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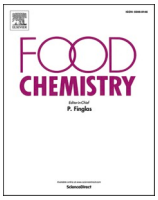

Analytical Methods

Novel ultrasound assisted extraction and d-SPE clean-up for the analysis of multiple legacy and emerging organic contaminants in edible fish

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ABSTRACT

Polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), novel brominated flame retardants (NBFRs), phthalate esters (PAEs) are pervasive environmental pollutants, posing threats to both ecosystems and human health. Although several analytical methods were developed for these compounds, they are not performed simultaneously. This study addresses the need for a sustainable, novel, analytical approach capable of simultaneously determining these diverse chemical classes in edible fish muscles. Employing ultrasound extraction coupled with dispersive solid-phase extraction (d-SPE) as a cleanup procedure, the method was compared to conventional techniques, revealing significant improvements. Analytical parameters were thoroughly assessed, and the innovative method demonstrated notable advantages, reducing extraction and purification times by approximately 74–80 % and solvent consumption by around 94–97 %. Applied to Mediterranean Sea fish samples, the results underscore the method's potential as a viable, sustainable alternative to traditional approaches, promising enhanced efficiency and reduced environmental impact.

1. Introduction

Legacy and emerging organic pollutants are one of the major global issues due to the considerable risk they pose to the environment and human health. In the aquatic environment, owing to their lipophilic and persistent properties, these organic compounds remain susceptible to aquatic organisms through breathing, ingestion, or body surfaces and consequently bio-accumulate throughout the food chain. Thus, the consumption of these contaminated aquatic organism may pose serious concern to human health (Vuković et al., 2018; Azcune et al., 2022; Guo & Kannan, 2015).

Among legacy organic contaminants, polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs) have been extensively used over the past century for various purposes (ATSDR (Agency for Toxic Substance and Disease Registry), 2017). As persistent organic pollutants (POPs), they are susceptible to long-range transport and bioaccumulation in biota, due to their high affinity for adipose tissue, thereby causing potential toxic effects on aquatic organisms (Corsolini & Sarà, 2017; Mitra et al., 2019). Recently, emerging organic contaminants, such as additives including Novel Brominated Flame Retardants (NBFRs) and phthalates (PAEs) have attracted the interest of the scientific community, with a large number of studies evidencing their presence at significant levels in the environment (Venier et al., 2015). NBFRs are newly produced flame retardants, ~~do~~ not share a similar chemical structure, but all containing Br-C bonds (Venier et al., 2015). There is limited research characterizing NBFRs, making it challenging to assess potential exposure risks and understand their environmental behaviors (Iqbal et al., 2017), ~~but~~ because they are suspected to have bioaccumulative, adverse effects and persistent properties similar to other (European Food Safety Authority (EFSA), 2012). Monitoring these organic contaminants in edible fish allows the governmental agencies to deal with be informed of the risks involved by consuming fish contaminated with POPs and other chemicals by establishing safe consumption advisories and risk areas (Zuiderveen et al., 2020).

On the other hand, PAEs are utilized as additives to enhance the flexibility and durability of plastics (Lithner et al., 2011). As they are not chemically bonded to plastics, PAEs can easily desorb and leach into the

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environment and be detected in various environmental matrices (Domínguez-Morueco et al., 2014). They can also be absorbed by organisms through the ingestion of plastics, and have the potential to cause toxic effects, for example acting as endocrine disruptors even at low concentrations (Diamanti-Kandarakis et al., 2009).

The selection of an optimal sample processing and extraction method are crucial for the measurement of these contaminants in edible fishes; thus the analyst must judiciously make choices based on specific needs and resource availability (Ali et al., 2019). Established extraction methods such as Soxhlet and Accelerated Solvent Extraction (ASE) are commonly utilized for PCBs, PBDEs, and NBFRs followed by GC analysis (Gilmour et al., 2021; Lewis et al., 2020; Li et al., 2019; Liu et al., 2011; Ma et al., 2020; Munschy et al., 2011; Panseri et al., 2019; Shang et al., 2016), in some instances, these chemicals also involve the use of ultrasound assisted extraction (UAE) as an extraction technique (Kelly et al., 2008; Teil et al., 2014; van der Schyff et al., 2021; Zhang et al., 2015), while it is frequently used for the extraction of PAE from fishes, involving analysis in either GC or LC (Gu et al., 2014; Hu et al., 2016; Teil et al., 2014). The analytical detection of these substances poses several challenges, including but not limited to the presence of these contaminants at low concentrations, the complexity of matrices, and the high analytical costs (Santhi et al., 2012). Recently multi-residual extraction methods have shown to be able to detect a wide range of contaminants with high extraction efficiency and optimal selectivity (Campanale et al., 2021). Furthermore, this approach follows the direction of the scientific community to place increasing importance toward sustainability of analytical procedure (Lucena Raphael, 2022). There has been a growing emphasis on promoting environmentally friendly practices within the field with the use of greener solvents and actively seeking ways to reduce energy consumption during extraction and purification processes (López-Lorente et al., 2022).

As the demand for fast, efficient and sustainable extraction methods continues to grow, quick, easy, cheap, effective, robust and safe extraction (QuEChERS) and ultrasound-assisted extraction are likely to remain at the forefront of innovative extraction techniques. QuEChERS extraction is an advanced and versatile analytical method that combines liquid–liquid extraction and dispersive solid-phase extraction to efficiently isolate a wide range of analytes from complex matrices (Chamkasem et al., 2016). In addition, UAE has attracted increasing interest in analytical chemistry due to its remarkable efficiency in rapidly extracting numerous compounds from food and environmental samples, exhibiting extraction efficiency comparable to traditional classical techniques (Picó, 2013).

The aim of this study was to develop and optimize a novel analytical method capable of simultaneously extracting, purifying and analyzing four distinct classes of compounds: PCBs, PBDEs, NBFRs, PAEs from edible fish samples, determining the most efficient and reliable approach. This will be achieved by comparing novel techniques and conventional methods commonly used in environmental analysis, using Soxhlet or ultrasonic extraction combined with solid-phase dispersion extraction (d-SPE) for a cost-effective, efficient and environmental friendly approach. Therefore, the novelty of the work was to improve the analysis of these analytes in terms of sustainability, while maintaining high analytical efficiency, in terms of recovery and other analytical parameters. Furthermore, the SANTE guidelines for food samples will be followed for the validation of the methods (SANTE 11312/2021, 2021). The study will assess the environmental impact of these analytical methods, recognizing the importance of sustainability in scientific methodologies. Subsequently, the most valuable method was applied to quantify the concentration of these contaminants in bogue (*Boops boops*) and European pilchard (*Sardina pilchardus*) from the Mediterranean Sea.

2. Materials and methods

2.1. Samples

European pilchard (*Sardina pilchardus*) and bogue (*Boops boops*) specimens were captured using a lampara net, a specialized fishing net, by professional fishermen operating in FAO Geographical Sub-Area 9 as part of the Plastic Busters MPAs project. Only adult specimens were selected for the study. After the capture, biological parameters (total length of specimen (cm), fork length (cm) and total weight (g)) were recorded for each fish. Dorsal fillets were carefully collected, wrapped in pre-cleaned aluminum foil to prevent any potential contamination, and stored at −20 °C, until laboratory analysis. A pool of muscle samples, comprising 2 g of muscle tissue from each specimen of the two species, was used to evaluate and compare the effectiveness of different extraction and clean-up procedures. This pooling strategy was employed to mitigate the inherent variability that may result from individual samples within each species. Indeed, the resulting composite pool represented a more comprehensive and representative sample set. Furthermore, individual analysis of ten specimens of bogue (*Boops boops*) and six European pilchard (*Sardina pilchardus*) was conducted to implement the most effective method in a realistic environmental scenario. This approach allowed the assessment of the method's practical applicability in a real-world context.

2.2. Preparation of calibration standards

Preparation of calibration standards, quality control samples and stock solutions of analytes were prepared in hexane and stored at –20 °C. The working solution concentrations for PCBs ranged from 0.10 to 150 ng/g, for PBDE and NBFRs from 0.3 to 125.0 ng/g and for PAEs from 1.0 to 600.0 ng/g. The limit of detection (LOD) and quantification (LOQ) were evaluated by replicated (n = 5) analysis of procedural blanks (Table S1). The LOD was based on a calibration slope 3.8σ / slope (10σ/ slop for LOQ), where σ is the standard error of the regression adopting the method proposed by the European Union (Wenzl et al., 2016). Calibration curves were performed using 6 standards in triplicate for each analyte. The linearity of each calibration curve was determined using least squares linear regression analysis, without a weighing factor. The lines were acquired in triplicate, independently prepared, by three different operators on three different days. Linear regression using the least-squares method was then applied and the acceptability of the linearity assumption was verified according to the following criteria: correlation coefficient R^2, for the chosen confidence level of 99 % and having 6 points the data population, the critical value for the correlation coefficient is $R^2 \geq 0.959$, a value far exceeded by the R^2 values obtained for each congener. For all the classes of compounds, the highest and lowest concentrations of the calibration curve also represented the limits of the linearity range.

2.3. Extraction and clean-up procedure

Three different extraction and purification strategies (Fig. 1) were performed and compared to determine the level of legacy (PCBs, PBDEs) and emerging (NBFRs and PAEs) organic contaminants in edible fish samples. The extraction techniques encompassed Soxhlet extraction or UAE, with subsequent purification steps employing either silica gel columns or d-SPE (containing primary secondary amine sorbent (PSA), C18, Bulk Carbograph (GBC) and magnesium sulphate) as illustrated in Fig. 1.

Further methods description of the materials and reagents used in the laboratory for sample preparation and analysis are provided in the supplementary data (SI).

For all methods, about 0.5 g of the pool samples were spiked with 100 μL of ^{13}C-PCB (9L, 37L, 79L, 11L, 162L, 194L, 206L), 100 μL ^{13}C-PBDE (28L, 47L, 99L, 100L, 153L, 154L, 183L), 100 μL deuterated

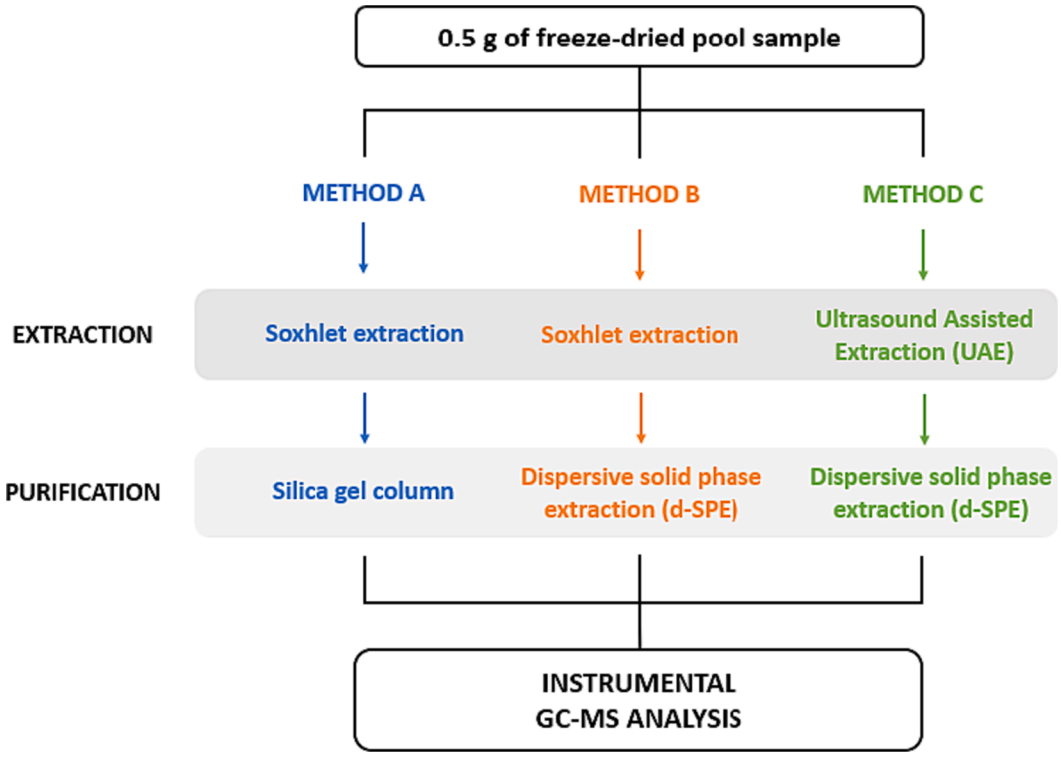

Fig. 1. The flowchart shows the different steps for each method. A and B have the same extraction step, but different clean-up. The method C has an UAE and a d-SPE clean-up.

phthalates IS-MIX (DMP-d4; DEP-d4; DIBP-d4; DBP-d4; BBzP-d4; DCHP-d4; DEHP-d4; DNOP-d4) and 100 μL of a mixture of NBFR containing pentabromotoluene (PBT),3-dibromopropyl-2,4,6-tribromophenyl ether (DPTE), hexabromobenzene (HBBZ), 1,2-bis(2,4,6-tribromophenoxy) ethane (BTBPE), tetrabromophthalate (BEHTBP), 2-ethylhexyl 2,3,4,5-tetrabromobenzoate (EHTBB), and pentabromoethylbenzene (PBEB). The concentrations of the standard solutions added in the samples are described in the SI (Table S2). For method A, the sample was placed in a cellulose thimble and Soxhlet extracted for 12 h with 200 mL of a DCM: *n*-hexane 3:1 (v/v) mixture. After Soxhlet extraction the sample was concentrated by Rotavapor (Strike 300, Steroglass; R-200, BÜCHI) to 11 mL and an aliquot of 1 mL of the extracts was used to determine lipid contents gravimetrically.

The remaining10 mL of the sample were cleaned-up, using a multi-layer silica gel column (length 190 mm, I.D. 22 mm) packed from the bottom as follows: glass wool, 2 g of activated silica, 2 g of acid silica, 2 g of activated silica and anhydrous Na_2SO_4. The column was rinsed with 100 mL of *n*-hexane. After conditioning, samples were loaded and eluted with 200 mL of hexane. Purified extracts were concentrated to a volume of about 5 mL, transferred in a test tube and blown down until 100 μL under a gentle stream of ultrapure nitrogen and analyzed by gas chromatography-mass spectrometry (GC–MS).

In Method C, 0.5 g of homogenized dried sample were weighted and then homogenized with 2 g of Na_2SO_4. The mixture was transferred in a glass test tube followed by addition of 100 μL of the same standards of Method A. 4 mL of extractive mixture 3:1 (v/v) dichloromethane: *n*-hexane solution was added to the sample and then Vortex shacked for 1 min. The test tube was placed in an ultrasonic bath for 20 min at 25 °C followed by centrifugation for 5 min (3000 rpm at room temperature). Then, the supernatant was transferred to a glass tube and 3 mL of extraction solvent were added to the remaining residue, extracted with a vortex for 5 min and centrifuged for 5 min (3000 rpm at room temperature). The supernatant was transferred to the previous glass tube and the whole step was repeated for a third time. The sample obtained from the extraction procedure was purified by using Agilent Technologies Bond Elute d-SPE. Once transferred into the tube it must be shaken with vortex for 1 min and then centrifuged for 5 min (4000 rpm at room temperature). Method B is a combination of method A and C: the extraction step involves the use of Soxhlet as in A, while the purification step involves the use of d-SPE. The final extracts referring to method B and C, were reduced in volume with a gentle stream of ultrapure nitrogen to be analysed by GC–MS. ^{13}C-PCB was added and used as the internal recovery standard.

2.4. Lipid content

The lipid content was determined gravimetrically for all three methods: an aliquot of the extracted sample, prior to the clean-up step, was taken and dried in an oven at 70 °C. When all the solvent was evaporated, the lipid content was determined. The Grubbs test was used to detect the presence of outliers. The standard deviation (s_r) and the relative standard deviation (RSD) % were calculated on 5 repetitions of the sample. Of the 45 replicates (15 per method), only 3 measurements were outliers (Table 1).

2.5. GC–MS analysis

The selection of gas chromatography-mass spectrometry (GC–MS) for the identification of target compounds in our study is based on its separation efficiency for distinguishing between closely related compounds and its precise quantification capabilities when applied to

Table 1
The table shows the different average lipid content in dry weight (ww) calculated from the three methods application.

Sample	Method	Average lipid content ww %	S_r	*RSD %*
Fish pool	A	9.342E-01	1.191E−01	1.275E+01
	B	9.313E-01	1.226E−01	1.446E+01
	C	1.508	2.662E−01	1.741E+01

environmental samples (Figs. S1–S4).

The MS system was operated in the selected ion monitoring (SIM) mode. The assignment of the peaks was confirmed by mass spectral libraries (i.e., NIST). An Agilent Technologies 6890N (G1530N) gas chromatograph, an Agilent Technologies 5973 inert mass spectrometer, J & amp; Agilent DB-5MS (30 m, 0.250 mm, 0.25 μm) gas chromatograph column was used for the detection and quantification of PCBs and J & amp; Agilent DB-5MS (15 m, 0.250 mm, 0.10 μm) gas chromatography column for NBFRs and PBDEs. For PCBs were used the following operating conditions: ion source set at 290 °C, quadrupole temperatures at 150 °C and the temperature program was 100 °C, 16 °C/min to 190 °C, 8 °C/min to 290 °C, 24 °C/min to 310 °C, held for 2 min, total run time 20.96 min. The injection was performed with 1 μL in the splitless mode; Helium was used as the carrier gas at a constant flow rate of 1 mL/min. For NBFRs and PBDEs the operating conditions were the same of the PCBs analysis but different GC oven temperature program: initial temperature at 90 °C, held for 1 min, 20 °C/min to 220 °C, 10 °C/min to 300 °C. Regarding the PAEs HP-5MS 30 m, 0.250 mm, 0.25 μm gas chromatograph column were used, ion source set at 290 °C, quadrupole temperatures at 150 °C and the temperature program was 80 °C, 20 °C/min to 240 °C, 15 °C/min to 310 °C, total run time 18.6 min. The injection was performed with 1 μL in the splitless mode; Helium was used as the carrier gas at a constant flow rate of 1 mL/min.

2.6. Analytical parameters

To compare the analytical methods (A, B, and C) applied to edible fish samples, all procedures were validated following the acceptable criteria by SANTE guidelines (SANTE 11312/2021, 2021). Linearity, matrix effect (ME), trueness in term of recoveries, intra and inter-day precision and limit of quantification (LOQ) were evaluated. The linearity of the methods was evaluated by spiking labeled internal standards at different concentrations in 0.5 g dry weight of fish samples. Precision was assessed evaluating intra-day and inter-day precision (repeatability and reproducibility, respectively) and the results were expressed as RSD %, the RSD was acceptable if lower than 20 %). The limit of detection (LOD) and limit of quantification (LOQ) were evaluated by replicated (n = 5) analysis of procedural blanks. Moreover, it was evaluated the ME and the residual standard deviations (F-test at the 95 % confidence level). Finally, recoveries were determined for all labeled internal standards spike levels within the same workday and the results were considered acceptable with a recovery ranging from 70 to 120 %.

The recovery percentage was calculated from the apparent recovery (AR) (%) and ME (%):

$$R(\%) = ME(\%) + AR(\%)$$

Where the AR is an observed value, derived from an analytical procedure by means of a calibration graph, divided by reference value, i.e., the ratio between the concentration found and that added to the sample in the initial sample preparation step (Gohshi & Müller, 2002). Moreover, for each matrix, a blank was prepared to subtract the natural content of the different compounds in each material.

2.7. Selectivity and matrix effect

Selectivity is the ability to uniquely determine the analyte in the presence of other components that are expected to be present, identifying and quantifying the PCBs, PBDEs and NBFRs from all other compounds present. Any peak detected at the retention time of the analytes with an area greater than 20 % of the analyte at the LOQ or 5 % of the internal standard was considered significant interference (Nosal et al., 2021). ME, defined as the combined influence of all the non-analyte components of the analyte signal, were assessed by comparing the peak areas of spiked matrix and spiked blank solvent:

$$ME\% = \left(\frac{S_m}{S_s} * 100\right) - 100$$

Where S_m and S_s are the slopes of calibration lines in matrix and in procedural blank, respectively (Scordo et al., 2020). Deviation in peak area >20 % compared with the neat injection solvent would be considered significant.

3. Results and discussion

3.1. PCBs, PBDEs and NBFRs

To discuss the advantages and disadvantages of all the methods, the analytical parameters for the compound classes studied were evaluated and the results compared (Table 2). To evaluate these parameters, two concentrations were chosen, a 'high' one, corresponding to 75.0 ng/g and a 'low' one, corresponding to 0.5 ng/g. Intra-day RSD% values were obtained by injecting both high and low concentration levels five times, while the inter-day RSD (%) values were obtained by injecting the two concentration levels on five consecutive days. The RSD% values were below the threshold values set by EUR 24815 EN 2011, for all concentrations used for the repeatability study. In particular, for high concentration the values were below 17 % (max 16.8 % for PCBs, 16.5 % for PBDEs and 14.9 for NBFRs); while for low concentrations they maximum for each class of compounds are: 11.8 % for PCBs, 11.2 % for PBDEs and 13.9 for NBFRs. The RSD% values obtained were all below these values, so the repeatability of the method was acceptable. Method A did not allow the identification and assessment the repeatability parameters for all NBFRs. Indeed, only PBT, PBEB, DPTE, HBBZ were assessable at 0.5 ng/g and PBT, PBEB, DPTE, HBBZ, EHTBB at 75.0 ng/g. For Method B and C, it was possible to determine the repeatability for all NBFRs, the only compound that could not be evaluated was EHTBB with Method B at 0.5 ng/g. Therefore, for Method B and C, almost all data on NBFRs were acceptable. To evaluate the linearity of the calibration curve in the matrix, the same linearity range of the calibration curve in solvent was applied in the final extract for each method. The R^2 values calculated for all methods and for all congeners of both compound classes turn out to be greater than 0.990, therefore the ME did not impact on linearity. To calculate the standard deviation and thus the RSD, 5 repetitions were performed for each concentration.

The recovery of each analyte from fish samples was determined at low and high concentrations by comparing the peak area ratios (analytical peak area: internal standard peak area) of experimental to theoretical samples. To assess the recovery a blank sample was spiked with standard, the methods were applied and finally it was analyzed in GC–MS. The results were compared with the standards signal added in a solvent blank.

The analytical parameters assessment was performed at two concentrations, 'low' (0.5 ng/g) and 'high' (75 ng/g) (Table S4). For all three methods under study, ME values were calculated for all classes of compounds in the calibration curve range; PCBs always showed a negative ME, while PBDEs showed a positive ME. In the case of PCBs, the slope value of the line in matrix was smaller than the slope value of the line in solvent. This means that the concentration values recorded for the calibration curve in matrix were lower than the values recorded for the calibration curve in solvent. For PBDEs, on the other hand, the opposite behavior is observed, the slope of the calibration line in matrix being greater than that in solvent. In any case, for both PCBs and PBDEs, the absolute value of this parameter never exceeds the critical value of 20 %.

Interestingly, the absolute values of ME for method A are on average lower than for methods B and C. This difference could be due to the different clean-up method used. The components contained in the d-SPE used in method B and C could be responsible for this increase. The background noise in the chromatographic profiles may have influenced the calculated ME values.

Table 2
The table shows the repeatability intra and inter-day from the low and high concentration as RSD % and the correlation coefficient (R^2) of calibration curve in matrix.

Compound	Method A				R2	Method B				R2	Method C				R2
	Spiked Conc. 0.5 ng/g		Spiked Conc. 75.0 ng/g			Spiked Conc. 0.5 ng/g		Spiked Conc. 75.0 ng/g			Spiked Conc. 0.5 ng/g		Spiked Conc. 75.0 ng/g		
	RSD intra-day (%)	*RSD inter-day (%)*	*RSD intra-day (%)*	*RSD inter-day (%)*		*RSD intra-day (%)*	*RSD inter-day (%)*	*RSD intra-day (%)*	*RSD inter-day (%)*		*RSD intra-day (%)*	*RSD inter-day (%)*	*RSD intra-day (%)*	*RSD inter-day (%)*	
PCB-79L	4.250	6.390	5.100	2.789	0.9994	5.522	9.613	6.246	1.112E+01	0.9978	7.228	1.510E+01	7.057	1.175E+01	0.9966
PCB-111L	5.416	6.288	6.499	3.112	0.9993	6.613	1.514E+01	6.657	1.173E+01	0.9979	8.438	1.682E+01	6.774	1.084E+01	0.9996
PCB-162L	2.044	8.424	2.452	2.624	0.9979	7.452	1.268E+01	5.279	9.124	0.9979	5.578	1.487E+01	6.830	9.157	0.9942
PCB-194L	2.704	5.850	2.974	2.153	0.9991	5.829	1.148E+01	7.073	1.066E+01	0.9934	6.363	1.676E+01	5.835	1.145E+01	0.9965
PCB-206L	2.241	8.178	2.465	2.438	0.9992	6.666	1.267E+01	5.827	1.177E+01	0.9968	7.768	1.504E+01	6.761	8.004	0.9941
BDE−28L	8.748E−01	9.248	9.623E−01	2.824	0.9993	7.112	9.002	5.625	8.571	0.9987	8.138	1.162E+01	5.735	8.158	0.9987
BDE−47L	1.232	5.138	1.356	3.044	0.9994	8.471	1.154E+01	4.907	8.256	0.9998	9.131	1.354E+01	5.328	8.013	0.9968
BDE−99L	8.914E−01	8.289	9.805E−01	2.582	0.9989	5.572	9.528	4.472	8.123	0.9992	5.044	6.562	5.928	8.482	0.9968
BDE−100L	8.867E−01	7.205	1.059	2.215	0.9989	5.423	1.164E+01	4.840	8.735	0.9977	7.573	1.210E+01	5.274	9.075	0.9979
BDE−153L	7.559E−01	7.424	8.315E−01	2.447	0.9992	4.843	1.102E+01	5.682	8.015	0.9965	8.556	1.333E+01	6.183	9.785	0.9964
BDE−154L	8.664E−01	8.046	9.530E−01	2.659	0.9989	4.213	9.212	4.614	9.283	0.9988	6.464	1.649E+01	6.428	1.122E+01	0.9969
BDE−183L	9.396E−01	8.916	8.837E−01	2.778	0.9987	7.348	1.122E+01	4.643	7.372	0.9966	5.528	1.237E+01	8.184	1.113E+01	0.9959
PBT	4.797	7.290	5.277	2.927	0.9987	8.148	1.349E+01	5.057	6.201	0.9976	3.174	4.146	1.619E+01	1.148E+01	0.9981
PBEB	3.186	5.557	3.505	3.675	0.9982	3.471	1.440E+01	4.327	8.105	0.9984	7.176	9.518	4.735	1.383E+01	0.9972
DPTE	3.849	7.503	1.146	4.666	0.9983	1.248	1.166E+01	5.028	6.125	0.9970	5.273	8.723	4.350	1.052E+01	0.9984
HBBZ	2.875	6.033	3.163	1.367	0.9999	8.197	1.382E+01	5.222	6.073	0.9957	5.600	1.111E+01	9.628	1.229E+01	0.9976
EHTBB	n.a	n.a	5.128	3.500	0.9978	n.a	n.a	4.828	1.001E+01	0.9954	n.a	n.a	8.853	1.237E+01	0.9982
BTBP	n.a	n.a	n.a	n.a	0.9975	7.131	1.486E+01	4.644	6.217	0.9958	6.863	8.734	7.512	1.172E+01	0.9972
BEHTBP	n.a	n.a	n.a	n.a	0.9923	7.627	1.138E+01	4.213	7.017	0.9979	4.825	5.580	6.445	1.118E+01	0.9986

The reason for such should be studied and investigated in the future, observing how the background noise varies with the different types of d-SPE, to see how the ME changes and to understand which interferer actually increases this phenomenon.

The lowest recovery was recorded for PBEB at a concentration of 75.0 ng/g in method A and corresponds to 57.3 % (Fig. 2). This result is lower than the minimum acceptable (70 %), therefore, using the Method A is necessary correcting for recovery. For EHTBB at L1, it was not possible to calculate the recovery even when applying methods B and C as the compound was probably not extracted, retained, or underwent changes during sample treatment and cleanup. Furthermore, it was not possible to determine the recovery of DPTE, BTBP and BEHTBP and at low and high concentrations with method A, because none were recovered. On the other hand, the same three analytes at L2 were recovered by applying methods B and C at low and high concentrations. The recoveries for Method B at low and high concentrations were 116.1 % and 69.1 % for DPTE, 121.6 % and 84.1 % for BTBP, 105.5 % and 85.0 % for BEHTBP; while for Method C at low and high concentrations were 107.4 % and 75.9 % for DPTE, 83.6 % and 80.9 % for BTBP, 92.5 % and 91.2 % for BEHTBP. Therefore, at least for these two compounds the clean-up step using d-SPE allowed to recover the analytes regardless of the extraction technique used. For method C, higher average values are consistently achieved, hovering 100 %, perfectly centered within the acceptable range. The remaining recovery results fell within the range of 70 and 120 %, making them acceptable as well.

3.2. PAEs

After the successful validation of the mentioned classes of compounds, a next step involved the implementation and validation of method C, specifically designed for the determination of PAEs. The decision to extend the application of Method C to PAEs was based on the similarity of chemical and physical characteristics shared between the different classes of compounds. By using Method C in bogue fillet and European sardine from the Mediterranean Sea, it was possible to simultaneously analyse all four classes of compounds, thus improving efficiency and resource utilization.

The recoveries for all congeners showed acceptable values, ranging from 70 to 120 %. To check the repeatability of the method, the RSD value (%) was used, using 5 repetitions for both concentrations. Ac, ME and linearity were result compliant with the validity criteria used. RSD values was borderline acceptable for all analytes except DPrP, which had a lower average recovery than the other analytes (see Table 3).

3.3. Methods' efficiency

One of the aims of analytical processes is to reduce the environmental impact, analysis costs and the health risk for the laboratory personnel, as much as possible. Sustainability of analytical processes is one of the goals of green chemistry, which Europe incentives with its policies and the scientific community acknowledges (Gałuszka et al., 2013; Koel, 2016). In order to highlight the different sustainability degree of the three methods, solvent consumption and analysis time were compared. About solvent, DCM consumption for Method C was reduced by 94 % compared to Method A and Method B, while hexane consumption was reduced by 98.8 % and 94 %, respectively. Furthermore, considering the goals of green chemistry, the lower solvent consumption not only reduce the analysis cost but also decreases the impact on laboratory personnel health, as DCM and hexane are carcinogenic and neurotoxic solvents, respectively (Lanska, 1999; Liu et al., 2013).

Finally, the sample preparation time was also compared (Fig. 3), and method C was found to be the most advantageous, as it does not involve Soxhlet continuous extraction methods and solvent reductions by rotavapor, providing a time decrease of approximately 97 % compared to method A and 94 % compared to Method B (Table S5).

Therefore, from the results obtained and in line with the advantages

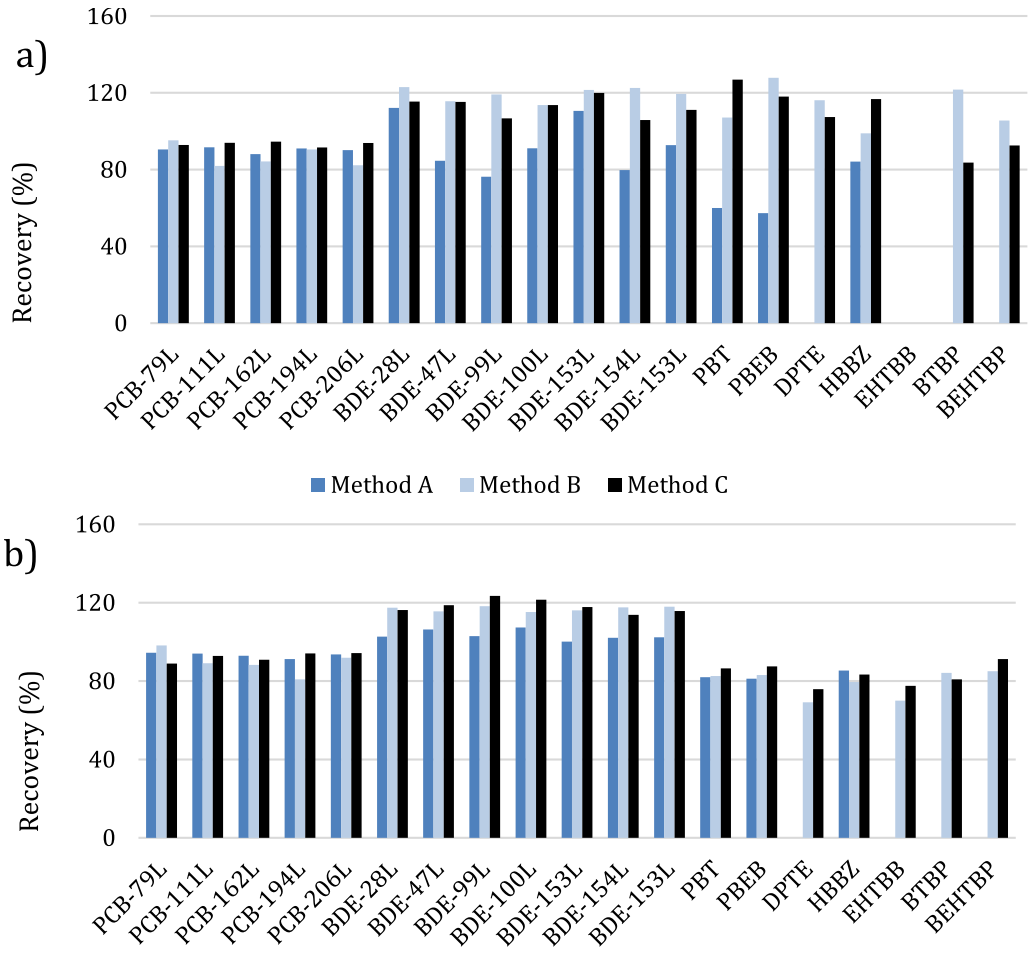

Fig. 2. Recovery % obtained from AR and ME for PCBs, PBDEs and NBFRs congeners at low and high concentration 0.5 ng/g (a) and 75.0 ng/g (b) respectively.

Table 3
The table show the AR (%), ME (%), Ac (%) and the RSD intra and inter-day. The AR and Ac were calculated for a low (10.0 ng/g) and high (500.0 ng/g) point of the calibration curve for deuterated phthalates (d^4).

	R^2	R (%)		RSD (%) intra-day		RSD (%) inter-day		ME (%)	AR (%)		Ac (%)	
		10 ng/g	500 ng/g	10 ng/g	500 ng/g	10 ng/g	500 ng/g		10 ng/g	500 ng/g	10 ng/g	500 ng/g
DMP	0.9983	1.056E+02	8.167E+01	7.315	9.255	7.812	1.016E+01	−1.334E+01	1.189E+02	9.502E+01	1.126E+02	1.163E+02
DEP	0.9989	1.063E+02	7.864E+01	6.414	8.687	6.946	9.452	−1.831E+01	1.246E+02	9.695E+01	1.172E+02	1.233E+02
DPrP	0.9999	7.759E+01	8.518E+01	8.128	7.558	9.028	8.720	2.435	7.516E+01	8.275E+01	9.686E+01	9.714E+01
DBP	0.9986	1.210E+02	9.341E+01	7.107	7.582	8.208	8.666	−1.187E+01	1.329E+02	1.053E+02	1.098E+02	1.127E+02
BBzP	0.9967	1.388E+02	1.452E+02	6.218	7.423	7.133	7.835	1.535E+01	1.542E+02	1.606E+02	1.111E+02	1.106E+02
DChP	0.9988	1.006E+02	1.028E+02	6.829	6.156	7.861	7.507	2.102E+01	1.216E+02	1.238E+02	1.209E+02	1.204E+02
DEHP	0.9977	1.077E+02	9.916E+01	8.113	7.890	9.126	8.150	1.764E+01	1.253E+02	1.168E+02	1.164E+02	1.178E+02
DNOP	0.9998	1.002E+02	8.351E+01	8.544	7.114	9.167	7.834	2.038E+01	1.206E+02	1.039E+02	1.203E+02	1.244E+02

also presented by other works using QuERChERS methods in place of traditional ones (Pedersen et al., 2023), method C is the method closest to the principles of process sustainability and green chemistry.

3.4. Method application

To assess the suitability of method C in fish samples, a set of 10 samples of bogue and 6 samples of European pilchard from the Mediterranean Sea were selected for analysis. Prior to analysis, these samples were freeze-dried, followed by the application of Method C. The implementation of this new extraction method yielded promising outcomes, successfully detecting considerable levels of contaminants from all four classes within the samples (Table S6).

The indicator PCBs, PCB-28 and 52, showed the highest concentrations in BB samples and similar concentrations of PCB-118, -138, -180. PCB-101 was found at higher concentrations than LOD in only 3 BB samples. The PCBs concentrations in these fish species of the Mediterranean Sea were comparable with the concentrations found in other similar studies (Bartalini et al., 2020; Corsolini et al., 2007; Storelli et al., 2012). For all samples, among dl-PCB the highest concentration was of PCB-118, which was considered an indicator PCB (Table S6).

To BB samples the octa and Deca mixture compound concentrations (BDE-183, -209) were lower than LOD. Finally, among NBFRs, only PBEB was found at concentrations higher than the LOD in 4 samples. The

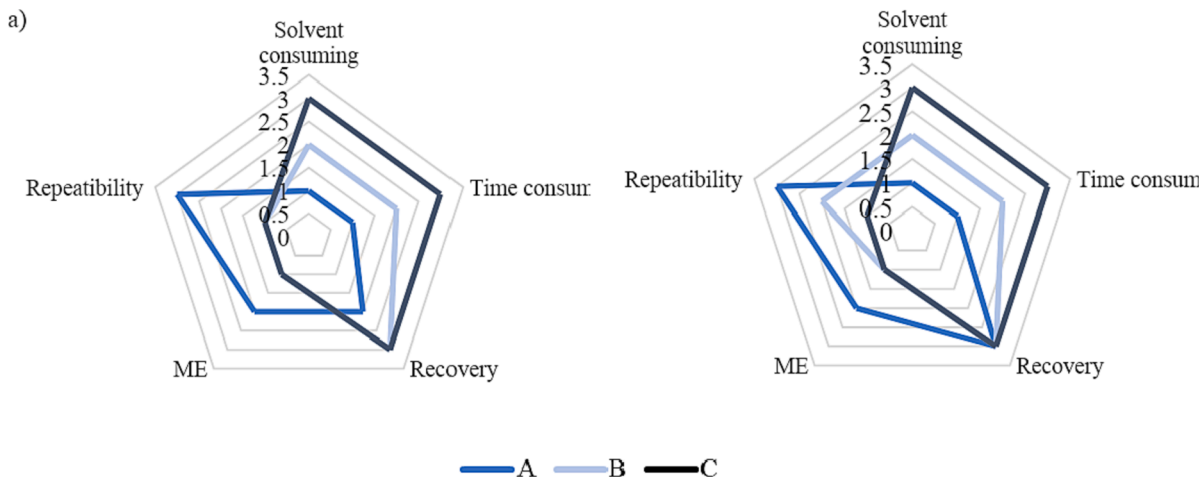

Fig. 3. The figure shows the values of each of the 5 categories considered, assigning an increasing value as the quality of the data. a) represents the average parameters at low (0.5 ng/g) and b) high concentrations (75.0 ng/g). The allocation criterion is described in SI (Table S5).

analyte peaks resulted in high resolution and low background noise, this allowed for effective evaluation using method C. Again, for SP species, the background noise did not allow the identification of some NBFRs and PBDEs. All analyte concentrations in SP samples were higher than in BB. About PCBs, in the 5 analyzed samples, PCB 153 was the most abundant with concentrations between 1.788E+02 ng/g and 1.771E+03 ng/g ww. Among the samples, SP-2 appears to be the one with the lowest concentrations of PCBs. Furthermore, the levels of dl-PCB were also higher than those of BB. In general, the determination of PCBs did not encounter problems during the chromatography analysis and the peaks of the analytes present were well resolved. For the PBDEs of the penta mixture, BDE-28 was found in the SP-05 sample at 3.3 ng/g ww, the highest concentration (Table S6). In the same sample, traces of BDE-99 and 183 were also found. However, in the other samples the presence of BDE-47, -100, -99, -154 was found. Instead, BDE-153 and BDE-209 ~~was~~ were found only in the SP_03 sample at 1.219E+01 ng/g and 8.715 ng/g ww Traces higher than the LOD of the octa mixture (BDE-183) were found in SP-03, −04, −05, −06 PBDE concentrations in the analysed samples were compared with other fish samples from the Mediterranean Sea and the results, considering the variability of concentrations in biotic samples, were similar (Ben Ameur et al., 2013; Borghesi et al., 2009; Koenig et al., 2013; Pizzini et al., 2015). Among the NBFRs, PBEB and DPTE were the most concentrated: 2.816E+01 and 4.239E+01 ng/g ww, respectively. PBT was found only in SP-5, which showed the presence of all NBFRs analyzed. The other analytes present in all SPs were EHTBB and HBBZ with concentrations between 1.421 and 1.171E+01 ng/g ww and between 1.501 and 9.037 ng/g ww, respectively (Table S6). The chromatographic profiles of SP samples showed lower resolution for BDE and the NBFRs at the end of the chromatographic column. Consequently, the low resolution was affected by the evaluation of the presence of these analytes.

Focusing on the results of phthalates, the analysis highlights the presences of 9 out of 11 analytes investigated, DChP and DINP were below the detection limit in all samples (Table S6). The contamination fingerprint identified corresponds to observations made in other fish species in a different region of the Mediterranean Sea (Rios-Fuster et al., 2022).

In bogue, the most abundant PAEs was DEHP followed by DIBP and DBP. Moving on to the European pilchard, the analysis demonstrated elevated concentrations of several analytes compared to the bogue fishes. DEP, DAP, DIBP, DEHP, BBzP, and DNOP were all detected (Table S6).

The presence of these compounds in the fillets of these fish can be attributed to the potential leaching of these plasticizer by microplastics ingestion during their life cycle (Paluselli et al., 2019).

4. Conclusions

This study focuses on the comparison of three analytical methods for the extraction and purification of PCBs, PBDEs, NBFRs and PAEs in edible biotic matrices. The evaluation considered different validation parameters for these compounds. All three methods showed compliance with the method validation parameters for PCBs, PBDEs, 5 out of 7 NBFRs and PAEs. The traditional approach with Soxhlet extraction and silica gel purification (method A) showed lower MEs and higher reproducibility compared to the other methods tested. Average recovery rates were similar for all three methods. However, the choice of a method is not only determined by statistical considerations, but also involves an analytical trade-off between efficiency and sustainability of the process. In this regard, ultrasonic extraction combined with dispersive solid phase extraction (d-SPE) as a clean-up method offers a suitable compromise that meets these requirements by significantly reducing extraction and clean-up times by approximately 74–80 % and solvent consumption by approximately 94–97 %. The method also minimises sample handling and uses user-friendly instrumentation. Furthermore, it optimises the use of laboratory space by allowing the simultaneous handling of multiple samples.

In addition, the analytical parameters for some NBFRs could not be reliably determined using method A. While precision and repeatability parameters generally favour method A, methods B and C still show acceptable performance in these aspects. Further investigations are needed to evaluate the effectiveness of these methods on samples with higher lipid content.

In conclusion, the proposed novel method involving ultrasonic extraction and d-SPE represents a viable alternative to the traditional method, offering improved efficiency and sustainability in the analysis of PCBs, PBDEs, NBFRs and PAEs in edible biotic matrices. The presence, concentration and significance of contaminants, both emerging and legacy, were identified in bogue and European pilchard, commercially valuable species. Such data underlines the importance of monitoring and managing the presence of pollutants substances for the protection of both the marine environment and humans, and underline the need to develop a methodology capable of efficiently and optimally co-extracting these substances. The C method developed in this study has the potential to be used not only in routine monitoring of fish species but also in environmental investigations.

CRediT authorship contribution statement

Saul Santini: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data

curation, Conceptualization. **Matteo Baini:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Tania Martellini:** Writing – review & editing, Writing – original draft, Visualization, Validation, Conceptualization. **Matteo Bissoli:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Matteo Galli:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Margherita Concato:** Writing – review & editing, Formal analysis, Data curation. **Maria Cristina Fossi:** Writing – review & editing, Visualization, Validation, Supervision, Funding acquisition, Conceptualization. **Alessandra Cincinelli:** Writing – review & editing, Visualization, Validation, Supervision, Funding acquisition, 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.foodchem.2024.138582.

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