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
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]. 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 (sulfadoxine-d3, SO-D3; sulfamethoxazole-d6, SFX-d6), 3 tetracyclines (tetracycline, TC; chlorotetracycline, CTC; oxytetracycline, OTC), 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 sulfadimethoxine-d3, SO-D3; sulfadimethazine-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 μg mL⁻¹ in acetonitrile and stored at −20 °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 (m/z) | Product ion (m/z) | Collision energy (V) | RT (min) | LOD (µg kg⁻¹) | LOQ (µg kg⁻¹) | linearity (µg kg⁻¹) | Recovery (1*LOD) (%) | RSD (%) | Recovery (5*LOD) (%) | RSD (%) | Recovery (10*LOD) (%) | RSD (%) |
|----|---------------------------|------|---------------------|------------------|----------------------|----------|--------------|--------------|---------------------|----------------------|---------|----------------------|---------|----------------------|---------|
| 1  | sulfisoxazole (SFZ) ESI+  | 268.0| 156.0* / 108.0      | 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       | 0.3–30       | 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       | 0.3–30       | 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       | 0.3–30       | 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       | 0.3–30       | 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       | 0.3–30       | 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       | 0.3–30       | 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       | 0.3–30       | 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       | 0.3–30       | 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       | 0.3–30       | 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       | 0.3–30       | 93            | 1.3               | 95                   | 1.3     | 98                   | 3.6     |
| 12 | sulfaquinuoxaline (SOX) ESI+ | 301.0| 156.0* / 108.0      | 15/20            | 3.57                 | 0.1       | 0.3–30       | 94            | 2.0               | 96                   | 2.0     | 98                   | 5.1     |
| 1  | enrofloxacin (EFX) ESI+    | 360.2| 316.1* / 301.1      | 22/20            | 3.12                 | 0.1       | 0.3–30       | 96            | 1.7               | 98                   | 2.1     | 99                   | 3.9     |
| 2  | ciprofloxacin (CFX) ESI+   | 332.0| 288.0* / 301.1      | 18/22            | 3.06                 | 0.1       | 0.3–30       | 100           | 0.6               | 102                  | 1.5     | 105                  | 5.7     |
| 3  | norfloxacin (NFX) ESI+     | 320.1| 276.1* / 233.1      | 20/25            | 3.03                 | 0.1       | 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       | 0.3–30       | 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       | 0.3–30       | 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       | 0.3–30       | 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       | 0.3–30       | 90            | 7.2               | 98                   | 6.5     | 101                  | 5.1     |
| 1  | chloramphenicol (CAP) ESI− | 321.0| 152.1* / 301.1      | 19/12            | 3.47                 | 0.004     | 0.012       | 0.012–2       | 93            | 4.7               | 95           | 4.0 | 99                   | 7.8     |
| 2  | Thiampenicol (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)
| ID | Compound | Mode | Precursor ion (m/z) | Product ion (m/z) | Collision energy (V) | RT (min) | LOD (µg kg⁻¹) | LOQ (µg kg⁻¹) | Linearity (µg kg⁻¹) | Recovery (%) | RSD (%) | Recovery (%) | RSD (%) | Recovery (%) | RSD (%) |
|----|----------|------|-------------------|------------------|---------------------|---------|---------------|--------------|-----------------|--------------|--------|--------------|---------|--------------|--------|
| 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    |
| MGs | 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    |
| TCa | 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 | 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 | 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.
Homogenized fish muscle (5.00 g) were weighed into a 50 mL centrifuge tube and the ISs (100.0 μL) 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 NH₄OH/H₂O/MeOH (1:95:5, v/v/v) in MAX mode, 1 mL H₂O/MeOH (95:5, v/v) in HLB mode, and 1 mL FA/H₂O/MeOH (1:95:5, v/v/v) in MCX mode, respectively. And then the cartridges were dried and eluted with 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 N₂. After re-dissolving in 1.0 mL acetonitrile/water (10:90 v/v), the extract was filtered through a 0.22 μm organic membrane syringe filter and transferred to an 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 × 50 mm, 1.7 μm). 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 μL 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 used as the IS for the analysis of EFX and FLX.
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 21317-2007), determination of malachite green and crystal violet residues in aquatic product by LC-MS/MS method and HPLC method (No. GB 29685-2013), 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 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 NH4OH/H2O/MeOH (1:95:5, v/v/v) in MAX mode, 1 mL H2O/MeOH (95:5, v/v) in HLB mode, and 1 mL FA/H2O/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 μg kg⁻¹). Linearity for the calibration graphs by matrix-matched to the fish samples were found between 0.3 and 30 μg kg⁻¹ for SAs and FQs; from 0.012 to 2 μg kg⁻¹ for CAP; from 0.12 to 20 μg kg⁻¹ for TAP and FGC; from 0.006 to 8 μg kg⁻¹ for MGs; from 0.6 to 30 μg kg⁻¹ for TCs; from 6 to 100 μg kg⁻¹ for AB; from 0.006 to 10 μg 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 at the retention times of the target compounds, respectively. In this method, satisfied LODs and LOQs were obtained at 0.002–2 μg kg⁻¹ and 0.006–6 μg 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-throughput 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).](image-url)
be EFX and CFX. The concentration of EFX and CFX was 6.2 μg kg⁻¹ and 2.5 μg 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 μg kg⁻¹ EFX and 2.7 μg 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 (CVEFX = 5.3%, CVCFX = 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 to 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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**REFERENCES**

1. Mock, T. S.; Francis, D. S.; Jago, M. K.; Glencross, B. D.; Smullen, R. P.; Keast, R. J. S.; Turchini, G. M. Altered levels of shorter vs long-chain omega-3 fatty acids in commercial diets for market-sized Atlantic salmon reared in seawater – effects on fatty acid composition, metabolism and product quality. *Aquaculture* 2019, 499, 167–77, https://doi.org/10.1016/j.aquaculture.2018.09.020.

2. Lazado, C. C.; Sween, L.; Soleng, M.; Pedersen, L.-F.; Timmerhaus, G. Crowding reshapes the mucosal but not the systemic response repertoires of Atlantic salmon to peracetic acid. *Aquaculture* 2020, https://doi.org/10.1016/j.aquaculture.2020.735830.

3. Nunes, K. S. D.; Assalin, M. R.; Vallim, J. H.; Jonsson, C. M.; Queiroz, S. C. N.; Reyes, F. G. R. Multiresidue method for quantification of sulfonamides and trimethoprim in tilapia fillet by liquid chromatography coupled to quadruple time-of-flight mass spectrometry using QuEChERS for sample preparation. *J. Anal. Methods Chem.* 2018, 2018, 4506754, https://doi.org/10.1155/2018/4506754.

4. Leonard, B. Fish and Fishery Products: Hazards And Controls Guidance, 4th ed.; DIANE Publishing, 2011.

5. EFSA. Malachite Green in Food. Retrieved November 30, 2017, from https://www.efsa.europa.eu/en/press/news/160727. 2017.

6. XiaoDong, P.; PingGu, W.; Wei, J.; Binjie, M. Determination of chloramphenicol, thiamphenicol, and florfenicol in fish muscle by matrix solid-phase dispersion extraction (MSPD) and ultra-high pressure liquid chromatography tandem mass spectrometry. *Food Control* 2015, 52, 34–8, https://doi.org/10.1016/j.foodcont.2014.12.019.

7. Commission, E. Commission regulation (EU) No 1430/94 of 22 June 1994 amending Annexes I, II, III and IV of Council Regulation (EEC) No 2377/90 laying down a community procedure for the establishment of maximum residues limits of veterinary medicinal products in foodstuffs of animal origin. *Off. J. Eur. Commun.* 1994, L156, 6–8.

8. Commission, E. Commission Decision 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. *Off. J. Eur. Commun.* 2003, L221, 8–36.

9. Commission, E. Commission regulation (EU) No 2003/181/EC of 13 March 2003 amending Decision 2002/657/EC as regards the setting of minimum required performance limits (MRPLs) for certain residues in food of animal origin. *Off. J. Eur. Commun.* 2003, L71, 17–8.

10. Epstein, R. L.; Henry, C.; Holland, K. P.; Dreas, J. International validation study for the determination of chloramphenicol in bovine muscle. *J. AOAC Int.* 1994, 77, 570–6.

11. Alechaga, E.; Moyano, E.; Galceran, M. T. Ultra-high performance liquid chromatography-tandem mass spectrometry for the analysis of phenicol drugs and florfenicol-amine in foods. * Analyst* 2012, 137, 2486–94.

12. Juan-García, A.; Font, G.; Picó, Y. Determination of quinolone residues in chicken and fish by capillary electrophoresis-mass spectrometry. *Electrophoresis* 2006, 27, 2240–9, https://doi.org/10.1002/elps.200500868.

13. Wanschel, A. C. B. A. Monitoring of phenicol drugs and florfenicol-amine in meats. *Drug Test. Anal.* 2016, 8, 556–64, https://doi.org/10.1002/dta.2007.

14. Arancibia, V.; Valderrama, M.; Rodríguez, P.; Hurtado, F.; Segura, R. Quantitative extraction of sulfonamides in meats by supercritical methanol-modified carbon dioxide: A foray into real-world sampling. *J. Sep. Sci.* 2003, 26, 1710–6.

15. Sindam. Sindicato Nacional da Indústria de Produtos para a Saúde Animal; Compendio de Produtos Veterinários, 2016.

16. Administration, U.F.a.D. Code of Federal Regulations (CFR) 6, Title 21, 2009.

17. Commission, E. *Off. J. Eur. Union* 2010, L15, 1.

18. Ministry of Agriculture of the People’s Republic of China No.235 Announcement Standard. No.235 Announcement Standard, 2002.

19. Granja, R. H. M. M.; de Lima, A. C.; Patel, R. K.; Salerno, A. G.; Wanschel, A. C. B. A. Monitoring of florfenicol residues in fish muscle by HPLC-UV with confirmation of suspect results by LC-MS/MS. *Drug Test. Anal.* 2012, 4, 125–9, https://doi.org/10.1002/dta.1362.

20. Courlet, P.; Alves Saldanha, S.; Cavassini, M.; Marzolini, C.; Choong, E.; Caijka, C.; Günthard, H. F.; André, P.; Buclin, T.; Desfontaine, V., et al. Development and validation of a multiplex UHPLC-MS/MS assay with stable isotopic internal standards for the monitoring of the plasma concentrations of the antiretroviral drugs bictegravir, cabotegravir, doravirine, and rilpivirine in people living with HIV. *J. Mass Spectrom.* 2020, 55, e4506, https://doi.org/10.1002/jms.4506.

21. Kim, D. H.; Yoo, Y. S.; Yoo, H. J.; Choi, Y. J.; Kim, S. A.; Sheen, D.-H.; Lee, S. K.; Lim, M. K.; Cho, K. Analysis of hair and plasma samples for Methotrexate (MTX) and metabolite using high-performance liquid chromatography triple quadrupole mass spectrometry (LC-MS/MS) detection. *J. Mass Spectrom.* 2020, n/a, e4648, https://doi.org/10.1002/jms.4648.

22. Martinez Bueno, M. J.; Herrera, S.; Uclés, A.; Agüera, A.; Hernando, M. D.; Shimelis, O.; Rudolfsson, M.; Fernándezalba, A. R. Determination of malachite green residues in fish using molecularly
imprinted solid-phase extraction followed by liquid chromatography-linear ion trap mass spectrometry. *Anal. Chim. Acta* **2010**, *665*, 47–54.

23. Xie, X.; Wang, B.; Pang, M. D.; Zhao, X.; Xie, K. Z.; Zhang, Y. Y.; Wang, Y. J.; Guo, Y. W.; Liu, C. J.; Bu, X. N., et al. Quantitative analysis of chloramphenicol, thiamphenicol, florfenicol and florfenicol amine in eggs via liquid chromatography-electrospray ionization tandem mass spectrometry. *Food Chem.* **2018**, *269*, 542–8, https://doi.org/10.1016/j.foodchem.2018.07.045.

24. Lawal, A.; Wong, R. C. S.; Tan, G. H.; Abdulrazif, A. M. A. Multi-pesticide residues determination in samples of fruits and vegetables using chemometrics approach to QuEChERS-dSPE coupled with ionic liquid-based DLLME and LC-MS/MS. *Chromatographia* **2018**, *81*, 759–68, https://doi.org/10.1007/s10337-018-3511-7.

25. Cho, S.; Elaty, A. M. A.; Park, K. H.; Park, J.; Assayed, M. E.; Jeong, Y.; Park, Y.; Shim, J. Simple multiresidue extraction method for the determination of fungicides and plant growth regulator in bean sprouts using low temperature partitioning and tandem mass spectrometry. *Food Chem.* **2013**, *136*, 1414–20.

26. Gao, L.; Qin, D.; Huang, X.; Wu, S.; Chen, Z.; Tang, S.; Wang, P. Determination of pesticides and pharmaceuticals from fish-cultivation water by parallel solid-phase extraction (SPE) and liquid chromatography-quadrupole time-of-flight mass spectrometry (LC-QTOF-MS). *Anal. Lett.* **2019**, *52*, 983–97, https://doi.org/10.1080/00032719.2018.1509676.

27. Gao, L.; Qin, D. L.; Song, W. U.; Huang, X. L.; Chen, Z. X.; Tang, S. Z.; Huang, L.; Wang, P. Detection of six pesticides in fishery waters by liquid chromatography tandem mass spectrometry, Chinese J. Fish. **2017**, *30*, 44–8.

28. Siwek, M.; Noubar, A. B.; Erdmann, R.; Niemeyer, B.; Galusynsky, B. Application of mixed-mode oasis MCX adsorbent for chromatographic separation of selenomethionine from antarctic krill after enzymatic digestion. *Chromatographia* **2008**, *67*, 305–8.

29. Tao, Y.; Chen, D.; Chao, X.; Yu, H.; Yuanhu, P.; Liu, Z.; Huang, L.; Wang, Y.; Yuan, Z. Simultaneous determination of malachite green, gentian violet and their leuco-metabolites in shrimp and salmon by liquid chromatography–tandem mass spectrometry with accelerated solvent extraction and auto solid-phase clean-up. *Food Control* **2011**, *22*, 1246–52, https://doi.org/10.1016/j.foodcont.2011.01.025.

30. Cheng, Y. F.; Phillips, D. J.; Neue, U. Simple and rugged SPE method for the determination of tetracycline antibiotics in serum by HPLC using a volatile mobile phase. *Chromatographia* **1997**, *44*, 187–90, https://doi.org/10.1007/BF02466454.

31. Malintan, N. T.; Mohd, M. A. Determination of sulfonamides in selected Malaysian swine wastewater by high-performance liquid chromatography. *J. Chromatogr. A* **2006**, *1127*, 154–60, https://doi.org/10.1016/j.chroma.2006.06.005.

32. Chen, X. H.; Yao, X. P Simultaneous determination of four fluoroquinolones in honey by high performance liquid chromatography with mass spectrometry. *Chinese J. Health Lab. Technol.* **2007**, *9*.

33. Hyung, S. W.; Lee, C. H.; Kim, B. Development of certified reference materials for accurate determination of fluoroquinolone antibiotics in chicken meat. *Food Chem.* **2017**, *229*, 472–8, https://doi.org/10.1016/j.foodchem.2017.02.112.

34. Communities, E. Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. *Off. J. Eur. Commun.* **2002**, *221*, 8–36.

35. Mor, F.; Sahindokuyucu, K. F.; Ozdemir, G.; Oz, B. Determination of sulfonamide residues in cattle meats by the Charm-II system and validation with high performance liquid chromatography with fluorescence detection. *Food Chem.* **2012**, *134*, 1645–9.

36. Pan, X. D.; Wu, P. G.; Jiang, W.; Ma, B. J. Determination of chloramphenicol, thiamphenicol, and florfenicol in fish muscle by matrix solid-phase dispersion extraction (MSPD) and ultra-high pressure liquid chromatography tandem mass spectrometry. *Food Control* **2015**, *52*, 34–8.

37. Wang, G.; Wang, B.; Zhao, X.; Xie, X.; Xie, K.; Wang, X.; Zhang, G.; Zhang, T.; Liu, X.; Dai, G. Determination of thiamphenicol, florfenicol and florfenicol amine residues in poultry meat and pork via ASE–UPLC–FLD. *J. Food Compos. Anal.* **2019**, *81*, 19–27.

38. Ramos, M.; Munoz, P.; Aranda, A.; Rodriguez, I.; Diaz, R.; Blanca, J. Determination of chloramphenicol residues in shrimps by liquid chromatography-mass spectrometry. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **2003**, *791*, 31–8, https://doi.org/10.1016/S1570-0232(03)00186-7.

39. Premaratne, J. M. K. J.; Satharasinghe, D. A.; Gunasena, A. R. C.; Munasinghe, D. M. S.; Abeynayake, P. Establishment of a method to detect sulfonamide residues in chicken meat and eggs by high-performance liquid chromatography. *Food Control* **2017**, *72*, 276–82, https://doi.org/10.1016/j.foodcont.2015.12.012.

40. Liu, Y.; Yang, H. L.; Yang, S.; Hu, Q. W.; Cheng, H. B.; Liu, H. Y.; Qiu, Y. S. High-performance liquid chromatography using pressurized liquid extraction for the determination of seven tetracyclines in egg, fish and shrimp. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **2013**, *917*, 11–7, https://doi.org/10.1016/j.jchromb.2012.12.036.

41. Tsai, M. Y.; Lin, C. F.; Yang, W. C.; Lin, C. T.; Hung, K. H.; Chang, G. R. Health risk assessment of banned veterinary drugs and quinolone residues in shrimp through liquid chromatography-tandem mass spectrometry. *Appl. Sci. Basel* **2019**, *9*.

42. Guidi, L. R.; Santos, F. A.; Ribeiro, A. C. S. R.; Fernandes, C.; Silva, L. H. M.; Gloria, M. B. Quinolones and tetracyclines in acuiculture fish by a simple and rapid LC-MS/MS method. *Food Chem.* **2018**, *245*, 1232–8.