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A simple, fast and sensitive screening LC-ESI-MS/MS method for antibiotics in fish

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ABSTRACT

The objective of this study was to develop and validate a fast, sensitive and simple liquid chromatography–electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) method for the screening of six classes of antibiotics (aminoglycosides, beta-lactams, macrolides, quinolones, sulfonamides and tetracyclines) in fish. Samples were extracted with trichloroacetic acid. LC separation was achieved on a Zorbax Eclipse XDB C18 column and gradient elution using 0.1% heptafluorobutyric acid in water and acetonitrile as mobile phase. Analysis was carried out in multiple reaction monitoring mode via electrospray interface operated in the positive ionization mode, with sulfaphenazole as internal standard. The method was suitable for routine screening purposes of 40 antibiotics, according to EC Guidelines for the Validation of Screening Methods for Residues of Veterinary Medicines, taking into consideration threshold value, cut-off factor, detection capability, limit of detection, sensitivity and specificity. Real fish samples (n=193) from aquaculture were analyzed and 15% were positive for enrofloxacin (quinolone), one of them at a higher concentration than the level of interest (50 µg kg^{-1}), suggesting possible contamination or illegal use of that antibiotic.

1. Introduction

Aquaculture is one of the food-producing systems with the highest growth in the world and today it accounts for nearly 50% of the world's food fish [1]. However, intensive systems of animal food production are favorable to the spread of infectious diseases due to high population density. This is specially so in aquaculture, as the aquatic environment is prone to disease proliferation. In addition, abrupt physico-chemical changes in the aquatic environment and inappropriate management practices can directly affect the health of the fish [2]. For these reasons, the use of antibiotics in aquaculture is a common practice in the treatment of diseases. In addition, antibiotics can be used as prophylactic agents to avoid or prevent diseases and also as a feed additive to promote growth and increase feed efficiency [3–5].

Many antibiotics are allowed for use in aquaculture worldwide, and varying classes are permitted in different countries. As examples, tetracycline, oxytetracycline (tetracyclines), oxolinic acid, flumequine, enrofloxacin (quinolones), amoxicylin (β-lactam), erythromycin (macrolide), sulfadimethoxine (sulfonamide), ormetoprim (diaminopyrimidine) and florfenicol (amphenicol) can be cited. The first two are the most widely used [6,7]. Antibiotics are administered through the diet or are released directly into surface waters and, after metabolism, antibiotics and/or their metabolites can end up in tissues or can be excreted through urine and feces. Therefore, there can be accumulation of antibiotics in water and sediments which can contaminate the aquatic ecosystem [8,9]. In addition, some antibiotics from intensive livestock can also be released into the environment and reach water resources [9–12].

Abbreviations: CCβ, detection capability; CE, collision energy; CRLs, Community Reference Laboratories; CXP, collision cell exit potential; DP, declustering potential; ELISA, enzyme-linked immunosorbent assay; ESI, electrospray ionization; FDA, Food and Drug Administration; HFBA, heptafluorobutyric acid; HPLC, high-performance liquid chromatography; HRMS, high-resolution mass spectrometry; LC, liquid chromatography; LOD, limit of detection; MRL, maximum residue limit; MRM, multiple reaction monitoring; MS, mass spectrometry; MS/MS, tandem mass spectrometry; PVDF, polyvinylidene fluoride; STC, screening target concentration; TCA, trichloroacetic acid; Tv, threshold value; UHPLC, ultra-high performance liquid chromatography; UV, ultraviolet

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Table 1
Antibiotics included in the study and respective Maximum Residue Limit (MRL), screening target concentration and concentrations of stock solutions.

Class/analyte	Concentration		
	MRL ($\mu g\,kg^{-1}$)	Screening target ($\mu g\,kg^{-1}$)	Stock solution ($\mu g\,mL^{-1}$)
Aminoglycosides			
Amikacin	500[a]	250	200
Apramycin	500[a]	250	200
Dihydrostreptomycin	500[c]	250	200
Gentamicin	500[a]	250	200
Hygromycin	500[a]	250	200
Kanamycin	500[a]	250	200
Neomycin	500[b]	250	200
Paromomycin	500[c]	250	200
Spectinomycin	500[b]	250	200
Streptomycin	500[c]	250	200
Tobramycin	500[a]	250	200
Beta-lactams			
Ampicillin	50[a]	25	200
Cefazolin	50[a]	25	200
Oxacillin	300[c]	150	200
Penicillin G	50[a]	25	200
Penicillin V	25[a]	12.5	200
Macrolides			
Clindamycin	100[b]	50	100
Erythromycin	100[b]	50	100
Lincomycin	200[b]	100	100
Spiramycin	200[c]	100	100
Tilmicosin	100[c]	100	100
Tylosin	100[c]	100	100
Virginiamycin	200[b]	100	100
Quinolones			
Ciprofloxacin	100[a]	50	100
Danofloxacin	100[b]	50	100
Difloxacin	300[a]	150	100
Enrofloxacin	100[a]	50	100
Flumequine	600[a]	300	100
Marbofloxacin	100[b]	50	100
Nalidixic acid	20[a]	20	100
Norfloxacin	100[b]	50	100
Oxolinic acid	20[a]	20	100
Sarafloxacin	30[a]	15	100
Sulfonamides			
Sulfachloropyridazine	100[a]	50	250
Sulfadiazine	100[a]	50	250
Sulfadimethoxine	100[a]	50	250
Sulfadoxine	100[a]	50	250
Sulfamerazine	100[a]	50	250
Sulfamethazine	100[a]	50	250
Sulfamethoxazole	100[a]	50	250
Sulfamethoxypyridazine	100[a]	50	250
Sulfaphenazole (IS)	–	–	
Sulfaquinoxaline	100[a]	50	250
Sulfathiazole	100[a]	50	250
Sulfisoxazole	100[a]	50	250
Tetracyclines			
Chlortetracycline	200[a]	100	200
Doxycicline	200[a]	100	200
Oxytetracycline	200[a]	100	200
Tetracycline	200[a]	100	200

IS–internal standard.
[a] Brasil [44].
[b] Codex [45].
[c] EC [43].

The inappropriate and abusive use of antibiotics can be a potential public health hazard once their residues can remain in the fish muscle [13]. For example, residues of tetracyclines and sulfonamides [14], chloramphenicol [5,15], oxytetracycline [16,17], enrofloxacin [18,19] and florfenicol [16,17,20] have been detected in fish. Furthermore, it can remain in the water and sediment from aquaculture systems. Indeed, Monteiro et al. [16,17] detected oxytetracycline, tetracycline and florfenicol in different fish farms and tetracycline antibiotics were found in river sediments.

Among health hazard issues to man, antibiotics in food can induce allergic reactions in some sensitive individuals. Furthermore, it can compromise human intestinal and immune systems, lead to the appearance of bacterial resistance in humans and animals, and affect the environment selecting the most resistant bacteria [4,5,13]. Several regulatory agencies established Maximum Residue Limits (MRL) for antimicrobials in food of animal origin (Table 1), and concentrations above the MRL are inappropriate for human consumption.

In order to warrant public health safety and to maintain competitiveness in international trade, the monitoring of antibiotics in fish and other foods of animal origin is needed. Therefore, sensitive and reliable analytical methods for the determination of multi-antibiotics in food are required. The effective control of antibiotics in foods requires the combination of cost effective and high sample throughput screening methods, followed by confirmation and quantification of suspect samples [5,21]. Liquid chromatography coupled to mass spectrometry in tandem (LC-MS/MS) has been used in the analysis of multi-antibiotics in food, both for screening and quantitative methods [14,17,22–32]. Analytical methods using bioassay techniques or sensitive microorganisms are widely used as screening methods [33]. However, the use of LC-MS/MS for screening purposes is becoming popular as it can provide good specificity, sensitivity, and low rate of false-positive samples [23,29,34–36]. Through determination of the cut-off factor in a screening method, it is possible to evaluate if the sample contains or not the antibiotic in a concentration above MRL [37]. Since in most of the cases the samples are expected to comply, reports can be issued faster for samples which comply, whereas samples with cut-off factor above MRL should be further analyzed by quantitative methods [21].

LC-MS/MS methods for the analysis of more than five classes of antibiotics are available for milk [22,25,28,29,31], eggs [29], honey [38], meat [26,29,30,39], liver [27] and fish [20,33,40–42]. However, most of the multiclass methods available for the screening of antibiotics in fish are, in general, laborious and limited to a few antimicrobials. Therefore, the objective of this study was to develop a simple, sensitive and fast screening method for multiple classes of antimicrobials in fish muscle.

2. Experimental

2.1. Material

2.1.1. Chemicals and reagents

LC-MS grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany); heptafluorobutyric acid (HFBA) was from Fluka (Buchs, Switzerland) and trichloroacetic acid (TCA) was from Vetec (Rio de Janeiro, Brazil). Ultra-pure water was obtained from a Milli-Q purification apparatus (Millipore, Bedford, MA, USA).

All the antibiotics were of high purity grade (> 99.0%). They included aminoglycosides, beta-lactams, macrolides, quinolones, sulfonamides, and tetracyclines, in a total of 49 compounds. They were purchased from Sigma-Aldrich (St. Louis, MO, USA), Fluka (Buchs, Switzerland) and Dr. Ehrenstorfer (Augsburg, Germany). Sulfaphenazole, the internal standard, was purchased from Sigma-Aldrich (St. Louis, MO, USA). The shelf-lives of the antibiotics were carefully considered and varied from 3 to 12 months.

Each standard was accurately weighed and transferred to a 50-mL volumetric flask and used to prepare methanolic stock solutions (Table 1) at concentrations varying from 100 to 250 $\mu g\,mL^{-1}$. Beta-lactams and aminoglycosides were dissolved in ultra-purified water, and 1 mL of 1 $mol\,L^{-1}$ NaOH was added to quinolone standard solutions to enhance solubility. 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

0.125 μg mL^{-1} to 3.0 μg mL^{-1}. The internal standard (sulfaphenazole) solution was prepared at 0.5 μg mL^{-1} in ultra-purified water. All the working solutions were kept at −10 °C and prepared fresh monthly, except beta-lactams, which were prepared weekly.

2.1.2. Samples

Blank samples of Nile tilapia used in the validation process were collected at two farms from the state of Minas Gerais, Brazil, where none of the studied antimicrobials were used. A total of 193 fish muscle samples from fish farms under federal inspection were obtained: 172 from the state of Minas Gerais and 21 from the state of Pará, Brazil. The samples from Minas Gerais included 149 Nile tilapia (*Oreochromis niloticus*) and 23 trout (*Oncorhynchus mykiss*); whereas the samples from Para included 9 Nile tilapia (*Oreochromis niloticus*) and 12 tambaqui (*Colossoma macropomum*).

2.2. LC-MS/MS analysis

Liquid chromatography was performed in an Agilent 1200 Series HPLC (Agilent Technologies Inc., Santa Clara, CA, USA) coupled to a Triple Quadrupole Mass Spectrometer detector API 5000 AbSciex (Life Technologies Corporation, CA, USA). A Zorbax Eclipse XDB C18 (150×4.6 mm, 1.8 μm, Agilent Technologies, CA, USA) column was used. To establish optimum conditions for the chromatographic separation of all compounds and to achieve a short running time, several chromatographic parameters were investigated, including composition and flow rate of the mobile phase, gradient elution, injection volume and column temperature.

Mass spectrometer parameters were also optimized for each compound separately by direct infusion of individual standard solutions at concentrations ranging from 50 to 100 μg L^{-1} in MeOH. The best precursor and product ions, declustering potential (DP), collision energy (CE) and collision cell exit potential (CXP) were established. Electrospray ionization (ESI) generated the ions in a positive mode. Multiple reaction monitoring (MRM) was used and two transitions were selected: the most intense transition for quantifications and the second most intense for confirmation purposes. Each chromatographic run was divided into scan events with a scan time of 90 s for each transition. The analytical system control, acquisition and data processing were performed using Analyst software, version 1.5.1, from AbSciex (Life Technologies Corporation, CA, USA).

2.3. Sample preparation

The method used for extraction of the antibiotics from the samples was adapted from that described by Gaugain-Juhel et al. [22]. A schematic diagram for sample preparation is indicated in Fig. 1. Briefly, 2.0 g (wet weight) of ground and homogenized fish muscle was weighted in a 50-mL polypropylene centrifuge tube. Then, 200 μL of internal standard (sulfaphenazole at 0.5 μg mL^{-1}) and 800 μL of deionized water were added. The sample was vortexed for 30 s and after standing for 10 min at room temperature, 8 mL of 5% TCA was added. The sample was homogenized in an ultra-turrax for 20 s, placed in a shaker for 10 min, and centrifuged at 2700×*g* for 12 min at 4 °C. The extract was filtered through a PVDF membrane with 0.45 μm pore size (Millipore, Bedford, MA, USA) immediately prior to LC-MS/MS analysis.

2.4. Validation of the method

The fitness of the screening method optimized for the analysis of antibiotics in fish was evaluated according to the Guidelines for the Validation of Screening Methods for Residues of Veterinary Medicines (Initial Validation and Transfer)-Community Reference Laboratories (CRLs) 20/1/2010 [37]. The following parameters were evaluated: threshold value (T_V), cut-off factor (F_m), detection capability (CCβ), limit of detection (LOD), sensitivity and specificity.

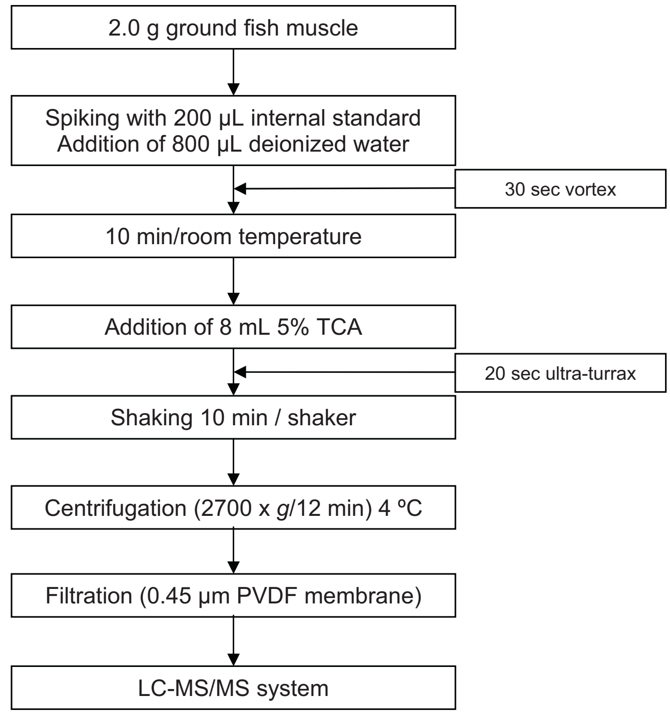

Fig. 1. Sample preparation for screening analysis of six classes of antimicrobials in fish muscle.

2.4.1. Threshold value

The threshold value (T_v) was determined by analyzing twenty blank samples of fish muscle extracted according to the procedure described in item 2.3. The analytical response (chromatographic peak area) of the blank sample at the retention time (±10%) of each analyte was determined in each chromatogram for both quantitation and confirmation transitions. The mean and the estimated standard deviation of the noise were calculated. T_v was calculated according to Eq. (1).

$$T_v = B + 1.64 \times S_B \quad (1)$$

where B and S_B are, respectively, the mean and the standard deviation of the chromatographic peak areas of blank samples at the retention time of each analyte.

2.4.2. Cut-off factor

The cut-off factors (F_m) were calculated by using twenty blank samples of fish muscle spiked with the screening target concentration (STC), which is half of the MRL concentration based on Brazilian legislation for fish and other matrices (chicken, pork and meat) when not available for fish and European legislations [43–45], except for nalidixic acid, oxolinic acid, tilmicosin and tylosin (STC=1.0×MRL) (Table 1). The samples were analyzed at the same day and this step was repeated in a different day to obtain forty independent data. Peak area was determined for each analyte (n=40) for both transitions of quantification and confirmation. Means and estimated standard deviations were calculated for each analyte and the cut-off factor was estimated according to Eq. (2).

$$F_m = D - 1.64 \times Sd \quad (2)$$

where D and Sd are, respectively, the mean and the standard deviation of the chromatographic peak areas. It means statistically that 95% of the samples spiked at the level of interest should give an analytical response above this value.

2.4.3. Detection capability

The detection capability (CCβ) was estimated from the comparison of threshold values and cut-off factors. When the cut-off factor is above the threshold value, CCβ is considered as definitely below the level of interest (0.5×MRL, in this case). On the other hand, when the cut-off factor is below the threshold value, more than 5% of the samples will be considered as negative samples and, consequently, CCβ is really above the level of interest [37].

2.4.4. Limit of detection (LOD)

The limit of quantification (LOD) was estimated by extracting and analyzing by LC-MS/MS 20 blank samples of fish muscle. LODs for each analyte (one for each *m/z* transition – quantification and confirmation) were calculated as the mean concentration of the blank samples in the retention time of each analyte plus three times the standard deviation of the blank concentration. The LOD for each analyte was ascribed as the higher of the two values, in most cases from the confirmation *m/z* transition.

2.4.5. Sensitivity and specificity

To calculate the sensitivity (%), twenty samples were spiked with all antibiotics at 0.5 x MRL concentration, extracted and analyzed by LC-MS/MS. The instrument response for peak area (R_{an}) for each analyte was compared to the cut-off factor and if $R_{an} > F_m$, the sample was considered non-compliant (positive), i.e., it contains a concentration above 0.5×MRL. However, if $R_{an} < F_m$, the sample was considered compliant (negative), i.e., it contains a concentration below 0.5×MRL.

The method sensitivity was estimated from Eq. (3) and it must be higher than 95% to ensure a β error below 5%. In this case, all the samples are positive because they were spiked at a 0.5×MRL concentration.

$$Sens.(\%) = \frac{Number\ of\ samples\ considered\ positive}{Number\ of\ samples\ really\ positive\ (20)} x100 \tag{3}$$

To determine specificity of the method, e.g. its ability to detect unambiguously a specific analyte from a complex matrix, the blank chromatograms at the retention time of each studied analyte were carefully evaluated in order to verify possible interferences.

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 ± 5%) for each analyte individually are shown in Table 2. The chromatographic conditions for the screening method were optimized to provide the shortest possible run of all analytes of interest with appropriate resolution. The mobile phase composition which provided best results was phase A – 0.1% of heptafluorobutyric acid (HFBA) 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 flow rate and injection volume were 0.6 mL min^{-1} and 10 μL, respectively and the column temperature was set at 35 °C. Total chromatographic run lasted 15 min

The presence of two chromatographic peaks, one for each *m/z* transition – quantification and confirmation, eluting at the same retention time allowed the unequivocal identification of each analyte. Each chromatographic peak presented a signal-to-noise ratio (S/N) equal to 3 under these conditions [23]. As can be noticed, several sulfonamides exhibit the same quantification and confirmation ions. However, as the precursor ion differs among them, distinction of each of them is allowed. Sulfadimethoxine and sulfadoxine had the same quantification and confirmation ions but they had also similar precursor ions (311.1 and 311.0, respectively), which could lead to mistaken identification of these two substances. However, because of the different retention time windows observed for these compounds (9.17–9.60 and 8.15–8.57, respectively), the correct identification of each antibiotic was possible.

The total ion chromatograms obtained for all analytes in solvent (water) and in the fish matrix are indicated in Fig. 2. The run had a total time of 15 min and all analytes eluted within 12 min. The shortest retention time was observed for sulfadiazine (5.58–6.00 min), which has highest affinity with the aqueous phase and lowest interaction with the stationary phase. On the other hand, the longest retention time was observed for oxacillin (11.00–11.60 min).

The high specificity and sensitivity of the triple quadrupole mass analyzer allowed the detection of the 40 analytes in only one chromatographic run. To assess specificity, 20 blank samples of fish muscle of different origins were analyzed and no chromatographic peak was detected in these samples at the retention time corresponding to each analyte, indicating a specificity of 100% for all the analytes. Both quantification and confirmation transitions (*m/z*) were used to confirm promptly a positive response. The extraction procedure proposed provided good quality chromatograms, suggesting its efficiency for the extraction and the analytes concentration.

3.2. Screening method validation

During validation of a screening method, it is important to find global conditions to detect all of the analytes simultaneously. The method has to present sufficient sensitivity to detect all the targeted analytes at least at the level of interest, which is 0.5×MRL. Furthermore, qualitative methods of analysis must have the capability of a high sample throughput and the ability to detect all targeted analytes with a false-compliant rate below 5% (β error) at the level of interest. In the case of suspected non-compliant results, these must undergo confirmation by a confirmatory method [46].

The results of CCβ, LOD, sensitivity, and the comparison between threshold value and cut-off factor (F_m/T_v) are presented in Table 3. The cut-off factor (the analytical response – peak area in this case – indicating that a sample contains a substance with a concentration equal to or higher than the level of interest) was compared to threshold value (the minimal analytical response above which the sample will be truly considered positive) to evaluate CCβ.

According to the protocol for validation of screening methods [37], detection capability (CCβ) of screening methods can be evaluated only when the cut-off factor is above the threshold value. When this condition is achieved, CCβ is considered as definitely below the level of interest (0.5×MRL, in this case). On the other hand, when the cut-off factor is below the threshold value, more than 5% of the positive samples will be considered as negative samples and, consequently, CCβ is really above the level of interest and the analyte cannot be analyzed by the method with 95% of confidence.

Among the 48 antibiotics analyzed, 40 attended the criteria established by EC [46] and EC [37], e.g., CCβ was truly below the level of interest tested during validation (0.5×MRL) and the screening method was efficient in detecting all 40 analytes which presented $F_m > T_v$, with 95% of significance and a false-compliant rate of 5%. In general, all these analytes showed low LODs values (minimum concentration of a given analyte that can be detected with a reasonable statistical confidence), indicating that the method is capable of detecting low concentrations of these antibiotics.

The eight antibiotics which did not attend EC [46] and EC [37] included erythromycin, spiramycin, tylosin, virginiamycin, ampicillin, oxacillin, penicillin G and penicillin V. These compounds did not have cut-off factors above threshold value (e.g., $F_m < T_v$), which indicates that CCβ values for these analytes were higher than 0.5×MRL and also that more than 5% of the non-compliant samples can show a compliant result (false negative). Although sensitivities for these analytes at 0.5×MRL concentration were satisfactory (> 95%), most of them had high LODs values (sometimes above the MRL). Therefore, even though

Table 2
Optimized spectrometric conditions – precursor ion, confirmation transition (C) and quantification transitions (Q), declustering potential (DP), entrance potential (EP), collision energy (CE), collision cell exit potential (CXP) and retention time windows (RTW) – for each analyte in the screening method.

Class/analyte	Precursor ion (*m/z*)	Quantification/confirmation ion (*m/z*)	DP	EP	CE	CXP	Retention time windows RTW[a] (min)
Aminoglycosides							
Amikacin	586	163 (Q)/245 (C)	60	10	53/21	14/20	7.80–8.13
Apramycin	540	217 (Q)/378 (C)	82	10	35/25	12/12	8.22–8.54
Dihydrostreptomycin	584	263 (Q)/246 (C)	120	10	42/54	12/12	7.43–7.75
Gentamicin	464.3	322.6 (Q)/160.2 (C)	50	10	20/20	12/12	8.41–8.92
Hygromycin	528	352 (Q)/177 (C)	50	10	25/25	12/12	7.31–7.63
Kanamycin	485	163 (Q)/205 (C)	70	10	35/35	12/12	7.88–8.21
Neomycin	615.3	161.3 (Q)/293.50 (C)	120	10	41/35	8/18	8.50–9.01
Paromomycin	616.2	293.1 (Q)/163.2 (C)	91	10	33/55	18/10	8.19–8.50
Spectinomycin	351	207 (Q)/189 (C)	66	10	31/33	12/12	6.74–7.09
Streptomycin	582	263 (Q)/246 (C)	157	10	45/51	12/12	7.39–7.83
Tobramycin	468	163 (Q)/324 (C)	100	10	20/20	12/8	8.27–8.58
Beta-lactams							
Ampicillin	350	106 (Q)/160 (C)	50	10	20/20	12/12	7.77–8.10
Cefazolin	455	323 (Q)/156 (C)	50	10	15/23	12/12	7.15–7.48
Oxacillin	402	160 (Q)/243 (C)	50	10	18/18	12/12	11.00–11.60
Penicillin G	335.4	176.3 (Q)/160.2 (C)	70	10	21/21	10/10	9.59–10.40
Penicillin V	351.1	160.1 (Q)/192 (C)	66	10	15/17	8/12	10.00–11.10
Macrolides							
Clindamycin	425.3	126.4 (Q)/377.2 (C)	75	10	43/27	22/10	9.09–9.35
Erythromycin	734.5	158.2 (Q)/576.7 (C)	66	10	43/27	14/8	10.10–10.80
Lincomycin	407	126 (Q)/359 (C)	60	10	40/26	12/12	7.39–7.68
Spiramycin	422.5	174.3 (Q)/101.3 (C)	56	10	31/25	16/8	9.33–9.72
Tilmicosin	869.5	174.4 (Q)/696.5 (C)	56	10	63/57	10/34	10.20–10.50
Tylosin	916.6	174.4 (Q)/772.4 (C)	115	10	55/43	6/20	9.88–10.80
Virginiamycin	526.5	355.2 (Q)/109 (C)	76	10	25/47	26/10	8.15–11.80
Quinolones							
Ciprofloxacin	332	314 (Q)/231 (C)	61	10	30/47	12/12	8.03–8.33
Danofloxacin	358	340 (Q)/255 (C)	60	10	33/50	10/10	8.18–8.26
Difloxacin	400	356 (Q)/299 (C)	100	10	35/40	10/10	8.98–9.30
Enrofloxacin	360	342 (Q)/286 (C)	72	10	30/50	12/12	8.42–8.72
Flumequine	262.1	244 (Q)/202 (C)	44	10	25/45	12/12	10.6–11.00
Marbofloxacin	363	345 (Q)/320 (C)	70	10	30/22	10/10	7.89–7.98
Nalidixic acid	233	215 (Q)/187 (C)	42	10	30/35	12/12	10.40–10.80
Norfloxacin	320	302 (Q)/231 (C)	60	10	33/50	12/12	7.89–8.20
Oxolinic acid	262	244 (Q)/216 (C)	53	10	25/40	12/12	8.92–9.28
Sarafloxacin	386	368 (Q)/348 (C)	50	10	30/40	12/12	8.82–9.15
Sulfonamides							
Sulfachloropyridazine	285	156 (Q)/92 (C)	51	10	21/39	12/12	7.82–8.26
Sulfadiazine	251	156 (Q)/108 (C)	53	10	22/30	12/12	5.58–6.00
Sulfadimethoxine	311.1	156 (Q)/108 (C)	50	10	23/37	12/12	9.17–9.60
Sulfadoxine	311	156 (Q)/108 (C)	60	10	25/40	12/12	8.15–8.57
Sulfamerazine	265	156 (Q)/92 (C)	60	10	35/35	12/12	6.22–6.59
Sulfamethazine	279	156 (Q)/108 (C)	50	10	25/36	12/12	6.73–7.11
Sulfamethoxazole	254	108 (Q)/92 (C)	60	10	35/35	12/12	8.23–8.68
Sulfamethoxypyridazine	281	156 (Q)/108 (C)	60	10	25/35	12/12	7.04–7.42
Sulfaphenazole (IS)	315	156	50	10	30	12	9.35–9.45
Sulfaquinoxaline	301	156 (Q)/108 (C)	50	10	23/40	12/12	9.19–9.61
Sulfathiazole	256	156 (Q)/108 (C)	53	10	20/34	12/12	6.15–6.51
Sulfisoxazole	268	156 (Q)/113 (C)	46	10	21/23	12/12	8.55–8.99
Tetracyclines							
Chlortetracycline	479.2	98.2 (Q)/275 (C)	61	10	67/55	12/12	9.31–9.64
Doxycicline	445	428 (Q)/154.2 (C)	55	10	25/40	12/12	9.51–9.82
Oxytetracycline	461.3	201.1 (Q)/283.2 (C)	41	10	59/53	12/12	8.07–8.40
Tetracycline	445	410 (Q)/427 (C)	55	10	27/25	12/12	8.44–8.77

[a] RTW, retention time ± 5% (n=20).

the method demonstrates ability to monitor these compounds, it is not capable of detecting them in concentrations below the MRL. Further studies at concentrations above the 0.5xMRL can be undertaken to determine the difference between this level and CCβ.

3.3. Screening of farm fish samples

The samples collected from Brazilian fish farms were analyzed using the validated screening method for the presence of the 40 antibiotics that attended the criteria established by EC [46] and EC [37]. Twenty nine samples (15% of 193 fish samples) were positive for enrofloxacin, both tilapia and trout, from the state of Minas Gerais. None of the samples from the state of Para, both Nile tilapia and 'tambaqui', had positive results. This could result from the fish farming practices prevalent in Para. Due to the large availability of fresh water from rivers, the fishes are usually cultivated in cages inside the rivers or in large tanks (lower fish densities), which reduces the risk of spread of diseases, thereby reducing the need of antibiotics. Overall, the low occurrence of antibiotics in farm fishes can reflect the good practices adopted in most of the farms, which results in lower need for the use of antibiotics.

Among the 29 positive samples, three were trout samples from the south of Minas Gerais and 26 samples were Nile tilapia also from Minas Gerais, but different regions (metropolitan region of Belo Horizonte, 'Central Mineira' and 'Zona da Mata'). Only one sample of Nile tilapia had analyte concentration above the cut-off factor, which

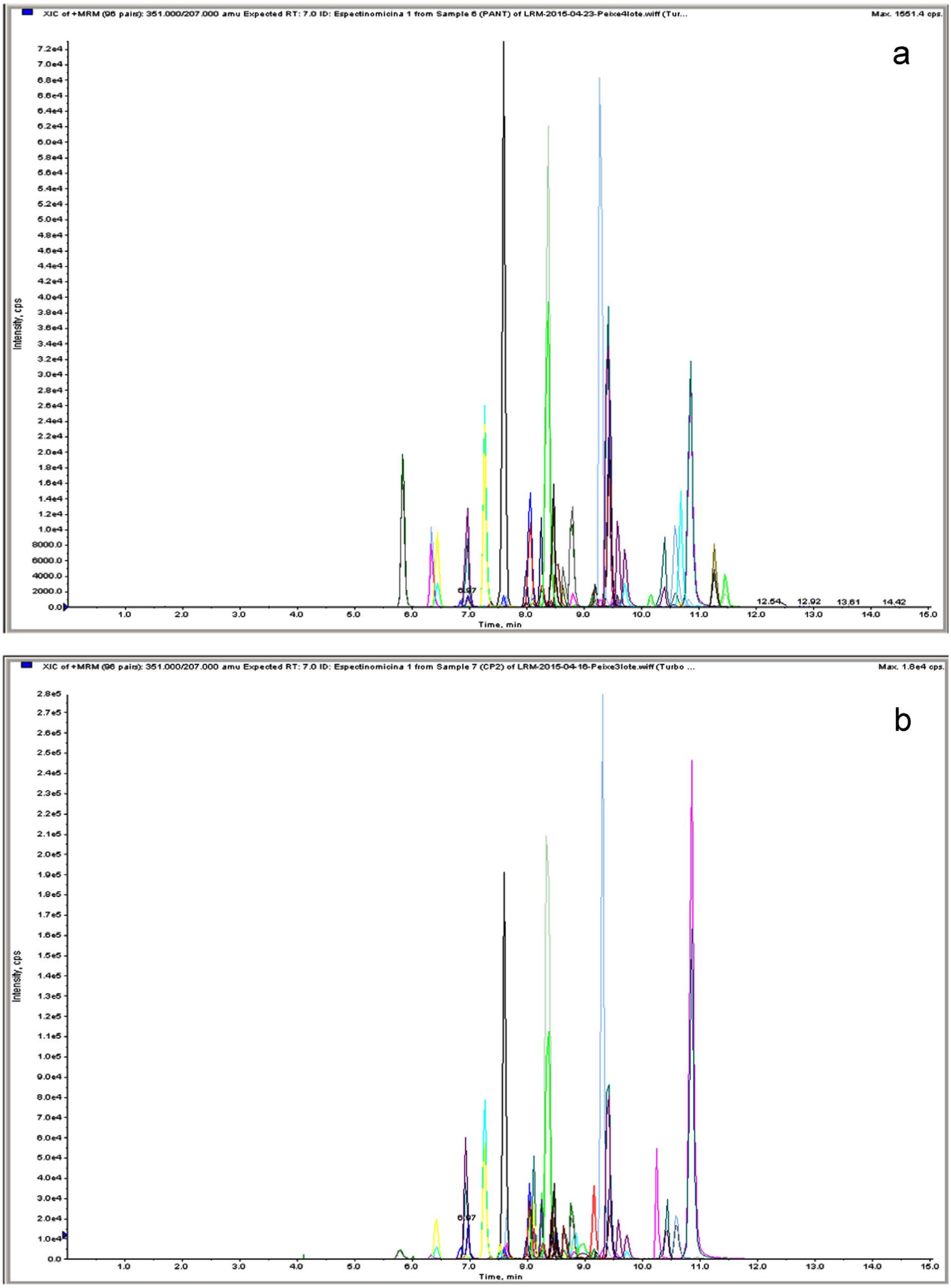

Fig. 2. Total ion chromatogram of six classes of antibiotics (a) in water and (b) in the fish matrix extract. Chromatographic conditions: mobile phases A – 0.1% heptafluorobutyric acid (HFBA) in water and B – acetonitrile, at a gradient elution: initial time – 90% A; 7.0 min – 50% A; 11.0 min – 50% A; 12.0 min – 90% A; and 15 min – 90% A at a constant flow rate – 600 µL min^{-1}.

means that this sample contained enrofloxacin in a concentration higher than the level of interest, which is 50 µg kg^{-1}. The other 28 samples had trace levels of enrofloxacin (< 50 µg kg^{-1}) and they should be submitted to a quantitative method for confirmation. These samples were positive for enrofloxacin below the cut-off factor.

Even though the use of enrofloxacin is forbidden in aquaculture in several countries, including Brazil [44,47,48], it was present in fish. Enrofloxacin is a fluoroquinolone antimicrobial agent with broad spectrum of activity available in the market for veterinary use and also allowed for use in aviculture in some countries [44,48]. In 2005, FDA

Table 3
Limit of detection (LOD), detection capability (CCβ), sensitivity (sens.) and the comparison of cut-off factor and threshold value (F_m/T_v) for each antibiotic residue in the validated screening method.

Class/analyte	LOD (μg kg^{-1})	Quantification transition			Confirmation transition		
		F_m/T_v	CCβ (μg/kg)	Sens. (%)	F_m/T_v	CCβ (μg/kg)	Sens. (%)
Aminoglycosides							
Amikacin	1.62[b]	$F_m > T_v$	< 250	95	$F_m > T_v$	< 250	100
Apramycin	3.15[a]	$F_m > T_v$	< 250	100	$F_m > T_v$	< 250	95
Dihydrostreptomycin	1.91[b]	$F_m > T_v$	< 250	95	$F_m > T_v$	< 250	95
Gentamicin	3.50[b]	$F_m > T_v$	< 250	100	$F_m > T_v$	< 250	100
Hygromycin	29.16[a]	$F_m > T_v$	< 250	95	$F_m > T_v$	< 250	100
Kanamycin	4.11[b]	$F_m > T_v$	< 250	95	$F_m > T_v$	< 250	95
Neomycin	3.32[b]	$F_m > T_v$	< 250	100	$F_m > T_v$	< 250	100
Paromomycin	3.67[a]	$F_m > T_v$	< 250	95	$F_m > T_v$	< 250	95
Spectinomycin	20.29[b]	$F_m > T_v$	< 250	100	$F_m > T_v$	< 250	100
Streptomycin	6.98[b]	$F_m > T_v$	< 250	100	$F_m > T_v$	< 250	95
Tobramycin	2.49[a]	$F_m > T_v$	< 250	100	$F_m > T_v$	< 250	100
Beta-lactams							
Ampicillin	**0.83**[b]	$\mathbf{F_m < T_v}$	***> 25***	100	$\mathbf{F_m < T_v}$	***> 25***	100
Cefazolin	1.88[b]	$F_m > T_v$	< 25	100	$F_m > T_v$	< 25	100
Oxacillin	**95.77**[b]	$\mathbf{F_m < T_v}$	***> 150***	100	$\mathbf{F_m < T_v}$	***> 150***	100
Penicillin G	**119.60**[b]	$\mathbf{F_m < T_v}$	***> 25***	100	$\mathbf{F_m < T_v}$	***> 25***	100
Penicillin V	**26.89**[b]	$\mathbf{F_m < T_v}$	***> 12,5***	100	$\mathbf{F_m < T_v}$	***> 12,5***	100
Macrolides							
Clindamycin	0.40[b]	$F_m > T_v$	< 50	100	$F_m > T_v$	< 50	100
Erythromycin	**5.84**[a]	$\mathbf{F_m < T_v}$	***> 50***	100	$\mathbf{F_m < T_v}$	***> 50***	100
Lincomycin	1.60[b]	$F_m > T_v$	< 100	100	$F_m > T_v$	< 100	100
Spiramycin	**74.24**[a]	$\mathbf{F_m < T_v}$	***> 50***	100	$\mathbf{F_m < T_v}$	***> 50***	100
Tilmicosin	1.22[b]	$F_m > T_v$	< 100	95	$F_m > T_v$	< 100	95
Tylosin	**13.29**[b]	$\mathbf{F_m < T_v}$	***> 100***	100	$\mathbf{F_m < T_v}$	***> 100***	95
Virginiamycin	**22.86**[b]	$\mathbf{F_m < T_v}$	***> 100***	100	$\mathbf{F_m < T_v}$	***> 100***	100
Quinolones							
Ciprofloxacin	0.56[b]	$F_m > T_v$	< 50	95	$F_m > T_v$	< 50	95
Danofloxacin	1.74[a]	$F_m > T_v$	< 50	100	$F_m > T_v$	< 50	100
Difloxacin	3.42[a]	$F_m > T_v$	< 150	95	$F_m > T_v$	< 150	100
Enrofloxacin	1.24[a]	$F_m > T_v$	< 50	100	$F_m > T_v$	< 50	100
Flumequine	9.09[a]	$F_m > T_v$	< 300	95	$F_m > T_v$	< 300	95
Marbofloxacin	10.02[a]	$F_m > T_v$	< 50	95	$F_m > T_v$	< 50	95
Nalidixic acid	0.82[b]	$F_m > T_v$	< 20	95	$F_m > T_v$	< 20	100
Norfloxacin	0.50[b]	$F_m > T_v$	< 50	100	$F_m > T_v$	< 50	95
Oxolinic acid	6.28[a]	$F_m > T_v$	< 20	100	$F_m > T_v$	< 20	100
Sarafloxacin	1.71[a]	$F_m > T_v$	< 15	95	$F_m > T_v$	< 15	100
Sulfonamides							
Sulfachloropyridazine	6.06[a]	$F_m > T_v$	< 50	95	$F_m > T_v$	< 50	100
Sulfadiazine	0.39[b]	$F_m > T_v$	< 50	100	$F_m > T_v$	< 50	100
Sulfadimethoxine	1.20[a]	$F_m > T_v$	< 50	100	$F_m > T_v$	< 50	95
Sulfadoxine	0.20[a]	$F_m > T_v$	< 50	100	$F_m > T_v$	< 50	100
Sulfamerazine	1.19[a]	$F_m > T_v$	< 50	95	$F_m > T_v$	< 50	95
Sulfamethazine	0.19[a]	$F_m > T_v$	< 50	95	$F_m > T_v$	< 50	100
Sulfamethoxazole	1.30[a]	$F_m > T_v$	< 50	100	$F_m > T_v$	< 50	95
Sulfamethoxypyridazine	0.54[b]	$F_m > T_v$	< 50	100	$F_m > T_v$	< 50	100
Sulfaquinoxaline	0.55[b]	$F_m > T_v$	< 50	95	$F_m > T_v$	< 50	95
Sulfathiazole	0.71[b]	$F_m > T_v$	< 50	100	$F_m > T_v$	< 50	95
Sulfisoxazole	1.78[b]	$F_m > T_v$	< 50	100	$F_m > T_v$	< 50	100
Tetracyclines							
Chlortetracycline	34.76[a]	$F_m > T_v$	< 100	100	$F_m > T_v$	< 100	100
Doxycicline	2.69[b]	$F_m > T_v$	< 100	100	$F_m > T_v$	< 100	95
Oxytetracycline	2.60[a]	$F_m > T_v$	< 100	95	$F_m > T_v$	< 100	95
Tetracycline	3.64[b]	$F_m > T_v$	< 100	95	$F_m > T_v$	< 100	100

Analytes that do not meet the requirements for inclusion in the screening method are shown in bold.
[a] Estimated from the data arising from the quantification *m/z* transition.
[b] Estimated from the data arising from the confirmation *m/z* transition.

[6] withdrew approval of its use in poultry because it could select for fluoroquinolone resistant Campylobacter. However, enrofloxacin is still approved for use in some food producing animals and companion animals [47]. It is important to consider that there could be several sources of fish contamination with antibiotics besides its administration. In the case of enrofloxacin, its use as a veterinary antibiotic, in aviculture for example, can result in its release in the environment through waste streams by which fish may be contaminated. Another source could be the direct use of enrofloxacin in aquaculture, either due to misinformation or on purpose. However, the source of contamination should be determined and educational programs implemented to warrant fish quality. Due to the health hazard associated with antibiotics abuse, there should be continuous monitoring of antibiotics in fish to warrant human health and international trade.

4. Conclusions

A screening LC-MS/MS method was optimized for the simultaneous determination of 40 antibiotics from six different classes, including aminoglycosides, beta-lactams, macrolides, quinolones, sulfonamides and tetracyclines, in fish muscle. Extraction was performed with TCA. A C18 column was used along with a gradient elution of 0.1% HFBA in water:acetonitrile. A single run of 15 min was capable of determining the presence of the compounds.

Sample preparation was simpler and faster when compared with other methods for multiclass antibiotic analysis in fish found in literature, which is desirable for routine methods. The developed method was validated according to the Guidelines for the Validation of Screening Methods for Residues of Veterinary Medicines (Initial Validation and Transfer)-Community Reference Laboratories (CRLs) 20/1/2010 and it satisfactorily fulfilled the established criteria for 40 antibiotics in fish. The method was successfully applied to real samples. Twenty nine (15%) of the 193 samples analyzed were positive for one of the 40 antibiotics (enrofloxacin), which is not allowed for use in aquaculture in Brazil. Only one sample had a concentration of enrofloxacin above the cut-off factor (50 µg kg^{-1}). This sample should proceed to quantification using a quantitative method to verify its real concentration. The low occurrence of antibiotics in farm fish suggests that there is a responsible management of aquaculture.

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