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Simultaneous pressurized liquid extraction and clean-up for the analysis of polybrominated biphenyls by gas chromatography–tandem mass spectrometry

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ABSTRACT

This paper describes a fast and simple pressurized liquid extraction (PLE) method combined with gas chromatography coupled to ion trap tandem mass spectrometry (GC–ITMS-MS) for the determination of polybrominated biphenyls (PBBs) in fish samples. The method is based on a simultaneous extraction/clean-up step to reduce analysis time and solvent consumption. The effect of several PLE operating conditions, such as solvent type, extraction temperature and time, number of cycles, and lipid retainer, was optimized to obtain maximum recovery of the analytes with the minimum presence of matrix-interfering compounds. The best conditions were obtained at 100 °C with n-hexane using 15 g of silica modified with sulphuric acid (44%, w/w) as sorbent for lipid removal. Quality parameters of the GC–ITMS-MS method were established, achieving good linearity ($r > 0.998$), between 1 and 500 ng ml^{-1}, and low instrumental limits of detection (0.14–0.76 pg injected). For the whole method, limits of detection ranging from 0.03 to 0.16 ng g^{-1} wet weight and good precision (RSD < 16%) were obtained.

1. Introduction

Polybrominated biphenyls (PBBs) are a large group of brominated flame retardants (BFRs) that have been used as additives in textiles, electric and electronic components, plastics, clothing, building materials and other commercial products to decrease the likelihood and intensity of fire [1]. PBBs are easily released into the environment owing to their lack of covalent link to the polymeric material or textile with which they are mixed to improve fireproof properties [2,3]. Despite their merits in fire protection, the threat of PBBs was recognized in 1973 after the Michigan disaster, when they were accidentally mixed with cattle feed [4–6]. Shortly after this event, in 1974, PBB producers in the USA voluntarily ceased to manufacture these compounds and this action was followed worldwide few years later. In spite of a continuous reduction of the annual production of PBBs, the continued presence of these compounds in biological and environmental samples has been reported [7–11]. The toxicological concern about exposure to PBBs is related to their potential disruptor endocrine and, in some cases, to carcinogenic properties [12,13]. In addition, the combustion of PBBs generates compounds of higher toxicity such as polybrominated dibenzo-*p*-dioxins (PBDDs) and dibenzofurans (PBDFs) [14,15].

The analysis of PBBs is currently performed using methods similar to those applied for polychlorinated biphenyls (PCBs) and other persistent organic pollutants. These methods generally involve the extraction of the analytes from solid matrices using classical techniques such as Soxhlet [16–18], solvent extraction [19,20] or sonication [21,22], followed by extensive clean-up procedures to remove matrix-interfering compounds. The main drawback of these techniques is that they often require large amounts of solvents and are usually time-consuming. Pressurized liquid extraction (PLE) has become one of the most promising techniques to address these drawbacks and it has been successfully applied to the analysis of organic pollutants [23,24,28]. However, in most of the PLE applications reported, an exhaustive clean-up of the extracts before analysis by gas chromatography is required. In an attempt to decrease sample handling time and to increase sample throughput, several authors propose selective pressurized liquid extraction (SPLE) using sorbents such as modified silica, Florisil or alumina [26–29]. In recent years, SPLE has been developed for the analysis of persistent organic pollutants such as PCBs [26–28,30–34], polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/Fs) [25,35,36], polybrominated diphenylethers (PBDEs) [32,37–42], polychloronaphtalenes (PCNs) [43,44], polycyclic aromatic hydrocarbons (PAHs) [45–48] and many other compounds [25,49] in environmental and food samples. To our knowledge, PBB extraction by PLE using on-line clean-up has not been described. Therefore one of the objectives of the present study was to develop a simultaneous extraction/clean-up method based on selective PLE for the analysis of PBBs.

Gas chromatography coupled to mass spectrometry, working in either electron ionization (EI-MS) or negative ion chemical ionization (NICI-MS) modes, is the technique most commonly used

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for the analysis of PBBs. Of these, NICI-MS is by far the most popular approach because it provides higher sensitivity than EI-MS. Nevertheless, NICI-MS offers lower selectivity because the dominating bromine isotope ions (m/z 79 and 81), which are often the base peak in full scan mass spectra, are selected for quantification [50]. In contrast, EI provides higher quality structural information, giving the molecular ions and the sequential losses of bromine atoms [51–53]. Recently, to overcome the lack of selectivity of the GC–NICI-MS method for the analysis of PBBs, a triple-quadrupole mass analyzer working in tandem mode has been used [54–56]. Nevertheless, PBDE mass interferes on the labelled PBBs, making difficult the quantification. Gas chromatography coupled to high resolution mass spectrometry (GC–HRMS) in EI mode provides the most selective method and reasonable sensitivity for the determination of these compounds [57–59]. However, GC–HRMS is relatively expensive and requires skilled personnel and heavy maintenance on a regular basis to ensure proper functioning. Gas chromatography coupled to ion-trap tandem mass spectrometry (GC–ITMS-MS) has also been proposed for the analysis of PBBs, but a few numbers of congeners has been studied [60–63].

Here we developed a simple and rapid PLE method in combination with gas chromatography coupled to GC–ITMS-MS for the analysis of PBBs at low concentrations in fish samples. A one-step extraction and clean-up method was optimized in order to reduce the analysis time and solvent consumption. For this purpose, several sorbents for lipid removal were studied and PLE parameters were optimized. In addition, MS–MS working conditions that provided maximum sensitivity and selectivity for the analysis of PBBs were established. Finally, quality parameters were determined and the applicability of the method was evaluated.

2. Experimental

2.1. Chemicals and standards

Individual analytical-reagent grade PBB congeners were supplied with a purity higher than 99% by AccuStandard (New Haven, CT, USA): 4,4′-dibromobiphenyl (BB-15), 2,4′,5-tribromobiphenyl (BB-31), 2,2′,4,5′-tetrabromobiphenyl (BB-49), 2,2′,5,5′-tetrabromobiphenyl (BB-52), 3,3′,4,4′-tetrabromobiphenyl (BB-77), 2,2′,4,5′,6-pentabromobiphenyl (BB-103), 2,2′,4,4′,5,5′-hexabromobiphenyl (BB-153), and 2,2′,4,4′,6,6′-hexabromobiphenyl (BB-155). Individual stock standard solutions of each PBB congener at 10 $\mu g\,ml^{-1}$ were prepared in isooctane. In addition, individual standard solutions of 3,3′,5,5′-tetrabromobiphenyl (BB-80), 2,2′,4,5,5′-pentabromobiphenyl (BB-101) and 3,3′,4,4′,5,5′-hexabromobiphenyl (BB-169) at a concentration of 10 $\mu g\,ml^{-1}$ in isooctane were obtained from Dr. Ehrenstorfer (Augsburg, Germany). A standard solution containing the eleven PBB congeners was prepared in isooctane from the respective individual stock standard solutions at a concentration of 1 $\mu g\,ml^{-1}$ of each compound. A standard solution of isotopically $^{13}C_{12}$-labelled CBs 28, 52, 101, 138, 153, 180 and 209 (MBP-MXE), supplied by Wellington Laboratories (Guelph, Canada), at 5 $\mu g\,ml^{-1}$ of each congener was used as internal standard for quantification by isotopic dilution. In addition, a standard solution of isotopically $^{13}C_{12}$-labelled BDE 139 at a concentration of 2.5 $\mu g\,ml^{-1}$, supplied by Wellington Labs, was used as syringe standard for recovery determination. Seven calibration standard solutions containing a mixture of the eleven PBB congeners at concentrations ranging from 1 to 500 $ng\,ml^{-1}$ and the isotopically labelled internal standards for recovery and quantification at 100 $ng\,ml^{-1}$ were prepared by dilution of the corresponding standard stock solutions in isooctane.

Dichloromethane, acetone, isooctane and n-hexane of residue analysis grade and sulphuric acid of analytical reagent grade (95–97%) were purchased from Merck (Darmstadt, Germany). Sodium sulphate (p.a., purity >99%), silica gel (Gel 60) and Florisil were also obtained from Merck. Before use, silica gel and Florisil were baked at 400 °C for 10 h and 675 °C for 12 h, respectively. Silica gel modified with sulphuric acid (44%, w/w) was prepared by slowly adding an appropriate amount of sulphuric acid to the silica at room temperature. Glass microfibre filters GF/A for covering PLE cell caps were provided by Whatman (Maidstone, UK). All glass materials were cleaned with AP-13 Extran alkaline soap (Merck, Darmstadt, Germany) for 24 h, rinsed consecutively with Milli-Q water and acetone, and dried overnight before use.

2.2. Samples and sample treatment

A set of five fish samples (trout, salmon, horse mackerel, sardine and gilthead seabream) purchased from a local supermarket were selected for the analysis of PBBs using the PLE and GC–ITMS-MS method. These fish samples were selected to cover a wide range of lipid content and are among those most frequently found in the Spanish diet [64]. Trout and salmon were of aquaculture origin, while horse mackerel, sardine and gilthead were caught in the Mediterranean Sea. Initially, each fish was washed and the non-edible parts were removed to obtain clean tissues. The edible part of the fish was triturated, homogenized, frozen and lyophilized. The dried sample was then ground in a glass mortar to obtain a fine powder, which was preserved in glass vials and stored at 4 °C in a dry place in darkness before analysis.

A standard reference material, SRM 1945 (whale blubber), obtained from the National Institute of Standards and Technology (NIST, Boulder, CO, USA), was used to validate the whole method. This reference material is certified for selected PCBs, organochlorine pesticides and PBDEs, although information about the presence of the BB-153 has been reported by Zhu and Hites [65].

2.3. On-line extraction and clean-up of fish samples using PLE

The simultaneous extraction and clean-up of the samples was performed on an ASE-100 Accelerated Solvent Extraction System (Dionex, Sunnyvale, CA, USA). Before PLE extraction, 1 g of the freeze-dried fish sample was spiked with isotopically $^{13}C_{12}$-labelled PCBs and was kept overnight at room temperature to equilibrate. The sample was then mixed with sodium sulphate at a Na_2SO_4/fish ratio of 3:1 (w/w) in a mortar until a homogenous mixture was obtained. Florisil and silica modified with sulphuric acid (44%, w/w) were tested as sorbents for lipid removal. The extraction cell was loaded by inserting two cellulose filters into the cell outlet, followed by the sorbent material and the sample, using anhydrous sodium sulphate to fill up the dead volume of the extraction cell. After sealing the cell with the top cell cap, the extraction cell was placed in the ASE system. Samples were extracted at 100 °C with n-hexane as extraction solvent and silica modified with sulphuric acid (44%, w/w) as lipid retainer, applying 3 static cycles of 5 min each and a flush volume of 60%. Further details about the optimization of the extraction parameters are described in the results and discussion section. The final extracts were then solvent-reduced to approximately 2 ml, adding isooctane as a keeper. Afterwards, the final volume of the extracts was carefully concentrated under a gentle nitrogen stream to ca. 50 μl. The extracts were then analyzed by GC–ITMS-MS after the addition of isotopically $^{13}C_{12}$-labelled BDE 139 used as syringe standard.

The lipid content of fish samples was determined by PLE (without sorbent for lipid removal) of an additional sub-sample and further gravimetric measurements. A solvent mixture of n-hexane:dichloromethane (1:1, v/v) was used. The percentage of lipids determined for the fish samples (wet weight) was as follows: 0.7% for trout, 2.1% for horse mackerel, 10.1% for gilthead, 9.8% for sardine and 14.6% for salmon.

Table 1
MS–MS transition selected and excitation CID voltage for each homologue group of PBBs and $^{13}C_{12}$-PCBs in GC–ITMS-MS method.

Time window (min)	Homologue	Compound	MS–MS transition		Excitation voltage (V)
			Precursor ion (*m*/*z*)	Product ions (*m*/*z*)	
7.0–11.0	Di-BB	BB-15	312 $[M+2]^{+\bullet}$	152 $[M-2Br]^{+\bullet}$	1.5
	$^{13}C_{12}$-tri-CB	$^{13}C_{12}$-CB 28	270 $[M+2]^{+\bullet}$	198 + 200 $[M-2Cl]^{+\bullet}$	1.3
	$^{13}C_{12}$-tetra-CB	$^{13}C_{12}$-CB 52	304 $[M+2]^{+\bullet}$	232 + 234 $[M-2Cl]^{+\bullet}$	1.3
11.0–14.3	Tri-BB	BB-31	390 $[M+2]^{+\bullet}$	230 + 232 $[M-2Br]^{+\bullet}$	1.8
	$^{13}C_{12}$-penta-CB	$^{13}C_{12}$-CB 101	338 $[M+2]^{+\bullet}$	266 + 268 $[M-2Cl]^{+\bullet}$	1.4
14.3–18.2	Tetra-BB	BB-49	470 $[M+4]^{+\bullet}$	389 + 391 $[M-Br]^{+}$	1.3
		BB-52	470 $[M+4]^{+\bullet}$	389 + 391 $[M-Br]^{+}$	1.3
	$^{13}C_{12}$-hexa-CB	$^{13}C_{12}$-CB 138	372 $[M+2]^{+\bullet}$	300 + 302 $[M-2Cl]^{+\bullet}$	1.4
		$^{13}C_{12}$-CB 153	372 $[M+2]^{+\bullet}$	300 + 302 $[M-2Cl]^{+\bullet}$	1.4
18.2–24.1	Tetra-BB	BB-77	470 $[M+4]^{+\bullet}$	310 + 312 $[M-2Br]^{+\bullet}$	2.0
		BB-80	470 $[M+4]^{+\bullet}$	310 + 312 $[M-2Br]^{+\bullet}$	2.0
	Penta-BB	BB-101	548 $[M+4]^{+\bullet}$	467 + 469 $[M-Br]^{+}$	1.6
		BB-103	548 $[M+4]^{+\bullet}$	467 + 469 $[M-Br]^{+}$	1.7
	$^{13}C_{12}$-hepta-CB	$^{13}C_{12}$-CB 180	406 $[M+2]^{+\bullet}$	334 + 336 $[M-2Cl]^{+\bullet}$	1.5
24.1–33.5	Hexa-BB	BB-153	628 $[M+6]^{+\bullet}$	547 + 549 $[M-Br]+$	2.0
		BB-155	628 $[M+6]^{+\bullet}$	547 + 549 $[M-Br]^{+}$	2.1
33.5–42.0	Hexa-BB	BB-169	628 $[M+6]^{+\bullet}$	466 + 468 $[M-2Br]^{+\bullet}$	2.4
	$^{13}C_{12}$-deca-CB	$^{13}C_{12}$-CB 209	510 $[M+4]^{+\bullet}$	438 + 440 $[M-2Cl]^{+\bullet}$	1.5
	$^{13}C_{12}$-hexa-BDE	$^{13}C_{12}$-BDE 139	656 $[M+6]^{+\bullet}$	496 + 498 $[M-2Br]^{+\bullet}$	1.2

2.4. GC–MS instrumentation

The GC–ITMS-MS analyses of PBBs were carried out using a Trace GC 2000 Series gas chromatograph coupled to a GCQ/Polaris ion trap mass spectrometer (ThermoFinnigan, Austin, TX, USA) equipped with an AS2000 autosampler. The chromatographic separation of PBBs was performed using a DB-5MS (5% phenyl, 95% methylpolysiloxane) fused-silica capillary column (J&W Scientific, Folsom, CA, USA), 30 m × 0.25 mm I.D., 0.25 μm of film thickness. The oven temperature was programmed from 140 °C (held for 2 min) to 210 °C at 20 °C/min (held for 1 min) and then to 300 °C at 2.5 °C/min (held for 10 min). Helium was used as carrier gas at a constant flow-rate of 1 ml/min held by electronic pressure control at 140 °C. Injector temperature was kept at 280 °C and 1 μl of samples and standards were injected in splitless injection mode (1 min). The MS operating conditions were the following: EI mode using automatic gain control (AGC) with electron energy of 70 eV and an emission current of 250 μA. The transfer line and ion source temperatures were kept at 280 °C and 200 °C, respectively. The instrument was tuned using perfluorotributylamine (FC-43) to achieve the highest sensitivity and performance in both MS and MS/MS mode. Electron multiplier voltage was set to 1350 V (10^5 gain) by automatic tuning. In MS–MS mode, for native PBBs the $[M+4]^{+\bullet}$ ions of the molecular cluster ions of each homologue group were selected as precursor ion, except for di-BBs and tri-BBs ($[M+2]^{+\bullet}$ ions) and hexa-BBs ($[M+6]^{+\bullet}$ ions) (Table 1). For $^{13}C_{12}$-labelled PCBs, $[M+2]^{+\bullet}$ ion of the molecular cluster ions was selected as precursor ion except for $^{13}C_{12}$-CB 209 for which $[M+4]^{+\bullet}$ was used. For $^{13}C_{12}$-BDE 139, the $[M+6]^{+\bullet}$ ion was chosen. The corresponding product ions $[M-2^{79}Br]^{+\bullet}$ and $[M-^{79}Br^{81}Br]^{+\bullet}$ for PBBs 15, 31, 77, 80, 169 and $^{13}C_{12}$-BDE 139 were monitoring for quantitative purposes. For PBBs 49, 52, 101, 103, 153 and 155, the $[M-^{79}Br]^{+}$ and $[M-^{81}Br]^{+}$ ions were selected as product ions while for the isotopically labelled PCBs the $[M-2^{35}Cl]^{+\bullet}$ and $[M-^{37}Cl^{35}Cl]^{+\bullet}$ ions were monitored. After optimization, the resonance excitation voltages, which produce the maximum yield in abundance of the corresponding product ions for all the compounds, ranged between 1.2 and 2.4 V (Table 1). The MS–MS acquisition method was time-programmed in six segments to determine di- to hexa-BBs. Xcalibur version 2.0 software was used for data acquisition and processing of the results.

Quantification of PBBs was carried out using seven isotopically $^{13}C_{12}$-labelled PCB congeners as internal standards (MBP-MXE) because not all individual $^{13}C_{12}$-labelled PBBs are commercially available to quantify by isotope dilution and moreover interferences between $^{13}C_{12}$-labelled PBBs and PBDEs have been described [54]. The recoveries of both PBBs and $^{13}C_{12}$-PCBs determined by the standard addition were higher than 90% demonstrating that $^{13}C_{12}$-PCBs could be used as surrogate standards. On the other hand, $^{13}C_{12}$-labelled BDE 139 was used as syringe internal standard.

2.5. Quality control criteria

Quality control was performed through the analysis of procedural blanks and replicate analysis of samples. Procedural blanks (both instrumental and method) were routinely performed during the analysis, and a quality control fish sample (a salmon sample with non-detectable amounts of PBBs spiked at 1 ng g^{-1}, w/w) was analyzed to ensure that the whole method was maintained under control and to rule out any cross-contamination between samples. Recovery rates of the labelled $^{13}C_{12}$-PCBs, which were used as surrogate standard, higher than 90% and precisions of the whole method lower than 20% were established as acceptance criteria. A daily sensitivity and calibration tests were carried out to check the performance of the GC–ITMS-MS method. In addition, limits of detection (LODs), defined as the concentration of the analyte that produces a signal to noise ratio greater than 3, were periodically determined analyzing standard solutions of PBBs at a low concentration level (1.0 ng ml^{-1}) and blank fish samples spiked at 0.3 ng g^{-1} (w/w) level. To confirm the identification of PBBs the following restrictive criteria were applied: (a) the signal-to-noise ratio should be greater than 3 for each congener, (b) the isotope ratios between the two monitored product ions should be within ±20% of the theoretical value, and (c) the retention time of each congener should be within the margin ±2 s of those observed for standards. Quantification of PBBs was carried out by isotope dilution using response factors relative to the $^{13}C_{12}$-PCBs.

3. Results and discussion

3.1. Optimization of the GC–ITMS-MS method

The EI-MS fragmentation pattern of PBBs is characterized by the presence of intense molecular cluster ions and fragment ions corresponding to successive losses of bromine atoms from the molecular ions, similar to the pattern observed for PCBs [66]. The most abundant fragment ions for PBB congeners 49, 52, 101, 103, 153 and 155 were the $[M-Br]^{+}$ ions while for PBBs 15, 31, 77, 80 and 169

Table 2
Instrumental quality parameters of the GC–ITMS-MS method for the analysis of polybrominated biphenyls (PBBs).

Compound	Linearity	LODs	Precision			
	Correlation coefficient (R)	pg injected	Low level (10.0 ng ml^{-1})		Medium level (100.0 ng ml^{-1})	
			Run-to-run[a] (RSD%)	Day-to-day[b] (RSD%)	Run-to-run[a] (RSD%)	Day-to-day[b] (RSD%)
BB-15	0.9994	0.17	3	4	3	3
BB-31	0.9998	0.36	3	4	3	4
BB-52	0.9998	0.15	4	7	3	5
BB-49	0.9994	0.14	4	7	4	7
BB-80	0.9993	0.49	4	6	3	4
BB-77	0.9998	0.41	5	8	3	3
BB-103	0.9998	0.28	3	5	3	4
BB-101	0.9995	0.23	5	8	3	4
BB-155	0.9998	0.18	4	4	3	3
BB-153	0.9998	0.18	4	5	3	4
BB-169	0.9985	0.76	4	8	5	6

[a] $n = 5$.
[b] $n = 5$ replicates × 3 days..

they were the $[M-2Br]^{+\bullet}$ ions. Therefore, the most intense ion of the molecular cluster ions of each homologue group was selected as precursor ion while the fragment corresponding to the loss of one bromine atom (^{79}Br or ^{81}Br) or two bromine atoms ($2^{79}Br$ or $^{79}Br^{81}Br$), depending on the PBB congener, were selected as product ions for MS–MS studies (see Table 1).

MS–MS operating conditions were optimized to maximize sensitivity and selectivity of GC–ITMS-MS to detect these compounds. The parameters optimized were those related to the isolation and excitation of the precursor ions and the storage of the product ions. For these experiments, a standard mixture of PBB congeners (100 ng g^{-1}) was used. The effect of the mass isolation window on the selective isolation of the precursor ions was first examined from 1 to 3 m/z. To achieve maximum selectivity, an isolation window of 1 m/z was chosen for all PBBs. In addition, optimum isolation time for the precursor ion was found to be 10 ms. The effect of the CID resonant excitation voltage on the product ion yield was then also studied from 0.6 V to 3.0 V in 0.2 V steps. The optimum excitation voltages for each congener are given in Table 1. We then studied the excitation time between 5 ms and 30 ms in 5-ms steps. In this case, optimum CID excitation time was obtained at 15 ms. Using the optimum CID conditions, additional experiments were performed to optimize the excitation energy, which is related to the stability of the product ions and can be controlled by the q_z parameter. The influence of this parameter on the fragmentation yield and the stabilization of the product ions were studied at three q_z levels, 0.225 (low), 0.300 (medium) and 0.450 (high). Maximum abundances of the product ions were observed at the highest q_z value (0.450). Values for $^{13}C_{12}$-labelled PCBs and $^{13}C_{12}$-labelled BDE 139 standards are also given in Table 1.

Quality parameters of the GC–ITMS-MS method were determined using standard solutions. In order to determine the linearity, seven standard calibration solutions ranging from 1.0 to 500 ng ml^{-1} for PBBs and containing isotopically $^{13}C_{12}$-labelled PCB congeners and $^{13}C_{12}$-labelled BDE 139 at a concentration of 100 ng ml^{-1} were used. Good linearity in the concentration range was obtained for all the compounds, with correlation coefficients higher than 0.9985 (Table 2). Limits of detection (LODs), defined as the concentration that produces a signal to noise ratio (S/N) greater than 3, were determined experimentally using standard solutions at low concentrations (1.0 ng ml^{-1}). Under these conditions, instrumental LODs ranged from 0.14 to 0.76 pg injected (Table 2). For run-to-run and day-to-day precisions, two PBB standard mixtures, one at a low (10.0 ng ml^{-1}) and another at a medium (100.0 ng ml^{-1}) concentration were analyzed in quintuplicate on one day and on three days, respectively. Good run-to-run and day-to-day precisions were achieved with RSDs (%) between 3 and 5% and from 3 to 8%, respectively (Table 2). In view of these results, we consider that GC–ITMS-MS shows enough sensitivity and precision to be used for the analysis of PBBs.

3.2. Optimization of the simultaneous extraction and clean-up PLE procedure

To simplify sample handling and to reduce the extraction time for the analysis of PBBs in fish samples, we examined the capability of PLE to simultaneously perform in-cell extraction and clean-up. Initial experiments were conducted to optimize PLE parameters such as lipid retainer, type of extraction solvent, number of extraction cycles, extraction temperature and static extraction time with the aim to obtain the maximum efficiency for the extraction of PBBs with the minimum presence of interfering compounds. To this end, two fish samples with non-detectable amounts of PBBs and a different lipid content (0.7% for trout and 14.6% for salmon) spiked with the selected PBB congeners at a concentration of 1.3 ng g^{-1} (w/w) and the $^{13}C_{12}$-labelled PCBs were used. The spiked samples were kept overnight at room temperature to equilibrate before PLE extraction and then mixed with anhydrous sodium sulphate (Na_2SO_4/fish ratio of 3:1, w/w). Initially, Florisil and silica modified with sulphuric acid (44%, w/w) were selected as sorbents because they are often used in conventional analytical methodology for the clean-up of fish extracts in PCB and PBDE analysis [26,30,37,41]. Several amounts of Florisil, from 10 g to 18 g in a 33-ml cell and between 15 g and 30 g in a 100-ml cell, were tested using 1 g of freeze-dried fish, an extraction temperature of 100 °C, a solvent mixture of n-hexane:dichloromethane (9:1, v/v), 3 static cycles of 5 min each and a flush volume of 60%. At these conditions, using a 33-ml cell and the maximum amount of Florisil that can be loaded in the extraction cell (18 g), the extracts obtained were not suitable for GC–ITMS-MS analysis because of the presence of high amounts of lipids. Increasing the amount of Florisil to 30 g in a 100-ml cell, extracts clean enough for the GC–ITMS-MS determination of PBBs were obtained. These conditions were similar than those obtained in a previous work for PBDE analysis by SPLE using Florisil as lipid retainer [41]. We then examined the influence of the extraction temperature, static extraction time and the number of cycles on the recovery of the analytes. In general, an increase on the extraction temperature produces an enhancement of the extraction efficiency of the analytes [25,49], but also increases the co-extraction of interfering compounds. Therefore, a temperature allowing high analyte extraction efficiency with minimum amounts of interfering compounds from co-extracted material is required. To this end, extraction temperature from 80 to 120 °C, extraction time from 3 to 10 min and between 1 and 5 cycles, using a flush volume of 60% and

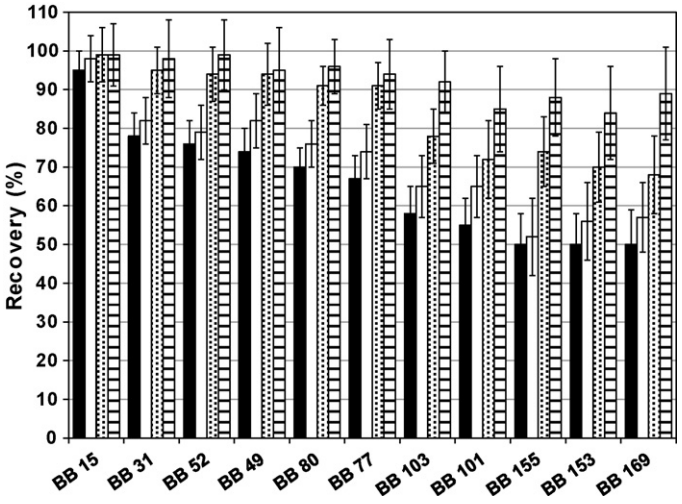

Fig. 1. Effect of the PLE solvent composition (n-hexane (■) and mixtures n-hexane:dichloromethane: 9:1 (□), 8:2 (▨) and 6:4 (▭), v/v) on the PBB recoveries ($n = 3$) for the spiked trout sample using Florisil as adsorbent for lipid removal. Error bars correspond to the standard deviation ($n = 3$).

a mixture of n-hexane:dichloromethane (9:1, v/v) were studied. An extraction temperature of 100 °C, a static extraction time of 5 min in combination with four cycles provided the best results. Finally, we examined the effect of the extraction solvent that provided the highest extraction yield with the minimum presence of lipids in the final extract. For this purpose, n-hexane and three mixtures of n-hexane:dichloromethane (9:1, 8:2 and 6:4, v/v) were tested. A rise in the proportion of dichloromethane in the extraction solvent increased the recovery of the target compounds up to >85% (Fig. 1). Nevertheless, percentages of dichloromethane higher than 40% also produced a considerable increase in the extraction of interfering compounds. Therefore, we chose n-hexane:dichloromethane (6:4, v/v) as extraction solvent. Using the above mentioned PLE procedure, the volume of the extract was always in ca. 230 ml and the whole extraction/clean-up procedure was accomplished in approximately 35 min. Table 3 shows the recoveries (%) obtained for the two spiked fish samples using Florisil as lipid retainer under the optimized PLE conditions. High extraction efficiencies were obtained, with recoveries higher than 91%.

In order to reduce both the final volume of the extracts and the amount of sorbent, silica modified with sulphuric acid was studied for lipid removal. The use of this sorbent in the extraction cell has shown to be very useful for the analysis of PCBs in complex matrices such as feed and food samples [26,27]. The relationship between fish lipid content and the amount of sorbent was studied. For fish samples with a low lipid content (<2.5% of lipids), such as trout or horse mackerel, the use of 10 g of silica/44% H_2SO_4 (w/w) in a 33-ml PLE cell and 1 g of freeze-dried sample was enough to obtain clean extracts. Nevertheless, for fish with medium and high lipid content (10–15% of lipids), such as sardine, gilthead seabream and salmon, the extracts obtained were not suitable for GC–ITMS-MS analysis because of the presence of small amounts of lipids, which drastically affected instrument response. For these samples, at least 15 g of silica/44% H_2SO_4 (w/w) was required to obtain extracts free of lipids. Therefore, for a general method that is applicable to a wide range of fish samples, we propose the use of 15 g of modified silica. The influence of extraction temperature (from 80 to 120 °C), static extraction time (from 3 to 10 min) and number of cycles (from 1 to 5 cycles) on the extraction efficiency of the target compounds was optimized as described previously for Florisil. In addition, n-hexane and mixtures of n-hexane:dichloromethane (9:1, 8:2 and 6:4, v/v) were tested as extraction solvents. The highest efficiency in the PLE extraction of PBBs was obtained at 100 °C using n-hexane as extraction solvent. These conditions agreed with those reported in the literature for PCB [25,26,28,32,33], while mixtures of n-hexane/dichloromethane are preferred for PBDEs [25,32,41]. For the quantitative recovery of the analytes, 3 static cycles of 5 min each and a flush volume of 60% were required. Under these conditions, recoveries of the target compounds were from 91% to 99% (Table 3). Using these conditions, the whole extraction/clean-up procedure was accomplished in approximately 24 min and the extract volume was about 65 ml. Although both sorbents for lipid removal, Florisil and silica/H_2SO_4 (44%, w/w), allowed high recoveries of the PBB congeners, the modified silica gel required a shorter extraction time and less solvent. In addition, less sorbent was required (15 g of modified silica versus 30 g of Florisil) for the clean-up of the extracts. Therefore, silica gel modified with H_2SO_4 (44%, w/w) is proposed for the simultaneous extraction and clean-up of PBBs in fish samples.

3.3. Performance of the PLE-GC–ITMS-MS method

To evaluate the performance of the developed method, a reference material, SRM 1945 (whale blubber), was analyzed. This reference material does not provide certified concentration values for PBBs but Zhu and Hites [65] reported a reference value for BB-153, which is the most predominant PBB congener found in environmental and biota samples [67–71]. Triplicate analyses of the SRM 1945 were carried out using the PLE-GC–ITMS-MS developed method. As a result, a concentration of 4.41 ± 0.43 ng g^{-1} wet weight (w/w) was found for BB-153 which agreed with that

Table 3
PBB recoveries obtained from two fish samples using Florisil and silica modified with sulphuric acid (44%, w/w) as sorbent for lipid removal in the PLE procedure.

Compound	Recovery (%)			
	Trout (0.7% lipids)		Salmon (14.6% lipids)	
	Florisil Mean ± s.d.[a]	Silica/H_2SO_4 (44%) Mean ± s.d.[a]	Florisil Mean ± s.d.[a]	Silica/H_2SO_4 (44%) Mean ± s.d.[a]
PBB-15	99 ± 8	99 ± 6	92 ± 9	96 ± 10
PBB-31	98 ± 10	92 ± 11	96 ± 8	94 ± 12
PBB-52	99 ± 9	97 ± 12	98 ± 10	95 ± 14
PBB-49	95 ± 11	96 ± 9	93 ± 9	94 ± 12
PBB-80	96 ± 7	93 ± 8	95 ± 9	96 ± 10
PBB-77	94 ± 9	97 ± 8	96 ± 11	94 ± 8
PBB-103	92 ± 8	91 ± 12	94 ± 10	93 ± 14
PBB-101	85 ± 11	91 ± 14	88 ± 9	96 ± 13
PBB-155	88 ± 10	91 ± 12	88 ± 13	95 ± 14
PBB-153	84 ± 12	94 ± 11	87 ± 12	92 ± 9
PBB-169	89 ± 12	91 ± 14	83 ± 13	91 ± 10

[a] $n = 3$.

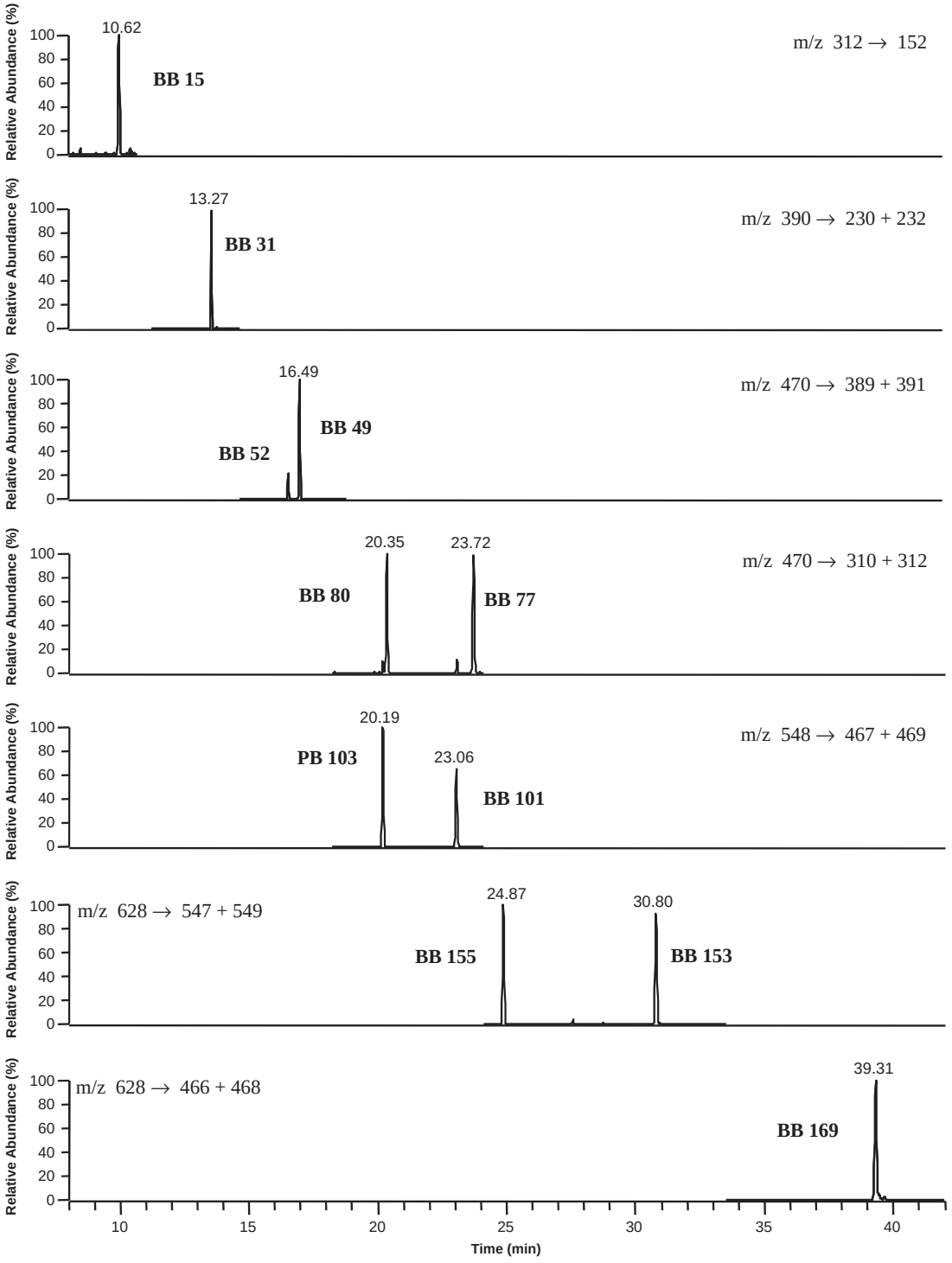

Fig. 2. GC–ITMS-MS chromatograms of PBB congeners obtained for the spiked sardine sample (about 1.70 ng g^{-1} wet weight) using the on-line clean-up PLE technique with silica modified with sulphuric acid (44%, w/w) as sorbent for lipid removal.

reported, 4.59 ± 0.39 ng g^{-1} (w/w) [65], achieving a relative standard deviation (RSD%) lower than 10%. These results demonstrate the suitability of the developed method for the analysis of PBBs in fish.

Limits of detection of the method (mLODs) were determined by spiking fish blank samples (sardine, gilthead seabream and salmon) at very low concentration levels (0.1–0.5 ng g^{-1}, w/w). The mLODs ranged from 0.03 to 0.16 ng g^{-1} (w/w) depending on the compound (Table 4) and are in agreement with those reported in the literature for BB-15 and BB-49 [72]. The values obtained in samples that varied in lipid content were very similar, thereby indicating that the on-line PLE clean-up method removed matrix interferences.

PBBs were analyzed in fish samples (sardine, trout, horse mackerel, gilthead seabream and salmon) but none of the samples showed detectable concentrations of PBBs. Therefore, to evaluate the performance of the method, three fish samples (sardine, gilthead seabream and salmon) were spiked with PBBs at concentrations ranging from 1.64 to 2.01 ng g^{-1} (w/w), in accordance with the concentrations reported in the literature for fish samples (0.23–3.1 ng g^{-1}, w/w) [67,68,73–75]. All analyses were performed

Table 4
Limits of detection of the method (mLODs), spiked levels and concentrations found ($n=3$) and their RSD (%), expressed in ng g^{-1} wet weight (w/w), obtained for each PBB congener in three selected fish samples using the PLE-GC–ITMS-MS method.

Compound	Sardine				Gilthead				Salmon			
	mLOD (ng g^{-1}, w/w)	Concentration (ng g^{-1}, w/w)			mLOD (ng g^{-1}, w/w)	Concentration (ng g^{-1}, w/w)			mLOD (ng g^{-1}, w/w)	Concentration (ng g^{-1}, w/w)		
		Spiked level	Mean	RSD (%) ($n=3$)		Spiked level	Mean	RSD (%) ($n=3$)		Spiked level	Mean	RSD (%) ($n=3$)
BB-15	0.14	1.67	1.70	8	0.16	1.68	1.31	8	0.13	1.90	1.82	9
BB-31	0.13	1.68	1.56	9	0.11	1.68	1.50	10	0.11	1.90	1.71	8
BB-52	0.10	1.70	1.50	8	0.13	1.70	1.58	8	0.13	1.93	1.62	7
BB-49	0.10	1.73	1.56	11	0.09	1.73	1.70	13	0.11	1.96	1.47	10
BB-80	0.08	1.71	1.73	10	0.10	1.71	1.66	16	0.14	1.94	1.53	10
BB-77	0.13	1.66	1.64	8	0.12	1.67	1.42	10	0.15	1.89	1.85	9
BB-103	0.10	1.77	1.65	8	0.11	1.77	1.66	9	0.08	2.01	1.49	7
BB-101	0.06	1.71	1.74	7	0.07	1.72	1.69	8	0.06	1.95	1.56	9
BB-155	0.06	1.75	1.61	7	0.08	1.75	1.59	7	0.05	1.99	1.89	8
BB-153	0.04	1.70	1.68	7	0.03	1.70	1.41	8	0.03	1.93	1.56	7
BB-169	0.10	1.64	1.48	7	0.12	1.64	1.56	7	0.12	1.86	1.58	9

in triplicate. For all target compounds, the found concentrations of PBBs were in good agreement with the spiked levels, providing relative standard deviations (RSD%) ranging from 7 to 16% (Table 4). Fig. 2 shows as an example the GC–ITMS-MS chromatograms corresponding to a sardine sample spiked at about 1.70 ng g^{-1} (w/w). As can be seen, the chromatograms obtained for PBBs in this sample using the GC–ITMS-MS technique show a high selectivity and good signal-to-noise ratio.

4. Conclusions

Here we developed a fast, simple and accurate on-line PLE extraction/clean-up method combined with GC–ITMS-MS for the analysis of PBBs in fish samples. The use of both silica gel modified with sulphuric acid (44%, w/w) (15 g in a 33-ml cell) and Florisil (30 g in a 100-ml cell) as sorbent for lipid removal provided quantitative recoveries of PBBs and extracts ready for GC–ITMS-MS determination. Nevertheless, a reduction in the extraction time, solvent consumption and the amount of adsorbent was obtained using modified silica for lipid removal. The optimization of the MS–MS parameters allowed maximum sensitivity and selectivity in the determination of PBBs, providing low limits of detection and good precision. The developed PLE method combined with GC–ITMS-MS can be proposed for the analysis of PBBs at low concentration levels in fish samples.

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