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Multi-Residue Determination of Sulfonamide and Quinolone Residues in Fish Tissues by High Performance Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

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ABSTRACT

A LC-MS/MS method was validated for the simultaneous quantification of 4 quinolones (oxolinic acid, enrofloxacine, ciprofloxacine, norfloxacine) and 4 sulfonamides (sulfamethoxypyridazine, sulfadoxine, sulfadimidine, sulfamerazine) on fish muscle following the European Union's (EU) criteria for the analysis of veterinary drug residues in foods. One gram of sample was extracted by acidic acetonitrile (0.5 mL of 0.1% formic acid in 7 mL of ACN), followed by LC-MS/MS analysis using an electrospray ionization interface. Typical recoveries of the 4 quinolones in the fish tissues ranged from 85 to 104%. While those of the sulfonamides ranged from 75 to 94% at the fortification level of 5.0 μg/kg. The decision limits (CCα) and detection capabilities (CCβ) of the quinolones were 1.35 to 2.10 μg/kg and 1.67 to 2.75 μg/kg, respectively. Meanwhile, the CCα and CCβ of the sulfonamides ranged from 1.62 μg/kg to 2.53μg/kg and 2.01μg/kg to 3.13 μg/kg, respectively.

Key words: quinolones, sulfonamides, LC-MS/MS, matrix effect

INTRODUCTION

Antimicrobials are widely administered to food-producing animals for purposes of treatment and prevention of diseases[1]. The extensive use of sulfonamides and quinolones can result in residues in aquatic products which are widely consumed all over the world.

Quinolones are broad-spectra antibacterial agents that act against gram-negative bacteria. Quinolones enter the bacterial cell by passive diffusion through water-filled protein channels in the membrane and inhibit bacterial growth by interfering with the enzyme DNA-gyrase[2].

Due to low minimum inhibitory concentration (MIC) for most fish pathogens and effective systemic distribution in fish *via* feed, quinolones have been widely used to treat systemic bacterial infections in fish[3,4]. In 1990, the European Union (EU) established maximum residue limits (MRLs) for quinolones in animal tissues for food safety[5]. The EU has also adopted a MRL of 100 μg/L in edible animal tissue[6]. In order to ensure food safety, Taiwan has set an MRL of 100 μg/kg for sulfadimethoxine and sulfamonomethoxine as well as a minimum required performance limit (MRPL) of 10 μg/kg

for sulfamerazine and sulfamethazine in aquatic products[7]. These limits require sensitive and specific methods to monitor and determine antibiotic residues in aquatic products.

LC-MS/MS is one of the most promising techniques for the analysis of antimicrobials in animal tissues, because it allows drug quantification and confirmation at trace levels. Recently, there are some reports on the determination of antimicrobials by liquid chromatography-tandem mass spectrometry (LC-MS/MS)[8-11]. However, these methods analyze only single class quinolones or sulfonamides. One of the problems in the determination of residue antimicrobials in aquatic products is sample treatment, due to the high protein and fat content in the matrix, which can interfere with analytical procedures. For the determination of sulfonamides, several methods have been reported, including liquid-liquid extraction and solid-phase extraction (SPE)[1,12,13]. Which also involve a step to precipitate the proteins. Recently, new procedures based on matrix solid-phase dispersion with hot water extraction have been proposed in order to simplify the extraction step[14]. Most of these methods was used to determine one class of drug. A few studies focused on the determination of several types of antimicrobials in food[15,16].

Less solvent usage, time saving and procedure simplification are important issues. QuEChERS (abbreviated from

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Quick, Easy, Cheap, Effective, Rugged and Safe) has been developed for the analysis of pesticides in foods[17-20]. These papers focused on pesticide analysis not including veterinary drug.

However, only a few papers have used this methodology (QuEChERS) for the determination of pharmaceuticals or veterinary drugs[21,22]. The purpose of this study was to develop a multi-residue method for the determination of quinolones and sulfonamides in fish. This method involves a simple extraction with acetonitrile without further clean up and analysis by high performance liquid chromatography connected with tandem mass spectrometry. The combination of LC-MS/MS and QuEChERS extraction provides a fast and simple method that can be executed by routine laboratories, which have to analyze large numbers of samples frequently and determine different classes of compounds.

MATERIALS AND METHODS

I. *Chemicals and Reagents*

Commercial standards of oxolinic acid (OXA), enrofloxacine (EFA), ciprofloxacine (CFA) and norfloxacine (NFA), were supplied by Fluka (Steinheim, Germany), while sulfamethoxypyridazine (SMP), sulfadimethoxine (SDT), sulfadimidine (SDD) and sulfamerazine (SMZ) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock standard solutions of individual compounds (1000 mg/L) were prepared by exact weighing of the powder and dissolving in 100 mL of methanol : water (50 : 50, v/v)(HPLC grade, Sigma), and were then stored at -25°C in the dark. A mixed working standard with a concentration of 0.1 mg/L of each compound was freshly prepared by appropriate dilutions of the stock solutions with methanol : water (50 : 50, v/v). Acetonitrile (HPLC grade, Merck) was supplied by Tidia. Formic acid (purity > 98%) were purchased from Merck. Ultrapure water was obtained from a Milli-Q Gradient water system (Millipore, Bedford, MA, USA).

II. *Samples*

Tilapia (*Oreochromis niloticus niloticus*), milkfish (*Chanos chanos*), eel (*Anguilla japonica*), bass (*Lates calcarifer*) and cat fish (*Silurus asotus*) samples were bought from Taiwan market. The fish was filleted, the skin and bones were removed, and the muscles were minced and frozen at -20°C before being analysis.

III. *Equipment*

Chromatographic analysis was performed using high performance liquid chromatography system (HPLC, Agilent 1100 Series) and separation was achieved using a reversed-phase C_{18} column (4.6 × 150 mm, 5 μm, Agilent, ZORBAX SB-C_{18}). The auto-sampler was equilibrated at 20°C. The mobile phase consisted of 0.5 mM of ammonium acetate and 0.05% formic acid in water (eluent A) and methanol (eluent B) at a flow rate of 700 μL/min. The gradient profile started at 90% of eluent A and decreased linearly to 10% in 12 min.

Mass spectrometric analysis was carried out using an API 4000 tandem quadrupole mass spectrometer (Applied Biosystem, USA). The instrument was operated using electrospray ionization (ESI) in the positive mode. ESI parameters were: capillary voltage of 5500 V and source temperature of 650°C. Collision-induced dissociation was performed using argon as the collision gas at the pressure of 4×10^{-3} mbar in the collision cell. The specific MS/MS parameters for each target analyte are shown in Table 1. Data acquisition was performed using Analyst 1.4.1 software from Applied Biosystems.

IV. *Matrix calibration curves*

Blank fish muscle samples were fortified with working standard solutions of OXA, EFA, CFA, NFA, SMP, SDT, SDD and SMZ to produce a calibration curves with points equivalent to 0.1, 0.2, 0.5, 1.0, 2.0, 5.0 and 10 μg/L of OXA, EFA, CFA, NFA, SMP, SDT, SDD and SMZ. Each of the same species of matrix, including tilapia, milkfish, frozen eel, bass and cat fish, was used for calibration. All samples were analysed on five different days. The calibration curves were obtained by plotting the recorded peak area versus the corresponding concentrations of the fortified samples. The linearity of the calibration curves were expressed by the correlation coefficient.

Table 1. Retention time windows and tandem mass spectrometry parameters for the selected antimicrobials

Compound	Retention Time (min)	Quantitation Transition (m/z)	Confirmation Transition (m/z)
Oxolinic acid (OXA)	9.40 ± 0.2	262.0 → 244.0	262.0 → 216.0
Enrofloxacine (EFA)	7.45 ± 0.3	360.0 → 342.0	360.0 → 316.0
Ciprofloxacine (CFA)	7.50 ± 0.4	332.0 → 314.0	332.0 → 288.0
Norfloxacine (NFA)	7.43 ± 0.4	320.0 → 302.0	320.0 → 276.0
Sulfamethoxypyridazine (SMP)	8.26 ± 0.3	281.0 → 156.0	281.0 → 126.0
Sulfadimethoxine (SDT)	8.95 ± 0.2	311.0 → 156.0	311.0 → 245.0
Sulfadimidine (SDD)	7.70 ± 0.1	279.0 → 186.0	279.0 → 124.0
Sulfamerazine (SMZ)	7.28 ± 0.1	265.0 → 156.0	265.0 → 172.0

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V. *Extraction Procedure*

Antimicrobials were extracted from fish tissue using an extraction procedure based on less organic solvent consuming methodology[18]. The procedure was as follows: 1.0 g of sample was weighed in a 50-mL polypropylene centrifuge tube. Point five milliliter of 1% formic acid water solution and 7.0 mL of acetonitrile were added, and the mixture was subjected to vortex for 30 s. Subsequently, the plastic tube with sample and solvent was shaken by a vertical shaker for 10 min. After centrifugation at 4,000 rpm for 6 min, the acetonitrile layer were transferred into a 15-mL glass tube. The extraction solvent was evaporated with N_2 stream under atmospheric pressure at 40°C to dryness. Two milliliter of *n*-hexane and 1.0 mL of a mixed solution of methanol and 0.01% formic acid aqueous solution (50 : 50, v/v) were added. After mixed well by vortex, the mixture was de-fat to dissolve the residues. After centrifugation at 11000 rpm for 5 min, 600 μL of the bottom layer was drawn and filtered through a 0.22-μm nylon filter (Agilent). Finally, 20 μL were injected into the LC-MS/MS system under the optimized analytical conditions.

RESULTS AND DISCUSSION

I. *Chromatographic Separation*

Chromatographic conditions were studied in order to achieve the best separation and retention for the analytes, bearing in mind that the major benefit of LC is the efficiency of the column, which provides narrow peaks and good separation.

First, several experiments were performed using different mobile phases consisting of methanol or acetonitrile as the organic phase and water with different concentrations of formic acid and acetic acid (0.01 to 0.5%). When acetonitrile was evaluated as the organic solvent in the mobile phase, retention time was observed to be decreased. However sensitivity was better with the use of methanol so methanol was selected for the separation of the selected antimicrobials in further experiments.

Several gradient profiles were tested, obtaining good response with the gradient described in the method section. Other parameters such as column temperature, flow rate and
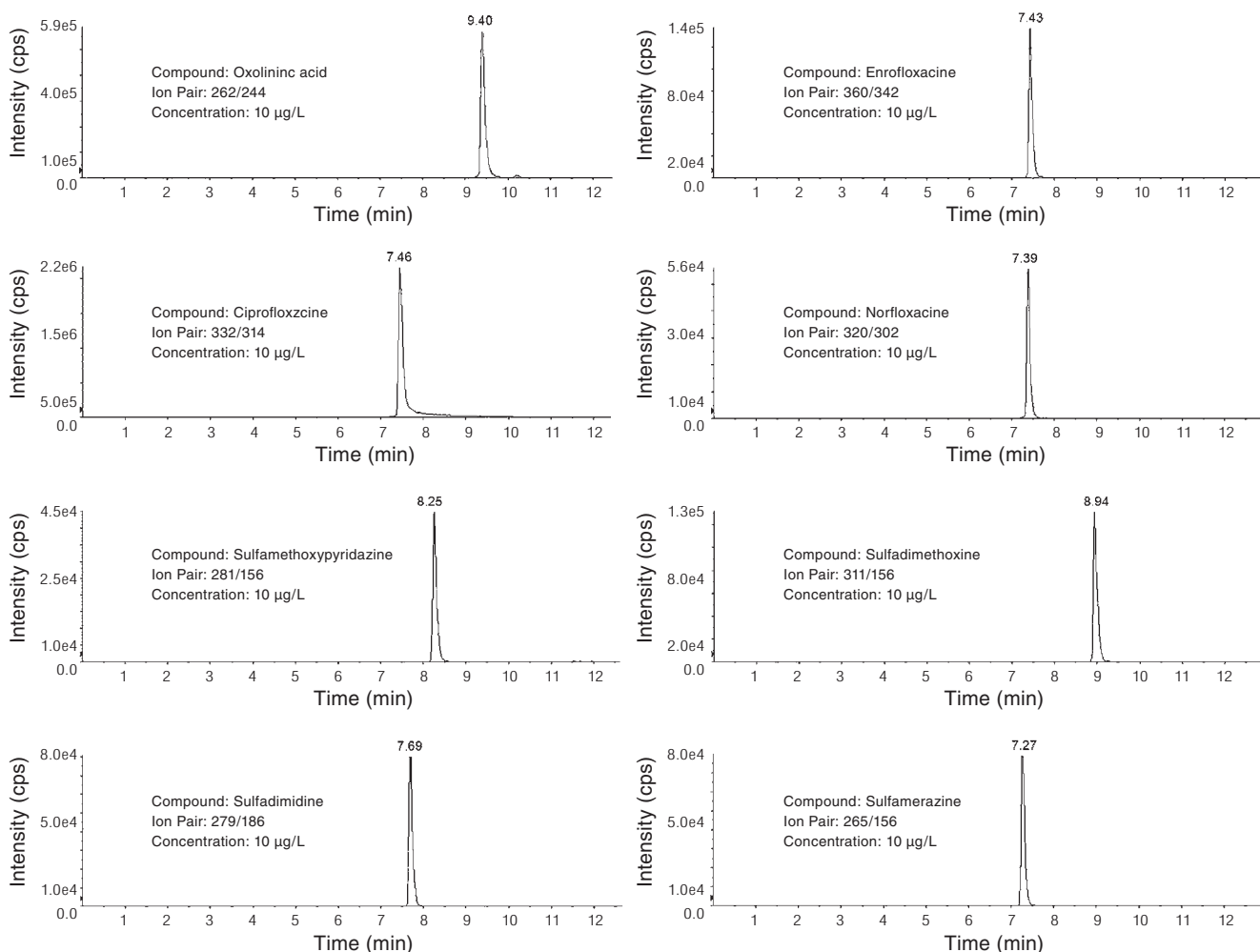

Figure 1. LC-MS/MS chromatograms from a spiked fish sample (10 μg/L) containing oxolininc acid, enrofloxacine, ciprofloxacine, norfloxacine, sulfamethoxypyridazine, sulfadimethoxine, sulfadimidine and sulfamerazine.

injection volume were studied in order to get a fast and reliable separation. The best results were observed at 25°C, 0.7 mL/min as the flow rate and 20 μL volume of injection. Under these conditions, the retention times of the analytes were constant, ranging from 7.28 min (SMZ) to 9.40 min (OXA). A representative chromatogram obtained from a standard mixture of the selected antimicrobials is shown in Figure 1. The calibration curves are shown in Figure 2.

II. *Mass Spectrometry*

The optimization of mass spectrometric parameters was performed by the infusion of a standard solution of 100 μg/L of each antimicrobial in a mixture of water : methanol (50 : 50) at a flow rate of 10 μL/min. The ESI probe in positive mode was selected as the ionization technique due to its sensitivity. First, full-scan spectra were acquired so as to select the most abundant *m/z* value, optimizing the parameters of ion path entrance, collisional focusing quadrupole, offset on collision cell quads and Q_3 entrance lens. In all cases, $[M+H]^+$ ions were found to be the most abundant and these ions were selected as the precursor ions. The most abundant product ions were selected for quantification purposes and a second one for confirmation. The MS/MS transitions for quantification and confirmation for each of the studied compounds are shown in Table 1. Good sensitivity was obtained when ESI in positive mode was applied.

III. *Optimization of the Extraction Procedure*

Sample preparation is often the most critical part of a multi-residue antibiotic method due to the different recoveries of the substances when extracted simultaneously. Furthermore, traditional strategies for the extraction of antimicrobials from milk involve a first step of precipitating the proteins with organic solvent, or in combination with strong acid such as trichloroacetic acid, followed by sample enrichment and clean-up with SPE. In order to simplify the conventional procedure, buffered QuEChERS procedure was evaluated[13,14]. As indicated previously, this has been mainly used for the extraction of different classes of pesticides but it has not been tested for the determination of veterinary drugs in food. Conventional QuEChERS implies a dispersive-SPE clean-up step, using PSA (primary secondary amine) as the
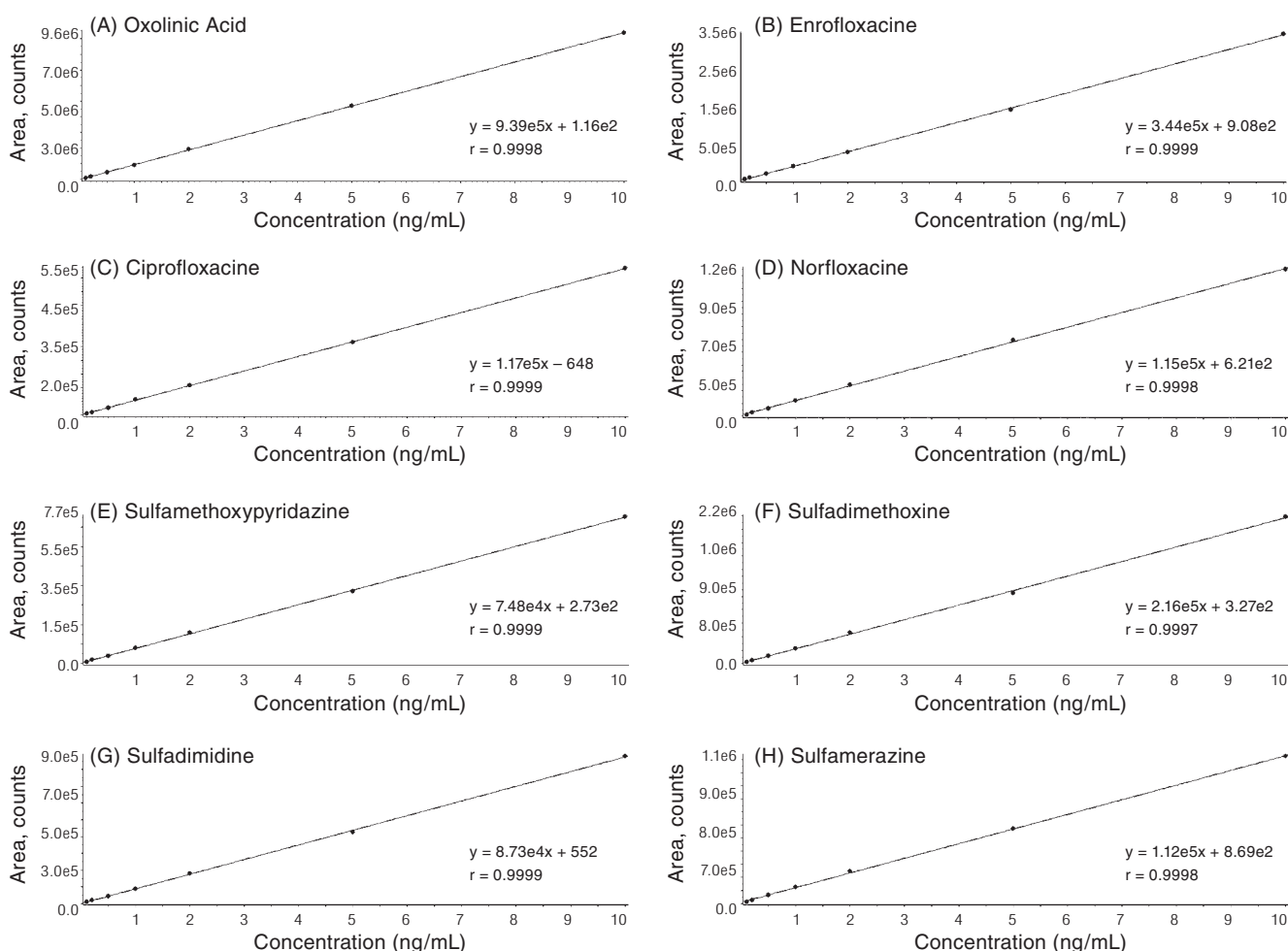

Figure 2. Calibration curves of oxolinic acid (OXA), enrofloxacine (EFA), ciprofloxacine (CFA), norfloxacine (NFA), sulfamethoxypyridazine (SMP), sulfadimethoxine (SDT), sulfadimidine (SDD) and sulfamerazine (SMZ) prepared with spiked concentration levels in fish muscle.

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sorbent material. In this work, SPE was not used, because clean extracts and consistent chromatographic responses were obtained. Therefore the sample treatment time was reduced. When the mixed solvent (0.5 mL of 1% sodium hydroxide aqueous solution and 7 mL of acetonitrile) was added for the extraction of the selected compounds, recoveries were low for SMP, SDT, SDD and SMZ, as can be observed in Table 3. Recoveries were high for CFA and NFA in milkfish muscle (Table 2). The control group used only 7 mL of acetonitrile for extraction. The results showed that recoveries were 80 - 172% for all antimicrobials in the tilapia muscle and 56 - 164% in the milkfish muscle. The organic solvent (1% formic acid in acetonitrile) was used for extraction, and recoveries were 85 - 104% and 75 - 101% for all antimicrobials in the tilapia and milkfish muscles, respectively. The addition of formic acid in the extraction procedure was advantageous for the protein precipitating in the sample tissue.

IV. *Evaluation of Matrix Effect*

When ESI is used as the ionization technique in mass spectrometry, one of the main problems is the signal suppression or enhancement of the analytes due to the other components present in the matrix (matrix effect). To evaluate this matrix effect, five different kinds of fish (tilapia, milkfish, eel, bass and cat fish) were selected and analysis was carried out by spiking a specific concentration level (5.0 µg/L) in the uncontaminated matrices in triplicate. Recoveries were observed in the range of 32 to 175%. The recoveries were compared (Table 3) and it was observed that matrix effect was noticed for some compounds such as CFA, NFA, SMP, SDT, SDD and SMZ, with a matrix enhancement effect for CFA and NFA. However there was no significant difference between different matrices ($p < 0.05$), so each kind of fish could be used as the representative matrix during routine analysis (data was not shown). In order to avoid the matrix effect, matrix-matched calibration standard curves were established to quantify antimicrobials in eel, milkfish, bass and tilapia as the representative matrix and spiked uncontaminated samples of fish at eight concentration levels between 0.1 and 10.0 µg/L were analyzed.

V. *Validation*

Method linearity was assayed by performing calibration curves (matrix-matched calibration) using fish samples spiked with the selected antimicrobials in the range from 0.1 to 10 µg/L. Calibration curves were obtained by least-squares

Table 2. Extraction recoveries of antimicrobials from tilapia and milkfish muscle. Extraction solvents: (a) 7 mL acetonitrile; (b) 7 mL acetonitrile with 0.5 mL 1% HCOOH aqueous solution; (c) 7 mL acetonitrile with 0.5 mL 1% NaOH aqueous solution

Compound	Recovery (%)					
	Tilapia			Milkfish		
	ACN	ACN+HCOOH	ACN+NaOH	ACN	ACN+HCOOH	ACN+NaOH
OXA	107 (7)	95 (7)	95 (2)	56 (2)	85 (3)	51 (1)
EFA	112 (9)	99 (9)	133 (8)	85 (3)	89 (5)	110 (3)
CFA	120 (12)	101 (9)	136 (3)	104 (5)	91 (3)	186 (6)
NFA	172 (19)	104 (8)	120 (8)	164 (8)	101 (4)	289 (15)
SMP	105 (10)	94 (7)	17 (9)	68 (4)	75 (2)	17 (1)
SDX	80 (8)	85 (6)	15 (6)	49 (3)	82 (3)	12 (2)
SDD	130 (7)	86 (7)	23 (7)	73 (5)	86 (2)	20 (3)
SMZ	140 (8)	92 (8)	21 (10)	86 (4)	87 (3)	19 (4)

* Standard deviation of repeatability are given in brackets (n = 5).

Table 3. Evaluation of matrix effects by comparing the recoveries using matrix-matched and solvent standards

Compound	Recovery (%)					
	Solvent (in methanol)	Tilapia	Milkfish	Frozen Eel	Bass	Cat Fish
OXA	104 (4.1)	48 (10.3)	44 (11.5)	65 (10.9)	29 (11.3)	50 (10.6)
EFA	100 (6.8)	21 (9.5)	39 (5.7)	70 (11.8)	72 (12.1)	44 (13.5)
CFA	97 (5.5)	40 (6.7)	20 (6.2)	175 (23.2)	17 (6.2)	48 (12.6)
NFA	98 (9.1)	53 (11.4)	48 (10.6)	164 (25.6)	17 (2.7)	34 (11.7)
SMP	99 (5.2)	15 (4.8)	16 (4.4)	17 (3.2)	15 (3.2)	12 (1.2)
SDT	103 (5.3)	19 (3.8)	18 (5.6)	19 (2.8)	14 (3.2)	13 (4.2)
SDD	103 (3.6)	10 (2.4)	23 (4.5)	16 (7.0)	11 (4.1)	14 (3.5)
SMZ	98 (5.8)	12 (2.9)	23 (3.5)	17 (5.4)	10 (5.1)	13 (2.9)

* Standard deviation of repeatability are given in brackets (n = 5).

Table 4. Validation parameters of the optimized LC-MS/MS method

Compound	Tilapia		Milkfish	
	CCα (µg/kg)	CCβ (µg/kg)	CCα (µg/kg)	CCβ (µg/kg)
OXA	1.89 (0.75)	2.34 (0.94)	1.69 (0.67)	2.09 (0.84)
EFA	2.49 (0.71)	3.08 (0.89)	2.13 (1.51)	2.64 (1.87)
CFA	2.27 (0.35)	2.81 (0.43)	2.36 (1.35)	2.92 (1.67)
NFA	2.74 (0.97)	3.39 (1.20)	1.98 (0.60)	2.44 (0.74)
SMP	2.66 (1.14)	3.29 (1.41)	3.22 (1.85)	3.98 (2.28)
SDX	2.19 (1.00)	2.71 (1.24)	3.05 (2.11)	3.77 (2.60)
SDD	2.21 (0.94)	2.74 (1.16)	3.34 (1.59)	4.13 (1.95)
SMZ	2.88 (1.45)	3.56 (1.80)	3.30 (1.94)	4.12 (2.38)

* Standard deviation given in brackets (n = 5).

linear regression analysis of the peak area which was relative with concentration. The response was linear in the assayed range and the determination coefficients were higher than 0.995 in all the cases.

The decision limit (CCα) and detection capability (CCβ) were calculated following the calibration curve procedure according to ISO 11843 (17)(Commission Decision 2002/657/EC 2002). CCα and CCβ were calculated by analyzing uncontaminated samples spiked at 0.1, 0.2, 0.5, 1.0, 2.0, 5.0 and 10 µg/L (Table 4). CCα ranged from 1.89 to 2.88 µg/kg and 1.69 to 3.34 µg/kg in tilapia and milkfish, respectively. CCβ ranged from 2.34 to 3.56 µg/kg and 2.09 to 4.13 µg/kg in tilapia and milkfish, respectively, which were lower than the MRLs established by the European Union[22]. Finally, the selectivity of the method was evaluated by analyzing uncontaminated control samples.

Although the acidic organic solvent can provoke an increase in the estimated detection capabilities (CCβ), sample dilution has several advantages such as reducing matrix effect of fish tissue and easy evaporation of extraction solvent. Furthermore, the extraction time of each sample was less than 5 min, so this approach could be used as a fast and reliable method for screening target antimicrobials in fish tissue.

CONCLUSIONS

A simple, sensitive and high-throughput method for multi-residue determination of several classes of quinolones and sulfonamides in fish samples was developed and validated. The extraction method was based on simple liquid extraction with 7 mL of acetonitrile (including 0.5 mL of 1% formic acid solution) and no further SPE step was necessary. In order to increase the recovery of sulfonamides, acetonitrile with formic acid should be added during the extraction procedure. Furthermore, protein precipitating was necessary in the extraction step and fat removal with *n*-hexane in the post-extraction step. In addition, the use of LC-MS/MS reduces analysis time and improves sensitivity and resolution, detecting and quantifying several classes of veterinary drugs satisfactorily in less than 15 min. Good validation

parameters such as linearity, recovery, precision, CCα and CCβ were obtained. Eight antimicrobials were determined with a single extraction and the proposed method could be applied in routine analysis. Therefore, the application of both described methodologies in routine analyses would be simple and quick, taking into account that the same sample extract could be analyzed by LC-MS/MS and the screening and confirmation for the presence of one or more of the analyzed quinolones and sulfonamides could be achieved.

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