


# A GC-EI-MS/MS Method for the Determination of 15 Polybrominated Diphenyl Ethers (PBDEs) in Fish and Shellfish Tissues

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**Abstract** This paper describes the development and validation of an analytical method to determine 15 polybrominated diphenyl ethers (PBDEs) in fish and shellfish tissues with gas chromatography coupled to triple quadrupole mass spectrometry using electron impact source (GC-EI-MS/MS). A QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe)-like procedure was applied; the extract was cleaned up on an acidic Extrelut-NT3 column connected on top of a silica cartridge and further purified with gel permeation chromatography (GPC). The SIM acquisition proved to yield better results than traditional MRM approach for octa-, nona-, and

deca-brominated congeners. The validation study was carried out in inter-laboratory conditions. The average recoveries were in the range 66–118%, with relative standard deviations generally lower than or equal to 22%. Quality assurance and quality control practices were thoroughly described and highlighted. These long and tedious practices are fundamental to obtain reliable quantitative data during food monitoring programs and, therefore, to support daily intake assessments. Finally, the procedure was applied to the analysis of marine fish samples collected in local markets. Mackerel and mullet were the most contaminated species.

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**Keywords** GC-EI-MS/MS · Polybrominated diphenyl ethers (PBDEs) · Fish · Shellfish · Validation study

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## Introduction

Polybrominated diphenyl ethers (PBDEs) are one of the classes of brominated flame retardants (BFRs) used as additives in a wide variety of commercial and industrial products (furnishings, textiles, paints, polymers, and electronic equipments) to retard or prevent the possible ignition of fire. They are usually added to plastics at concentrations between 5 and 30% by weight as additives not chemically bonded to the polymer (EFSA 2011). Their basic chemical structure consists of two phenyl rings variably substituted by bromine atoms and linked by an ether bond. Two hundred nine different molecules (congeners) may be synthesized, marketed in three major technical mixtures, and named penta-BDE, octa-BDE, and deca-BDE, according to their average bromine content. These lipophilic and poorly degradable organic pollutants are, therefore, released in the environment during production, use, and disposal of PBDEs containing products, causing

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bioaccumulation in animals and humans (Fromme et al. 2016). Literature data show a substantial evidence of PBDE contamination in foods of animal origin, as a result of their bio-accumulative properties (EFSA 2011). The diet represents the main route for human exposure and fish significantly contributes to the total human intake. Recently, the European Food Safety Authority (EFSA) requested an update on PBDEs occurrence in food, in order to review the scientific opinion on the dietary exposure and on human health risk assessment. The main concern is based on the evidence that these compounds could have dioxin-like properties and exhibit toxic effects on the endocrine system and thyroid function and delay early neurodevelopment of animals. Accordingly, in 2003 the European Union (EU) banned the use and applications of penta- and octa-BDE technical mixtures and, in 2008, the deca-BDE mixture, too (Dir EC 11/2003; Cases C-14/06/2008).

The need for reliable and effective validated analytical methods enabling the analysis of these contaminants in the various food matrices is evident. Many European countries such as Norway, Belgium, the Netherlands, and Sweden have already developed PBDE analytical procedures for a long time; on the contrary, in Italy, very few laboratories are able to analyze PBDEs, and therefore, the knowledge on the PBDE environmental and food contamination levels is poor.

In March 2014, the European Union issued Recommendation 118/2014 (EU 118/2014) in which Member States were encouraged to monitor five different classes of BFRs in food, in order to give a better estimation of human exposure upon food consumption. The Recommendation foresees the analysis of ten PBDE congeners of primary interest: BDE-28, BDE-47, BDE-49, BDE-99, BDE-100, BDE-153, BDE-154, BDE-183, and BDE-209. Usually, their determination is carried out using gas chromatography (GC) coupled with different detectors and sources like electron ionization high-resolution mass spectrometry (EI-HRMS), electron capture ionization (ECD), negative chemical ionization mass spectrometry (NCI-MS), and electron ionization tandem mass spectrometry (EI-MS/MS or EI-QqQ).

The aim of this work was the development of a method for the identification and quantification of PBDEs in fish and shellfish tissues in the light of the possible imminent setting of limits by European Union. The applied technique was GC-EI-MS/MS, available in most of EU official laboratories involved in contaminant analysis. The method allowed the simultaneous determination of 15 PBDEs (28, 47, 49, 66, 77, 85, 99, 100, 138, 153, 154, 183, 197), including the heaviest BDE-206 and BDE-209 in a single chromatographic run using a 15-m-long column. A QuEChERS-like (Quick, Easy, Cheap, Effective, Rugged, and Safe) extraction followed by a two-step cleanup and a large volume injection (10  $\mu$ L) enabled to reach limits of quantification generally below 10  $\text{pg g}^{-1}$  for most analytes. It must be underlined that

PBDE analysis is plagued with many analytical problems compared to other contaminant classes due to the low required limits of quantification ( $\leq 10 \text{ pg g}^{-1}$ ) and the well-known issue of the background laboratory contamination, since PBDEs are ubiquitous pollutants dispersed in dust. Therefore, laboratory decontamination procedures have been described thoroughly here together with all the quality control practices implemented in order to minimize and continuously monitor this phenomenon. The method was fully validated and applied to the analysis of 35 marine fish samples collected from local markets.

## Materials and Methods

### Reagents and Standards

Individual PBDE congeners 49, 66, 77, 85, 138, 197, 206, and the 77- $^{13}\text{C}_{12}$  and 138- $^{13}\text{C}_{12}$ -labeled mixture (purity >98%) were supplied by Wellington Laboratories (Guelph, Ontario, Canada). The Labeled Surrogate Stock Solution Mixture of Internal Standards (IS) (28, 47, 99, 100, 153, 154, 183, 209,  $^{13}\text{C}_{12}$ -labeled, purity 99%; code EO-5277), the Syringe Standard (SS) Mixture (77 and 138;  $^{13}\text{C}_{12}$ -labeled, purity 99%; code EO-5275), and the Native Stock Solution Mixture (28, 47, 99, 100, 153, 154, 183, 209, purity >98%; code EO-5278) were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). All the solvents were pesticide grade. Cyclohexane, dichloromethane, ethyl acetate, and *n*-hexane were supplied from Carlo Erba Reagents (Rodano, Milano, Italy). Fluka isooctane was from Sigma-Aldrich (Steinheim, Germany). Bio-Beads S-X3 200–400 mesh was purchased from Bio-Rad Laboratories (Switzerland), Extrelut-NT3 columns were supplied from Merck (Darmstadt, Germany), and the Isolute silica 2 g/6 mL columns from Biotage (Uppsala, Sweden). Magnesium sulfate and sodium chloride for the QuEChERS-like extraction were delivered from Sigma-Aldrich (Steinheim, Germany).

### Sample Preparation

Twenty grams of homogenized tissue (fish or molluscs) were spiked with the eight ISs (1 ng each and 10 ng of  $^{13}\text{C}_{12}$ -BDE-209), mixed with 5 mL of ultrapure water, and shaken vigorously with 15 mL of ethyl acetate in a polypropylene centrifuge tube. Subsequently, 3 g of sodium chloride and 6 g of anhydrous magnesium sulfate were added to the mixture. After further shaking and centrifuging, an aliquot of 10 mL was taken from the upper organic layer and reduced in volume at 35 °C under vacuum using the Genevac EZ-2 centrifuge (SP Scientific, Ipswich, Suffolk, UK). Solid phase extraction (SPE) Isolute 2 g/6 mL silica cartridges were conditioned with 12 mL of *n*-hexane/dichloromethane (3:1, v/v) and 4 mL of *n*-

hexane. The Extrelut NT-3 column was loaded with 3 mL of concentrated sulphuric acid, kept standing for about 1 h, and connected on top of the preconditioned silica cartridge. Both columns were washed with 7 mL of *n*-hexane. The sample residue was redissolved in  $3 \times 1$  mL of *n*-hexane, loaded on top of the Extrelut NT-3 column, and the analytes eluted with 7 mL of *n*-hexane, collecting all solvents in a 25-mL glass tube. The Extrelut column was then disconnected and the silica further eluted with 10 mL of *n*-hexane/dichloromethane (3:1, *v/v*). The solvent was reduced to ca. 0.5 mL at 40 °C under a gentle nitrogen stream. The extract was brought to 2 mL with cyclohexane/dichloromethane (1:1, *v/v*) and 1.5 mL were injected on top of a Bio-Beads S-X3 packed column (500 mm  $\times$  10 mm). The gel permeation chromatography (GPC) was run on a Gilson GPC system equipped with an ASPEC Xli for injection and fraction collection and a 307 HPLC system with UV-VIS detector (Gilson Wisconsin, USA). The mobile phase (cyclohexane/dichloromethane 1:1, *v/v*) was run at 1 mL min<sup>-1</sup>. The purification runtime was 50 min, and the fraction collected was between 25 and 43 min. The collected fraction was evaporated under a gentle nitrogen stream and finally dissolved in 250  $\mu$ L of the mixture of the two SS at 2 ng mL<sup>-1</sup> prior to GC injection.

#### Instrumental Determination by GC-EI-MS/MS

The analysis was performed on a 7000B GC-triple-quadrupole analyzer (MS/MS) system (Agilent Technologies (Palo Alto, CA, USA) equipped with 7890A GC oven, programmable temperature vaporization (PTV) inlet, and a 7693 series automatic liquid sampler. Ten microliters of the purified extract in isooctane were injected. The PTV operated in solvent vent mode, without CO<sub>2</sub>, cooling programming the temperatures ramps as follow: start at 50 °C, 0.5 min at 50 °C, ramp at 600 °C/min until 325 °C. Vent flow was 50 mL/min, pressure 5 psi for 0.5 min, purge flow to split vent was 50 mL/min after 2 min. The chromatographic separation was achieved in programmed temperature mode using helium as carrier gas at 1 mL min<sup>-1</sup> and the DB-5HT column (Agilent Technologies, 15 m  $\times$  250  $\mu$ m  $\times$  0.10  $\mu$ m). The oven temperature program was set as follows: 50 to 80 °C (15 °C/min), 80 to 220 °C (60 °C/min), 220 to 250 °C (30 °C/min), 250 to 270 °C (5 °C/min), and 270 to 325 °C (10 °C/min). The final temperature was maintained for 5 min. Measurements were carried out in multiple reaction monitoring (MRM) mode, except for BDE-197, BDE-206, and BDE-209 (Selected Ion Monitoring, SIM). Ion source, quadrupole and interface temperatures were 230, 150, and 320 °C, respectively. Dwell times were selected and acquisition windows adjusted to optimize the acquisition frequency to get at least ten data points per peak. Calibration and instrument tune were performed in the EI high-sensitivity autotune mode every 2 weeks. According to international rules, for each analyte

two MRM transitions were selected or three ions in case of SIM acquisition (EU 589/2014). The ionization energies were also optimized. Quantitation was performed only with one transition, the target transition; the qualifier transition was used only for identification purpose verifying the relative ion ratio. The ratio measured in the sample was compared to that measured in the standard solution at similar concentration. The retention times and the mass spectrometric parameters are in Table 1.

#### Method Validation

To date, the European Commission did not set neither maximum limits for PBDEs nor specific rules to assess method performances for their determination in food. Therefore, Regulation 589/2014 (EU 589/2014) laying down methods of sampling and analysis for the control of levels of dioxins and polychlorinated biphenyls (PCBs) in food has been followed to evaluate the fitness for purpose of the procedure here developed. In addition, the quantification limits (lower than or equal to 10 pg g<sup>-1</sup>) required by Recommendation 118/2014 (EU 118/2014) were taken into account. Accordingly, the validation scheme (spiking levels) was scheduled starting from this concentration, except for BDE-206 and BDE-209 for which this level was not considered technically achievable with the available equipment.

#### Linearity

The linearity was investigated injecting standard mixtures of the PBDE congeners at 0.05, 0.1, 0.2, 0.5, 1.0, 5.0 and 10 ng mL<sup>-1</sup> in isooctane (BDE-206 and BDE-209: tenfold more concentrated). The eight labeled surrogate internal standards were added at 2 ng mL<sup>-1</sup> (<sup>13</sup>C12-BDE-209: 20 ng mL<sup>-1</sup>).

#### Accuracy (Trueness and Precision)

Replicate analysis of uncontaminated Chilean mussels, i.e., with negligible PBDE contamination, was performed at six increasing concentrations (10, 20, 100, 200, 400, and 600 pg g<sup>-1</sup>) in inter-laboratory reproducibility conditions by the two laboratories of the Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche (Ancona and Perugia) using identical GC-EI-MS/MS equipments. For PBDE-206 and 209, the validation levels were systematically tenfold higher (100, 200, 1000, 2000, 4000, and 6000 pg g<sup>-1</sup>). Each laboratory analyzed three or four replicates per level in two different batches. Therefore, a total of 42 independent experiments (seven replicates for each of the six validation levels) were performed by each laboratory for a total of 84 replicates. Precision (repeatability and inter-lab reproducibility) was estimated at each level

**Table 1** GC-EI-MS/MS acquisition parameters

Congener	RT (min)	Precursor <sup>a</sup> ( <i>m/z</i> )	Product <sup>a</sup> ( <i>m/z</i> )	CE (V)	iLOD <sup>b</sup> (pg)	iLOQ <sup>b</sup> (pg)		
BDE-28	6.64	408	[M] <sup>+</sup>	248	[M-Br <sub>2</sub> ] <sup>+</sup>	20	0.2	0.5
		406	[M] <sup>+</sup>	246	[M-Br <sub>2</sub> ] <sup>+</sup>	21		
<sup>13</sup> C12-BDE-28		420	[M] <sup>+</sup>	260	[M-Br <sub>2</sub> ] <sup>+</sup>	16		
BDE-49	7.23	486	[M] <sup>+</sup>	326	[M-Br <sub>2</sub> ] <sup>+</sup>	24	0.2	0.5
		484	[M] <sup>+</sup>	324	[M-Br <sub>2</sub> ] <sup>+</sup>	24		
BDE-47	7.36	486	[M] <sup>+</sup>	326	[M-Br <sub>2</sub> ] <sup>+</sup>	24	0.2	0.5
		484	[M] <sup>+</sup>	324	[M-Br <sub>2</sub> ] <sup>+</sup>	24		
<sup>13</sup> C12-BDE-47		498	[M] <sup>+</sup>	338	[M-Br <sub>2</sub> ] <sup>+</sup>	10		
BDE-66	7.46	486	[M] <sup>+</sup>	326	[M-Br <sub>2</sub> ] <sup>+</sup>	34	0.2	0.5
		484	[M] <sup>+</sup>	324	[M-Br <sub>2</sub> ] <sup>+</sup>	24		
BDE-77	7.63	486	[M] <sup>+</sup>	326	[M-Br <sub>2</sub> ] <sup>+</sup>	24	0.3	1
		484	[M] <sup>+</sup>	324	[M-Br <sub>2</sub> ] <sup>+</sup>	17		
<sup>13</sup> C12-BDE-77		498	[M] <sup>+</sup>	338	[M-Br <sub>2</sub> ] <sup>+</sup>	24		
BDE-100	7.86	566	[M] <sup>+</sup>	406	[M-Br <sub>2</sub> ] <sup>+</sup>	17	0.3	1
		404	[M-Br <sub>2</sub> ] <sup>+</sup>	297	[M-CBr <sub>3</sub> O] <sup>+</sup>	34		
<sup>13</sup> C12-BDE-100		576	[M] <sup>+</sup>	416	[M-Br <sub>2</sub> ] <sup>+</sup>	38		
BDE-99	8.02	566	[M] <sup>+</sup>	406	[M-Br <sub>2</sub> ] <sup>+</sup>	28	0.3	1
		404	[M-Br <sub>2</sub> ] <sup>+</sup>	297	[M-CBr <sub>3</sub> O] <sup>+</sup>	35		
<sup>13</sup> C12-BDE-99		576	[M] <sup>+</sup>	416	[M-Br <sub>2</sub> ] <sup>+</sup>	38		
BDE-85	8.31	566	[M] <sup>+</sup>	406	[M-Br <sub>2</sub> ] <sup>+</sup>	17	0.3	1
		404	[M-Br <sub>2</sub> ] <sup>+</sup>	297	[M-CBr <sub>3</sub> O] <sup>+</sup>	34		
BDE-154	8.38	644	[M] <sup>+</sup>	484	[M-Br <sub>2</sub> ] <sup>+</sup>	20	0.3	1
		482	[M-Br <sub>2</sub> ] <sup>+</sup>	375	[M-CBr <sub>3</sub> O] <sup>+</sup>	39		
<sup>13</sup> C12-BDE-154		656	[M] <sup>+</sup>	496	[M-Br <sub>2</sub> ] <sup>+</sup>	26		
BDE-153	8.61	644	[M] <sup>+</sup>	483.8	[M-Br <sub>2</sub> ] <sup>+</sup>	20	0.3	1
		482	[M-Br <sub>2</sub> ] <sup>+</sup>	375	[M-CBr <sub>3</sub> O] <sup>+</sup>	39		
<sup>13</sup> C12-BDE-153		656	[M] <sup>+</sup>	496	[M-Br <sub>2</sub> ] <sup>+</sup>	26		
BDE-138	8.91	644	[M] <sup>+</sup>	484	[M-Br <sub>2</sub> ] <sup>+</sup>	20	0.3	
		482	[M-Br <sub>2</sub> ] <sup>+</sup>	375	[M-CBr <sub>3</sub> O] <sup>+</sup>	39		
<sup>13</sup> C12-BDE-138		656	[M] <sup>+</sup>	496	[M-Br <sub>2</sub> ] <sup>+</sup>	26		
BDE-183	9.18	722	[M] <sup>+</sup>	562	[M-Br <sub>2</sub> ] <sup>+</sup>	17	0.5	2
		562	[M-Br <sub>2</sub> ] <sup>+</sup>	455	[M-CBr <sub>3</sub> O] <sup>+</sup>	35		
<sup>13</sup> C12-BDE-183		734	[M] <sup>+</sup>	574	[M-Br <sub>2</sub> ] <sup>+</sup>	20		
BDE-197 <sup>b</sup>	9.82	802	[M] <sup>+</sup>				0.5	2
		800	[M] <sup>+</sup>					
BDE-206 <sup>b</sup>	10.90	722	[M-Br] <sup>+</sup>				2	7
		642	[M-Br <sub>3</sub> ] <sup>+</sup>					
BDE-209 <sup>b</sup>	12.42	720	[M-Br <sub>2</sub> ] <sup>+</sup>				5	17
		722	[M-Br <sub>2</sub> ] <sup>+</sup>					
<sup>13</sup> C12-BDE-209		802	[M-Br <sub>2</sub> ] <sup>+</sup>					
		800	[M-Br <sub>2</sub> ] <sup>+</sup>					
		720	[M-Br <sub>3</sub> ] <sup>+</sup>					
		812	[M-Br <sub>2</sub> ] <sup>+</sup>					

<sup>a</sup> In italics, the transition used as quantifier <sup>b</sup> iLOD and iLOQ: instrumental detection and quantification limits measured in solvent (injected pg); SIM acquisition

applying ANOVA (EURACHEM 2014). Trueness was measured by apparent recoveries, i.e., the recoveries obtained considering the ratio between the native analyte and its corresponding labelled internal standard.

### Method Limits

After the acquisition of four calibration curves, instrument limits of detection (iLODs) and quantification (iLOQ) were

estimated by multiplying the standard deviation (SD) measured at the first calibration level by three and by ten, respectively. Method LODs (MDLs) and LOQs (MQLs) were estimated by analyzing 60 procedural blanks acquired during the analysis of routine batches as described in our previous work (Piersanti et al. 2015).

### Quality Assurance/Quality Control (QA/QC)

It has been thoroughly demonstrated that the QA/QC program is fundamental to assure the reliability of PBDE analysis (Papke et al. 2004; Vonderheide 2009; Krol et al. 2012; Kalachova et al. 2013). If this is an obvious concept for all the chemical analysis, as mentioned before, the low levels of PBDE in food (parts per trillion) and the concentrations occurring in laboratory blanks exacerbate this aspect. In Table 2, the QC samples planned in each analytical batch are detailed (internal quality assurance). The two spiked samples used to build Shewhart and R-charts as well as the matrix-matched standard were prepared using blank mussels from Chilean coast. External quality assurance was guaranteed by the participation in inter-calibration exercises organized by the European Union Reference Laboratory for Dioxins and PCBs in Feed and Food (EURL, State Institute for Chemical and Veterinary Analysis of Food, Freiburg, Germany) in fish liver and fish liver oil during 2014 and by the Norwegian Institute of Public Health (NIPH) in salmon in 2015.

### Data Analysis

Stata 11.0 (StataCorp., College Station, TX, USA) was used for the data analysis. The differences among fish species were assessed by ANOVA. A *p* value of 0.05 (2-tailed) was chosen as criterion for statistical significance.

### Fish Sample Collection

After the validation study, the method was applied to the analyses of 35 marine fish. Fresh or frozen samples belonging to seven species were collected from local markets: six anchovy, five cod, four flounder, six mackerel, five mullet, six sole, and three tuna samples were analyzed. The species were selected taking into account the local population consumption habits.

## Results and Discussion

### Optimization of Sample Preparation

Preliminary experiments on the preparative method were carried out following the suggestion proposed by Kalachova et al. (2013) for the analysis of PBDEs in fish. The hydrophobic target analytes were transferred from aqueous sample

suspension (partition supported by inorganic salts) into ethyl acetate (modified QuEChERS). The organic phase was then purified on prepacked SPE-Si columns (2 g/6 mL). The conditioning, sample loading, and elution volumes were optimized using hexane as loading solvent and hexane/dichloromethane (3:1, *v/v*) for analytes recovering. However, the obtained chromatograms were not sufficiently clean and the whole method was not robust enough, indicating the need of a further purification. On the other hand, in order to improve the limits of quantification (MQLs), the amount of analyzed matrix was doubled compared to the Kalachova's method. Then a H<sub>2</sub>SO<sub>4</sub> acidic Extrelut-NT3 cartridge (large-pore diatomaceous material) was connected in sequence with the Si-SPE column obtaining better recovery and precision. Nevertheless, shifts in retention times were still noticed, indicating a not negligible matrix effect (Fig. 1). Finally, the introduction of a third cleanup step based on GPC allowed the achievement of the suitable robustness also for the most troublesome heavy congeners (BDE-206 and -209). Even Labadie et al. (2010) documented the detrimental effects of an insufficient sample purification on the stability of retention times.

The use of isotopic dilution methodology is always advisable for a procedure which needs to detect low parts per trillion involving laborious and long sample treatment. Therefore, the eight EFSA <sup>13</sup>C<sub>12</sub>-congeners were added at the beginning of the sample treatment: Their use enables quantification with automatic correction of analyte losses. For PBDEs without the corresponding labelled analogues (BDE-49, BDE-66, BDE-77, BDE-85, BDE-138, BDE-197, and BDE-206), a <sup>13</sup>C<sub>12</sub>-labelled congener with the same degree of bromination was used as IS. The two SSs were also introduced to correct for inter-injection fluctuations and to assess the recoveries of the eight labelled congeners. Moreover, in each analytical batch, a matrix-matched standard (an “uncontaminated” mussel sample processed along with the samples and finally spiked with the eight <sup>13</sup>C<sub>12</sub>-PBDE and the two SSs) was processed, too (Table 2).

### Optimization of the GC–MS/MS Parameters

The GC column was selected taking into account the thermal degradation of highly brominated congeners: a short column helps to prevent this phenomenon. The column should be also resistant to the high temperatures, necessary to elute the high boiling PBDEs. Thus, the 15-m DB-5HT capillary column ((5%-phenyl)-methylpolysiloxane, 0.25 mm i.d., 0.1 μm film thicknesses), resistant up to 400 °C, was chosen. The injection volume was optimized in order to introduce in the PTV injector as much purified extract as possible. It was experimentally demonstrated that 10 μL did not involve any detrimental effect on peak shapes, enhancing the instrumental response almost ten times in respect of the 1 μL injection; therefore, this volume was set in the final method.

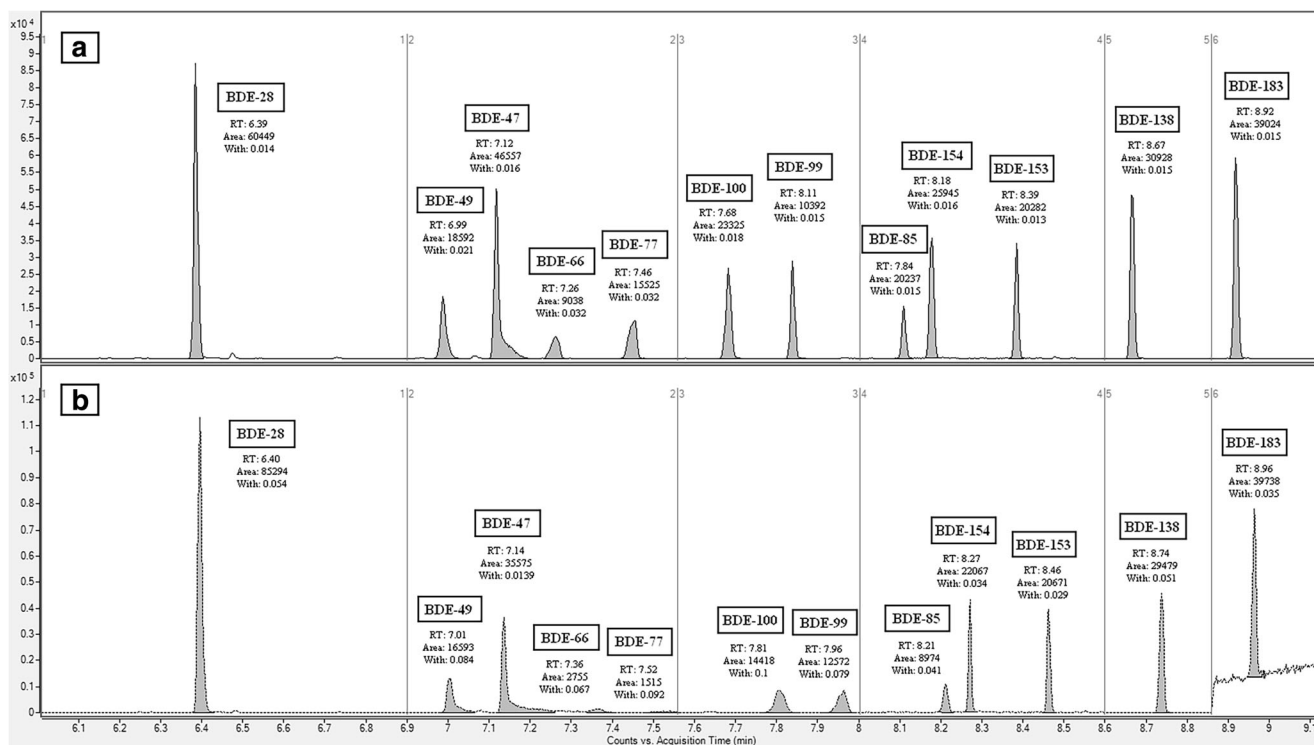
**Table 2** Internal QC program of each analytical batch (12 samples)

QC description	Frequency per batch	QC preparation <sup>a</sup>	Scope	Chart type
Calibration curve		Six-points solvent calibration curve	Quantification of unknown samples Instrument performance check	—
Matrix-matched standard	1	Twenty grams of blank mussels are taken through the entire analytical process. The 15 native congeners and the eight labeled ISs are added immediately prior to GC injection (concentration in vial 0.88 and 2.2 ng mL <sup>-1</sup> , respectively)	Quantification of IS recoveries	—
Procedural blank	2	Fifty microliters of a solution at 20 ng mL <sup>-1</sup> of the eight labelled ISs are added in a falcon tube without matrix and taken through the entire analytical process	Laboratory contamination control	X-chart
Duplicate spiking at 20 pg g <sup>-1</sup> of a blank sample	2	Fifty microliters of a solution at 20 ng mL <sup>-1</sup> of the ISs and 40 µL of a solution at 10 ng mL <sup>-1</sup> of the native congeners are added to 20 g of blank Chilean mussels and taken through the entire analytical process	Method accuracy evaluation	X- and R-chart

<sup>a</sup> For native BDE-206 and BDE-209 and labelled BDE-209-L, the concentrations were tenfold higher

In trace analysis of food, co-eluting isobaric matrix interferences usually make MRM the technique of choice to achieve method selectivity. In tandem mass spectrometry, two MRM transitions are universally required to have definitive confirmation of the compound identity (EU 589/2014). The EI spectra of tri- and tetra-brominated congeners are dominated by the ions corresponding to the molecular cluster

$[M]^+$ , whereas for the higher brominated compounds they are dominated by the cluster corresponding to the loss of two bromine atoms  $[M-Br_2]^+$  (Hites 2008). Therefore, for congeners BDE-28, BDE-49, BDE-47, BDE-66, and BDE-77 (tri- and tetra-brominated), the molecular ion was chosen as the precursor of both transitions (Table 1). For the highest brominated PBDEs (from five- to nona-),  $[M]^+ > [M-Br_2]^+$



**Fig. 1** **a** Chromatogram of a mussel sample after the optimization of the cleanup steps; **b** chromatogram of the same mussel sample before the optimization (without GPC purification)

and  $[M-\text{Br}_2]^+ > [M-\text{CBr}_n\text{O}]^+$  ( $n = 3$  or  $5$ ) were the most monitored transitions. Finally, for BDE-209, almost all the authors selected an ion from  $[M-\text{Br}_2]^+$  or  $[M-\text{Br}_4]^+$  cluster as precursor (Labadie et al. 2010; Sanchez-Avila et al. 2011; Cristale et al. 2012; Mackintosh et al. 2012; Kalachova et al. 2013; Garcia-Bermejo et al. 2016). An overview of the transitions reported in literature for the 15 congeners included in this work is shown in Table S1.

The acquisition method was optimized starting from Kalachova et al. (2013). Adjustments were carried out injecting the solutions of each individual standard (the 15 native PBDEs and the  $10^{13}\text{C}_{12}$ -labelled surrogate and syringe standards) in full-scan mode (50–1000  $m/z$ ). Precursor ions were subjected to different collision energies (5, 10, 15, 20, 30, and 40 eV) to generate the subsequent product ions. The transition with the highest intensity was selected as quantitative (Q) and a second one as confirmation (q). The relative intensity ratios expressed as percentage ( $q/Q \times 100$ ) were calculated using the MassHunter Workstation Software (Agilent Technologies). The maximum permitted tolerances adopted for abundance ratios of the selected mass fragments were the ones listed in Regulation 589/2014 (EU 589/2014).

It is worth to note that for BDE-197 (octa-brominated), BDE-206 (nona-brominated), and BDE-209 (deca-brominated), the SIM acquisition was preferred with respect to the MRM, enabling lower instrumental limits (Table 1). Since the highly brominated compounds fragment extensively in the EI source, they give low fragmentation yields in the collision cell of triple quadrupoles, and therefore, the MRM responses are scarce. In order to choose the best acquisition mode for the four heaviest congeners, comparative experiments were carried out in mussel samples spiked at levels close to the method quantification limits (Supplementary Material Table S2). Except for BDE-183, SIM acquisition gave better responses (S/N, signal-to-noise ratio) than MRM. For BDE-197, the second transition ( $m/z$  802  $>$   $m/z$  642) was not detectable at 10  $\text{pg g}^{-1}$ . Obviously, SIM is less selective, but method selectivity is affected by both the instrumental technology and the sample treatment protocol and the here adopted multiple clean-up strategy allowed sufficient selectivity also when SIM acquisition was applied. Finally, the pseudo multiple reaction monitoring (pseudo MRM) was experimented, too. In pseudo MRM, the two quadrupoles monitor the same  $m/z$ ; this technique was successfully applied, for example, in the PAH determination (Shang et al. 2014). The sensitivities using pseudo MRM were similar to those obtained with the application of SIM (data not shown).

### Quality Assurance/Quality Control (QA/QC)

PBDEs have been widely used as additives to avoid or slow down the ignition of fire in various consumer products, and they are widely distributed everywhere also in analytical

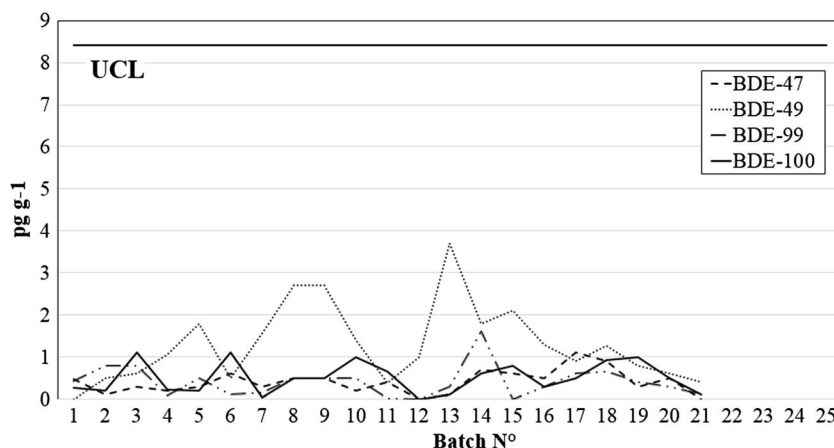
laboratories because of the building material used as well as the presence of electronic equipments in which BDEs have been added as flame retardants. Therefore, as a result of this “unavoidable” blank contamination, each laboratory has to deal with this tricky issue when starting with PBDE analysis. Additional labware, fume hood, instrument, and bench decontamination procedures were then implemented until the reaching of negligible levels compared to PBDE incurred concentrations (Piersanti et al. 2015). Moreover, during routine analysis, the laboratory contamination was continuously checked by procedural blanks (two within each analytical batch), as summarized in Table 2. The method repeatability was monitored building R-Charts of four congeners (BDE-49, BDE-47, BDE-99, and BDE-100) (Fig. 2). This kind of chart was built measuring the difference ( $r$ ) between the results obtained from the two QC samples spiked at 20  $\text{pg g}^{-1}$  (Table 2). The upper control limit was fixed following the fitness for purpose criterion (target value charts) (Funk et al. 2007), establishing as maximum acceptable difference the maximum relative standard deviation in repeatability conditions, i.e., 15% (Horwitz-Thomson equation). Hence,  $r = (2 \cdot \sqrt{2}) \cdot s_r = 2.8 \cdot 3 \text{ pg g}^{-1} = 8.4 \text{ pg g}^{-1}$ . As shown in Fig. 2, all the analytical batches were compliant; among the four congeners, BDE-49 demonstrated the highest variability due to the lack of the corresponding labelled IS. For X-chart (Shewhart chart), the control limits were fixed considering suitable apparent recovery values between 80 and 120% (from 16 to 24  $\text{pg g}^{-1}$ ). The spiking level was 20  $\text{pg g}^{-1}$  for all the congener except for BDE-206 and BDE-209 (200  $\text{pg g}^{-1}$ ). Also in this case, the congeners without the corresponding labelled IS showed lower accuracy (control limits were fixed between 70 and 130%).

### Method Validation

Good linear responses were observed in the range from 0.05 to 10  $\text{ng mL}^{-1}$  for all congeners, except for BDE-197 (0.2–10  $\text{ng mL}^{-1}$ ), BDE-206, and BDE-209 (0.5–50  $\text{ng mL}^{-1}$ ). The coefficients of determination ( $R^2$ ) were all higher than 0.999.

As mentioned, two kinds of limit were estimated: (i) instrument limits of detection and quantitation (iLODs and iLOQs), which represents the instrument performances; (ii) method limits of detection and quantitation (MDL and MQL), which are the “real” LOD and LOQ of the method when it is applied to real samples taking into account possible matrix effects, analyte losses during sample treatment and laboratory contamination (L'Homme et al. 2015). As listed in Table 1, the estimated iLODs and iLOQs were in the ranges 0.2–5 and 0.5–17 picograms injected, respectively. These values were obtained using the SD observed from replicate injections of the lowest calibration level: 0.05  $\text{ng mL}^{-1}$  for all congeners

**Fig. 2** R-charts of BDE-47, BDE-49, BDE-99, and BDE-100 evaluated in the two spiked samples at  $20 \text{ pg g}^{-1}$  carried out in each analytical batch



except BDE-197 ( $0.2 \text{ ng mL}^{-1}$ ), BDE-206 ( $0.5 \text{ ng mL}^{-1}$ ), and BDE-209 ( $0.5 \text{ ng mL}^{-1}$ ).

As expected, the iLODs of BDE-206 and BDE-209 were about one order of magnitude higher than the lightest congeners. The values obtained for tri- to hepta-brominated were generally similar to those reported by other researchers using the same technique (GC-EI-MS/MS), as summarized in Table S3 (Kalachova et al. 2013; Labadie et al. 2010; Cristale et al. 2012; Sanchez-Avila et al. 2011; Mackintosh et al. 2012; Baron et al. 2014; Garcia-Bermejo et al. 2016). It is worth to note that, for the heaviest congeners (BDE-197, BDE-206, and BDE-209), our iLODs/iLOQs values were generally lower, although an exact comparison with literature data was not feasible, as a result of the different evaluation approaches (signal-to-noise, S/N or SD of the lowest solution of calibration curve) (Garcia-Bermejo et al. 2016). As explained, these congeners were analyzed using SIM instead of MRM acquisition (Table 1). Comparing the two modes, for BDE-197 and BDE-206, MRM acquisition yielded iLODs of about 2 and 10 injected picograms, respectively, instead of 0.5 and 2 pg (SIM). For BDE-209, the first detectable peak was observed injecting 50 pg in MRM while the iLOD measured using SIM acquisition was 5 injected picograms. MDLs and MQLs (in matrix) reflected the effective sensitivity of the method when applied to real samples. However, in the case of PBDEs, background contamination of blanks should always be taken into account to set proper limits. Therefore, the MDLs/MQLs were estimated using the data of 60 procedural blanks as detailed in our previous paper (Piersanti et al. 2015). As a result, MDLs were from 2 to  $4 \text{ pg g}^{-1}$ , except for BDE-206 and BDE-209 (23 and  $38 \text{ pg g}^{-1}$ , respectively). Similarly, MQLs were from 4 to  $7 \text{ pg g}^{-1}$  (for BDE-206 and BDE-209, 43 and  $71 \text{ pg g}^{-1}$ , respectively).

The availability of an analyte-free matrix in which to realize the validation study is an important issue because the natural occurrence of PBDEs together with the background contamination can hamper the assessment of method accuracy mainly at the lowest levels ( $10\text{--}100 \text{ pg g}^{-1}$ ). At first,

commercial mussel samples were purchased from local markets to perform spiking experiments. However, at the low concentrations, for some congeners the incurred levels hampered the correct evaluation of method performances (Piersanti et al. 2014). Fortunately, later, PBDE-free commercial frozen mussels from Chilean coast were found, and therefore, they were used as bulk sample to carry out spiking experiments. The accuracy study results (84 experiments) are shown in Table 3. Apparent recoveries (R%) were between 66 and 118%. Recoveries of the surrogate-labelled standards were in the range 64–107%. The RSD<sub>r</sub> (relative standard deviation in repeatability conditions) and RSD<sub>R</sub> (relative standard deviation in intra-lab reproducibility conditions) were lower than or equal to 14 and 22%, respectively. Sporadic higher values were observed for those congeners without labelled analogues as IS, i.e., BDE-49, BDE-66, BDE-77, BDE-85, BDE-138, BDE-197, and BDE-206.

Since fish is naturally contaminated by PBDEs, the daily recoveries at  $50 \text{ pg g}^{-1}$  of the eight surrogate labelled standards (non-native congeners) were used to evaluate the method performances in fish muscles (Fig. 3). These data were recorded within the analyses of the 35 samples of the market survey (see the paragraph below) during 3 weeks in both laboratories involved in the project (Perugia and Ancona). The behavior of the eight ISs is sufficiently representative (from tri- to deca-brominated congeners) of that of native compounds, demonstrating the method applicability to various fish species (anchovy, cod, flounder, mackerel, mullet, sole, and tuna). The found recoveries ranged from 66 to 90% with RSDs of about 20% (Fig. 3). These performances were achievable thanks to the thorough sample cleanup which allows the analysis of fish muscles with different composition (mainly fat and protein contents).

Method accuracy was also checked, participating in two proficiency tests organized by the European Reference Laboratory (EURL) for Dioxins and PCBs and by the Norwegian Institute of Public Health (NIPH). The z-scores obtained were satisfactory, demonstrating the applicability of



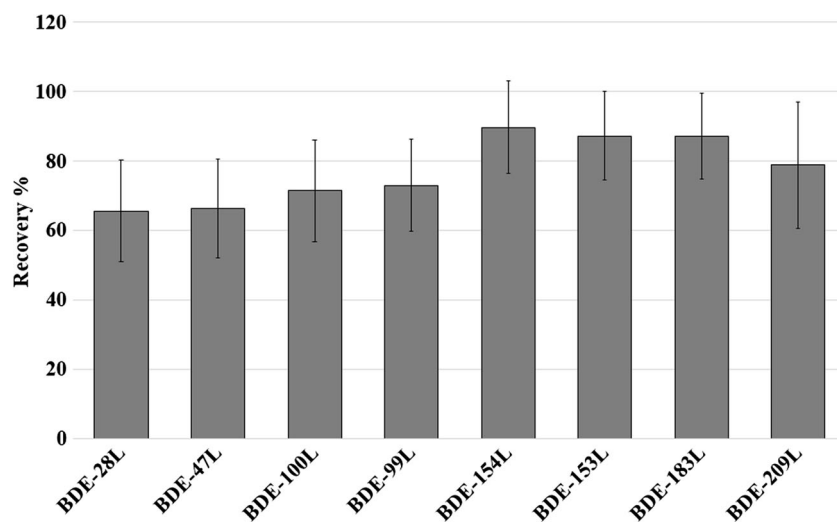
**Table 3** Validation results

BDE	Spiking level (pg g <sup>-1</sup> )	Mean (pg g <sup>-1</sup> )	RSD <sub>r</sub> (%)	RSD <sub>R</sub> (%)	R <sub>app</sub> (%)	IS recovery (%)	BDE	Spiking level (pg g <sup>-1</sup> )	Mean (pg g <sup>-1</sup> )	RSD <sub>r</sub> (%)	RSD <sub>R</sub> (%)	R <sub>app</sub> (%)	IS recovery (%)
28	10	9.5	4.9	6.3	96	79	154	10	9.7	7.1	7.9	97	91
	20	19	4.1	4.4	94	65		20	19	5.8	6.0	96	96
	100	100	2.3	6.4	100	69		100	100	5.1	5.6	100	107
	200	183	3.9	13	91	84		200	172	7.0	12	86	67
	400	382	1.6	11	95	77		400	363	6.6	9.3	91	64
	600	556	5.2	5.2	93	68		600	520	3.2	4.3	87	75
49	10	11	16	16	106		153	10	9.7	6.1	7.0	97	85
	20	22	4.5	14	111			20	19	3.3	5.4	96	87
	100	111	8.0	12	111			100	98	5.0	5.9	98	94
	200	169	8.5	15	84	-		200	188	5.2	16	94	72
	400	406	3.1	3.1	101			400	364	2.6	5.1	91	76
	600	642	1.9	5.2	107			600	541	8.3	11	90	85
47	10	11	12	12	105	86	138	10	10	14	15	102	
	20	19	11	11	94	83		20	19	5.8	9.1	94	
	100	86	3.8	7.1	86	84		100	104	8.0	9.0	104	-
	200	191	9.3	20	96	88		200	188	5.4	19	94	
	400	408	11	13	102	85		400	363	3.4	3.8	91	
	600	568	18	21	95	84		600	540	2.7	9.2	90	
66	10	9.2	8.1	10	92		183	10	10	8.9	9.5	104	84
	20	19	6.5	13	97			20	21	12	15	104	83
	100	86	3.5	21	89			100	101	4.7	5.7	101	91
	200	169	5.3	19	84	-		200	170	3.1	8.3	85	79
	400	398	3.9	15	99			400	373	2.4	15	93	82
	600	609	3.8	19	101			600	528	2.1	6.9	88	80
77	10	7.3	13	15	66		197	10	8.4	8.7	9.4	80	
	20	18	10	16	88			20	17	5.4	15	85	
	100	86	4.3	21	86			100	79	5.9	12	79	-
	200	178	7.4	12	89	-		200	178	2.6	9.4	89	
	400	396	2.6	24	99			400	411	4.4	24	103	
	600	594	6.4	11	99			600	581	5.9	18	97	
100	10	9.9	8.1	8.9	99	88	206	100	115	18	20	115	
	20	19	8.7	8.7	82	88		200	228	16	22	114	
	100	101	2.8	5.6	101	82		1000	1180	20	20	118	-
	200	173	7.6	18	86	88		2000	2148	7.4	7.4	107	
	400	344	3.4	9.3	86	84		4000	4256	4.3	14	106	
	600	486	7.1	11	81	98		6000	6291	7.7	20	105	
99	10	11	12	12	100	92	209	100	103	16	16	104	74
	20	21	4.9	8.5	93	90		200	199	10	15	100	81
	100	103	2.1	4.3	103	81		1000	1119	6.5	12	112	89
	200	160	8.9	16	80	89		2000	1969	5.5	14	98	92
	400	331	3.1	12	83	86		4000	3911	2.6	17	98	81
	600	488	7.7	8.2	81	87		6000	5716	1.7	19	95	79
85	10	11	6.6	7.5	111								
	20	20	14	14	100								
	100	109	4.8	20	109	-							
	200	194	5.0	5.0	97								
	400	434	2.9	9.0	109								
	600	623	4.0	9.3	104								

the procedure also for the analysis of very complex matrices such as cod liver, fish liver oil, and fat fish such as salmon (Supplementary Table S4). It is interesting to note that 36 laboratories participated in NIPH proficiency test (2015), but only 29 gave result for BDE-209 (consensus median: 22 pg g<sup>-1</sup>), and among these, seven gave unsatisfactory values (outliers). Moreover, for five laboratories, including ours, the consensus median value was lower than their MQL. On the

other hand, for BDE-183 (consensus median 2.9 pg g<sup>-1</sup>), all 36 laboratories answered correctly, with only two values removed as outliers. This evidence confirms the general difficulty in quantifying BDE-209 and in reaching MQL lower than or equal to 10 pg g<sup>-1</sup> as requested by Recommendation 118/2014 (EU 118/2014). At the end of the validation study, the procedure was accredited according to ISO/IEC 17025 standard (ISO/IEC 17025, 2005).

**Fig. 3** Recoveries at  $50 \text{ pg g}^{-1}$  of the eight surrogate labelled internal standards recorded in the 35 fish samples of the market survey

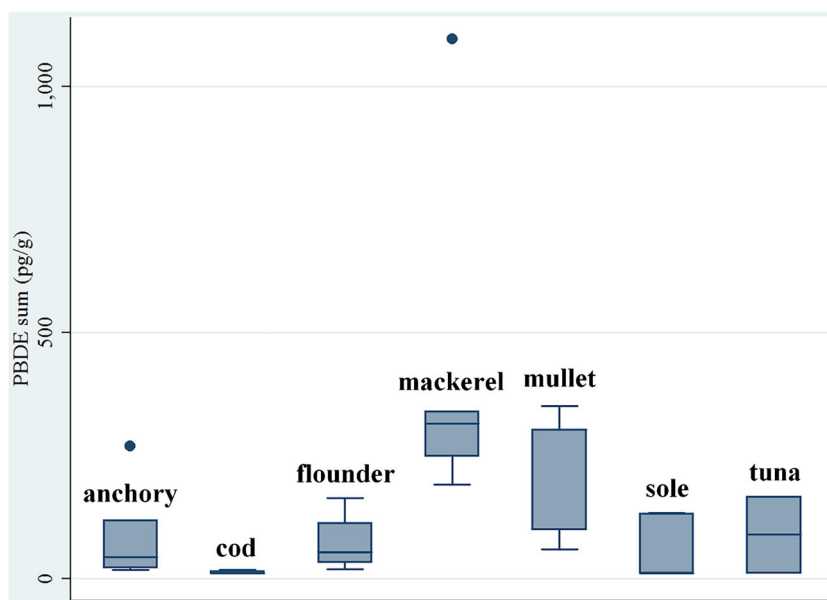


### Market Survey

Several studies have demonstrated that in European countries, the dietary intake is the main route of human exposure to PBDE. Among food, fish is the major source of PBDEs (Domingo 2012). Due to the lack of official limits and to intrinsic complexity of the measurement, at present few data are available about PBDE contamination of food produced or commercialized in Italy (EFSA 2011). Figure 4 shows the results obtained from the analyses of 35 marine fish samples harvested in local markets (individual data are reported in Table S5). Concentrations lower than MDL were arbitrarily set at 0.5 MDL to calculate PBDE sums (Labadie et al. 2010). Only eight congeners out of 15 were detected: BDE-28, BDE-47, BDE-49, BDE-66, BDE-99, BDE-100, BDE-153, and

BDE-154. The most frequently found (>MDL) were BDE-47, followed by BDE-100, BDE-154, BDE-49, and BDE-99. BDE-28, BDE-66, and BDE-153 were measured only in mackerel and in one sample of tuna fish (*Thunnus alalunga*). Hepta-, octa-, and nona-BDEs were never detected, which is consistent with previous findings: These congeners are usually present in fish at much lower concentrations than the lowest brominated ones. In all species, the dominant compound was BDE-47 (47%), which is usually the most abundant PBDE in fish, as a result of its highest bioconcentration factor (Bureau et al. 2006). The highest levels were measured in mackerel and mullet (mean PBDE sum equal to 418 and 222  $\text{pg g}^{-1}$  wet weight, respectively). Flounder, anchovy, and sole demonstrated lower contamination, whereas the lowest PBDE sum was detected in cod. A high variability was observed among

**Fig. 4** PBDE sums measured in the seven marine fish species collected from local market



the three collected tuna samples. This can be explained because tuna species includes 15 sub-species with very different sizes and longevity. In fact, the three specimens belonged to different sub-species: *Thunnus albacares*, *Thunnus alalunga*, and *Thunnus obesus*; this latter was the most contaminated (BDE sum 166 pg g<sup>-1</sup>). Then, it turns out that PBDE sum measured in mackerel was significantly higher ( $p < 0.05$ ) than that observed in anchovy (mean 86 pg g<sup>-1</sup>), sole (mean 53 pg g<sup>-1</sup>), and cod (mean 13 pg g<sup>-1</sup>). The sum found in flounder (mean 73 pg g<sup>-1</sup>) was at the limit of significance compared to mackerel ( $p = 0.05$ ). Pearson correlation coefficients indicated that the concentrations found significantly correlated to fat content of the seven collected fish species (EFSA 2011).

The ratio between PBDE-99 and PBDE-100 is considered an indicator of the organism metabolic capacity: High ratios indicate low capacity (Voorspoels et al. 2003; Perez-Fuentetaja et al. 2010; Munsch et al. 2011). Shellfish have a low metabolic capacity with PBDE-99/PBDE-100 ratios generally above 1.0 (Munsch et al. 2015). In our previous study carried out in 134 mussel samples, this ratio was 1.6 (Piersanti et al. 2015). On the other hand, for marine organisms, which are at a higher degree of the evolutionary ladder, PBDE-99/PBDE-100 ratios are below 1.0. The mean value here observed was about 0.5; interestingly, for four of the five mackerel, PBDE-99/PBDE-100 was higher than or equal to 1.0 (0.8, 1.0, 1.3, 1.5, and 1.9). The seven analyzed species were among the most consumed in Italy. Analogously, Pardo et al. (2014) reported the levels of PBDE in 25 different commercial marine fish and seafood collected in Valencia Region (Spain). They collected all the species here included, except sole and mullet. The measured concentrations were generally low (maximum PDBE upper bound sum 473 pg g<sup>-1</sup>), but it is worth to note that, after salmon, mackerel was the most contaminated species (PDBE sum 255 pg g<sup>-1</sup> upper bound).

## Conclusions

The protocol here carefully described can be easily converted to Standard Operating Procedure format and applied by expert routine laboratories. Satisfactory performance characteristics were obtained, except for the limits of nona- and decabrominated congeners with MQLs higher than those required by Recommendation 118/2014 (10 pg g<sup>-1</sup>). This is due to the worst intrinsic sensitivity of these heaviest compounds, and probably, at present, for common laboratories applying the GC-EI-MS/MS technique, the improvement of this performance is not feasible in practice. The method is labor- and time-intensive, needing multiple cleanup steps and high percentage of quality controls per batch (about 40%). This involves a high cost per sample, but in case of flame retardants analysis, the quality assurance practices have to be stressed.

Particular care must be paid to laboratory cleaning procedures, which can strongly affect method performances especially when concentrations lower than 100 pg g<sup>-1</sup> have to be quantified in real samples. Only these strict measures guarantee the quality of the data produced during food monitoring programs, efficiently supporting the daily intake assessments and political decisions.

Finally, the results of the analysis of 35 fish selected among the locally most consumed marine species show low PBDE levels (sum of PBDEs lower than 420 pg g<sup>-1</sup> wet weight) with the highest concentrations measured in mackerel and mullet, confirming that the species with higher fat contents are generally more contaminated (EFSA 2011).

## Compliance with Ethical Standards

**Conflict of Interest** Arianna Piersanti declares that she has no conflict of interest. Tamara Tavoloni declares that she has no conflict of interest. Eleonora Bastari declares that she has no conflict of interest. Carmela Lestingi declares that she has no conflict of interest. Sara Romanelli declares that she has no conflict of interest. Rosanna Rossi declares that she has no conflict of interest. Giorgio Saluti declares that he has no conflict of interest. Simone Moretti declares that he has no conflict of interest. Roberta Galarini declares that she has no conflict of interest.

**Human and Animal Rights** This article does not contain any studies with human participants or animals performed by any of the authors.

**Informed Consent** Informed consent is not applicable for this study.

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