



# Multi-residues determination of antimicrobials in fish tissues by HPLC–ESI–MS/MS method



Mamdouh R. Rezk, Safa'a M. Riad, Fatma I. Khattab, Hoda M. Marzouk\*

Analytical Chemistry Department, Faculty of Pharmacy, Cairo University, Kasr El-Aini St., 11562 Cairo, Egypt

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## ABSTRACT

A rapid, simple, sensitive and specific LC–MS/MS method was developed and validated for the simultaneous quantification of four antimicrobials commonly used in aquaculture, namely ciprofloxacin (CPX), trimethoprim (TMP), sulphadimethoxine (SDM) and florphenicol (FLOR) in fish tissues. The LC–MS/MS was operated under the multiple-reaction monitoring mode using electrospray ionization. Sample preparation involves simple liquid extraction step followed by post-extraction clean-up step with n-hexane. The purified extracts were chromatographed on Agilent Poroshell 120 EC, C<sub>18</sub> (50 mm × 3 mm, 2.7 μm) column by pumping an isocratic mobile phase consisting of 0.1% formic acid in water:0.1% formic acid in methanol (20:80, by volume) at a flow rate of 0.4 mL/min. A detailed validation of the method was performed as per FDA guidelines and the standard curves were found to be linear in the range of 1–100 ng/g for both CPX and TMP, 0.5–100 ng/g for SDM and 1–50 ng/g for FLOR. The intra-day and inter-day precision and accuracy of the results were within the acceptable limits. A run time of 1.5 min for each sample made it possible to analyze multiple fish tissue samples per day. The developed assay method was successfully applied for the detection of antimicrobials in real fish tissue samples obtained from different fish farms.

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## 1. Introduction

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

Sulphadimethoxine sodium (SDM) (Fig. 1a) is a member of sulphonamide group of drugs. Trimethoprim (TMP) (Fig. 1b) is one of the most widely used antibacterial additives that act synergistically in combination with sulphonamides. These combinations are commonly used in food-producing animals as growth promoters and as therapeutic and prophylactic drugs [2].

Florphenicol (FLOR) (Fig. 1c) is a broad spectrum, primarily bacteriostatic, antibiotic with a range of activity similar to that of chloramphenicol. However, florphenicol does not carry the risk of inducing human aplastic anaemia that is associated with chloramphenicol. Because of this, chloramphenicol has been banned

and florphenicol was permitted as a substitute for use in food-producing animals [3].

Ciprofloxacin hydrochloride (CPX) (Fig. 1d) is a broad-spectrum antibiotic belonging to the fluoroquinolone group. Fluoroquinolones have been shown to be very effective in combating various diseases in animal husbandry and aquaculture and are used extensively worldwide [4].

Several analytical methods have been reported in the literature for the determination of the studied drugs either individually or in combination with other drugs in different matrices. These methods include spectrophotometry [5], GC [6], HPLC [7–12], HPTLC [13], LC/MS [14–22], immunoassays [23,24] and capillary electrophoresis [25,26].

Low level doses of such antimicrobials in foodstuffs that may be consumed for long periods may lead to an increase in resistant bacterial strains. To protect human health, the European Union (EU) and the U.S. Food and Drug Administration (FDA) have established safe maximum residue limits (MRLs) for these drugs. The use of veterinary drugs is regulated through EU Council Regulation 2377/90/EC [27] that describes the procedure for establishing MRLs for veterinary medicinal products in foodstuffs of animal origin.

The EU established MRLs of 100 ng/g for CPX and SDM, 50 ng/g for TMP and 1000 ng/g for FLOR in fish [27]. These limits require sensitive and specific methods to monitor and determine

\* Corresponding author at: Kasr El-Aini St., ET-11562 Cairo, Egypt.

Tel.: +20 1066095952.

E-mail address: [h.marzouk@yahoo.com](mailto:h.marzouk@yahoo.com) (H.M. Marzouk).

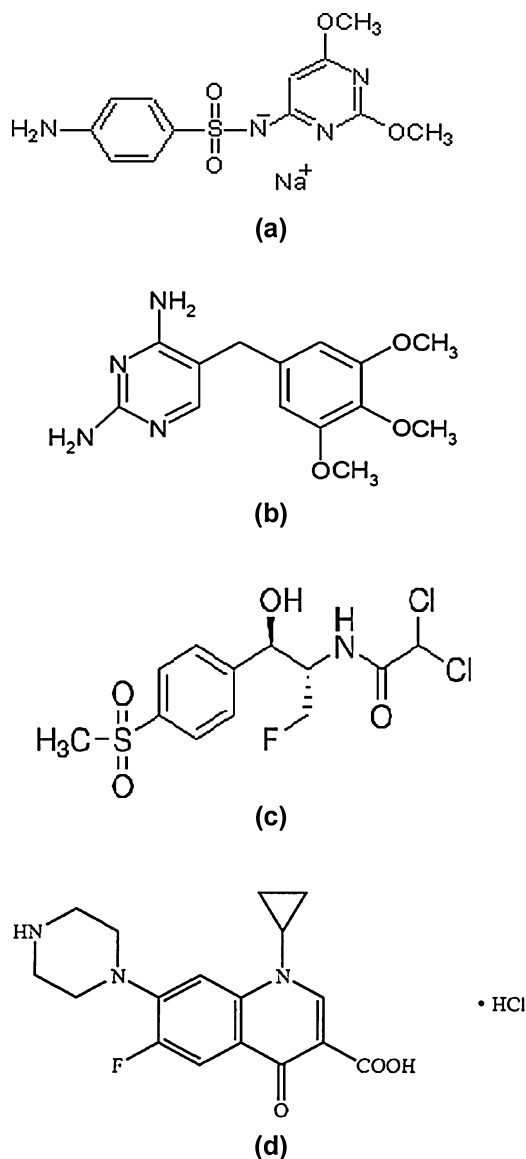


Fig. 1. Chemical structure of (a) sulphadimethoxine sodium, (b) trimethoprim, (c) florphenicol and (d) ciprofloxacin hydrochloride.

antimicrobial residues in aquatic products. Therefore, residue monitoring of antimicrobials plays an important role to guarantee the safety of food.

Large varieties of analytical approaches are used in the food industry. Chromatography combined with on line mass spectrometry is among the most sensitive and selective analytical methodologies. Food-based matrices being very complex, selectivity is a prime advantage, while sensitivity is a key issue to identify trace components. Since several years LC with MS detection has been used for confirmatory analysis because this detection method is more sensitive, selective and allows rapid and multi-residues determination in complex matrices and gives structural information [28].

The aim of the present study was to develop and validate a confirmative, simple, rapid and sensitive method for the simultaneous determination of CPX, TMP, SDM and FLOR in fish tissues. The developed method employs simple liquid extraction procedure followed by post-extraction clean-up step with n-hexane using high-performance liquid chromatography and detection by electrospray tandem mass spectrometry (HPLC–MS/MS). This method

was used to detect and estimate the concentrations of the studied drugs in real fish samples collected from different fish farms.

To the best of our knowledge, the present work is the first LC–MS/MS method for the simultaneous determination of the studied drugs that belong to different chemical classes in trace amounts in fish tissues.

## 2. Experimental

### 2.1. Instruments

Chromatographic analysis was performed using high performance liquid chromatography system (Agilent 1260 series). Chromatographic separation of analytes was carried out on a reversed-phase C<sub>18</sub> column (50 mm × 3 mm, 2.7 μm, Agilent, Poroshell 120 EC) using an isocratic mobile phase composed of 0.1% formic acid in water:0.1% formic acid in methanol (20:80, by volume) at a flow rate of 0.4 mL/min. The column temperature was maintained at 25 °C.

Mass spectrophotometric analysis was carried out using an ABSciex 4000 QTRAP® (hybrid triple quadrupole/linear ion trap) mass spectrophotometer. The instrument was equipped with electrospray ionization (ESI) operated in the positive ionization mode (PI) for CPX, TMP and SDM and in the negative ionization mode (NI) for FLOR. The source dependent parameters were maintained for both the analytes and internal standards (ISs): cone gas flow, 30 L/h; desolvation gas flow, 500 L/h; source temperature, 400 °C; and capillary voltage of 5.5 kV. The optimum values for compound dependent parameters like cone voltage and collision energy were set at 29 V and 10 eV for CPX; 33 V and 10 eV for TMP; 29 V and 10 eV for SDM and 10 V and 14 eV for FLOR, respectively. Detection of the ions was performed in the multiple reaction monitoring (MRM) mode. The specific MS/MS parameters for each target analyte are shown in Table 1. ABSciex software was used to control all parameters and data acquisition.

### 2.2. Reagents

Methanol, acetonitrile (HPLC grade) and formic acid (purity >98%) were purchased from Sigma–Aldrich. Ultrapure water was obtained from a Milli-Q Gradient water system (Millipore, Bedford, MA, USA).

### 2.3. Samples

#### 2.3.1. Pure samples

Standard sulphadimethoxine sodium and trimethoprim were kindly supplied by Pharma Swede, Cairo, Egypt. Sulphadimethoxine sodium purity was found to be 99.87 ± 0.854 (n = 6) according to the official HPLC method [29]. Trimethoprim purity was found to be 100.63 ± 0.777 (n = 5) according to the official non-aqueous titration [29]. Standard ciprofloxacin hydrochloride was kindly supplied by European Egyptian Pharmaceuticals. Its purity was found to be 99.70 ± 1.235% (n = 6) according to its official HPLC method [30].

Florphenicol was kindly supplied by Jiang Su Guo International Group Co. Ltd. (China) and its purity was found to be 99.41 ± 0.958 (n = 6) according to a reported HPLC method [31].

#### 2.3.2. Fish tissue samples

2.3.2.1. Experimentally collected samples (blank samples). A total number of 20 apparently healthy *Oreochromis niloticus* with an average weight of (150 ± 10 g, each) and length of (14 ± 2 cm, each) were randomly collected from a private freshwater farm at Abbassa–Sharkia, Egypt. The fish were stocked in full glass aquarium filled with chlorine free tap water and supplied with air pump

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

Analyte	$t_R$ (min)	Quantitation transition ( $m/z$ )	Confirmation transition ( $m/z$ )	ESI mode
CPX	0.162	333.998 → 316.20	333.998 → 290.20	ESI+
TMP	0.290	292.497 → 231.100	292.497 → 262.100	ESI+
SDM	0.45	310.821 → 156.200	310.821 → 92.200	ESI+
FLOR	0.745	355.918 → 336.100	355.918 → 185.100	ESI–

for aeration. The fish were fed (3% of their body weight) from commercial pellets containing at least 30% proteins. The fish were used as a control for antimicrobials spiking and fortification studies.

**2.3.2.2. Naturally collected samples (real samples).** A total number of 25 Nile Tilapia (*O. niloticus*) were collected alive from different private fish water farms at Kafr El-Sheikh (Farm 1), Sharkia (Farm 2), El-Behera (Farm 3), Fayoum (Farm 4) and Giza governorates (Farm 5), at a rate of 5 fish/fish farm.

These fish farms have suffered from previous bacterial infections and the fish were treated with a course of different antimicrobials. The fish were randomly collected at different periods post-treatment. The average weight of the collected fish was ( $150 \pm 5$  g, each) and an average length of ( $10 \pm 2$  cm, each).

The fish were transported alive to the lab and stocked in large tanks filled with water of the same source. The tanks were supplied with air pumps to maintain an accurate dissolved oxygen value.

Prior to analysis, fish samples were collected on dry ice and stored in the freezer for some time before analysis.

**2.3.2.3. Fish tissue samples pre-treatment procedure.** The collected fish were filleted, the skin and bones were removed, and the muscles were cut and frozen at  $-20^\circ\text{C}$  before being analyzed. Prior to analysis, frozen fish tissue samples were thawed overnight in a refrigerator. The muscle samples (100–150 g) were diced into small pieces.

## 2.4. Solutions

### 2.4.1. Stock standard solutions

Stock standard solutions of individual compounds (each, 100  $\mu\text{g}/\text{mL}$ ) were prepared by accurately weighing and dissolving the powder in 25 mL methanol:water (50:50, by volume) into four separate 100-mL volumetric flasks and the volume was completed to the mark with same solvent mixture. The prepared solutions were then stored at  $-20^\circ\text{C}$  and protected from light.

### 2.4.2. Working standard solutions

Appropriate dilutions were made in methanol:water (50:50, by volume) for the primary stock solutions to produce working standard solutions of (each, 1  $\mu\text{g}/\text{mL}$ ) on the day of analysis and these stocks were used to prepare the calibration curves.

## 2.5. Procedures

### 2.5.1. Calibrators and quality control samples

Calibration curves and quality control samples were prepared every time before sample analysis. Nine different working standard solutions of CPX, TMP, SDM and FLOR were prepared by accurately taking different volumes from its primary or secondary stock solutions with appropriate dilution into 10 mL with methanol:water (50:50, by volume) to prepare the calibrators and quality control samples. Calibration and QC samples were prepared by spiking 1 g blank fish tissue samples with working standard solutions of each; CPX, TMP, SDM and FLOR on the day of analysis as illustrated in Table 2.

### 2.5.2. Sample preparation and extraction procedure

Antimicrobials were extracted from fortified fish tissues using simple liquid extraction procedure. The detailed procedure was as follows: 1 g of blank fish tissue samples was weighed in a 20-mL glass centrifuge tube and fortified with known amounts of working standard solutions of CPX, TMP, SDM and FLOR. The fortified samples were then homogenized and left to stand for 15 min. A volume of 0.25 mL of 1% formic acid aqueous solution, 0.5 mL of acetonitrile and 0.5 mL of methanol were added to the fortified samples, and then subjected to vortex for 30 s. Subsequently, the glass tube with sample and solvent was shaken by a vertical shaker for 10 min. After centrifugation at 10,000 rpm for 10 min, the supernatant was transferred into a 15-mL glass tube. The extraction procedure was repeated three times and the four supernatants were pooled. A post-extraction clean-up step was performed by evaporating the extracting solvent to dryness with an eppendorf's evaporator at  $45^\circ\text{C}$  followed by reconstitution with 1 mL of the mobile phase and 2 mL of n-hexane were added. After mixed well by vortex, the mixture was de-fatted to dissolve the residues. Centrifugation at 10,000 rpm for 5 min was then carried out. A volume of 500  $\mu\text{L}$  of the bottom layer was drawn and filtered through 0.22- $\mu\text{m}$  nylon membrane filter (Agilent). A volume of 10  $\mu\text{L}$  was injected under the optimized analytical conditions.

### 2.5.3. Method validation

The method was validated to meet the acceptance criteria of industrial guidance for bioanalytical method validation [32,33].

**2.5.3.1. Specificity and selectivity.** The specificity of the method was determined by analyzing six different blank fish tissue samples to demonstrate the lack of chromatographic interference from the endogenous matrix components.

**2.5.3.2. Calibration curve.** Calibration curves were acquired by plotting the peak area ratio of the transition pair of analytes to that of IS against the corresponding concentration of calibration standards. CPX in concentration of 10 ng/mL was used as an internal standard (IS-1) for SDM, TMP and FLOR. While SDM in concentration of 0.5 ng/mL was used as internal standard (IS-2) for CPX. Matrix-matched calibration curves were prepared at six spiking

**Table 2**  
Preparation of calibrators and quality control samples for CPX, TMP, SDM and FLOR for the proposed LC/MS/MS method.

Final spiked concentration (ng/g)	Fish tissues	Prepared samples			
		FLOR	SDM	TMP	CPX
Calibrators	1 g	1	1	0.5	1
		10	5	5	12.5
		20	10	10	20
		50	30	25	30
		80	60	60	40
		100	100	100	50
QCL		3	3	1.5	3
QCM		45	45	45	22
QCH		90	90	90	45

levels over ranges of 1–100 ng/g for CPX, 1–100 ng/g for TMP, 0.5–100 ng/g for SDM and 1–50 ng/g for FLOR. The acceptance criterion for each back-calculated standard concentration was  $\pm 15\%$  deviation from the nominal value except at LLOQ which was set at  $\pm 20\%$ .

**2.5.3.3. Precision and accuracy.** Inter- and intra-assay precision and accuracy were determined by analyzing six replicates at the lower level of quantification (LLOQ) in addition to three different QC levels as described above on different days. The criteria for acceptability of the data  $\pm 15\%$  standard deviation (SD) from the nominal values and a precision  $\leq 15\%$  relative standard deviation (RSD).

**2.5.3.4. Extraction efficiency.** The recovery of CPX, TMP, SDM and FLOR was determined by comparing the responses of the analytes extracted from replicate QC samples at LQC, MQC and HQC with the response of analytes from post-extracted fish tissues standard samples at equivalent concentrations [34].

**2.5.3.5. Matrix effect.** The effect of fish tissue constituents over the ionization of analytes was determined by comparing the response of the post extracted fish tissues standard QC samples ( $n = 4$ ) with the response of analytes from neat samples at equivalent concentrations [35]. Matrix effect was determined at same concentrations for each analyte as in recovery experiment.

**2.5.3.6. Stability experiments.** The stability of analytes and IS in the injection solvent was determined periodically by injecting replicate preparations of processed samples up to 12 h (in autosampler) after the initial injection. The peak areas of the analytes and IS obtained at initial cycles were used as reference to determine the relative stability of the analytes at subsequent points. Stability of analytes in the fish tissues after 8 h exposure in an ice bath (bench top) was determined at three concentrations in six replicates. Samples were processed as described above. Samples were considered to be stable if assay values were within the acceptable limits of accuracy (i.e.  $\pm 15\%$  SD) and precision (i.e.  $\leq 15\%$  RSD) [32].

### 3. Results and discussion

#### 3.1. Optimization of sample preparation and chromatographic conditions

Sample preparation is often the most critical part of a multi-residue antimicrobial method in fish tissues due to different recoveries of substances when extracted simultaneously, in addition to the high protein and fat contents that interfere with the analytical procedures.

Simple liquid extraction procedure was carried out followed by partitioning with n-hexane to remove lipids. Efficient extraction as well as high recoveries was obtained by the use of an organic solvent mixture composed of acetonitrile:methanol and 0.1% formic acid (2:2:1, by volume). Simultaneous extraction of all antimicrobials in the fish tissues was performed in a single procedure, in spite of the different chemical natures of the analytes. The addition of formic acid in the extraction procedure was advantageous for protein precipitating in the sample tissues.

In this work, efficient simple liquid extraction made it possible to get clean extracts instead of using the multistep, sophisticated and time consuming solid phase extraction procedures [21,22].

As FLOR contains halogen atoms and hydroxyl group which have high electronegativities, high sensitivity could be obtained in NI mode, whereas SDM, TMP, CPX with amino groups are more sensitive in the PI mode. The protonated molecules  $[M+H]^+$  were selected as the precursor ions for CPX, TMP and SDM in PI mode and the deprotonated molecules  $[M-H]^-$  were selected as the precursor

ion for FLOR in the NI mode, as shown in Table 1. Detection of ions was performed in MRM mode by monitoring the transition pairs as described in Section 2. For each analyte, two different mass transitions were monitored. The most abundant fragment was used for quantification, while the second one was used for confirmation.

To optimize the proposed LC/MS/MS method, the effects of several chromatographic parameters were studied in order to achieve the best separation and retention for the analytes. These included the type of organic modifier, pH of aqueous solution and organic modifier – aqueous ratio. These parameters were optimized based on the peak shape, peak intensity/area, peak resolution and retention time for analytes on Agilent Poroshell 120 EC, HPLC C<sub>18</sub> (50 mm × 3 mm, 2.7  $\mu$ m) column.

Different mobile phases were tried. Finally, a mobile phase composed of 0.1% formic acid in methanol and 0.1% aqueous formic acid was chosen to separate the four antimicrobials in further experiments.

The best chromatographic conditions were achieved using an isocratic mobile phase system composed of 0.1% formic acid in water:0.1% formic acid in methanol (20:80, by volume). Other parameters such as column temperature and flow rate were studied in order to get a fast and reliable separation. The best results were observed at 25 °C and 0.4 mL/min as the flow rate. Under these conditions, all the analytes were eluted in the narrow range of retention times (0.16–0.75 min) which is advantageous to compensate the matrix effect as shown in Fig. 2.

#### 3.2. Mass spectrometry

The optimization of mass spectrophotometric parameters was performed by the infusion of a standard solution of 10 ng/mL of each antimicrobial in a mixture of water:methanol (50:50) at a flow rate of 0.4 mL/min. The ESI probe in positive mode was selected as the ionization technique for SDM, TMP and CPX and in the negative mode for FLOR. First, full-scan spectra were acquired so as to select the most abundant  $m/z$  value and optimizing the parameters. The most abundant product ions were selected for quantification purposes and others were used for confirmation. The MS/MS transitions for quantification and confirmation for each of the studied compounds are shown in Table 1.

#### 3.3. Method validation

##### 3.3.1. Selectivity

The selectivity of the proposed method was demonstrated by its ability to differentiate and quantify the analytes from endogenous components and co-extractives in fish tissues matrix. Fig. 2a shows the chromatograms of blank fish tissue sample at each transition

**Table 3**  
Intra-assay precision and accuracy of proposed LC/MS/MS method.

Compound	n	Concentration (ng/g)			
		Added	Measured	Recovery%	RSD%
CPX	6	3.00	2.84	94.67	2.89
	6	45.00	43.00	95.56	3.13
	6	90.00	86.65	96.28	2.78
TMP	6	3.00	2.85	95.00	1.97
	6	45.00	43.29	96.20	2.65
	6	90.00	88.36	98.18	3.45
SDM	6	1.50	1.41	94.00	2.10
	6	45.00	44.38	98.62	2.74
	6	90.00	85.77	95.30	3.21
FLOR	6	3.00	2.88	96.00	2.34
	6	22.00	21.46	97.54	1.87
	6	45.00	44.32	98.49	2.01

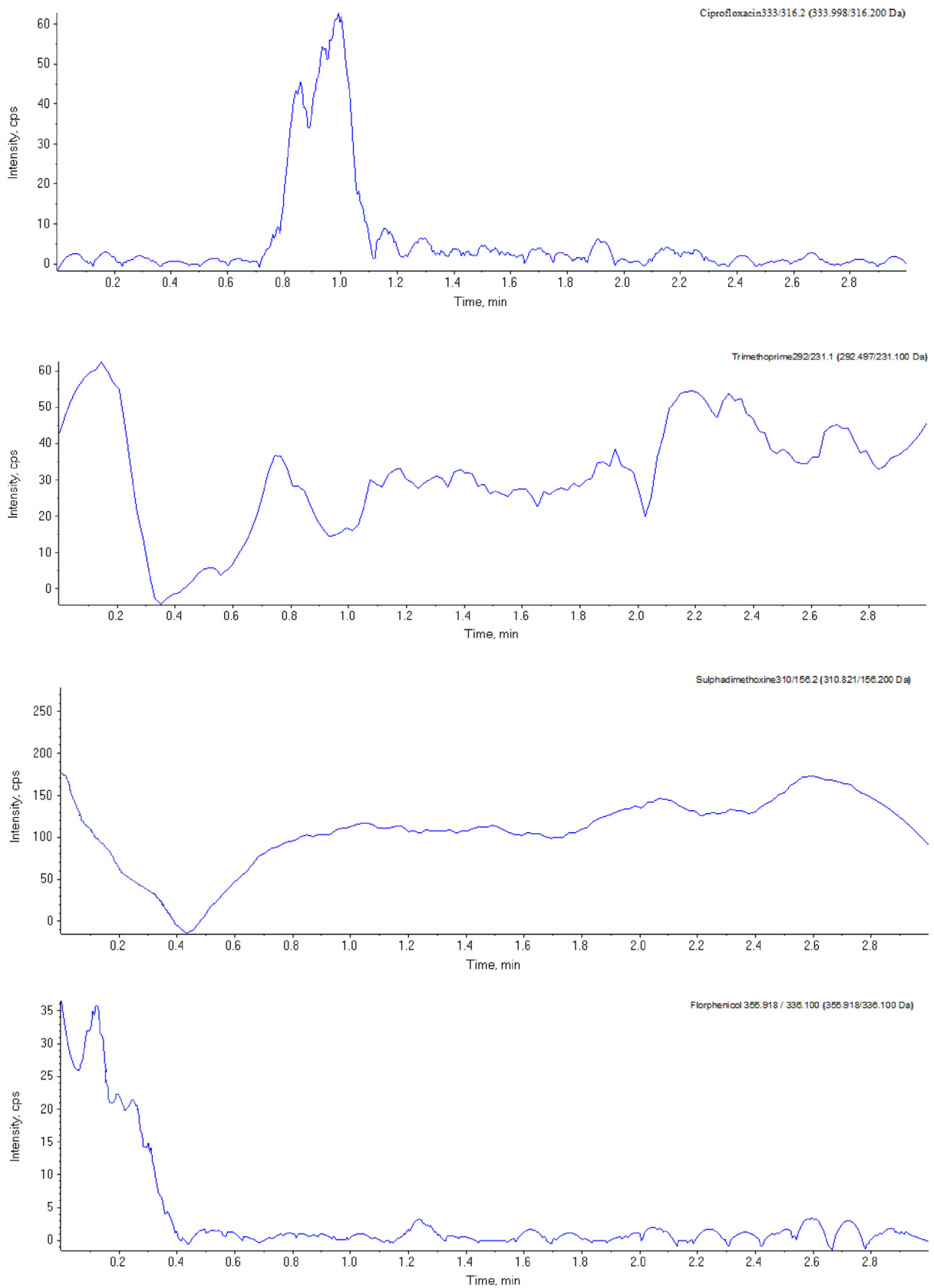


Fig. 2. LC-MS/MS chromatograms from (a) blank fish tissue samples and (b) spiked fish samples containing CPX, TMP, SDM and FLOR (10 ppb).

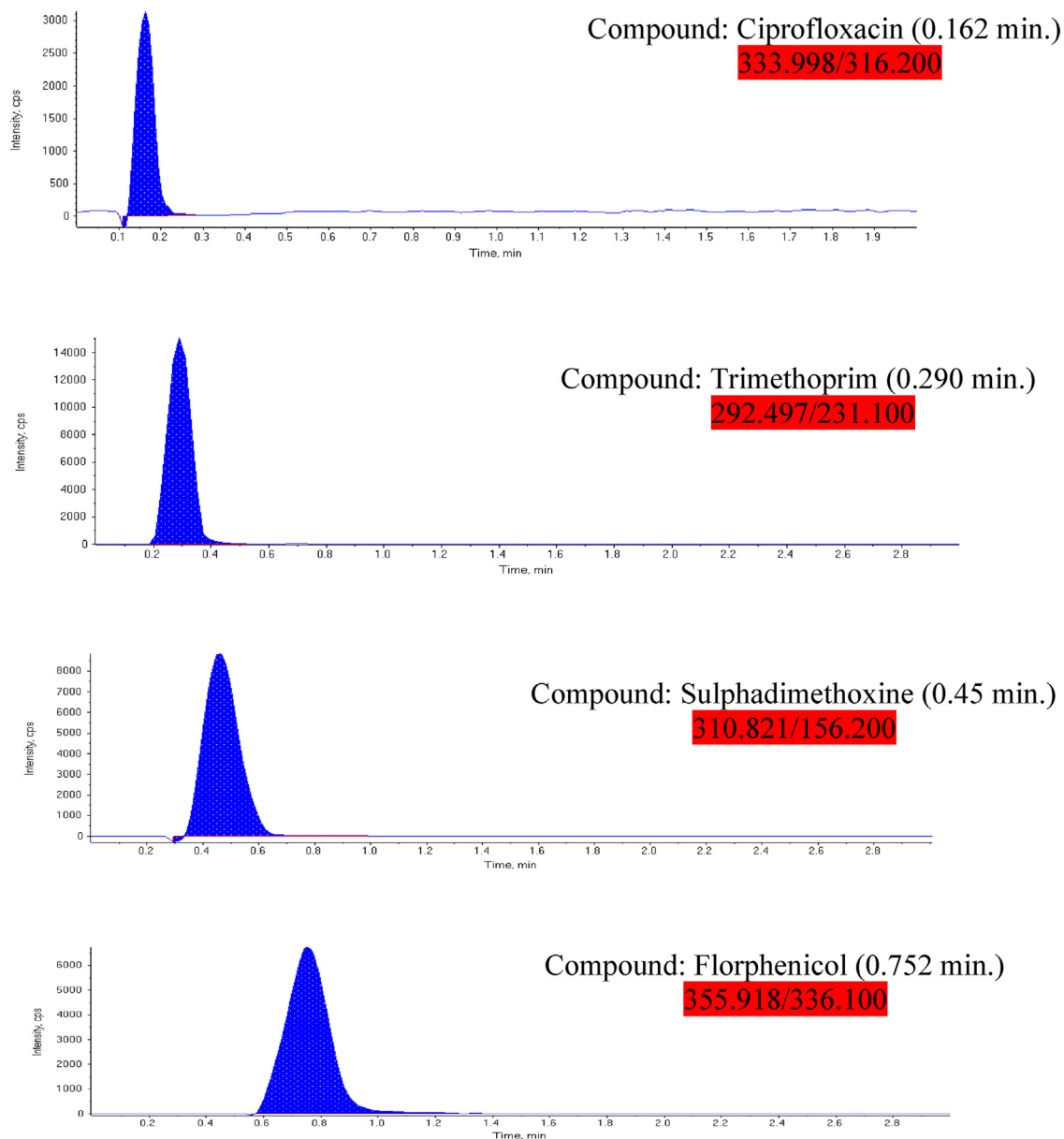


Fig. 2. (Continued)

**Table 4**  
Inter-assay precision and accuracy of the proposed LC/MS/MS method.

Compound	n	Concentration (ng/g)			
		Added	Measured	Recovery%	RSD%
CPX	6	3.00	3.10	103.33	2.13
	6	45.00	44.20	98.22	2.34
	6	90.00	89.15	99.06	1.89
TMP	6	3.00	2.89	96.33	1.65
	6	45.00	43.83	97.40	2.55
	6	90.00	87.98	97.72	2.73
SDM	6	1.50	1.45	96.67	1.34
	6	45.00	45.21	100.47	2.78
	6	90.00	92.34	102.60	1.28
FLOR	6	3.00	3.13	104.33	1.11
	6	22.00	22.36	101.63	3.11
	6	45.00	43.61	96.91	2.16

for the studied compound. The chromatograms for blank fish tissue samples spiked with the studied drugs are shown in Fig. 2b. The chromatograms were found to be free from interfering peaks. The method selectivity was demonstrated on six blank fish tissue samples; the chromatograms were found to be free from interfering peaks.

### 3.3.2. Linearity and limit of quantification

The calibration curves were linear in the studied range. The calibration curve equation is  $y = bx + c$ , where  $y$  represents analyte/internal standard peak area ratio and  $x$  represents the analyte concentration in ng/g. The mean equations of the calibration curve ( $n = 6$ ) obtained from 6 points were:  $y = 0.0126x - 0.003$ ,  $r = 0.9999$ ,  $y = 1.6694x + 4.9877$ ,  $r = 0.9999$ ,  $y = 17.848x - 0.901$ ,  $r = 0.9999$  and  $y = 23.469x + 6.0409$ ,  $r = 0.9995$  for CPX, TMP, SDM and FLOR, respectively.

The limit of quantification was 0.5 ng/g for SDM and 1 ng/g for CPX, TMP and FLOR.

**Table 5**  
Stability of CPX, TMP, SDM and FLOR by the proposed LC/MS/MS method.

Parameter	CPX Recovery%, RSD%	TMP Recovery%, RSD%	SDM Recovery%, RSD%	FLOR Recovery %, RSD%
(a) Short term stability of analyte in matrix at room temperature				
Spiked concentration level 1 <sup>a</sup>	93.77, 2.12	95.47, 3.24	95.07, 1.60	99.01, 0.54
Spiked concentration level 2 <sup>a</sup>	95.13, 1.97	94.77, 1.09	97.23, 1.73	98.73, 0.98
Spiked concentration level 3 <sup>a</sup>	96.23, 1.86	95.70, 1.06	96.99, 0.45	97.89, 1.42
(b) Post-preparative stability at 4 °C				
Spiked concentration level 1 <sup>a</sup>	98.76, 3.12	97.77, 2.65	97.95, 1.73	98.02, 1.72
Spiked concentration level 2 <sup>a</sup>	97.22, 1.11	98.01, 0.98	97.55, 1.77	98.72, 0.91
Spiked concentration level 3 <sup>a</sup>	97.55, 2.67	97.67, 0.52	98.77, 0.76	98.56, 0.61
(c) Long term stability of analyte in matrix at –20 °C				
Spiked concentration level 1 <sup>a</sup>	97.44, 2.92	97.88, 1.01	97.36, 0.45	96.99, 1.26
Spiked concentration level 2 <sup>a</sup>	98.21, 2.01	97.61, 0.92	98.67, 0.53	98.01, 0.83
Spiked concentration level 3 <sup>a</sup>	97.66, 1.03	98.51, 2.70	99.02, 2.09	97.01, 1.73

Spiked concentrations; CPX or TMP: level 1 = 3 ng/g; level 2 = 45 ng/g; level 3 = 90 ng/g.

SDM: level 1 = 1.5 ng/g; level 2 = 45 ng/g; level 3 = 90 ng/g and FLOR: level 1 = 3 ng/g; level 2 = 22 ng/g; level 3 = 45 ng/g.

<sup>a</sup> n = 6.

### 3.3.3. Precision and accuracy

The precision, characterized by the relative standard deviation, was 14.3%, 15.8%, 6.7% and 9.4% at LLOQ for CPX, TMP, SDM and FLOR, respectively, while the accuracy, expressed as the recovery percentage, was 92.3%, 91.6%, 94.1% and 93.7% for the four analytes at the LLOQ ( $n = 6$ ).

The intra-assay precision and accuracy results across three QC levels are shown in Table 3. The precision (RSD) ranged from 1.87 to 3.45% and accuracy was within 94.00 to 98.62% for the analytes. Similarly for inter-assay experiments, Table 4, the precision varied from 1.34 to 3.11 and the accuracy was within 96.33 to 104.33%.

### 3.3.4. Extraction efficiency

It became clear during the method development that the chemical natures of the four analytes were sufficiently different that obtaining high recoveries for all analytes would be unlikely. However, the extraction and clean-up procedures described here gave moderate to good recoveries for all the analytes investigated. The mean extraction recovery for the analyzed drugs was calculated at all QC levels. It varied from 92.32 to 93.45%, 97.12 to 97.78%, 96.54 to 97.01% and 95.48 to 95.94% for CPX, TMP, SDM and FLOR, respectively.

### 3.3.5. Matrix effects

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). The effect of fish tissues co-extractives over the ionization of analytes was determined by comparing the responses of the post extracted fish tissues standard QC samples ( $n = 4$ ) with the response of analytes from neat samples at equivalent concentrations. The relative standard deviation of peak area ratios (analyte/IS) was lower than 2% and the relative standard deviation of peak areas of individual components is lower than 4%, indicating no significant matrix effects.

### 3.3.6. Sample stability

Stability was concluded if the concentration change was less than 15% of the nominal concentration. The results are shown in Table 5.

The short term stability of analytes in fish tissue samples (with low, medium, and high quality control samples) was studied for a period of 6 h at room temperature (25 °C) and protected from direct light where the samples were stable under the studied conditions.

Three sets of spiked samples with low, medium and high concentrations of the four analytes were analyzed and left in the autosampler at 4 °C for 12 h. The samples were analyzed using

freshly prepared calibration samples. The processed samples were stable at room temperature for this period. The results are shown in Table 5.

The long term stability of frozen spiked fish tissue samples was examined after 2 weeks storage at –20 °C. The samples were stable under studied conditions and the results are shown in Table 5.

### 3.4. Application to real fish samples

The low limit of quantification permits the use of the method for the determination of the studied antimicrobials in real fish samples obtained from five different private aquaculture farms. Optimized method was applied for detection and quantification of the four antimicrobials in all different real samples. Results obtained from real fish samples analysis indicate the presence of CPX in Farms 3 and 5 in a concentration of  $5.67 \pm 0.24$  and  $20.34 \pm 0.92$  ng/g, respectively. FLOR residue was detected in Farm 1 in a concentration of  $70.85 \pm 1.67$  ng/g. SDM and TMP residues were not detected in any farm. The residues found were in a concentration below their MRLs established by the EU. These confirm the safety of such samples for human consumption.

## 4. Conclusions

The developed and validated HPLC–MS/MS method is a rapid, simple, precise and sensitive method for the simultaneous determination of ciprofloxacin, trimethoprim, sulphadimethoxine and florphenicol in fish tissues in trace levels that are much lower than their maximum residue limits established by EU. The precision and accuracy of the method are well within limits required for bioanalytical assays. The low limit of quantification permits the use of the method for the determination of the studied antimicrobials in real fish samples. Therefore, the proposed method will be useful and practical in the residue monitoring of ciprofloxacin, trimethoprim, sulphadimethoxine and florphenicol in fish tissues.

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