydrocarbons or polychlorinated dibenzofurans, among others (Krasnobaev et al., 2020; Schwarzenbach et al., 2010), the existing regulation for the so-called emerging pollutants is scarce (Geissen et al., 2015; Daughton, 2004; Dulio et al., 2018). The list of compounds and chemicals in this group is significantly large and is ever expanding with the introduction of new commercial chemicals, changes in use, and disposal of chemicals currently in widespread use. Moreover, social and scientific concern has increased due to the lack of knowledge on their bioaccumulative capacity and their toxicological effects in the environment and in human health. Within this context, several agencies and institutions have published different lists of emerging pollutants, including the US Environmental Protection Agency (O. US EPA, 2014) or the European Water Framework Directive (WFD) (European Commission, 2008). Within the European framework, the NORMAN network (https://www.norman-network.net/) has also elaborated different lists of suspect compounds in the environment.

Biota is one of the matrices employed to screen the presence of contaminants that enter the water bodies (van der Oost et al., 2003; Du et al., 2016; Díaz-Cruz et al., 2019) because chemicals can be bioaccumulated and biotransformed in wild organisms. Once accumulated, they can be biomagnified through the trophic chain and become xenobiotics of risk for humans through the consumption of fishery products (Arnot and Gobas, 2006; Ojemaye and Petrik, 2018; Justino et al., 2016). Despite of the ecological and health problems that may arise from the accumulation of pollutants in biota, few works in the literature carry out the determination of a wide range of emerging xenobiotics in aquatic organisms. Therefore, there is a need for the development of analytical methods capable of measuring a large amount of pollutants in these samples (Du et al., 2016; Barbieri et al., 2019a; Baduel et al., 2015; Du et al., 2017a; González-Gaya et al., 2018).

Determination of organic pollutants in biota samples includes extraction and clean-up steps. An exhaustive extraction requires a proper selection of the extraction solvent together with an energy source, including microwaves, ultrasounds or mechanical shaking, among others. For instance, extraction based on focused ultrasounds (Ziarrusta et al., 2016; Ziarrusta et al., 2017) has been successfully applied. Among the solvents used for the extraction of emerging pollutants, acetonitrile, methanol or buffered solutions of both organic solvents are commonly applied (Du et al., 2017a; Mijangos et al., 2019; Martínez-Piernas et al., 2018; Lohne et al., 2015). Some applications include the addition of salts such as sodium chloride or magnesium sulfate in order to enhance the extraction efficiency (Baduel et al., 2015). Besides, and due to the lack of selectivity of the extraction, a clean-up step is usually necessary in order to eliminate proteins and lipids in the final extracts and to minimize matrix effect in the detection. To that end, some methods include the addition of acids (formic, acetic) to the methanol solvent used as extractant (Mijangos et al., 2019) or the use of a cooling period of acetonitrile used as extractant (Baduel et al., 2015), in order to favour protein precipitation. Other protocols include further clean-up steps, including normal phase, reverse-phase and mixed-mode solid phase extraction (SPE) (Du et al., 2017a; Terzic and Ahel, 2011), and, recently, filters that remove selectively proteins and lipids are commercially available (Baduel et al., 2015).

Liquid chromatography coupled to low-resolution tandem mass spectrometry (LC-MS/MS) has been mostly applied for the target analysis of emerging pollutants (Gago-Ferrero et al., 2016; Hernández et al., 2012). However, the challenges within the determination of xenobiotics have evolved together with the development of new analytical instrumentation. The development of robust high resolution mass spectrometry, especially in tandem (such as quadrupole-Orbitrap), has evolved analytical chemistry from classical target analysis of a few tens to hundreds of target compounds to the suspect or non-target screening of thousands of chemicals, without the need of expensive analytical standards (Gago-Ferrero et al., 2016; Hernández et al., 2012; McEachran et al., 2018). The exact mass, together with the isotopic pattern, the fragmentation (obtained in tandem instruments) and chromatographic retention time, offer information for the identification of suspects and non-targets. Different annotation levels are defined according to the confidence in the identification of the compounds (Schymanski et al., 2014a), with the use of reference standards for the unequivocal identification of suspects.

Within this context, the aim of the present study was to optimize the extraction and clean-up conditions of a multi-target method (180 analytes) for the determination of emerging xenobiotics in fish muscle, together with the application of the developed method to suspect screening. In this sense, the efficiency of focused ultrasound solid-liquid extraction (FUSLE) using different solvents was tested. In addition, the use of normal phase and mixed-mode SPE, together with two commercially available filters was studied in the clean-up step, with the aim of obtaining an efficient extraction of the largest number of organic xenobiotics, while minimizing the matrix effect during the LC-q-Orbitrap analysis. The developed and validated method was further applied to both the multi-target and suspect analysis of xenobiotics in fishery products bought in local markets and to wild fish fished in the Urdaibai reserve of the biosphere estuary (Biscay, north of Spain).

## 2. Material and methods

### 2.1. Reagents and materials

The target analytes used during method optimization and target analysis are included in Table 1S (see Supplementary Information, SI), together with the purity of the standard, the commercial vendor and the solvent used for preparing the stock solution of each individual compound. Stock solutions were prepared in the 100–10,000 $\mu g \cdot g^{-1}$ range using methanol (99.9%, UHPLC-MS quality, Scharlab, Barcelona, Spain), acetonitrile (ChromAR HPLC, Macron Fine Chemicals, Avantor, USA), acetone (ChromAR HPLC), ethanol (ChromAR HPLC), dimethylsulfoxide (Applichem, Panreac, Bacelona, Spain) and/or Milli-Q water (< 0.05 $\mu S \cdot cm^{-1}$, Millipore 185, Millipore, Burlington, USA) depending on the target compound. Solutions in the 1–5 $\mu g \cdot g^{-1}$ range containing all the target compounds were prepared in methanol according to experimentation every week and all solutions were kept at −20 °C.

Acetonitrile, methanol, ethyl acetate, (ChromAR HPLC), Milli-Q water, formic acid (HCOOH, ≥98.0, Honeywell, Fluka, USA), ammonia ($NH_3$, 25%, Applichem, Panreac), anhydrous magnesium sulfate ($MgSO_4$, 99.5%, Alfa Aesar, Massachusetts, USA), trisodium citrate dihydrate ($Na_3Cit \cdot 2H_2O$, Panreac) and disodium hydrogen citrate sesquihydrate ($Na_2HCit \cdot 11/2H_2O$, 99%, Fluka) were used during extraction and/or clean-up steps.

Reverse-phase Chromabond HR-X sorbent (5 μm, 55–65 Å, Macherey-Nagel, Düren, Germany), Sepra ZT-WCX (30 μm, 85 Å, Phenomenex, California, USA) weak cation exchanger, Sepra ZT-WAX (30 μm, 85 Å, Phenomenex) weak anion exchanger, Florisil ($MgO_3Si$, 60–100 mesh, Sigma-Aldrich, San Luis, USA), alumina ($Al_2O_3$, 0.063–0.200 mm, 90% activated, Merck, Darmstadt, Germany) and silica ($SiO_2$, 0.063–0.200 mm, Merck) sorbents were used during the different SPE protocols. Captiva Enhanced Matrix Removal (EMR)-Lipid (300 mg, 3 mL, Agilent Technologies, Santa Clara, USA) and Captiva Non-Drip

(ND)-Lipid (100 mg, 3 mL, Agilent Technologies) filters were also tested for clean-up purposes.

### 2.2. Sample preparation

Halibut (*Hippoglossus hippoglossus*) bought in a local market was used during extraction and clean-up optimization steps. The method developed was applied for multi-target and suspect screening of hake (*Merluccius australis*), fish farmed gilt-head bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*) fillets, as well as prawns (*Penaeus sp.*) all bought in a local market. In the case of the fish fillets, at least three were pooled before sample preparation. In the case of prawns, three different bags containing approx. 360 g each, were pooled together. Besides, fillets of thicklip grey mullet (*Chelon labrosus*) collected in Urdaibai estuary (43°19′26.5"N, 2°40′25.0"W), close to the WWTP of Gernika (17,000 inhabitants, north of Spain), in August 2019 were also analysed using the developed method. Fish fillets from three individuals were separated with the use of a clean scalpel. Fish processing described herein was carried out according to the current regulations (procedure approval CEEA/380/2014/ETXEBARRIA LOIZATE) evaluated by the Bioethics Committee of UPV/EHU and approved by the Local Authority.

In all the cases, samples were ground, homogenized, freeze-dried separately in a Cryodos-50 laboratory freeze-dryer (Telstar, Terrassa, Spain) and stored in clean polypropylene bottles at −20 °C in the fridge until analysis.

### 2.3. Sample extraction

Optimization of the FLUSLE step was performed with spiked halibut aliquots (concentration in the final extract of approximately 200 ng $g^{-1}$ for the 180 target compounds). Under optimal conditions, 0.5 g of freeze-dried fish muscle were weighed, and 10 mL of pure acetonitrile were added to a 40 mL pre-cleaned polypropylene falcon tube. FUSLE was carried out at 0 °C in an ice bath for 2 min (with a pulsed on/off time of 0.8/0.2 s) and 20% amplitude using a 2 mm titanium microtip connected to a Bandelin Sonopuls HD 3100 sonifier (20 kHz, Bandelin Electronic, Berlin, Germany).

### 2.4. Protein precipitation

Once homogenization was done, samples were centrifuged for 15 min at 10,000 rpm (Centrifuge Allegra X-30R, F2402H, Beckman Coulter, High Wycombe, UK). The supernatant was quantitatively recovered and kept in the freezer at −20 °C for at least 12 h to favour protein precipitation. After protein precipitation, the supernatant was quantitatively recovered on a glass lab tube for the clean-up step.

### 2.5. Sample clean-up

For sample clean-up optimization, 0.5 g of halibut samples were extracted using acidified (1% HCOOH, pH = 2.0) acetonitrile. For each clean-up procedure, extracts were spiked with the 180 target compounds before ($n = 3$) or after ($n = 3$) the clean-up step in order to obtain a concentration of ca. 200 ng·$g^{-1}$ in the final extract. Then, the supernatant obtained after protein precipitation was evaporated to ~500 μL under a gentle stream of nitrogen (99.999%, Air Liquide, Benoît Potier, France) in a Turbovap LC Evaporator (Zymark, Biotage, Uppsala, Sweden), except for the Captiva EMR-Lipid filters, where the extract was evaporated to ~1.6 mL.

a) Mixed-mode SPE

In house-made 12 mL cartridges contained from top to bottom 300 mg of reverse-phase Chromabond HR-X sorbent, 100 mg of Sepra ZT-WAX and 100 mg of Sepra ZT-WCX (Schymanski et al., 2014a). Pre-concentrated extracts were diluted in 5 mL of Milli-Q water and loaded to the cartridges, previously conditioned using 5 mL of a 1:1 (v/v) methanol:ethyl acetate mixture followed by 5 mL of Milli-Q water. Next, cartridges were dried under vacuum and analytes were eluted with 6 mL of a 1:1 (v/v) methanol:ethyl acetate mixture containing 2% of ammonia followed by 6 mL of a 1:1 (v/v) methanol:ethyl acetate mixture containing 1.7% of HCOOH. Both eluates were recombined, evaporated to dryness under a flow of nitrogen at 40 °C in the Turbovap and re-dissolved in 200 μL of methanol.

b) Clean-up using normal phase SPE

Normal phase clean-up of the extracts was adapted from the literature (Du et al., 2017a). In house-made cartridges contained from top to bottom 7.5 g of alumina, 5.0 g of silica and 1.0 g of Florisil. After cartridge conditioning using 5 mL of acetonitrile, 0.5 mL of the extract were loaded onto the cartridge and analytes were eluted using 15 mL of methanol. Eluates were evaporated to dryness using a gentle stream of nitrogen at 40 °C in the Turbovap and re-dissolved in 200 μL of methanol.

c) Clean-up using Captiva EMR-Lipid filters

Clean-up using Captiva EMR-Lipid filters was carried out according to the supplier recommendations (https://www.agilent.com/cs/library/usermanuals/public/5991-8308EN.pdf). Briefly, 400 μL of Milli-Q water were added to the ~1.6 mL extract and the mixture was loaded onto the filter. After elution and in order to favour the recovery of hydrophobic compounds (log $K_{ow} > 3$), 400 μL of a (80:20, v/v) acetonitrile:Milli-Q water mixture were added and the filters were dried for 30 s. Finally, the cleaned-up extract was evaporated to dryness at 40 °C in the Turbovap and re-dissolved in 200 μL of methanol.

d) Clean-up using Captiva ND-Lipid filters

Clean-up using Captiva ND-Lipid filters was based on the supplier recommendations (https://www.crawfordscientific.com/media/wysiwyg//Literature/Sample_Prep/Agilent/Captiva-ND-and-ND-Lipids-Method-Guide.pdf). Under optimal conditions, after adding 1500 μL of acetonitrile containing 0.1% of HCOOH as crash solvent to the cartridge, the ~500 μL of extract were loaded and, with the help of a pipette, the mixture was five-fold mixed. After elution, the filter was dried, and the eluates were evaporated to dryness at 40 °C in the Turbovap and re-dissolved in 200 μL of methanol.

### 2.6. UHPLC-q-Orbitrap analysis

All samples and solutions were filtered using 0.22 μm polypropylene filters onto chromatography vials and kept in the freezer at −20 °C until analysis, which was performed using a Dionex Ultimate 3000 ultrahigh performance liquid chromatograph (UHPLC, Thermo Fisher Scientific, MA, USA) coupled to a high-performance Q Exactive Focus Orbitrap (q-Orbitrap, Thermo Fisher Scientific) mass analyzer with a heated electrospray ionization source (HESI, Thermo Fisher Scientific).

Analyte separation was performed in an ACE UltraCore 2.5 SuperPhenylHexyl (2.1 mm × 100 mm, 2.5 μm) column with a prefilter (2.1 mm ID, 0.2 μm) from Thermo-Fisher Scientific. Milli-Q water (A) and acetonitrile (B) were used as mobile phase, both containing 0.1% HCOOH and 5 mM ammonium acetate for positive and negative ionization modes, respectively. Column flow was set at 0.3 mL $min^{-1}$ and column was maintained at 35 °C. Gradient elution started with 85% A that changed to 70% A in 4 min, then to 50% A in 4 min and kept at this composition for 12 min. Then, the mobile phase composition was changed to 10% A in 10 min and kept for another 5 min lapse. Then, the mobile phase was changed to the starting conditions for 5 min.

External calibration of the q-Orbitrap mass analyzer was performed every three days using Pierce LTQ ESI (Thermo Fisher Scientific) calibration solutions. Measurements were performed in negative and positive

ionization in the Full scan - data dependent MS2 (Full MS-ddMS2) discovery acquisition mode in the *m/z* 70–1050 Da range. After a complete scan at a 70,000 full width at half maximum (FWHM) resolution at *m/z* 200, three scans were performed in the *m/z* 100–600 Da range at 17,500 FWHM at *m/z* 200 with an isolation window of 3.0 *m/z* with a normalized collision energy (NCE) of 30. The ddMS2 scans were run with an automatic intensity threshold and dynamic exclusion. ACG target was set at $5e^4$, and its minimum was set at $8.00e^3$.

With respect to the HESI parameters, spray voltage was set at 3.2 kV for positive and 3.5 kV for negative ionization modes. For positive ionization, the capillary temperature was set at 320 °C, the sheath gas at 40 arbitrary units (au), the auxiliary gas at 15 au and 310 °C and the sweep gas at 1 au. For negative ionization, the capillary temperature was set at 300 °C, the sheath gas at 40 arbitrary units (au), the auxiliary gas at 15 au and 280 °C and the sweep gas at 1 au.

Xcalibur 3.1 (Thermo Fischer Scientific) was used for instrument control. Target analysis and quantification was performed using the TraceFinder 4.1 software (Thermo Fischer Scientific), which contained a homemade database including the exact masses, isotopic pattern, retention time (included in Table S1) and MS2 fragmentation of all the target compounds. 5 ppm mass error was accepted for both the monoisotopic and fragment ions, while a 70% of fitting was allowed for the experimental and theoretical isotopic pattern. 60-s window was accepted for the retention time. Suspect analysis was carried out using the Compound Discoverer 2.1 (Thermo Fisher Scientific) software and mzCloud (Thermo Fischer Scientific) library was used as suspect list.

### 2.7. Validation and application to real samples

11-point external calibration was built in the instrumental limit of quantification ($LOQ_{inst}$) and 500 ng $g^{-1}$ range. Calibration points corresponding to 0.1 ng $g^{-1}$, 0.5 ng $g^{-1}$, 1 ng $g^{-1}$, 3 ng $g^{-1}$, 5 ng $g^{-1}$, 10 ng $g^{-1}$, 20 ng $g^{-1}$ and 50 ng $g^{-1}$ were injected in triplicate for $LOQ_{inst}$ calculation. A 6-point matrix-matched calibration was built in the procedural limit of quantification ($LOQ_{proc}$) and 500 ng $g^{-1}$ range using analyte-free halibut fillets. Calibration points corresponding to 10 ng $g^{-1}$, 20 ng $g^{-1}$ and 50 ng $g^{-1}$ were injected in triplicate for $LOQ_{proc}$ calculation. Both external and matrix-matched calibrations were repeated at least three times in the analysis sequence. Methanol was injected every 6 samples and several injections ($n = 5$) of the 250-ng $g^{-1}$ calibration level solution were done along the sequence in order to check for possible carryover and for the determination of the repeatability of the measurement, respectively.

Validation of the analytical method was carried out using halibut fillets that were spiked at three levels, 50 ng $g^{-1}$ (low), 100 ng $g^{-1}$ (medium) and 200 ng $g^{-1}$ (high), in triplicate. For absolute recovery calculation, external calibration was used. The accuracy of the method was determined by: (i) calculating the apparent recovery using matrix-matched calibration and (ii) determining the repeatability of the whole procedure ($n = 3$) at the three spiked levels.

## 3. Results and discussion

### 3.1. Optimization of the clean-up step

The four clean-up approaches described in Section 2.5 were tested in order to obtain an optimal recovery and a minimum matrix effect in the detection of the 180 target compounds. With this purpose, the efficiency of the clean-up step, defined as chromatographic peak area ratio obtained for each compound before and after the clean-up step, was calculated. The boxplot in Fig. 1(a) summarizes the clean-up efficiencies obtained for each strategy.

The clean-up based on normal-phase SPE rendered lower extraction efficiencies in comparison to the rest of studied strategies (Fig. 1(a)). As shown by the median, using normal-phase SPE the 50% of the compounds were recovered at 47%, whereas this value increased to

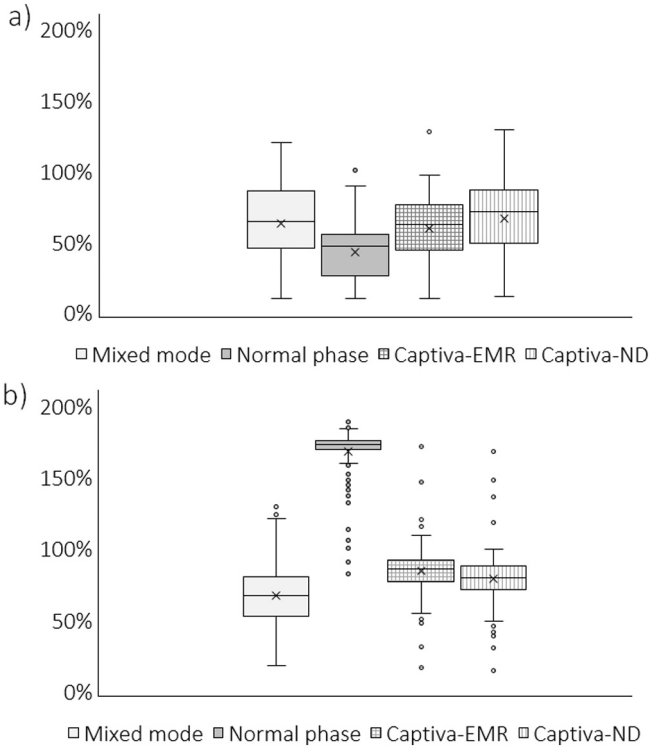


**Fig. 1.** Box-plot diagram of (a) the clean-up efficiency (%) and (b) the matrix effect observed at the detection step using the four clean-up approaches studied for the determination of 180 xenobiotics.

62–72% using the rest of the clean-up strategies. Moreover, 25% of the compounds showed recoveries <26% using normal-phase SPE and between 44 and 49% in the case of the rest of the approaches. The same percentage of compounds showed recoveries higher than 55%, whereas this value increased to 76–87% for the rest of the strategies. In order to study the matrix effect at the detection step, the chromatographic peak areas obtained for the samples spiked after the clean-up step were compared with those of the pure methanol standard solution containing target compounds at the expected concentration level (200 ng·$g^{-1}$) in the final extract. The results obtained as the ratio between the chromatographic peak areas of the different compounds in the samples versus the standard solution are summarized in Fig. 1(b). When recoveries were in the 70%–130% range, a lack of significant matrix effect was considered.

A high signal enhancement was obtained for most of the analytes, with 50% of the studied compounds showing values higher than 175% for the normal-phase SPE clean-up approach, which was, therefore, discarded. The rest of tested strategies showed better results in terms of a lack of matrix effect, showing the Captiva EMR and Captiva ND-Lipid filters the most satisfactory results. While an 88% (Captiva EMR) and a 79% (Captiva ND-Lipid) of the analytes showed no matrix effect at the detection step when the filters were used, only a 45% of the target compounds did not show it when mixed-mode SPE was performed. Concerning repeatability, a 96% of the analytes showed a relative standard deviation (RSD %) lower than 10% with the Captiva ND-Lipid filters, while this value decreased to an 86% for the Captiva EMR filters.

Taking into account all the above-mentioned, the Captiva ND-Lipid protocol was further studied in order to try to improve the results obtained for some target compounds that showed either a high matrix effect or a low efficiency. The crash-solvent nature (methanol versus acetonitrile) and the extract/crash-solvent ratio (1:3 and 1:5, v/v) were studied in a one-variable-at-a-time (OVAT) design approach. For a 95% of confidence level, there were no significant differences (*p*-value >.05) for the results obtained under the different conditions tested according to the one-way analysis of variance (ANOVA), and the

conditions proposed by the commercial report (https://www.crawfordscientific.com/media/wysiwyg//Literature/Sample_Prep/Agilent/Captiva-ND-and-ND-Lipids-Method-Guide.pdf) were further used.

### 3.2. Optimization of the focused ultrasound extraction step

The extraction time (2 and 4 min) and the extraction solvent nature were studied, while the rest of the conditions (number of on-off cycles, % of power, extraction temperature, solvent volume) were kept constant based on the previous experience of the research group (Ziarrusta et al., 2016; Ziarrusta et al., 2017; Mijangos et al., 2019). All the assays were performed in triplicate.

According to the ANOVA, no statistical differences ($p$-value >.05) were observed for the two different extraction times studied (data not shown), and, therefore, the extraction time was fixed in 2 min for the subsequent assays, resulting in a shorter processing time.

According to the literature, several extraction solvents were studied: pure acetonitrile (Baduel et al., 2015), acidified (1% HCOOH, pH = 2.0, acetonitrile (Ziarrusta et al., 2017), acidified (1% HCOOH) acetonitrile containing 0.2 g of $MgSO_4$ (pH = 4.0) (Baduel et al., 2015) and acetonitrile acidified with citrate (pH = 6.6) (Barbieri et al., 2019a; Mijangos et al., 2019). The results obtained regarding the 4 different extraction solvents are summarized in Fig. 2.

From the 180 compounds studied, 124 compounds were similarly extracted with any of the solvents used. Although 11 compounds (i.e., amiodarone, azelastine, desloratadine, dodemorph, eprosartan, fluvoxamine, memantine, nortriptyline, paroxetine, sertraline and spiroxamine) showed improved extraction recoveries in the presence of HCOOH, 43 compounds (i.e., acetochlor, alachlor, carbendazim, clonidine, glimepiride, nitrofurantoin, propamocarb, sulfadiazine, sulfapyridine, sulfathiazole or triethylphosphate, among others) were preferably extracted with pure acetonitrile. Besides, the addition of $MgSO_4$ did not improve the results in comparison with those obtained using pure acetonitrile in terms of either absolute recoveries or the number of compounds extracted. In the case of acetonitrile acidified with citrate, the results were equivalent to those obtained with pure acetonitrile. Hence, owing to the number of compounds that can be extracted with pure acetonitrile and the repeatability that can be achieved using this solvent (38% of compounds showed a RSD value lower than 10%, whereas the percentage decreased to around 8–17% of compounds in the rest of approaches).

Successive extractions (2 × 10 mL) were studied in order to improve extraction recovery and the number of compounds extracted. Two different approaches were followed. On the one hand, pure acetonitrile was used in the two successive extractions. On the other hand, pure acetonitrile extraction was followed by an extraction in acidified (HCOOH 1%, pH = 2.0) acetonitrile. The results are included in Fig. 3. An improvement (approx. a 20% in average) in extraction recovery was observed

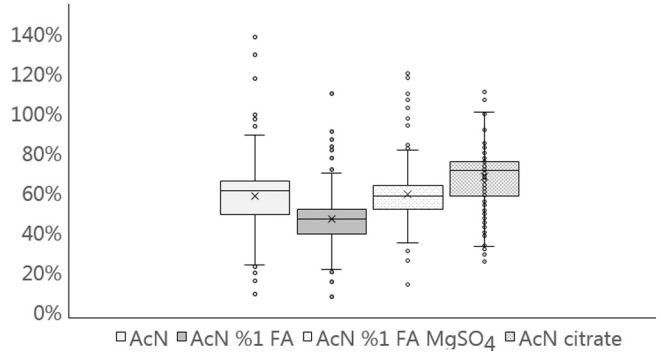


**Fig. 2.** Influence of the extraction solvent on the extraction efficiency. ACN states for acetonitrile and FA for formic acid.

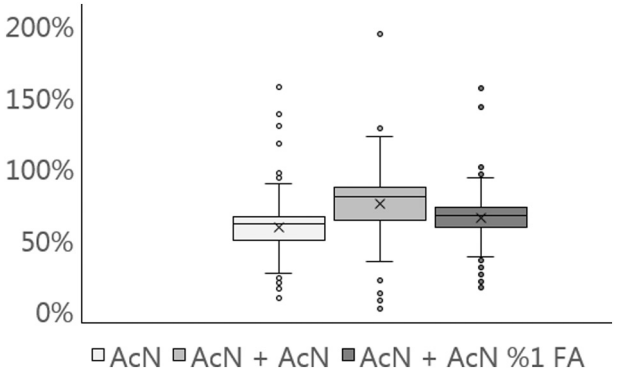


**Fig. 3.** Absolute recoveries for (a) a single extraction with acetonitrile, (b) two successive extractions with acetonitrile and (c) a single extraction with acetonitrile followed by a second extraction with acidified (HCOOH 1%, formic acid, FA) acetonitrile.

when successive extractions were performed but no improvement was observed for the number of analytes recovered. Actually, when the extracts obtained with pure and acidified (HCOOH 1%, pH = 2.0) acetonitrile were combined, the same analytes lost in the single extraction with acidified (HCOOH 1%, pH = 2.0) acetonitrile were missing, indicating a degradation of the analytes in the presence of a high concentration of HCOOH.

Taking into account the number of compounds preferably extracted and the small benefit of the performance of a second extraction, a single extraction in 10 mL of pure acetonitrile was considered for further validation.

### 3.3. Validation

a) Linearity and limits of quantification

Linearity for both external and matrix-matched calibrations are included in Table 2S in SI. $LOQ_{inst}$ and $LOQ_{proc}$ were established from the external and matrix-matched calibrations, respectively. Both $LOQ_{inst}$ and $LOQ_{proc}$ were estimated as the lowest calibration point that showed a precision (as RSD of three replicate injections) and a trueness (as the difference between the true concentration of the calibration solution and the concentration estimated from the calibration curve) lower than 30%, since the q-Orbitrap does not retrieve signal-to-noise ratios. The results obtained are included in Table 2S in SI and are expressed as the concentration in extract. The values obtained can be considered satisfactory if we take into account the large number (180) of compounds used for the validation, while similar works in the literature studied a much lower number (26) of compounds for method validation (Baduel et al., 2015; Du et al., 2017a).

b) Absolute and apparent recoveries

In the absence of certified reference materials and similar to other works in the literature (Barbieri et al., 2019a; Huerta et al., 2013; Dasenaki and Thomaidis, 2015), absolute and apparent recoveries were calculated using analyte-free halibut samples spiked at three different levels, 20 ng $g^{-1}$ (low), 100 ng $g^{-1}$ (medium) and 200 ng $g^{-1}$ (high) level, based on the linearity range, the $LOQ_{inst}$ and $LOQ_{proc}$, and the typical validation levels found in the literature (Barbieri et al., 2019a; Huerta et al., 2013; Dasenaki and Thomaidis, 2015). Analyte-free halibut aliquots were included together with the spiked samples in order to control the formation of artifacts during the extraction and clean-up steps and target analytes were not observed in those blanks. Absolute and apparent recoveries at the three spiking levels are included in Table 2S in SI.

Figs. 4(a) and 4(b) show a summary of the absolute and apparent recoveries. Absolute recoveries were in the 20%-161%, 20%

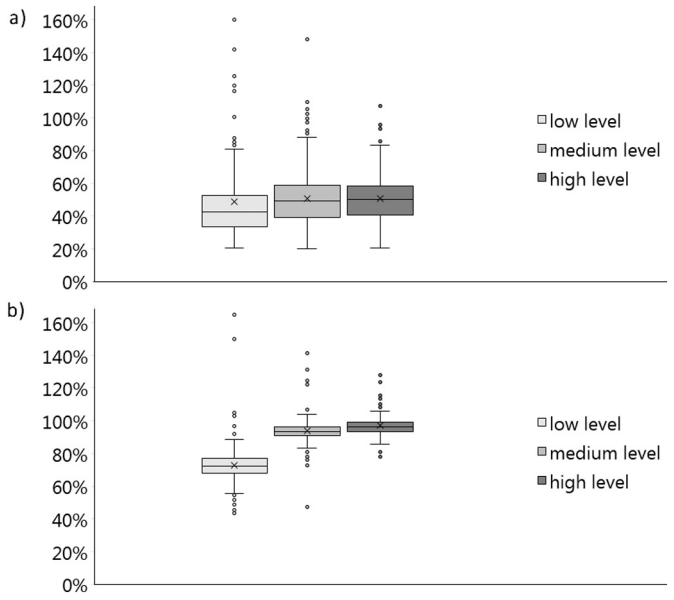


**Fig. 4.** Absolute (a) and apparent (b) recoveries.

-148% and 21%-107% ranges for the low, medium and high spiking levels, respectively, for 123 (low), 172 (medium) and 180 (high) number of compounds. The rest of the compounds showed either concentrations lower than $LOQ_{inst}$ or RSDs higher than 30% and, therefore, their absolute recoveries were not included in Table S2 in SI. As can be observed in Fig. 4(b), box plots were much narrower and all the analytes showed apparent recoveries in the 43% -105%, 73% -131% and 78%-128% range for the low, medium and high spiking levels, respectively, except for dichlorvos (at low, medium and high level) and exemestane (at medium level) when matrix-matched calibration was used. Labelled standards could also be tested in future studies as replacement of matrix-matched calibration but they should be selected carefully in order to cover such a large amount of compounds.

c) Instrumental and procedural precision

Both external ($RSD_{inst}$) and matrix-matched ($RSD_{proc}$) RSDs were estimated from the external and matrix-matched calibrations, respectively, for the three spiking levels (see Table S2). $RSD_{inst}$ in the 1% - 30%, 1% - 28% and 1% - 28% ranges were obtained for the validated compounds at the low, medium and high spiking levels, except for caffeine (34% at the low level), hexazinone (33% at the low level) and acetaminophen (32% at the medium level and 34% at the high level). Similarly, $RSD_{proc}$ values in the 1% - 30%, 1% - 17% and 2% - 24% ranges were obtained for the validated compounds at the low, medium and high spiking levels, except for PFOA (35% at the low level), benzothiazole (31% at the medium level) and acetaminophen (33% at the medium level and 34% at the high level).

d) Limits of identification

Instrumental limits of identification ($LOI_{inst}$) were defined as the lowest concentration that could be detected and unequivocally identified (Solliec et al., 2016). $LOI_{inst}$ was calculated using standard solution of pure standards in methanol in the 0.01–50 ng $g^{-1}$ range. For an unequivocal identification of the compounds a mass error of 5 ppm, an isotopic profile and fragmentation spectra fit of 70% and a ± 0.1 min error in the retention time were admitted. The values obtained using the Compound Discoverer 2.1 program are included in Table 2S as SI. For a 60% of the compounds $LOI_{inst}$ lower than 20 ng $g^{-1}$ were obtained. Again, those values can be considered satisfactory taking into account the number of validated compounds.

### *3.4. Target and suspect analysis of xenobiotics in different biological samples*

The developed method was applied to samples bought in a local market (hake, gilt-head bream, sea bass and prawn) and fished (thicklip grey mullet) at the Urdaibai estuary (Biscay, north of Spain). Both target quantification and suspect screening was performed in all the samples.

a) Target analysis

Multi-target analysis (180 compounds) validated in the present study was carried out using the TraceFinder 4.1 program. For analyte identification, 5 ppm mass accuracy both for the precursor and fragment ions, an 70% fit in the isotopic profile and fragmentation mass spectra and a shift of ±0.1 min in the retention time was allowed. The results obtained are summarized in Table 1. In the case of prawn sample, all targets were found bellow $LOQ_{proc}$.

In the three fish bought in the local market 2-hydroxybenzothiazole (anticorrosive agent) was determined. This compound has also been determined in estuarine water samples from the Basque Country (Mijangos et al., 2018). Besides, benzothiazole was determined in sea bass and gilt-head bream at ≈200 ng $g^{-1}$ concentration. In the case of the three fish collected at the Urdaibai estuary, benzothiazole was also determined in two of the thicklip grey mullets. Albendazole, mebendazole and PFOS were also detected in thicklip grey mullets from the Urdaibai estuary. Concentration for mebendazole was always lower than $LOQ_{proc}$. Albendazole was quantified at concentrations higher than $LOQ_{proc}$ in two of the fish and PFOS in only one.

b) Suspect screening

Suspect screening was performed using the Compound Discoverer 2.1 program, compounds included in the mzCloud library were used as suspect list (approx. 17,800 compounds) and the identification was performed as follows. First, only features with a Lorentzian chromatographic peak shape and a minimum peak area of $10^6$ were considered. Besides, the feature should be present in the three replicates performed for each sample and the group variance should be lower than 30%. The Compound Discoverer 2.1 program provided all the features that, according to their exact mass and isotopic profile, matched with one or several of the compounds in the suspect list. Then, fitting higher than 70% in the case of the fragmentation

**Table 1**
Concentrations (ng $g^{-1}$) found in samples bought in a local market (gilt-head bream, sea bass and hake) and fished (thicklip grey mullet) at the Urdaibai estuary with targeted analysis.

| Compound | Gilt-head bream | Sea bass | Hake | Thicklip grey mullet (1) | Thicklip grey mullet (2) | Thicklip grey mullet (3) |
|---|---|---|---|---|---|---|
| 2-hydroxybenzothiazole | 13 ± 3 | 31 ± 11 | 12 ± 2 | <$LOQ_{proc}$ | <LOQ | <$LOQ_{proc}$ |
| Benzothiazole | 200 ± 87 | 194 ± 16 | <$LOQ_{proc}$ | <$LOQ_{proc}$ | 143 ± 43 | 90 ± 21 |
| Albendazole | <$LOQ_{proc}$ | <$LOQ_{proc}$ | <$LOQ_{proc}$ | 28 ± 9 | 22 ± 6 | <$LOQ_{proc}$ |
| Mebendazole | <$LOQ_{proc}$ | <$LOQ_{proc}$ | <$LOQ_{proc}$ | <$LOQ_{proc}$ | <$LOQ_{proc}$ | <$LOQ_{proc}$ |
| PFOS | <$LOQ_{proc}$ | <$LOQ_{proc}$ | <$LOQ_{proc}$ | 3.8 ± 0.8 | <$LOQ_{proc}$ | <$LOQ_{proc}$ |

spectrum was considered. Finally, retention time was considered

**Table 2**
Annotated compound in samples bought in a local market (gilt-head bream, sea bass and hake) and fished (thicklip grey mullet) at the Urdaibai estuary with suspect screening, including the theoretical monoisotopic mass (Da), mass error (Da), proposed candidates, mzcloud library match (%), experimental retention time (min), retention time error (min) and annotation level.

| # | Theoretical monoisotopic mass (Da) | Δ mass (Da) | Molecular formula | Candidates | mz cloud | tRexp (min) | ΔTr (min) | Annot. level | Gilt head bream | Prawn | Sea bass | Hake | Thicklip grey mullet 1 | Thicklip grey mullet 2 | Thicklip grey mullet 3 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 99.06841 | 0.00022 | C5 H9 N O | 4-piperidone | 88.7 | 1.196 | 0.645 | 2a | | | | | * | * | * |
| 2 | 113.0846 | −0.00027 | C6 H11 N O | Caprolactam | 87.8 | 1.383 | −2.019 | 2a | * | * | * | * | | | |
| 3 | 129.15175 | -1E-05 | C8 H19 N | N,N-Diisoprpylethylamine (DIPEA) | 78.6 | 1.162 | −2.181 | 2a | | | | | | * | * |
| 4 | 135.01427 | 0.00016 | C7 H5 N S | Benzothiazole | 94.8 | 4.811 | 0.028 | 1 | * | * | | * | | * | * |
| 5 | 143.13101 | 3.0E-05 | C8 H17 N O | Valpromide | 90.5 | 4.507 | −2.430 | 2a | | | | * | | | |
| 6 | 158.14666 | −0.9999 | C9 H19 N O | 2,2,6,6-tetramethyl-1-piperidinol (TEMPO) | 94.9 | 6.771 | 2.400 | 2a | * | * | * | * | | | |
| 7 | 162.03169 | -9E-05 | C9 H6 O3 | 7-Hydroxycoumarine<br>4-Hydroxycoumarine | 76.2<br>75.8 | 6.682 | −0.616<br>0.008 | 3 | | | | | | * | * |
| 8 | 164.09496 | 0.00014 | C9 H12 N2 O | Fenuron | 96 | 2.951 | −2.089 | 2a | * | * | | * | | | |
| 9 | 181.00199 | −0.00011 | C8 H7 N S2 | 2-(Methylthio)benzothiazole | 86.9 | 10.024 | 0.056 | 2a | | | | | * | * | * |
| 10 | 206.16707 | −0.00076 | C14 H22 O | 2,6-di-tert-butylphenol<br>2,4-di-tert-butylphenol<br>4-tert-Octylphenol | 99.7<br>99.6<br>98.8 | 14.457 | −0.273<br>−0.273<br>0.135 | 3 | | * | * | * | * | | |
| 11 | 213.98648 | −0.00068 | C4 H F7 O2 | Perfluorobutanoic acid | 97.7 | 1.813 | 2.669 | 2a | * | * | * | | | | |
| 12 | 218.16707 | −0.0002 | C15 H22 O | Nootkatone | 77.9 | 12.448 | 2.109 | 2a | | | | | * | * | * |
| 13 | 218.18819 | 0 | C12 H26 O3 | Bis(2-butoxyethyl) ether | 97.8 | 9.046 | 0.666 | 2a | * | * | * | * | | | |
| 14 | 219.1048 | 2E-05 | C16 H13 N | N-Phenyl-2-naphthylamine<br>N-Phenyl-1-naphthylamine | 99.1<br>99.1 | 13.684 | 1.683<br>1.682 | 3 | * | * | * | * | | | |
| 15 | 222.08921 | -1E-05 | C12 H14 O4 | Diethyl phthalate<br>Monobutyl phthalate | 99.4<br>97.3 | 8.051 | −0.362<br>−0.370 | 3 | * | * | * | * | | | |
| 16 | 222.16198 | −0.00047 | C14 H22 O2 | 2,5-di-tert-butylhydroquinone | 88.4 | 11.954 | 0.046 | 2a | | | | | * | * | * |
| 17 | 229.16779 | −0.00019 | C12 H23 N O3 | Icaridin | 97.8 | 8.55 | 1.071 | 2a | | | | | | * | * |
| 18 | 237.10348 | −0.00028 | C9 H19 N O4 S | CAPSO | 72.9 | 2.513 | −1.686 | 2a | | | | | * | * | * |
| 19 | 238.14164 | −0.0001 | C10 H22 O6 | PEG n5 | 98 | 1.029 | 1.213 | 2a | * | * | * | * | | | |
| 20 | 250.17802 | -8E-05 | C12 H26 O5 | PPG n4 | 99.5 | 2.399 | −1.018 | 2a | * | * | * | * | | | |
| 21 | 265.0885 | −0.00026 | C12 H15 N3 O2 S | Albendazole | 95.5 | 6.029 | 0.029 | 1 | | | | | * | | * |
| 22 | 266.15518 | 0.00013 | C12 H26 O4 S | Dodecyl sulfate | 97.3 | 9.916 | 1.288 | 2a | | | | | | * | |
| 23 | 278.15181 | −0.00018 | C16 H22 O4 | Dibutyl phthalate<br>Diisobutyl phthalate<br>Mono(2-ethylhexyl) phthalate | 99<br>97.5<br>96.8 | 14.182 | 1.704<br>2.039<br>2.191 | 3 | * | * | * | * | | | |
| 24 | 282.16785 | −0.00023 | C12 H26 O7 | PEG n6 | 80.5 | 1.124 | 1.407 | 2a | * | * | * | * | | | |
| 25 | 294.18648 | 0.00046 | C14 H30 O4 S | Myristyl sulfate | 97.1 | 9.313 | −0.645 | 2a | * | * | * | * | | | |
| 26 | 295.09569 | −0.00026 | C16 H13 N3 O3 | Mebendazole | 86.2 | 6.389 | 0.029 | 1 | | | | | * | | * |
| 27 | 325.29808 | 0.07235 | C22 H47 N | Didecyldimethylammonium | 88.8 | 12.14 | 1.376 | 2a | | | | | * | * | * |
| 28 | 330.2096 | −0.00021 | C23 H26 N2 | Leucomalachite green | 86.1 | 8.402 | 0.438 | 2a | | | | | * | | |
| 29 | 414.20424 | −0.00018 | C24 H30 O6 | Bis(4-ethylbenzylidene)sorbitol | 98.3 | 9.324 | −2.919 | 2a | * | * | * | * | | | |
| 30 | 499.93749 | −466.93749 | C8 H F17 O3 S | Perfluorooctanesulfonic acid | 99.1 | 10.942 | 0.052 | 1 | | | | | * | * | * |

before confirmation: (i) When the pure standard was available, a deviation of ±0.1 min was admitted. (ii) When the pure standard was not available, an estimation of the theoretical retention time was performed based on the partition coefficient of the suspect compound at either pH = 3.0 (for compounds detected in the positive mode) or at pH = 7.0 (compounds detected in the negative mode), using a linear regression model that correlates the partition coefficient of the compounds and the experimental retention times of 217 compounds available at the laboratory. In the latter case, a ± 3 min window was admitted for positive identification. The compounds identified and their annotation level (Schymanski et al., 2014b) are included in Table 2. Figures 1S–5S in SI show the overlaid extracted ion chromatograms (EICs) for gilt head bream, hake, prawn, sea bass and thicklip grey mullet, respectively, for positive (a) and negative (b) ionization modes.

Up to date few studies report pharmaceuticals and PPCPs detected with suspect analysis approaches in biological matrixes, particularly in fish fillet (Baduel et al., 2015; Dwiyitno et al., 2015; Du et al., 2017b). Those few analyses include insecticides such as fipronil and DEET, pharmaceuticals like poloxalene, paramethanose or morforex, and different families of human products such as caffeine or cotinine, among many others. Those are of the same use profile and similar physicochemical characteristics as the ones identified here. Compounds found in this study and similar products have been targeted in previous reports, such as plastic related products (caprolactam, phthalates, polyethylenglycols) (Du et al., 2017b; Álvarez-Muñoz et al., 2015), pharmaceutical products (albendazole, mebendazole, valpromide) (Pereira Lopes et al., 2012; Zhang et al., 2011; Turnipseed et al., 2018) and pesticides or insects repellents (icaridin, myristyl sulfate, nootkatone) (Yao et al., 2016; Barbieri et al., 2019b).

## 4. Conclusions

A multitarget method able to determine up to 180 priority and emerging compounds in fish muscle was developed for the first time based on a FUSLE in cold acetonitrile and a clean-up using Captiva ND-Lipid filters. From the different solvents studied, pure acetonitrile and acidified (1% HCOOH) acetonitrile showed the largest differences in the compounds extracted, with 11 compounds showing improved extraction in acidified (1% HCOOH, pH = 2.0) acetonitrile and 43 in pure acetonitrile. Since successive extractions in pure acetonitrile followed by acidified (1% HCOOH, pH = 2.0) acetonitrile did not improve the results, a single extraction in pure cold acetonitrile was chosen as a consensus. In the case of the 4 tested clean-up techniques, the use of Captiva filters rendered improved results in terms of reduced matrix effect and higher precision compared with normal-phase and mixed-mode SPE. The absolute recoveries obtained were successfully improved using a matrix-matched calibration approach, but the use of labelled surrogate standards should be further studied in future works. The developed method was applied for both multitarget quantification and suspect screening of xenobiotics in fishery samples from a local market and the Urdaibai estuary and allowed the quantification of few compounds at ng $g^{-1}$ levels in real samples, such as food additives, antiparasitic drugs and PFOS. Moreover, suspect analysis revealed the presence of plastic related products (caprolactam, phthalates, polyethylenglicols), pharmaceutical products (albendazole, mebendazole, valpromide) and pesticides or insects repellents (icaridin, myristyl sulfate, nootkatone).

## CRediT authorship contribution statement

**M. Musatadi:** Investigation, Formal analysis, Writing - original draft. **B. González-Gaya:** Investigation, Formal analysis, Visualization. **M. Irazola:** Investigation, Formal analysis. **A. Prieto:** Formal analysis, Methodology, Visualization. **N. Etxebarria:** Supervision, Resources, Funding acquisition. **M. Olivares:** Supervision, Methodology, Conceptualization, Formal analysis, Visualization. **O. Zuloaga:** Supervision, Formal analysis, Writing - review & editing.

## Declaration of competing interest

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

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2020.139894.

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