Simultaneous Determination of Multiclass Antibiotic Residues in Nile Tilapia (*Oreochromis niloticus*) by LC/MS/MS

Application Note

Food Testing & Agriculture

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Abstract

A method was developed and validated for simultaneous assessment of 11 drugs of different antibiotic classes (chloramphenicol, oxytetracycline, tetracycline, chlortetracycline, sulfadimethoxine, sulfathiazole, sulfamethazine, enrofloxacin, ciprofloxacin, norfloxacin, and sarafloxacin) on Nile tilapia muscle (*Oreochromis niloticus*). The sample pretreatment process included extraction with 5 g of fish muscle, 1 mL of 0.1 M Na_2EDTA, and 24 mL of acetonitrile:water (0.1% formic acid) (70:30) with purification by Captiva cartridges. The compounds were determined in a single run. The limits of quantification (LOQs) were less than 4.3 µg/kg for all compounds, the recovery ranged from 83.8 to 110.1%, and accuracy was lower than 5.5%.

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Introduction

Aquaculture has an annual growth of approximately 9% in the world [1]. In Brazil, the growth production of fish farming only reached 60.2% between 2007 and 2009. Tilapia production increased 105% in seven years (2003–2009) [2]. This forced the aquaculture system to become increasingly dependent on chemical inputs. Because of the high stocking densities required, the organisms are under constant attacks, which increases the demand for chemicals, especially antibiotics. However, importing countries such as the European Community and the United States, impose increasingly restrictive limits for antibiotics residues.

As a result of misuse, antibiotic residues in products of animal origin are a concern to consumers. These residues can be toxic or cause allergic reactions in some hypersensitive individuals. In addition, low-level doses of antibiotic in foodstuffs consumed for long periods can lead to the spread of drug-resistant micro-organisms.

This study presents a rapid method using chemical filtration by Captiva cartridges and ESI LC/MS/MS. The main benefit of Captiva cartridges (p/n A5300002) is that they can easily be used for efficiently removing precipitated proteins and particulate matter.

Sulfadimethoxine-d6 deuterated was used as an internal standard to obtain more reliable results. The developed method was fully validated in terms of selectivity, linearity, accuracy, matrix effect, precision, and sensitivity according to the European Union Commission Decision 2002/657/EC [3].

Experimental

Chemicals

The solvents used were methanol and acetonitrile, HPLC grade (Tedia). The reagents used were formic acid (Vetec) and Na_2EDTA (Sigma-Aldrich), both analytical grade. The water used was purified with a Milli-Q system (Millipore USA). The analytical standards oxytetracycline 97% (OTC), tetracycline 97.5% (TC), chlortetracycline 93% (CTC), ciprofloxacin 99.5% (CFX), enrofloxacin 99.0% (EFX), sarafloxacin 97.2% (SAR), sulfathiazole 98.0% (STZ), norfloxacin 99% (NFX), and the internal standard sulfadimetoxina-d6 99.4% were acquired from Fluka Analytical. Sulfadimethoxine (SDM) and sulfamethazine (SMZ) were acquired from Chem Service, both with 99.5% purity. Chloramphenicol 98.5% (CAP) was acquired from Dr. Ehrestorfer.

Stock standard solutions of individual compounds (100 µg/mL) were prepared in methanol, and stored at –20 °C in an amber bottle for 6 months. A multicompound working standard solution (1,000 µg/L) was prepared by the appropriate dilution of the stock solutions with water, stored under refrigeration (T < 5 °C), and renewed weekly.

Sample preparation

A 5 g amount of sample was placed in a 50-mL screw-capped Teflon tube. Fifty microliters of sulfadimethoxine-d6 (1.0 µg/mL) was added as internal standard, then 1 mL of 0.1 M Na_2EDTA solution, and 24 mL of acetonitrile:water 70:30 with 0.1% formic acid. The mixture was homogenized for 5 minutes with a Marconi ultraturrax (MA102), then centrifuged in a Hitachi CF16RXII Centrifuge for 5 minutes at 1,370 xg. A 500 µL amount of supernatant was eluted in a Captiva ND cartridge (A5300002) using a Manifold Supelco Visiprep System, into a 2-mL vial and analyzed by LC/MS/MS.

LC/MS/MS method

LC conditions

Instrument	Agilent 1200 Infinity Series LC system		
Column	Agilent ZORBAX Eclipse Plus C18 (3 × 100 mm, 3.5 µm)		
Column temperature	30 °C		
Mobile phase	A) 0.1% Formic acid in water B) 0.1% Formic acid in acetonitrile		
Gradient	Time (min)	%A	%B
	0	95	5
	13	5	95
Flow rate	0.4 mL/min		
Injection volume	10 µL		

MS conditions

Instrument	Agilent 6430 Triple Quadrupole LC/MS System
Ionization mode	ESI (Positive)
Drying gas flow	10 L/min
Nebulizer	50 psi
Drying gas temperature	350 °C
Capillary voltage	4,000 V
Software	Agilent Mass Hunter(B.03.01)
Detection mode	Multiple Reaction Monitoring (MRM)

Validation and Quantification procedure

The validation of the proposed procedure was carried out in agreement with the criteria of Commission Decision 2002/657/EC considering the following parameters: specificity, limits of detection (LOD) and LOQ, precision, and recovery.

A matrix-matched calibration (MMC) was carried out with blank and spiked samples, with seven different concentration levels for 11 target compounds in the 5 to 400 µg/kg range. Each level of concentration was analyzed in triplicate.

Concentrations of the target compounds in the samples were determined using the internal standard method. Sulfadimethoxine-d6 deuterated was used as an internal standard to obtain more reliable results.

Results and Discussion

The monitored ions for each compound are listed in Table 1. The most intense transition was used as a quantifying ion and the second most intense was used as a qualifying ion for the confirmation of the analysis.

Table 1. Retention Time (RT) and MS/MS Conditions of the Selected Compounds

Compounds	RT (min)	Precursor ion	Production	Fragmentor energy (V)	Collision energy (V)
Chlortetracycline	10.610	479.1	462.2*	125	12
		479.1	444.1**	125	17
Oxytetracycline	9.685	461.2	426*	115	16
		461.2	201.1**	115	41
Tetracycline	9.944	445.2	410.2*	115	17
		445.2	154.2**	115	30
Sulfadimethoxine	11.982	311.1	156*	120	16
		311.1	108**	120	28
Sulfamethazine	10.127	279.1	186*	115	12
		279.1	156**	115	16
Sulfathiazole	9.128	256	156*	90	8
		256	108**	90	20
Ciprofloxacin	9.786	332.1	288.1*	125	13
		332.1	245.1**	125	22
Enrofloxacin	10.021	360.2	342.2*	132	17
		360.2	316.2**	132	16
Norfloxacin	9.695	320.1	302.1*	125	20
		320.1	231.0**	125	44
Sarafloxacin	10.560	386.1	342.1*	119	15
		386.1	299.1**	119	26
Chloramphenicol	11.563	323	305*	70	0
		323	275**	70	8
Sulfadimethoxine-d6	11.945	317.1	162.2*	65	20
		317.1	108.1**	65	28

*Transitions used for quantification
** Transitions used for qualifying
Compound in bold is an internal standard.

The antibiotic residues were identified using retention time and two MRM transitions. Chromatograms of the compounds with the transitions selected for the analysis are shown in Figure 1.

The main benefit of Captiva cartridges is that they can easily be used to efficiently remove precipitated proteins and particulate matter. This benefit was demonstrated by the selectivity of the method and verified with injections of blank fish samples (without antibiotics) and extracts fortified with antibiotics. The blank fish sample showed less than 10% interference at the practical limit of quantatition (LOQ) of 5 µg/kg, using the same retention times as those compounds that produce the best selectivity.

The concentration range (5–400 µg/kg) showed linearity indicated by the determination coefficients (R^2) greater than 0.99 for all compounds in matrix (Table 2). Figure 2 shows an example of the drug analysis results in Nile tilapia matrix using this method.

The results also demonstrate a wide range of analyses that can discover antibiotics in fish samples, and less than 10% dispersion of the points on the curve. The curves, signal-to-noise, and calculations were made by Agilent MassHunter Software (B.03.01).

The LOD and LOQ shown in Table 2 were adequate for the antibiotics analysis in fish.

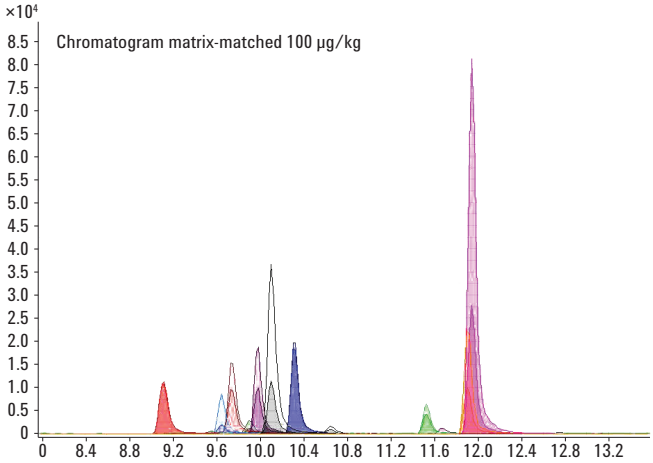

Figure 1. Total ion chromatograms (TIC) of the spiked fish sample (100 µg/kg).

Table 2. LOD, LOQ, and Determination Coefficient (R^2) of Antibiotics in Nile Tilapia Muscle

Compound	LOD (µg/kg)	LOQ (µg/kg)	R^2
Chlortetracycline	0.91	3.00	0.9992
Oxytetracycline	1.20	4.00	0.9994
Tetracycline	1.00	3.20	0.9994
Sulfadimethoxine	0.30	0.90	0.9995
Sulfamethazine	0.80	2.56	0.9992
Sulfathiazole	1.30	4.00	0.9990
Ciprofloxacin	0.40	1.20	0.9994
Enrofloxacin	0.50	1.50	0.9976
Norfloxacin	1.30	4.30	0.9992
Sarafloxacin	0.60	1.90	0.9986
Chloramphenicol	1.00	3.50	0.9992

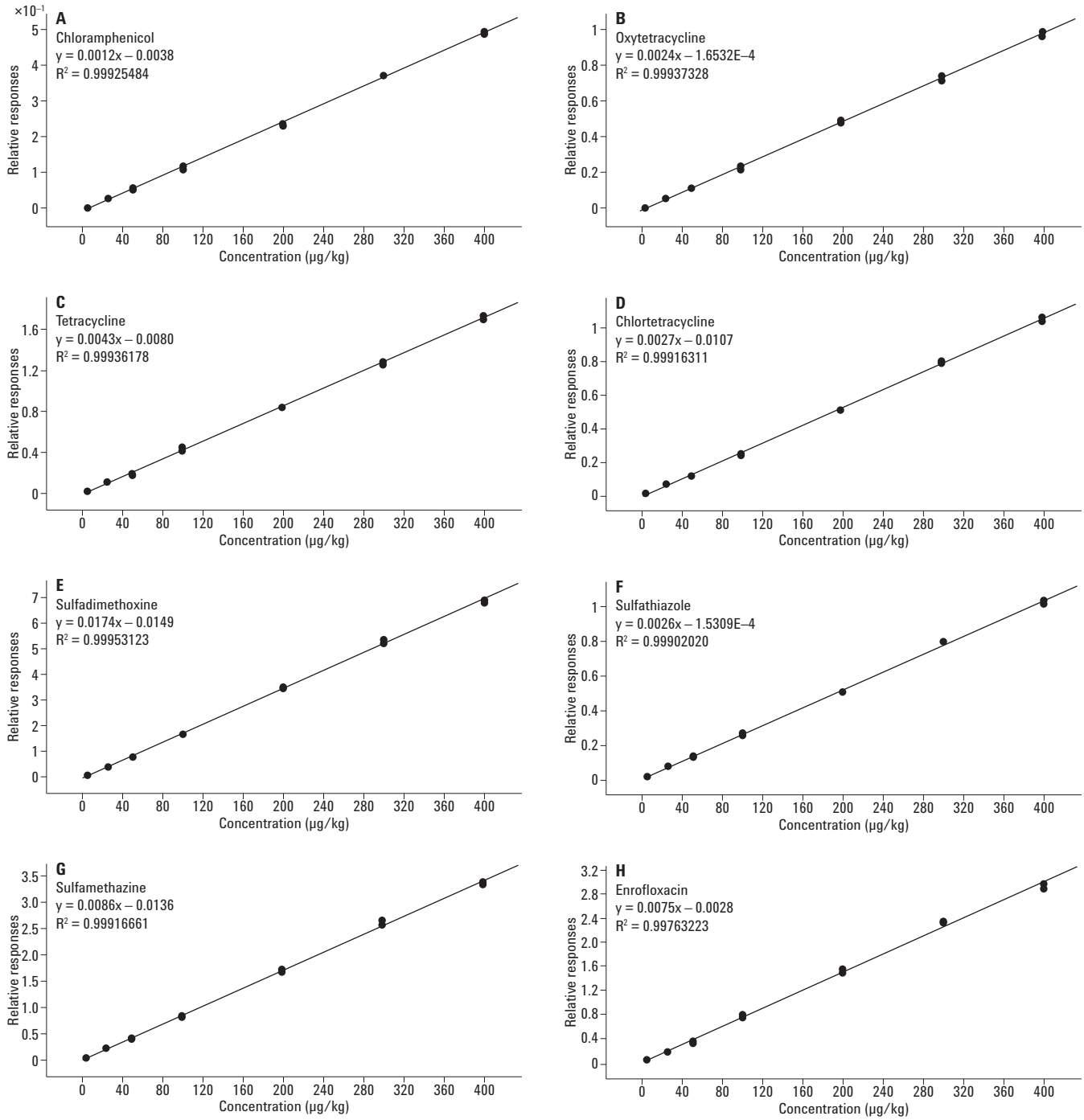

Figure 2. Calibration curves of chloramphenicol (A), oxytetracycline (B), tetracycline (C), chlortetracycline (D), sulfadimethoxine (E), sulfathiazole (F), sulfamethazine (G), and enrofloxacin (H) from 5.0 to 400 µg/kg in fish sample. *Continued next page.*

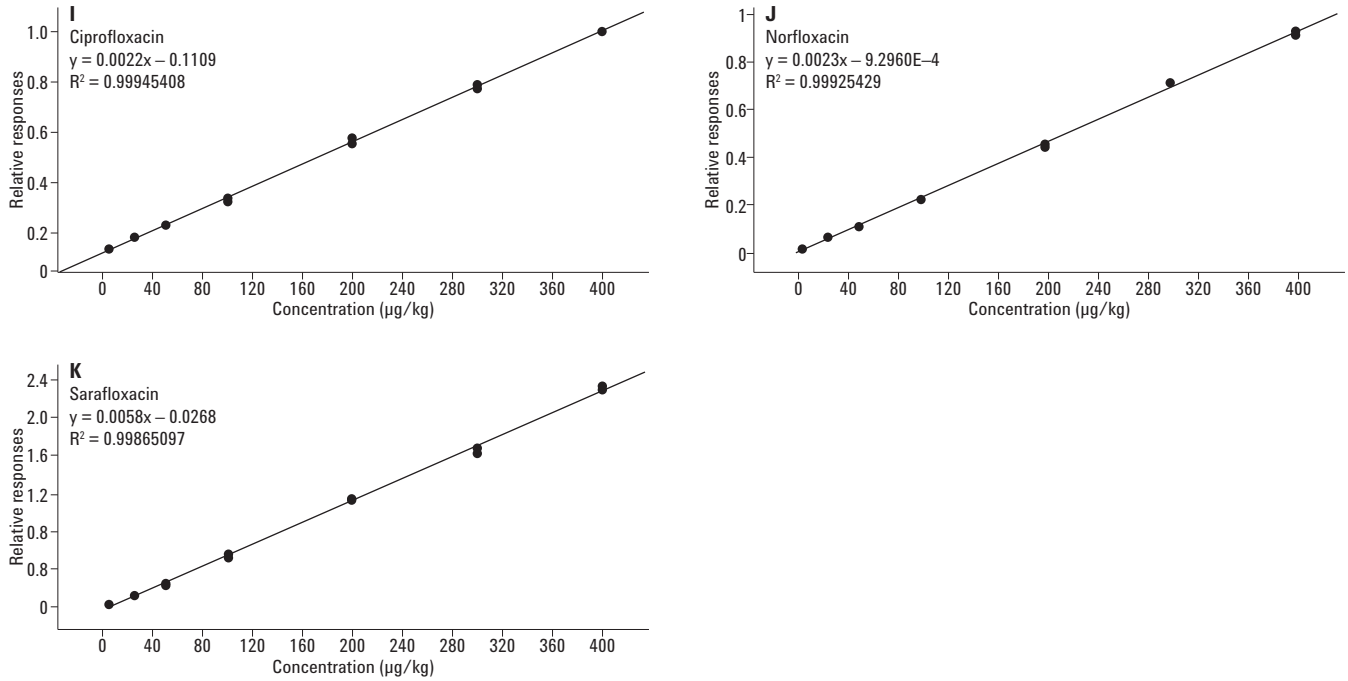

Figure 2. Calibration curves of ciprofloxacin (I), norfloxacin (J), and sarafloxacin (K) from 5.0 to 400 µg/kg in fish sample.

Precision and accuracy expressed in terms of recovery from Nile tilapia muscle were studied by analyzing spiked samples at the concentrations 50, 100, and 200 µg/kg. Intra-day precision was studied by seven replicate measurements at the concentration levels mentioned above. The results are presented in Table 3. Inter-day precision was established during routine operation of the system over a period of 30 days by seven replicates at a concentration of 100 µg/kg. The results obtained were between 2.8 and 10.3% and considered acceptable.

Table 3. Percentage of Recoveries and Intra-Day Precision (Relative Standard Deviation) for the Three Fortification Levels in Fish, and Inter-Day Precision for Level 100 µg/kg

Compound	50 µg/kg	RSD (%)	100 µg/kg	RSD (%)	200 µg/kg	RSD (%)	Inter-day RSD (%)
Chlortetracycline	108.1	3.7	100.8	4.5	87.5	7.7	6.1
Oxytetracycline	103.8	4.6	96.8	11.5	86.3	8.3	8.5
Tetracycline	106.4	4.5	107.1	5.4	93.0	7.4	6.2
Sulfadimethoxine	86.5	6.0	89.3	9.3	96.0	4.8	7.8
Sulfamethazine	99.3	4.1	94.9	7.7	97.6	5.0	5.6
Sulfathiazole	92.5	3.3	92.3	7.2	95.8	5.8	7.7
Ciprofloxacin	87.9	6.5	87.2	8.6	82.8	6.2	9.1
Enrofloxacin	98.4	6.6	108.8	4.7	95.4	8.3	8.5
Norfloxacin	96.0	10.6	100.0	9.4	87.5	5.1	7.8
Sarafloxacin	98.1	3.0	90.0	5.4	90.7	4.4	4.7
Chloramphenicol	93.2	5.2	90.5	13.2	94.5	5.5	10.3

The recovery was calculated as the ratio of the mass of analyte found in the spiked sample to the spiked mass and was expressed as a percentage. The developed method proved to be precise and accurate, and can be used for the determination of antibiotics with reliability.

Conclusions

This application note details the development of a method for the selective determination of different antibiotic classes in Nile tilapia muscle. The method uses triple quadrupole LC/MS, and is characterized by high accuracy, precision, and sensitivity. It allows the identification and quantification of the target compounds in low parts-per-billion ranges in fish farms.

The extraction procedure proposed was simple, and the sample treatment did not require an additional cleanup step to provide satisfactory recoveries.

References

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