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Determination of organophosphorus flame retardants in fish by freezing-lipid precipitation, solid-phase extraction and gas chromatography-mass spectrometry

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ABSTRACT

An analytical method has been developed for measuring 12 organophosphorus flame retardants (PFRs) in fish tissue samples. After the Soxhlet extraction of PFRs with dichloromethane. The experimental parameters of the clean-up were systematically optimized. Methanol was found to be a more effective solvent than acetonitrile used in freezing-lipid precipitation. Methanol (5%) in ultrapure water, was finally selected to perform solid-phase extraction (SPE, Oasis HLB cartridge), with mean lipid removal efficiency of 94% after freezing-lipid precipitation. Further purification followed by 200 mg of Z-Sep and C18 dispersant to eliminate the remaining interferences. Quantification was performed using gas chromatography-mass spectrometry in selective ion monitoring mode. The recovery, precision, and the method detection limits (MDLs) were verified by spiking experiments. All chemicals except triethyl phosphate (TEP) showed satisfactory recoveries in the range of 73–107% and 56–108% in the spiked blanks samples and spiked fish tissue samples, respectively. MDLs for PFRs in the biological samples ranged from 0.004 to 0.059 ng/g. The proposed method successfully applied to the determination of PFRs in real fish samples with recoveries of four internal standards varying from 75 to 97%. The results demonstrated that the proposed method is highly effective for analyzing PFRs in fish samples.

1. Introduction

Organophosphorus flame retardants (PFRs) have been widely used as flame retardants in commercial products such as electronic devices, and have also been applied as plasticizers or additives in lubricants [1]. There has been a huge increase in the demand and production of PFRs because they are regarded as appropriate alternatives for brominated flame retardants (BFRs). Over the last few decades, PFRs have been found to be ubiquitous in abiotic environments such as air [2], soil [3], water [4], dust [5,6] and sediment [7,8]. However, studies examining PFRs in biota are scarce and have only started emerging in the last few years [9,10].

Available data for PFRs in biota samples are limited. The major reason is the lack of an efficient and systematic pretreatment method. PFRs contain ester bonds in their chemical structures which lead to less persistence properties and different bioaccumulation capacity compared to other persistent flame retardants. The structural differences among PFRs result in a variety of chemical and physical properties, from highly lipophilic (log K_{OW} = 10.6 for Trioctyl phosphate) to highly hydrophilic (log K_{OW} = −9.8 for Tetrakis(hydroxymethyl) phosphonium sulfate) [1]. Meanwhile, the levels of PFRs in biota are influenced by degradation/transformation processes such as metabolism [11], which decreases the concentrations of these compounds in living organisms. Therefore, a highly efficient extraction method is needed due to the low concentration of PFRs in biological samples.

Several approaches have been developed to eliminate the co-extracted lipid interferences for organism samples, including pressurized liquid extraction using aqueous solution and solid-phase microextraction [12], on-line turbulent flow chromatography [13], matrix solid-phase dispersion [14], and gel permeation chromatography and silica gel cleanup[15]. These methods are either time-consuming and organic solvent-consuming or have high equipment requirements. Meanwhile, conventional cleanup

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methods, such as basic or acidic treatment like saponification cannot be applied to PFR analysis since PFRs are prone to degradation under extremely acidic or basic conditions.

Due to their low melting points, lipid components can be easily separated from many compounds such as organophosphorus insecticides [16], chlorinated pesticides [17], and phenols [18] by freezing-lipid filtration. The cleanup step enables efficient removal of lipids extracted from biological samples without significant loss of the target compounds and no much organic solvents are consumed.

Up to date, the method of freezing-lipid filtration was not conducted on the PFR analysis in organisms. In this study, a combination of freezing-lipid precipitation and solid-phase extraction was developed for determining PFRs in biological samples containing high levels of lipids. The developed method was validated and applied to detect PFRs in fish samples from the Pearl River Delta.

2. Materials and methods

2.1. Standards and reagents

Triethyl phosphate (TEP), tri-iso-propyl phosphate (TiPP), tri-*n*-propyl phosphate (TnPP), tri-*n*-butyl phosphate (TnBP), tri(2-chloroethyl) phosphate (TCEP), tri(2-chloro-isopropyl) phosphate (TCPP), tris(2-butoxyethyl) phosphate (TBEP), tri(2-chloro,1-chloromethy-ethyl) phosphate (TDCP), tri(2-ethylhexyl) phosphate (TEHP), 2-ethylhexyl diphenyl phosphate (EHDPP), triphenyl phosphate (TPhP), and tri-cresyl phosphate (TCrP) [19] were purchased from AccuStandard (New Haven, CT, USA), as well as TnPP-D_{21}, TnBP-D_{27}, TCPP-D_{18}, TPhP-D_{15}, TCEP-D_{12}, and TDCP-D_{15}.

Oasis HLB cartridges (200 mg, 6 mL) were purchased from Waters (Milford, Massachusetts, USA). Z-Sep and C18 sorbents were purchased from Supelco (Bellefonte, PA, USA). Methanol, dichloromethane, acetonitrile, and hexane (chromatography grade) were purchased from Oceanpk (Sweden). Ethyl acetate was obtained from Honeywell (USA).

2.2. Sample collection

Fish samples, including plecostomus (*Hypostomus plecostomus*), tilapia (*Tilapia nilotica*), mud carp (*Cirrhinus molitorella*), and catfish (*Silurus asotus*), were collected in the Pearl River Delta as described in a previous study [20]. All the collected samples were freeze-dried, triturated, wrapped in aluminum foil, sealed in zip bags and stored at −20 ° until analysis.

2.3. Sample extraction and cleanup

After being spiked with surrogate standards (TnPP-D_{21}, TnBP-D_{27}, TCPP-D_{18}, TPhP-D_{15}, 100 ng each), 2 g of the lyophilized catfish sample (with a wet weight of 8.8 g) was Soxhlet-extracted with 200 mL of dichloromethane for 24 h. The extract was preconcentrated and transferred to a 10 mL centrifuge tube, where the solvent was concentrated to near dryness, under gentle nitrogen flow, and reconstituted in a polar organic solvent. Three different solvents were tested: ethyl acetate, methanol, and acetonitrile. The polar organic solvent extract was then stored in the freezer at −20 ° for 2 h to freeze the lipids. Most of them were precipitated on the bottom of the tube as a condensed mass. The supernatant was collected in a 500 mL flat bottom flask and 300 mL of ultrapure water was added. Different organic solvent volumes (5 and 10% of organic solvent/ ultrapure water) were also tested. The mixture was subsequently purified and fractionated by SPE on an Oasis HLB cartridge, which was activated separately with 4 mL each of ethyl acetate, methanol, and ultrapure water. After loading the mixture on the cartridge, the cartridge was dried for about 20 min under a gentle nitrogen stream, and was eluted with two aliquots of 4 mL of ethyl acetate. The remaining water and the residual lipids from the elution were removed with anhydrous sodium sulfate and 200 mg of Z-Sep/C18 (1:1) dispersant. After evaporation to near dryness, the liquid was re-dissolved in 200 μL of n-hexane. TCEP-D_{12} (100 ng) and TDCP-D_{15} (100 ng) were added as recovery standards, prior to instrumental analysis.

TnPP-D_{21}, TnBP-D_{27}, TCPP-D_{18}, and TPhP-D_{15} were added as internal standards. TnPP-D_{21} was used for TEP, TiPP, and TnPP quantification, whereas TnBP-D_{27} was used for TnBP and TCEP quantification, and TCPP-D_{18} was used for TCPP and TDCP quantification. Finally, TPhP-D_{15} was used for TBEP, TPhP, EHDPP, TEHP, and TCrP quantification. TCEP-D_{12} was used as a recovery standard for TnPP-D_{21} and TnBP-D_{27}, and TDCP-D_{15} was used as a recovery standard for TCPP-D_{18} and TPhP-D_{15}.

2.4. Instrumental analysis

PFR analysis were carried out with a Shimadzu 2010 gas chromatograph (GC) equipped with a DB-5 capillary column (30 m × 0.25 mm × 0.25 μm; SGE Analytical Science) and coupled to a mass spectra detector (MSD) . It was operated in selective ion monitoring (SIM) mode, with two characteristic ions acquired for each compound [6]. The GC temperature program was set at 70 °C and held for 2 min, increased at 15 °C/min to 300 °C, and then held at 300 °C for 10 min. Sample injection (1 μL) was performed using the splitless mode with injector temperature of 290 °C. The carrier gas was Helium, at a flow rate of 1 mL/min. The temperatures of the interface, ion source, and injector were 290, 200, and 290 °C, respectively.

2.5. Determination of the lipid content for fish samples

After Soxhlet-extraction, the extract was concentrated and the volume was adjusted to 10 mL. An aliquot of the extract (1/10) was used to determine the lipid content by gravimetrical method, while the rest of the extract was used for PFRs determination by the developed method. The frozen lipid eliminated after freezing-lipid precipitation was also determined by gravimetric measurement. The average lipid contents of the plecostomus, tilapia, mud carp, and catfish were 2.91 ± 0.592, 2.01 ± 0.268, 1.83 ± 1.10,2.40 ± 0.581(%,w/w), respectively.

2.6. Quality assurance (QA) and quality control (QC)

In consideration of PFRs are widespread used and are likely to be present in various lab equipment, any plastic and rubber material was avoided to be used to minimize possible contamination of the samples during storage, sampling, extraction and transport. All the glassware were baked at 450 ° for 5 h and rinsed with acetone, dichloromethane and *n*-hexane orderly. Anhydrous sodium sulfate was heated at 450 ° for 5 h and stored in glass drying vessel. The connecting pipe and cock of the SPE device were also rinsed with three kinds of reagents orderly.

Measures quality was controlled and assured by spiking of surrogate standards into all samples and regular analysis of procedural blanks, spiked blanks, spiked matrices, and triplicate samples. PFRs-spiked fish tissue samples and blank samples were repeatedly (n = 3) analyzed during the development of the proposed method and a procedural blank for each batch of 12 samples was processed. In the procedural blank only traces of TCEP and TCPP were found. Instrumental QC included regular injection of the solvent blank and the standard solution (spiked with 500 ng/mL of PFRs). The standard solution was injected three times within a day and this solution was injected everyday to monitor the stability of instrument. The RSDs for the intra-day were in the range from 2.7% for

Table 1
Removal efficiency of lipids extracted from fish tissue samples by a freezing-lipid filtration method.

	Content (%)	Fish weight (dw/g)	Extraction lipid (g)	Freeze lipid (g)	Removal efficiency (%)
Methanol in water	5%–1	2.0	0.37	0.35	95
	5%–2	2.0	0.33	0.32	97
	5%–3	2.0	0.30	0.27	90
	10%–1	2.0	0.34	0.28	82
	10%–2	2.0	0.30	0.25	83
	10%–3	2.0	0.32	0.27	84
Acetonitrile in water	5%–1	2.0	0.30	0.25	83
	5%–2	2.0	0.32	0.27	84
	5%–3	2.0	0.33	0.25	76
	10%–1	2.0	0.32	0.30	94
	10%–2	2.0	0.29	0.24	83
	10%–3	2.0	0.31	0.25	81

TnBP to 8.6% for TCPP. The RSD for the inter-day ranged from 3.5% for TCEP to 9.7% for TPhP.

This method was validated by calculating the recovery, precision, linear range, method detection limits (MDLs) and method quantification limits (MQLs). Precision was evaluated as the relative standard deviation (RSD) of replicate measurements. The RSDs were less than 15% (n = 3) for all the target chemicals. The linear concentration range of the GC–MS method increasing from 2.0 to 2000 ng/g with 10 spiking levels of PFRs under the optimized conditions. The MDLs was defined as 3 times of the standard deviations for spiked blank, the MQLs was set as the mean value of target compounds detected in procedure blanks plus three times of standard deviations. For the undetectable compounds in blanks, the MQLs were estimated as a signal to noise ratio (S/N) of 10. It's the limitation of this method that since no certified reference materials are available for PFR in fish or organisms and inter-laboratory calibration was not conducted on PFRs in organisms.

3. Results and discussion

3.1. Optimization of the clean-up conditions

Given the variation on chemical and physical properties of PFRs and the requirement of lipid removement prior to analysis, we used freezing-lipid precipitation as the clean-up step after the Soxhlet extraction with dichloromethane. Generally, the lipid content of fish tissue in terms of net mass is about 15%, being mainly composed of phospholipids and triacylglycerolipids [21]. Freezing-lipid precipitation can be used to eliminate a large amount of lipids from the matrix, considerating the low melting point of triacylglycero lipids. However, lipids have high solubility in non-polar solvents such as dichloromethane and can re-dissolved in them during supernatant collection at room temperature. Therefore, other organic solvents, where lipids have lower solubility, were used.

In the present study, ethyl acetate, methanol, and acetonitrile were tested. When ethyl acetate was used, the lipids could still be re-dissolved rapidly when the temperature increased. Thus, optimization was conducted between methanol and acetonitrile in subsequent procedures.

Most of the lipids in both methanol and acetonitrile solutions were precipitated when the extract was stored in the freezer at −20 °C for 2 h (Table 1), whereas PFRs were soluble even in cold methanol or acetonitrile solvents. The supernatant was immediately collected to prevent lipids melting. Freezing-lipid precipitation was repeated two times to improve the extraction yield of PFRs. Two solvent volumes were used to obtain optimal lipid removal and PFRs recovery. As shown in Tables 1 and 2, more than 80% of the lipids were eliminated without any significant loss of the PFRs. Low volume methanol (5% of organic solvent/ ultrapure water) showed the highest lipid removal efficiency of up to 97%, with a mean of 94%. Since the target recoveries are not significantly different between methanol and acetonitrile solvents, or between 5 and 10% solvent volumes, 5% methanol was selected.

Table 2
Recoveries of PFRs in fish tissue samples for different solvents and solvent volumes after freezing-lipid precipitation and HLB solid-extration.

Chemicals	Methanol/water		Acetonitrile/ water	
	5%	10%	5%	10%
TiPP	51 ± 6.0	59 ± 9.8	55 ± 5.9	30 ± 4.0
TnPP	91 ± 2.0	83 ± 5.6	105 ± 13	89 ± 17
TnBP	112 ± 8.4	123 ± 8.9	121 ± 15	115 ± 13
TCEP	68 ± 1.5	59 ± 13	65 ± 10	48 ± 12
TCPP	105 ± 14	82 ± 13	115 ± 14	107 ± 14
TDCP	107 ± 12	102 ± 16	100 ± 4.3	92 ± 8.2
TBEP	103 ± 13	114 ± 10	107 ± 16	100 ± 10
TPhP	108 ± 0.80	104 ± 12	102 ± 4.1	96 ± 4.8
EHDPP	108 ± 12	98 ± 19	92 ± 3.6	89 ± 6.7
TEHP	62 ± 3.5	49 ± 12	48 ± 9.9	47 ± 14
TCrP	110 ± 6.1	107 ± 19	103 ± 5.6	94 ± 9.3

After freezing-lipid precipitation, the extract still contained up to 20% of the original lipid content, unable for GC–MS analysis. A further clean-up step using SPE was performed in the present study. An Oasis HLB cartridge and Z-Sep/DSC18 mixture sorbent (200 mg, 1:1, w/w) were employed to eliminate the remaining lipid interferences in the extracts. HLB cartridges have been widely used for purification of sediment and water samples for the detection of organophosphate compounds, providing excellent results and recoveries [8,12] and dispersive SPE (d-SPE) has been reported as an efficient cleanup method for lipid removal [9,22]. Z-Sep and C18 have been applied to achieve satisfactory cleanup for PFRs detection in food samples [23] and eggs [24]. Large amounts of these expensive sorbents are required for high lipid content samples. In order to reduce the sorbent usage, in the present study, the d-SPE process was performed for further cleanup after freezing-lipid precipitation and HLB cartridge were applied, which removed most interferences from lipids. After these further purification steps, the analytes reached the requirements for analysis by GC/MS.

3.2. Method validation

The linearity of the GC–MS method was tested with standard mixtures at 10 levels of concentration. Correlation coefficients from 0.9913 to 0.9998 were obtained for PFRs (Table 3). The MDLs and MQLs of the development method ranged from 0.004 to 0.059 ng/g and 0.027 to 0.55 ng/g, respectively.

The performance of the developed method was verified by spiked matrixes and blanks experiments. 2 g samples of dry fish muscle were spiked with 100 ng of surrogate standards and 40 ng of 12 PFR standards. The samples were repeatedly (n = 3) extracted,

Table 3
The Linear range, correlation coefficient, method detection limits (MDLs) and method quantification limits (MQLs) of the proposed method, as well as recoveries (%) of PFRs in spiked blank and fish samples.

No.	Chemicals	Linear range(ng/ml)	R^2	Recovery ± RSD (%)			MQL(ng/g)	MDL(ng/g)
				The proposed method		The comparative method		
				Spiked blank samples	Spiked fish samples	Spiked fish samples		
1	TiPP	2.0-2000	0.9985	78 ± 5.7	56 ± 3.2	67 ± 2.7	0.039	0.012
2	TnPP	2.0-2000	0.9991	107 ± 1.1	108 ± 7.4	93 ± 13	0.095	0.029
3	TnBP	2.0-2000	0.9958	106 ± 0.13	92 ± 2.3	40 ± 10	0.051	0.015
4	TCEP	2.0-2000	0.9942	95 ± 2.6	80 ± 5.2	111 ± 5.8	0.34	0.010
5	TCPP	2.0-2000	0.9998	94 ± 1.2	95 ± 1.9	105 ± 12	0.55	0.004
6	TDCP	2.0-2000	0.9990	105 ± 3.5	89 ± 6.3	42 ± 8.6	0.154	0.046
7	TBEP	2.0-2000	0.9913	93 ± 2.8	90 ± 1.5	19	0.074	0.022
8	TPhP	2.0-2000	0.9997	105 ± 0.74	106 ± 1.8	111	0.043	0.013
9	EHDPP	2.0-2000	0.9982	75 ± 0.92	101 ± 2.4	167	0.021	0.006
10	TEHP	2.0-2000	0.9986	73 ± 0.11	63 ± 6.0	22	0.027	0.008
11	TCrP	2.0-2000	0.9997	79 ± 1.6	83 ± 3.5	121	0.198	0.059
12	TnPP-d21			97 ± 2.7	75 ± 5.3	52 ± 4.4		
13	TnBP-d27			103 ± 6.7	96 ± 11	89 ± 10		
14	TCPP-d18			106 ± 3.5	97 ± 12	227 ± 60		
15	TPhP-d15			90 ± 8.1	84 ± 7.8	160 ± 7.0		

purified and analyzed under the optimized condition of the method .

The means and the RSDs of the recoveries are listed in Table 3. As indicated, the recoveries of four surrogate standards ranged from 90 ± 8.1% to 106 ± 3.5% in the spiked blanks, and from 75 ± 5.3% to 97 ± 12% in the spiked fish samples. The recoveries of PFRs in the spiked blank were between 73 ± 0.11% and 108 ± 1.1%, and were between 56 ± 3.2% and 108 ± 7.4% in the spiked fish samples. However, no TEP was detected in either the spiked blank or fish samples using this method; this is a consequence of the volatility of TEP, which could not be quantified accurately due to significant losses during concentration of the extracts by solvent evaporation [6]. The relatively low recoveries of TiPP could also be attributed to this reason as published data have reported [6].

In view of the recoveries of PFRs and the removal of interferences, freezing-lipid precipitation and SPE cleanup were effective for the reliable confirmation and quantitative analysis of PFRs in biological samples with high lipid contents. This method uses little solvent and simplifies the cleanup process, which largely reduces the possibility of blank contamination. Only TCEP and TCPP were identified in the procedural blanks, at 0.33 ng/mL and 0.54 ng/mL, respectively.

3.3. Comparison with traditional chromatographic column cleanup method

Pressurized liquid extraction using aqueous solution and solid-phase microextraction [12], on-line turbulent flow chromatography [13], matrix solid-phase dispersion [14], and gel permeation chromatography and silica gel cleanup [15] have been previously used to determine PFRs in biota samples. These methods provide reasonable results with suitable recoveries, accuracy or detection limits, but are either time-consuming or need to use large quantities of organic solvents to remove the lipids. In consideration of equipment requirements and the laboratory conditions, we only conducted a comparison between the conventional chromatographic column cleanup method and the method proposed in the present study.

Conventional chromatographic column cleanup strategies, developed to eliminate co-extracted lipid interferences, typically include gel permeation chromatography(GPC) [10,15] and SPE on cartridges with alumina, florisil, silica, and/or combinations of these three materials [25]. A brief description of the conventional chromatographic column cleanup, which involved a lipid removement by GPC and a cleanup by composite silica column, is given here. After Soxhlet-extraction, the extract was concentrated to 1 mL and then was subjected to gel permeation chromatography using a glass column packed with 40 g of SX-3 Bio-Beads (Bio-Rad Laboratories, Hercules, CA, USA), and eluted with dichloromethane/n-hexane (1:1, v/v) for lipid removal. Eluate from 80 to 150 mL containing PFRs was collected and concentrated to 2 mL. The extract was further purified on a 1 cm i.d. multilayer silica column packed with neutral silica, alumina, and florisil (6 cm, 10 cm, and 5 cm, respectively). The fraction containing the targets was eluted using 40 mL of ethyl acetate.

A total amount of 6 spiked fish samples were analyzed. 3 spiked fish samples were treated with the method proposed by the present study (method 1) and the remaining 3 spiked fish samples were treated with the conventional chromatographic column cleanup method (method 2).

The final extract from method 2 used to instrumental analysis showed faint yellow colour and obvious lipid particles could be found when the extract was stored at −20 °C. At the same time, the final extract from method 1 was almost colorless and transparent. The lipid removal efficiency of the method 1 was higher than that of method 2. This could be further confirmed by the chromatograms. The total ion chromatograms (TIC) of spiked fish samples for two methods are shown in Fig. 1a. Method 1 has a lower baseline in the range from 10 to 17 min compared with method 2. During instrument analysis of samples treated by method 1, the instrument kept stable. However, the matrix interferences were a serious problem for the method 2 and the chromatographic column was polluted soon. Additionally, method 1 showed relatively satisfactory recoveries for all targets expect for TEP. The recoveries of PFRs were between 59 ± 2.8% and 113 ± 5.9%, while four internal standards were between 73 ± 11% and 96 ± 4.5%, which were similar to the aforementioned developed experiment, verifying the robustness and good repeatability of the proposed method. The recoveries of PFRs in the method 2 ranged from 19% to 167%, and from 52 ± 4.4% to 227 ± 60% for four surrogate standards. The recoveries of four surrogate standards excess 200%, which exhibited the interferences of matrix.

Additionally, we found that regardless of the column packing material (alumina, florisil, silica, and/or combinations of these three materials), the lipids could not be easily separated from the PFRs. To elute PFRs from the column, dichloromethane or ethyl acetate is needed as the elution solvent, which results in lipid co-extraction. The poor performance of the method 2 may be mainly explained with the experimental conditions not being optimum. However, the result of the present study indicates that the proposed method

Table 4
Concentrations of PFRs in biota samples.

Location/ samples	Concentration (ng/g lw)												
	TEP	TiPP	TnPP	TnBP	TCEP	TCPP	TDCP	TBEP	TPhP	EHDPP	TEHP	TCrP	∑PFRs
Zhongtang plecostomus	ND	3.82	47.0	11.7	13.5	ND	ND	7.06	42.3	ND	14.6	ND	140
	ND	4.66	57.5	13.7	19.5	ND	3.79	ND	63.4	ND	12.7	ND	175
	ND	5.94	68.1	17.6	18.3	ND	ND	ND	85.0	ND	13.9	ND	209
Shatian Tilapia	ND	3.85	187	42.6	ND	ND	ND	8.10	16.3	ND	62.8	ND	321
	ND	7.34	255	31.9	ND	ND	ND	22.9	32.3	ND	83.6	ND	433
	ND	7.76	200	94.6	ND	ND	ND	22.4	54.4	ND	96.1	ND	475
Gaoming Mud carp	ND	13.8	73.3	91.3	ND	23.5	ND	4.69	ND	ND	78.2	ND	285
	ND	15.0	73.8	65.4	ND	28.9	ND	9.38	ND	ND	93.2	ND	286
	ND	18.8	70.5	61.3	6.51	26.3	ND	7.88	ND	7.25	88.2	ND	287
Shitan Catfish	ND	4.43	15.1	22.2	6.34	ND	ND	1.19	19.3	6.95	56.3	10.3	142
	ND	4.79	16.0	22.4	6.11	ND	ND	9.46	20.3	5.12	52.8	9.66	147
	ND	4.75	16.8	22.4	6.33	ND	ND	ND	19.2	4.26	53.7	8.71	136

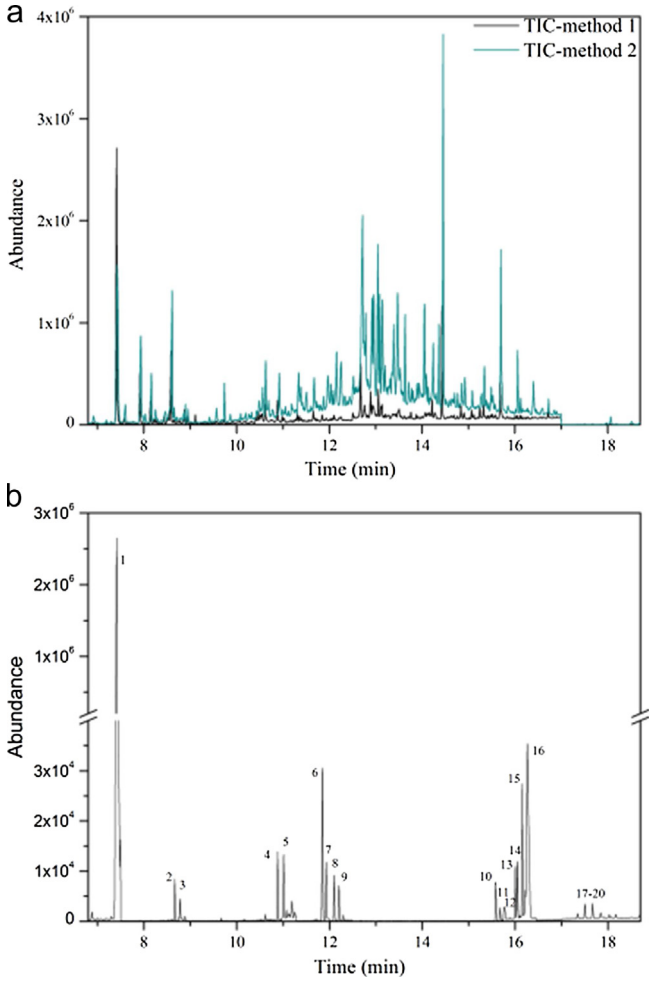

Fig. 1. (a) Total ion chromatograms (TIC) of spiked fish extracts purified by (method 1) Oasis HLB cartridge after freezing-lipid precipitation; (method 2) gel permeation chromatography column (GPC) and composite silica cartridge. (b) SIM chromatogram of the proposed method.Peak identities: 1,TiPP; 2,TnPP-D21; 3,TnPP; 4,TnBP-D27; 5,TnBP; 6,TCEP-D12; 7,TCEP; 8,TCPP-D18; 9,TCPP; 10,TDCP-D15; 11,TDCP; 12,TBEP; 13,TPhP-D15; 14,TPhP; 15,EHDPP; 16,TEHP; 17–20,TCrP1-4.

is efficient in lipid remove from the extracts. And the proposed method consumes less organic solvent and cost less labor and time.

3.4. Application to biological samples

To examine the applicability of our method, we investigated the presence of PFRs in some fish muscle samples of plecostomus (*Hypostomus plecostomus*), tilapia (*Tilapia nilotica*), mud carp (*Cirrhinus molitorella*), and catfish (*Silurus asotus*) collected from the Pearl River Delta.

PFRs were detected in all samples and levels are presented as lipid weight concentrations (Table 4). Among of the 12 PFRs, the predominant pollutants were TnPP, TnBP, TEHP, TCEP, and TPhP. Total concentrations of PFRs ranged from 136 to 475 ng/g lipid weight (lw), from 15.1 to 255 ng/g lw for TnPP, from 11.7 to 94.6 ng/g lw for TnBP, from 12.7 to 96.1 ng/g lw for TEHP, from 6.11 to 19.5 ng/g lw for TCEP and from 16.3 to 85.0 ng/g lw for TPhP.

Levels of PFRs in the present study are slightly lower than those reported in aquatic life in previous studies. Kim et al[26] detected 9 PFRs in 20 species collected from Manila Bay (the Philippines) and the total concentrations ranged from 190 to 1900 ng/g lw. TEHP, TEP, and TnBP are the main contributors to the total PFR contents in the Philippines environment. Ma et al. [15]. detected PFRs in catfish and grass carp from the Pearl River (Guangdong Province),with concentrations of predominant pollutants between 43.9 and 2950 ng/g lw for TnBP, 82.7 and 4690 ng/g lw for TCEP, 62.7 and 883 ng/g lw for TCPP, and 164 and 8840 ng/g lw for TBEP. Domestic and international studies have reported similar results to our research in terms of the major contaminants.

Considering the relatively low recoveries of TEP and TiPP, their levels might been underestimated as these compounds could not be exactly quantified as a result of losses during the clean-up step. Although much lower levels of PFRs were found in living organisms than in abiotic substances, the exposure of PFRs in living organisms is not negligible with the increasing usage of PFRs in the future. Therefore, it is necessary to pay more attention to PFR pollution in order to understand the potential environmental and human health risks of these compounds.

4. Conclusion

We have developed a method to analyze PFRs in biota matrices. The method has a high removal efficiency of lipids extracted from fish samples. The freezing-lipid precipitation method, combined with SPE steps (HLB and sorbent), is simple and organic solvent-saving and it provided satisfactory final results as well. Hence, the method can be used as a rapid screening tool for the determination of PFRs in fish, on the basis of GC/MS analysis with deuterium-labeled internal standards. Further work to improve the method

and explore the possibility of applying it to the determination of PFRs in other living organisms is recommended.

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