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"Development of an analytical method for the determination of polybrominated diphenyl ethers in mussels and fish by gas chromatography - inductively coupled plasma mass spectrometry"

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Highlights

- Analytical procedure for the determination of PBDEs in fish and mussels was developed
- PBDEs were extracted with TMAH and addition of Tris-citrate buffer and iso-octane
- Concentrations of PBDEs in the organic phase (iso-octane) were determined by GC-ICP-MS
- The procedure developed is simple, accurate, repeatable, reproducible and sensitive
- In fish and mussel samples BDE 47, BDE 100 and BDE 99 were commonly detected

Abstract

Six congeners of polybrominated diphenyl ethers (PBDEs): BDE 28, BDE 47, BDE 99, BDE 100, BDE 153 and BDE 154, were determined by a reliable and sensitive analytical method based on gas chromatography coupled to inductively coupled plasma mass spectrometry (GC-ICP-MS) in mussel and fish tissue samples. For their extraction, 30 min of ultrasound-assisted extraction with a 25% aqueous solution of tetramethylammonium hydroxide (TMAH) and an additional 2 h of mechanical shaking with tris(hydroxymethyl)aminomethane (Tris)-citrate buffer and iso-octane were applied. An effective cleaning, with minor solvent consumption, was achieved by passing the extract through a column filled with Florisil. PBDEs in the organic phase were quantified by GC-ICP-MS. Accuracy checks were performed by analyzing reference materials NIST SRM 2974a (freeze-dried mussel tissue) and SRM 1946 (fresh fish tissue homogenate) samples with a standard addition calibration method and by comparative analysis with species-specific isotope-dilution GC-ICP-MS. Good agreement of results between the determined and certified values were obtained (recoveries lied between 94 and 105%). Limits of detection (LODs) expressed on wet weight (ww) basis were 0.003 ng g\(^{-1}\) for BDE 28, 0.006 ng g\(^{-1}\) for BDE 47, 0.008 ng g\(^{-1}\) for BDE 99, 0.004 ng g\(^{-1}\) for BDE 100, 0.005 ng g\(^{-1}\) for BDE 153 and 0.009 ng g\(^{-1}\) for BDE 154. The analytical method was applied for the determination of PBDEs in marine mussels and fish samples from the northern
Adriatic Sea and fish samples from the Sava River. Among the six PBDEs congeners determined, BDE 47, BDE 100 and BDE 99 were commonly detected in the samples analysed.

**Keywords:**
Polybrominated diphenyl ethers
Mussel and fish tissue
Gas chromatography-inductively coupled plasma mass spectrometry
Isotope dilution mass spectrometry

1. **Introduction**

Polybrominated diphenyl ethers (PBDEs) are a group of chemicals that have been on the market since the 1960s. They have been widely used as flame retardants in a variety of commercial products, such as polyurethane foams, electronic equipment, plastics, building materials and textiles [1]. PBDEs do not form chemical bonds to the matrix of the flame-retarded product and can be easily leached into the environment during their manufacturing and during the use or after the disposal of products that contain them [2,3]. Hence, current major sources of PBDE release into the environment are from waste processing and separation, sewage sludge disposal and landfill leachate outflows. Their occurrence has been demonstrated worldwide in sediments, soils, surface waters, sewage sludge, outdoor or indoor air, house dust and biota [4-7]. They can even be detected in samples taken from the deep ocean and remote sites in Arctic [8] and Antarctic environments [9].

PBDEs belong to a class of hydrophobic, poorly degradable persistent organic pollutants that tend to adsorb onto particulate matter, bio-accumulate in fatty tissues and bio-magnify through the food web [10]. They are frequently present in different aquatic organisms and mammals [10], including humans, where they have been detected in blood, adipose tissue and
breast milk. Studies have demonstrated that exposure to PBDEs can cause adverse health effects in experimental animals and humans, including the disruption of growth and the immune and endocrine (thyroid hormone, for example) systems, as well as neurodevelopmental delays [11,12]. Accordingly, the production and use of technical penta-, octa- and deca-BDE mixtures has been restricted by law in the EU [13,14] and partially banned in the USA. Despite the bans, PBDEs will continue to be released into the environment from existing large reservoirs of PBDE-containing products for many years to come and remain a pollution problem in the foreseeable future.

The human population is mainly exposed to PBDEs through the diet and dust ingestion [10,15]. The diet is the predominant exposure pathway for Europeans [16,17]. In a study carried out by Voospoels et al.[18] a positive correlation between fish consumption and concentrations of PBDEs in blood serum was established. Fish consumption contributed approximately 40%–50% of the total dietary intake of PBDEs. The European Union Water Framework Directive (WFD) included six PBDE congeners (BDE 28, BDE 47, BDE 99, BDE 100, BDE 153 and BDE 154) in the group of priority hazardous substances [19]. Their regular monitoring is recommended by the WFD in order to prevent pollution of the aquatic environment. The Environmental Quality Standard (EQS) limit concentration for the sum of the six PBDE congeners in the biota (relating to fish), is set to 8.5 ng/kg wet weight (ww) [19]. The analysis of mussels, which are known bioindicator organisms used for monitoring organic and inorganic pollutants in aquatic environments, and fish samples provides valuable information about the risks associated with human exposure to PBDEs through the diet [20]. Thus, reliable and very sensitive analytical methods for the determination of PBDEs in fish and mussel tissues are needed in order to evaluate PBDE pollution of the aquatic environment and the consequent risk to human health.
Extraction of PBDEs from fish and mussel tissue sample matrices has been performed in recent years by using Soxhlet extraction [7,21,22], accelerated solvent extraction [23-25], ultrasound-assisted extraction [26] or microwave-assisted extraction [23,27]. After extraction, the content of PBDEs is determined with high sensitivity by using advanced instrumental techniques. Those based on the separation of PBDEs by gas chromatography (GC) [28] coupled with different highly developed detector systems, such as high resolution mass spectrometry [27,29], mass spectrometry operating in the negative chemical ionization mode [7,16,26] or electron capture detectors [22-24] are almost exclusively applied. Alternatively, inductively coupled plasma mass spectrometry (ICP-MS), which possesses good sensitivity and excellent selectivity for bromine determination, even in the presence of compounds containing sulfur or chlorine that can interfere with the detector systems listed above, can be used for the detection of the GC-separated PBDEs [30,31].

The aim of this work was to develop a simple analytical procedure that requires minimal sample preparation for a rapid, sensitive and reliable determination of the six PBDEs listed in the WFD in mussel and fish samples by GC-ICP-MS. For this purpose, the influence of different extracting agents (25% aqueous solution of tetramethylammonium hydroxide (TMAH), 0.5 mol L\(^{-1}\) acetic acid in methanol (MeOH) and 0.1 mol L\(^{-1}\) hydrochloric acid (HCl) in MeOH) and the subsequent simultaneous addition of tris(hydroxymethyl)aminomethane (Tris)-citrate buffer (co-extracting agent) and iso-octane on the extraction efficiency was studied when applying different modes of extraction (mechanical shaking, microwave- and ultrasound-assisted extraction). The extraction step was optimized by analyses of National Institute of Standards and Technology standard reference material, NIST SRM 2974a freeze-dried mussel tissue. A rapid and low solvent-consuming clean-up step, which included passing the extract through a column filled with Florisil, was also tested. The accuracy of the analytical method developed was checked by analyzing NIST SRM 2974a and NIST SRM
1946 (Lake Superior fish tissue) samples by the standard addition calibration method and performing a comparative analysis with the species-specific isotope-dilution (ID) GC-ICP-MS method. To demonstrate the applicability of the GC-ICP-MS analytical procedure developed for the determination of PBDEs in real samples, mussels and marine fish samples from the northern Adriatic Sea and fish samples from the Sava River were analyzed.

2. Experimental

2.1. Instrumentation

The analysis of PBDEs was carried out on an Agilent 6890 GC Agilent Technologies (Santa Clara, CA, USA) equipped with an Agilent 6890 Series Autosampler Injector. The GC was coupled to an Agilent 7700x ICP-MS via a heated transfer line and fitted with a 15 m × 0.25 mm DB-5MS capillary column (film thickness 0.25 µm) coated with 5% phenylmethylpolysiloxane (Agilent J&W Scientific, Palo Alto, CA, USA). Hyphenated instrumental set-up was controlled by Agilent MassHunter software.

For the separation of PBDEs on a 15-m column, the following GC temperature program was applied: the temperature was raised from 120 °C to 300 °C at a heating rate of 30 °C min⁻¹ and held there for 5 min. The inlet temperature and the transfer line were held at 280 °C. Helium at a flow rate of 1.5 mL min⁻¹ was used as the carrier gas. The injection mode was splitless and the injection volume 2 µL. The operating parameters of the GC-ICP-MS instrumental set-up are presented in Supplementary Table S1. Their optimisation is described in our previous work [32].

Mechanical shaking of the samples during the extraction procedure was performed on a Vibromix 40 orbital shaker (elliptical table shaker) Tehtnica (Železniki, Slovenia), the
ultrasound-assisted extraction of the samples on a 550D VWR International ultrasonic bath
(West Chester, PA, USA), and the microwave-assisted extraction on a CEM MARS 5
microwave acceleration reaction system CEM (Matthews, NC, USA). Centrifugation of the
sample extracts was carried out on Hettich Universal 320 Centrifuge Hettich GmbH & Co.,
KG (Tuttlingen, Germany). Tissue samples were homogenised in a OmniBlend V – 1.5 Litre
BPA FREE blender OmniBlend Australia Pty Ltd (Centennial Byron Bay, Australia).
Samples were stored at -20 °C in a freeze drier FN6192PW Gorenje (Velenje, Slovenia).

2.2. Reagents and materials

All the reagents used were of analytical reagent grade. MilliQ water (18.2 MΩ cm)
Milipore (Bedford, MA, USA) was used for the preparation of all the aqueous solutions.
Individual standards of seven BDE congeners (28, 47, 77, 99, 100, 153 and 154) at a
concentration of 50 μg mL⁻¹ were purchased from Cambridge Isotope Laboratories Inc.
(Andover, MA, USA). Standard stock solutions of the PBDEs were prepared in iso-octane at
concentration levels of 5 μg mL⁻¹ and stored in the dark at 4 °C. Working standard solutions
were prepared daily in acetone. ⁸¹Br isotopically enriched BDEs (BDE 28, BDE 47, BDE 99,
BDE 100, BDE 153 and BDE 154) were obtained from ISC Science (Oviedo, Spain) and were
used for the calculation of BDE concentrations by isotope dilution (ID) GC-ICP-MS. The
compositions of the ⁸¹Br enriched BDE 28, BDE 47, BDE 99 and BDE 153 determined for
isotopes 81 and 79 were 99.53% and 0.47%, respectively. The compositions of the ⁸¹Br
enriched BDE 100 for isotopes 81 and 79 were 68.5% and 31.5%, and of ⁸¹Br-enriched BDE
154, 76.3% and 24.7%, respectively.

The standard reference material 2974a (freeze-dried mussel tissue) and the standard
reference material 1946 (Lake Superior fish tissue) were purchased from the National Institute
of Standards & Technology (NIST) (Gaithersburg, MD, USA). Tetramethylammonium hydroxide (TMAH), hydrochloric acid (HCl), acetone, acetic acid, citric acid monohydrate, 25% potassium hydroxide (KOH) and tris(hydroxymethyl)aminomethane (Tris) were obtained from Merck (Darmstadt, Germany).

Hexane, methanol (MeOH) and iso-octane (2, 2, 4-trimethylpentane) were purchased from J. T. Baker (Deventer, Holland). Tris-citrate buffer (pH 6.0) was prepared daily from a 0.2 mol L$^{-1}$ solution of Tris with the appropriate addition of citric acid. The columns used to clean up the extract were Strata FL-PR Florisil (170 μm, 80A) 500 mg/3 mL, obtained from Phenomenex, Inc. (Torrance, CA, USA).

2.3. Cleaning procedure

To avoid contamination, all the glassware was rinsed three times with tap water, soaked in 10% nitric acid for 48 h, rinsed three times with tap water and three times with MilliQ water and heated at 400 °C for at least 4 h. Prior to use, all the glassware was rinsed with hexane and acetone and dried at room temperature.

2.4. Sample preparation

For method development, standard reference material NIST SRM 2974a (freeze-dried mussel tissue) was used. To check the accuracy of the developed analytical procedure PBDEs were determined in two reference materials, NIST SRM 2974a and NIST SRM 1946 (frozen fish tissue homogenate), which are certified for all six BDEs analysed. The latter was analysed to experimentally prove that the extraction of PBDEs from mussel tissue matrix is also applicable to their efficient extraction from fish sample matrix. To verify the applicability of
the method, PBDEs were determined in marine mussels and marine and freshwater fish samples (sampled at several sampling locations during the year 2015 in the northern Adriatic Sea and the Sava river, respectively). Mussels from mariculture located in the Slovenian part of the northern Adriatic Sea were purchased in a local store. In the laboratory, mussels were rinsed with fresh water and methanol. Shells were removed and the whole mussel tissues homogenised in a blender. Analysis was carried out using the pooled fresh sample. Fish samples were filleted, homogenized in a blender, lyophilized and stored in the dark at -20 °C until analysis.

2.5. Recommended analytical procedure

Lyophilized fresh mussel or fresh fish sample (0.25 g or 1.5 g, respectively) was weighed into a glass reactor vessel to which BDE 77 (internal standard), $^{81}$Br-enriched PBDE spike and 10 mL of 25% aqueous solution of TMAH were added. The vessels were closed and ultrasound-assisted extraction applied for 30 min at power of 700 W. Then, 50 mL of Tris-citrate buffer solution (pH 6) and 2 mL of iso-octane were added and the samples mechanically shaken for 2 h (20 °C, 300 rpm). The organic phase (iso-octane), which contained the dispersed emulsion, was collected into a 10-mL glass vial with a Pasteur pipette. To separate the emulsion from the organic phase, 0.5 mL of 25% potassium hydroxide (KOH) in MeOH was added and the sample centrifuged for 5 min at 5000 g. The clear organic phase was collected and passed through a 3-mL column packed with 500 mg of Florisil (preconditioned with 1 mL of iso-octane) to remove lipids from the matrix. The column was washed with additional 2 mL of iso-octane and the entire organic phase concentrated under a gentle nitrogen flow to a final volume of approximately 30 μL. Finally, the concentrated extract was collected in a 2-mL amber vial with a Pasteur pipette and 2 μL injected onto the
GC column. Analysis was carried out by GC-ICP-MS and the concentrations of PBDEs calculated by both the standard addition calibration method and ID GC-ICP-MS procedure based on the equations provided by Rodríguez-González et al. [33]. Blank samples were processed following the analytical method described. If not stated otherwise, all the analyses were carried out in triplicate. A flow chart of the analytical method used is presented in Supplementary material (Fig. S1).

3. Results and discussion

3.1. Optimization of the extraction procedure

The main difference between environmental (fresh or sea water, soils or dust) and biological (fish or mussel) sample matrices is the higher content of organic matter, proteins and lipids in the later. Hence, to release the hydrophobic PBDEs from samples with such a complex matrix, an appropriate digestion/extraction method must be applied. Such methods can be relatively time- and solvent-consuming, not sensitive enough for the purpose of their intended use, and with poor recovery for a particular BDE congener. Mussels are normally composed of around 80%, 15%, 2% and 1% water, proteins, fat and ash, respectively, whereas fish fillets consist of 66%–81%, 16%–21%, 0.2%–25% and 1.2%–1.5% water, proteins, lipids and ash, respectively [34]. Due to their high lipid content, they should be removed prior to gas chromatographic separation of the PBDEs. The clean-up techniques mostly reported are saponification, concentrated sulphuric acid treatment, gel permeation chromatography and column chromatography with suitable adsorbents [35]. However, these procedures usually require large amounts of organic solvents, the retention capacity of the columns used is limited and the loss of analytes can occur, whereas the GC-ICP-MS determination of PBDEs requires no other elimination of co-extracted interfering substances.
in the clean-up step, except the removal of lipids, which can damage the GC column. So, to develop a sensitive and reliable analytical procedure for the determination of the selected PBDEs in mussels and fish and samples by GC-ICP-MS, the parameters that influence the efficiency of their extraction must first be optimized. In this work, optimization of the analytical method was performed on freeze-dried mussel tissue reference material SRM 2974a, for which certified or reference values for all six PBDEs had been determined. SRM 2974a (0.25 g) was weighed into a glass reactor vessel and spiked with BDE 28, BDE 47, BDE 99, BDE 100, BDE 153, BDE 154 and BDE 77 (200 ng g\(^{-1}\) each) as an internal standard. The spiking procedure consisted of the addition of a standard in acetone to a sample, homogenization of the resulting slurry and sample incubation in a fume hood until complete evaporation of the acetone.

3.1.1. Influence of different extraction agents on extraction efficiency

The most efficient extraction agents was chosen for the extraction of PBDEs from mussels after a series of experiments in which 10 mL of a given extraction agent (25% aqueous solution of TMAH, 0.5 mol L\(^{-1}\) acetic acid in MeOH or 0.1 mol L\(^{-1}\) HCl in MeOH) was added to glass reactor vessels containing 0.25 g of the spiked, freeze-dried mussel tissue. These reagents were used since they have been already successfully applied in the extraction procedure of organotin compounds from mussel tissue [36]. The sample was mechanically shaken for 2 h. Subsequently, 50 mL of Tris-citrate buffer, as the co-extracting agent, aiming to further improve the extraction efficiency, and 2 mL of iso-octane were simultaneously added and the sample, which was mechanically shaken for another 2 h. It has been experimentally proven that the addition of Tris-citrate buffer facilitates the extraction of PBDEs from the matrix and substantially alleviates the separation of the organic and aqueous phases [32]. Nevertheless, after mechanical shaking, dispersed emulsion was still partly
present in the organic phase. To separate the emulsion from iso-octane, 25% KOH in MeOH was added as described in Paragraph 2.5. and concentrations of PBDEs in the clear organic phase determined by GC-ICP-MS. Data from these experiments are presented in Fig. 1.

As can be seen from Fig. 1 (analysis of spiked, freeze-dried mussel tissue), similar analytical signal intensities, which indicate the extraction efficiency, for all the BDE congeners, including the internal standard BDE 77, were obtained regardless the extraction agent applied. On the contrary, in the analysis of fresh fish samples, significant variations of analytical signal intensities were observed. These data were not shown since in some samples a thick slurry was formed, which prohibited us from collecting an optimal quantity of organic phase in the case of 0.5 mol L\(^{-1}\) acetic acid in MeOH or 0.1 mol L\(^{-1}\) HCl in MeOH, respectively.

Thus, on the basis of our experimental observations and results, we concluded that, for the application of the developed analytical procedure to both freeze-dried and fresh samples, the 25% aqueous solution of TMAH is the most efficient extraction agent.

3.1.2. Influence of different modes of extraction on extraction efficiency

Three modes of extraction, namely mechanical shaking (20 °C, 300 rpm), microwave-assisted extraction (40 °C, 60 °C or 90 °C, 15% of 1600 W) and ultrasound-assisted extraction (20 °C, 240 W), were applied over different time intervals and their performances compared during optimization of the extraction of PBDEs from mussel and fish samples.

3.1.2.1. Extraction by applying mechanical shaking. To 0.25 g of spiked SRM 2974a (freeze-dried mussel tissue sample) 10 mL of 25% aqueous solution of TMAH was added. The sample was, in the first step, mechanically shaken (20 °C, 300 rpm) for 2 h. In the second step,
Tris-citrate buffer (50 mL) and iso-octane (2 mL) were added and the sample shaken for different periods from 0.25 to 16 h. Results of these experiments are presented in Fig. 2A.

*Insert Fig. 2. around here*

The results presented in Fig. 2A show that constant maximum values of analytical signal intensities for all the PBDEs analysed were reached when the sample was first shaken with 25% aqueous solution of TMAH for 2 h, followed by shaking in Tris-citrate buffer and iso-octane for a minimum of two additional hours. Similarly to the second step, the time of extraction was optimized for the first step of extraction, varying the time interval from 0.25 to 16 h in the first step, while keeping the time of extraction in the second step constant (2 h). These results are presented in Fig. 2B and show that the optimal duration of mechanical shaking in the first extraction step is 2 h. Thus, the optimal total extraction time of the two-step mechanical shaking extraction method was 2 h (shaking with 25% aqueous solution of TMAH) + 2 h (subsequent shaking after the addition of Tris-citrate buffer and iso-octane).

3.1.2.2. Ultrasound-assisted extraction. The closed-glass reactors, which contained 0.25 g of spiked, freeze-dried mussel tissue (SRM 2974a) sample and 10 mL of 25% aqueous solution of TMAH, were subjected to ultrasound-assisted extraction for 15, 30 or 60 min. Then, the Tris-citrate buffer and iso-octane were added and the sample mechanically shaken for 2 h in the second step. Corresponding results, which are presented in Fig. 3A, demonstrate that the analytical signal intensities were increased with time and became constant when 30 min of ultrasound-assisted extraction was applied. The maximum values of analytical signal intensities were slightly higher than those obtained after extraction with mechanical shaking.

3.1.2.3. Microwave-assisted extraction. As in the other two modes of extraction, microwave-assisted extraction was carried out in two steps. In the first step, closed vessels containing 0.25 g of spiked, freeze-dried mussel tissue (SRM 2974a) sample and 10 mL of 25% aqueous solution of TMAH were subjected to microwave-assisted extraction at different temperatures.
(40 °C, 60 °C or 90 °C). The following programme was used: power 15% of 1600 W, ramp to selected final temperature (40 °C, 60 °C or 90 °C) in 1 min and hold at that temperature for 3 min. After cooling, the suspensions were transferred to glass reactors, Tris-citrate buffer and iso-octane were added and samples were mechanically shaken for 2 h. By comparison of the analytical signal intensities presented in Fig 3B, it can be seen that extraction of the PBDEs from the sample matrix is slightly less efficient at 40 °C than that at 60 °C or 90 °C.

Insert Fig. 3. around here

To summarize section 3.1.2. (compare analytical signal intensities in Figs. 2 and 3), it can be deduced that, among all the modes of extraction compared, microwave-assisted extraction is the least and ultrasound-assisted the most efficient for the extraction of PBDEs from a freeze-dried mussel sample matrix. This result is consistent with outcomes of our previous work where the efficiency of microwave extraction for the extraction of PBDEs from sewage sludge was found to be inferior to the tested alternatives (mechanical shaking, ultrasound-assisted extraction) [30].

Once the extraction agents and relevant extraction conditions were optimized for mussels (SRM 2974a), it was experimentally proven that, among the extraction agents tested for mussels, only a 25% aqueous solution of TMAH can also be efficiently applied for the extraction of PBDEs from a fresh fish sample matrix (NIST SRM 1946). When different modes of extraction were applied under their corresponding optimal parameters, experimental results showed that PBDEs can be extracted from this sample matrix by mechanical shaking or ultrasound-assisted extraction with almost identical efficiency, whereas microwave-assisted extraction was less efficient.

Because the ultrasound-assisted extraction applied in the first step exhibits several advantages, such as being the most robust, time efficient and easiest to handle, the extraction method consisting of 30 min of ultrasound-assisted extraction with 25% aqueous solution of
TMAH in the first step, followed by 2 h of mechanical shaking with Tris-citrate buffer and iso-octane in the second step was chosen as the optimal method for lyophilised mussel and fresh fish sample matrices.

3.2. Performance of the analytical procedure

The performance of the optimised analytical procedure (paragraph 2.5.) for the simultaneous speciation analysis of six BDE congeners in freeze-dried mussel tissue (SRM 2974) and fresh fish tissue (SRM 1946a) was evaluated by the determination of its limits of detection and quantification, linearity, repeatability and reproducibility of measurement and by an accuracy check.

3.2.1. Limits of detection and quantification, linearity, repeatability and reproducibility of measurement

The limits of detection (LODs) and limits of quantification (LOQs) for the determination of six BDE congeners were calculated on the basis of three times (3s) and ten times the standard deviation (10s), respectively, of the signal intensities of the blank sample, measured in eight replicates. To estimate the linearity of the calibration curves, SRM 2974a and SRM 1946 were spiked with six PBDEs in concentrations ranging from 0.05 to 5 ng g\(^{-1}\) for each BDE. The results are presented in Table S2. Under the optimized analytical procedure, low LODs and LOQs were obtained. LODs for six BDE congeners calculated on ww and dry weight (dw) basis ranged from 0.003 ng g\(^{-1}\) to 0.009 ng g\(^{-1}\) and from 0.01 to 0.031 ng g\(^{-1}\), respectively, while LOQs from 0.011 ng g\(^{-1}\) to 0.030 ng g\(^{-1}\) and from 0.038 ng g\(^{-1}\) to 0.105 ng g\(^{-1}\), respectively. Correlation coefficients of the calibration curves (R) for the PBDEs determined were better than 0.997.
The repeatability of the proposed methodology was evaluated by applying six independent analysis of sample reference materials SRM 2974a and SRM1946 by injecting the extracts on the same day. The same approach was used to test the reproducibility of measurement by performing analysis of six independent sample reference materials SRM 2974a and SRM1946 over a period of 7 days. These data are presented in Tables S3 and S4, respectively. Considering the complex matrix of mussel and fish tissues, good measurement repeatability was obtained for the PBDEs analyzed (relative standard deviations (RSDs) from 3.0% to 5.7%), whereas the measurement reproducibility was slightly worse (RSDs from 5.4% to 8.3%).

3.2.2. Accuracy check

The efficiency of the extraction and accuracy of the analytical procedure applied were evaluated by analysis of the standard reference materials NIST SRM 2974a and NIST SRM 1946. The samples were analysed with GC-ICP-MS using the standard addition method for quantification. The recoveries calculated as the ratio between the certified and determined PBDE concentrations are presented in Supplementary Table S5. It can be seen from Table S5 that the recoveries lay between 94% and 105%, indicating a high extraction efficiency. It can be further seen that the determined concentrations of PBDEs are in good agreement with the certified values. The accuracy of the analytical procedure was also verified by the species-specific ID-GC-ICP-MS method applied for quantification. For this purpose, $^{81}$Br-enriched BDEs were used and the PBDE concentrations determined by ID using the procedure and equations reported by Rodríguez-González et al. [33]. These results are presented in Supplementary Table S6.
They indicate that the differences between results determined by GC-ICP-MS and those determined by ID-GC-ICP-MS did not exceed 6%, further confirming the accuracy of the analytical procedure.

Typical chromatograms corresponding to working standard solutions (60 ng L\(^{-1}\)) of all the PBDEs analyzed, which were used to depict the retention time of a given BDE, the standard NIST SRM 2974a, and the standard NIST SRM 1946 are presented in Fig. 4 as Fig. 4A, Fig. 4B and Fig. 4C, respectively. It can be seen that the retention times obtained for the PBDE standard solution match those of both NIST standards. The chromatographic peaks are well resolved, another indication of the high selectivity of the analytical procedure developed.

*Insert Fig. 4. around here*

On the basis of the results presented in this paragraph, it can be concluded that the analytical procedure developed is suitable for its intended use (the determination of six PBDEs in mussel and fish samples, freeze-dried or fresh). When compared with the GC-MS procedures cited in the literature, it is characterized by faster sample preparation, a simpler clean-up step, lower solvent consumption, very good recoveries and low LODs.

3.3. *Analysis of PBDEs in blue mussels and fish samples from the northern Adriatic Sea and fish samples from the Sava River*

In order to confirm the feasibility of the newly developed GC-ICP-MS analytical procedure for determination of the content of PBDEs in fish and mussels, blue mussels and fish samples from the northern Adriatic Sea (Slovenia) and Chub River fish samples from the Sava River taken at different locations (Slovenia, Croatia and Serbia) were analysed. These samples are commonly analysed in biomonitoring of particular toxic substances in aquatic
environments or to monitor food safety. All the samples analysed were collected in 2015. The results are presented in Table 1.

The PBDE concentrations in mussels and fish samples calculated on the ww and dw basis ranged from below LODs to 0.54 ng g\(^{-1}\) and from below LODs to 1.61 ng g\(^{-1}\) for a given BDE congener, respectively, while the \(\Sigma PBDEs\) from 0.24 ng g\(^{-1}\) to 0.83 ng g\(^{-1}\), respectively. All six PBDE congeners listed in the WFD were detected, confirming their presence in the aquatic environment from which samples were taken. This is the first evidence of the presence of PBDEs in fish from this part of the Europe. The concentrations of the \(\Sigma PBDEs\) from the present study were of comparable orders magnitude or lower than those reported in the literature for other European countries. Among the PBDEs determined, BDE 47 was the most abundant. Its concentrations ranged from 0.09 and 0.54 ng g\(^{-1}\) ww. Beside BDE 47, BDE 100 and BDE 99 were commonly detected. A similar pattern of concentrations of different BDE congeners was also observed by other authors, who’s results on the \(\Sigma PBDE\) concentrations in fish (ng g\(^{-1}\) ww) from the different sites in Europe are summarized in Table 2.

Our first results suggest that PBDEs are ubiquitous in the aquatic environment in this part of Europe. However, to evaluate the extend of pollution by PBDEs, more fresh and marine water biological samples, including fish and mussels from regular monitoring programmes, should be analysed, especially since the \(\Sigma PBDE\) concentration in the fish samples analysed was above the environmental quality standard (EQS) for biological samples (EQS\(_{biota}\) for the sum of BDE congeners 28, 47, 99, 100, 153 and 154 is set to be lower than 0.0085 ng g\(^{-1}\) (ww basis)).

4. Conclusions
A simple, sensitive, fast and robust analytical method was developed for reliable determination of six PBDE congeners (BDE 28, BDE 47, BDE 99, BDE 100, BDE 153 and BDE 154) in mussels and fish samples by GC-ICP-MS. The most efficient extraction of PBDEs, from both mussels and fish samples, was achieved when 30 min of ultrasound-assisted extraction with 10 mL of 25% aqueous solution of TMAH, followed by 2 h of mechanical shaking with 50 mL of Tris-citrate buffer and 2 mL of iso-octane, was applied. This extraction approach has the advantages of being robust, time efficient and easiest to handle.

The removal of lipids from the organic phase was performed by passing the extract through a column filled with Florisil. This clean-up step is simpler than those needed prior to detection of PBDEs by MS techniques. Quantification of the PBDEs extracted in iso-octane was performed by GC-ICP-MS using a standard addition calibration method and by species-specific ID-ICP-MS. Results of analyses of the NIST SRM 2974a (freeze-dried mussel tissue) and the NIST SRM 1946 (frozen fish tissue homogenate) reference materials were in agreement with the certified values, confirming the accuracy of the analytical procedure. Good repeatability and reproducibility of measurements were obtained (RSDs from 3.0% to 8.3%). The analytical method is sensitive, with LODs for six PBDEs ranging from 0.003 to 0.009 ng g⁻¹ ww (0.010 to 0.031 ng g⁻¹ dw). In general, RSDs are better and LODs lower or in the same range as those reported in the cited literature.

The procedure developed was successfully applied for the determination of the six PBDEs in mussels and fish samples from the Slovenian part of the northern Adriatic Sea and fish from the Sava River. The concentrations of the ΣPBDEs and the observed PBDE profiles from the present study are within the same magnitude of order or lower than those reported for other European countries. Due to its simplicity, sensitivity and reliability, the method
developed can be used to assess the levels of PBDE pollution in the biota samples in an attempt to enhance ecosystem monitoring strategies and food safety.

Acknowledgements

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References


**Figure Captions**

**Fig. 1.** Influence of different extraction agents on signal intensities in mussel tissue sample (NIST SRM 2974a) spiked with PBDEs (200 ng g\(^{-1}\)) obtained by applying mechanical shaking and speciation analysis with GC-ICP-MS. Results represent the average ± standard deviation of three replicates. Samples were shaken for 2 h. Tris-citrate buffer and iso-octane were added and samples shaken for another 2 h.

![Graph showing signal intensities for different extracting agents and PBDEs](image)

**Fig. 2.** Influence of different modes of extraction on signal intensities in mussel tissue sample (NIST SRM 2974a) spiked with PBDEs (200 ng g\(^{-1}\)) obtained by applying mechanical shaking and speciation analysis with GC-ICP-MS. Results represent the average ± standard deviation of three replicates. (A) Samples were shaken with 10 mL of 25% aqueous solution of TMAH for 2 h. Tris-citrate buffer and iso-octane were added and samples shaken again for different time intervals (0.25–6 h). (B) Samples were shaken with 10 mL of 25% aqueous solution of TMAH for different times (0.25–16 h). Tris-citrate buffer and iso-octane were added and samples shaken again for 2 h.
Fig. 2. Influence of different modes of extraction on signal intensities in mussel tissue sample (NIST SRM 2974a) spiked with PBDEs (200 ng g⁻¹) obtained by applying ultrasound- and microwave-assisted extraction and speciation analysis with GC-ICP-MS. Results represent the average ± standard deviation of three replicates.

(A) Samples were supplemented with 10 mL of 25% aqueous solution of TMAH and subjected to ultrasound-assisted extraction for 15, 30 and 60 min. Tris-citrate buffer and iso-octane were added and samples mechanically shaken for next 2 h.
(B) Samples were supplemented with 10 mL of 25% aqueous solution of TMAH and subjected to microwave-assisted extraction at 40, 60 and 90 °C for 4 min. Tris-citrate buffer and iso-octane were added and samples mechanically shaken for next 2 h.

Fig. 3.

Fig. 4. Chromatograms of (A) the mixture of six BDE congeners and internal standard BDE 77 in isooctane (60 ng mL\(^{-1}\) each), (B) the freeze-dried mussel tissue sample (NIST SRM 2974a) spiked with internal standard BDE 77 and (C) the frozen fish tissue homogenate sample (NIST SRM 1946) spiked with internal standard BDE 77. The organic phase of the
samples was concentrated to 30 µL and the GC-ICP-MS procedure applied. Chromatograms were recorded at m/z 79.

Fig. 4.
Table 1
Concentrations of PBDEs in fish and mussel samples from the Northern Adriatic Sea and from the Sava River determined by GC-ICP-MS expressed in ng g\(^{-1}\) on ww and dw basis.

<table>
<thead>
<tr>
<th>Location</th>
<th>Species</th>
<th>BDE congener (ng g(^{-1}) ww)</th>
<th>BDE congener (ng g(^{-1}) dw)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>BDE 28</td>
<td>BDE 47</td>
</tr>
<tr>
<td>Litija (SI)</td>
<td>chub</td>
<td>0.04</td>
<td>0.50</td>
</tr>
<tr>
<td>Čatež (SI)</td>
<td>chub</td>
<td>&lt; LOD</td>
<td>0.19</td>
</tr>
<tr>
<td>Zagreb (HR)</td>
<td>chub</td>
<td>0.04</td>
<td>0.54</td>
</tr>
<tr>
<td>Beograd (RS)</td>
<td>chub</td>
<td>0.02</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>sardine pilchard</td>
<td>0.11</td>
<td>0.53</td>
</tr>
<tr>
<td>Slovenia</td>
<td>seabass</td>
<td>0.05</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td>whiting</td>
<td>0.07</td>
<td>0.14</td>
</tr>
<tr>
<td>Slovenia</td>
<td>blue mussel</td>
<td>0.03</td>
<td>0.09</td>
</tr>
</tbody>
</table>

SI – Slovenia  
HR – Croatia  
RS – Serbia
Table 2
Comparison of $\sum_6$PBDE (BDE 28, BDE 47, BDE 99, BDE 100, BDE 153, BDE 154) concentrations (ng g$^{-1}$ ww) in fish samples among European countries in last 18 years. All the compared concentrations were determined in fish muscle tissues.

<table>
<thead>
<tr>
<th>Location</th>
<th>Sampling year</th>
<th>Species</th>
<th>$\sum_6$PBDE (ng g$^{-1}$ ww)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Finland Sea</td>
<td>2007-2008</td>
<td>farmed fish</td>
<td>0.9 - 1.7*</td>
<td>[37]</td>
</tr>
<tr>
<td>Baltic Sea</td>
<td>2004-2006</td>
<td>herring, salmon</td>
<td>1.2 - 2.5</td>
<td>[38]</td>
</tr>
<tr>
<td>Belgium Sea</td>
<td>2003</td>
<td>plaice, whiting</td>
<td>0.06 - 6.1</td>
<td>[39]</td>
</tr>
<tr>
<td>German rivers</td>
<td>2013</td>
<td>bream</td>
<td>0.14 – 18</td>
<td>[40]</td>
</tr>
<tr>
<td>Czech Republic Elbe River</td>
<td>2002-2003</td>
<td>chub, bream, trout</td>
<td>2 – 18</td>
<td>[29]</td>
</tr>
<tr>
<td>Norway lakes</td>
<td>1998-2004</td>
<td>brown trout</td>
<td>0.3 – 23</td>
<td>[41]</td>
</tr>
<tr>
<td>Italy</td>
<td>2011-2012</td>
<td>halibut, blue fish, trout</td>
<td>0.03 - 0.5</td>
<td>[25]</td>
</tr>
</tbody>
</table>

*dw basis, moisture 12%