Eur Food Res Technol (2012) 235:295–301
DOI 10.1007/s00217-012-1758-z

ORIGINAL PAPER

Development of a cleanup method for polybrominated diphenyl ether (PBDE) in fish by freezing-lipid filtration

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Received: 5 April 2012 / Revised: 4 May 2012 / Accepted: 13 May 2012 / Published online: 5 June 2012

Abstract A new cleanup method for the determination of brominated flame retardants with an emphasis on polybrominated diphenyl ethers (PBDEs) has been developed for fish tissue sample. This method effectively reduces the sample pretreatment time, labor and required less solvent quantities relative to conventional methods. Freezing-lipid filtration procedure removes approximately 90 % of the lipids in the extract without any significant loss of the PBDEs. A multilayered silica gel column was used for further cleanup of the extracts after freezing-lipid filtration. Multilayered silica gel column chromatography eliminated most of the co-extracted interferences, such as residual lipids and fatty acids. The extracts were analyzed after cleanup by high-resolution gas chromatography/high-resolution mass spectrometry using the isotope dilution method. Tissue samples with 1.6–8.0 and 8.0–40 ppb of PBDE were analyzed using both the sulfuric acid treatment and freezing-lipid filtration cleanup methods in order to evaluate the method performance. Sulfuric acid treatment did not detect 2,4-DiBDE, whereas freezing-lipid filtration detected 2,4-DiBDE but at 50 % recovery. To compare the method, WMF-01 was analyzed via both the sulfuric acid treatment and freezing-lipid filtration cleanup.

Keywords Polybrominated diphenyl ethers (PBDEs) · Fish · Freezing-lipid filtration · Solid-phase extraction · High-resolution gas chromatography/high-resolution mass spectrometry

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Introduction

Polybrominated diphenyl ethers (PBDEs) are a group of brominated flame retardants (BFRs) that have been manufactured in big quantities and used in a large variety of consumer goods [1]. Their chemical structure is similar to both polychlorinated dibenzo-*p*-dioxins and polychlorinated biphenyls (PCBs), and their toxicity has been studied [2]. PBDEs are both persistent and lipophilic. These properties influence their bioaccumulation in the fatty tissues of organisms and enrichment throughout the food chain [3]. Studies have indicated that PBDEs may be potent competitors of thyroxine (T4) and could disrupt normal thyroid hormone functions in humans [4, 5]. A number of recent studies indicate that the degradation of highly brominated PBDEs, such as the commonly produced decabromodiphenyl ether, can lead to a variety of less brominated congeners [6, 7]. Current data suggest that less brominated congeners are more toxic than highly brominated ones [8, 9]. These compounds spread ubiquitously as environmental contaminants and have been detected in a wide range of environmental samples and humans [10–13].

The development of analytical methods for PBDEs has been described. New extraction techniques for PBDE analysis have been developed and involve organic solvent extraction, supercritical fluid extraction (SFE) [14, 15],

microwave-assisted extraction (MAE) [16] and pressurized liquid extraction (PLE) [17, 18], followed by further cleanup via adsorption chromatography [19] or SPE [20]. Most of these analyses have concentrated on only a few specific PBDE congeners. However, a simple and rapid method for the ultra-trace quantification of individual congeners is required to determine the extent of environmental exposure. PBDEs in biological samples are largely associated with the lipid fraction. The procedures commonly used to eliminate lipid interference are saponification, concentrated sulfuric acid treatment, gel permeation chromatography [21], column chromatography with suitable adsorbents [22] and various cleanup methods [23, 24]. However, controlling the conditions for saponification is critical. High temperature or too long saponification time may degrade highly brominated PBDEs and PBBs [25, 26], which leads to underestimating their concentration and overestimating of less brominated PBDEs and PBBs. Recent reports have recommended using the freezing-lipid filtration method to eliminate large quantities of lipids from biological samples for an efficient cleanup. This method has been used for the successful determination of chlorinated pesticides, anabolic steroids, synthetic hormones and PCDD/Fs in biological matrices [27–29]. Most lipids can be extracted from biological samples through freezing-lipid filtration. The extract can be further purified via impregnated multilayer silica gel columns containing neutral, acidified and basic silica [30].

In the present study, the new freezing-lipid filtration method is applied to the congener-specific determination of 27 different PBDEs, including the mono- to deca-BDE congeners, for monitoring biological samples.

Materials and methods

Standards and reagents

All organic solvents were of ultra-residue grade (J.T. Baker, Philipsburg, NJ, USA). Anhydrous sodium sulfate and sulfuric acid from E. Merck (Darmstadt, Germany) were used.

An isotope internal standard method typically used to quantify dioxin concentrations in environmental samples was adopted for PBDE analysis.

All PBDE standard solutions were purchased from Wellington Laboratories (ON, Canada). A mixed native PBDE standard solution (BDE-CVS-E) containing 1 ng/μl of BDE-3, 7, 15, 17, 28, 47, 49, 66, 71, 77, 85, 99, 100, 119 and 126; 2 ng/μl of BDE-138, 153, 154, 156, 183, 184, 191, 196 and 197; and 5 ng/μl of BDE-206, 207 and 209 was prepared. Five standard solutions were prepared with PBDE concentrations of 1, 5, 20, 100 and 400 ng/mL for each of BDE-3, 7, 15, 17, 28, 47, 49, 66, 71, 77, 85, 99, 100, 119 and 126; 2, 10, 40, 200 and 800 ng/mL for each of BDE-138, 153, 154, 156, 183, 184, 191, 196 and 197; and 5, 25, 100, 500 and 2,000 ng/mL for each of BDE-206, 207 and 209. All calibration standard solutions contained 100 ng/mL of each of the eleven ^{13}C-labeled PBDEs ($^{13}C_{12}$-BDE-3, 15, 28, 47, 99, 153, 154, 183, 197, 207 and 209) as internal standards. A 200 ng/mL injection standard of $^{13}C_{12}$-BDE-138 was used for this study.

A ^{13}C-labeled internal standard solution (MBDE-MXE) contained 100 ng/mL of $^{13}C_{12}$-BDE-3, 15, 28, 47 and 99; 200 ng/mL of $^{13}C_{12}$-BDE-153, 154, 183 and 197; and 500 ng/mL $^{13}C_{12}$-BDE-207 and 209.

Samples

Mackerel samples were purchased from a grocery store in Seoul. A pooled sample, weighing approximately 100 g, was homogenized and stored below −20 °C pending analysis. Aliquots of the samples that did not contain PBDEs were used for spiking experiments. The spiked samples were then stored in a tightly closed amber glass bottle at room temperature for 24 h. The lipid content in 10 g of the fish samples ranged from 10 to 15 % according to gravimetric determination.

Reference fish tissue was purchased for organic contaminant analysis to verify the method (WMF-01, Wellington Laboratories Inc., ON, Canada).

Sample extraction and delipidation

A 10 g ground sample was added to 30 g of powered anhydrous sodium sulfate, and 1–5 ng of ^{13}C-labeled PBDE internal standards was added prior to extraction. The extraction was performed by sonicating twice for 10 min with 100 mL of *n*-hexane. Both freezing-lipid filtration and sulfuric acid treatment were used to remove any lipids. During freezing-lipid filtration, the extract was dissolved in 50 mL of acetonitrile and stored at −24 °C for 40 min to freeze the lipids. Most of the lipids were suspended as clusters and easily removed via filtration. The lipid removal (%) was calculated from the lipid residue after freezing-lipid filtration. The detailed freezing-lipid filtration procedure has been described previously [28–30]. Sulfuric acid treatment was performed following EPA method 1614 for comparison with the freezing-lipid filtration method. After eliminating the lipids, the sample was concentrated for addition to the multilayered silica gel column.

Sample cleanup

The semiautomated cleanup system (Dioxin cleanup system DPU-8B, GL Sciences, Japan) was used to eliminate co-extracted interferences.

The concentrated extract was sequentially subjected to multilayer silica gel chromatography on a multilayer silica gel column (glass reservoir with 25 mm inner diameter and 15 cm long) packed from bottom to top with 2 g of activated silica, 4 g of basic silica (2 %, w/w), 2 g of anhydrous sodium sulfate, 10 g of acidic silica (44 %, w/w) and 2 g anhydrous sodium sulfate as shown in Fig. 1. The eluent was evaporated to dryness under a gentle stream of nitrogen for the derivatization step.

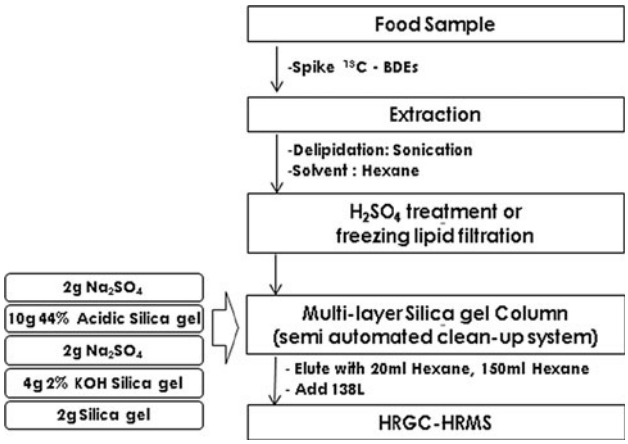

Fig. 1 Schematic diagram for analytical procedure

High-resolution gas chromatography/high-resolution mass spectrometry analysis

Instrumental analysis was performed using a JEOL (Akishima, Tokyo, Japan) MStation JMS-700D high-resolution mass spectrometer (B/E configuration) equipped with an Agilent (Palo Alto, CA, USA) 6890 Plus gas chromatograph.

A 15 m × 0.25 mm capillary GC column coated with a DB-5 HT stationary phase (film thickness of 0.1 μm) was used. Samples were injected in splitless mode with an injector temperature of 280 °C and an initial column temperature of 120 °C. After 1 min, the temperature was

Table 1 Recoveries and RSD of PBDEs in fish spiked with 1.6–8.0 pg/g obtained via sulfuric acid treatment and freezing-lipid filtration

Isomer	IUPAC No.	Conc. (pg/g)	Sulfuric acid treatment Recovery (RSD %)	Freezing-lipid filtration Recovery (RSD %)
4-MonoBDE	3	1.6	87.0 (6.7)	83.6 (8.7)
2,4-DiBDE	7	1.6	ND	51.7 (11.2)
4,4′-DiBDE	15	1.6	97.0 (18.5)	95.9 (12.6)
2,2′,4-TrBDE	17	1.6	77.8 (10.9)	91.9 (7.3)
2,4,4′-TrBDE	28	1.6	99.5 (19.3)	76.5 (10.2)
2,2′,4,4′-TeBDE	47	1.6	118.2 (4.1)	82.0 (8.6)
2,2′,4,5′-TeBDE	49	1.6	98.0 (19.9)	80.4 (7.2)
2,3′,4,4′-TeBDE	66	1.6	86.3 (11.8)	83.6 (4.2)
2,3′,4′,6-TeBDE	71	1.6	100.8 (13.1)	88.7 (19.8)
3,3′4,4′-TeBDE	77	1.6	98.1 (11.1)	100.6 (15.0)
2,2′,3,4,4′-PeBDE	85	1.6	89.5 (14.4)	98.4 (19.6)
2,2′,4,4′,5-PeBDE	99	1.6	85.7 (17.6)	74.0 (9.9)
2,2′,4,4′,6-PeBDE	100	1.6	91.7 (12.9)	96.8 (4.3)
2,3′,4,4′,6-PeBDE	119	1.6	95.5 (10.7)	100.1 (11.4)
3,3′,4,4′,5-PeBDE	126	1.6	97.7 (13.9)	102.7 (13.2)
2,2′,3,4,4′,5′-HeBDE	138	3.2	86.4 (17.5)	76.5 (3.4)
2,2′,4,4′,5,5′-HeBDE	153	3.2	80.7 (1.6)	83.6 (1.1)
2,2′,4,4′,5,6′-HeBDE	154	3.2	70.0 (5.5)	72.9 (2.5)
2,3,3′,4,4′,5-HeBDE	156	3.2	100.0 (6.5)	94.7 (15.3)
2,2′,3,4,4′,5′,6-HpBDE	183	3.2	119.3 (17.9)	112.0 (10.6)
2,2′,3,4,4′,6,6′-HpBDE	184	3.2	90.8 (12.9)	115.8 (16.3)
2,3,3′,4,4′,5′,6-HpBDE	191	3.2	103.1 (7.9)	113.5 (17.4)
2,2′,3,3′,4,4′,5,6′-OcBDE	196	3.2	90.4 (19.8)	116.1 (17.2)
2,2′,3,3′,4,4′,6,6′-OcBDE	197	3.2	117.5 (19.9)	116.1 (11.1)
2,2′,3,3′,4,4′,5,5′,6-NoBDE	206	8	104.8 (8.7)	112.4 (13.7)
2,2′,3,3′,4,4′,5,6,6′-NoBDE	207	8	102.7 (19.7)	88.2 (16.1)
DeBDE	209	8	89.5 (6.6)	79.6 (4.0)

Table 2 Recoveries and RSD of PBDEs in fish spiked with 8.0–40.0 pg/g obtained via sulfuric acid treatment and freezing-lipid filtration

Isomer	IUPAC No.	Conc. (pg/g)	Sulfuric acid treatment Recovery (RSD %)	Freezing-lipid filtration Recovery (RSD %)
4-MonoBDE	3	8	77.7 (16.1)	83.6 (7.1)
2,4-DiBDE	7	8	ND	52.4 (0.9)
4,4′-DiBDE	15	8	85.6 (5.8)	86.6 (15.4)
2,2′,4-TrBDE	17	8	82.3 (4.6)	91.1 (16.9)
2,4,4′-TrBDE	28	8	86.6 (10.7)	85.8 (11.5)
2,2′,4,4′-TeBDE	47	8	101.6 (16.8)	92.6 (7.7)
2,2′,4,5′-TeBDE	49	8	106.1 (6.7)	105.5 (12.4)
2,3′,4,4′-TeBDE	66	8	93.9 (9.7)	98.0 (12.5)
2,3′,4′,6-TeBDE	71	8	115.6 (5.8)	110.9 (6.5)
3,3′4,4′-TeBDE	77	8	96.2 (17.7)	99.8 (7.2)
2,2′,3,4,4′-PeBDE	85	8	99.8 (16.3)	108.1 (12.4)
2,2′,4,4′,5-PeBDE	99	8	116.1 (4.4)	110 (9.9)
2,2′,4,4′,6-PeBDE	100	8	93.6 (4.3)	100.9 (7.5)
2,3′,4,4′,6-PeBDE	119	8	97.7 (13.1)	112.0 (9.4)
3,3′,4,4′,5-PeBDE	126	8	80.0 (20.0)	89.6 (10.6)
2,2′,3,4,4′,5′-HeBDE	138	16	82.6 (10.9)	79.0 (13.3)
2,2′,4,4′,5,5′-HeBDE	153	16	79.6 (16.7)	101.0 (16.1)
2,2′,4,4′,5,6′-HeBDE	154	16	88.5 (14.5)	78.7 (13.6)
2,3,3′,4,4′,5-HeBDE	156	16	88.1 (14.7)	84.2 (13.2)
2,2′,3,4,4′,5′,6-HpBDE	183	16	100.8 (4.6)	94.1 (7.5)
2,2′,3,4,4′,6,6′-HpBDE	184	16	97.1 (12.3)	84.1 (18.3)
2,3,3′,4,4′,5′,6-HpBDE	191	16	103.2 (6.6)	91.2 (8.5)
2,2′,3,3′,4,4′,5,6′-OcBDE	196	16	101.3 (7.2)	83.5 (17.0)
2,2′,3,3′,4,4′,6,6′-OcBDE	197	16	96.7 (15.7)	86.4 (15.8)
2,2′,3,3′,4,4′,5,5′,6-NoBDE	206	40	87.3 (13.1)	93.8 (10.6)
2,2′,3,3′,4,4′,5,6,6′-NoBDE	207	40	95.4 (13.1)	97.7 (10.1)
DeBDE	209	40	110.0 (10.5)	104.3 (11.0)

ramped at 10 °C/min up to 330 °C, 5 °C/min down to 235 °C and 3 °C/min up to 310 °C, which was held for 5 min. The ion source operated at 260 °C, the electron energy was 38 eV, and the mass spectrometer was tuned to a mass resolution of 10,000. When the signal-to-noise ratio (S = N) for a given peak was less than 3, congeners were recorded as not detected (ND) for quantification.

Fortified recovery studies

To compare the delipidation, 10 g of fish samples was spiked with 8–40 and 1.6–8 ng/g of the mixed 27 native PBDEs standard solution (BDE-CVS-E). Each concentration was analyzed 5 times. The fish used in this experiment were purchased from a grocery store, and control experiments were performed. Samples used in this experiment containing three times the average measured concentration were removed from the sample pool.

Results and discussion

Extraction and cleanup

The efficiency of the extraction methods and solvents was determined. The extraction methods used were Soxhlet extraction and sonication extraction with hexane and dichloromethane as extractants. The results of these different sample processing steps indicated that hexane was suitable for extraction whereas extraction by dichloromethane was not found. The crude extract requires cleaning as many compounds (e.g., humic acids, lipids) can be co-extracted with the analytes. Biota extracts usually contain high lipid concentrations that should be removed by either destructive or nondestructive methods prior to gas chromatographic separation of the BFRs. Sulfuric acid treatment, applied either directly to the extract [25, 31–34] or via an impregnated silica column [35–38], is the most

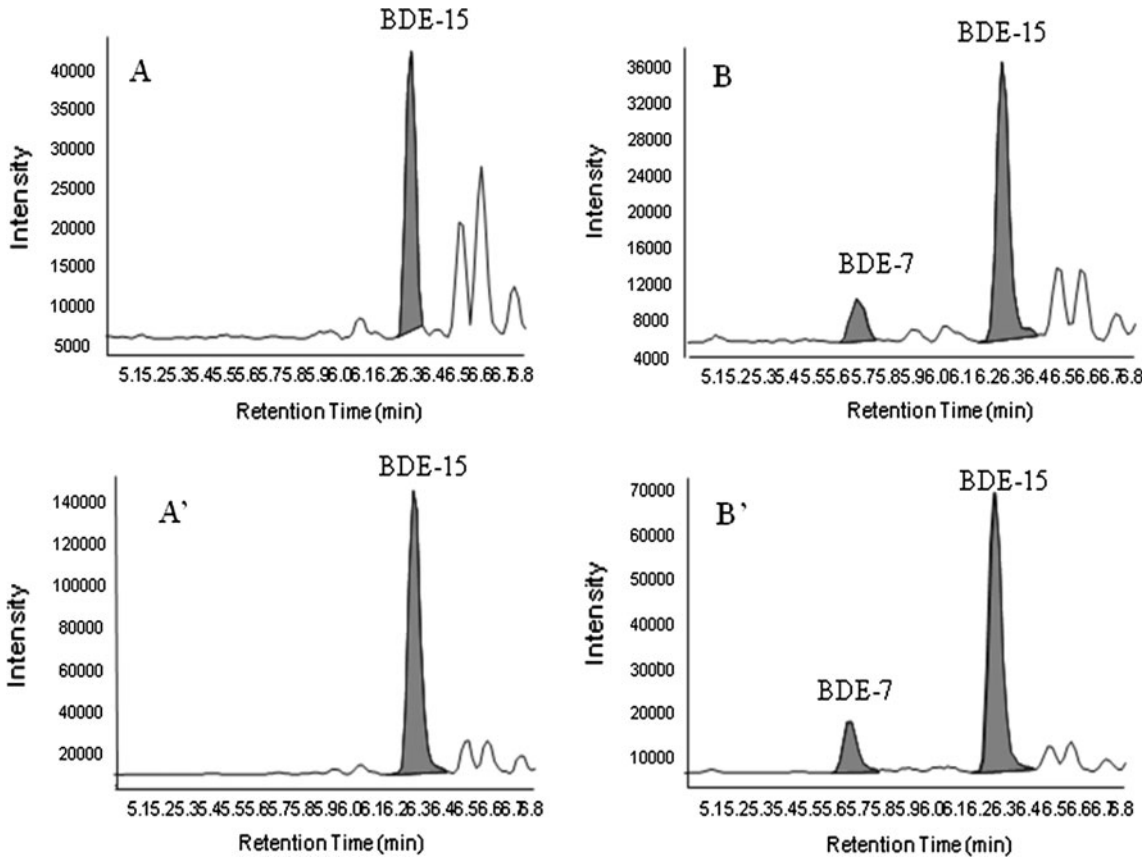

Fig. 2 Chromatogram of DiBDE (BDE-7, BDE-15) in fish. **a**, **a′** Sulfuric acid treatment, **b**, **b′** freezing-lipid filtration. *Upper* fish spiked with 1.6–8.0 pg/g; *bottom* fish spiked with 8.0–40.0 pg/g

common destructive lipid removal method. It has been shown that PBDEs are stable under strong acidic conditions [25, 31, 32, 39]. Furthermore, lipid destruction via concentrated acid may allow for the removal of a greater quantity of lipids than adsorption chromatography. Our results support the evidence that the direct use of concentrated acid requires several extraction and centrifugation steps, which make the procedure labor intensive and time-consuming. In contrast, acidified silica gel should be handled with extreme care, and inhalation of the fine, acid-containing particles should be avoided.

Freezing-lipid filtration has been used to rapidly eliminate lipids extracted from biota sample PBDEs analysis. On the basis of the significant differences between the melting points of lipids (below approximately 40 °C) and PBDEs (below approximately 72 °C), PBDEs in the extract can be separated from lipids. Through this process, approximately 90 % of the lipids in the extraction solvent could be eliminated without any significant loss of the PBDEs. The elution study of the column cleanup was performed using a ^{13}C-labeled internal standard solution (MBDE-MXE) and checking the recovery of each congener. PBDE recoveries in the column elution study ranged from 26.8 to 146.3 %.

Method evaluation

To compare the performance of these methods, 2 and 10 µl of the BDE-CVS-E standard (80–400 ng/mL), which contains the 27 different PBDEs congeners, was added to separate 10 g fish tissue samples for an equivalent concentration of 1.6–8.0 and 8.0–40 ppb in the tissue samples, respectively, and analyzed using both the sulfuric acid treatment and freezing-lipid filtration cleanup methods.

Tables 1 and 2 as well as Fig. 2 present the recoveries for each of the spiked native congeners from the 2 and 10 µl BDE-CVS-E standard solutions (80–400 ng/mL) obtained via sulfuric acid treatment and freezing-lipid filtration cleanup.

The recoveries obtained using freezing-lipid filtration were nearly equivalent to those for sulfuric acid cleanup with the exception of 2,4-DiBDE. The detection of 2,4-DiBDE after sulfuric acid treatment was unsuccessful, whereas it was detected after freezing-lipid filtration in the

Table 3 The concentration of PBDEs in WMF-01 obtained via sulfuric acid treatment and freezing-lipid filtration

Isomer	IUPAC No.	Cert. (pg/g)	Sulfuric acid treatment (pg/g)	Freezing-lipid filtration (pg/g)
2,4,4′-TrBDE	28	3,214 ± 290	3,065	3,006
2,2′,4,4′-TeBDE	47	123,200 ± 24,800	102,331	121,714
2,2′,4,4′,5-PeBDE	99	37,500 ± 4,220	36,542	35,560
2,2′,4,4′,6-PeBDE	100	35,870 ± 14,500	40,521	31,861
2,2′,4,4′,5,5′-HeBDE	153	17,040 ± 8,000	10,887	17,614
2,2′,4,4′,5,6′-HeBDE	154	19,790 ± 2,880	17,030	18,454
2,2′,3,4,4′,5′,6-HpBDE	183	532 ± 400	509	579

detection but with a 50 % recovery. 2,4-DiBDE can be easily lost during the cleanup process. Analysis of low brominated compounds in biological samples via the freezing-lipid filtration cleanup method can be more effective than the sulfuric acid treatment. The overall time required for the freezing-lipid filtration procedure was about 3 h; oppositely, 1 day is required for the sulfuric acid treatment. Freezing-lipid filtration also possesses the advantage of requiring a smaller quantity of reagents than required for sulfuric acid treatment.

Method verification

To verify the method, WMF-01 was analyzed using both the sulfuric acid treatment and freezing-lipid filtration cleanup methods. Table 3 presents the PBDE concentrations in WMF-01 obtained via both the sulfuric acid treatment and freezing-lipid filtration cleanup. 2,4,4′-TrBDE, 2,2′,4,4′-TeBDE, 2,2′,4,4′,5-PeBDE, 2,2′,4,4′,6-PeBDE, 2,2′,4,4′,5,5′-HeBDE, 2,2′,4,4′,5,6′-HeBDE and 2,2′,3,4,4′,5′,6-HpBDE were analyzed. Identical total PBDE concentrations were found for both the sulfuric acid treatment and freezing-lipid filtration cleanup. For analyzing low brominated compounds in biological samples, freezing-lipid filtration can be more effective than sulfuric acid treatment.

Conclusions

The freezing-lipid filtration method was evaluated and verified for the efficient removal of lipids extracted from fish samples and WMF-01 for the analysis of PBDEs. This method was rapid, simple and economically cleanup that had several advantages over conventional sample treatments. The analytical results for the elimination of lipids from biological samples obtained after freezing-lipid filtration were nearly equivalent to those for others, official methods. This method can be applied as a rapid screening tool for the determination of PBDEs in biological samples, especially in samples containing high levels of lipids and as it allows for the treatment of larger sample sizes to effectively eliminate lipids.

Acknowledgments This work was supported by outstanding researchers program of Korea Research Foundation (T31750).

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