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Simultaneous solid phase extraction coupled with liquid chromatography tandem mass spectrometry and gas chromatography tandem mass spectrometry for the highly sensitive determination of 15 endocrine disrupting chemicals in seafood

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ABSTRACT

This study aimed to develop a sensitive and reliable multi-residue method for the determination of trace amounts of endocrine disrupting chemicals including five phthalate esters (PAEs), five monoalky phthalate esters (MPEs), four alkylphenols (APs) and bisphenol A (BPA) in seafood. Ultrasonic liquid extraction was selected for extraction based on acetonitrile, instead of frequently-used n-hexane, due to its lower background of PAEs. Application of solid phase extraction (SPE) with primary secondary amine (PSA, 1 g/6 mL) cartridge achieved the relatively low matrix effects for MPEs and BPA in seafood. To our knowledge, it is the first study reporting about simultaneous extraction and purification of PAEs, MPEs, APs and BPA in biota samples. To obtain the maximum sensitivity, both liquid chromatography tandem mass spectrometry (LC–MS/MS) and gas chromatography tandem mass spectrometry (GC–MS/MS) were applied for analysis. This method was validated and tested on fish, mollusk and prawn. Sufficient linearity was verified by Mandel's fitting test for the matrix-matched calibrations used in this study for MPEs, APs and BPA, between 0.5 ng/g and 200 ng/g or 400 ng/g. And correlation coefficients of all calibrations suppressed 0.99 for all analytes. Good recoveries were obtained, ranging from 60% to 127% for most compounds. The sensitivity was good with method detection limits (MDLs) of 0.015–2.2 ng/g wet weight (ww) for all compounds. Most MDLs are much lower than those in previous reports. The sensitive method was then applied on real fish, mollusk and prawn samples from the Yangtze River Delta sea area (China), and all the target compounds were detected with the maximum concentrations of PAEs, MPEs, APs and BPA up to 219.3 ng/g ww, 51.4 ng/g ww, 62.0 ng/g ww and 8.6 ng/g ww, respectively.

1. Introduction

Phthalate esters (PAEs), alkylphenols (APs) and bisphenol A (BPA), which consist of one or two benzene rings, are typical compounds of endocrine disrupting chemicals (EDCs). They are manufactured mostly as additives or precursors in products such as plastics, surfactants and resin [1,2]. Because of their huge output, wide dispersive use and low removal efficiency, these compounds are found in many matrices and cannot be avoided in our environment. Occurrence of PAEs, APs and BPA in environmental matrices, especially in water and food, has aroused considerable concern [3]. Previous reports showed that many EDCs are likely to cause reproductive, teratogenic and developmental toxicities even at low concentrations [4]. In recent years, study of the main primary metabolites of PAEs, monoalky phthalate esters (MPEs), has also been carried out because PAEs can be easily degraded in vivo [5] and MPEs could reflect the internal exposure of biota to PAEs [6]. Many countries have strengthened regulations on use of PAEs, APs and BPA. In China, the quality limits for PAEs in food and food additives are set at 1.5 mg/kg for di (2-ethylhexyl) phthalate (DEHP) and 0.3 mg/kg for di-n-butyl phthalate (DBP) according to the announcement of Chinese Ministry of Health in June, 2011. In 2010, Canada first listed BPA as toxic compound [7]. The European Council Directive (2003/53/EC) on the marketing and use of nonylphenol (NP) intends to decrease its consumption [8].

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The relatively low concentrations of PAEs, MPEs, APs and BPA in marine biota and the complexity of matrices make the enrichment and cleanup steps unavoidable prior to instrumental analysis. To the present, some sample pretreatment methods, like ultrasonic extraction (USE) [9], coupled with gel permeation chromatography (GPC) [10] or solid phase extraction (SPE) [11] have been frequently used for extraction and purification of PAEs, MPEs, APs and BPA in seafood. Furthermore, many studies carried out simultaneous sample pretreatment of these compounds in water, urine, food and biota. Mortazavi et al. [12] used Soxhlet extractor to extract 4-nonylphenol (4-NP), octylphenol (OP) and BPA in fish for 10 h with dichloromethane followed with purification process based on silica gel column. Niu et al. [13] applied liquid extraction based on acetonitrile followed with on-line SPE system to pretreat NP, OP and BPA in cereals. Sun et al. [14] developed temperature controlled ionic liquid dispersive liquid–liquid microextraction to pretreat DEHP and mono-2-ethylhexyl ester (MEHP) in human urine. Wang et al. [15] and Chen et al. [16] used SPE to prepare urine samples for the determination of MPEs and BPA. Bono-Blay et al. [17] used SPE cartridge to extract PAEs, APs and BPA in water for more than 1 h. However, to the best of our knowledge, none of these researches has reported about the simultaneous sample preparation of PAEs, MPEs, APs and BPA in seafood before. The sample pretreatment is always time-consuming and may take about 61% of the whole chemical analysis time [18]. It required a long time to pretreat PAEs, MPEs, APs and BPA separately in seafood. In order to shorten the pretreatment period, simultaneous sample pretreatment method for these compounds in seafood is needed.

In addition, many high sensitive analytical techniques, like liquid chromatography tandem mass spectrometry (LC–MS/MS) and gas chromatography tandem mass spectrometry (GC–MS/MS) [19,20], have been used for analysis of these analyses in recent years. GC–MS/MS has been frequently used for the analysis of PAEs due to the high volatility. As for MPEs, APs and BPA, the low volatility limits the direct application of GC–MS/MS. Although derivation steps can help to enhance the volatility, they are time-consuming and also, however, result in the transformation of MPEs [5]. Thus LC–MS/MS is always applied in analysis of MPEs, APs and BPA [16,19]. Simultaneous detection would be more convenient, but we would still use different techniques to obtain low method detection limits (MDLs).

This study aimed to develop a simple, sensitive and robust method to determine PAEs, MPEs, APs and BPA in seafood. Simultaneous sample pretreatment of 15 target compounds including five PAEs, five MPEs, four APs and BPA, based on USE followed by SPE purification, were developed, and various operational conditions such as selection of extraction solvent and SPE cartridge, elution of SPE and the gradient of liquid phase were optimized. USE was applied to the extraction procedure in this study because USE with glass centrifuge tube got the least blank contamination of PAEs compared to other extraction techniques with plastic materials. Acetonitrile replaced frequently-used n-hexane [21] and dichloromethane [12] in USE because of the high background levels of PAEs in n-hexane in our study and the toxicity of dichloromethane. As for SPE procedure, separate elution on PSA cartridge reduced matrix effects for MPEs and BPA, significantly. Reversed-phase high performance liquid chromatography/electrospray ionization in negative mode coupled triple quadrupole mass spectrometry (HPLC/ESI-MS/MS) was selected for the analysis of MPEs, APs and BPA, and gas chromatography/electron impact ionization in positive mode coupled triple quadrupole mass spectrometry (GC/EI-MS/MS) was selected for PAEs to obtain the maximum sensitivity. In addition, the developed method was validated by assessing linearity, precision and accuracy as well as the matrix effect, and further applied to seafood samples, including fish, prawn and mollusk, from coastal areas of Zhejiang Province in China.

2. Experiment

2.1. Reagents and materials

GC grade acetonitrile was supplied by Scharlau (Barcelona, Spain). HPLC grade methanol (MeOH), acetone, acetic acid (99% purity) and formic acid (96% purity) were purchased from Tedia (OH, USA). Analytical reagent ammonia solution (25–28% purity) and sodium chloride (NaCl) were supplied by Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Ultrapure water was produced by a Millipore Milli-Q system (MA, USA).

The divinylbenzene/N-vinylpyrrolidone copolymer (Oasis HLB, 3 cc/60 mg) glass cartridges were purchased from Waters (MA, USA); the primary secondary amine (ProElut PSA, 1 g/6 mL) glass cartridges were supplied by Dikma (Beijing, China); AccuBond Florisil cartridges were purchased from Agilent Technologies (CA, USA).

The analytical standards dimethyl phthalate (DMP) (99.5% purity), diethyl phthalate (DEP) (99.5% purity), DBP (99.0% purity), DEHP (98.5% purity), di-n-octyl phthalate (DNOP) (99.5% purity), mono-methyl ester (MMP) (98.0% purity), mono-n-butyl ester (MBP) (98.0% purity), MEHP (91.0% purity) were obtained from Dr. Ehrenstorfer (Augsburg, Germany). Mono-ethyl ester (MEP) and mono-n-octyl ester (MNOP) were supplied by Accustandard (CA, USA) and Toronto Research Chemicals Inc. (Ontario, Canada), respectively. BPA, 4-NP, 4-n-nonylphenol (4-n-NP), 4-octylphenol (4-OP) and 4-tert-octylphenol (4-t-OP) were purchased from Sigma–Aldrich (Poole, UK), and the purities are all above 98%.

Individual standard stock solutions of the 15 target compounds with concentration of 1000 mg/L were all prepared in acetonitrile and stored in a refrigerator in the dark at −20 °C. Standard native solutions were prepared from the stock solutions by acetonitrile dilution and eight points were set at 0.5, 5, 10, 25, 50, 100, 200 and 400 μg/L for working solutions. Matrix-matched standard solutions were also obtained to compensate for matrix effects at 0.5, 5, 10, 25, 50, 100, 200 and 400 ng/g by spiking extracts of blank matrix, including fish, prawn and mollusk. These solutions were all stored in the dark at −4 °C prior to use.

All glasswares used in experiment were washed by acetone twice and NaCl was baked at 500 °C for 5 h to elude the contained target compounds prior to use.

2.2. Instrumentation

2.2.1. LC–MS/MS analysis

Separation and quantification of MPEs, APs and BPA were performed by using a liquid chromatography (1200 series, Agilent Technologies, CA, USA), equipped with an automatic injector (CTC analytics, Swiss), coupled to a triple-stage quadrupole mass spectrometer (API5000 QTrap, ABSciex, USA). The injection volume was set at 10 μL. As for APs, the chromatographic separation was carried out with a Waters Xbridge C_{18} column (5 μm, 100 mm × 2.1 mm (i.d.)) (Waters, MA, USA) at a flow rate of 0.25 mL/min. The column temperature was kept at 40 °C. The mobile phases were (A) 0.3% ammonium hydroxide in MilliQ water and (B) MeOH with the following gradient: from 20% to 65% (B) in 2 min; from 65% to 100% (B) in 4 min; keeping 100% (B) for 6 min; from 100% to 20% (B) in 0.2 min; keeping 20% (B) for 6.8 min. The column for BPA and MPEs was an Agilent Eclipse XDB C_8 column (5 μm, 100 mm × 4.6 mm (i.d.)) (Agilent technologies, CA, USA). And column was kept at 30 °C. The mobile phase compositions were: (A) 0.01% acetic acid in MilliQ water; (B) acetonitrile. The solvents were mixed as follows: 0 min,

Table 1
Retention time (t_R), precursor and product ions, collision energy (CE), declustering potential (DP), dwell time of the target compounds, ionized in negative (ESI^-) mode for LC–MS/MS detection.

Compounds	t_R (min)	Precursor ion (Da)	Product ions (Da)	DP(V)	CE(V)	Dwell time (ms)
MAEs						
MMP	3.12	179.3	107	−70	−15	50
			77	−55	−24	50
MEP	3.71	193.3	77	−67	−24	50
			121	−45	−15	50
			147	−72	−16	50
MBP	5.24	221.3	77.1	−53	−24	50
			149.2	−80	−14	50
MEHP	7.81	277.3	134.1	−116	−21	50
			127.1	−109	−22	50
			233.3	−109	−16	50
MNOP	8.00	277.4	203.3	−124	−24	50
			119.2	−115	−35	50
			121.1	−116	−24	50
APs						
4-t-OP	8.98	205.4	133.4	−153	−30	50
			118.1	−154	−53	50
4-OP	9.42	205.6	106.1	−135	−24	50
			119	−91	−47	50
4-NP	9.31	219.4	133.4	−66	−42	50
			147.3	−64	−30	50
4-n-NP	9.70	219.4	106.1	−116	−27	50
BPA	5.90	227.4	133.4	−108	−35	50
			212.3	−120	−25	50

40% (B); 8 min, 100% (B); 12 min, 100% (B); 15 min, 40% (B); 19 min 40% (B).

Mass analysis was performed with an ESI source in the negative (ESI^-) ion mode for APs, MPEs and BPA. Nitrogen was used as the nebuliser gas. The optimum MS parameters were: curtain gas, 35 psi; collision gas, 4 psi; ion source gas 1, 60 psi; ion source gas 2, 60 psi; ionspray voltage, −4500 V; temperature, 450 °C. All analyses were analyzed in multiple reaction monitoring (MRM) mode. The LC–MS/MS settings and parameters of ESI source were optimized by manual infusion of standards with a syringe pump. The optimized parameters are summarized in Table 1.

2.2.2. GC–MS/MS analysis

The GC–MS/MS analysis of PAEs was performed on a 7000 Triple Quad–MS/MS system (Agilent Technologies, CA, USA) on EI positive mode, equipped with an Agilent 120 atuosampler. The instrument control, data acquisition and data analysis are performed with Agilent GC–MS/MS MassHunter software. An Agilent HP-5 MS capillary column, 30 m × 0.25 mm (i.d.) × 0.25 mm film thickness, was selected. The GC oven temperature was initially held at 65 °C for 1 min, ramped at 20 °C/min to 220 °C, then ramped at 5 °C/min to 290 °C and held for 3 min. Splitless injection with 1 μL was set, with the let temperature of 260 °C. The auxiliary temperature was at 300 °C. The ion source temperature was at 230 °C. All PAEs were analyzed in MRM mode. The parameters of mass spectrometer were improved based on a previous research [22].

2.3. Sample preparation and clean-up

The homogenized fresh sample (2 g) was weighed into a 15 mL glass centrifuge tube with crew crap. 2 mL of ultra-pure water was added into the tube which was then shaken by a Vortex Genie (Scientific industries INC, NY, USA) for 30 s to disperse the sample. 10 mL of acetonitrile was added, and the tube was immediately manually shaken for 1 min and bathed in ultrasonic water for 15 min. After addition of 1 g NaCl, the tube was shaken and centrifuged (2500 rpm, 5 min). Upper acetonitrile layer was transferred into a new glass tube. This process was repeated and the supernatants were combined and dried to 1.5 mL under a gentle stream of nitrogen at the temperature of 38 °C. Vortex the tube and dissolve the residue before purification.

ProElut PSA (1 g/6 mL) cartridge was conditioned by 5 mL of acetonitrile. Then the concentrated extracts were loaded into the cartridge at a flow rate of gravity. PAEs and APs were first eluted into a clean 10 mL glass centrifuge tube by passing through 5 mL of acetonitrile (Solution 1). The second elution was performed by 6 mL of formic acid–MeOH (3/97, v/v), and the eluent was collected in another glass tube (Solution 2). Solution 1 and Solution 2 were both evaporated near to 200 μL separately under a gentle stream of nitrogen at a temperature of 38 °C. Residues were then reconstituted with 1 mL acetonitrile, vortexed for 1 min and filtered through 0.22 μm nylon syringe filter prior to LC–MS/MS and GC–MS/MS analysis.

2.4. Method validation

The method was validated to evaluate its performance in accordance with a conventional validation procedure that includes the following parameters: linearity, MDLs, matrix effects, accuracy and precision. Analysis of PAEs by GC–MS/MS was calibrated by native standards at eight points of 0.5–400 μg/L (0.5, 5, 10, 25, 50, 100, 200, 400 μg/L) and the correlation coefficients (R^2) were evaluated. Analysis of MPEs, APs and BPA by LC–MS/MS was calibrated by matrix-matched standards, which were carried out by spiking extracts of blank matrix (fish, mollusk, prawn) with native standard solutions to compensate for the matrix effects. And its linearity was evaluated by the Mandel's fitting test according to a previous report [23].

MDLs were determinated based on the procedures described in a regulation of US Government Printing Office [24]. Samples

with different concentrations (0.1–5 ng/g ww) were analyzed and the ones that could get a signal-to-noise (peak to peak) ratio of three were considered to be at the levels near MDLs. Then the batch included seven replicates was set for each level as well as other three replicates of reagent blank used as background values. MDL was calculated as 3.14 SD (standard deviation), within a 95% confidence interval ($n = 7$).

Because of the lack of an absolute PAEs-free matrix, samples in quality control (QC) were needed. A total of 30 samples including low QC samples at 5 ng/g, medium QC samples at 25 ng/g, high QC samples at 50 ng/g and reagent blanks were analyzed. Trace concentrations of DEHP, with average level of 8.18 ± 0.25 μg/L, were detected in reagent blanks, whereas other compounds were not detected in reagent blanks. Therefore, sample concentrations would be subtracted from blank values for DEHP.

The recovery assays were carried out to investigate accuracy and precision of the method. It was evaluated by analyzing QC samples which were prepared by spiking extracts of blank seafood samples (in original samples) with native standard mixtures at three levels (5, 25 and 50 ng/g). Six replicates were set for the spiked samples and three replicates were set for blank samples to subtract the trace amounts of target compounds in blank samples. The precision of the method was described as repeatability, expressed as the relative standard deviations (RSDs), and were determined by both intra-day assays during 1 day and inter-day assays during 3 days.

3. Results and discussion

3.1. Optimization of sample pretreatment

3.1.1. Optimization of the extraction procedure

The analysis of complex matrices, like biota samples, needs rigorous sample preparation to obtain a stable and sensitive analysis. In addition, the physical and chemical differences between target analyses make the optimization of pretreatment more delicate, with estimated log octanol–water partition coefficients ($\log K_{ow}$) at 4.12–4.48 for APs [25], 1.46–8.54 for PAEs [26], 1.37–4.73 for MPEs [5] and 3.32 for BPA [27]. The extraction period is based on liquid extraction principle. Organic solvents could allow the extraction of target compounds from matrix, and the simple step might also reduce the levels of background contamination. According to references, n-hexane and acetonitrile were frequently used for liquid extraction of these compounds due to the fine recoveries. Lin et al. [28] and Ferrara et al. [29] selected n-hexane for the extraction of PAEs and APs respectively in seafood with the recovery range of 71–106%; while acetonitrile-based method was chosen for the extraction of BPA in fish by Wei et al. [11] with the recoveries of BPA-*d*16 ranged from 76% to 79%. On the other hand, background values of PAEs in n-hexane and acetonitrile were compared in our study. Results indicated that n-hexane-based method had higher levels of background contamination, especially for DEHP (nine times higher) and DBP (two times higher). And the levels in acetonitrile are no more than 5 μg/L. Furthermore, recoveries of acetonitrile-based method were satisfied and sufficient (70–120%) for all target compounds. Thus, acetonitrile was chosen as the extraction solvent.

Several volumes of acetonitrile (10 mL and 20 mL) were subsequently tested. Results indicated that the volume of 20 mL had good recoveries in the range of 70–111%. So the volume of 20 mL was chosen.

3.1.2. Optimization of the clean-up procedure

SPE cartridge is often used for cleanup procedure of PAEs, MPEs, APs and BPA according to previous reports. Tsumura et al. [30] used SPE based on florisil and PSA cartridges for purification of PAEs in food. Florisil cartridges were necessary in the pretreatment of fish [9] and PSA has a weak anion exchange function which could extract polar compounds from samples containing much non-polar substances like fat and protein [31]. Blount et al. [32] applied Oasis HLB in the pretreatment of MPEs in human urine. It is reported that the HLB cartridges showed the best recoveries of phenolic and steroidal EDCs in water [33]. The sorbents of Oasis HLB act as hydrogen acceptors and have the unique ability to retain a wide range of compounds. To obtain satisfied cleanup effects for sample pretreatment, all cartridges mentioned above were tested in fish, prawn and mollusk matrix in this work. As is shown in Fig. 1, PSA cartridges showed the best performance at retention and purification of 15 target compounds in all matrices. Although satisfied results of most MPEs, APs and BPA occurred when florisil cartridges were applied in prawn matrix treatment, poor results were obtained for PAEs (the recoveries of PAEs <40%). And the recoveries of PAEs and MPEs were also below 40% in all matrices when HLB cartridges were used. Thus, PSA cartridges were chosen for the clean-up procedure.

PSA with n-hexane and acetonitrile elution were compared. PAEs and APs were eluted from PSA cartridges by n-hexane and acetonitrile easily, and both of them obtained good results with the recoveries of 80–110% and 70–110%, respectively. Although elution of n-hexane obtained better recoveries, the high levels of background pollution made n-hexane omitted (see in Section 3.1.1). Therefore, acetonitrile was selected as the elution solvent for PAEs and APs. As for MPEs and BPA, both n-hexane and acetonitrile obtained low recoveries (<15%). This may due to the stronger ionic bond between MPEs and BPA and PSA cartridges. This limitation was overcome by adding formic acid in solvents. After optimizing the percentage of formic acid in MeOH and acetonitrile, MeOH obtained better recoveries for all analyses with less volume of formic acid than acetonitrile. After further optimization, 5 mL of acetonitrile followed by 6 mL of formic acid–MeOH (3 + 97, v/v) were selected as the elutes finally.

3.2. LC–MS/MS conditions

LC–MS/MS were selected for MPEs, APs and BPA but not for PAEs in this study because of the pollution from liquid plastic piping systems especially for DBP, DEHP and DNOP. The optimization of MS/MS conditions for MPEs, APs and BPA was performed by the direct infusion of 50 μg/L mixed standard solutions via the syringe pump at a flow rate of 10 μL/min prior to entering the ESI source. For all the compounds, precursor ions (Q1) corresponding to the protonated molecules $[M+H]^+$ in positive mode and $[M-H]^-$ in negative mode were both scanned. Scan results indicated that good sensitivity could be achieved in negative mode for APs, MPEs and BPA. It means that APs, MPEs and BPA respond in negative mode following the deprotonation of the carboxylic functional group. Then diagnostic fragment ions were selected and all mass-spectrometer parameters were optimized. The most stable and intense fragment ion is used for quantification, and the second transition is used for confirmation. The dwell times are established to obtain at least 12 data points across the narrowest peak in the window. The selected MRM transitions as well as the individual declustering potentials (DP) and collision energies (CE), which are optimized in MRM mode, are shown in Table 1.

The separation effectiveness of HPLC columns is an important factor which would influence the peak shape and response of target compounds greatly. With the injection of 10 μL of MPEs, APs and BPA mixture standard solutions at the concentration of 50 μg/L, three kinds of HPLC columns, including Waters X-Bridge C_{18} column (100 mm × 2.1 mm i.d., 5 μm), Agilent Zorbax Eclipse XDB C_8 column (150 mm × 4.8 mm i.d., 5 μm) and Agilent ZORBAX SB C_{18} column (150 mm × 4.8 mm i.d., 5 μm), were compared to select the suitable columns (details see Fig. S1 in Supplementary Material).

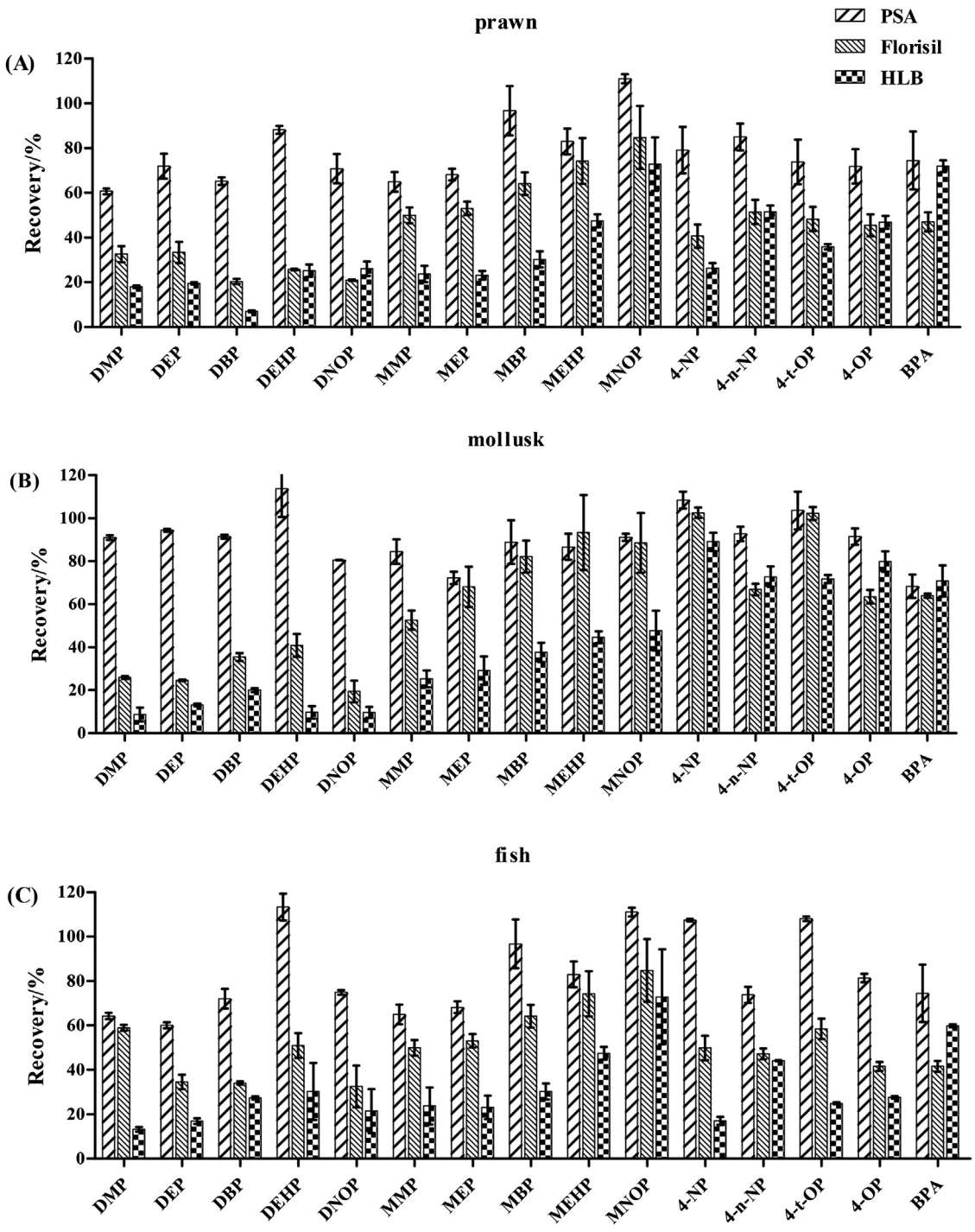

Fig. 1. Effect of different SPE sorbents (PSA, florisil and HLB) for targeted analytes in prawn (A), mollusk (B) and fish samples (C) at 50 ng/g ww level ($n = 3$).

Although XDB C_8 column could produce sharp peaks, X-Bridge C_{18} column was still chosen as the analytical column for APs as the responses and separation were better than those of XDB C_8 column. Comparison of three chromatograms of MPEs indicated that XDB C_8 column would obtain the most sharp and highest response of MPEs, especially for MMP, MEP, BPA and MBP.

In addition, mobile phase is also an important factor influencing ionization efficiency in ion source and then finally affecting the resolution and sensitivity of APs, MPEs and BPA. Therefore, the composition of mobile phases (MeOH–water and acetonitrile–water) and the concentration of additives (acetic acid and ammonium hydroxide) were also compared. The optimum separation conditions were established by injecting 10 μL of mixture standard solutions (spiked at 50 μg/L) (details see Fig. S2 in Supplementary Material). In comparing MeOH/water and acetonitrile/water as the mobile phase, the former gave higher signal and better separation for APs while the later got better results for MPEs and BPA. MeOH was chosen for APs and acetonitrile was selected for MPEs and BPA. In further, concentrations of additives were optimized. MeOH/0.1% acetic acid, MeOH/0.1% ammonia hydroxide and MeOH/water were compared for APs. Although responses of APs under MeOH/water were twice as large as those under MeOH/ammonia hydroxide, the former were not reliable especially in complicated matrix. Ammonia hydroxide in water could improve the *S*/*N* ration [19].

Table 2
Regression equations, correlation coefficients (R^2), and test values (TV) for the *F* test of Mandel's fitting test for the matrix-matched calibration curves in different matrices (within a confidence level of 99%).

Compounds	Matrix	Regression equation	R^2	Linear range (ng/g)	TV
4-t-OP	Fish	$y = 14{,}800x - 111{,}000$	0.9918	0.5–400	16.77
	Mollusk	$y = 10{,}000x - 30{,}300$	0.9976	0.5–400	10.33
	Prawn	$y = 17{,}400x - 43{,}300$	0.9986	0.5–200	6.43
4-OP	Fish	$y = 14{,}100x - 48{,}500$	0.9977	0.5–200	11.83
	Mollusk	$y = 18{,}500x - 8860$	0.9997	0.5–400	7.54
	Prawn	$y = 16{,}200x - 24{,}500$	0.9995	0.5–400	1.59
4-NP	Fish	$y = 8660x + 131{,}000$	0.9965	0.5–400	34.10
	Mollusk	$y = 7080x + 129{,}000$	0.9984	0.5–400	10.71
	Prawn	$y = 10{,}000x + 16{,}4000$	0.9980	0.5–400	5.87
4-n-NP	Fish	$y = 27{,}100x - 182{,}000$	0.9944	0.5–400	21.32
	Mollusk	$y = 42{,}100x - 78{,}500$	0.9988	0.5–400	9.62
	Prawn	$y = 54{,}500x - 82{,}100$	0.9995	0.5–400	0.59
MMP	Fish	$y = 1710x - 2660$	0.9996	0.5–200	4.51
	Mollusk	$y = 2290x + 380$	0.9997	0.5–400	1.90
	Prawn	$y = 1990x - 3790$	0.9976	0.5–400	2.29
MEP	Fish	$y = 5400x - 7740$	0.9998	0.5–400	1.22
	Mollusk	$y = 6440x + 12{,}500$	1.0000	0.5–400	0.60
	Prawn	$y = 5750x - 2620$	0.9995	0.5–200	1.45
MBP	Fish	$y = 6110x + 21{,}100$	0.9993	0.5–400	7.14
	Mollusk	$y = 8100x + 19{,}300$	0.9996	0.5–400	8.42
	Prawn	$y = 7100x + 6620$	0.9997	0.5–200	1.24
MEHP	Fish	$y = 4190x + 3100$	0.9991	0.5–400	5.46
	Mollusk	$y = 4950x + 122{,}000$	0.9990	0.5–400	4.70
	Prawn	$y = 508x + 84400$	0.9990	0.5–400	0.41
MNOP	Fish	$y = 190x + 176$	0.9990	0.5–400	0.70
	Mollusk	$y = 339x + 1910$	0.9993	0.5–400	5.62
	Prawn	$y = 324x + 2670$	0.9992	0.5–400	0.43
BPA	Fish	$y = 1870x - 2150$	0.9999	0.5–400	2.06
	Mollusk	$y = 1380x - 893$	0.9997	0.5–400	4.35
	Prawn	$y = 1910x - 2890$	0.9997	0.5–400	6.71

Table 3
Comparison of method detection limit (MDL) of compounds focused in previous studies and our study.

Analytes	Matrix	Pretreatment	Detection system	MDL in previous study (ng/g ww)	Reference	MDL in our study (ng/g ww)
DMP	Fish	ULE[a] with SPE (deactivated alumina)	LC–MS/MS	1.8	[28]	0.23
DEP	Fish	ULE[a] with SPE (deactivated alumina)	LC–MS/MS	1.6	[28]	0.64
DBP	Fish	ULE[a] with SPE (deactivated alumina)	LC–MS/MS	4.2	[28]	0.37
DEHP	Fish	ULE[a] with SPE (deactivated alumina)	LC–MS/MS	4.2	[28]	0.034
DNOP	Fish	ULE[a] with SPE (deactivated alumina)	LC–MS/MS	4.2	[28]	2.2
MMP	Fish mollusk	ASE[b] with SPE (Oasis MAX)	LC–MS/MS	0.39	[5]	1.0
MEP	Fish mollusk	ASE[b] with SPE (Oasis MAX)	LC–MS/MS	0.99	[5]	0.30
MBP	Fish mollusk	ASE[b] with SPE (Oasis MAX)	LC–MS/MS	0.42	[5]	0.099
MEHP	Fish mollusk	ASE[b] with SPE (Oasis MAX)	LC–MS/MS	0.05	[5]	0.019
MNOP	Fish mollusk	ASE[b] with SPE (Oasis MAX)	LC–MS/MS	0.14	[5]	0.82
4-NP	Fish mollusk prawn	Liquid extraction	GC–MS	12.4	[34]	0.16
4-n-NP	NA[c]	NA[c]	NA[c]	NA[c]	NA[c]	0.060
4-OP	Fish mollusk prawn	Liquid extraction	GC–MS	0.5	[34]	0.015
4-t-OP	NA[a]	NA[c]	NA[c]	NA[c]	NA[c]	0.48
BPA	Fish	ULE[a] with SPE (Oasis HLB)	LC–MS/MS	0.5	[11]	0.37

[a] Ultrasonic liquid extraction.
[b] Accelerated solvent extraction.
[c] Data not available.

Table 4
Validation parameters: recoveries (*R*), intra and inter day precisions (relative standard deviation, RSD) at three concentrations (5 ng/g, 25 ng/g, 100 ng/g).

Matrix	Compounds	*R* (%)			Intra-day precision RSD (%, *n* = 6)			Inter-day precision RSD (%, *n* = 3)		
		5 ng/g	25 ng/g	50 ng/g	5 ng/g‘	25 ng/g	50 ng/g	5 ng/g	25 ng/g	50 ng/g
Fish	DMP	69	57	64	19	3	13	2	19	9
	DEP	94	68	76	14	3	13	0.4	4	1
	DBP	80	82	101	24	9	8	2	12	4
	DEHP	77	119	106	17	7	7	10	21	0.3
	DNOP	125	118	112	7	10	7	14	2	7
	MMP	77	69	64	12	12	9	34	22	20
	MEP	72	72	63	10	9	6	25	19	15
	MBP	107	105	74	8	5	13	33	6	2
	MEHP	96	82	69	10	13	10	2	11	18
	MNOP	86	92	78	14	11	15	4	11	18
	4-NP	95	77	79	9	9	14	14	35	14
	4-n-NP	73	60	76	13	8	11	17	14	14
	4-OP	98	73	84	5	12	15	8	32	17
	4-t-OP	71	67	62	14	10	9	1	14	11
	BPA	97	103	94	7	12	14	17	0.6	16
Mollusk	DMP	70	68	83	4	9	18	6	7	1
	DEP	83	68	83	22	10	17	15	1	1
	DBP	82	96	96	15	15	15	6	14	14
	DEHP	73	87	117	14	5	5	3	10	0.6
	DNOP	114	91	93	8	9	17	35	7	3
	MMP	115	111	102	13	5	6	31	14	22
	MEP	112	104	101	8	2	5	12	5	7
	MBP	127	98	103	10	5	3	18	4	17
	MEHP	120	68	75	5	2	10	52	13	14
	MNOP	96	72	98	9	17	13	5	9	20
	4-NP	88	97	92	12	11	17	34	14	13
	4-n-NP	111	101	91	5	4	11	12	10	4
	4-OP	119	90	93	16	9	12	11	6	2
	4-t-OP	95	84	81	8	10	12	1.3	3	3
	BPA	112	108	86	3	2	10	6	12	12
Prawn	DMP	85	87	60	24	9	14	11	5	8
	DEP	107	102	63	5	7	11	5	5	2
	DBP	116	99	73	18	13	15	10	6	4
	DEHP	110	78	117	17	7	3	43	5	8
	DNOP	84	100	79	17	6	15	17	1	48
	MMP	24	29	33	6	2	12	25	8	28
	MEP	57	53	64	4	7	19	16	2	0.3
	MBP	66	75	77	7	13	12	26	15	12
	MEHP	100	92	94	6	5	6	18	11	20
	MNOP	103	120	112	5	11	4	16	2	5
	4-NP	78	97	96	6	11	11	3	26	4
	4-n-NP	70	80	81	10	8	4	5	3	4
	4-OP	70	92	88	14	9	7	2	6	4
	4-t-OP	70	88	87	14	10	13	0.6	4	2
	BPA	67	71	76	15	5	9	10	9	2

After further improvement, MeOH/0.3% ammonia hydroxide was selected as the mobile phase of APs. Only acetonitrile/acetic acid and acetonitrile/water were compared because the pH range of XDB C_8 column is below 7. Results indicated that acetonitrile/acetic acid could separate MPEs well. Finally, acetonitrile/0.1% acetic acid in water (v/v) was selected as the mobile phase of MPEs and BPA (details see Fig. S2 in Supplementary Material). The use of gradient elution was necessary to achieve optimum separation for all compounds, and the retention times and ions of all analyses (Table 1) could be determined from the chromatogram obtained.

3.3. Method validation

Eight points of standard calibration curve was obtained with the concentrations ranged from 0.5 to 400 μg/L for PAEs. The linearity was fine with R^2 more than 0.99 for PAEs. Matrix-matched calibration was prepared from 0.5 to 200 ng/g or 400 ng/g for MPEs, APs and BPA. The factors like regression equation, R^2, linear range and test value (TV) for the *F* test of Mandel's fitting test were shown in Table 2. TV was calculated with residual standard deviations of both linear and 2nd calibration function. Eight concentration levels were set and each was analyzed in duplicate ($n = 6$). If $TV \leq F$, it could indicate that this calibration has sufficient linearity, and vice versa [23]. As is shown in Table 2, the calibrations have sufficient linearity with all TVs < *F* (34.12) and values of $R^2 > 0.99$.

As is shown in Table 3, MDLs ranged from 0.015 to 2.2 ng/g ww in general. MDLs of PAEs were between 0.034 and 0.64 ng/g ww except that of DNOP with the value of 2.2 ng/g ww. For other compounds, MDLs were no more than 1.0 ng/g ww. These MDLs were compared with those reported in the literatures (Table 3). Ferrara et al. [34] reported MDLs of 12.4 ng/g ww for 4-NPand 0.5 ng/g ww for 4-OP which were much more higher than those in our study with the values of 0.16 ng/g ww and 0.015 ng/g ww, respectively. Lin et al. [28] got MDLs of five PAEs (DMP, DEP, DBP, DEHP and DNOP) at the range of 1.8–4.2 ng/g ww whereas the values ranged from 0.034 to 2.2 ng/g ww were got in our study. Blair et al. [5] reported MDLs of MMP, MEP, MBP, MEHP and MNOP with the range of 0.05–0.99 ng/g ww which were similar to those in our study with the values of 0.019–1.0 ng/g ww. Comparisons above show that method in our study has higher sensitivity.

Table 5
Evaluation of the matrix effect.

Compound	Slope[a]	RSD (%)
MMP	0.992 ± 0.021	1.2%
MEP	1.007 ± 0.055	4.7%
MBP	1.065 ± 0.043	2.1%
MEHP	0.987 ± 0.008	0.1%
MNOP	0.916 ± 0.053	4.4%
4-t-OP	0.571 ± 0.198	88.5%
4-OP	0.828 ± 0.089	55.4%
4-NP	0.711 ± 0.020	21.3%
4-n-NP	0.586 ± 0.337	83.6%
BPA	1.160 ± 0.004	0.9%

[a] Within 95% confidence intervals.

The results of this research confirm that the developed method has satisfactory precision and accuracy for the determination of PAEs, MPEs, APs and BPA in seafood samples. As we can see in Table 4, the mean recoveries of all analyses were between 60% and 120%, except MMP in mollusk matrix, with the recoveries between 24% and 33%, which were in consistent with previous report [5]. The low recoveries of MMP may be due to its strong hydrophilicity ($\log K_{ow}$ 1.37 [5]) which would result in the low recoveries of acetonitrile extraction. The mean recoveries ranged from 24% to 127% for MPEs, while they were from 60% to 125% for PAEs, except DMP in fish matrix at 25 ng/g which had mean recovery of 57%, and from 60% to 119% for APs, and, finally, the mean recoveries for BPA ranged from 67% to 112%. With regard to intra-day precision (Table 4), good results were obtained, with RSDs of less than 20% except for DBP in fish, DEP in mollusk matrix and DMP in prawn matrix at 5 ng/g, which exhibited RSDs of 24%, 22% and 24%, respectively. The intra-day precisions were less than 15% for many of the investigated compounds. The inter-day precisions (Table 4) were less than 25% for most compounds except 4-NP, 4-OP and MMP in fish, 4-NP, DNOP and MMP at 5 ng/g in mollusk, which displayed RSDs ranged from 32% to 35% and from 31% to 35% respectively, and DEHP in prawn at 5 ng/g with RSD of 43%. Trace concentrations of DEHP were often detected in reagent blanks, with average level of 8.18 ± 0.25 ng/g. The highest values of RSD for DEHP may be due to the high background values of blank samples which would finally decrease stability of the method.

PAEs, MPEs, APs and BPA in analysis by GC–MS/MS and LC–MS/MS were calibrated by native standards firstly. It indicated that the recoveries of PAEs ranged from 60% to 120%, whereas those of MPEs, APs and BPA analyzed by LC–MS/MS were low (<20% for most compounds). Matrix effect is common phenomenon in analysis of LC–MS. It could be studied by plotting analytes to internal standard peak areas in two different matrix and the values of slope and intercept were taken to evaluate the matrix effect [35]. Actually, it is difficult to find a suitable internal standard and isotope standards were also not selected due to the huge price in this study. Based on the previous method [35], matrix effects here were assessed by plotting the analyte peak areas of two matrix (seafood and acetonitrile) at four concentration levels and RSDs of analytes peak areas in three samples (fish, mollusk and prawn). The slope (within confidence interval at 95%) and RSDs are listed in Table 5. If the value of 1 for slope and 0% for RSD mean no matrix effect. It indicated that matrix effects for MPEs and BPA were low with the values of slope ranging from 0.916 to 1.065 and RSD below 4.7%, respectively. This may due to the separate elution procedures on PSA cartridge. However, the suppressed matrix effects of APs are strong with the low values of slope ranging from 0.571 to 0.828 and RSDs more than 21.3%. Although usage of internal standard is the typical and common method to solve matrix effects [36], it is difficult to find the appropriate compounds and 15 isotopes are also expensive in our study. Matrix-matched calibration method might help to compensate for matrix effects in our study. Dong et al. [31] successfully used matrix-matched standards to eliminate matrix effects with good recoveries of 70.0–108% and RSDs less than 20.9% for all analytes. After the further linearity verification by the Mandel's fitting test, matrix-matched standards were used for the quantification for APs, and in order to get accurate data, MPEs and BPA were also quantified by matrix-matched calibration.

3.4. Application to real samples

In order to test the applicability of this method, several fresh seafood samples (six fish, six prawn and six mollusk samples), which were from the Yangtze River Delta sea area (China), were pretreated and analyzed by the procedures described in Sections 2.2 and 2.3. Results showed that PAEs, MPEs, APs and BPA were all detected in seafood samples. Specially, PAEs were found with the highest concentrations compared to other compounds, with the concentrations in the range of 5.0–46.3 ng/g ww in fish, 3.3–219.3 ng/g ww in mollusk and 5.0–57.3 ww ng/g in prawn. The MPEs also had high levels in these samples, with the concentrations ranging from 1.2 to 20.4 ng/g ww in fish, 0.8–51.4 ng/g ww in mollusk and 0.5–26.7 ng/g ww in prawn. The levels of APs were from 0.8 to 5.7 ng/g ww in fish, 0.5–62.0 ng/g ww in mollusk and 0.7–18.2 ng/g ww in prawn. For BPA, concentrations ranged from below MDL to 0.8 ng/g ww in fish, 1.2–8.6 ng/g ww in mollusk and 1.2–2.7 ng/g ww in prawn.

4. Conclusions

In the present study, a sensitive and convenient method was established for the determination of PAEs, MPEs, APs and BPA in seafood including fish, mollusk and prawn. The developed simultaneous sample pretreatment method includes acetonitrile based ultrasonic extraction and SPE purification with PSA. To our knowledge, it is the first report about simultaneous pretreatment of PAEs, MPEs, APs and BPA in biota samples. This pretreatment method reduced the blank contamination of PAEs, with background levels between ND and 8.18 ng/g, and obtained low matrix effects for MPEs and BPA. Performance of this method was satisfactory, with mean recoveries between 60% and 127% and intra-day precisions less than 15% as well as inter-day precisions less than 25% for most compounds. The MDLs ranged from 0.016 to 2.2 ng/g ww, most of which were lower than those reported in previous studies. Analysis of the real biota samples from the Yangtze River Delta sea area (China) demonstrated that the developed method was highly sensitive and all the target compounds were detected. This method meets the requirement of multi-residue analysis for APs, BPA, PAEs and MAEs in seafood samples.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2014.06.024.

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