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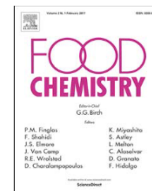

Quinolones and tetracyclines in aquaculture fish by a simple and rapid LC-MS/MS method

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ABSTRACT

The determination of antimicrobials in aquaculture fish is important to ensure food safety. Therefore, simple and fast multiresidue methods are needed. A liquid chromatography tandem mass spectrometry method was developed and validated for the quantification of 14 antimicrobials (quinolones and tetracyclines) in fish. Antimicrobials were extracted with trichloroacetic acid and chromatographic separation was achieved with a C18 column and gradient elution (water and acetonitrile). The method was validated (Decision 2002/657/EC) and it was fit for the purpose. Linearities were established in the matrix and the coefficients of determination were ≥0.98. The method was applied to Nile tilapia and rainbow trout (n = 29) and 14% of them contained enrofloxacin at levels above the limit of quantification (12.53–19.01 μg.kg^{-1}) but below the maximum residue limit (100 μg.kg^{-1}). Even though prohibited in Brazil and other countries, this antimicrobial reached fish. Measures are needed to ascertain the source of this compound to warrant human safety.

1. Introduction

Aquaculture is an important system of fish production, which is growing worldwide faster than any other animal food-producing sectors (FAO, 2010; Romero, Feijoo, & Navarrete, 2012). The contribution of aquaculture to total fish production (including nonfood uses) has grown from 13.4% in 1990 to 42.2% in 2012 (FAO, 2014). Its relative contribution to the total amount of fish produced for human consumption ranged from 5% in 1962, to 37% in 2002 and to 49% in 2012 (FAO, 2014; Santos & Ramos, 2016).

Although aquaculture has many advantages, the fast growth of this production system has resulted in concerns over fish quality and safety. Fish production adopts intensive and semi-intensive practices, in which high densities of animals in small spaces can occur, substantially increasing the risk of spreading disease among fish and resulting in high-mortality rates (EFSA, 2008; Quesada, Paschoal, & Reyes, 2013b; Santos & Ramos, 2016). The dissemination of diseases in aquaculture is also due to inadequate management and poor environmental conditions, including feeding levels, removal and restocking, and inadequate nutrition (Quesada et al., 2013b). Therefore, the use of antimicrobial

Abbreviations: CCα, decision limit; CCβ, detection capability; CE, collision energy; CV, coefficient of variation; CXP, collision cell exit potential; DP, declustering potential; ESI, electrospray ionization; FDA, Food and Drug Administration; HPLC, high-performance liquid chromatography; LC, liquid chromatography; LOQ, limit of quantification; MRL, maximum residue limit; MRM, multiple reaction monitoring; MS, mass spectrometry; MS/MS, tandem mass spectrometry; PVDF, polyvinylidene fluoride; TCA, trichloroacetic acid; VL, validation level

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agents in aquaculture is needed, as it can help in the treatment and prevention of infectious diseases. In this way, antibiotics are used as therapeutic, prophylactic or metaphylactic agents and for growth promotion purposes (Quesada et al., 2013b; Romero et al., 2012).

The most commonly used antibiotics in aquaculture worldwide are tetracycline, oxytetracycline (tetracyclines), oxolinic acid, flumequine, sarafloxacin, enrofloxacin (quinolones), amoxicillin (β-lactam), erythromycin (macrolide), sulfadimethoxine (sulfonamide), ormetoprim (diaminopyrimidine) and florfenicol (amphenicol) (Quesada et al., 2013b). Each country has its own legislation regarding the approval of antibiotics and concentrations for use in aquaculture. In Brazil, only two antimicrobials are licensed for aquaculture – florfenicol and oxytetracycline (SINDAM, 2016). Maximum residue limits (MRL) for antibiotics in food are established by many regulatory agencies around the world, including the European Union (EU), the United States Food and Drug Administration (FDA), the Brazilian Ministry of Agriculture, Livestock and Supply (MAPA), as well as the Codex Alimentarius and the European Medicines Agency (EMEA) to ensure the quality and safety of consumer products (Brasil, 2015; CODEX, 2014; EC, 2010; Quesada et al., 2013b; Rezk, Riad, Khattab, & Marzouk, 2015). MRL values for quinolones and tetracyclines in fish are described in Table 1. The low limits (μg.kg^{-1}) established for these antibiotics require sensitive and specific methods to monitor and determine unequivocally antimicrobial residues in aquatic products.

Based on this information, it is important to monitor the presence of antibiotics in fish in order to allow international trade and to protect consumers from health hazards. The presence of such residues in food can be responsible for toxic effects, allergic reactions in individuals with hypersensitivity and can also result in the development of resistant strains of bacteria (Freitas, Barbosa, & Ramos, 2013). Indeed, in recent years, bacterial resistance has become a worldwide concern and food-producing animals are a potential source of antibiotic resistant bacteria in humans. As a result, there is increasing pressure on laboratories responsible for ensuring the safety of food for human consumption regarding the development of reliable and sensitive methods for the analysis of antibiotic residues in food (Cháfer-Pericás, Maquieira, & Puchades, 2010).

Liquid chromatography tandem mass spectrometry (LC-MS/MS) is a powerful technique for the analysis of antibiotics because of its high specificity and sensitivity (Monteiro et al., 2015). Many studies were undertaken using LC-MS/MS to detect one or a few antibiotics in fish (Dickson, 2014; Fedorova, Nebesky, Randak, & Grabic, 2014; Freitas et al., 2014; Gbylik, Posyniak, Mitrowska, Bladek, & Zmudzki, 2013; Hernando, Mezcua, Suarez-Barcena, & Fernandez-Alba, 2006; Mendoza et al., 2012; Monteiro et al., 2015; Quesada, Paschoal, & Reyes, 2013a; Rezk et al., 2015; Samanidou, Evaggelopoulou, Trotzmüllerb, Guob, & Lankmayrb, 2008; Santos et al., 2005; Wu, Chen, Mao, Lu, & Wang 2012). However, most of the methods available include a laborious sample preparation step, which increases analysis time and the consumption of reagents, generating large amounts of residues in the environment. In this context, there is a need for simple, fast and reliable quantitative methods for the analysis of antibiotics in aquaculture fish.

The aim of the present study was to develop and validate a simple, rapid and sensitive quantitative method for the simultaneous determination of quinolones and tetracyclines in fish and to analyze fish samples from aquaculture.

Table 1
Maximum residue levels (MRL) of quinolones and tetracyclines in fish established by different regulatory agencies.

Class/Antibiotic	Maximum residue levels – MRL (μg.kg^{-1})/regulatory agency		
	Brazil (Brasil, 2015)	CODEX (CODEX, 2014)	European Union (EC, 2010)
Quinolones			
Ciprofloxacin[a]	Sum equal to 100	n.e.	Sum equal to 100
Danofloxacin	n.e.	n.e.	100
Difloxacin	300	n.e.	300
Enrofloxacin[a]	Sum equal to 100	n.e.	Sum equal to 100
Flumequine	600	500 (trout)	600
Nalidixic acid	20	n.e.	n.e.
Oxolinic acid	20	n.e.	100
Sarafloxacin	30	n.e.	30
Tetracyclines[b]	Sum equal to 200		
Chlortetracycline		n.e.	100
Doxycycline		n.e.	n.e.
Oxytetracycline		200	100
Tetracycline		n.e.	100

Maximum residue levels (MRL) has not been established for the quinolones Marbofloxacin and Norfloxacin in the above mentioned regulatory agencies.
n.e. – not established.
[a] Sum of ciprofloxacin and enrofloxacin.
[b] Sum of all tetracyclines.

2. Material and methods

2.1. Material

2.1.1. Chemicals and reagents

LC-MS grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). Trichloroacetic acid (TCA) and sodium hydroxide (NaOH) were from Vetec (Rio de Janeiro, RJ, Brazil). Ultra-pure water was obtained from a Milli-Q purification apparatus (Millipore, Bedford, MA, USA).

All antibiotics were of high purity grade (> 99.0%). They included four tetracyclines (chlortetracycline, doxycycline, oxytetracycline and tetracycline) and ten quinolones (ciprofloxacin, danofloxacin, difloxacin, enrofloxacin, flumequine, marbofloxacin, nalidixic acid, norfloxacin, oxolinic acid and sarafloxacin), a total of 14 compounds. They were purchased from Sigma-Aldrich (St. Louis, MO, USA), Honeywell Fluka (Bern, Switzerland) and Dr. Ehrenstorfer (Augsburg, Germany). The respective shelf-lives were carefully considered (5 months for tetracyclines and 6 months for quinolones).

Each standard was accurately weighed and transferred to a 50-mL volumetric flask and used to prepare methanolic stock solutions at concentrations of 100 μg.mL^{-1} for quinolones and 200 μg.mL^{-1} for tetracyclines. To enhance solubility, 1 mL of 1 mol.L^{-1} NaOH was added to quinolone standard solutions. Individual stock solutions were stored at −10 °C. Working standard solutions were obtained by dilution of each stock solution in ultra-purified water, at concentrations varying from 0.15 μg.mL^{-1} to 3.0 μg.mL^{-1} for quinolones and to 1.0 μg.mL^{-1} for tetracyclines. All working solutions were kept at −10 °C and prepared fresh monthly.

2.1.2. Samples

Blank samples of Nile tilapia, used for method validation, were collected from two farms in the state of Minas Gerais, Brazil, where none of the studied antimicrobials were used. A total of 29 samples of Nile tilapia (*Oreochromis niloticus*) and rainbow trout (*Oncorhynchus mykiss*) from fish farms in the state of Minas Gerais, Brazil, were used.

2.2. LC-MS/MS analysis

A 1200 Series HPLC (Agilent Technologies Inc., Santa Clara, CA, USA) liquid chromatograph coupled to a Triple Quadrupole Mass Spectrometer detector API 5000 AbSciex (Framingham, MA, USA) was used to separate and to quantify the antibiotics. A Zorbax Eclipse XDB C18 (150 × 4.6 mm, 5.0 μm) column, from Agilent Technologies (Santa Clara, CA, USA), was used. The composition and flow rate of the mobile phases, gradient elution, injection volume, and column temperature were investigated to establish optimum conditions for the chromatographic separation of all compounds and to achieve a short running

time. The final condition was: phase A – 0.1% heptafluorobutyric acid in water and phase B – acetonitrile at a gradient elution of: initial time – 90% A; 7.0 min – 50% A; 11.0 min – 50% A; 12.0 min – 90% A; and 15 min – 90% A at a constant flow rate of 600 µL.min^{-1}. The injection volume was 10 µL and the column temperature was set at 35 °C.

The best conditions for precursor and product ions, collision energy (CE), collision cell exit potential (CXP) and declustering potential (DP) were established through the optimization of each compound separately by direct infusion of individual standard solutions at concentrations ranging from 50 to 100 ng.mL^{-1} in methanol. Electrospray ionization (ESI) in positive mode and multiple reaction monitoring (MRM) were used and the two most intense transitions were selected for quantification and confirmation purposes. Analyst software, version 1.5.1 from AbSciex (Framingham, MA, USA), was used for system control, acquisition and data processing.

2.3. Sample preparation

The method was adapted from another one applied to milk (Gaugain-Juhel et al., 2009). The optimized parameters were: TCA concentration, and vortexing and centrifugation times. Briefly, the final condition was: 2.0 g of ground and homogenized fish muscle was weighed in a 50-mL polypropylene centrifuge tube. Then, 1 mL of ultrapure water was added. The sample was vortexed for 30 s and after standing for 10 min at room temperature, 8 mL of 0.5% TCA was added. The sample was homogenized in an ultra-turrax for 20 s, placed in a shaker for 5 min, and centrifuged for 10 min at 2700 × g at 4 °C. The extract was filtered through a 0.45 µm pore size PVDF membrane (Millipore, Bedford, MA, USA) immediately prior to LC-MS/MS analysis.

2.4. Maximum residue limit and validation level

Maximum residue limit (MRL) values used were based on Brazilian and European legislation for fish (Brasil, 2015; EC, 2010). When it was not available for fish, MRL established for other matrices, e.g. chicken, pork and cattle (CODEX, 2014), were used. Validation levels (VL) were set as 0.5xMRL concentrations, except for nalidixic acid and oxolinic acid (VL = 1.0 × MRL).

2.5. Validation of the method

The fitness of the optimized method for the analysis of ten quinolones and four tetracyclines in fish was evaluated according to the Commission Decision 2002/657/EC (EC, 2002). The following parameters were determined: calibration curves, accuracy, precision, decision limit (CCα), detection capability (CCβ), specificity and limit of quantification.

2.5.1. Calibration curves

The existence of matrix effect was assumed by observation of the chromatogram of the analytes in solvent and in matrix. Therefore, the calibration curves were all constructed in the matrix extract.

Calibration curves were constructed using blank fish samples spiked with six concentrations (0.25 × VL, 0.50 × VL, 0.75 × VL, 1.0 × VL, 1.25 × VL, 1.5 × VL) of each antibiotic. The ranges for each analyte used in the calibration curves are described in Table 2.

For each analyte, a plot of instrument response versus concentration was built and the linear equation and the correlation coefficient (fit degree) were calculated by least squares linear regression analysis. A calibration curve was run every day of samples' analysis.

2.5.2. Accuracy and precision

Known levels of analytes were added to a blank matrix to determine accuracy and precision (repeatability and reproducibility). Eighteen aliquots of the blank matrix were selected and three groups of six aliquots each were fortified with 0.5, 1.0 and 1.5 times the validation levels described in Table 2. The samples were analyzed and the concentrations for each aliquot and for each level were calculated. Then, accuracy was calculated as described in Eq. (1) (EC, 2002):

$$\text{Accuracy} = 100 \times \text{mean of concentration found/fortification level} \quad (1)$$

Repeatability was established through evaluation of the coefficient of variation and the standard deviation for each level (0.5 × VL, 1.0 × VL and 1.5 × VL). The analyses were carried out on three different days with different analysts in order to evaluate reproducibility. Mean concentration, standard deviation and coefficient of variation (%) were calculated for the fortified samples of each analyst (EC, 2002).

2.5.3. Specificity

Twenty different blank samples of fish muscle were analyzed to evaluate the specificity of the method. The existence of any interference (possible peaks) that could affect detection in the range of retention time of the target analytes was investigated.

2.5.4. Decision limit (CCα) and detection capability (CCβ)

The decision limit (CCα) was established by testing twenty blank samples fortified at the validation level. According to the Commission Decision 2002/657/EC (EC, 2002), CCα should be evaluated at the MRL level. However, it was calculated at 0.5 × MRL levels (except for oxolinic and nalidixic acids) in order to allow the detection of lower concentrations. The decision limit (α = 5%) was equal to the validation level plus 1.64 times the corresponding standard deviation (EC, 2002).

In order to determine CCβ, twenty blank samples were fortified at CCα for each antibiotic. CCβ (β = 5%) was equal to CCα plus 1.64 times the corresponding standard deviation (EC, 2002).

2.5.5. Limit of quantification (LOQ)

The limit of quantification was determined as the lowest concentration of the analyte that could be determined with acceptable accuracy and precision. It was considered as the first analyte concentration at the calibration curve (AOAC, 1998).

2.6. Analysis of fish samples

Aquaculture fish samples (n = 29) were analyzed, including 26 Nile tilapia (*Oreochromis niloticus*), from different regions of Minas Gerais (metropolitan region of Belo Horizonte, 'Central Mineira' and 'Zona da Mata'), and three rainbow trout (*Oncorhynchus mykiss*), from the south of Minas Gerais. These samples were positive for enrofloxacin in a previous screening study, which investigated the occurrence of several antimicrobials from different classes (Guidi et al., 2017). The optimized and validated method was used. Calibration curves were run simultaneously every day of analysis.

3. Results and discussion

3.1. Optimization of the LC-MS/MS procedure

The optimized spectrometric parameters and the retention time windows (equal to retention time ± 3 times standard deviation) for each analyte individually are shown in Table 3. The chromatographic conditions were optimized to provide the shortest possible run of all analytes of interest with appropriate resolution. After investigating different compositions and gradients of the mobile phase, the one which provided best results was phase A – 0.1% heptafluorobutyric acid in water and phase B – acetonitrile at a gradient elution of: initial time – 90% A; 7.0 min – 50% A; 11.0 min – 50% A; 12.0 min – 90% A; and 15 min – 90% A at a constant flow rate of 600 µL.min^{-1}. The injection volume was 10 µL and the column temperature was set at 35 °C.

The chromatographic run had a total time of 15 min and all analytes eluted within 12 min. The shortest retention time was observed for

Table 2
Maximum residue levels (MRL), validation levels (VL), range of calibration curves concentrations and analytical curves equations in the matrix (y = peak area, x = analyte concentration in $\mu g.kg^{-1}$) and determination coefficients (R^2) of each antibiotic of the quantification method during the validation of the method for the analysis of quinolones and tetracyclines in fish by LC-MS/MS.

Class/Analyte	MRL ($\mu g.kg^{-1}$)	VL ($\mu g.kg^{-1}$)	Range of concentration in calibration curves ($\mu g.kg^{-1}$)	Equations (R^2)
Quinolones				
Ciprofloxacin	100[a]	50	12.5–75.0	y = 3931.4x − 3068.9 (0.98)
Danofloxacin	100[b]	50	12.5–75.0	y = 3197.4x − 3320 (0.99)
Difloxacin	300[a]	150	37.5–225.0	y = 190.98x − 360 (0.98)
Enrofloxacin	100[a]	50	12.5–75.0	y = 5259x + 177.78 (0.99)
Flumequine	600[a]	300	75.0–450.0	y = 8281.9x + 6888.9 (0.99)
Marbofloxacin	100[b]	50	12.5–75.0	y = 1460.6x − 922.22 (0.98)
Nalidixic acid	20[a]	20	5.0–30.0	y = 2087.4x + 2297.8 (0.98)
Norfloxacin	100[b]	50	12.5–75.0	y = 2625.5x − 4588.9 (0.99)
Oxolinic acid	20[a]	20	5.0–30.0	y = 13712x − 3155.6 (0.996)
Sarafloxacin	30[a]	15	3.75–22.50	y = 2212.8x − 606.67 (0.99)
Tetracyclines	Sum equal to 200[a]		25.0 – 150.0	
Chlortetracycline		100		y = 488.91x + 164.44 (0.98)
Doxycycline		100		y = 3538.3x + 4377.8 (0.99)
Oxytetracycline		100		y = 939.66x − 1486.7 (0.98)
Tetracycline		100		y = 2709.1x − 5088.9 (0.99)

MRL – Maximum residue limit; VL – validation level; R^2 – determination coefficient.
[a] (Brasil, 2015).
[b] (CODEX, 2014).

marbofloxacin (7.89–7.98 min), which had the highest affinity to the aqueous phase and lowest interaction with the stationary phase. On the other hand, the longest retention time was observed for flumequine (10.6–11.00 min).

Typical chromatograms (extracted ion chromatograms) obtained from fish muscle samples spiked with all tested antibiotics at the validation level are shown in Fig. 1. These chromatograms were obtained by selecting the quantification transition for each analyte (Table 3). The high specificity and sensitivity of the triple quadrupole mass analyzer allowed the detection of the 14 analytes in only one chromatographic run. Both quantification and confirmation transitions (*m/z*) were used to promptly confirm a positive response. As can be observed in the chromatograms (Fig. 1), the proposed extraction procedure provided peaks with good resolution, suggesting its efficiency in the extraction of

Table 3
Range of retention times and optimized spectrometric conditions – precursor (Q1), quantification (Q) and confirmation (C) ions, declustering potential (DP), entrance potential (EP), collision energy (CE) and collision cell exit potential (CXP) – for each analyte of the quantification method during analysis of quinolones and tetracyclines by LC-MS/MS.

Class/Analyte	Retention times range (min)*	Q1 (*m/z*)	Q3 (*m/z*)		DP	EP	CE	CXP
			Q	C				
Quinolones								
Ciprofloxacin	7.98–8.28	332.0	314.0	231.0	61	10	30/47	12/12
Danofloxacin	8.26–8.34	358.0	340.0	255.0	60	10	33/50	10/10
Difloxacin	8.93–9.25	400.0	356.0	299.0	100	10	35/40	10/10
Enrofloxacin	8.31–8.61	360.0	342.0	286.0	72	10	30/50	12/12
Flumequine	10.46–10.86	262.1	244.0	202.0	44	10	25/45	12/12
Marbofloxacin	7.97–8.07	363.0	345.0	320.0	70	10	30/22	10/10
Nalidixic acid	10.22–10.86	233.0	215.0	187.0	42	10	30/35	12/12
Norfloxacin	7.85–8.17	320.0	302.0	231.0	60	10	33/50	12/12
Oxolinic acid	8.85–9.21	262.0	244.0	216.0	53	10	25/40	12/12
Sarafloxacin	8.77–9.11	386.0	368.0	348.0	50	10	30/40	12/12
Tetracyclines								
Chlortetracycline	8.56–8.90	479.2	98.2	275.0	61	10	67/55	12/12
Doxycycline	9.47–9.79	445.0	428.0	154.2	55	10	25/40	12/12
Oxytetracycline	7.97–8.31	461.3	201.1	283.2	41	10	59/53	12/12
Tetracycline	8.36–8.70	445.0	410.0	427.0	55	10	27/25	12/12

Q1 – precursor ion; Q – quantification ion; C – confirmation ion; DP – declustering potential; EP – entrance potential; CE – collision energy; CXP – Collision Cell Exit Potential; * Retention time range (mean of retention time ± 3sd) (n = 15).

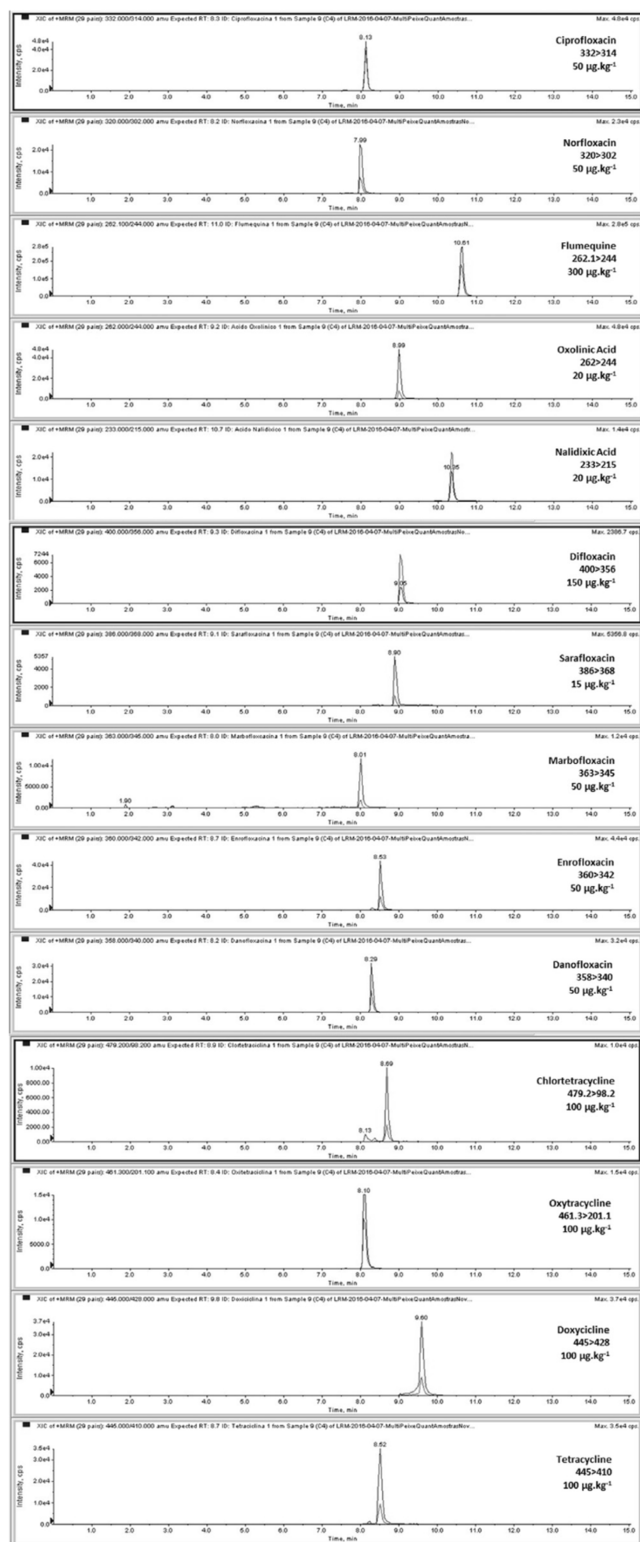

Fig. 1. Extracted Ion Chromatogram (XIC) for both the precursor and quantification (Q1 > Q) ions showing retention times for blank fish muscle sample spiked with each investigated quinolone and tetracycline at the validation level during LC-MS/MS analysis.

the analytes at the desired concentration.

3.2. Method validation

3.2.1. Analytical curves, accuracy, repeatability, reproducibility

Equations and determination coefficients (R^2) of quinolones and tetracyclines in the matrix are indicated in Table 2. The data fitted a linear regression model with R^2 above 0.98 and, therefore, adequate linearity within the working range for all analytes.

The mean concentration of analytes in spiked samples, the coefficients of variation (CV) of repeatability and reproducibility and accuracy are indicated in Table 4. Accuracy was evaluated by means of recovery of known amounts of each analyte added to a blank matrix. According to the Commission Decision 2002/657/EC (EC, 2002), when analyte concentration is between 1 and 10 µg.kg^{-1}, the acceptable range of recovery must be between 70% and 110%; when analyte concentration is higher than or equal to 10 µg.kg^{-1}, the acceptable range of recovery must be between 80% and 110%. As the mean recovery for all the studied analytes fitted this criterion, the accuracy of the method was considered adequate.

According to the Commission Decision 2002/657/EC (EC, 2002), the maximum CV allowed for "in house" reproducibility for the considered MRLs is 20% for all analytes, except for concentrations above 150 µg.kg^{-1}, in which the maximum CV allowed is 15%. Repeatability maximum CV must be between 1/2 and 2/3 of the CV of reproducibility. Then, the maximum CVs for repeatability were 13.3% and 10%, respectively. As CVs for all the analytes fit these criteria, the method was considered precise in the analysis of fish muscle.

3.2.2. Specificity

Blank samples (n = 20) of fish muscle were analyzed to evaluate the presence of interference in the expected retention time of each analyte. The absence of interference above a signal-to-noise ratio of 3 at the range of retention time of the target compounds was verified. There were no interferences that could compromise detection and identification of the compounds and the method was considered specific for all the studied analytes.

3.2.3. Limit of quantification (LOQ), decision limit (CCα) and detection capability (CCβ)

The results for LOQ, CCα and CCβ for each antibiotic are indicated in Table 5. Limits of quantification were established as the first point of calibration curves and varied from 3.75 to 75.0 µg.kg^{-1}. Decision limits (CCα) varied from 17.87 to 323.20 µg.kg^{-1} and indicate that samples with concentrations above these values are considered positive with an error $\alpha = 5\%$. Detection capability (CCβ) varied from 20.75 to 346.40 µg.kg^{-1}. Detection capability is the lowest analyte content that can be detected, identified and/or quantified in a sample with a probability of error β. In the case of substances with an established allowed limit, the detection capability is the concentration at which the method is able to detect concentrations at this limit with a statistical certainty of 1-β (EC, 2002). Although these parameters do not present criteria for upper limits, determined values were considered satisfactory, once they were below or close to the MRL.

3.3. Analysis of fish samples

The validated method was used in the analysis of 29 samples of fish collected from farms, three rainbow trout and 26 Nile tilapia samples, from the state of Minas Gerais, Brazil. These samples were positive for enrofloxacin in a previous screening test.

None of the tetracyclines and quinolones, except enrofloxacin, was detected in the samples, confirming that the screening method previously used was reliable at 5% significance, i.e., no false negative samples were found.

However, enrofloxacin was detected in every positive sample analyzed in the screening method; therefore, no false positive samples were found. Among tilapia and rainbow trout samples analyzed, only four Nile tilapia samples had enrofloxacin at concentrations equal or above the LOQ (12.5 µg.kg^{-1}): 12.53, 12.62, 12.92, and 19.01 µg.kg^{-1}. The remaining 25 samples had trace levels of enrofloxacin, below LOQ. Therefore, in every sample this antimicrobial was present at levels

Table 4
Mean concentration, coefficients of variation of repeatability (CV_r) and reproducibility (CV_R) and accuracy obtained for quinolones and tetracyclines in fish by LC-MS/MS.

Class/Analyte	Spiking level (μg.kg^{-1})	Mean concentration ± sd (μg.kg^{-1})	Precision (%)		Accuracy (%)
			CV_r	CV_R	
Quinolones					
Ciprofloxacin	25	25.7 ± 0.7	6.7	2.6	102.8
	50	50.3 ± 1.0	6.9	1.9	100.5
	75	76.8 ± 3.2	5.9	4.1	102.4
Danofloxacin	25	24.5 ± 3.4	7.7	13.8	97.8
	50	49.0 ± 2.4	8.2	4.8	98.0
	75	70.3 ± 5.1	8.9	7.2	93.8
Difloxacin	75	74.3 ± 2.9	5.0	3.9	99.1
	150	149 ± 4.1	6.9	2.7	99.3
	225	220 ± 14	7.1	6.2	97.8
Enrofloxacin	25	22.3 ± 2.6	9.2	11.6	89.3
	50	45.9 ± 5.3	8.7	11.5	91.8
	75	69.0 ± 9.7	9.6	14.0	92.0
Flumequine	150	153 ± 6.0	4.6	3.9	102.6
	300	301 ± 13	6.0	4.2	100.7
	450	434 ± 5.2	5.2	4.2	96.3
Marbofloxacin	25	25.7 ± 0.9	6.3	3.5	102.6
	50	50.0 ± 2.7	6.7	5.4	99.0
	75	75.0 ± 4.2	4.4	5.6	99.9
Nalidixic acid	10	9.90 ± 0.3	5.8	2.5	99.0
	20	20.0 ± 0.3	6.7	1.6	100.0
	30	29.1 ± 1.7	6.4	5.8	96.8
Norfloxacin	25	24.4 ± 1.5	6.0	6.1	97.6
	50	48.8 ± 2.9	6.0	5.8	97.6
	75	73.5 ± 5.8	6.5	7.9	98.0
Oxolinic acid	10	10.1 ± 0.4	5.7	3.5	101.3
	20	20.2 ± 0.3	6.6	1.4	101.2
	30	29.4 ± 0.3	6.8	0.9	97.9
Sarafloxacin	7.5	7.78 ± 0.3	7.1	3.8	103.7
	15	14.7 ± 0.2	6.4	1.1	97.7
	22.5	21.2 ± 0.8	9.3	3.8	94.3
Tetracyclines					
Chlortetracycline	50	50.9 ± 2.3	8.3	4.4	101.8
	100	99.9 ± 3.4	5.8	3.4	99.9
	150	143 ± 6.4	6.5	4.5	95.6
Doxycycline	50	50.2 ± 1.5	4.9	2.9	100.4
	100	99.6 ± 6.1	4.0	6.1	99.6
	150	149 ± 3.8	4.9	2.5	99.5
Oxytetracycline	50	50.2 ± 2.8	7.0	5.6	100.4
	100	102 ± 3.5	7.8	3.4	102.3
	150	143 ± 6.2	6.4	4.3	95.2
Tetracycline	50	50.1 ± 2.6	5.1	5.1	100.2
	100	102 ± 3.9	7.4	3.8	101.7
	150	140 ± 6.0	7.3	4.3	93.5

n = 18; sd – standard deviation; CV_r – coefficient of variation of repeatability; CV_R – coefficient of variation of reproducibility.

Table 5
Limit of quantification (LOQ), decision limit (CCα) and detection capability (CCβ) obtained for quinolones and tetracyclines in fish by LC-MS/MS.

Class/Analyte	LOQ (μg.kg^{-1})	CCα (μg.kg^{-1})	CCβ (μg.kg^{-1})
Quinolones			
Ciprofloxacin	12.5	55.6	61.3
Danofloxacin	12.5	56.4	62.9
Difloxacin	37.5	167	183
Enrofloxacin	12.5	58.5	67.0
Flumequine	75.0	323	346
Marbofloxacin	12.5	53.6	57.1
Nalidixic acid	5.0	23.9	27.8
Norfloxacin	12.5	55.2	60.3
Oxolinic acid	5.0	22.4	24.8
Sarafloxacin	3.75	17.9	20.8
Tetracyclines			
Chlortetracycline	25.0	111	122
Doxycycline	25.0	107	115
Oxytetracycline	25.0	111	121
Tetracycline	25.0	111	123

LOQ – limit of quantification; CCα – decision limit; CCβ – detection capability.

below the MRL established by the legislation – 100 μg.kg^{-1} (Brasil, 2015).

The screening method previously used (Guidi et al., 2017) suggested that enrofloxacin concentration was above the screening target (50 μg.kg^{-1}) in one sample, which was not confirmed in this quantitative method. This result reinforces the need to use quantitative method following screening results, to confirm occurrence and concentration of analytes.

Quesada et al. (2013a) failed to detect four fluoroquinolones including enrofloxacin in 13 samples of pacu and 18 of tilapia from the state of São Paulo, Brazil. Monteiro et al. (2015) did not find fluoroquinolones in tilapia from the state of São Paulo, Brazil; however, they detected florfenicol, tetracycline and oxytetracycline in fish from five different farms. Quantitative information regarding quinolones and tetracyclines in fish from farms from the state of Minas Gerais was reported for the first time.

Enrofloxacin is an effective antimicrobial agent with a broad spectrum of activity. It was detected in the fish samples even though its use is not allowed in aquaculture in Brazil and several other countries (Brasil, 2015; Kim, Kim, Yoon, Chun, & Cerniglia, 2012; SINDAM, 2016). However, it is available in the market for veterinary use and also

allowed for use in aviculture in some countries, including Brazil (Brasil, 2015; SINDAM, 2016). Although FDA withdrew the approval for the use of enrofloxacin in poultry in 2005 because it could select for fluoroquinolone resistant Campylobacter, it is still approved for use in some food producing animals and companion animals (Kim et al., 2012). It is important to consider that residues of antibiotics can reach fishes by several routes of contamination. The use of enrofloxacin in aviculture for example can result in its release in the environment through waste streams by which fish may be contaminated. Also, the illegal direct use of enrofloxacin in aquaculture, either due to misinformation or on purpose, could be another important source of contamination. Furthermore, the availability of enrofloxacin as a veterinary antibiotic facilitates its acquisition and possible illegal use. In order to guarantee fish quality, human health and international trade, it is necessary to determine the source of contamination and to implement educational programs to prevent health hazard associated with antibiotic abuse.

4. Conclusions

A quantitative LC-MS/MS method was optimized for the simultaneous quantification of 14 antibiotics (10 quinolones and 4 tetracyclines) in fish muscle. Extraction was performed with 0.5% TCA and separation was achieved with a C18 column and a gradient elution of water:acetonitrile. All the compounds eluted in a single run of 15 min. Sample preparation was simpler and faster when compared with other methods for multiclass antibiotic analysis in fish found in the literature, which is desirable for routine methods.

The method was validated according to Commission Decision 2002/657/EC and it satisfactorily fulfilled the established criteria for the 14 antibiotics in fish. The method was successfully applied to real samples. Four out of 29 samples of Nile tilapia had enrofloxacin at concentrations above the LOQ (12.5 $\mu g.kg^{-1}$), with concentrations ranging from 12.53 to 19.01 $\mu g.kg^{-1}$. The remaining 25 samples had trace levels of enrofloxacin below LOQ. Every sample had levels of enrofloxacin below the MRL established by legislation. However, it is important to elucidate the source of contamination to protect consumer's health. The low occurrence of antibiotics in farm fish suggests responsible management of aquaculture in the places where the samples were collected.

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