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Research Article

Determination of polybrominated diphenyl ethers in aquatic animal tissue using cleanup by freezing-dispersive liquid–liquid microextraction combined with GC-MS

A method for the determination of polybrominated diphenyl ethers (PBDEs) in aquatic animal tissue was developed, and it is based on cleanup by freezing-dispersive liquid–liquid microextraction (DLLME) for the pretreatment of samples with fat content. Aquatic animal tissue homogenate was extracted with acetone, and then frozen in a freezer at -80°C . Fat in aquatic animal tissue was isolated from the analytes, *i.e.* PBDE congeners dissolved in acetone and fat formed floccules at low temperature. The supernatant was extracted using the DLLME technique. Under the optimum DLLME conditions, 0.75 mL supernatant with 33 μL chlorobenzene was added into 5.0 mL pure water, and subsequently cloudy solution was formed. After centrifuging, 1.0 μL lower phase was subjected to GC-MS analysis. Recovery test was performed at fortified concentrations of 5.0–2500 $\mu\text{g}/\text{kg}$. The detection results showed that the recoveries for each target analyte ranged from 75.3 to 127.8%. The repeatability of the proposed method by spiking aquatic animal samples at 10 $\mu\text{g}/\text{kg}$ for PBDEs, expressed as RSD, $n = 5$, varied between 4.3 and 10.3% ($n = 5$). LOD of the proposed method for aquatic animal tissue samples were between 2.4 and 4.9 $\mu\text{g}/\text{kg}$ for all the analytes.

Keywords: Aquatic animal tissue / Cleanup by freezing-dispersive liquid–liquid microextraction / GC-MS / Polybrominated diphenyl ethers
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1 Introduction

Flame retardants, added to polymers, are used in plastic, textiles, electronic circuitry and other materials to prevent fires [1, 2]. The most used brominated flame retardants are polybrominated diphenyl ethers (PBDEs), hexabromocyclodecane, tetrabromobisphenol-A, and polybrominated biphenyl brominated flame retardants (except tetrabromobisphenol-A) appear to be fairly nonvolatile, lipophilic, and bioaccumulated in biota and humans, which are similar to other persistent organic pollutants [3, 4].

PBDEs have been widely detected in various environmental compartments with increasing concentrations over time [5–8]. Seven PBDE congeners (BDE-28, 47, 66, 99, 100, 153, and 154) were found in all samples, which included seven species of marine fish (conger eel, flounder, gray

mullet, horse mackerel, red sea bream, sea bass, and yellowtail), and the most abundant PBDE congener was BDE-47 [9]. In the food marker-basket, fish had the highest average sum of PBDEs levels (BDE-28, 47, 99, 100, 153, 154, and 183; 460 pg/g), followed by dairy products and eggs (260 pg/g), fast food, and meat products (86 pg/g) [10]. The total concentration of PBDEs in mussels was 13.8 ng/g lipid in Qingdao coastal sea [11]. A few PBDE congeners (BDE-47, 99, 100, and 28) were found in crustacean muscles (spider crab, edible crab, velvet swimming crab, and Norway lobster) from coastal waters Brittany and Normandy (France) [12]. The total concentration of PBDEs ranged from 0.38 to 9.19 ng/g wet weight in mussels (*Mytilus coruscus* and *M. edulis*) and oysters (*Crassostrea gigas*) [13].

As the worldwide environmental contaminants, several methods have been developed for the determination of PBDEs in environmental samples. There are accelerated solvent extraction [14], matrix solid-phase dispersion [15], microwave-assisted extraction (MAE) [16], headspace solid-phase microextraction [17], and dispersive liquid–liquid microextraction (DLLME) [18]. DLLME was first proposed by Assadi *et al.* in 2006 for the determination of polycyclic

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Abbreviations: DCM, dichloromethane; DLLME, dispersive liquid–liquid microextraction; EI, electron impact ionization; MAE, microwave-assisted extraction; PBDEs, polybrominated diphenyl ethers

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aromatic hydrocarbons, which is based on a ternary component solvent extraction system including extraction solvent, dispersive solvent and aqueous sample containing analyte of interest [19]. It is attracting more and more people's attention due to its superior advantages of high enrichment factor, high recovery, low cost, rapidity and easy operation. Up to now, this method has been widely applied in analyzing the organophosphorus pesticides [20], chlorophenols [21], and polychlorinated biphenyls [22]. In addition, its application has been extended to trace elemental analysis in environmental water samples [23–25]. However, DLLME is widely performed in the preparation of environmental water samples, and rarely applied in analyzing biological samples, especially aquatic animal samples (such as fish, snail, frog, and so on) because of their relatively complicated matrices.

In the present work, cleanup by freezing was used to remove interferences, DLLME was applied in extracting PBDEs, and GC-MS was used to determine PBDE congeners. The combination of cleanup by freezing and DLLME technique was proved to be an efficient pretreatment method for the determination of PBDEs in aquatic animal samples. The factors influencing DLLME efficiency were optimized in detail. Cleanup by freezing-DLLME technique shows great potential in analyzing trace pollutants in aquatic animal samples.

2 Materials and methods

2.1 Reagents and standards

All PBDEs solutions in isooctane (50 mg/L) were obtained from Accustandard (New Haven, CT, USA). Because of the limited water solubility of isooctane, acetone was selected as intermediate solvent. Hundred microlitres of each PBDE solution was mixed and evaporated under a gentle nitrogen flow. Then, the residue was redissolved in 5 mL acetone to obtain a standard stock solution with a concentration of 1 mg/L. The working standard solutions were prepared by serial dilutions of the stock solution with ultra Milli-Q water (Millipore, Molsheim, France) prior to analysis. The HPLC-grade acetonitrile was purchased from Merck Company (Darmstadt, Germany). Acetone, hexane, dichloromethane (DCM), carbon tetrachloride, 1,1,2,2-tetrachloroethane, and chlorobenzene were all of analytical grade and redistilled prior to use. Aquatic animal tissue (snail, frog, and fish) was collected from a local supermarket (Wenzhou, China).

2.2 Instrumentation

The determination of PBDEs was carried out using an Agilent 6890 GC-5950C MS (Agilent Technologies, Wilmington, DE, USA) under electron impact ionization (EI) conditions. The GC was equipped with a split-splitless injector held constant at 290°C. The chromatographic separation was achieved using a HP-5 capillary column

(30 m × 0.25 mm id, 0.25 µm film thickness, Agilent Technologies), which was maintained at 110°C for 2 min, and then ramped at 30°C/min to 220°C for 6 min, further ramped at 3°C/min to 280°C for 3 min. The MS was operated in EI mode with selecting ion monitoring for the quantification. Ion masses 323.9, 325.8 were selected for BDE-47, masses 403.7, 401.8 for BDE-99, 100, 85, and masses 481.7, 483.6 for BDE-153, 154 [26]. In EI mode, the MS source temperature was 250°C and the electron energy was 70 eV.

2.3 Sample preparation

Aquatic animal tissue (snail, frog, and fish), collected from a local supermarket (Wenzhou, China), was wrapped round with sterilized aluminum foil, and transported to the laboratory in a hermetical cold box with ice bags. The whole fish without skin was pooled, the bone of frog was discarded, and the snail was deshelled. The muscles of the fish, frog, and snail samples were mixed uniformly, wrapped round with foil, and stored at –20°C, respectively. After being freeze-dried, every kind of dried muscle was homogenized in a stainless steel blender. The recovery test was undertaken for target concentrations ranging from 5 to 100 µg/kg in the homogeneous muscles for each PBDE congener. The spiking solution was allowed to equilibrate with the homogenized tissue sample for 3 h prior to extraction. An aliquot (1 g) of homogeneous muscles was extracted with 10 mL of acetone for 30 min by shaking after adding 4 g of anhydrous sodium sulfate (heated at 650°C for 8 h). The extract was collected, concentrated to 5 mL under a gentle nitrogen flow, and then frozen in a freezer at –80°C and 0.75 mL supernatant was subjected to DLLME procedure.

2.4 DLLME procedure

A 5.0 mL aqueous solution was placed in a 10 mL screw cap conical bottomed glass test tube and 0.75 mL of acetone extract containing 33.0 µL chlorobenzene (extraction solvent) was injected rapidly into the aqueous solution. A cloudy solution, resulting from the dispersion of the fine chlorobenzene droplets in the aqueous solution, was formed in the test tube. After extracting for a few seconds, the mixture was then centrifuged for 3 min at 3000 rpm. The dispersed fine chlorobenzene droplets were deposited at the bottom of the conical test tube. A 1.0 µL lower phase was removed by 10.0 µL microsyringe and injected into GC-MS.

3 Results and discussion

3.1 Selection of organic solvent for the extraction of PBDEs from aquatic animal tissue

In this study, to simplify the procedure, the solvent should be used as an extraction solvent for the aquatic animal

tissue, and also as the disperser solvent in the DLLME step. The solvent mixtures (hexane/acetone or hexane/DCM) are the most used solvents for extraction of PBDEs from biological tissue and have demonstrated acceptable recoveries [27]. Acetonitrile and acetone were often used as the disperser solvent in DLLME procedure. Therefore, acetonitrile and acetone were investigated as the extraction solvent for the aquatic animal tissue and compared with the solvent mixtures (hexane/DCM, 1:1 v/v) under the same conditions (fish sample, 1 g; added concentration of each PBDE congener, 50 µg/kg; extraction solvent volume, 10 mL; shaken extraction, 30 min). The cleanup procedure (sulphuric acid treatment) for the extract was as follows: the extract was collected in screw cap conical bottomed glass test tube and concentrated under a gentle nitrogen flow rate at room temperature; the residue was dissolved in 1.0 mL hexane and mixed with 300 µL concentrated sulphuric acid; the lower phase was removed after centrifuging at 3000 rpm for 1 min; the addition of concentrated sulphuric acid and centrifugation was in triplicate. The final extract was concentrated to 100 and 1.0 µL was injected into GC-MS. As listed in Table 1, acetone has the best recoveries, and it is also the cheapest solvent among acetonitrile, hexane, and DCM. Therefore, acetone was chosen as the extraction solvent of PBDEs from aquatic animal tissue and used as the disperser solvent in subsequent processes.

3.2 Selection of the cleanup technology

GPC is widely used for the primary cleanup of biological samples [28, 29]. However, this technology needs a lot of organic solvents, and often an additional cleanup step is used for further purification of the extract. Sulphuric acid treatment, either directly to the extract [26, 30] or *via* impregnated silica column [31–33], is the most commonly applied lipid destructive removal method. However, this kind of treatment needs several extraction and centrifugation steps, thus making the procedure laborious and time-consuming. On the contrary, acidified silica gel should be handled with extreme care and inhalation of fine acid containing particles should be avoided. To avoid the shortcomings of the above methods, the acetone extract

Table 1. Efficiency of different extraction solvents evaluated of PBDEs from fish sample

| Compounds | Recovery (%) | | |
|-----------|------------------|--------------------|-----------------------------------|
| | Hexane/DCM (1:1) | CH ₃ CN | CH ₃ COCH ₃ |
| BDE 47 | 99.7 | 79.4 | 100.5 |
| BDE100 | 93.6 | 82.3 | 106.7 |
| BDE 99 | 102.7 | 85.4 | 116.6 |
| BDE 85 | 95.9 | 79.9 | 103.7 |
| BDE154 | 94.3 | 81.5 | 110.1 |
| BDE153 | 91.2 | 79.6 | 108.2 |

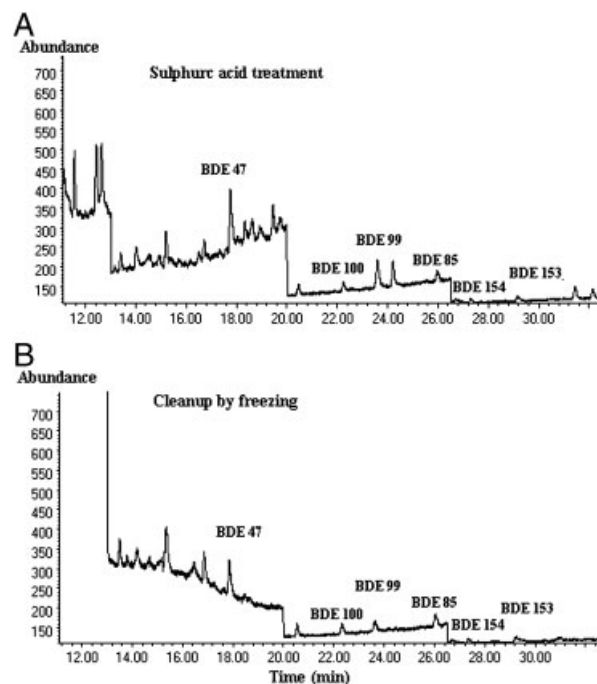


Figure 1. Chromatograms attained from DLLME with sulphuric acid treatment (A) and cleanup by freezing technology (B).

was frozen in a freezer at -80°C . The fat formed floccules, and the supernatant was collected and applied in further DLLME procedure. To investigate the effect of cleanup by freezing, it was compared with the sulphuric acid treatment. Figure 1 shows two chromatograms from DLLME, which were attained by using two kinds of cleanup technology under the following constant conditions (fish sample, 1 g; added concentration of each PBDE congener, 5 µg/kg; water, 5 mL; acetone (disperser solvent), 1 mL; chlorobenzene (extraction solvent), 33 µL). Obviously, the two kinds of cleanup technology had the fine and similar cleanup efficiencies. Considering the shortcomings of GPC and sulphuric acid treatment, cleanup by freezing was applied in subsequent experiments.

3.3 Optimization of DLLME

3.3.1 Effect of type of the extraction solvent of DLLME

Three kinds of organic solvents (chlorobenzene, tetrachloroethane, and carbontetrachloride) were chosen as extraction solvent in DLLME, because they have the properties of higher density than water, high extraction capability for analytes, and low solubility in water. A series of sample solutions were tested using 1.0 mL acetone, containing different volumes of the extraction solvent (chlorobenzene, 33 µL; tetrachloroethane, 26 µL; carbontetrachloride, 42 µL; concentration of each PBDE congener, 1.0 ng/mL) to achieve a 20 µL volume of the lower phase. As can be seen from Table 2, chlorobenzene had high extraction recovery

Table 2. Effect of the type of extraction solvent on the recovery obtained from DLLME^{a)}

| Compounds | Recovery (%) | | |
|-----------|---|---|---|
| | Chlorobenzene mean \pm SD (<i>n</i> = 3) | Tetrachloroethane mean \pm SD (<i>n</i> = 3) | Carbontetrachloride mean \pm SD (<i>n</i> = 3) |
| BDE 47 | 118.3 \pm 4.3 | 86.1 \pm 3.9 | 118.6 \pm 10.0 |
| BDE 100 | 114.7 \pm 9.7 | 87.2 \pm 0.9 | 65.2 \pm 5.4 |
| BDE 99 | 95.0 \pm 7.8 | 88.4 \pm 8.8 | 82.4 \pm 11.1 |
| BDE 85 | 118.3 \pm 9.6 | 106.4 \pm 1.1 | 71.7 \pm 2.0 |
| BDE 154 | 110.9 \pm 4.5 | 106.3 \pm 4.2 | 50.3 \pm 10.5 |
| BDE 153 | 123.6 \pm 0.5 | 110.1 \pm 7.8 | 85.7 \pm 10.7 |

a) Extraction conditions: water sample volume, 5.00 mL; disperser solvent (acetone) volume, 1.00 mL; extraction solvent volume, 33 μ L chlorobenzene, 26 μ L 1,1,2,2-tetrachloroethane, 42 μ L carbon tetrachloride; sedimented phase volume, 20 \pm 0.5 μ L; room temperature; concentration of each PBDE congener, 1.0 ng/mL.

(95.0–123.6%) and also its toxicity is the lowest among the three studied organic solvents. Therefore, chlorobenzene was selected as the extraction solvent in DLLME.

3.3.2 Effect of volume of the extraction solvent of DLLME

To examine the effect of the extraction solvent volume, 1.0 mL acetone containing different volumes of chlorobenzene (33, 43, 53, 63, and 73 μ L) was subjected to the same DLLME procedures (water sample volume, 5 mL; concentration of each PBDE congener, 1.0 ng/mL). By increasing the volume of chlorobenzene from 33 to 73 μ L, the volume of lower phase increases from 20 to 74 μ L. As can be seen from Fig. 2, EF decreased with the increasing volume of chlorobenzene. Therefore, in order to obtain higher enrichment factor and lower detection limit, 33 μ L of chlorobenzene was selected as the volume of extraction solvent in DLLME.

3.3.3 Effect of volume of the disperser solvent of DLLME

In order to examine the effect of volume of disperser solvent (acetone), the volume of lower phase was kept constant (20 μ L) and the volume of acetone and chlorobenzene was changed simultaneously. The different volumes of acetone 0.5, 0.75, 1.00, 1.25, and 1.50 mL was applied concomitantly with 30, 31, 33, 32, and 36 μ L of chlorobenzene, respectively. The other experimental conditions were kept constant (water sample volume, 5 mL; concentration of each PBDE congener, 1.0 ng/mL). It can be seen from Fig. 3 that 0.75 mL acetone has the highest recovery among all of the different volumes. Therefore, 0.75 mL was selected as the volume of acetone.

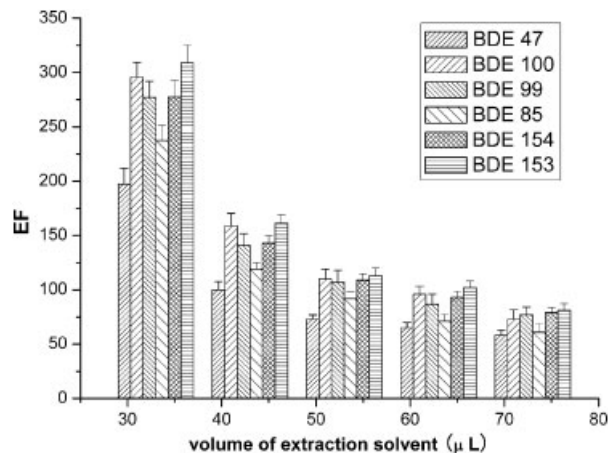


Figure 2. Effect of the volume of extraction solvent (chlorobenzene) on the enrichment factor of PBDEs obtained from DLLME. Extraction conditions: water sample volume, 5.00 mL; disperser solvent (acetone) volume, 1.00 mL; extraction solvent volume, 33, 43, 53, 63, and 73 μ L; room temperature.

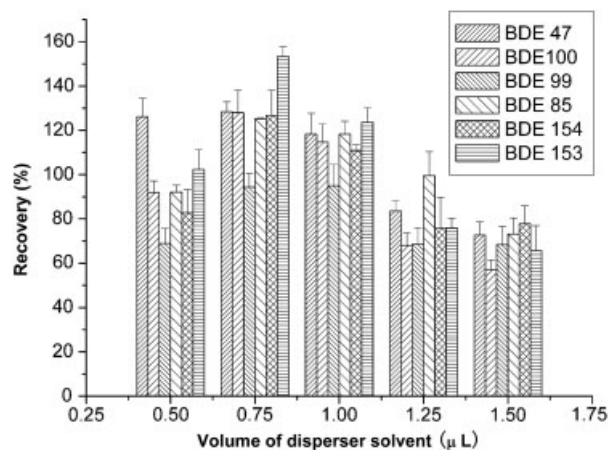


Figure 3. Effect of the volume of disperser solvent (acetone) on the recovery of PBDEs obtained from DLLME. Extraction conditions: water sample volume, 5.00 mL; disperser solvent (acetone) volume, 0.50, 0.75, 1.00, 1.25, and 1.50 mL; extraction solvent (chlorobenzene) volume 30, 31, 33, 32, and 36 μ L; room temperature.

3.3.4 Effect of salt

The influence of ionic strength was evaluated at 0–6% w/v NaCl levels under the following constant conditions (water sample volume, 5 mL; dispersive solvent (acetone) volume, 0.75 mL; extraction solvent (chlorobenzene) volume, 33 μ L; concentration of each PBDE congener, 1.0 ng/mL). The result showed that salt addition had no significant effect on extraction recovery (Fig. 4), which was in accordance with other reports [34, 35]. Therefore, all the extraction experiments were carried out without adding salt.

In total, the optimum DLLME conditions were as follows: water sample volume, 5 mL; dispersive solvent (acetone) volume, 0.75 mL; extraction solvent (chlorobenzene) volume, 33 µL; no addition of salt; 3.0 min centrifugation at 3000 rpm; room temperature.

3.4 Quantitative analysis

To investigate the linearity of cleanup by freezing-DLLME, a series of standard solutions containing different concentrations of PBDEs were tested in deionized water under the optimum conditions. The characteristics of calibration curves are summarized in Table 3. The linearity of calibration curve was observed in the range of 5.00–2500.00 µg/kg for BDE 47, 100, 99, 85, 154, and 153. The coefficients of correlation (r^2) ranged from 0.9988 to

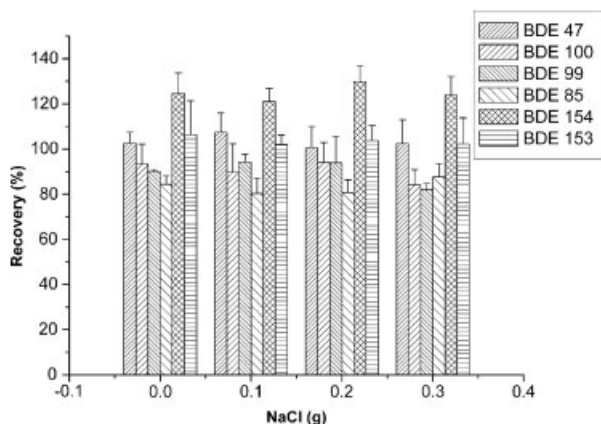


Figure 4. Effect of the addition of NaCl on the recovery of PBDEs obtained from DLLME. Extraction conditions: water sample volume, 5.00 mL; disperser solvent (acetone) volume, 0.75 mL; extraction solvent (chlorobenzene) volume, 33 µL; addition of NaCl, 0.0, 0.1, 0.2, and 0.3 g; room temperature.

0.9998. The method precision was evaluated by carrying out five independent measurements of the studied compounds at 10 µg/kg. The results showed that the RSDs ranged from 4.3 to 10.3%. The LOD, based on S/N of 3 ranged from 2.4 to 4.9 µg/kg.

3.5 Analytical performance

The proposed cleanup by freezing-DLLME technology was applied in the determination of PBDEs in several aquatic animal tissue samples to elucidate the applicability and reliability of this method. The detection results showed the six PBDEs were all at below detectable level (<2.4 µg/kg). The spiking recoveries of the target PBDEs in the real samples fortified at the different concentrations (5, 20, and 100 µg/kg of each PBDE congener) are summarized in Table 4. Acceptable recoveries (75.3–127.8%) and

Table 3. Quantitative results of PBDEs from aquatic animal tissue samples by DLLME^{a)}

| Compounds | RSD (%) $n = 5$ | LR (µg/kg) | r^2 | LOD (µg/kg) |
|-----------|-----------------|------------|--------|-------------|
| BDE 47 | 10.3 | 5.00–2500 | 0.9998 | 3.1 |
| BDE 100 | 5.3 | 5.00–2500 | 0.9998 | 2.7 |
| BDE 99 | 9.3 | 5.00–2500 | 0.9994 | 2.4 |
| BDE 85 | 4.3 | 5.00–2500 | 0.9988 | 2.8 |
| BDE 154 | 7.2 | 5.00–2500 | 0.9993 | 4.2 |
| BDE 153 | 7.1 | 5.00–2500 | 0.9995 | 4.9 |

a) Extraction conditions: water sample volume, 5.00 mL; disperser solvent (acetone) volume, 0.75 mL; extraction solvent volume, 33 µL chlorobenzene; sedimented phase volume, 20 ± 0.5 µL; room temperature.

RSDs, the addition concentration of each PBDE congener in aquatic animal samples was 10 µg/kg; LR, liner range; r^2 , coefficients of correlation; LOD, limit of detection for $S/N = 3$.

Table 4. Relative recoveries and standard deviations of PBDEs from aquatic animal tissue by cleanup by freezing-DLLME

| Compounds | Frog | | | Snail | | | Fish | | |
|-----------|---------------|--------------------------------|-----------------------|---------------|--------------------------------|-----------------------|---------------|--|-----------------------|
| | Added (µg/kg) | Found (RSD%, $n = 3$) (µg/kg) | Relative recovery (%) | Added (µg/kg) | Found (RSD%, $n = 3$) (µg/kg) | Relative recovery (%) | Added (µg/kg) | Found (RSD%, $n = 3$) (µg/kg ¹) | Relative recovery (%) |
| BDE 47 | 5.00 | 5.75 (10.5) | 115.3 | 20.00 | 15.06 (5.5) | 75.3 | 100.00 | 123.8 (10.4) | 123.8 |
| BDE 100 | 5.00 | 4.20 (10.1) | 84.0 | 20.00 | 19.98 (9.5) | 99.9 | 100.00 | 127.8 (6.7) | 127.8 |
| BDE 99 | 5.00 | 3.85 (6.9) | 76.9 | 20.00 | 17.46 (10.2) | 87.3 | 100.00 | 133.3 (8.0) | 123.3 |
| BDE 85 | 5.00 | 5.10 (9.9) | 102.0 | 20.00 | 20.02 (8.6) | 100.1 | 100.00 | 126.7 (6.9) | 126.7 |
| BDE 154 | 5.00 | 5.55 (6.9) | 111.2 | 20.00 | 25.50 (9.5) | 127.5 | 100.00 | 116.4 (10.8) | 116.4 |
| BDE 153 | 5.00 | 5.85 (9.5) | 117.0 | 20.00 | 21.26 (9.2) | 106.3 | 100.00 | 102.5 (9.2) | 102.5 |

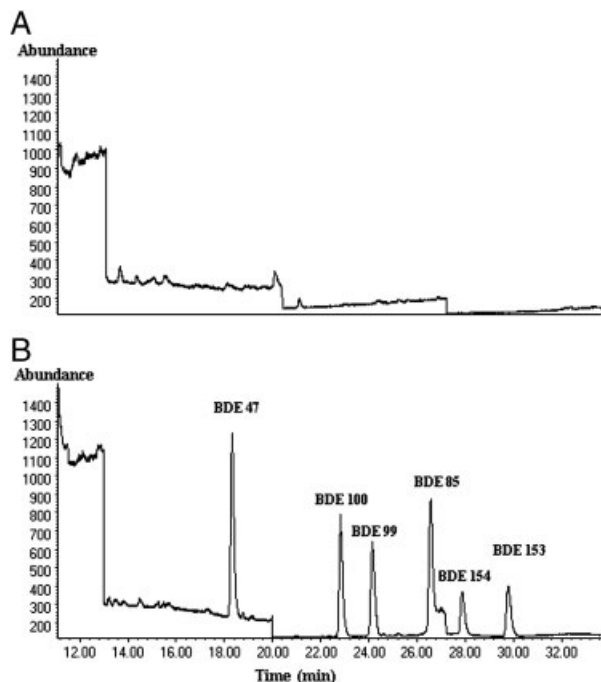


Figure 5. Chromatograms attained from the fish (A) and the spiked fish samples (B) at the concentration level of 100 $\mu\text{g}/\text{kg}$ for BDE 47, 100, 99, 85, 154, and 153.

Table 5. Comparison with other conventional methods

| Method | Solvent consumption (mL) | Extraction time | LODs ($\mu\text{g}/\text{kg}$) | Reference |
|---------------------------|--------------------------|-----------------|----------------------------------|--------------------|
| Cleanup by freezing-DLLME | 10 | 3.5 h | 2.4–4.9 | Represented method |
| SE | 80 | 12 h | 0.01 | [37] |
| MAE | 25 | 15 min | 0.5–1.2 | [36] |

repeatability (5.5–10.8%) were obtained. Figure 5 depicts the chromatograms attained from the fish (A) and the spiked fish samples (B) at the concentration level of 100 $\mu\text{g}/\text{kg}$ for BDE 47, 100, 99, 85, 154, and 153 according to the presented method.

3.6 Comparison with conventional methods

The represented method was proved to be an efficient pretreatment method for trace PBDE congeners in aquatic animal tissue by comparing with the conventional methods (Table 5). A similar LODs level can be achieved with the represented method relative to MAE [36]. As for aquatic animal samples, only 10 mL extraction solvent is required, which is much less than that of Soxhlet extraction (SE, 80 mL) [37] and MAE (25 mL). In addition, the extraction

time is much shorter than that of SE (12 h). Although MAE needs a shorter extraction time (10–20 min) than the represented method, it requires expensive MAE instrument, which is about 30 000 dollars in the current prices. However, the represented method does not need expensive equipment, and it is a stable, simple, low cost and environment-friendly pretreatment technique.

4 Concluding remarks

A new pretreatment technique termed as FC-DLLME has been described for the analyses of PBDE congeners at trace levels in aquatic animal samples. In conventional application, DLLME is widely used for the analysis of organic pollutants in environmental water samples with relatively clean matrices. As far as our information goes, this is the first report on the use of FC-DLLME for the determination of PBDEs in aquatic animal samples with relatively complex matrices. Compared with other conventional pretreatment methods (*e.g.* SE and MAE) for the determination of aquatic animal samples, the presented method possesses the following advantages such as simplicity, rapidity, easy operation, no expensive instrument required and low detection limit. On the basis of the former considerations, we can conclude that FC-DLLME has great application potential in determining trace organic pollutants in complicated animal samples with fat content.

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