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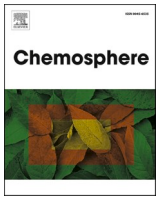

Optimization of the ultrasound-assisted extraction method for determining high production volume chemicals in fish liver and skin samples

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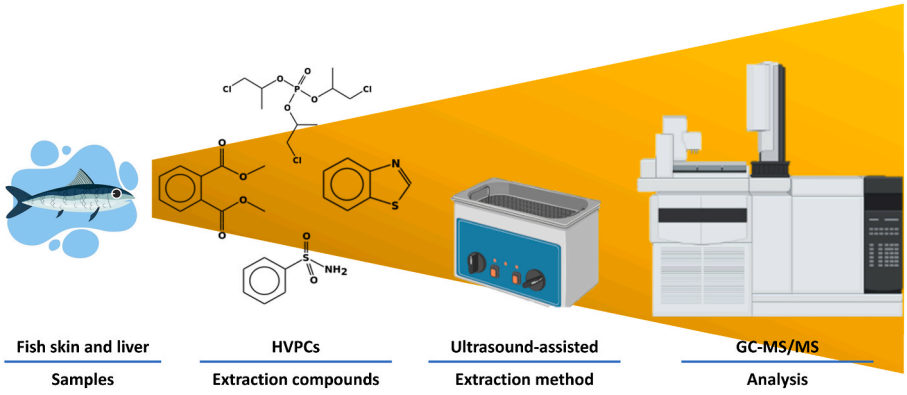
HIGHLIGHTS

- HPVC have been determined in fish and liver samples.
- A method based on ultrasound-assisted extraction was optimized.
- Most HPVC were present in the samples.
- Benzothiazoles and benzosulfonamides determined for the first time in fish and liver.

GRAPHICAL ABSTRACT



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ABSTRACT

The aquatic ecosystem is one of the most delicate environments, housing a diverse range of organisms, including fish, all of which are exposed to a wide variety of pollutants. The accumulation of these harmful substances in fish, which are part of the human diet, presents a significant health risk to humans. In our study, we have optimized an extraction technique to determine the presence of 25 high production volume chemicals in liver and skin samples taken from commonly consumed fish species. We have employed ultrasound-assisted extraction in conjunction with gas chromatography tandem mass spectrometry to achieve this goal. Apparent recoveries of the method ranged from 50% to 111% for both sample types with some exceptions such as most of the benzosulfonamides and benzothiazole. Additionally, the method's detection and quantification limits varied from 0.1 to 1.7 ng g^{-1} (dry weight, d.w) and 0.2–4.5 ng g^{-1} (d.w), respectively. Our investigation focused on three frequently consumed fish species in Tarragona: sea bass, sea bream, and turbot. Almost all of the samples we analysed contained traces of contaminants, with phthalates being the most commonly detected. The highest concentrations were observed for diethyl phthalate, with levels peaking at 8350 ng g^{-1} (d.w.). Organophosphate esters, such as triethyl phosphate and tributyl phosphate, also showed notable presence, with peak concentrations of 93.6 and 34.0 ng g^{-1} (d.w.), respectively.

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1. Introduction

Research on contaminants in biota, particularity fish, has garnered increasing attention due to potential health risks tied to their consumption. Most investigations focus on the hazards linked to consuming fish flesh (Castro et al., 2020a, 2020b; Jia et al., 2019), with only a limited number delving into the accumulation of chemicals in various organs. Among these studies, the liver typically exhibits the highest concentrations, followed by the kidney, muscle, intestine, and ovaries (Hou et al., 2017). The liver and intestines play crucial roles in the digestion and absorption of nutrients, making them essential for understanding the bioaccumulation process in fish. Additionally, these studies help assess the overall quality of the aquatic environment (Raskovic et al., 2011). Furthermore, specific studies (Rakers et al., 2010) explore fish skin as a versatile tissue with essential functions, including chemical protection and physical sensory activities. Moreover, it serves as a primary defence barrier against pathogens, offering insights into exposure to environmental contaminants.

Research on chemicals in various fish species remains limited. It is imperative to consider the tissue-specific distribution of contaminants since they initially accumulate in the bloodstream and are subsequently transported to different tissues based on their characteristics (Kojadinovic et al., 2007). Factors such as fish growth patterns, lipid content, dietary habits, the aquatic environment, seasonal variations, trophic levels, gender, size, and resorption rate significantly influence contaminant accumulation (Altindağ and Yiğit, 2005; Yilmaz and Yilmaz, 2007). Typically, at the top of the food chain are expected to accumulate more contaminants through bioaccumulation compared to those lower down the food chain. Benthic fish species tend to contain higher levels of contaminants than pelagic species, as contaminants tend to accumulate more in sediments and detritus (Kalantzi et al., 2013).

Many contaminants find their way into water bodies, particularly High Production Volume Chemicals (HPVC), which pose a significant concern due to their widespread use in both industrial and household products, thereby impacting aquatic organisms. Organophosphate esters (OPEs), benzothiazoles (BTHs), benzosulfonamides (BSAs), and phthalates (PAEs) are among the HPVC produced in quantities exceeding 1000 tons annually (Organisation for economic co-operation and development, 2004). In recent years, organophosphate esters have gained popularity as substitutes for brominated flame retardants (BFRs) and plasticizers in applications like furniture, electronics, textiles, primarily for fire prevention or enhanced plasticity. Benzothiazoles and benzosulfonamides have diverse industrial and consumer product uses, including vulcanization accelerators, dyes, biocides, and pharmaceutical synthesis (Trabalón et al., 2017). Phthalates, the most widely used plasticizers globally (Wittassek et al., 2011), are prevalent in various applications such as toys, food packaging, and inks. Many of these contaminants ultimately find their way into the sea, either directly from industries or their products (Gao and Wen, 2016). In the sea, contaminants come into contact with biota like fish, posing a significant environmental problem due to their presence, toxicity, and tendency to accumulate through respiration, adsorption, and ingestion (Castro et al., 2020a). The presence and accumulation of these contaminants in biota pose a health risk to humans (Organisation for economic co-operation and development, 2004), as their consumption can lead to severe health issues, including carcinogenic and endocrine-disrupting effects.

Numerous studies have explored contaminants in various parts of fish (Valton et al., 2014; Petersen et al., 2011; Mcgoldrick et al., 2014), yet few address processes such as bioaccumulation (Kim et al., 2011), and there is limited information on skin and liver (Kaczyński et al., 2017; Souza Caldas et al., 2013; Choo et al., 2018). In crucian carp, OPEs have been detected, with the highest levels identified in the liver (Choo et al., 2018). The combined concentrations of nine OPEs ranged from 6.22 to 18.1 ng g^{-1} (wet weight, w.w.) in the liver, 4.23–7.75 ng g^{-1} (w.w.) in muscle, and 3.08–7.70 ng g^{-1} (w.w.) in gonads. Roach liver collected from the Orge River in France exhibited the highest concentrations of PAEs (Valton et al., 2014):, diethyl phthalate (DEP) 2001 ± 2400 ng g^{-1} (d.w.), isobutyl phthalate (DiBP) 2126 ± 4099 ng g^{-1} (d.w.), di-(2-ethylhexyl)-phthalate (DEHP) 3052 ± 3854 ng g^{-1} (d.w.), di-n-octyl phthalate (DnOP) 653 ± 1285 ng g^{-1} (d.w.) and dimethyl phthalate (DMP) 386 ± 667 ng g^{-1} (dry weight, d.w.)

While these compounds have been identified through chromatographic techniques in various environmental samples like water and air, their accurate and precise determination in biological samples remains challenging. Gas chromatography coupled with tandem mass spectrometry (GC-MS/MS) has demonstrated excellent sensitivity and specificity for these chemical determinations. However, matrix interference often complicates trace determination in lipid-rich biological samples (Castro et al., 2020a; Trabalón et al., 2017).

Several extraction techniques are available for analysing organic contaminants in fish, including solid-liquid extraction (SLE) (Qu et al., 2017), ultrasound-assisted extraction (USAE) (Qu et al., 2017; Lorenzo et al., 2018), matrix solid-phase dispersion (MSPD) (Souza Caldas et al., 2013), microwave-assisted extraction (MAE) (Batista et al., 2001), pressured liquid extraction (Hidalgo-Serrano et al., 2020), and QuEChERS (Quick, Easy, Cheap, Effective, Rigged, and Safe) (Castro et al., 2020a; Hidalgo-Serrano et al., 2021).

After the extraction process, a purification step becomes necessary due to the matrix's complexity. One of the most commonly employed clean-up methods involves solid-phase extraction (SPE). For instance, Choo et al. (Gao and Wen, 2016) utilized these techniques to detect OPEs in fish skin and liver samples. Oasis HLB cartridges were used to identify PAEs in fish liver and muscle samples using USAE, followed by purification with solid-phase extraction (SPE) employing Florisil cartridges (Valton et al., 2014). Certain researchers have employed dispersive SPE (d-SPE), such as Hidalgo et al. (Hidalgo-Serrano et al., 2021), who analysed fish muscle samples via QuEChERS using d-SPE with C_{18} as a cleaning technique. For tissue analysis, many authors use primary secondary amine sorbent (PSA); however, other sorbents like ENVI-Cab, Discovery DSC-18, Z-Sep, and HLB-SPE have also found application (Chu and Letcher, 2015). An alternative method gaining popularity today is the cleanup using the LipiFiltr® cartridge (Castro et al., 2020a), which specifically targets lipid elimination and is widely used in food analysis. Various authors have also proposed lipid removal through freezing to eliminate substantial lipid content (Kaczyński et al., 2017; Hidalgo-Serrano et al., 2021; Ahn et al., 2006). For instance, this approach has been applied in the determination of chlorinated pesticides, polychlorinated dibenzo-p-dioxins, and furans in fish samples (Ahn et al., 2006).

The primary objective of this research is to pioneer an ultrasound-assisted extraction method for the determination of 25 high production volume chemicals in fish liver and skin samples. The HPVCs will be determined by gas chromatography-tandem mass spectrometry (GC-MS/MS). Multiple fish species will be assessed to certain the presence of HVPCs.

2. Materials and methods

2.1. Chemicals and reagents

The following organophosphate esters (OPEs) were determined: tri-iso-butyl phosphate (TiBP), triphenyl phosphate (TPP), triethyl phosphate (TEP), tributyl phosphate (TBP), 2-ethylhexyl-diphenyl-phosphate (EHDPP), tris (2-chloroethyl)-phosphate (TCEP), tris (2-ethylhexyl)-phosphate (TEHP), tritolyl phosphate (TTP) and tris (2-chloroisopropyl) phosphate (TCPP). Tributyl phosphate deuterated ($TBP\text{-}d_{27}$) was used as the corresponding internal standard. The following phthalic acid esters (PAEs) were determined: di-n-octyl phthalate (DnOP), di-(2-ethylhexyl)-phthalate (DEHP), isobutyl phthalate (DiBP), di-(2-ethylhexyl)-adipate (DEHA), diethyl phthalate (DEP) and dimethyl phthalate (DMP). And di-(2-ethylhexyl) phthalate deuterated (DEHP-d4) was used as the corresponding internal standard.

The following benzosulfonamides (BSAs) were determined: para-toluesulfonamide (p-TSA), ortho-toluesulfonamide (o-TSA), N-methyl-para-toluesulfonamide (Me-p-TSA), benzosulfonamide (BSA) and N-ethyl-para-toluesulfonamide (Et-p-TSA). We used ortho-toluesulfonamide deuterated (TSA-p-d4) as the corresponding internal standard. The following benzothiazoles (BTHs) were determined: 2-aminobenzothiazole (NH_2BT), 2-(methylthio)-benzothiazole (MeSBT), 2-hydroxybenzothiazole (OHBT), benzothiazole (BT) and 5-chlor-1-benzothiazole (ClBT). Benzothiazole deuterate ($BT\text{-}d_{27}$) was used as the corresponding internal standard. All the standards were sourced from Sigma Aldrich (Sant Louis, USA).

We sourced ultrapure water from a Synergy purification System manufactured by Millipore (Massachusetts, USA). Ethyl acetate, acetonitrile and acetone were used with a purity exceeding 99.9%, were provided by J.T. Baker Chemicals (Deventer, The Netherlands). The QuEChERS (Standard Method Originals OR) extraction kit was obtained from Sharlab S.L. (Barcelona, Spain), LipiFiltr® extraction filters were obtained by Carlo Erba Reagents (Barcelona, Spain) and Florisil was obtained by Sigma Aldrich (Sant Louis, USA). Helium and nitrogen gas purity from the GC-MS/MS instrument were 99.999% from Carburos Metálicos (Barcelona, Spain).

2.2. Sample preparation

Three different fish species were analysed: sea bass (*Dicentrarchus labrax*), turbot (*Scophthalmus rhombus*) and sea bream (*Sparus aurata*). These species were selected due to their popularity among the local population as commonly consumed fish. Fish of these three species of Mediterranean origin were bought from the central market in Tarragona (de Agricultura and Alimentación, 2021). The skin and liver were separated and frozen at −24 °C. Frozen samples were lyophilized in the miVac Duo system from Genevac (Ipswich, United Kingdom). The resulting samples were then crushed and homogenized into a fine powder.

2.3. Sample extraction and clean-up

Due to the ubiquity of these compounds, we tool several preventive measures in laboratory to reduce samples contamination. Whenever possible, glass material was used to avoid plastic-related contamination. All material was also washed in an isopropanol ultrasonic bath for 30 min before use. During each sampling sequence, a procedural blank (extraction without sample) was used to observed whether contaminants were present during the extraction methods.

2.3.1. Ultrasound-assisted extraction method

For the liver and skin samples, 5 mL of ACN was combined with 0.1 g of each sample, followed by sonication in a Branson 2510 ultrasonic cleaner (Darmstadt, Germany) for 45 min. The resultant mixture was then frozen at −24 °C for 40 min. The supernatant was taken up with a glass pipette and passed thought a 0.22 μm PTFE filter connected in series with a LipiFiltr® cleaning cartridge using a 10 mL syringe. The extract was gathered in a 20 mL vial and subjected to evaporation under nitrogen gas to approximately one drop in volume. Ultimately, 100 μL of a 1 ppm internal standard solution was added to the content, and the volume was adjusted to 2 mL using ethyl acetate.

2.3.2. QuEChERS method

The USAE method was compared with a QuEChERS method based on a previously developed method (Castro et al., 2020a). Briefly, 0.1 g of freeze-dried sample was placed in a 50 mL centrifuge tube. Subsequently, 10 mL of ultrapure water and 10 mL of acetonitrile were included. The mixture was agitated for 1 min in a vortex. QuEChERS (Standard Method Originals OR) reagents comprising 4 g magnesium sulphate and 1 g sodium chloride were introduced and agitated for 3 min. Subsequently, they were subjected to centrifugation at 4000 revolutions per minute for 5 min using the Hettich Universal 32R centrifuge (Tuttlingen, Germany). The liquid supernatant was removed with a glass pipette and passed through a LipiFiltr® cartridge employing a 10 mL syringe. This was then transferred to 20 mL vials and evaporated under nitrogen gas until nearly dry. Following these, 100 μL of a 1 ppm internal standard solution was added for a final concentration of 50 μg L^{-1}. The sample was reconstituted with ethyl acetate to a final volume of 2 mL by previously passing it through a 0.22 μm PTFE filter.

2.4. Gas chromatography-mass spectrometry in tandem

The GC-MS/MS parameters were adapted from a previous study (Castro et al., 2020a). An Agilent 7890A Gas Chromatograph system, connected to a triple quadrupole mass spectrometer supplied by Agilent Technologies (California, USA). The instrument employed in conjunction with a ZB-50 column (30 m × 0.25 mm i.d. x 0.25 μm film thickness) from Phenomenex (California, USA). The temperature for the oven initiated at 50 °C for 3 min, then increased at a rate of 30 °C/min until reaching 300 °C, where it held form 2 min. Helium served as the carrier gas at a steady flow rate of 1.2 mL/min. The injection was 25 μL using a high-volume injection (Agilent multimode input MMI) in solvent ventilation mode, commencing at an initial temperature of 55 °C for 0.41 min and then ramping up to 350 °C at 600 °C/min for 5 min. The total time analysis was 30 min with a solvent delay of 3 min. The triple quadrupole mass spectrometer functioned in electron ionization mode at 70 eV, with the following temperature settings for the ionization source, quadrupole 1 and quadrupole 2: 280 °C, 150 °C and 150 °C, respectively.

Tandem mass spectrometry (MS/MS) was employed to identify target compounds in MRM mode, utilizing one quantifier transition and two qualifying transitions for each compound. A comprehensive summary of all MS/MS parameters can be found in Table S1 in the Supplementary Material. Quantification was carried out via internal standard calibration.

3. Results and discussion

3.1. Ultrasound-assisted extraction

The first with the initial USAE conditions taken from Ahn et al. (2007), yielded low recoveries due to the high matrix effect. Therefore, before optimizing the USAE conditions, such as solvent and analysis time, we optimized the cleaning step because of the complexity of the matrix. The clean-up step was very important for reducing interfering compounds, especially lipids. Two cleaning methods were tested: a cleaning with Florisil as detailed in Ahn et al. (2007), and a cleaning with LipiFiltr® cartridges, which are very suitable for lipid removal (Castro et al., 2020a; Hidalgo-Serrano et al., 2021). Florisil or magnesium metasilicate ($MgSiO_3$) is an adsorbent widely used for sample preparation in chromatographic analysis and for clean-up, compound isolation and purification (Herrero et al., 2013; Nácher-Mestre et al., 2010). We evaluated LipiFiltr® cartridges as an alternative for the clean-up step, as good results have been obtained in previous studies with fish muscle samples (Castro et al., 2020a; Hidalgo-Serrano et al., 2021). LipiFiltr® is a simple one-step tool that traps unwanted lipids and fats, leading to a purified extract suitable for chromatographic analysis. There is no data available on the composition of the abasement that encapsulated in the LipiFiltr®.

To facilitate results comparison, we calculated the apparent recoveries (R_{app}) of the method employing both cleaning steps. The R_{app} was calculated by taking into account the signal of spiked samples with the signal of an un-spiked samples. To optimize the clean-up and extraction process, 0.1 g of liver or skin was spiked at 2000 ng g^{-1} (d.w.). To achieve this, 5 mL of MeOH was introduced and subjected to 30 min of sonication. The resulting extract was cooled to −24 °C to eliminate lipids, and the supernatant underwent further purification via clean-up method that included supernatant filtration. The analysis was

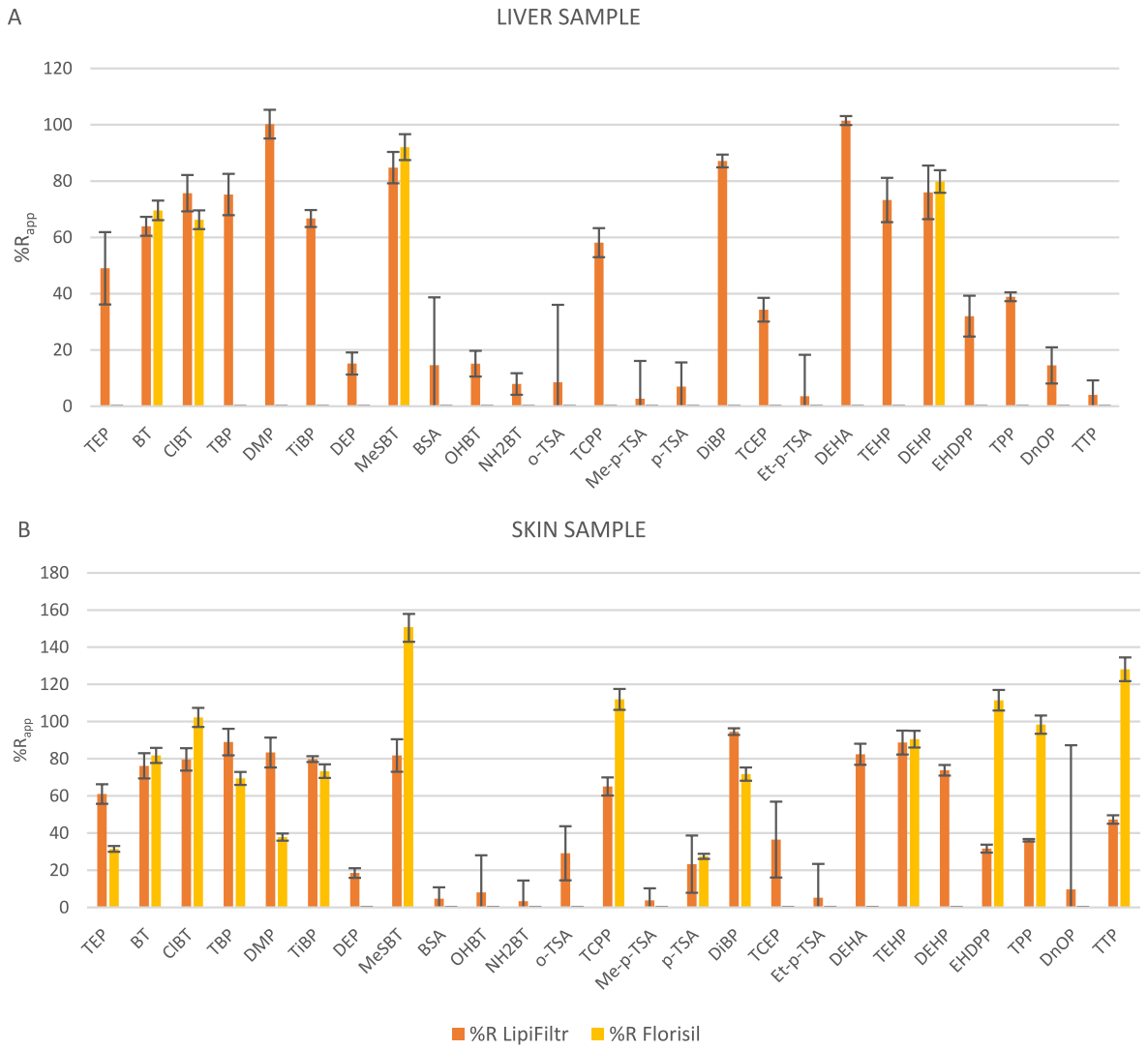

Fig. 1. Apparent recoveries (%) of the compounds when different clean-up strategies were applied to liver (A) and skin (B) samples.

conducted in triplicate for each clean-up approach applied to both skin and liver samples, with the R_{app} results presented in Fig. 1. In all tests, non-spiked fish liver and skin samples were analysed to subtract the response of the compounds present in the samples.

As illustrated in Fig. 1A, using Florisil, low R_{app} were obtained for all compounds except BT, ClBT, MeSBT and DEHP in liver samples. In skin samples, Fig. 1B, the R_{app} obtained were generally better than in liver samples, except for some compounds. Although Florisil gave good results in the analysis of the edible part of the fish in some studies (Ahn et al., 2007; Nácher-Mestre et al., 2010), in the samples of the present study (skin and liver), these compounds did not show good recoveries.

Using LipiFiltr®, better R_{app} was obtained for most compounds, between 32 and 100%, except for the benzosulfonamides and for NH_2BT, OHBT, DnOP and DEP, which were below 15%. Equal results were obtained for both liver and skin samples, except for the TTP, with low R_{app}, less than 5% in the liver samples. Notably, the R_{app} values obtained with LipiFiltr® clean-up method were higher compared to those obtained with Florisil. Thus, LipiFiltr® was selected as the optimal clean-up method. It should be noted that benzosulfonamides, benzothiazoles and TTP recoveries are low and need to be optimized.

3.1.1. Extraction solvent

Once the cleaning step had been optimized, we then optimized the USAE method parameters, like the type of solvent and the sonication time in order to increase the low R_{app} of some compounds. The initial method was implemented using methanol, and to improve the R_{app} two additional solvents were evaluated: ethyl acetate and acetonitrile. Two solvents of different polarities to methanol were selected to cover a wider range of solvent polarity.

A total of 5 mL of ACN or EtAc was introduced and subjected to 30 min of sonication. The sample was then cooled to −24 °C, and the resulting supernatant was filtered through a 0.22 μm PTFE filter and passed thought a LipiFiltr® cartridge. The extraction was executed in triplicate, and the corresponding R_{app} results are illustrated in Fig. 2.

Using ethyl acetate, high R_{app} was obtained for liver samples (Fig. 2A) for most of the compounds except for most of the benzosulfonamides and DEP, OHBT, NH_2BT, DEHA, DEHP and TTP. Results in the skin samples were notably similar (Fig. 2B). However, the R_{app} did not improve for the compounds that showed low R_{app} with methanol, such as benzothiazoles and benzosulfonamides.

Using acetonitrile, higher R_{app} were obtained for benzosulfonamides and benzothiazoles compared to using ethyl acetate or methanol and similar for other compounds. R_{app} of 23–111% for liver and 22–88% for skin were observed, except o-TSA, for which a R_{app} of only 18% was achieved. Therefore, acetonitrile was selected as extraction solvent due to its higher R_{app} for most compounds. Higher R_{app} were also observed for benzosulfonamides and benzothiazoles although the values obtained cannot be considered good enough.

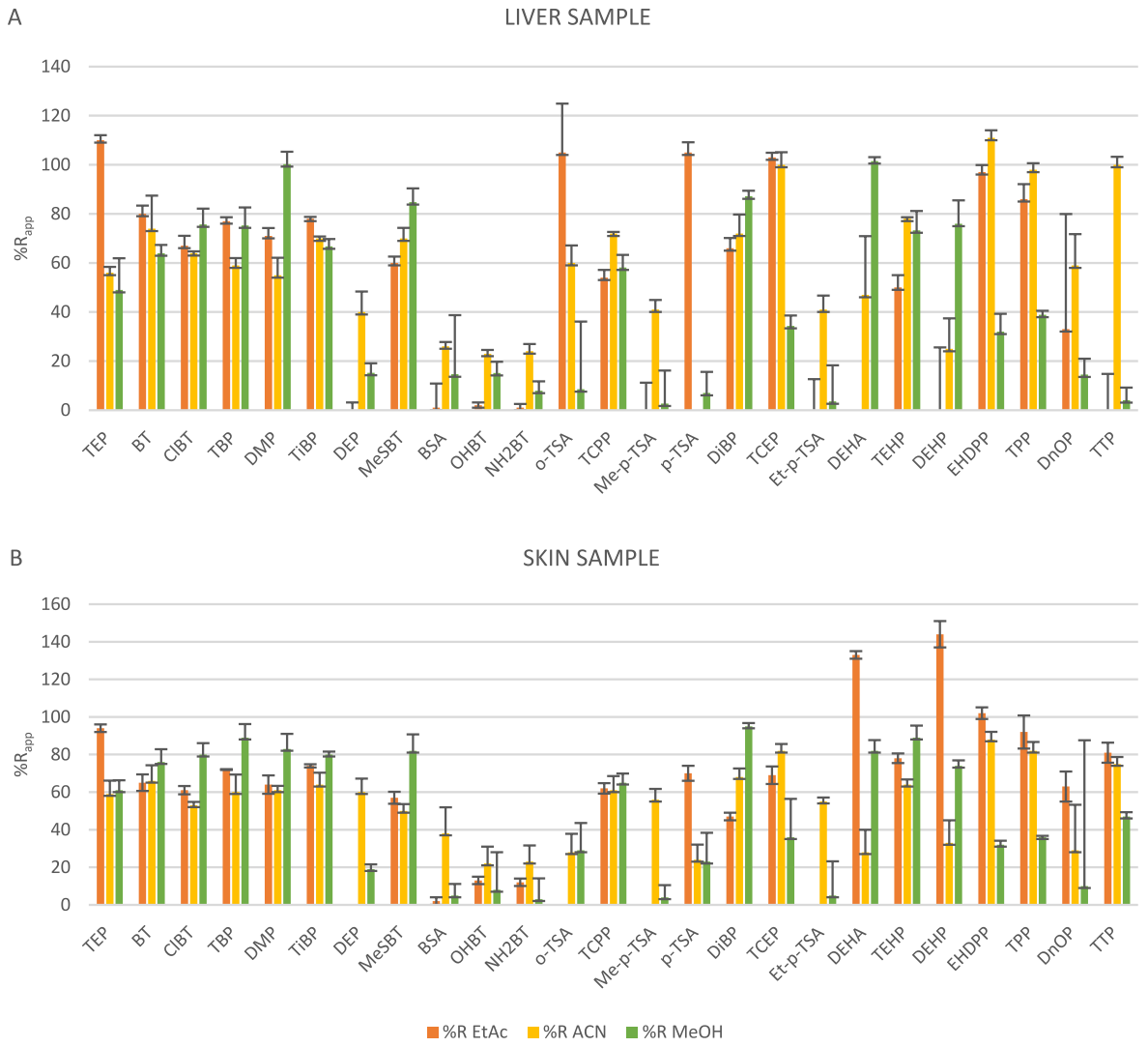

Fig. 2. Apparent recoveries (%) of the HVPC in liver (A) and skin (B) samples using different solvents.

Several authors use sonication extraction for the determination of OPEs in biota, with the difference being the extraction solvent. Some use acetone: hexane (1:1, v/v) as extraction solvent with recoveries between 48 and 102% (Sala et al., 2019) and others use ACN as extraction solvent with recoveries between 46 and 112% (Zheng et al., 2020). Other authors, on the other hand, use dichloromethane in Soxhlet as an extraction method with recoveries between 56 and 108%, also in biota samples (Liu et al., 2019). Both recoveries are very similar and also with those calculated in this article.

Other compounds, such as benzothiazoles and benzosulfonamides are less studied and not much information can be found in previous studies for comparison.

3.1.2. Sonication time

The sonication time was optimized. It was initially 30 min and was increased to 45 min to try to obtain better R_{app}, also reduced to 15 min to optimize the extraction time. An amount of 0.1 g was added to the extraction solvent (ACN) and left for 15 min, 30 min and 45 min in the ultrasonic bath. Subsequently, an identical procedure to that outlined in Section 3.1.1 was executed.

Using 15 min, good R_{app} were obtained except for the compounds OHBT and NH_2BT, which were less than 10% for both sample types. These results were not as high as those obtained using 30 min of sonication. In general, higher R_{app} were obtained in 45 min especially for the compounds DEP, OHBT and NH_2BT. A sonication duration of 45 min was selected as it showed a small increase in R_{app} without significantly extending the analysis time. The optimization was satisfactory for the most of compounds, but it is worth noting the high complexity of the matrix analysed.

3.2. Method validation

The USAE method underwent validation encompassing aspects such as the linear range, apparent recovery, method limit of quantification, method detection limit and repeatability. The instrumental linear range was 0.01–250 μg L^{-1}, with good linearity (R^2>0.999) for most compounds. The R_{app} was then determined experimentally by adding 1000 ng g^{-1} (n = 5) to 0.1 g of skin and liver samples. For the compounds found in the sample, the concentration of the compounds in the original sample were taken into account in the calculations.

The %ME (n = 5) values were calculated by spiking 1000 ng g^{-1} (d.w) in sample extracts and reconstituting the extract to 2 mL, then comparing the response of the analytes with those of a standard solution. A positive ME value means enhancement of the signal with a negative means suppression.

Method detection limits of (MDL) and method quantification limits (MQL) for each compound were determined considering their instrumental detection (S/N = 3) and quantification limits (the first

Table 1
Quality parameters of the method.

Compounds	*%R_{app} (n = 5)*		*Repeatability (%RSD) (n = 5)*		*%ME (n = 5)*		*MQL ($ng{\cdot}g^{-1}$)(d.w.)*	*MDL ($ng{\cdot}g^{-1}$)(d.w.)*
	skin	*liver*	*skin*	*liver*	*skin*	*liver*	*liver*	*liver*
TEP	59	56	11	3	28	45	0.8	0.2
BT	66	74	10	9	−2	−5	0.8	0.3
ClBT	53	64	10	4	3	5	0.4	0.2
TBP	60	59	7	1	−15	−7	0.8	0.2
DMP	61	55	9	5	−13	−8	0.3	0.2
TiBP	64	70	4	2	−7	−1	0.8	0.2
DEP	60	40	6	6	−1	−3	0.3	0.2
MeSBT	50	70	12	7	−8	−7	0.4	0.2
BSA	38	26	10	8	9	−46	1.3	0.3
OHBT	22	23	18	16	−12	−2	4.5	0.7
NH_2BT	23	24	17	18	−9	−2	2.2	0.7
o-TSA	28	60	9	15	31	16	35.7	17.9
TCPP	61	72	5	4	24	2	0.8	0.2
Me-p-TSA	56	41	7	1	−50	−50	0.9	0.2
p-TSA	24	18	18	4	−31	−49	2.1	0.4
DiBP	68	72	10	3	−16	−14	0.3	0.1
TCEP	82	100	3	6	4	6	0.2	0.1
Et-p-TSA	55	41	9	0	−43	−1	0.9	0.2
DEHA	28	47	20	19	47	−13	0.7	0.4
TEHP	64	78	3	4	−21	−19	0.3	0.2
DEHP	33	25	40	38	−2	−1	0.6	0.3
EHDPP	88	111	6	7	56	39	0.2	0.1
TPP	82	98	5	6	40	27	0.6	0.1
DnOP	29	59	20	4	16	14	1.7	0.7
TTP	75	100	10	7	25	25	0.7	0.1

Table 2
Apparent recovery (%) for the QuEChERS method.

Compounds	%R_{app} (n = 5)	
	skin	liver
TEP	45	53
BT	29	52
ClBT	82	140
TBP	84	68
DMP	69	73
TiBP	81	90
DEP	18	13
MeSBT	75	102
BSA	19	14
OHBT	9	2
NH_2BT	3	2
o-TSA	7	–
TCPP	74	90
Me-p-TSA	3	21
p-TSA	8	46
DiBP	117	101
TCEP	83	111
Et-p-TSA	4	26
DEHA	48	80
TEHP	100	75
DEHP	58	52
EHDPP	45	43
TPP	41	38
DnOP	–	106
TTP	46	39

%RSD (n = 5) below 22% for liver and below 20% for skin samples.

concentration within the instrumental linear range), respectively. These calculations took into consideration the sample treatment and the R_{app} values. Repeatability (n = 5) was also assessed and expressed as the relative standard derivation (%RSD).

Table 1 illustrates that satisfactory R_{app} values for most of the compounds were obtained for each matrix ranging from 55 to 111% for liver and from 50 to 89% for skin with some exceptions such as benzosulfonamides that had low R_{app}. The liver sample is a more complex matrix than the skin sample, so it obtained slightly different results between matrices. Although low recoveries were obtained for a few compounds, it should be taken into account the high number of compounds and the complexity of the samples. The MQL and MDL were between 0.2 and 4.5 ng g^{-1} (d.w) and between 0.1 and 1.7 ng g^{-1} (d.w) respectively, except for o-TSA. Repeatability was always lower than 20% (%RSD) for skin and liver samples, except for the compound DEHP. A %ME of between −50 and 56% was obtained for all compounds.

3.3. Comparison with a QuEChERS extraction

The results obtained from USAE method were compared with those of a QuEChERS method, as detailed in Section 2.3.2, which had previously been applied to the same compounds in fish muscle samples. Table 2 shows the results of the R_{app} for the QuEChERS method. The R_{app} for the QuEChERS method obtained good values for most of the compounds, except for the benzosulfonamides and the compounds OHBT and NH_2BT in liver and skin samples, the same that showed low R_{app} using USAE. It was also observed that o-TSA in the liver samples and DnOP in the skin samples were not extracted. For these compounds, the R_{app} using QuEChERS were below 9% for both sample types. For example, the compounds OHBT and NH_2BT had a R_{app} of 9% and 3% in skin and 2% in liver, while with the USAE method R_{app} values were around 23%. Therefore, using USAE the results showed an improvement in the R_{app} of these compounds. Another example of improvement using USAE was found for benzosulfonamides such as o-TSA and Me-p-TSA. These compounds using QuEChERS showed values of 7% and 3% in skin and 21% for Me-p-TSA in liver (o-TSA was not extracted in liver as we commented). When using USAE method, the R_{app} for o-TSA and Me-p-TSA were higher in both skin (28 and 56%, respectively) and liver samples (60 and 41%, respectively).

Fig. 3 depicts a comparison of the matrix effect (%ME) results between the two methods. Only the results of the %ME in the liver samples are displayed, as the skin samples results exhibited as similar trend. The %ME results for the liver samples using the QuEChERS method were between −28 and 43% except for the compounds TEP, OHBT and NH_2BT, which were 77, -82 and −79%, respectively. Using the USAE method the %ME is much lower for most of the compounds, especially for the compounds OHBT and NH_2BT. The results range between −50 and 56% for all compounds. The USAE method is preferred as it has a lower %ME and higher R_{app}. The USAE method allows us to extract most

Fig. 3. Matrix effect (%) of the HVPC in liver samples using different extraction methods.

Table 3
Occurrence of HPVC in fish liver samples. Concentrations (n = 3) are in ng·g^{-1} (d.w).

Compounds	Turbot		Sea bass		Sea bream	
	sample 1	sample 2	sample 1	sample 2	sample 1	sample 2
Benzothiazoles						
BT	53.5	40.6	96.1	78.6	37.4	46.9
OHBT	120.7	114.9	n.d	101.4	n.d	n.d
NH_2BT	n.d	47.3	n.d	n.d	n.d	n.d
Benzosulfonamides						
BSA	n.d	118.2	3.0	n.d	n.d	<MQL
Me-p-TSA	13.6	92.1	17.0	76.9	n.d	16.6
p-TSA	124.6	25.9	29.2	117.9	16.0	68.1
Et-p-TSA	n.d	8.4	n.d	n.d	n.d	n.d
Organophosphates esters						
TEP	93.6	64.4	64.0	78.1	64.0	36.3
TBP	7.2	10.8	6.6	8.1	6.9	6.2
TiBP	3.4	30.1	4.3	11.5	4.1	6.9
TCEP	n.d	2.3	0.7	n.d	n.d	n.d
TEHP	n.d	9.5	n.d	1.7	n.d	n.d
TPP	n.d	16.9	n.d	8.4	n.d	4.1
TTP	<MQL	3.1	1.6	<MQL	<MQL	6.4
Phthalate acid esters						
DMP	8.1	27.8	10.1	26.1	22.5	19.0
DEP	422.9	2888.3	1053.8	4702.9	1202.6	2644.6
DiBP	39.2	338.5	69.5	103.9	60.2	59.2
DnOP	n.d	692.9	n.d	n.d	n.d	n.d

<MQL: less than the quantification limit of the method.
n.d: not detected.

Table 4
Occurrence of HPVC in fish skin samples. Concentrations (n = 3) are in ng·g^{-1} (d.w).

Compounds	*Turbot*		*Sea bass*		*Sea bream*	
	sample 1	sample 2	sample 1	sample 2	sample 1	sample 2
Benzothiazoles						
BT	n.d	n.d	n.d	n.d	1.7	n.d
Benzosulfonamides						
BSA	n.d	118.2	3.0	n.d	n.d	<MQL
Me-p-TSA	n.d	n.d	n.d	3.3	n.d	n.d
p-TSA	n.d	28.6	n.d	5.5	n.d	2.3
Organophosphates esters						
TEP	20.7	n.d	34.9	n.d	46.9	n.d
TBP	6.6	7.9	5.5	14.7	7.2	34.0
TiBP	3.0	3.9	2.7	9.0	3.2	10.6
TCEP	n.d	n.d	n.d	<MQL	n.d	1.9
TEHP	2.0	n.d	n.d	n.d	n.d	18.2
EHDPP	n.d	n.d	n.d	n.d	n.d	12.7
TPP	7.0	45.7	7.3	66.1	12.3	65.7
TTP	n.d	10.6	2.6	12.9	4.6	3.3
Phthalate acid esters						
DMP	0.7	0.9	1.9	4.5	2.7	n.d
DEP	412.6	6393.2	750.8	8350.0	978.0	2039.1
DiBP	23.7	26.0	34.6	146.9	43.8	43.4
DEHA	774.0	1209.1	178.6	2171.8	101.1	100.2
DEHP	n.d	2788.8	201.5	6347.5	80.1	21.8

<MQL: less than the quantification limit of the method.
n.d: not detected.

of the compounds with satisfactory apparent recoveries except for a few compounds such as p-TSA. Therefore, USAE is a very suitable method to determine most of the selected HPVC in fish liver and skin samples. The USAE method involves a longer analysis time than the QuEChERS method but less manipulation. This is due to the freezing time of 45 min, which the QuEChERS method does not have.

There are few studies on the determination of these compounds in liver samples and very few for skin samples. In the case of liver, the recoveries obtained for OPEs by the USAE method can be compared with those of other studies, giving similar recoveries. In the study of Liu et al., (Li et al., 2022) fish viscera samples were analysed by QuEChERS method, bit recoveries for most compounds were higher than 140%. In other study, fish viscera, specifically liver, kidneys and gills, were also analysed from an accelerated solvent extraction with dichloromethane/hexane (1:1, v/v) and recoveries for OPEs between 71 and 113% were obtained (Bekele et al., 2021).

3.4. Occurrence of HPVC in liver and skin samples

The USAE method was employed to analyse these compounds in two liver and two skin specimens of each fish species (turbot, sea bream, and sea bass). The presence of contaminants was confirmed by comparing the retention time, the presence of the qualifier ion transitions, and the ratio of quantifier/qualifier transitions compared with that obtained with a standard solution.

Tables 3 and 4 show the concentrations of the triplicate analysis of liver and skin samples of each fish species, expressed on a dry weight basis. The table only includes the compounds that have been found in the samples.

In liver, the highest phthalate concentrations were found for the DEP

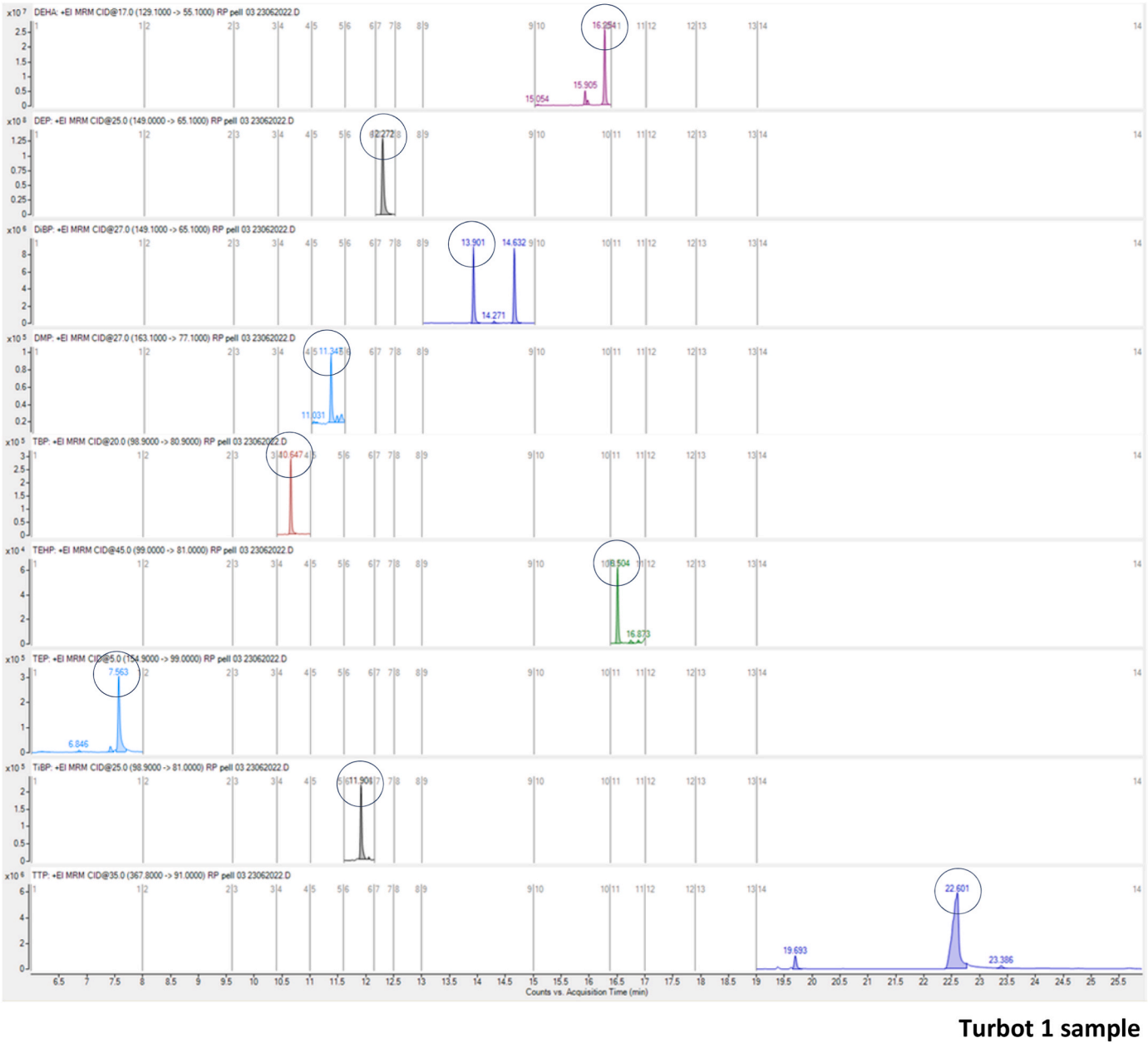

Fig. 4. Quantifier ion transitions for a turbot skin sample in MRM mode.

of the sea bass species. The other phthalates obtained concentrations between 8.1 and 27.8 ng g^{-1} (d.w) for the DMP and between 39.2 and 338.5 ng g^{-1} (d.w) for the DiBP. The DnOP was only detected in one sample with a concentration of 692.9 ng g^{-1} (d.w). The results for phthalates in liver are lower than those found in the paper by Valton et al. (2014), as they found concentrations of 386 ± 667 ng g^{-1} (d.w) for DMP, 2126 ± 4099 ng g^{-1} (d.w) for DiBP, 653 ± 1285 ng g^{-1} (d.w) for DnOP, and 3052 ± 3854 ng g^{-1} (d.w) for DEHP. An exception is DEP, with concentrations of 2001 ± 2400 ng g^{-1} (d.w) lower than the concentrations of 412.6–4702.9 ng g^{-1} (d.w) obtained in the present study.

In the liver, organophosphate esters such as TEP, TBP, TiBP and TCEP were also determined at lower concentrations in all the samples analysed. Some compounds were not detected in any liver sample: ClBT, MeSBT, o-TSA, TCPP, DEHA, DEHP and EHDPP. The compound TEP stands out with a wet weight concentration of 93.6 ng g^{-1} (d.w) (corresponding to 58.5 ng g^{-1} (w.w)), which is higher than that found in the paper by Choo et al. (2018), which reported concentrations of 3.62–8.77 ng g^{-1} (w.w) for this compound. In contrast, other compounds such as TCEP showed very similar concentrations in this study of between 0.7 and 2.3 ng g^{-1} (d.w) (corresponding to 0.5–0.9 ng g^{-1} (w.w)). Choo et al. found concentrations of between 1.44 and 2.70 ng g^{-1} (w.w).

In skin, there was a high concentration of DEP in all the samples, and the maximum was 8350 ng g^{-1} (d.w) in sea bass. As expected, phthalates, which are also found in the sea samples (Gimeno et al., 2003; Paluselli et al., 2018), showed the highest concentrations in the skin samples, probably due to contamination during transport and handling for fish packaging. Organophosphate esters such as TBP, TiBP and TPP were found in smaller concentrations in all the samples. Of the benzothiazoles, only compound BT was detected. The following compounds were also not detected in any sample: o-TSA, TCPP, Et-p-TSA and DnOP. We could not find any HVPC results in the literature in fish skin samples for comparison. As an example, the chromatogram in Fig. 4 shows the quantifier ion transitions of the compounds found in a turbot skin sample.

Comparing the results for the skin and liver samples we can see that if

we exclude the major compound (DEP), the sum of the remaining compounds (ΣHPVC) in the liver is about 483 ng g^{-1} (d.w.), much higher than in the skin, which reached 102 ng g^{-1} (d.w.). The contaminant bioaccumulated more in the liver than in the skin, resulting in a higher concentration of contaminants to be found.

Looking at the individual species, the liver samples of turbot have a sum of concentrations of 875 ng g^{-1} (d.w.), followed by sea bass 381 ng g^{-1} (d.w.) and sea bream 192 ng g^{-1} (d.w.). The difference in concentrations can be related to the size of the fish and its diet, since the larger species, such as turbot, have more contaminants. The turbot diet is more connected to the ground, and mainly consists of bivalves, which are filtering organisms and have a high capacity for bioaccumulation of contaminants. The other species, sea bass and sea bream, have a similar diet with the exception that sea bream also feeds on algae, which could explain its lower concentrations.

In the skin samples, sea bream, sea bass and turbot have a similar sum of concentrations of 125 ng g^{-1} (d.w.), 102 ng g^{-1} (d.w.) and 80 ng g^{-1} (d.w.), respectively. The turbot can camouflage itself and spends most of the time under the sand, which could lead to a lower contamination of its skin; however, it should be taken into account that the skin is also contaminated from the handling of the fish, especially with phthalates.

4. Conclusions

An USAE-based analytical method followed by GC-MS/MS was optimized for determining 25 HPVC in fish liver and skin samples. The method provided good repeatability and MQL and MDL at low ng·g^{-1} levels. Compared with a QuEChERS-based method, the USAE method provides better R_{app} for liver and skin, especially for compounds such as benzosulfonamides and benzothiazoles, although the recovery values are not very high due to difficulty of the complexity of the matrix.

Three different species (sea bass, sea bream and turbot) were analysed and some HPVCs were determined. In the skin samples, phthalates dominated over the other contaminants, probably due to contamination during transport and packaging. The liver samples showed a higher bioaccumulation of the contaminants and therefore more contaminants were found. Excluding the compound with the highest concentrations, DEP, the ΣHPVC had a value of 483 ng g^{-1} (d.w), similar than the one found in the skin samples, which was 102 ng g^{-1} (d.w). Comparing the species, turbot was the fish with the highest ΣHPVC in the liver, possibly due to its larger size and its feeding type, followed by sea bass then sea bream. In the skin samples, sea bream and sea bass had higher compound concentrations than turbot, but they all had fairly similar results.

CRediT authorship contribution statement

Sílvia Borrull: Writing - original draft, Validation, Resources, Investigation, Formal analysis. **Francesc Borrull:** Supervision, Funding acquisition. **Rosa Maria Marcé:** Writing - review & editing, Supervision, Methodology, Conceptualization. **Eva Pocurull:** Writing - review & editing, Supervision, Methodology, Conceptualization.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2024.141273.

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