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Multiclass analysis of antimicrobial drugs in shrimp muscle by ultra high performance liquid chromatography-tandem mass spectrometry



Saksit Susakate^a, Saranya Poapolatthep^a, Chanat Chokejaroenrat^b,
Phanwimol Tanhan^a, Jana Hajslova^c, Mario Giorgi^d, Kanista Saimek^e,
Zhaowei Zhang^f, Amnart Poapolatthep^{a,*}

^a Department of Pharmacology, Faculty of Veterinary Medicine, Kasetsart University, Bangkok 10900, Thailand

^b Department of Environmental Technology and Management, Faculty of Environment, Kasetsart University, Bangkok 10900, Thailand

^c Department of Food Analysis and Nutrition, Faculty of Food and Biochemical Technology, University of Chemistry and Technology, Prague 6-Dejvice, Czech Republic

^d Department of Veterinary Sciences, University of Pisa, Via Livornese, San Piero a Grado, 56122, Pisa, Italy

^e Department of Laboratory, Charoen Pokphand Foods Public Company Limited, Klang, Rayong 21170, Thailand

^f Oil Crops Research Institute of the Chinese Academy of Agricultural Sciences, Wuhan 430062, China

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ABSTRACT

A reliable, selective and rapid multiclass method has been developed for the simultaneous determination of 55 antibacterial drug residues in shrimp muscle samples by ultra high performance liquid chromatography-tandem mass spectrometry. The investigated compounds comprise of eight different classes, namely fluoroquinolones, sulfonamides and synergistic agents, tetracyclines, macrolides, lincosamides, penicillins, nitroimidazole and amphenicols. A simple liquid extraction procedure was developed consisting of extraction with a mixture of acetonitrile and ethylenediaminetetraacetic acid (EDTA), followed by a defatting step with *n*-hexane. Chromatographic conditions were optimized, obtaining a running time <10 min. Mean recoveries ranged from 74.3% to 113.3%. For precision test, relative standard deviations (RSD, %) were lower than 15.0% and 24.0% for repeatability and reproducibility, respectively. Limits of detection and quantification ranged from 1.0 to 5.0 ng/g and 3.0–10.0 ng/g, respectively. Finally, the method was applied to real samples and the results demonstrated that enrofloxacin, ciprofloxacin, pefloxacin and doxycycline were quantifiable in shrimp samples.

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* Corresponding author. Department of Pharmacology, Faculty of Veterinary Medicine, Kasetsart University, 50 Ngamwongwan Road, Chatuchak, Bangkok 10900, Thailand.

E-mail addresses: fvetamp@ku.ac.th, fvetamp@hotmail.com (A. Poapolatthep).

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1. Introduction

Shrimp farming has grown significantly over recent decades, production now exceeds the global catch of all marine shrimps [1]. In 2010, the value of shrimp production accounted for approximately 15% of the total value of globally traded fishery products which made shrimps the largest single commodity. In terms of economic value, shrimp markets continue to show positive growth [2]. However, shrimp farming has faced serious disease outbreaks from viral, bacterial and parasitic infections causing significant economic loss [3]. For this reason, antimicrobial drugs have been widely employed to prevent and treat infectious disease. The use of these agents in aquatic animal production has been an issue in the past, and is of increasing concern for consumers in many countries. The misuse of these compounds including extra label and inappropriate use, can promote the persistence of drugs in animal products. The presence of antimicrobial drug residues in animal products can cause severe adverse health problems for consumers, such as allergic reactions in hypersensitive individuals. Moreover, prolonged exposure of low level doses of antimicrobial drugs may result in development of resistance in pathogenic bacteria, which can transfer from foods to humans, and initiate gene mutations [4–7]. There are several groups of antimicrobial drugs commonly applied in current veterinary practice, such as fluoroquinolones (FQs), sulfonamides (SAs), tetracyclines (TCs), amphenicols (APs), penicillins (PENs), macrolides (MCs), lincosamides (LINs), nitroimidazoles (NIs), and synergistic agents [8]. In addition, the usage of multiple compounds or multiple classes of drug has been noted in food-producing animals. Due to perceived economic benefit, farmers freely use antimicrobial drugs despite their limited knowledge about these agents [5,9,10]. To ensure food safety and protect consumer health, European Union (EU) has defined maximum residue limits (MRLs) for residues of veterinary drugs in food [11]. These limits require the development of sensitive, specific, accurate and precise methods for analysis of the trace residues in food. There have been a number of reports on the variety of chemical analysis techniques used for detecting multiple classes of antimicrobial residues in food of animal origin. Microbiological assays, based on growth inhibition in microorganisms of interest, are commonly used to determine the presence of antimicrobial residues because these methods are somewhat simple, fast and inexpensive. However, these methods generally cannot separate between members of a class of drug, lack sensitivity, and can only provide semi-quantitative measurements of the total amount of residues. These methods sometimes give false-positive results by detecting drug residues at a level far below the officially mandated safe levels [12–14].

Given these limitations, high performance liquid chromatography (HPLC) operated with tandem mass spectrometry (MS/MS) has become the predominant technique for detecting multiple drug classes. It combines analyte separation with structural information, for monitoring antibacterial residues in food matrices such as shrimp. The method requires simple sample pretreatment, provides rapid sample throughput, unambiguous identification and reliable confirmation. Interference is reduced, especially when multiple

reaction monitoring (MRM) mode is used [15,16]. Nowadays, the use of ultra high performance liquid chromatography (UHPLC) shows a variety of advantages in relation to HPLC in terms of selectivity, sensitivity and resolution. In addition, it has features such as reduced time required for analysis, this is likely to be attractive for laboratories running large numbers of routine samples [17–20]. Despite this, the method has limitations for the simultaneous determination of antibacterial drugs from different classes in complex biological matrices, such as shrimp muscle, mainly due to differences in physicochemical properties of the drugs [17,21]. Hence, it is a challenge to develop an UHPLC-MS/MS method, which can simultaneously detect multiple residues, and multiple classes of antimicrobial drugs in shrimp products. To date, only a small number of methods for detecting and quantifying multiple classes of drug in shrimp muscle are available. Villar–Pulido et al. developed a fast liquid chromatography time-of-flight mass spectrometry (LC-TOFMS) method for simultaneous quantification of 7 residues of selected antibiotics and other veterinary drugs (benzalkonium chloride, ethoxyquin, leucomalachite green, malachite green, meben-dazole, sulfathiazole and trimethoprim) in shrimp. The selected extraction method was QuEChERS. Recovery rates were in the range of 58–133%. Although it can be applied for screening and quantification purposes, it cannot be used as a confirmatory method because of the requirements of legislation [15] positive findings need to be confirmed using a MS/MS detector [22]. Storey et al. proposed the HPLC-MS/MS screening method for the detection and identification of 26 veterinary drugs in fish and shrimp. The analytes included 13 sulfonamides, trimethoprim, three fluoroquinolones, three quinolones, three triphenylmethane dyes, two leuco dye metabolites, and one hormone. In this method, tissue is mixed with ethylenediaminetetraacetic acid (EDTA)-Mcllvaine buffer, double-extracted with acetonitrile, p-toluenesulfonic acid (p-TSA acid) and N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride (TMPD) [23]. Kim et al. described a multi-class, multi-residue analytical method based on UHPLC-MS/MS detection, this was developed for the screening and confirmation of 28 veterinary drugs and metabolite residues in fish and shrimp. To achieve fast and simultaneous extraction of various analytes, a simple and generic liquid extraction procedure using EDTA-ammonium acetate buffer and acetonitrile, without clean-up step, was applied to sample preparation. Mostly, the recoveries were in the range of 60–110%, and precision, expressed as the relative standard deviation (RSD), was in the range of 5–15% [24]. To the best of our knowledge, this work is the first UHPLC-ESI-MS/MS method for the simultaneous determination of drugs belonging to different physiochemical classes in trace amounts in shrimp muscle.

This research aimed to develop and validate a rapid, effective UHPLC-MS/MS and simple liquid-extraction method for detection of 55 compounds in shrimp muscle. These compounds consisted of 52 antimicrobial drugs from 8 different families, namely Fluoroquinolones (FQs), Sulfonamides (SAs), Tetracyclines (TCs), Macrolides (MCs), Lincosamides (LINs), Penicillins (PENs), Nitroimidazoles (NIs) and Amphenicols (APs), and included 3 synergistic agents, namely, trimethoprim, ormetoprim and dapsone.

Table 1 – The MS/MS parameters of the selected antibacterial drugs.

Compound	Abbreviated name	Precursor ion	Product ion	Fragmentator (V)	Collision energy (eV)	Retention time (min)
Fluoroquinolones	FQs					
Enrofloxacin	ENR	360.2	316.2	156	16	3.68
			286.1	156	36	
Ciprofloxacin	CIP	332.1	231.1	150	40	3.49
			288.2	150	20	
Difloxacin	DIF	400.1	356.2	156	16	3.97
			299.1	156	32	
Sarafloxacin	SAR	386.1	368.1	150	20	3.92
			299.1	150	40	
Norfloxacin	NOR	320.0	302.1	150	20	3.42
			231.1	150	40	
Danofloxacin	DAN	358.2	340.1	151	20	3.58
			82.1	151	48	
Ofloxacin	OFL	362.2	318.2	150	20	3.44
			261.1	150	40	
Gatifloxacin	GAT	376.3	261.0	130	36	3.82
			289.0	130	23	
Moxifloxacin	MOX	402.2	384.2	150	20	4.09
			96.1	150	40	
Pefloxacin	PEF	334.0	316.1	132	19	3.46
			290.1	132	19	
Marbofloxacin	MAR	363.2	72.2	144	28	3.35
			320.1	144	12	
Fleroxacin	FLE	370.1	326.1	150	20	3.45
			58.1	150	40	
Sparfloxacin	SPA	393.1	349.0	140	20	3.96
			292.0	140	23	
Orbifloxacin	ORB	396.0	352.0	120	15	3.76
			295.0	120	22	
Oxolinic acid	OXO	262.1	202.0	114	32	6.59
			215.9	114	29	
Flumequine	FLU	262.1	202.0	108	32	6.59
			244.1	108	12	
Nalidixic acid	NAL	233.1	215.1	150	20	6.29
			104.0	150	40	
Sulfonamides	SAs					
Sulfadiazine	SDZ	251.1	92.1	96	28	3.16
			156.0	96	8	
Sulfadimethoxine	SDMX	311.1	156.0	128	16	5.27
			92.1	128	36	
Sulfamerazine	SMR	265.1	92.1	114	28	3.60
			156.0	114	12	
Sulfamethazine	SMZ	279.1	186.1	132	12	3.92
			92.1	132	32	
Sulfamethoxazole	SMXZ	254.1	92.1	108	24	4.58
			156.0	108	12	
Sulfaquinoxaline	SQX	301.1	156.0	118	16	5.27
			92.0	118	32	
Sulfachloropyridazine	SCP	285.0	156.0	108	12	4.39
			92.1	108	24	
Sulfadoxine	SDX	311.1	156.0	126	16	4.58
			92.1	126	32	
Sulfamethoxypyridazine	SMPZ	281.1	156.0	120	12	3.94
			92.1	120	32	
Sulfamoxole	SMX	268.1	92.0	150	20	3.74
			108.0	150	20	
Sulfonamides	SAs					
Sulfamethizole	SMTZ	271.0	156.0	150	10	3.89
			108.0	150	20	
Sulphisoxazole	SFX	268.1	92.0	150	40	4.77
			108.0	150	20	
Sulfathiazole	STZ	256.0	92.1	102	28	3.31
			156.0	102	12	

Table 1 – (continued)

Compound	Abbreviated name	Precursor ion	Product ion	Fragmentator (V)	Collision energy (eV)	Retention time (min)
Sulfaphenazole	SPZ	315.1	158.1	150	40	5.42
			92.0	150	40	
Sulfapyridine	SPD	250.1	92.0	150	20	3.43
			108.0	150	20	
Sulfamonomethoxine	SMM	281.0	155.9	120	17	4.22
			126.0	120	21	
Synergistic agents	SYNs					
Trimethoprim	TMP	291.2	230.1	151	20	3.38
			123.1	151	24	
Ormetoprim	OMP	275.2	123.0	122	25	3.54
			259.0	122	25	
Dapsone	DAP	249.1	92.0	151	28	4.29
			156.0	151	10	
Tetracyclines	TCs					
Oxytetracycline	OTC	461.0	426.0	120	20	3.50
			444.0	120	10	
Tetracycline	TC	445.2	410.1	150	20	3.67
			98.1	150	40	
Chlortetracycline	CTC	479.1	444.0	120	20	4.22
			462.0	120	12	
Doxycycline	DC	445.2	428.1	113	16	4.36
			321.1	113	28	
Macrolides	MCs					
Erythromycin	ERY	734.5	158.1	171	32	5.13
			576.3	171	16	
Tilmicosin	TIL	869.6	174.1	294	48	4.43
			696.4	294	44	
Tylosin	TYL	916.5	174.1	228	44	5.63
			101.0	228	56	
Josamycin	JOS	828.5	174.1	228	36	6.91
			109.0	228	56	
Spiramycin I	SPI	843.5	101.0	150	52	3.97
			43.2	150	68	
Lincosamides	LINs					
Lincomycin	LIN	407.2	126.1	150	24	3.10
			359.2	150	16	
Clindamycin	CLI	425.2	126.1	150	20	4.25
			377.2	150	20	
Penicillins	PENs					
Ampicillin	AMP	350.0	106.0	113	16	3.26
			160.0	113	4	
Oxacillin	OXA	402.0	160.0	113	8	6.91
			243.0	113	8	
Dicloxacillin	DIC	470.0	160.0	113	10	7.36
			311.0	113	6	
Penicillin G	PEN G	335.1	160.0	65	6	5.82
			176.1	65	6	
Nitroimidazoles	NIs					
Dimetridazole	DMZ	142.1	96.1	100	16	3.10
			81.1	100	28	
Metronidazole	MNZ	172.1	128.0	98	12	2.74
			82.1	98	24	
Ronidazole	RNZ	201.1	140.1	80	8	3.08
			55.2	80	20	
Amphenicols	APs					
Florfenicol	FF	356.0	185.1	141	12	4.65
			336.0	141	4	

2. Materials and methods

2.1. Chemicals and reagents

All antimicrobial standards were purchased from Sigma–Aldrich (St. Louis, MO, USA). The investigated analytes are listed in Table 1. Stock standard solutions of each compound at concentration 100 µg/mL were prepared in the appropriate solvents. Sulfonamides, synergistic agents, macrolides and amphenicols were prepared in acetonitrile. Penicillins were prepared in acetonitrile:water (1:1, v/v), and the other classes of antimicrobial drugs were dissolved in methanol. The solutions were stored in the dark at –20 °C away from light for three months (except penicillins, these were kept at 4 °C). Cocktail working standard solutions of each group were prepared by combining each of individual stock solution and diluting with acetonitrile to a final concentration of 1 µg/mL, and they were stored at 4 °C in the dark. These solutions were re-prepared weekly. Suitable dilutions were also prepared to facilitate spiking for both the validation process and the routine analysis. All reagents were of analytical grade with the exception of chemicals used for the mobile phase, which were of HPLC grade. Acetonitrile, methanol and formic acid were HPLC grade, and were purchased from RCI Labscan (Bangkok, Thailand), Honey well (New Jersey, USA) and Merck (New Jersey, USA), respectively. Ethyl acetate and *n*-hexane were purchased from Avantor (Seoul, Korea). Ethylenediamine-tetra-acetic acid disodium salt (Na₂EDTA) and sodium sulfate (Na₂SO₄) were purchased from Merck (Schiphol-Rijk, Netherlands). Ultrapure water with 18.2 MΩ/cm was obtained from a Millipore Milli-Q system (Bedford, MA, USA).

2.2. Samples and sample preparation

Shrimp samples were collected during October–December 2017 from local market in Thailand, and they were confirmed to be free of selected drug residues by UHPLC-MS/MS. The head, shell, tail and intestinal organs of the shrimp were removed prior to analysis. Muscle tissues were blended using a Moulinex mixer, and stored at –20 °C until analysis. Then, the targeted residues were extracted from shrimp muscle using simple liquid extraction modified from Freitas A. et al. [25]. Briefly, the optimized procedure was as follows; 1.0 g of homogenized sample was weighed into a 50 mL plastic centrifuge tube followed by addition of 1 g of Na₂SO₄ anhydrous, 800 µL of 0.05 M EDTA solution, 3 mL of *n*-hexane and 4 mL of acetonitrile, respectively. Then, the mixture was homogenized using a homogenizer until it was homogeneous sample. It was centrifuged at 4000 rpm (2688 × *g*), 4 °C for 10 min. After centrifugation, the acetonitrile layer was transferred to a 15 mL glass tube, and the pellet was re-extracted by addition of 3 mL of *n*-hexane, and 4 mL of acetonitrile. The sample was shaken for 5 min, and centrifuged again, as described above. The acetonitrile layer was then transferred to the glass tube before the total acetonitrile layer was evaporated to dryness at 40 °C under a nitrogen stream. Finally, the residue was dissolved with 0.5 mL of a mixture of 0.1% formic acid solution and acetonitrile (1:1, v/v). The extract was filtered by passing through a 0.22 µm Nylon membrane filter

(Sartorius AG, Goettingen, Germany) and then injected into the unit for UHPLC-MS/MS.

2.3. Instrumental and chromatographic conditions

UHPLC analysis was performed using an Agilent 1290 infinity (Agilent Technologies, Waldbronn, Germany) consisting of a binary pump, a degasser, a column oven and an auto sampler. The chromatographic separation was achieved on a ZORBAX RRHD Eclipse plus C18 column (100 × 2.1 mm, 1.8 µm particle size). The column was maintained at 40 °C. The mobile phase consisted of 0.1% formic acid in water (mobile phase A) and acetonitrile (mobile phase B). The gradient program of the mobile phase was as follows: 5% B (initial for 0.5 min), 5–35% B (5 min) and 95% B (10 min). The column was re-equilibrated for 5 min between injections. The flow rate was 0.3 mL/min while the injection volume was 5 µL. The chromatographic run time was 10 min.

The mass spectrometer used was a triple quadrupole analyzer (6460 triple, Agilent Technologies, Waldbronn, Germany) equipped with an electrospray ionization source run in both positive and negative ion modes under the multiple reaction monitoring (MRM) mode with two separate chromatographs analyzed per sample. The ionization source parameters were optimized as follows: capillary voltage 3500 V, gas temperature 300 °C, gas flow rate 8 L/min and nebulizer pressure 45 psi. The parameters (for the mass spectrometer) were optimized as shown in Table 1.

2.4. Validation procedure

Performance characteristics of the optimized method was determined to assess the efficiency of this analytical method by investigating the selectivity, matrix effect, linearity, limit of detection, limit of quantitation, accuracy, precision and applicability in accordance with the European Commission regulation for the performance of analytical methods [15].

3. Results and discussion

3.1. Optimization of UHPLC-MS/MS conditions

For multi-class analytical method, UHPLC-MS/MS was developed for simultaneous analysis in the single run with positive and negative electrospray ionization mode. For both ionization modes, the best sensitivity for all drugs was determined to provide the highest signals for quantification and confirmation. It was difficult to simultaneously evaluate 55 antimicrobial drugs with a short dwell time. Thus, dynamic multiple reaction monitoring (d-MRM) was used. The advantage of d-MRM is that it allows the MS/MS system to be focused directly on the expected retention time for an analyte in a defined retention time window. The sensitivity can be enhanced since optimal dwell times can be automatically achieved under d-MRM by reducing the number of concurrent ion transitions [26].

To achieve maximum response, standard solutions (0.5 µg/mL) for individual analytes were injected directly into the quadrupole mass spectrometer without the analytical column

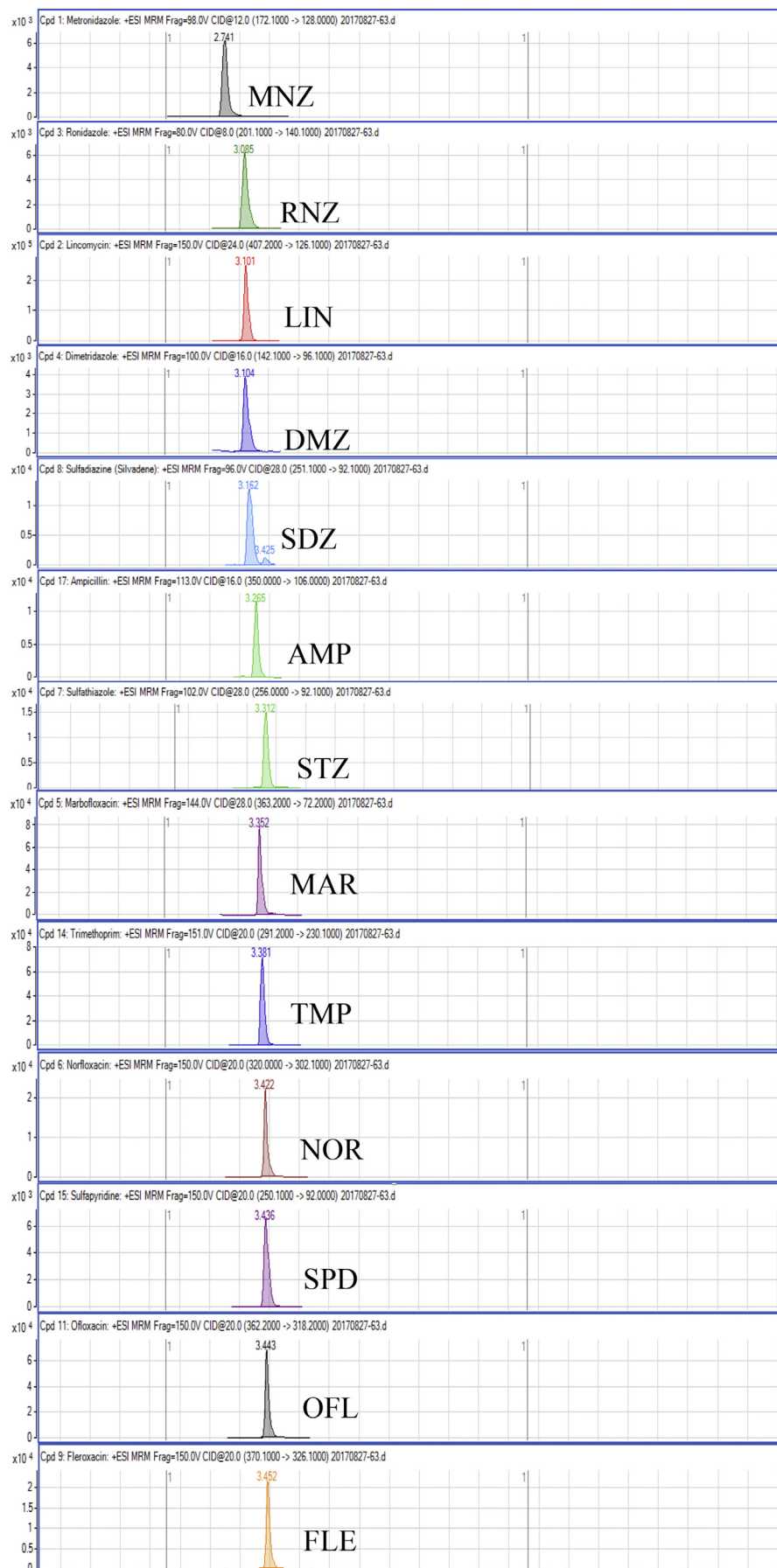


Fig. 1 – UHPLC-MS/MS chromatograms of investigated antibacterial drugs spiked at 15 ng/g for nitroimidazoles and 50 ng/g for the other compounds in a blank shrimp muscle sample.

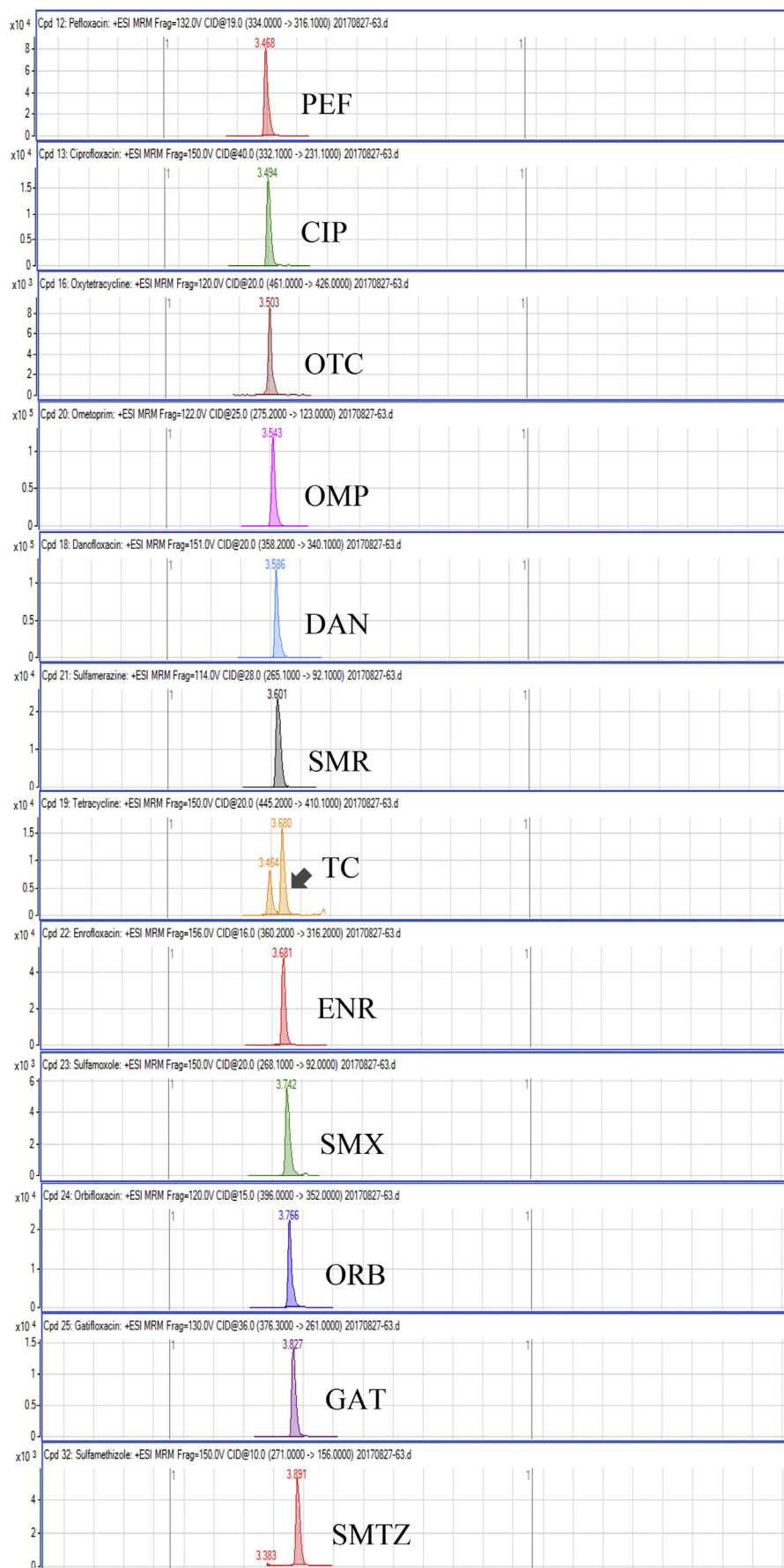


Fig. 1 – (continued).

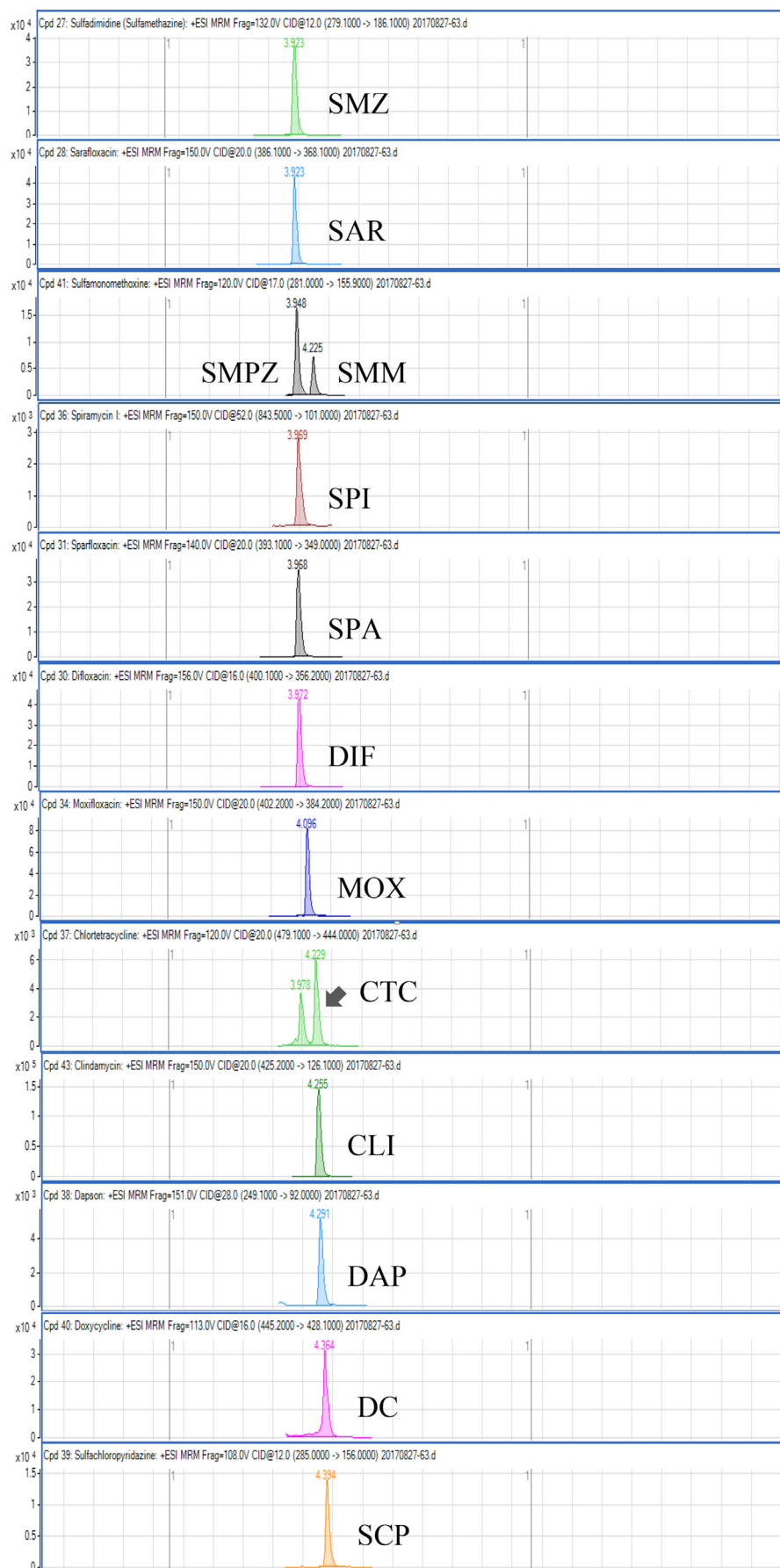


Fig. 1 – (continued).



Fig. 1 – (continued).

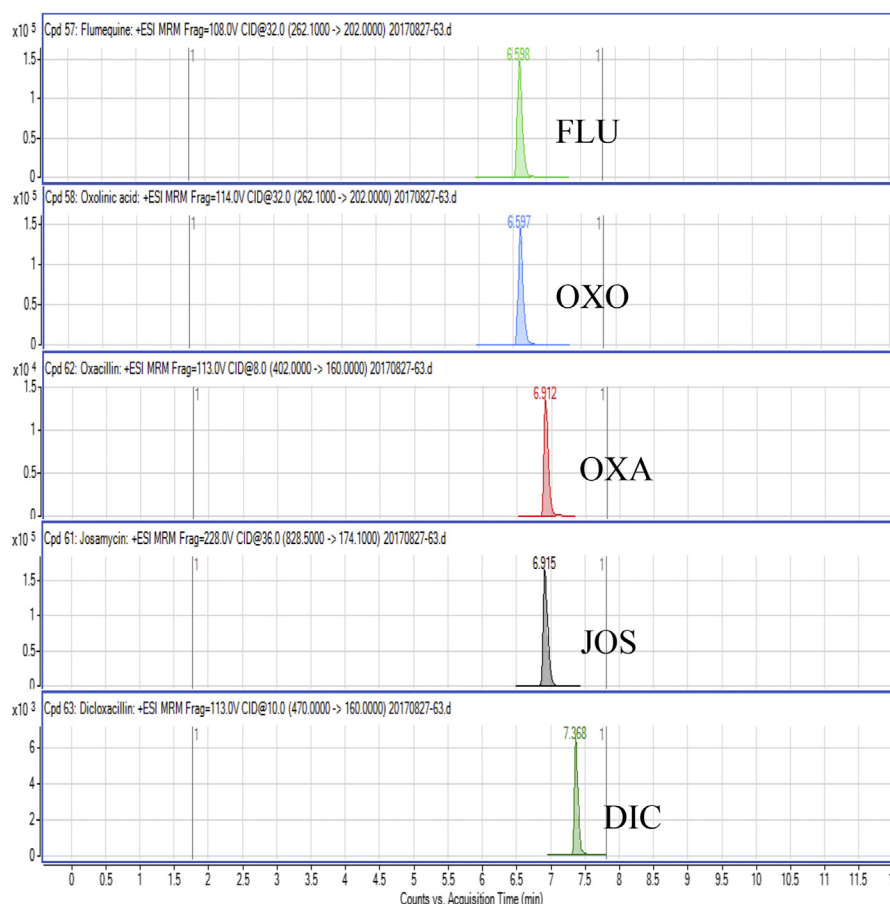


Fig. 1 – (continued).

connected and detected in full scan ion detection mode to obtain two transitions per compound as precursor and product ions. The deprotonated molecular ion $[M-H]^-$ was selected as the precursor ion for amphenicols whereas the rest of the compounds were detected as the protonated molecular ion $[M+H]^+$. Proposed mass fragmentation patterns of investigated antimicrobial drugs using the method described below and the results obtained in this work concur with previous works that have already been discussed [27–31].

For FQs, the most intense fragment ion observed is $[MH-H_2O]$. The loss of CO_2 and the further loss of piperazinic ring fragments (C_2H_5N for SAR, C_3H_7N for DIF and C_4H_9N for ENR) are other product ions for FQs with a piperazinyl ring. Oxolinic acid (OXO) and flumequine (FLU), which have no piperazinyl ring, showed less fragmentation. Besides the $[MH-H_2O]$ ions at m/z 216 and 202 are the most intense fragments observed for OXO and FLU, respectively. For SAs, two characteristic fragment ions with m/z 156 and 92 were observed. The former corresponds to the common molecular fragment for all SAs, the p-sulfoaniline group, and the ion with m/z 92 was the result of the loss of the sulfonyl group from this structure. TCs have a structure formed by an octahydrotracene-2-carboxamide skeleton. Characteristic fragmentation patterns for the tetracyclines studied are the loss of water or ammonia. MCs are 12-, 14- or 16-membered macrocyclic lactone with sugar moieties attached to them by a glycosidic

bond. In general, the loss of sugar moiety is the most intense fragment ion observed. Also, the sugar specific group can form product ions, which is demonstrated by the presence of ions at m/z 174 and 158 corresponding to the two sugar moiety [orthodesosamine + H] $^+$ and [desosamine + H] $^+$, respectively. LCs are a group of monoglycosides containing an amino acid-like side chain. Both LINs, lincomycin (LIN) and clindamycin (CLI) shared a fragment of m/z 126 corresponding to the loss of the monoglycoside with amino acid-like side chain ring. Characteristic fragmentation ions for the LINs studied are the loss of thiomethyl group $[M-SCH_3]^+$ on monoglycoside ring. The basic structure of PENs consists of a thiazolidinic ring condensed on a β -lactam ring with a lateral chain. All PENs studied share a fragment of m/z 160 corresponding to the thiazolidinic ring. The characteristic fragment is the result of the presence of the ion formed due to the loss of this fragment $[M + H - 159]$. Another characteristic fragment is the result of the loss of the carboxylic group from the 160 fragment to obtain a fragment of m/z 114. NIs are a group with a 5-nitroimidazole cyclic ring. Characteristic fragmentation patterns for the NIs studied are due to the loss of the chemical group at the side-chain on the imidazole ring. The characteristic is the presence of the ion formed due to the loss of nitro group. FF is a representative of the amphenicol group as it contains a p-sulfomethyl phenyl ring bonded with a fluoropropaneidol moiety and a dichloroacetamide side chain. The product ion was dominated by

neutral loss of HF to give $[M-HF]^-$ ions and another characteristic fragment is due to the loss of dichloroacetamide side chain.

Different mobile phases consisting of methanol or acetonitrile, and acidified water with different concentrations of formic acid (0.01% and 0.1%, v/v) were tested to provide the best chromatographic results. The final result showed 0.1% formic acid in water (as aqueous phase) and acetonitrile (as organic phase) provided the best overall sensitivity. In order to shorten the analysis time, UHPLC technique was performed using conditions as described in section 2.3. Due to the differences in physiochemical properties of antimicrobial drugs, a gradient program from 95% aqueous phase to 95% acetonitrile was applied to elute 55 compounds within 10 min.

Regarding the results obtained from the optimization of UHPLC-MS/MS conditions, there are five isomeric pairs of compounds that have the mass transitions in common (OXO/FLU, tetracycline (TC)/doxycycline (DC), sulfadimethoxine (SDMX)/sulfadoxine (SDX), sulfamonomethoxine (SMM)/

sulfamethoxyypyridiazine (SMPZ), sulfamoxole (SMX)/sulphisoxazole (SFX). For OXO/FLU, they were eluted at the same time, but they could be differentiated by specific confirmation ions in the MRM mode. The remaining isomeric compounds could be separated by retention time, and the separation of these compounds was excellent under the developed conditions. Fig. 1 shows a chromatogram of the investigated antimicrobial drugs (quantifier transition ion only, is shown), obtained by spiking a blank sample at 15 ng/g for NIs and 50 ng/g for the other compounds.

3.2. Optimization of the extraction procedure

Sample preparation is an important part of a multiclass analysis because of the different properties of the antimicrobial drugs that must be extracted simultaneously. A small number of methods for detecting and quantifying multi classes of drug in shrimp are available. Villar–Pulido et al. developed a fast liquid chromatography time-of-flight mass spectrometry (LC-TOFMS) method for simultaneous quantification of 7 residues of selected antibiotics and other

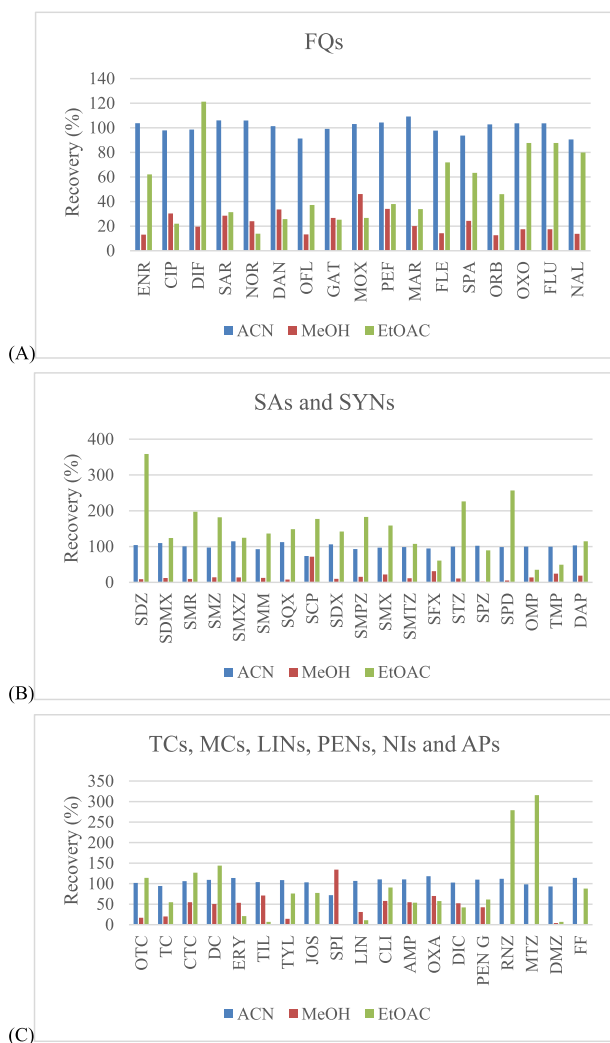


Fig. 2 – Effect of different extraction solvents in the solid–liquid extraction procedure for shrimp muscle samples: (A) – fluoroquinolones; (B) – sulfonamides; (C) – the rest of other groups.

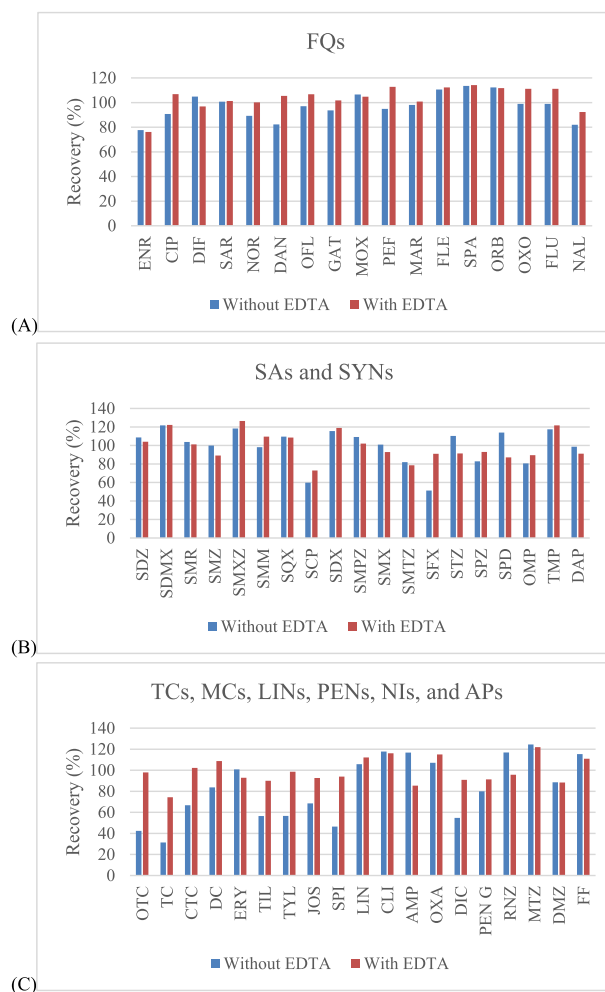


Fig. 3 – Effect of EDTA addition on extraction recovery of selected antibacterial drugs from shrimp muscle sample: (A) – fluoroquinolones; (B) – sulfonamides; (C) – the rest of other groups.

veterinary drugs (benzalkonium chloride, ethoxyquin, leucomalachite green, malacite green, mebendazole, sulfathiazole and trimethoprim) in shrimp and used acetonitrile as solvent followed by a clean-up step with primary secondary amine (QuEChERS). Recovery rates were in the range of 58–133% [22]. Storey et al. proposed the HPLC-MS/MS screening method for the detection and identification of 26 veterinary drugs in fish and shrimp. The analytes included 13 sulfonamides, trimethoprim, three fluoroquinolones, three quinolones, three triphenylmethane dyes, two leuco dye metabolites, and one hormone. In this method, tissue is mixed with ethylenediaminetetraacetic acid (EDTA)-Mcllvaine buffer, double-extracted with acetonitrile, p-toluenesulfonic acid (p-TSA acid) and N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride (TMPD) [23]. Kim et al. described a multi-class, multi-residue analytical method based on UHPLC-MS/MS detection, this was developed for the screening and confirmation of 28 veterinary drugs and metabolite residues in fish and shrimp. To achieve fast and simultaneous extraction of various analytes, a simple and generic liquid extraction procedure using EDTA-ammonium acetate buffer and acetonitrile, without clean-up step, was applied to sample preparation. Mostly, the recoveries were in the range of 60–110%, and precision, expressed as the relative standard deviation (RSD), was in the range of 5–15% [24]. It is difficult to obtain good recoveries, and minimize the loss of all analytes during the extraction step. Multi-step and complex sample clean-ups can result in loss of some target compounds. Therefore a simple extraction method, described previously in section 2.2, was employed as the extraction method in this work and the recovery values of this work were higher than the previous studies [22–24].

Three organic solvents including acetonitrile, methanol and ethyl acetate were evaluated for use in sample extraction.

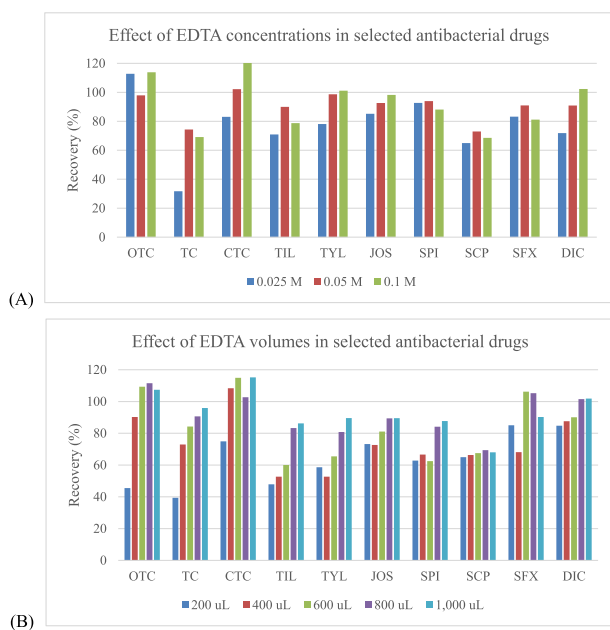


Fig. 4 – Effect of concentrations and volumes of EDTA in the selected antibacterial drugs: (A) – Effect of EDTA concentrations; (B) – Effect of EDTA volumes.

Acetonitrile showed the best extraction efficiency, and provided satisfied recovery values that were better than those for methanol and ethyl acetate for all drugs (Fig. 2). EDTA can be used in addition to improve the recovery of some antimicrobial drugs since these drugs can form chelation complexes with cations present in solution. However, these complexes can be lost during extraction procedure [32,33]. The EDTA, a quelating agent, was added, especially to compete with antibacterial drugs as tetracyclines and macrolides. It is known that these compounds can form complexes with the bivalent and trivalent cations present in the sample extraction solution which can lead to significant losses of those compounds during the procedure. Thus, EDTA can be used to improve the recovery of some antimicrobial drugs. From our experiments, the recovery was somewhat low for tetracyclines (oxytetracycline (OTC), TC and chlortetracycline (CTC)), macrolides (tilmicosin (TIL), tylosin (TYL), josamycin (JOS) and spiramycin

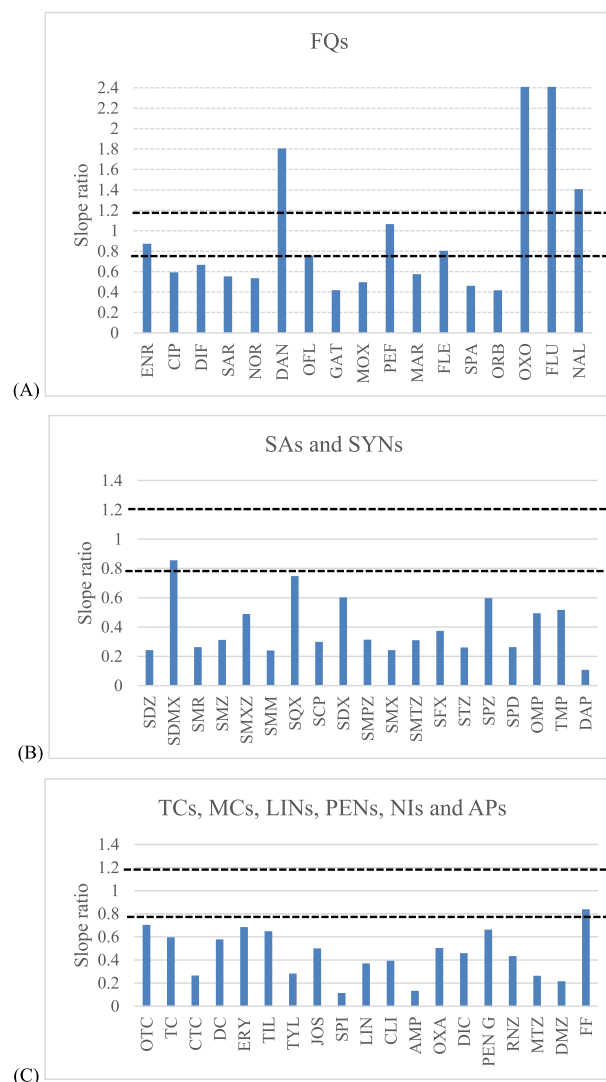


Fig. 5 – Slope ratios for all antibacterial drugs between matrix-matched and solvent calibration. The acceptable range of slope ratios between 0.8 and 1.2 has been plotted: (A) – fluoroquinolones; (B) – sulfonamides; (C) – the rest of other groups.

Table 2 – Maximum residue limit (MRL), limit of detection (LOD) and limit of quantification (LOQ) of the optimized UHPLC-MS/MS method for simultaneous determination of antimicrobial drugs.

Compound	MRL (ng/g)	LOD (ng/g)	LOQ (ng/g)
Fluoroquinolones			
ENR	100 ^a	2.0	5.0
CIP	100 ^a	2.0	5.0
DIF	300	2.0	5.0
SAR	– ^b	2.0	5.0
NOR	– ^b	2.0	5.0
DAN	100	2.0	5.0
OFL	– ^b	2.0	5.0
GAT	– ^b	2.0	5.0
MOX	– ^b	2.0	5.0
PEF	– ^b	2.0	5.0
MAR	– ^b	2.0	5.0
FLE	– ^b	2.0	5.0
SPA	– ^b	2.0	5.0
ORB	– ^b	2.0	5.0
OXO	100	2.0	5.0
FLU	200	2.0	5.0
NAL	– ^b	2.0	5.0
Sulfonamides			
SDZ	100 ^c	2.0	5.0
SDMX	100 ^c	2.0	5.0
SMR	100 ^c	2.0	5.0
SMZ	100 ^c	2.0	5.0
SMXZ	100 ^c	2.0	5.0
SQX	100 ^c	2.0	5.0
SCP	100 ^c	2.0	5.0
SDX	100 ^c	2.0	5.0
SMPZ	100 ^c	2.0	5.0
SMX	100 ^c	2.0	5.0
SMTZ	100 ^c	2.0	5.0
SFX	100 ^c	2.0	5.0
STZ	100 ^c	2.0	5.0
SPZ	100 ^c	2.0	5.0
SPD	100 ^c	2.0	5.0
SMM	100 ^c	2.0	5.0
Synergistic agents			
TMP	50	2.0	5.0
OMP	– ^b	2.0	5.0
DAP	– ^d	2.0	5.0
Tetracyclines			
OTC	100	2.0	5.0
TC	100	2.0	5.0
CTC	100	2.0	5.0
DC	– ^b	2.0	5.0
Macrolides			
ERY	200	2.0	5.0
TIL	50	2.0	5.0
TYL	100	2.0	5.0
JOS	– ^b	2.0	5.0
SPI	– ^b	2.0	5.0
Lincosamides			
LIN	100	2.0	5.0
CLI	– ^b	2.0	5.0
Penicillins			
AMP	50	2.0	5.0
OXA	– ^b	2.0	5.0
DIC	300	2.0	5.0
PEN G	50	2.0	5.0
Nitroimidazoles			
DMZ	– ^d	1.0	3.0
MNZ	– ^d	1.0	3.0

Table 2 – (continued)

Compound	MRL (ng/g)	LOD (ng/g)	LOQ (ng/g)
RNZ	– ^d	1.0	3.0
Amphenicols			
FF	100	5.0	10.0

^a MRL expressed as sum of ENR and CIP.^b MRL not established for this compound in the matrix evaluated.^c MRL expressed as sum of all sulfonamides.^d MRL cannot be established (prohibited drug).

I (SPI)), sulfonamides (sulfachlorpyridazine (SCP) and sulphisoxazole (SFX)) and penicillins (dicloxacillin (DIC)). To avoid this situation, EDTA was also added during the extraction step, whereas the other antimicrobial drugs had satisfactory recovery (Fig. 3). Given this finding, several concentrations (from 0.025 to 0.1 M) and volumes (from 200 to 1000 µL) of EDTA were examined. The results showed that the concentrations at 0.05 M and 0.1 M included the volumes at 800 µL and 1000 µL demonstrated the acceptable recovery for effecting antibacterial drugs. However, the volume of 800 µL (0.05 M) found to be the most appropriate for this procedure (Fig. 4).

3.3. Method validation

The method was validated in shrimp muscle matrix based on European Commission Decision 2002/657/EC [15].

3.3.1. Selectivity

The selectivity was evaluated by analyzing 20 blank shrimp muscle samples using the developed method. The selectivity of this method was good with no interferences, above a signal to noise ratio of 3, at the retention time of each compound.

3.3.2. Matrix effect

A major factor to avoid when ESI is used as the ionization technique in mass spectrometry, is the signal suppression or enhancement of the targeted drugs due to the interferences present in the matrix (matrix effect). To evaluate matrix effect, several standards in pure solvents and in the blank shrimp muscle matrix from the investigated range (3–15 ng/g for NIs, 10–50 ng/g for APs and 5–50 ng/g for the rest of antimicrobial drugs) were analyzed. The slopes obtained in the calibration with the post extracted shrimp muscle were compared with those obtained with solvent standard. Then, matrix/solvent slope ratios for each compound were obtained (Fig. 5) considering a signal enhancement or suppression effect as acceptable if the slope ratio ranged from 0.8 to 1.2. Slope ratios with values higher than 1.2 or lower than 0.8 indicate a strong matrix effect [34]. A matrix effect was demonstrated for most of the investigated compounds, 4 and 47 compounds showed signal enhancement and suppression, respectively, except for enrofloxacin (ENR), pefloxacin (PEF), sulfadimethoxine (SDMX) and florfenicol (FF). Therefore, the matrix-matched calibration (MMC) curves were used for quantitative analysis and compensated this phenomenon.

3.3.3. Linearity

Linearity was evaluated using MMC at five concentration levels ranging from 3 to 15 ng/g for NIs, 10–50 ng/g for APs and

Table 3 – Accuracy and precision for antimicrobials determination in optimal UHPLC-MS/MS conditions for spiked shrimp muscle samples.

Compound	Recovery (%)			Interday precision (RSD %)		
	5 ng/g	20 ng/g	50 ng/g	5 ng/g	20 ng/g	50 ng/g
Fluoroquinolones						
ENR	94.8 (7.9) ^a	107.6 (6.0)	98.9 (4.8)	11.5	5.8	6.4
CIP	111.8 (8.2)	97.3 (4.4)	88.35 (5.4)	16.0	11.5	11.4
DIF	106.9 (8.6)	107.2 (5.7)	96.2 (3.2)	13.9	9.8	8.3
SAR	103.9 (10.0)	93.2 (4.5)	89.6 (4.8)	18.1	6.3	6.8
NOR	107.1 (7.9)	97.4 (4.6)	91.3 (5.3)	17.4	8.7	8.9
DAN	105.1 (10.1)	98.1 (4.0)	89.4 (4.5)	18.5	12.7	14.2
OFL	109.5 (6.4)	99.8 (4.9)	87.3 (4.4)	18.5	7.8	11.3
GAT	109.8 (7.4)	103.7 (4.7)	97.0 (6.2)	22.7	8.9	10.6
MOX	108.9 (8.0)	105.4 (3.5)	97.9 (7.1)	18.7	14.8	11.0
PEF	94.2 (10.7)	100.1 (3.2)	96.6 (6.7)	20.4	10.6	17.2
MAR	104.9 (8.7)	104.6 (3.1)	99.8 (5.2)	14.7	12.8	11.2
FLE	96.9 (11.2)	103.7 (4.5)	92.5 (5.0)	23.0	8.4	9.7
SPA	107.8 (11.7)	97.7 (9.5)	87.1 (4.0)	12.0	13.7	11.3
ORB	104.7 (9.6)	99.8 (9.2)	90.0 (7.3)	19.7	6.6	7.5
OXO	113.1 (5.1)	97.2 (9.2)	84.7 (6.7)	20.6	12.7	10.4
FLU	102.5 (11.3)	106.9 (10.1)	98.4 (6.8)	21.1	12.8	11.0
NAL	97.6 (5.8)	92.3 (8.3)	82.2 (4.4)	21.6	14.1	12.2
Sulfonamides						
SDZ	87.5 (5.2)	97.0 (7.5)	94.0 (10.8)	10.1	8.1	10.0
SDMX	77.9 (5.9)	79.0 (4.1)	77.8 (5.3)	14.3	11.8	10.2
SMR	87.7 (5.1)	103.8 (8.1)	95.9 (11.2)	19.7	7.8	10.0
SMZ	84.0 (6.9)	90.5 (8.0)	83.7 (12.7)	14.2	7.6	8.9
SMXZ	93.3 (8.9)	92.9 (6.4)	90.1 (5.9)	22.5	6.1	7.0
SQX	78.6 (5.2)	76.7 (5.7)	77.7 (4.5)	17.6	9.9	10.0
SCP	84.2 (12.7)	97.4 (4.1)	95.3 (9.1)	16.0	8.0	13.8
SDX	82.4 (9.0)	89.6 (3.7)	83.7 (5.5)	17.4	8.8	8.5
SMPZ	74.9 (4.7)	88.4 (7.0)	83.1 (11.5)	17.8	10.4	7.2
SMX	81.4 (10.0)	86.1 (6.1)	82.1 (11.6)	17.4	7.9	10.7
SMTZ	83.2 (13.2)	106.4 (8.2)	101.9 (10.0)	21.8	10.5	9.1
SFX	90.6 (9.7)	99.9 (5.6)	101.0 (3.8)	18.1	16.7	18.9
STZ	85.6 (4.8)	94.3 (5.3)	93.6 (9.1)	13.6	5.0	5.5
SPZ	85.2 (9.8)	77.6 (5.8)	77.8 (6.2)	23.2	11.1	7.9
SPD	80.1 (6.1)	93.0 (7.0)	91.6 (10.8)	18.1	8.0	7.8
SMM	103.3 (7.7)	99.8 (4.4)	95.4 (9.8)	10.7	5.9	6.6
Tetracyclines						
OTC	95.3 (9.6)	91.4 (5.4)	87.6 (5.3)	20.4	14.3	10.3
TC	101.1 (8.3)	102.0 (7.5)	91.8 (6.3)	19.9	8.8	7.3
CTC	111.0 (7.7)	108.4 (6.7)	104.1 (7.4)	10.9	8.5	7.6
DC	102.1 (5.4)	99.0 (5.1)	94.7 (9.8)	17.4	7.5	9.6
Macrolides						
ERY	104.1 (10.2)	110.6 (7.0)	111.1 (6.3)	15.6	13.4	14.0
TIL	105.9 (11.2)	106.0 (4.2)	102.1 (2.7)	13.5	12.2	16.3
TYL	77.0 (10.4)	74.3 (6.5)	78.9 (8.5)	12.0	11.9	7.9
JOS	86.2 (14.5)	86.8 (10.4)	89.6 (7.2)	16.6	10.0	9.1
SPI	84.0 (11.6)	83.2 (7.6)	89.7 (10.6)	18.9	20.8	12.3
Lincosamides						
LIN	113.3 (5.0)	112.9 (3.4)	107.3 (4.6)	12.4	7.5	7.7
CLI	99.2 (5.8)	112.5 (2.9)	108.4 (9.7)	9.7	5.9	7.7
Penicillins						
AMP	98.3 (7.5)	83.0 (5.7)	80.1 (6.8)	11.1	11.0	10.4
OXA	93.4 (7.5)	94.6 (4.2)	94.9 (4.7)	22.7	9.2	9.4
DIC	82.6 (8.6)	88.6 (5.7)	86.8 (3.3)	22.0	16.1	14.8
PEN G	96.9 (5.8)	94.8 (7.3)	97.8 (8.4)	18.9	16.4	14.6
Nitroimidazoles^b						
DMZ	90.8 (7.0)	101.3 (10.5)	96.6 (11.5)	20.2	11.9	11.8
MNZ	86.3 (2.8)	110.0 (4.4)	105.3 (3.5)	12.4	8.2	8.5
RNZ	96.8 (8.2)	110.3 (4.9)	104.5 (3.0)	8.6	4.8	6.2

(continued on next page)

Table 3 – (continued)

Compound	Recovery (%)			Interday precision (RSD %)		
	5 ng/g	20 ng/g	50 ng/g	5 ng/g	20 ng/g	50 ng/g
Amphenicols^c						
FF	110.8 (7.0)	111.3 (6.7)	105.5 (6.3)	16.6	12.5	13.7

^a Intraday precision expressed as % RSD.

^b Evaluated at 3, 7.5 and 15 ng/g for low, medium and high concentration levels, respectively.

^c Evaluated at 10, 30 and 50 ng/g for low, medium and high concentration levels, respectively.

5–50 ng/g for the rest of the antimicrobial drugs. The linearity of an analytical procedure is its ability (within a given range) to obtain test results that are directly proportional to the concentration (amount) of analytes in the sample. Five-point calibration curves were plotted as the peak area (y) of each antimicrobial drug against its concentration ($y = mx \pm c$) at the concentration ranges mentioned above. The internal standard was not used for this procedure due to the differences in physiochemical properties of antibacterial drugs in multi-residues method. Therefore, it was difficult to find the suitable internal standard for all analytes. The calibration curves showed good linearity with determination coefficient (r^2) higher than 0.99 in all the cases.

3.3.4. Limits of detection (LODs) and limits of quantification (LOQs)

LODs and LOQs were calculated by analyzing the spiked samples at low level concentrations. LODs were determined as the lowest concentration of the analyte for which a signal-to-noise (S/N) ratio was 3:1 whereas, S/N ratio was 10:1 for LOQs. The results are shown in Table 2. The LODs and LOQs of the compounds of interest ranged from 1 to 5 ng/g and 3–10 ng/g, respectively. It indicated that the proposed method showed good sensitivity because the LODs and LOQs of all compounds were lower than the MRL established by EU [11].

3.3.5. Recovery and precision

Recovery and intraday precision (repeatability) were performed by preparing spiked samples at three different concentration levels (3, 7.5, 15 ng/g for NIs, 10, 30, 50 ng/g for APs and 5, 20, 50 ng/g for the rest of the compounds), and analyzed using ten replicates for each concentration level in one day. Inter-day precision (reproducibility) was evaluated

by testing spiked samples at the same concentration levels on five different days (six spiked samples per day and per concentration level). Table 3 shows that good results were obtained from all tested compounds at the three different concentration levels with recoveries ranging from 74.3% (TYL at 20 ng/g) to 113.3% (LIN at 5 ng/g). For precision evaluation, we found that the relative standard deviations (RSDs) for all the investigated levels were lower than 15.0% and 24.0% for repeatability and reproducibility, respectively. All these results were in accordance with the acceptance criteria, according to the Commission Decision 2002/657/EC [15]. It can be concluded that the developed method was accurate and precise for the multiclass analysis of antimicrobial drugs in shrimp muscle.

3.3.6. Applicability

The validated method was used to determine the presence and to quantify the concentrations of antimicrobial drug residues in shrimp muscle samples obtained from local supermarkets in Thailand during October–December 2017. To ensure the quality of the results when the proposed method was applied, an internal quality control was tested in every batch of samples. This quality control consisted of a matrix-matched calibration, a reagent blank, and a spiked blank sample at 7.5 ng/g for NIs, 30 ng/g for APs and 20 ng/g for the rest of compounds. Furthermore, the retention time and the ion ratio of detected antimicrobial drug residues in real samples were compared to those of corresponding calibration standards in the same batch to confirm the identity of the detected compounds using the criteria established by Commission Decision 2002/657/EC [15]. The 30 shrimp muscle samples were analyzed for the 55 antimicrobial drugs studied by simultaneous determination using UHPLC-MS/MS. Four of 30 shrimp muscle samples were greater than LOQs. Most

Table 4 – Concentration of antimicrobial drug residues (ng/g) found in real samples (total of 30 samples).

Antibacterial drugs	Sample 8	Sample 11	Sample 16	Sample 17	Sample 18	Sample 19	Sample 30
Fluoroquinolones-							
ENR	–	–	<LOQ	<LOQ	<LOQ	<LOQ	9.8
CIP	–	–	1169.2	1426.9	–	–	–
OFL	–	–	–	–	–	<LOQ	–
PEF	–	–	11.0	12.7	–	–	–
Sulfonamides-							
SMXZ	–	–	<LOQ	<LOQ	–	–	–
Tetracyclines-							
DC	–	83.1	–	–	–	–	–
Lincosamides							
CLI	<LOQ	–	–	–	–	–	–

antimicrobial residues were detected at levels lower than the LOQ, except for ENR, CIP, PEF and DC which were higher than the LOQ in some shrimp muscle samples. Our results of antimicrobial drug residues in shrimp muscle samples are in line with the previous study [35]. In addition, multiple antimicrobials were detected in the shrimp samples, CIP/PEF being the most common mixture. Nevertheless, only CIP was detected in two samples at levels higher than the MRL (100 ng/g) established by EU [11]. The detected compounds as well as the residue concentrations in four positive samples are summarized in Table 4.

4. Conclusions

A simple, cheap, fast, reliable and sensitive multiclass analytical method was developed for the simultaneous determination of antimicrobial drugs (55 compounds from eight different families) in shrimp muscle samples using simple liquid extraction with UHPLC-MS/MS. This method can detect antimicrobial drugs within a single run and was demonstrated to be an excellent tool for unambiguous identification of selected compounds using the retention time of quantification and confirmation ions. The method was validated according to international guidelines and satisfactory validation data were obtained for linearity, LODs, LOQs, recovery and precision. In addition the developed method was successfully applied to real samples, and the results of antimicrobial drug residues showed that ENR, CIP, PEF and DC were quantifiable in shrimp samples. CIP residue concentration was higher than the MRL established by EU, indicating inappropriate treatment and lack of adequate withdrawal times might be an issue in some Thai shrimp production facilities. However, further studies with a larger sample size are needed to confirm antimicrobial residues in shrimp muscles for assuring food safety.

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