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Determination of 24 personal care products in fish bile using hybrid solvent precipitation and dispersive solid phase extraction cleanup with ultrahigh performance liquid chromatography-tandem mass spectrometry and gas chromatography-mass spectrometry

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ABSTRACT

Personal care products (PCPs) are ubiquitous in aquatic environments owing to the continuous discharge of domestic wastewater from highly urbanized regions. These PCPs can be adsorbed by fish and thereafter usually enter the bile of the fish through biliary excretion. In this study, a sensitive method based on a combination of hybrid solvent precipitation and dispersive solid phase extraction (d-SPE) purification was developed to simultaneously extract and detect 24 PCPs, namely, 16 biocides, 4 synthetic musks, and 4 benzotriazoles, from fish bile. Hybrid precipitation on solid phase extraction (SPE) tubes was applied to remove phospholipids and proteins, and a d-SPE procedure was used for further purification. The extraction solvents for the hybrid precipitation/SPE tubes and d-SPE materials were optimized. The method performance for bile samples both with and without enzyme hydrolysis using β-glucuronidase/arylsulfatase were validated. The 24 PCPs in fish bile were spiked with standard concentrations of 10 ng/mL, 20 ng/mL, 100 ng/mL, and 200 ng/mL to evaluate recoveries, which ranged from 70 to 120% for 16, 16, 22, and 21 analytes with hydrolysis, respectively, and 70–120% for 14, 15, 23, and 23 analytes without hydrolysis, respectively. The quantification limits for target PCPs were in the range 0.26–7.38 ng/mL [excluding musk xylene (MX) and musk ketone (MK)] and 0.20–9.48 ng/mL (excluding MX and MK) for bile samples with and without enzyme hydrolysis, respectively. After enzyme hydrolysis, 12 PCPs were detected in bile from fish collected from the Yangtze River, with a maximum detected concentration of 460 ng/mL, for triclosan (TCS). The hydrolysis reaction indicated that high percentages of glucuronide and sulfate metabolites for some PCPs, i.e. four parabens and TCS, existed in the bile.

1. Introduction

Currently, personal care products (PCPs) are indispensable to the daily life of most people. The active ingredients in PCPs, such as biocides, synthetic musks, and benzotriazoles, have elicited concerns from both scientific researchers and the public because of their potential adverse effects on animals and humans [1–3]. After use, PCP compounds are released directly to aquatic environments or discharged to municipal wastewater treatment plants (WWTPs) [4]. Owing to incomplete removal in WWTPs [1,4], PCPs have been frequently detected in effluents [5,6], surface water [2,7], and sediments [7]. Consequently, aquatic organisms inhabiting these environments may bioaccumulate PCPs. In previous studies, ecotoxicity seldom has been evaluated via the internal exposure of pollutants in aquatic organisms, compared to external environmental exposure [8]. However, internal exposure in the biological compartments of aquatic organisms is vital for assessing toxicity mechanisms for certain contaminants [9,10].

In fish, biliary excretion after liver metabolism is an important removal mechanism for many environmental contaminants

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[11,12]. Therefore, fish bile is often used as an indicator of internal exposure and uptake of xenobiotic substances in biota [13], especially for hydrophilic contaminants, which are readily excreted via the bile compartment [11]. In addition, hydrophobic compounds also can be biotransformed to more water-soluble forms, by either hydroxylation or conjugation in the liver, and then excreted via bile [11,13]. Glucuronidation and sulfation are the main conjugation processes and result in contaminants existing in bile in conjugated form [14]. Enzyme-assisted hydrolysis can release conjugated contaminants and facilitate the determination of most parent compounds in fish bile [15,16]. Several studies have reported the concentrations of PCPs in fish muscle and liver tissues in their free forms [17–19], whereas the concentrations of PCPs in bile are seldom reported, either in free or conjugated form. Because PCPs in fish bile may be present in either free or conjugated form, comparisons of concentrations of these PCP forms in bile is meaningful for characterizing the uptake and metabolism mechanisms for PCPs in biota [11,15].

Bile comprises complex biological matrices, in which biological compounds, including phospholipids, proteins, cholates, pigments (bilirubin and biliverdin), and cholesterol, are present [20,21]. The existence of phospholipids and some other substances in biofluids have been reported to suppress the response of analytes in the electrospray ionization (ESI) mode of liquid chromatography-mass spectrometry [22,23]. Hence, an effective sample pretreatment method is necessary to remove matrix interferences and achieve sensitive and rigorous detection of PCPs in bile. Conventional solid phase extraction (SPE) has been a widely used approach for purifying matrices and concentrating analytes in fish bile and is performed by loading aqueous samples onto solid sorbents in an SPE cartridge and then eluting the analytes with appropriate organic solvents. For example, estrogenic compounds and some pharmaceuticals were extracted from bile via the SPE method using Oasis HLB cartridges [24,25] and Strata X-AW cartridges [26]. Some PCPs, such as triclosan (TCS), tonalide (AHTN), and galaxolide (HHCB), were also extracted from fish bile with HLB or Plexa cartridges [27,28].

Analysts continually strive for faster analyses, shorter run times, lower limits of detection, and fewer matrix effects by adequately removing interference from samples. To extract pollutants from fish bile, the above SPE approaches usually require long pretreatment times and display low removal efficiencies because of biological matrix interferences [29,30]. Usually, matrix components in biological samples are quite different from those in water samples. Hence, some special biological matrix removal SPE cartridges have been used to purify biofluids, which can selectively retain biological matrix interferences, such as phospholipids, proteins, and pigments, but allow analytes to pass [31]. These analytes can be collected directly without extra elution processes. This simple and efficient method of hybrid precipitation/SPE plates has been used to remove phospholipids and proteins from plasma samples [32,33]. The method has also been applied to cleaning other types of biological fluid samples for pharmaceuticals, through combination with extra purification processes such as dispersive solid phase extraction (d-SPE) [34].

The objective of this study was to develop a pretreatment, i.e., extraction and purification method by combining hybrid precipitation/SPE tubes and d-SPE kits, for extraction and analysis of 24 PCPs in fish bile samples. The target PCP compounds comprised 16 biocides, 4 synthetic musks, and 4 benzotriazoles. Various parameters, i.e., extraction solvent, elution ratio, and d-SPE materials, were tested to obtain optimal matrix removal efficiency for bile samples. Then, PCP concentrations were analyzed in the extracts using ultrahigh performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) and gas chromatography-mass spectrometry (GC–MS). In addition, to compare the conjugated and free PCP forms, the bile was treated with or without enzyme hydrolysis using β-glucuronidase/aryl-sulfatase. The method was validated for precision, repeatability, recoveries, method qualitative limits, method quantification limits, and matrix effects. The developed method was applied to investigate the concentrations of these 24 PCPs in bile from fish collected from the Yangtze River, China.

2. Experimental

2.1. Standards, reagents, and materials

The basic and supplier information for all 24 analytical standards (purity ≥ 95%) is listed in Table S1 (Supplementary materials). The abbreviations of the target compounds are displayed in Table 1. The selection of internal standards is based on the use of corresponding isotope-labeled analogs or labeled standards with similar retention time. The 12 internal standards (purity ≥ 95%) were supplied by several vendors. Imazalil-D_5, thiabendazole-D_6, fluconazole-D_4, AHTN-D_3, and musk xylene-D_{15} were supplied by Dr. Ehrenstorfer GmbH (Augsburg, Germany). Ketoconazole-D_8, miconazole-D_5, and methylparaben-D_4 were obtained from Campro Scientific (Berlin, Germany). Clotrimazole-D_5 was purchased from Toronto Research Chemicals (North York, Canada). Propylparaben-D_4 was obtained from CDN Isotopes (Pointe-Claire, Canada). $^{13}C_{12}$-TCS and triclocarban-D_7 (TCC-D_7) were supplied by Cambridge Isotope Laboratories (Andover, USA). The positive control standard of triclosan O-β-D-glucuronide sodium salt was purchased from Toronto Research Chemicals (North York, Canada), and the hydrolysis enzyme β-glucuronidase/aryl-sulfatase, isolated from *Helix pomatia*, was supplied by CNW Technologies (Dusseldorf, Germany). The tricaine methanesulfonate was obtained from CNW Technologies (Dusseldorf, Germany).

HPLC-grade solvents of acetonitrile and methanol were supplied by Merck (Darmstadt, Germany) and the dichloromethane and dimethyl sulfoxide were purchased from CNW Technologies (Dusseldorf, Germany). Reagents of formic acid, ammonium acetate, acetic acid (AA), and sodium acetate (CH_3COONa) were purchased from CNW Technologies (Dusseldorf, Germany). All materials for phospholipid and other matrices removal were commercially available. The Captiva ND^{Lipid} Protein and Phospholipid Removal tubes and d-SPE materials, kit b: anhydrous magnesium sulfate/PSA/GCB and kit c: anhydrous magnesium sulfate/PSA/C18, were obtained from Agilent Technology (Palo Alto, USA). The d-SPE materials, kit a: Z-Sep/C18, was supplied by Sigma Aldrich (Santa Clara, USA). Ultrapure water was prepared with a Milli-Q water purification system (Millipore, United Kingdom).

Stock solutions (0.1 g/L or 1 g/L) for individual standards and internal standards were prepared in methanol. Individual or mixed working solutions were prepared via proper dilution of each stock solution with methanol to various final concentrations. Stock and working solutions for all standards and internal standards were stored at −20 °C. A 0.2 M acetate buffer (pH 5) was prepared with a mixture of 0.2 M AA and 0.2 M CH_3COONa ($V_{AA}/V_{CH3COONa}$ = 3/7), prepared in ultrapure water. The hydrolysis enzyme β-glucuronidase/aryl-sulfatase (100,000 units/mL) was stored in its original solution form at 4 °C, and enzyme work solution (10,000 units/mL) was diluted with 0.2 M acetate buffer (pH 5) before use.

2.2. Study area and sample collection

Bile samples were obtained from fish in the Yangtze River, China, which is the largest river in China in terms of mean annual water discharge, 900 km^3/y [35]. Sampling campaigns were carried out in July, the wet season, and in November, the dry season, of 2013.

Table 1
Mass transitions and retention times for the target compounds (biocides and benzotriazoles) in UPLC–MS/MS.

Compounds	Abbr[a]	R.T. (min)[b]	Precursor ions (*m/z*)	Product ions (*m/z*)[c]	Frag. (eV)[d]	CE (eV)[e]	IS[f]
Biocides (LC–MS/MS, ESI+)							
DEET	DEET	6.489	192.1	91.1	45	36	IMZ-D_5
				119.1		16	
Icaridin	ICD	7.049	230.2	67.1	45	40	IMZ-D_5
				130.1		12	
Carbendazim	CBD	2. 620	192.1	132.1	115	36	TBD-D_6
				160.1		16	
Thiabendazole	TBD	3.312	202.0	131.1	150	36	TBD-D_6
				175.0		28	
Fluconazole	FCZ	2.516	307.1	70.1	45	44	FCZ-D_4
				220.1[a]		16	
Ketoconazole	KCZ	6.748	531.2	81.1	220	88	KCZ-D_8
				135.1		44	
Climbazole	CBZ	7.560	293.1	41.1	115	44	IMZ-D_5
				69.1		20	
Clotrimazole	CTZ	8.964	345.1	165.1	45	40	CTZ-D_5
				277.1		4	
Miconazole	MCZ	9.743	415.0	123.0	170	80	MCZ-D_5
				159.0		36	
Itraconazole	ICZ	10.045	705.2	159.0	225	96	KCZ-D_8
				392.2		40	
Imazalil-D_5 (IS)[f]	IMZ-D_5	5.922	302.1	42.1	145	100	–
				46.1		36	
Thiabendazole-D_6 (IS)	TBD-D_6	3.222	208.1	136.1	160	40	–
				180.1		28	
Fluconazole-D_4 (IS)	FCZ-D_4	2.515	311.1	172.1	120	24	–
				223.1		16	
Ketoconazole-D_8 (IS)	KCZ-D_8	6.698	539.2	82.1	45	56	–
				119.1		52	
Clotrimazole-D_5 (IS)	CTZ-D_5	8.902	350.2	169.7	45	32	–
				282.1		4	
Miconazole-D_5 (IS)	MCZ-D_5	9.681	420	127.0	175	60	–
				164.0		28	
Biocides (LC-MS/MS, ESI-)							
Methylparaben	MP	3.080	151.0	92.1	110	16	MP-D_4
				136.0		8	
Ethylparaben	EP	4.582	165.1	92.1	105	16	MP-D_4
				137.1		8	
Propylparaben	PP	6.239	179.1	92.1	115	20	PP-D_4
				136.0		8	
Butylparaben	BP	6.762	193.1	92.1	120	20	PP-D_4
				136.0		8	
Triclocarban	TCC	7.657	313	126.1	86	13	TCC-D_7
				160.0		5	
Triclosan	TCS	7.767	286.9	35.1	65	1	$^{13}C_{12}$-TCS
Methylparaben-D_4 (IS)	MP-D_4	3.033	155.1	96.1	100	16	–
				140.1		8	
Propylparaben-D_4 (IS)	PP-D_4	6.228	183.1	96.1	125	20	–
				140.1		12	
Triclocarban-D_7 (IS)	TCC-D_7	7.629	320.0	130.1	107	13	–
				163.0		5	
$^{13}C_{12}$-Triclosan (IS)	$^{13}C_{12}$-TCS	7.767	299.0	35.1	65	1	–
Benzotriazoles (LC-MS/MS, ESI+)							
Benzotriazole	BT	2.425	120.1	65	135	44	TBD-D_6
				39		20	
5-methyl-1H-benzotriazole	5-TT	3.419	134.1	77	120	28	TBD-D_6
				51		48	
5-chloro-1H-benzotriazole	CBT	4.164	154	98.9	140	24	TBD-D_6
				72.9		40	
5,6-dimethyl-1H-benzotriazole	XT	4.502	148.1	77	130	28	TBD-D_6
				51		56	
Thiabendazole-D_6 (IS)	TBD-D_6	3.222	208.1	136.1	160	40	–
				180.1		28	

[a] Abbreviation.
[b] R.T., retention time.
[c] Numbers with underline are the quantification ions.
[d] Frag., fragmentor voltage.
[e] CE., Collision energy.
[f] IS., Internal standard.

As shown in Fig. S1 (Supplementary materials), four sampling sites located in the highly urbanized area of the Yangtze River basin were selected: C1 and C2 located in the middle reach and C3 and C4 in the lower reach. Because many big cities, including Yichang, Wuhan, Nanjing, and Zhenjiang, are located along the Yangtze, large quantities of treated and untreated domestic sewage are discharged into

its tributaries or mainstream. Detailed geographic and population information for the selected sites is listed in Table S2.

Using fishing nets, 35 fish samples were collected during two sampling campaigns; the biological characteristics of the fish samples are listed in Table S3. After collection, all fish were transported to the lab within one hour, in an aeration tank with source water from each site. Upon arrival, the live fish were anesthetized with tricaine methanesulfonate and sacrificed via rapid dissection. Bile was removed from the fish gall bladders using a syringe and then stored in cryogenic tubes at −80 °C until extraction. Fish collection and ethical care complied with the recommendations of Animal Care Quality Assurance in China.

2.3. Sample extraction

In this study, the extraction method for the 24 PCPs in fish bile was developed with hybrid precipitation/SPE tubes and d-SPE kits, and the processing parameters, including solvent type, solvent amount, and d-SPE materials, were optimized. The optimized pretreatment procedures were as follows. Before extraction, 100 μL of each bile sample was equally separated into two 50 μL duplicates, to investigate the concentrations of each PCP in conjugated (glucuronide or sulfate metabolites) and free (unconjugated) forms [13]. For samples undergoing enzyme hydrolysis, 50 μL of bile was aliquoted into sample vials and spiked with 20 ng of each internal standard dissolved in 40 μL of 0.2 M acetate buffer (pH 5) solution and 10 μL of enzyme work solution. Then, sample vials were incubated at 37 °C for 4 h. For samples not undergoing enzyme hydrolysis, 50 μL of bile was aliquoted into sample vials and spiked with 20 ng of each internal standard dissolved in 40 μL of 0.2 M acetate buffer (pH 5) and 10 μL of acetate buffer solution. Then, the two bile sample aliquots were transferred separately into hybrid precipitation tubes (Captiva ND^{Lipid} Protein and Phospholipid Removal tubes), previously loaded with 500 μL acetonitrile (containing 1% AA, *v*/*v*). The tubes were subjected to a vortex for 30 s, and then protein and phospholipid interferences were isolated from the acetonitrile solvent with a 25–50 kPa vacuum. The acetonitrile eluents were collected into polyethylene d-SPE tubes containing anhydrous magnesium sulfate/PSA/C18. Subsequently, 200 μL acetonitrile (containing 1% AA, *v*/*v*) was loaded into the Captiva tubes to flash the wall bile residue, and the eluates were collected together. An additional 5.2 mL of acetonitrile, containing 1% AA, was added to the d-SPE tubes, which then were shaken by hand for 1 min. After centrifugation at 2364 *g* for 10 min, 3 mL of supernatant was transferred to a glass tube and gently evaporated to nearly dry under a nitrogen stream. Extracts were redissolved in 200 μL methanol and stored at −20 °C. The final extracting solution was separated into two equal parts with an exchange methanol/water solvent (50:50, *v*/*v*) and dichloromethane, for UPLC-MS/MS and GC–MS analysis, respectively. Fig. 1 illustrates the flow chart for the extraction procedures.

2.4. Instrumental analysis

The quantitative analysis of 16 biocides and 4 benzotriazoles in the fish bile samples was performed via UPLC-MS/MS [Agilent 1200 series ultrahigh performance liquid chromatograph coupled to an Agilent 6460 triple-quadrupole mass spectrometer equipped with an ESI source (Agilent, Palo Alto, USA)] in both positive and negative multiple reaction monitoring (MRM) modes. The separation was completed with an Agilent SB-C18 column (100 mm × 3.0 mm, 1.8 μm particle size). The column temperature was maintained at 40 °C, and the injection volume was 5 μL for each sample. For the positive mode, the mobile phases were 0.05% (*v*/*v*) formic acid – 5 mmol/L ammonium acetate in ultrapure water (A) and methanol (B), with the following gradients: 50% B at 0 min, stepped to 80% B in 5 min, increased to 90% B in 1.5 min, and held for 5 min. The flow rate was maintained at 0.3 mL/min, and a post run rest of 5 min was implemented before the next injection for column equilibration. For the negative mode, the mobile phases were ultrapure water (A) and methanol (B), with the following gradient: 50% B at 0 min, increased to 56% B in 3 min, stepped to 90% B in 1 min, and held for 4.5 min. The flow rate was 0.35 mL/min, and a post-run rest of 5 min was implemented before the next injection for column equilibration. The LC parameters and ESI source conditions are summarized in Table S4 (Supplementary materials).

The quantitative analysis of four synthetic musks in the fish bile samples was conducted via GC–MS [Agilent 6890N series gas chromatograph coupled to an Agilent 5975B series mass spectrometer equipped with an electron impact (EI) source (Agilent, Palo Alto, USA)] in selective ion monitoring (SIM) mode. The separation was accomplished using a DB-5MS UI column (30 m × 0.25 mm, 0.25 μm film thickness), with helium as the carrier gas, at 1.0 mL/min. The GC oven temperature was programmed with the following gradients: 80 °C at 0 min, increased to 170 °C at a rate of 15 °C/min, programmed to 185 °C at a rate of 1 °C/min, stepped to 300 °C at a rate of 20 °C/min, and held at 300 °C for 5 min. The temperatures of the injection inlet, transfer line, ionization source, and mass analyzer were set at 280 °C, 300 °C, 230 °C, and 150 °C, respectively. The injection volume was 2.0 μL in splitless mode. The mass spectrometry parameters for UPLC-MS/MS (fragmentor voltage, collision energy, precursor ions, and product ions) are shown in Table 1, and the characteristic ions for each compound used in GC–MS are listed in Table 2.

2.5. Quantification and method validation

The quantitative analysis of 24 PCPs was based on isotope dilution quantitation, by comparing the peak area ratio of product ions (within ± 20%) and retention times (within ± 0.5 min) with calibration standards. For UPLC-MS/MS calibration, serial concentrations (0.1, 0.25, 0.5, 1, 2.5, 5, 10, 25, 50, 100, and 200 ng/mL) of 20 standard analyte mixtures and 10 internal standard mixtures with a uniform concentration of 50 ng/mL were prepared in methanol/water (50:50, *v*/*v*). For GC–MS calibration, four standard analyte mixtures at concentrations of 1, 5, 10, 20, 50, 100, and 200 ng/mL and two internal standards mixtures with a uniform concentration of 50 ng/mL were prepared in dichloromethane. All sample analysis processes complied with strict quality control procedures. For analyzing each batch of samples, a solvent blank, procedure blank, and independent check standard (100 ng/mL standard solution) were injected in sequence every twenty injections to assess carryover, background contamination, and system performance. The 24 target PCPs were not detected in the solvent blank and procedure blank, and the concentrations of the independent check standards were within 20% of expected values.

A positive control for enzyme hydrolysis in the bile samples was made for each extraction process to examine hydrolysis efficiency by spiking 200 ng triclosan *O*-*β*-D-glucuronide, i.e., 20 μL of the 10 μg/mL working solution, into a blank bile sample. After undergoing the same incubation as the bile samples from the wild fish, an equivalent TCS concentration was determined in the extract. The hydrolysis efficiencies from triclosan *O*-*β*-D-glucuronide to free TCS by *β*-glucuronidase/aryl-sulfatase were 92–94% (n = 6). The standards of 24 analytes were also mixed with blank bile and stand overnight, so that the conjugated analytes may be formed under the catalysis with bioactive enzymes in bile. Then, samples were hydrolyzed and extracted with the optimized procedures. The recoveries for 24 analytes were at the range of 78–138%, indicating that conjugation forms of analytes in bile can be effectively hydrolyzed by *β*-glucuronidase/aryl-sulfatase.

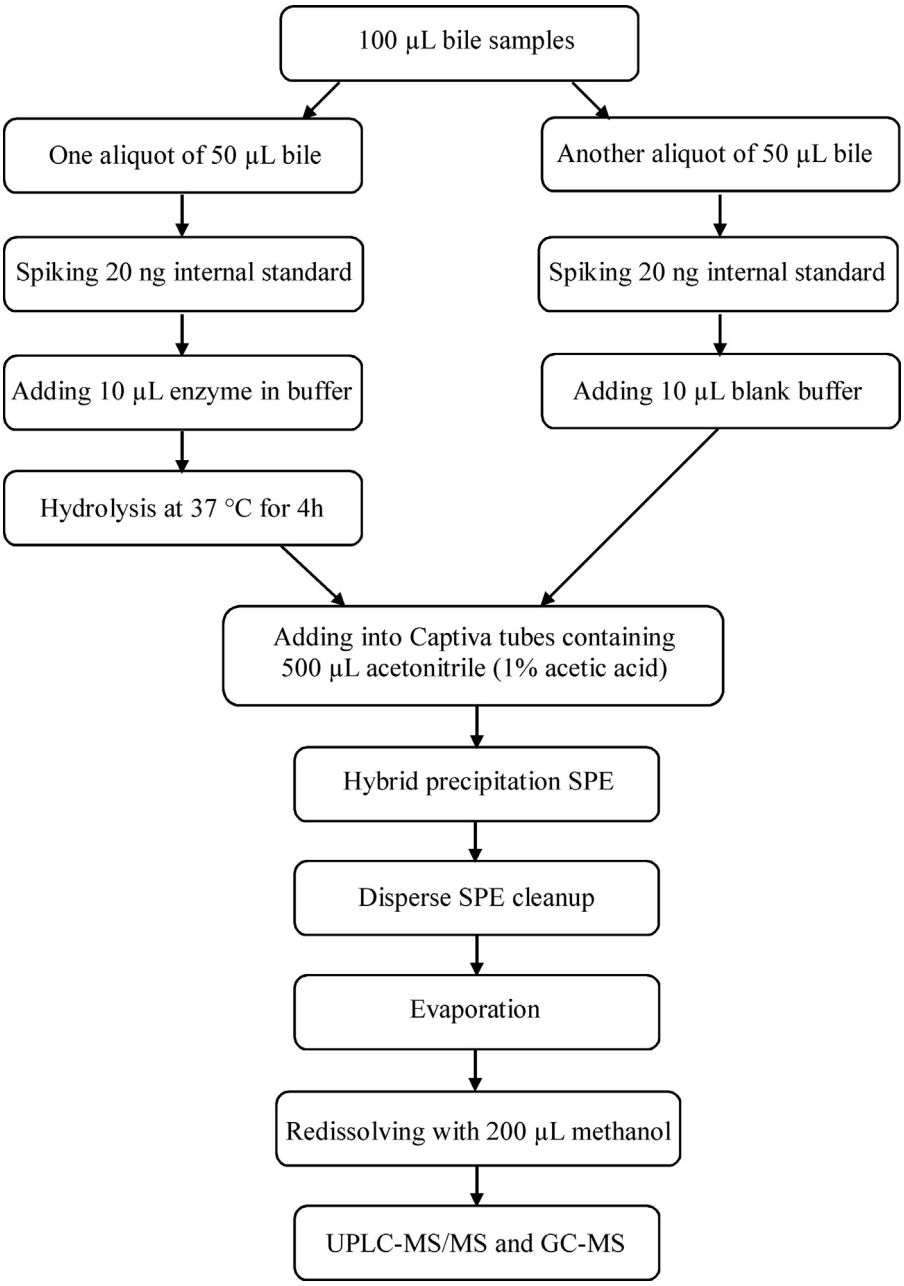

Fig. 1. Flow chart shows the extraction and purification procedures of PCPs in fish bile.

Table 2
Mass transitions and retention times for the target compounds (synthetic musks) in GC–MS.

Compounds	Abbr[a]	R.T. (min)[b]	Characteristic ions (*m/z*)			IS[c]
Tonalide	AHTN	13.749	243.2	258.2	187.1	AHTN-D_3
Galaxolide	HHCB	13.502	243.2	258.2	213.2	AHTN-D_3
Musk xylene	MX	13.633	282	297		MX-D_{15}
Musk ketone	MK	17.555	279.1	294.1	280.1	MX-D_{15}
Tonalide-D_3 (IS)	AHTN-D_3	13.677	246.2	261.2	190.1	
Musk xylene-D_{15} (IS)	MX-D_{15}	13.241	294.2	293.2		

[a] Abbreviation.
[b] R.T., retention time.
[c] Internal standard.

Recovery experiments were accomplished using four different concentrations of standards (10, 20, 100, and 200 ng/mL) and a uniform concentration of internal standards (200 ng/mL), which were spiked into bile taken from carp fish from an aquatic products market, with each spiking concentration measured in triplicate. Matrix effects were evaluated by comparing the slopes of standard solutions prepared in solvent with those in bile extracts that had undergone the optimized extraction processes. Detailed

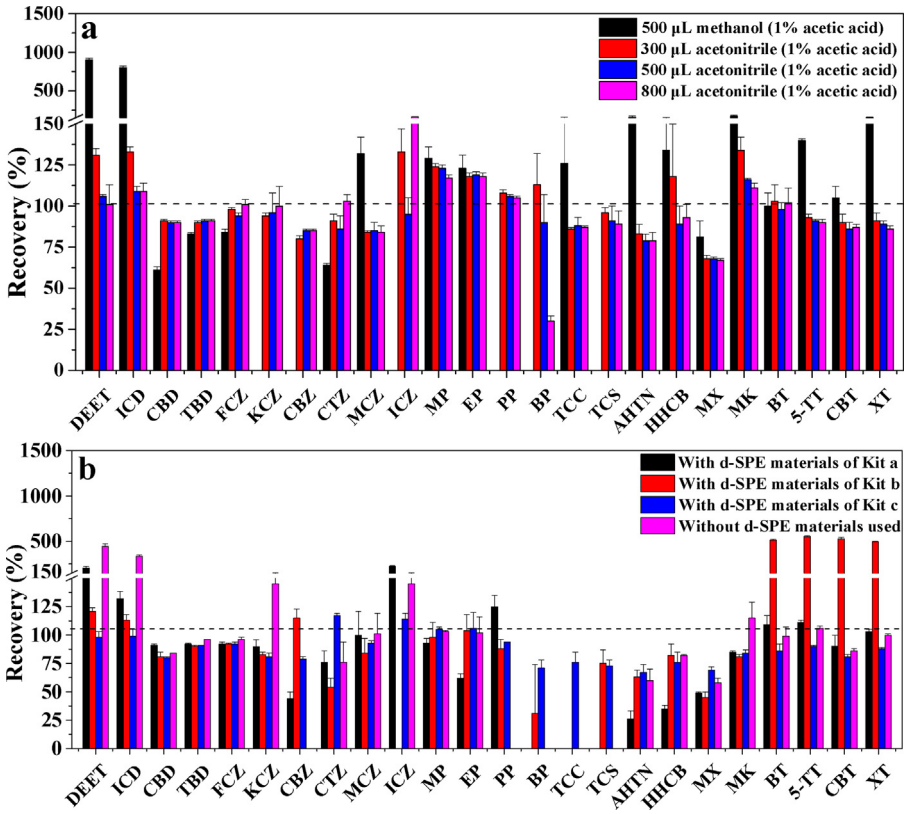

Fig. 2. The recoveries [mean (%) ± standard deviation (%), n = 3] of target compounds for bile samples with different extraction solvent (Fig. 2a), and the recoveries with different d-SPE kits [kit a (300 mg C18 + 120 mg Z-Sep), kit b (855 mg MgSO4 + 150 mg PSA + 45 mg GCB) and kit c (900 mg $MgSO_4$ + 150 mg PSA + 150 mg C18)] tested (Fig. 2b).

descriptions and calculations of recovery and matrix effects are given in Text S1 and S2. The method detection limits (MDLs) and method quantification limits (MQLs) were defined as the minimum detectable amount of an analyte from the bile matrix, using the bile samples with minimum spiking concentrations. The MDLs and MQLs were calculated using 3 and 10 times the signal-to-noise (*S/N*) ratios (peak height based) near the target peaks, respectively. For data collection and handling (such as *S/N* and concentration calculation), the software MassHunter (Agilent, Palo Alto, USA) for UPLC-MS/MS and MSD ChemStation (Agilent, Palo Alto, USA) for GC–MS were used.

3. Results and discussion

3.1. Optimization of the extraction processes for fish bile samples

The Captiva ND^{Lipid} Protein and Phospholipid Removal tube (Agilent) has been reported to effectively retain precipitated proteins and phospholipids in plasma or blood [36]. Fish bile samples contain similar biological matrices as blood samples do, such as phospholipids and proteins. Hence, SPE tubes can be utilized to remove these matrices. Meanwhile, matrices, such as bile pigments (bilirubin and biliverdin) and bile acids, can be purified further using d-SPE materials that have been effective for purifying pharmaceuticals in bile samples [34]. Two key factors, namely precipitation solvents (solvent type and amount) and d-SPE materials, were optimized using the common carp bile samples. Bile samples were hydrolyzed using β-glucuronidase/aryl-sulfatase to release potential conjugated analytes before loading them into the removal tube, and non-enzyme hydrolysis was compared simultaneously. Using isotope-labeled internal standards can also compensate for matrix interferences to a certain extent [23,37].

3.1.1. Optimization of solvent types and volume

Selecting an extraction solvent is very important for improving the extraction efficiency of analytes and reducing the nonspecific extraction of matrix components [23]. Methanol and acetonitrile are two commonly used solvents for precipitating proteins in serum [31], and the precipitated proteins can be physically removed using a Captiva ND^{Lipid} Protein and Phospholipid Removal tube through a frit filter [32]. In this study, two types of solvents: methanol (containing 1% AA) and acetonitrile (containing 1% AA), were tested. The recoveries of the 24 PCPs in the bile samples with the different solvents are shown in Fig. 2a.

As shown in Fig. 2a, the recoveries of the 24 PCPs in bile samples were much better for acetonitrile (containing 1% AA) than for methanol (containing 1% AA). For acetonitrile, the recoveries of 24 PCPs ranged from 69% to 123%. However, for methanol, abnormal recoveries of many target analytes were observed because more interferences in bile were extracted with methanol than with acetonitrile. For example, the recoveries of DEET and ICD were much higher than 120%, which was probably due to the strong ion suppression of their internal standard IMZ-D_5, but not for those two target analytes on the ESI source, which resulted in the overestimation of the final analyte concentrations [17]. Besides, the recoveries of KCZ, CBZ, ICZ, PP, BP, and TCS were almost zero, owing to the strong ion suppression of those target analytes on the ESI source. The images in Fig. S2 show that the extracts using methanol as a solvent were deeper in color than those using acetonitrile, as more bile pigments were extracted by methanol than by acetonitrile. Previous work has also shown that methanol displayed significantly stronger extraction efficiency of phospholipids in the biofluid plasma than did acetonitrile [38], which caused serious ion suppression effects on the target PCPs in the ESI mass spectrometry source [23]. Thus, acetonitrile was selected as the extraction solvent.

Table 3
The recoveries (mean (%) ± standard deviation (%), n = 3), method detection limits (MDLs), and method quantitation limits (MQLs) of biocides, synthetic musks and benzotriazoles in fish bile with enzyme and without enzyme hydrolysis.

	With enzyme process						Without enzyme process						Linearity(R^2)
Compounds	**10 ng/mL**	**20 ng/mL**	**100 ng/mL**	**200 ng/mL**	**MDLs[b]**	**MQLs**	**10 ng/mL**	**20 ng/mL**	**100 ng/mL**	**200 ng/mL**	**MDLs**	**MQLs**	
Biocides													
DEET	**147 ± 13**[a]	**132 ± 4**	112 ± 4	112 ± 3	0.27	0.88	**142 ± 3**	**125 ± 4**	116 ± 1	115 ± 4	0.12	0.41	0.9996
ICD	110 ± 4	111 ± 4	107 ± 4	108 ± 4	0.16	0.55	115 ± 2	109 ± 3	113 ± 2	112 ± 3	0.25	0.76	0.9999
CBD	84 ± 1	73 ± 2	82 ± 2	**69 ± 1**	0.24	0.8	84 ± 3	**69 ± 2**	80 ± 1	72 ± 3	0.24	0.80	0.9996
TBD	93 ± 3	87 ± 3	86 ± 1	88 ± 1	0.08	0.26	92 ± 1	86 ± 1	86 ± 1	87 ± 4	0.13	0.42	0.9995
FCZ	103 ± 9	101 ± 2	86 ± 1	91 ± 2	0.51	1.70	92 ± 2	97 ± 2	85 ± 2	88 ± 2	0.80	2.67	0.9995
KCZ	74 ± 7	**64 ± 1**	78 ± 2	75 ± 2	1.46	4.88	**67 ± 6**	**65 ± 10**	77 ± 1	77 ± 3	1.23	4.12	0.9984
CBZ	76 ± 2	**69 ± 1**	78 ± 4	76 ± 4	0.24	0.81	74 ± 3	73 ± 7	81 ± 1	77 ± 5	0.24	0.80	0.9997
CTZ	78 ± 7	**67 ± 4**	78 ± 6	74 ± 7	0.87	2.92	77 ± 6	**68 ± 5**	83 ± 7	79 ± 6	0.83	2.75	0.9990
MCZ	**66 ± 4**	**61 ± 2**	88 ± 2	71 ± 1	0.60	2.01	**65 ± 5**	**61 ± 5**	83 ± 1	75 ± 4	0.70	2.34	0.9999
ICZ	**123 ± 10**	120 ± 10	**149 ± 12**	**126 ± 5**	1.67	5.56	109 ± 7	119 ± 6	**147 ± 9**	**121 ± 8**	0.76	2.54	0.9988
MP	107 ± 8	104 ± 1	92 ± 6	94 ± 1	0.30	0.99	90 ± 8	87 ± 5	92 ± 1	92 ± 2	0.26	0.85	0.9987
EP	**68 ± 7**	82 ± 6	96 ± 5	96 ± 7	0.16	0.52	**65 ± 8**	84 ± 6	96 ± 6	98 ± 1	0.10	0.32	0.9986
PP	78 ± 1	84 ± 4	88 ± 1	91 ± 1	0.10	0.34	71 ± 2	78 ± 2	87 ± 1	87 ± 5	0.06	0.20	0.9997
BP	**60 ± 7**	81 ± 4	88 ± 8	71 ± 9	0.15	0.49	**56 ± 6**	**61 ± 9**	118 ± 7	71 ± 8	0.22	0.73	0.9999
TCC	81 ± 5	**69 ± 6**	95 ± 3	86 ± 6	0.15	0.49	71 ± 10	**63 ± 8**	109 ± 2	78 ± 6	0.11	0.38	0.9988
TCS	73 ± 11	**68 ± 8**	88 ± 3	77 ± 1	0.87	2.90	**65 ± 7**	**61 ± 7**	85 ± 8	78 ± 4	0.96	3.18	0.9981
Synthetic musks													
AHTN	**63 ± 8**	**66 ± 8**	88 ± 5	75 ± 3	0.54	1.79	**64 ± 7**	**62 ± 8**	81 ± 5	78 ± 4	0.28	0.94	0.9930
HHCB	113 ± 11	93 ± 6	96 ± 4	**69 ± 2**	0.35	1.15	**132 ± 5**	88 ± 8	91 ± 4	71 ± 5	0.31	1.04	0.9940
MX	**na**[c]	87 ± 4	104 ± 1	78 ± 1	3.23	10.7[d]	**na**	95 ± 6	101 ± 1	98 ± 5	3.90	12.9	0.9960
MK	**na**	98 ± 8	**123 ± 10**	90 ± 3	3.12	10.4	**na**	90 ± 4	99 ± 2	113 ± 8	3.53	11.7	0.9950
Benzotriazoles													
BT	118 ± 4	101 ± 4	104 ± 3	102 ± 4	2.21	7.38	117 ± 6	106 ± 1	107 ± 3	105 ± 4	2.84	9.48	0.9995
5-TT	95 ± 4	98 ± 4	93 ± 2	96 ± 2	0.45	1.50	98 ± 2	95 ± 2	95 ± 1	96 ± 4	0.48	1.61	0.9999
CBT	95 ± 4	85 ± 7	83 ± 1	85 ± 1	0.81	2.70	99 ± 6	85 ± 3	84 ± 1	86 ± 6	0.45	1.49	0.9995
XT	92 ± 1	93 ± 4	85 ± 1	89 ± 1	1.36	4.55	90 ± 2	92 ± 2	85 ± 1	90 ± 3	0.62	2.07	0.9996

[a] Bold numbers in the table represent those recoveries outside the range of.70%–120%.
[b] The unit of MDLs and MQLs was ng/mL.
[c] Data not available.
[d] The MDLs and MQLs values of MX and MK were calculated based on the spiking concentration of 20 ng/mL.

Table 4
The comparison of method quantitation limits (MQLs) for some PCPs in this work and previously published works.

Compounds	Extraction/elute solvent	Pretreatment technology	MQLs		Reference
			In other work	In this work	
KCZ	Methanol	Pressurized liquid extraction	<1 ng/mL	4.12 ng/mL	[45]
TCS	Hexane/methyl-t-butyl-ether (*v*/*v* = 2:1)	Liquid-liquid extraction	10 ng/mL	2.90 ng/mL	[15]
TCS	Methanol	Solid phase extraction	0.55 ng/mL	2.90 ng/mL	[27]
AHTN	Mthyl acetate	Solid phase extraction	28 ng/mL	0.94 ng/mL	[28]
HHCB	Mthyl acetate	Solid phase extraction	89 ng/mL	1.04 ng/mL	[28]

The volume of solvent used can also influence the removal efficiency of proteins and phospholipids, thus acetonitrile solvent volumes (containing 1% AA) of 300 μL, 500 μL, and 800 μL were compared. As shown in Fig. 2a, the recoveries of ICZ were 134% and 162% for acetonitrile at 300 μL and 800 μL, respectively, and the recoveries of BP for acetonitrile at 800 μL was as low as 30%. Yet, for 500 μL of acetonitrile (containing 1% AA), the recoveries of ICZ and BP were 90% and 80%, respectively, suggesting good recoveries for these two chemicals using that volume of acetonitrile as the solvent. The recoveries of the 24 PCPs were in the ranges 68–134%, 69–123%, and 30–162% for acetonitrile (containing 1% AA) volumes of 300 μL, 500 μL, and 800 μL, respectively. As a result, 500 μL of acetonitrile (containing 1% AA) was chosen as the optimum hybrid precipitation and extraction solvent.

3.1.2. Optimization of d-SPE materials

An additional purification process was applied to remove interferences, such as cholates, pigments (e.g., bilirubin and biliverdin), and cholesterol, in bile [20,21]. The PSA and C18 sorbents remove polar matrix components, e.g., organic acids, polar pigments, and nonpolar interferences [39]. The GCB and Z-Sep sorbents remove pigments and phosphate in biological matrices [40,41]. Thus, three d-SPE sorbent kits, kit a (containing 300 mg C18 and 120 mg Z-Sep), kit b (containing 855 mg $MgSO_4$, 150 mg PSA, and 45 mg GCB), and kit c (containing 900 mg $MgSO_4$, 150 mg PSA, and 150 mg C18), were tested. The bile extracts without d-SPE purification were also compared.

Fig. 2b shows the recoveries of the target compounds in the bile samples using the different d-SPE materials. The CBZ, PP, BP, TCC, and TCS were undetectable because of strong matrix interferences when no d-SPE material was used for further purification, indicating that it is necessary to purify the extracts before instrumental analysis. When kit a (Z-Sep/C18) or kit b (anhydrous $MgSO_4$/PSA/GCB) were used as d-SPE sorbents, the target compounds ICZ, BP, TCC, and TCS were undetectable, which may be due to the adsorption of these compounds on Z-Sep or GCB. When kit b (anhydrous $MgSO_4$/PSA/GCB) was used, the recoveries of the four benzotriazoles were much higher than 120%, which is related to the strong retention ability of GCB on the internal standard TBD-D_6 [17]. Similar to this result, strong retention by Z-Sep of some analytes based on Lewis acid-base interactions has been reported [42], and the strong adsorption ability by GCB of planar compounds was also investigated [43]. When kit c (anhydrous $MgSO_4$/PSA/C18) was used as the d-SPE material, the recoveries of the 24 PCPs in the

bile were in the range of 67–113%, which is acceptable for detecting these compounds in bile. Thus, kit c (anhydrous $MgSO_4$/PSA/C18) was selected as the d-SPE material.

The newly developed method utilizing phospholipid–protein removal tubes, i.e., Captiva ND^{Lipid} Protein and Phospholipid Removal tubes, combined with d-SPE materials achieved simultaneous extraction and purification of 24 PCPs from fish bile samples. The total treatment time for the whole procedure was <60 min per batch of 24 samples, excluding the enzyme hydrolysis time.

3.2. Quantification and method evaluation

To quantify trace organic contaminants using UPLC-MS/MS and GC–MS, an isotope dilution quantitation method is desirable to compensate for potential processing errors and matrix effects [17]. An isotope-labeled analog of the analyte is an ideal internal standard because this analog co-elutes exactly with the analyte and experiences the same suppression/enhancement effects as the target analyte [37]. According to the chemical properties and retention time of target analytes, 12 isotope-labeled internal standards for the 24 target PCPs were used in this study: IMZ-D_5, TBD-D_6, FCZ-D_4, KCZ-D_8, CTZ-D_5, MCZ-D_5, MP-D_4, PP-D_4, TCC-D_7, $^{13}C_{12}$-TCS, AHTN-D_3, and MX-D_{15} (Tables 1 and 2) However, it is difficult to obtain the corresponding labeled internal standards for every target analyte, therefore, isotope-labeled compounds with similar properties and retention times to the analytes were selected, i.e., IMZ-D_5 and TBD-D_6 [44].

Good linearity for standard calibration curves in the ranges 0.1–200 ng/mL for UPLC-MS/MS and 1–200 ng/mL for GC–MS was obtained, with coefficients of determination (R^2) >0.993. The intra-day and inter-day precision were also calculated according to an analysis of standard mixtures, at concentrations of 100 ng/mL, seven times within a day and within one week, respectively [44]. The intra-day repeatability, expressed as the relative standard deviation (RSD), for the 24 target PCPs ranged from 0.2% to 5.38%, whereas the inter-day repeatability (RSD) ranged from 0.6% to 10.5% (Table S5).

Table 3 displays the recoveries, MDLs, and MQLs of the developed method for bile samples with and without enzyme hydrolysis treatment. For most of the 24 target PCPs, the recoveries were in the range 70–120% for the four spiking concentrations (10, 20, 100, and 200 ng/mL) in the bile samples, with or without enzyme hydrolysis. For bile samples with enzyme hydrolysis, the recoveries of the 24 PCPs ranged 60–147% (MX and MK were not included), 61–132%, 78–149%, and 69–126% for the spiking concentrations of 10 ng/mL, 20 ng/mL, 100 ng/mL, and 200 ng/mL, respectively. For bile samples without enzyme hydrolysis, the recoveries of the 24 PCPs were in the ranges 56–142% (MX and MK were not included), 61–125%, 77–147%, and 71–121% for spiking concentrations of 10 ng/mL, 20 ng/mL, 100 ng/mL, and 200 ng/mL, respectively. Fig. 3 provides the chromatograms of the 24 PCPs in the fish bile samples for the recovery experiment with spiking concentrations of 100 ng/mL.

The MDL and MQL values for a majority of the 24 target PCPs were <3 ng/mL and 10 ng/mL, respectively, except for MX and MK. The MQL values ranged 0.26–10.7 ng/mL and 0.2–12.9 ng/mL for the samples with and without enzyme hydrolysis, respectively. As shown in Table 4, the MQL values of KCZ in this study were higher compared to those reported in a previous study in which pressurized liquid extraction was used [45]. The MQL values of TCS in this study were lower than those reported in other studies, in which the liquid-liquid extraction method was used [15], and comparable with results using the conventional SPE method [27]. The MQL values for AHTN and HHCB in the present study were lower than those in a previous study, in which the SPE method was used [28].

Table 5
The matrix effects (%) of biocides, synthetic musks and benzotriazoles in fish bile with enzyme and without enzyme hydrolysis.

Compounds	With enzyme process	Without enzyme process
Biocides		
DEET	−18	−20
ICD	−19	−20
CBD	**−25**[a]	**−28**
TBD	−14	−17
FCZ	−17	−18
KCZ	−19	−20
CBZ	−20	−19
CTZ	−18	−18
MCZ	−16	−12
ICZ	−6	−5
MP	−20	−18
EP	−17	−20
PP	−18	−15
BP	−15	−7
TCC	−8	−7
TCS	−18	−6
Synthetic musks		
AHTN	−18	−13
HHCB	**−29**	**−31**
MX	14	17
MK	11	16
Benzotriazoles		
BT	−14	−10
5-TT	−17	−19
CBT	**−26**	**−21**
XT	**−25**	**−22**

[a] Bold numbers in the table represent those matrix effect outside the range of ±20%.

3.3. Matrix effects

Matrix effects are common when determining analytes in environmental and biological samples because of the co-extraction of matrix components [17]. Table 5 provides the matrix effects for the 24 PCPs in the bile samples with and without enzyme hydrolysis. The matrix effects for 24 target PCPs were ranged from −31% (HHCB) to 17% (MX). It can be seen that the matrix effects for most of target PCPs were at the range of ±20%, except for CBD, HHCB, CBT and XT. Isotope dilution quantitation is desirable for compensating for matrix effects in biological or environmental samples with relatively effective preprocessing [37], especially for those PCPs with their corresponding isotope-labeled analog used as internal standards. However, for CBD, HHCB, CBT and XT without corresponding isotope-labeled internal standards, the high matrix effects probably due to that the trend of matrix interference were not in accordance with their internal standards. So, for CBD, HHCB, CBT and XT with matrix effects outside ±20%, the matrix-matched calibrations were used to quantify these analytes in real bile samples.

3.4. Application to environmental samples

The developed hybrid precipitation SPE and d-SPE purification was used for the simultaneous analysis of the 24 target PCPs in bile from fish collected from the Yangtze River. Some pharmaceuticals, such as fluoxetine, carbamazepine, and norfluoxetine, have been reported in fish bile in conjugated forms [13], thus the enzyme β-glucuronidase/aryl-sulfatase was used to release conjugated PCPs to their free forms in this study [15]. Table 6 shows the concentrations of PCPs in the bile samples from fish of Yangtze River with and without enzyme hydrolysis, and Fig. 4 shows the chromatograms of the detected PCPs in the bile from fish of Yangtze River. For bile samples with hydrolysis treatment, 12 PCPs were found, at concentrations in the range of <0.49–460 ng/mL. For the same bile samples without hydrolysis, 11 PCPs were detected, at concentra-

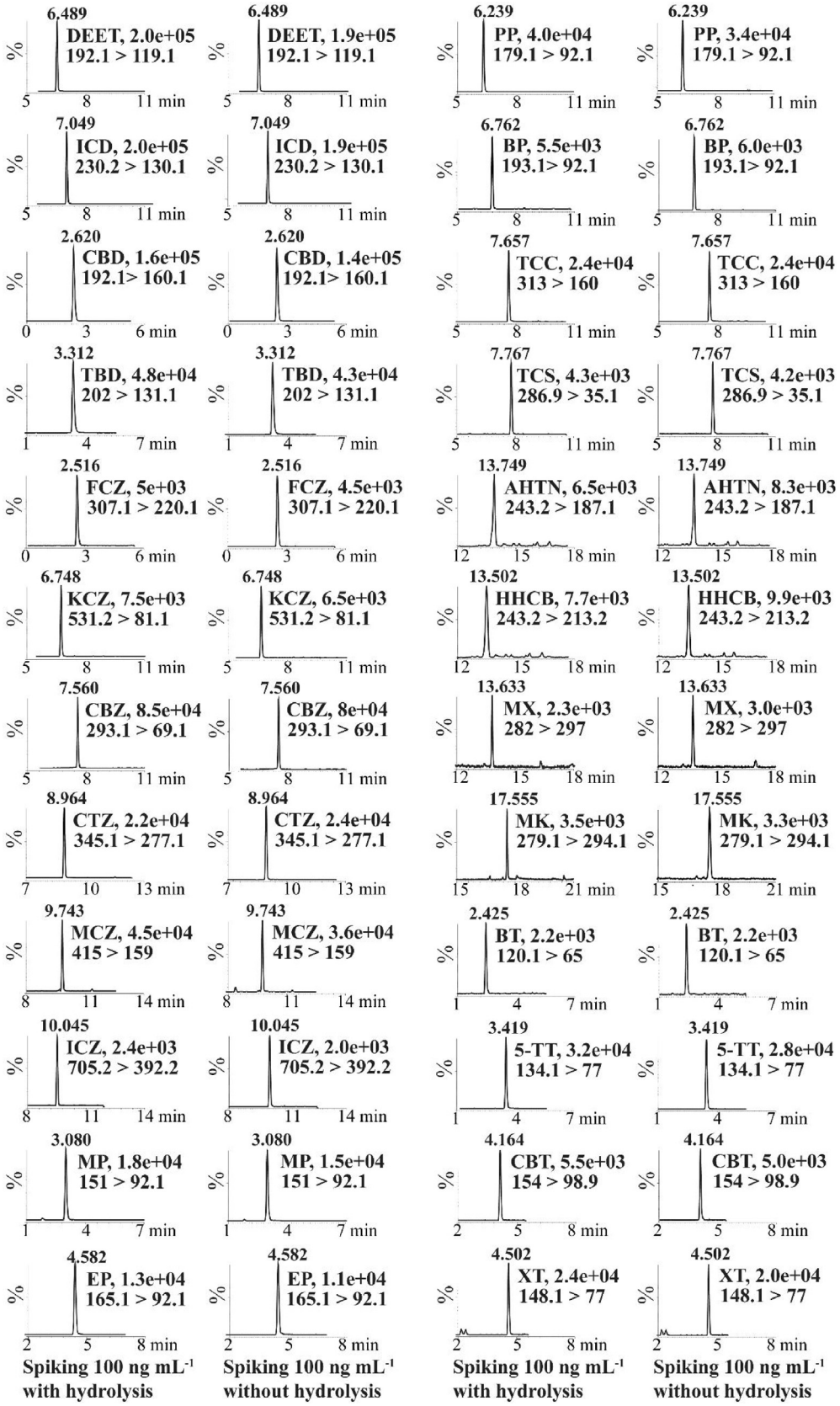

Fig. 3. The chromatograms of 24 target compounds in bile samples at the spiking concentration of 100 ng/mL.

tions in the range of <0.38–122 ng/mL. As shown in Table S6, all of these detected PCPs were commonly found in the surface water of corresponding sampling sites in Yangtze River, suggested that fish bile can be used as indicator on the monitoring of PCPs in the aquatic environment [11]. And previous study showed that most of these 11 PCPs were also detected in liver and muscle tissues of

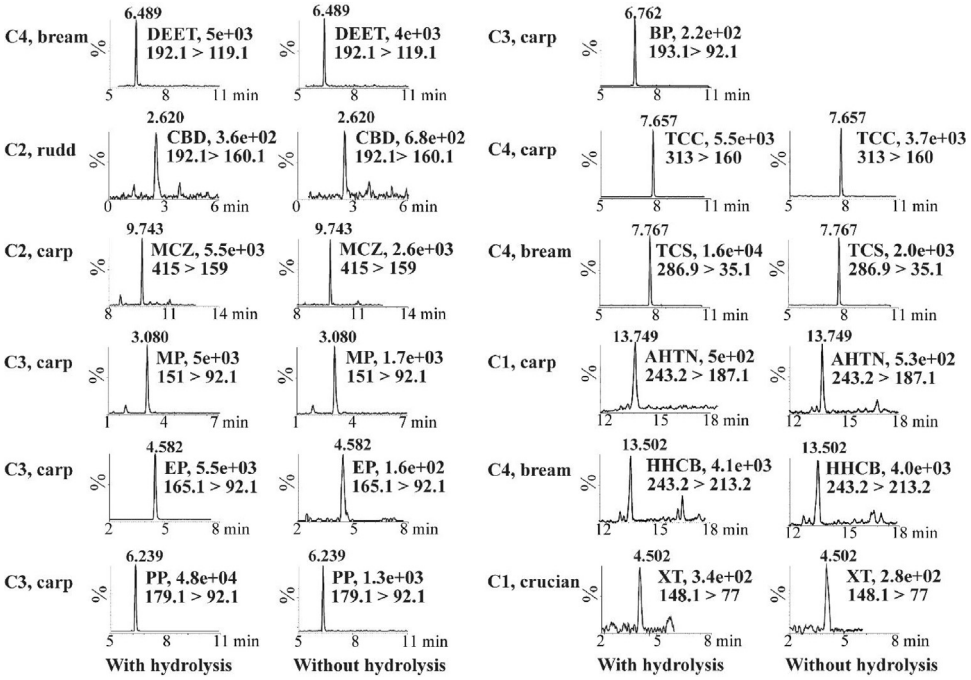

Fig. 4. The chromatograms of some detected compounds in bile of fish collected from Yangtze River of sites C1, C2, C3 and C4, with and without enzyme hydrolysis.

Table 6
Concentration (ng/mL) of target compounds in the bile of fish collected from Yangtze River with enzyme and without enzyme hydrolysis.

Compounds	With enzyme				Without enzyme			
	D.F[a]	Range	Mean	Median	D.F	Range	Mean	Median
Biocides								
DEET	100	1.81-5.33	3.21	3.10	100	1.68–5.06	3.04	2.92
CBD	3	0–2.34	<0.8	nd[b]	3	0–3.96	<0.8	nd
MCZ	14	0–13.6	<2.01	nd	11	0–12.8	<2.34	nd
MP	100	8.17–21.9	12.5	11.3	100	6.84–21.0	10.1	9.60
EP	77	0–31.6	4.07	3.06	49	0–6.71	1.62	nd
PP	100	2.19–112	9.12	3.06	91	0–10.0	2.64	2.30
BP	11	0–4.42	<0.49	nd	0	nd	nd	nd
TCC	6	0–14.2	<0.49	nd	6	0–9.38	<0.38	nd
TCS	100	7.84–460	57.6	23.9	89	0–64.0	8.27	5.60
Synthetic musks								
AHTN	49	0-12.6	4.40	nd	46	0–16.3	4.71	nd
HHCB	91	0–95.7	32.4	24.1	100	<1.04–122	39.7	31.1
Benzotriazoles								
XT	9	0–<4.55	0.20	nd	9	0–2.95	<2.07	nd

The data of "nd" (not detected) were identified as zero for the calculation of mean, median and range of concentrations, and the data less than MQLs values were substituted by 1/2 MQLs. The data "nd" were not counted but data below the MQLs were included for the statistics of detection frequencies.

[a] D. F: Detection frequency (%).

[b] nd: Not detected.

fish collected from Yangtze River [46], so the detection of parent or metabolites of PCPs in fish bile indicated the uptake trend of these PCPs by fish [13]. For the concentrations of these 12 detected PCPs in the bile samples with or without enzyme hydrolysis, the non-parametric Kruskal-Wallis test (K-W test) indicated that no obvious differences among the four sampling sites were observed ($p > 0.05$), and no significant differences existed among three common fishes ($n > 3$): carp, bream, and crucian (K-W test, $p > 0.05$). This may be because the number of fish samples was limited.

For bile samples with hydrolysis treatment, Fig. S3 shows that the concentrations of three parabens (MP, EP, PP) and TCS were higher than those for the same bile samples without hydrolysis. BP was sporadically detected in bile with hydrolysis treatment but not found in bile without hydrolysis. After enzyme hydrolysis, the concentrations of MP, EP, PP, and TCS were up to 3, 10, 23, and 28 times higher, respectively, than those in the corresponding non-enzyme hydrolyzed samples. The median percentages of conjugates (glucuronides + sulfates) to the total amounts of MP, EP, PP, and TCS were 16%, 28%, 40%, and 79%, respectively. These results suggest that the four parabens and TCS can conjugate with glucuronide and sulfate compounds in fish bile, which is in agreement with previous studies of the four parabens and TCS in urine or the liver in those conjugated forms [47,48]. In contrast, for the other seven detected PCPs, i.e., DEET, CBD, MCZ, TCC, AHTN, HHCB, and XT, no significant differences in the bile samples between enzyme hydrolysis and non-enzyme hydrolysis were observed (Kolmogorov-Smirnov test, $p > 0.05$), indicating they have no glucuronide/sulfate conjugates in the fish bile. For the 12 detected PCPs, only five compounds with structures containing the hydrophilic benzene hydroxyl group can generate glucuronide or sulfate metabolites with the dehydration–condensation reaction [4]. In general, using the hydrolysis enzyme in the proposed

extraction and cleanup method facilitates PCP determination in bile samples with free and conjugated forms.

4. Conclusion

A sensitive hybrid precipitation and d-SPE cleanup method was developed and validated for the simultaneous extraction of 24 PCPs in fish bile. The target compounds were determined via UPLC-MS/MS and GC–MS. High sensitivity, satisfactory recoveries, and acceptable matrix effects were obtained for most of the target compounds. The proposed method was applied to detect target PCPs in bile from fish collected from the Yangtze River. Differing concentrations for parabens and TCS in the same bile sample with and without enzyme hydrolysis indicated that conjugated forms of some PCPs should be considered.

Notes

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.chroma.2018.04.003.

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