Multi-pharmaceuticals analysis in fish by continuous series solid-phase extraction coupled with UPLC-MS/MS

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ABSTRACT

Pharmaceuticals which are widely used in aquatic can easily migrate into the environment and aquatic animals, and can increase the risk of drug resistance and allergic symptoms if consumed by humans. In order to achieve high-throughput analysis of pharmaceuticals with different physical and chemical properties from complex matrices, we developed a new method for various types pharmaceuticals in fish and shrimp tissue. Series solid-phase extraction (s-SPE) with different adsorbents was selected for extracting and purifying analytes with different paddings. s-SPE were combined with ultra performance liquid chromatography triple quadruple tandem mass spectrometry (UPLC-MS/MS) for the detection of 30 pharmaceuticals antibiotics in fish samples. This method was stabilized and reliable to determinate the pharmaceuticals in fish and shrimp samples. As the method combined multiple Chinese national standards method, it could be easily treat the multi-pharmaceuticals from the fish and shrimp samples once time. It provided for both quantitative and qualitative methods and they could be applied to single- or multi-residue methods.

KEYWORDS

series solid-phase extraction (s-SPE), ultra performance liquid chromatography triple quadruple tandem mass spectrometry (UPLC-MS/MS), multi-pharmaceuticals, fish, food safety

INTRODUCTION

Fish is a common source of animal protein for people. It also contains long chain ω -3-fatty acids such as eicosapentaenoic acid and docosahexaenoic acid, as well as vitamins and minerals which are beneficial for a healthy diet [1]. Due to the advantages of aquaculture, many fish were fed in limited space to meet the current demand. However, fish growing in crowded environments induce stress and increase susceptibility to disease [2]. In order to prevent the occurrence of diseases, the use of chemicals as a prophylactic treatment has become a common practice and this resulted in concerns over fish quality and safety [3].

Malachite green is one of the commonly used antifungal and antiparasitic agents in aquaculture. However, due to its toxic effects and potential carcinogenesis, this chemical is not permitted to be used in any animal or food for human consumption in the US [4] and the EU [5]. Chloramphenicol was banned in some countries including China, USA and member states of European Union (the EU) [6–10]. Presently, synthetic analogues, the substitute of chloramphenicol such as thiamphenicol and florfenicol are proposed and widely used in

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veterinary medicine [11]. However, these substitute also pose the risk to human health. Sulfonamide antibiotics (SAs), tetracyclines (TCs) and fluoroquinolones (FQs) are the most commonly used antibiotics in aquaculture worldwide [12]. These antibiotics are synthetic substrates with broad-spectrum antimicrobial activity, and are used mainly for human and veterinary infectious diseases treatment. At the moment, the residues of antibiotics have been present in worldwide environment samples including the water sample [13]. Toxicological studies have shown that SAs can lead to blood disorders, liver damage, cancer and tumours [14]. Each country has its own legislation regarding the approval of antibiotics and concentrations for the use in aquaculture. For example, in Brazil, only two antimicrobials florfenicol and oxytetracycline are licensed for aquaculture [15]. To ensure food safety and human health, many countries, including the USA [16], the European Union [17], and China [18] have established maximum residue limits (MRLs) for antibiotics in edible tissues. Therefore, it is important to monitor the presence of pharmaceuticals in fish in order to allow international trade and protect consumers from health hazards.

Some analytical techniques such as LC-UV and LC-fluorescence detection (FLD) have been developed [19]. Nevertheless, most of these techniques provide a way for semi-qualitative showing low selectivity. Liquid chromatography tandem mass spectrometry (LC-MS/MS) is a powerful technique for the analysis of pharmaceuticals because of its high specificity and sensitivity [20, 21]. Many studies were undertaken to detect one or a few pharmaceuticals in fish using LC-MS/MS [22, 23]. However, the sample preparation technique is the bottleneck for determination multi-pharmaceuticals in complex matrix. Most of the methods require a laborious sample preparation step in the analysis of multi-pharmaceuticals with different properties, which increases analysis time and the consumption of reagents, and generates large amounts of residues to the environment. Under such circumstances, there is a need for a simple, fast and reliable quantitative method for the analysis of multi-pharmaceuticals in fish.

Several approaches including solid-phase extraction (SPE), gel permeation chromatography (GPC), solid-phase microextraction (SPME), QuEChERS, dispersive-solid phase extraction (d-SPE) and matrix solid-phase dispersion (MSPD) have been attempted to eliminate lipids and co-extracted interference from fatty food extracts. Among them, QuEChERS is the most used method for the analysis of multi-pesticides in the fruit and vegetable samples [24]. However, it is not applicable to fish sample due to the co-extractive interference of lipids, fats, fatty acids and proteins in the matrix. SPE is mostly used for the analysis of a series of analytes with similar properties and provide an appreciably high signal/noise ratio [25]. However, due to the selectivity of adsorbents in SPE, the analytes with different properties could not be enriched by one single adsorbent. Thus, for the multi-class multi-residue analytes, various types of adsorbents should be applied in SPE.

The aim of present work is to develop and optimize a sensitive and accurate analytical method for the high-

throughput analysis of multi-pharmaceuticals in fish tissues based on a series solid-phase extraction (s-SPE) cleanup procedure and LC-MS/MS. Different SPE cartridges, HLB, MAX, MCX were applied in order to minimize interferences and achieve high recovery values. Thirty pharmaceuticals residues including 12 SAs, 7 FQs, 3 amide-alcohols, 2 malachite greens, 3 tetracyclines and 3 other drugs were chosen to evaluate the analytical performance of this method. In addition, the linearity and working range, accuracy and precision, LODs and LOQs were also validated. The combination of s-SPE with LC-MS/MS detection modes has already been demonstrated to be successful for the determination of multi-pharmaceuticals in fish samples.

EXPERIMENTAL

Materials and reagents

The HLB, MAX, MCX SPE cartridge were bought from Waters Co. (Milford, MA, USA). Filter membrane (0.22 μm), methanol, acetonitrile, formic acid, ammonium hydroxide and centrifuge tube provided by Anpel Co., Ltd. (Shanghai, China) were used to filter the extracts before injection into the chromatographic system. Deionized water was collected from a Milli-Q-Plus water purifier (Millipore, Bedford, MA, USA). The 30 pharmaceuticals standard including 12 SAs (sulfisoxazole, SFZ; sulfathiazole, ST; sulfamonomethoxine, SMX; sulfamethoxazole, SMZ; sulfamethizol, SMZO; sulfamethazine, SM2; Sulfamerazine, SM1; sulfadoxine, SO; sulfamethazine, SMZX; sulfadiazine, SDZ; sulfachloropyridazine, SCP; sulfaquinoxaline, SOX), 7 FQs (enrofloxacin, EFX; ciprofloxacin, CFX; norfloxacin, NFX; ofloxacin, OFX; pefloxacin, PEF; lomefloxacin, LOM; fleroxacin, FLX), 3 amide-alcohols (chloramphenicol, CAP; thiamphenicol, TAP; florfenicol, FFC), 2 malachite greens (malachite green, MG; leucomalachite green, LMG), 3 tetracyclines (tetracycline, TC; oxytetracycline, OTC; chlorotetracycline, CTC) and 3 other drugs (ivermectin, IV; abamectin, AB; erythromycin, ERY) and 8 internal standards (ISs) including sulfadoxine-d3, SO-D3; sulfadimethoxine-d6, SFX-D6; norfloxacin-d5, NFX-D5; ciprofloxacin-d8, CFX-D8; enrofloxacin-d5, EFX-D5; chloramphenicol-d5, CAP-D5; malachite green-d5, MG-D5; leucomalachite green-d6, LMG-D6 were obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany).

Standard solutions

Individual stock solutions of standards (Table 1) were prepared at a concentration of 10 $\mu\text{g mL}^{-1}$ in acetonitrile and stored at $-20\text{ }^{\circ}\text{C}$ for use and renewed monthly. All working solutions were prepared daily by further dilution of the individual stock solutions.

Sample preparation

To detect residues of pharmaceuticals, the fish samples were obtained from a market in the Harbin of China. Those samples were cut into small pieces and crushed with a

Table 1. The properties of the 30 compounds (including 12 SAs, 7 FQs, 3 TCs, 2 MGs, 3 CAPs, 3 Others drugs)

	ID	compound	mode	Precursor ion (<i>m/z</i>)	Product ion (<i>m/z</i>)	Collision energy (V)	RT (min)	LOD ($\mu\text{g kg}^{-1}$)	LOQ ($\mu\text{g kg}^{-1}$)	linearity ($\mu\text{g kg}^{-1}$)	Spiking level (1*LOD)		Spiking level (5*LOD)		Spiking level (10*LOD)	
											Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
SAs	1	sulfisoxazole (SFZ)	ESI+	268.0	156.0*/108.0	13/16	3.46	0.1	0.3	0.3–30	93	2.6	95	2.2	97	6.5
	2	sulfathiazole (ST)	ESI+	256.0	156.0*/108.0	15/20	2.74	0.1	0.3	0.3–30	93	3.2	95	3.2	96	4.4
	3	sulfamonomethoxine (SMX)	ESI+	281.0	156.0*/215.1	20/15	3.27	0.1	0.3	0.3–30	96	4.3	97	4.0	100	9.1
	4	sulfamethoxazole (SMZ)	ESI+	254.0	156.0*/108.0	15/20	3.40	0.1	0.3	0.3–30	94	3.3	96	2.9	98	6.1
	5	sulfamethizol (SMZO)	ESI+	271.0	156.0*/108.0	15/20	3.14	0.1	0.3	0.3–30	97	2.6	99	2.9	101	3.0
	6	sulfamethazine (SM2)	ESI+	279.0	156.0*/186.0	20/16	3.11	0.1	0.3	0.3–30	97	2.7	98	2.8	101	7.0
	7	Sulfamerazine (SM1)	ESI+	265.0	156.0*/172.0	15/15	2.88	0.1	0.3	0.3–30	94	4.0	96	4.7	99	4.6
	8	sulfadoxine (SO)	ESI+	311.0	156.0*/108.0	18/26	3.37	0.1	0.3	0.3–30	103	3.3	104	3.0	105	4.6
	9	sulfamethazine (SMZX)	ESI+	311.0	156.0*/108.0	15/20	3.57	0.1	0.3	0.3–30	95	5.3	97	4.6	98	7.4
	10	sulfadiazine (SDZ)	ESI+	251.0	156.0*/108.0	15/20	1.95	0.1	0.3	0.3–30	98	2.9	99	2.7	101	6.5
	11	sulfachloropyridazine (SCP)	ESI+	285.0	156.0*/108.0	15/25	3.32	0.1	0.3	0.3–30	93	1.3	95	1.3	98	3.6
	12	sulfaquinoxaline (SOX)	ESI+	301.0	156.0*/108.0	15/20	3.57	0.1	0.3	0.3–30	94	2.0	96	2.0	98	5.1
FQs	1	enrofloxacin (EFX)	ESI+	360.2	316.1*/245.0	22/20	3.12	0.1	0.3	0.3–30	96	1.7	98	2.1	99	3.9
	2	ciprofloxacin (CFX)	ESI+	332.0	288.0*/245.0	18/22	3.06	0.1	0.3	0.3–30	100	0.6	102	1.5	105	5.7
	3	norfloxacin (NFX)	ESI+	320.1	276.1*/233.0	20/25	3.03	0.1	0.3	0.3–30	95	3.3	97	2.4	99	6.1
	4	ofloxacin (OFX)	ESI+	362.1	318.2*/261.1	18.24	3.04	0.1	0.3	0.3–30	104	1.4	99	3.3	102	5.2
	5	pefloxacin (PEF)	ESI+	334.1	316.2*/290.2	22.18	3.05	0.1	0.3	0.3–30	94	1.3	96	7.5	98	4.8
	6	lomefloxacin (LOM)	ESI+	352.1	265.1*/308.2	18.21	3.10	0.1	0.3	0.3–30	98	6.1	96	3.1	98	4.2
	7	fleroxacin (FLX)	ESI+	370.1	326.2*/269.1	18.25	3.05	0.1	0.3	0.3–30	90	7.2	98	6.5	101	5.1
CAPs	1	chloramphenicol (CAP)	ESI–	321.0	152.1*/257.1	19/12	3.47	0.004	0.012	0.012–2	93	4.7	95	4.0	99	7.8
	2	Thiamphenicol (TAP)	ESI–	354.0	185.1*/290.1	20/13	3.04	0.04	0.12	0.12–20	95	4.8	97	4.3	100	6.4

(continued)

Table 1. Continued

	ID	compound	mode	Precursor ion (<i>m/z</i>)	Product ion (<i>m/z</i>)	Collision energy (V)	RT (min)	LOD (μg kg ^{−1})	LOQ (μg kg ^{−1})	linearity (μg kg ^{−1})	Spiking level (1*LOD)		Spiking level (5*LOD)		Spiking level (10*LOD)	
											Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
MGs	3	Florfenicol (FFC)	ESI−	355.9	335.9*/185.0	10/16	3.40	0.04	0.12	0.12–20	95	4.7	97	3.6	98	6.7
	1	malachite green (MG)	ESI+	329.2	313.2*/208.1	36/42	4.04	0.002	0.006	0.006–8	96	4.0	97	3.7	101	7.8
	2	leucomalachite green (LMG)	ESI+	331.2	316.2*/239.1	22/30	5.10	0.002	0.006	0.006–8	94	2.5	95	2.7	97	7.0
TCs	1	tetracycline (TC)	ESI+	445.2	427.2*/410.2	14/20	3.12	0.2	0.6	0.6–30	92	3.1	98	2.7	99	3.1
	2	oxytetracycline (OTC)	ESI+	461.2	426.2*/443.2	18/13	3.06	0.2	0.6	0.6–30	93	2.3	100	2.7	102	3.8
	3	chlorotetracycline (CTC)	ESI+	479.1	462.1*/444.1	16/20	3.32	0.2	0.6	0.6–30	95	2.5	89	5.5	91	4.7
Others	1	ivermectin (IV)	ESI+	897.5	329.3*/753.5	50.42	5.96	0.2	0.6	0.6–30	87	3.6	94	3.5	95	4.9
	2	abamectin (AB)	ESI+	895.5	327.3*/449.4	50.46	5.24	2	6	6–100	91	1.7	96	3.7	98	5.6
	3	erythromycin (ERY)	ESI+	734.5	158.2*/576.4	25.18	3.53	0.002	0.006	0.006–10	92	2.2	95	1.6	96	3.7
ISs	1	sulfadoxine-d3 (SO-D3)	ESI+	314.1	156.0*	20	3.36	–	–	–	–	–	–	–	–	–
	2	sulfadimethoxine-d6 (SFX-D6)	ESI+	317.0	156.0*	23	3.55	–	–	–	–	–	–	–	–	–
	3	norfloxacin-d5(NFX-D5)	ESI+	325.1	307.0*	18	3.02	–	–	–	–	–	–	–	–	–
	4	ciprofloxacin-d8(CFX-D8)	ESI+	340.2	322.2*	20	3.05	–	–	–	–	–	–	–	–	–
	5	enrofloxacin-d5(EFX-D5)	ESI+	365.0	321.0*	20	3.11	–	–	–	–	–	–	–	–	–
	6	chloramphenicol-d5(CAP-D5)	ESI−	326.0	157.0*	19	3.46	–	–	–	–	–	–	–	–	–
	7	malachite green-d5(MG-D5)	ESI+	334.2	318.2*	38	4.04	–	–	–	–	–	–	–	–	–
	8	leucomalachite green-d6(LMG-D6)	ESI+	336.2	239.1*	32	5.06	–	–	–	–	–	–	–	–	–

*Was the quantitative ion pair.

grinder. Homogenized fish muscle (5.00 g) were weighed into a 50 mL centrifuge tube and the ISs (100.0 μ L) was added. Extractant were added after shaking for several seconds. Samples were extracted in an ultrasonic bath for 20 min at room temperature followed by centrifugation at 5,000 rpm for 5 min. The extraction process was repeated three times with 10.00 mL extractant of acetonitrile, acidified acetonitrile and ammonium acetonitrile, respectively. All the extracts were combined and concentrated by rotary evaporation, and re-dissolved in 10 mL 30% MeOH. Subsequently, the reconstituted extract was passed through the s-SPE cartridge, including the HLB cartridge (pH = 7), MCX cartridge (pH = 6), and MAX cartridge (pH = 8), respectively (pH was adjusted with acetic acid or ammonium hydroxide). The MAX, HLB and MCX cartridges were activated and equilibrated before use with 2 mL methanol and 2 mL deionized water, respectively. The rinsing agents were 1 mL $\text{NH}_4\text{OH}/\text{H}_2\text{O}/\text{MeOH}$ (1:95:5, v/v/v) in MAX mode, 1 mL $\text{H}_2\text{O}/\text{MeOH}$ (95:5, v/v) in HLB mode, and 1 mL $\text{FA}/\text{H}_2\text{O}/\text{MeOH}$ (1:95:5, v/v/v) in MCX mode, respectively. And then the cartridges were dried and eluted with 3 mL methanol/acetic acid (100:1, v/v) for MAX cartridge, 3 mL methanol for HLB cartridge, and 3 mL methanol/ammonium hydroxide (100:1, v/v) for MCX cartridge, respectively. All the eluents were combined in a bottle and evaporated to dryness at 37 °C in a water bath and 10 psi pressure of N_2 . After re-dissolving in 1.0 mL acetonitrile/water (10:90 v/v), the extract was filtered through a 0.22 μ m organic membrane syringe filter and transferred to a autosampler vial. Blank fish samples and spiked fish samples were extracted under the same condition. Spiked fish samples were prepared by adding the proper amounts of standard solutions.

LC-MS/MS analysis

The analysis was performed on a Waters ACQUITY UPLC H-Class system (Waters, Milford, MA, USA), which was

equipped with a quaternary pump and a heated column compartment. The separation of analytes was achieved on a Waters ACQUITY UPLC BEH C18 column (2.1 $\times$ 50 mm, 1.7 μ m). Mobile phase A consisted of 5 mmol L^{-1} ammonium acetate in 0.1% formic acid, and mobile phase B was acetonitrile. The chromatographic conditions were 5% B in 0–1 min, from 5 to 95 % B in 1–3.5 min, 95% B in 3.5–6.6 min, from 95 to 5% B in 6.6–6.7 min, 5% B in 6.7–8 min, and the curve was set at 6. The column heater was set at 30 °C. The flow rate was 300 $\mu\text{L min}^{-1}$.

Mass spectrometry detection was performed using a triple quadrupole MS (Xevo TQ-S, Waters, Milford, MA, USA) in positive and negative ionization mode with an ESI ion source. The source parameters were optimized as follows. Capillary was set at 3.00 kV. Cone was set as 20 V. Desolvation temperature was set at 450 °C. Desolvation gas was set at 800 L/hour. Cone was set as 250 L/hour. Nebuliser was set as 7.0 bar. Source temperature was set as 150 °C. Collision gas flow was set at 0.14 mL min^{-1} .

The data were collected in multiple reaction monitoring (MRM) mode, and the detail information including precursor ion, product ion and collision energy was shown in Table 1. Instrument control, data acquisition, and data processing were performed by Masslynx 4.1 software. The schematic diagram of extraction and data analysis process was shown in Fig. 1.

The amount of 30 pharmaceuticals were quantitated by both IS and external standard calibration curve. Blank fish sample was spiked with a series of concentrations of standards in order to establish the calibration curves. Sulfadoxine-d3 (SO-D3) was used as the IS for the analysis of ST, SMX, SMZ, SMZO, SM2, SM1, SO, SMZX, SDZ and SOX. Sulfadimethoxine-d6 (SFX-D6) was used as the IS for the analysis of SFZ and SCP. Norfloxacin-d5 (NFX-D3) was used as the IS for the analysis of NFX, OFX, PEF and FLX. Ciprofloxacin-d8 (CFX-D8) was used as the IS for the analysis of CFX and LOM. Enrofloxacin-d5 (EFX-D5) was

Fig. 1. The schematic diagram of extraction and data analysis process

used as the IS for the analysis of EFX. Malachite green-d5 (MD-D5) was used as the IS for the analysis of MG. Leucomalachite green-d6 (LMG-D6) was used as the IS for the analysis of LMG. Chloramphenicol-d5 (CAP-D5) was used as the IS for the analysis of CAP, TAP and FFC. TC, OTC, CTC, IV, AB, ERY were quantified by using external standard method.

RESULTS AND DISCUSSION

Optimization of the sample pretreatment conditions

The spiked fish (*Gadus morhua*) samples were prepared as previously described. Thirty pharmaceuticals with different properties were selected (Table 1). The extraction experiment was performed using three replicates. The effect of extraction solvents, extraction time and eluent solution were optimized for the cleanup procedure to obtain the satisfied recoveries. When one condition was changed, the others were fixed at their optimum values.

Effect of extraction solvents. The choice of extraction solvents was of great importance for extraction efficiency. The extraction solvents should have satisfied specific properties such as excellent extraction efficiency, no interference with the analytes and cheap. Thus, we selected acetonitrile, acidified acetonitrile and ammonium acetonitrile as extractant for the analytes with different properties according to a series of standard method of China. These standards including determination of sulfonamides and quinolones residues in aquatic products by LC-MS/MS method (No. 1077th-1-2008), determination of tetracyclines residues in food of animal origin by LC-MS/MS method and HPLC method (No. GB/T 21317-2007), determination of malachite green and crystal violet residues in aquatic product by LC-MS/MS method and HPLC method (No. GB/T 19857-2005), determination of chloramphenicol, thiamphenicol, and florfenicol residues in edible animal muscles, liver and aquatic products by LC-MS/MS method (No. GB/T 20756-2006), determination of avermectin and ivermectin residues in aquatic products by HPLC method (No. GB 29695-2013), and determination of erythromycin residues in aquatic products by LC-MS/MS method (No. GB 29684-2013).

Effect of extraction time. Ultrasonically assisted extraction time of 10, 15, 20, 25 and 30 min were investigated. As shown in Fig. 2, the extraction efficiency was increased with extraction time over the range of 5–20 min, and reached a maximum at 20 min. The extraction equilibrium was almost reached after 20 min. Therefore, 20 min was selected in the following experiments.

Effect of the cartridge in s-SPE. Multiple compounds could not be extracted by one single SPE due to the different polarities and chemical properties [26, 27]. The MCX and MAX was based on the same polymeric structure as HLB, and contains the functional groups on the surface. The surface were patented mixed-mode polymeric sorbents with reversed

Fig. 2. Effect of extraction time for the recoveries of 30 pharmaceuticals. The plots represent the average recoveries of the 12 SAs, 7 FQs, 3 CAPs, 2 MGs, 3 TCs and 3 Others.

phase and strong cation- or anion-exchange functionalities attached to a polymer backbone of poly (divinyl-benzene-co-N-vinylpyrrolidone) [28]. MGs, CAP, FFC and TAP have been reported to be extracted by MCX [23, 29], TCs and SAs by HLB [30, 31], and FQs by MAX [32, 33]. Thus, in the present study, a s-SPE with different adsorbents (including HLB, MAX, and MCX) was used for extracting and purifying the multi-class multi-residue pharmaceuticals in fish sample. Then the different steps of the SPE cleanup were optimized.

Effect of the rinsing agents volume. It is well known that the target analytes and some impurities could be retained on the SPE column. In order to reduce the impurities, the washing solution with a moderate elution strength to not reduce the recoveries was selected. After loading the extract onto SPE column, washing step was performed including 1 mL $\text{NH}_4\text{OH}/\text{H}_2\text{O}/\text{MeOH}$ (1:95:5, v/v/v) in MAX mode, 1 mL $\text{H}_2\text{O}/\text{MeOH}$ (95:5, v/v) in HLB mode, and 1 mL $\text{FA}/\text{H}_2\text{O}/\text{MeOH}$ (1:95:5, v/v/v) in MCX mode. Different volumes (1, 3, and 5 mL) of washing solution were tested. It revealed that there were no losses of analytes when 1 mL washing solution was selected.

Effect of the eluent in s-SPE. In order to achieve a better recovery of the adsorbed analytes, the eluent solvent were selected according to the standards method of China as above, previous study [26] and also the specific interaction forces between paddings and pharmaceuticals. Thus, 3 mL methanol/acetic acid (100:1, v/v), 3 mL methanol and 3 mL methanol/ammonium hydroxide (100:1, v/v) were used as the eluent solvent for MAX, HLB and MCX cartridge, respectively. Finally, the eluent was evaporated to near dryness at 37 °C under the nitrogen stream and re-dissolved in the initial mobile phase composition to obtain a good peak shape in UPLC.

Effect of the purification. The effect of s-SPE purification was examined by LC-UV (Waters ACQUITY UPLC I-Class

with UV detector). The chromatographic condition was the same as UPLC-MS/MS, and the data was collected by Empower 3 workstation. The blank *G. morhua* was confirmed by Chinese national standard for determination of SAs, FQs, CAPs, MGs, AB, IV, ERY and TCs. The absorbance profile was set at 254 nm as the elution was detected at 254 nm in gel permeation chromatograph. As could be seen in Fig. 3A and B, the purified extract solution using s-SPE method was cleaner than that without any purification. Since there were no co-elution compounds at the retention time of the analytes after s-SPE purification, the qualitative and quantitative results would not be affected. The purification and enrichment process would also improve sensitivity in MRM mode. Furthermore, the examination method of purification effect in this study could be applied to examine other methods in food analysis.

Validation of the methodology

Linearity. The linearity of the UPLC-MS/MS method for determination of pharmaceuticals in fish sample was evaluated by matrix-matched calibration curve which was prepared by spiking blank fish samples at different concentration levels (from 0.006 to 100 $\mu\text{g kg}^{-1}$). Linearity for the calibration graphs by matrix-matched to the fish samples were found between 0.3 and 30 $\mu\text{g kg}^{-1}$ for SAs and FQs; from 0.012 to 2 $\mu\text{g kg}^{-1}$ for CAP; from 0.12 to 20 $\mu\text{g kg}^{-1}$ for TAP and FFC; from 0.006 to 8 $\mu\text{g kg}^{-1}$ for MGs; from 0.6 to 30 $\mu\text{g kg}^{-1}$ for TCs; from 6 to 100 $\mu\text{g kg}^{-1}$ for AB; from 0.006 to 10 $\mu\text{g kg}^{-1}$ for ERY. Characteristic parameters for regression equations of the UPLC-MS/MS method obtained by least squares treatment of the results are given in Table 1.

Precision and accuracy. Precision and accuracy of the UPLC-MS methods were tested at three different concentration spiking levels for each compound under the same experimental condition. The experiment was repeated three times within a day (intra-day precision), and three times on different three days (inter-day precision). The value for intra-day precision and inter-day precision studies were less than 9.1%, as shown in Table 1.

Detection and quantitation limit. Each pharmaceutical was confirmed by comparison of the signal intensity ratios of the two transitions (quantification and confirmation) obtained

from samples with spiked blank fish samples. Confirmation was considered reliable if the ratio was within the criteria laid down in the European Commission Decision [34]. Limit and quantitation of detection (LOQ and LOD) were defined as the lowest concentration with a signal-to-noise ratio no less than 10 and 3 at the retention times of the target compounds, respectively. In this method, satisfied LODs and LOQs were obtained at 0.002–2 $\mu\text{g kg}^{-1}$ and 0.006–6 $\mu\text{g kg}^{-1}$, as shown in Table 1.

Comparison of the proposed method with others

Compared with the China standard method 1077th-1-2008, GB/T 21317-2007, GB/T 19857-2005, GB/T 20756-2006, GB 29695-2013 and GB 29684-2013, lower LODs were obtained with the proposed method. It had advantages such as simplicity in the determination of multi-pharmaceutical and low cost for sample preparation. Moreover, high-throughout pharmaceutical determination could be processed using this method. As could be seen in Table 2, the recoveries, linear dynamic ranges, and relative standard deviations in this study were similar or better than the methods described in the literature. In summary, the developed s-SPE method showed high potential for routine analysis of multi-pharmaceuticals in monitoring fish samples.

Application to real samples

Finally, the proposed method was successfully applied to the analysis of these analytes from shrimp and fish samples, including *Penaeus chinensis*, *Scophthalmus maximus*, *Ctenopharyngodon idellus*, *Carassius auratus* and *Cyprinus carpio* bought from market in Harbin, China. Among them, EFX and CFX were found in one *S. maximus* sample, as could be seen in Fig. 4. The ion ratios of two transition reactions of two analytes (EFX and CFX) in spiked blank sample were 0.32 and 0.59, respectively. And the ion ratios of EFX and CFX in *S. maximus* sample were 0.30 and 0.55, respectively. The criteria set by EU guidelines was as follows: If the expected ratio was more than 0.5, the observed ratio should be within 20%. If the expected ratio was from 0.2 to 0.5, the observed ratio should be within 25%. If the expected ratio was from 0.1 to 0.2, the observed ratio should be within 30%. If the expected ratio was less than 0.1, the observed ratio should be within 50%. According to the law of the ion ratios, the peak in 3.12 min and 3.06 min was confirmed to

Fig. 3. The chromatogram of blank *Gadus morhua* without s-SPE purification (A) or with s-SPE purification (B).

Table 2. Comparison of this method with other methods for determination of pharmaceuticals

Sample	Pharmaceutical numbers	Sample preparation	Determination	LOD ($\mu\text{g kg}^{-1}$)	LOQ ($\mu\text{g kg}^{-1}$)	Recovery (%)	RSD (%)	References
cattle meats	7	extraction by ultrasonic purification by n-hexane	LC-FLD	8–15	13–25	45–81	<6	[35]
fish	3	MSPD extraction	UPLC-MS/MS	20–60	110–160	84–100	<12	[36]
poultry meat	3	accelerated solvent extraction	UPLC-FLD	1.0–4.2	3.0–11.4	81–98	4.6	[37]
fish	5	molecularly imprinted SPE	UPLC-MS/MS	0.003–0.020	0.005–0.025	23–118	12	[22]
eggs	4	ultrasonic	UPLC-MS/MS	0.04–0.5	0.1–1.5	91–108	11.3	[23]
shrimps	1	C18 SPE cartridge	LC-MS	0.02	0.2	101–110	7.1	[38]
chicken meat and eggs	7	liquid-liquid extraction	HPLC- DAD	108–116	129–140	86–108	15	[39]
egg, fish and shrimp	7	pressurized liquid extraction	HPLC-UV	5.0–10.0	10.0–15.0	76–104	11	[40]
shrimp	14	vortex mixed	LC-MS/MS	–	0.25–1	75–101	14.6	[41]
fish	14	homogenized	LC-MS/MS	–	13–19	89–103	14	[42]
fish and shrimp	30	Extraction by ultrasonic purification by s-SPE	UPLC-MS/MS	0.002–0.2	0.006–0.6	87–105	9.1	this study

Fig. 4. The total ion chromatogram of 30 pharmaceuticals and 6 internal standards (A), and the extract ion chromatogram of the real sample (B).

be EFX and CFX. The concentration of EFX and CFX was $6.2 \mu\text{g kg}^{-1}$ and $2.5 \mu\text{g kg}^{-1}$ determined by the proposed method. The concentration was also determined by the standard method (1077-1-2008), and the value of EFX and CFX was $6.0 \mu\text{g kg}^{-1}$ EFX and $2.7 \mu\text{g kg}^{-1}$, respectively. The value was below the maximum residue limits. This revealed that the performance of this method was similar to the standard method ($\text{CV}_{\text{EFX}}=3.0\%$, $\text{CV}_{\text{CFX}}=3.8\%$). Furthermore, the proposed method could be used for the analysis of more kinds of pharmaceuticals.

CONCLUSIONS

An efficient and high-throughput method for the determination of 30 pharmaceuticals in fish by s-SPE coupled with UPLC-MS/MS was established and verified. The method combined a series of Chinese national standard methods (including but not limited 1077th-1-2008; GB/T 21317-2007; GB/T 19857-2005; GB/T 20756-2006; GB 29695-2013 and GB 29684-2013). The s-SPE and UPLC-MS/MS showed good analytical performance in compound determination.

The analytes were well purified from the complex matrix using s-SPE cleanup procedure. Then the redissolved solution was isolated and detected using the UPLC-MS/MS based on the selectivity of the MRM transitions. The developed protocol was successfully applied to fish samples, and it provides a new approach for risk assessment and fishery accident identification.

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