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Simultaneous determination of selected veterinary antibiotics in gilthead seabream (*Sparus Aurata*) by liquid chromatography–mass spectrometry

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Abstract

A method was optimised and validated for simultaneous monitoring of several drugs of different classes of antibiotics such as quinolones (oxilinic acid and flumequine), tetracyclines (oxytetracycline), sulfonamides (sulfadiazine) and trimethoprim in fish muscle and skin. The method is based on solid–liquid extraction without further sample clean up followed by liquid chromatography–mass spectrometry (LC–MS) determination with electrospray ion source (ESI) in positive mode. The limits of quantification (LOQs) were lower than 20 μg/kg for all compounds and repeatability, expressed as relative standard deviations (RSD), were lower than 15%. Therefore, the LC–MS method was successfully applied for the quantitative determination of antibiotics in gilthead sea bream muscle and skin and oxytetracycline in medicated fishes.

Keywords: Multiclass; Antibiotics; Liquid chromatography; Mass spectrometry; Gilthead sea bream

1. Introduction

Aquaculture production has notably increased in the last decades, mainly thanks to intensive farming. Together with market globalisation, this gives rise to the spreading of several fish diseases, increasing the demand for veterinary drugs for aquatic species. Many classes of antibiotics are commonly used in aquaculture worldwide to treat infections caused by a variety of bacterial pathogens of fish [1]. Thus, antibacterial agents used for treatment of fish diseases, include sulfodiazine, oxytetracycline, and oxolinic acid among others [2]. However, the potential hazards associated with the presence of these products in edible tissues from aquaculture include allergies, toxic effects, acquisition of drug resistance in pathogens in the human body [3] as well as their potential carcinogenic character [4]. So there is a global concern about the consumption of low levels of antimicrobial residues in aquatic foods and the effects of these residues on human health. In this sense, the European Union has established maximum residue limits (MRLs) for these compounds in food-producing animal tissues in order to ensure human food safety [5].

These limits require the development of sensitive and specific methods for the determination of antibiotic residues in food. Several published papers have proposed different methods based on immunoassay techniques [6,7]. These methods generally do not distinguish among members of a given class of antibiotics, provide only semiquantitative measurements of residues and sometimes give rise to false positives [8]. Nevertheless they are still used because of their simplicity and low-cost although other techniques must be used in order to confirm the results obtained by bioassay techniques. For this reason, other techniques, such as chromatography or electrophoresis [9], have been proposed to overcome these shortcomings, and liquid chromatography (LC) is the most frequently approach used. In relation to detection methods, they are diverse and include UV [10,11] or fluorescence detection [12,13]. However, Public Health Agencies, based on European Union guidelines, rely on the detection by mass spectrometry (MS) for confirmation of antibiotics in foodstuffs [14], considering that it provides more reliable identification and confirmation of these analytes than conventional detectors. Bearing in mind that most antibiotics are thermally labile and low-volatile compounds, liquid

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chromatography coupled to MS (LC–MS) and tandem mass spectrometry (LC–MS/MS) have become the most popular techniques for the determination of these analytes during the last few years [3,8,15–19], using electrospray (ESI) or atmospheric pressure chemical ionisation (APCI) sources [20,21]. In this sense, LC coupled to ESI has become a very valuable technique for multiresidue analysis, because it is more sensitive, selective and allow rapid and multiresidue determination in complex matrices, providing structural information [22].

Thus, many LC–MS methods have been developed for antibiotic groups such as sulfonamides [20,23], tetracyclines [24,25] and quinolones [22,26], although there is still a challenge to develop multiresidue methods, which are relatively scarce [27,28].

Furthermore, one of the main problems involved in multiresidue antibiotic analysis in real samples is the tediousness and complexity of the procedures required for the extraction, cleanup and preconcentration of the matrix analytes before instrumental analysis. Most of the extraction methods are time consuming and costly, involving extraction techniques such as liquid-liquid extraction [29], solid phase extraction (SPE) [30,31], matrix solid phase dispersion (MSPD) [32], and pressurized liquid extraction [33], including several steps such as elution, evaporation and sample resuspension, and clean-up [4]. Besides, the effective extraction and analysis of multiple classes of compounds is still a significant challenge in multi-class residue method development since the wide range of polarities, solubilities and pK_as of antibiotics, so new extraction procedures should be developed.

The aim of this work was to develop a method for the simultaneous determination of selected veterinary antibiotics, including 2 fluoroquinolones (oxolinic acid and flumequine), one tetracycline (oxytetracycline) and one sulfonamide (sulfadiazine), that are widely used in veterinary medicine as well as trimethoprim, a dihydrofolate reductase inhibitor, which is commonly used in the combination with sulfonamides as potentiator. The method involves a simple and rapid extraction procedure and the determination of several antibiotics in gilthead seabream (S*parus aurata*) from fish farm by LC–MS, considering the use of single quadrupole allows the method to be more widely adapted, since LC–MS is currently more common than LC–tandem MS in many routine laboratories.

2. Experimental

2.1. Chemicals and reagents

Oxytetracycline hydrochoride (>98.5%), flumequine (>99.0%), sulfadiazine (>99.5%), trimethoprim (>99.5%) and oxolinic acid (>98.0%) were all from Dr. Ehrenstorfer (Augsburg, Germany). Stock standards solutions of individual compounds (with concentrations between 200 and 300 mg/L) were prepared by exact weighing of the powder and dissolved in 100 mL of methanol (HPLC grade, Panreac, Barcelona, Spain), which were then stored at −20 °C in the dark. A multicompound working standard solution at a concentration of 10 mg/L of each compound was prepared by appropriate dilutions of the stock solutions with methanol and stored in screw-capped glass tubes at −20 °C in the dark. This solution was stable for 3 weeks, after which it was replaced by a new fresh solution.

One molar citrate acid solution at pH 4 was prepared by dissolving citric acid (Panreac) in water and pH was adjusted with NaOH 1 M (Panreac). EDTA–Na_2 was obtained from Merck (Darmstadt, Germany) and acetonitrile was purchased from Panreac. Other reagents were of analytical reagent grade. Cartridges Oasis HLB 200 mg from Waters (Milford, Massachusetts) and 500 mg C_{18} Sep-Pak cartridges (Milford, MA, USA) were used for cleanup during optimisation of the extraction procedure. Ultrapure water was obtained from a Milli-Q Gradient water system (Millipore, Bedford, USA).

2.2. Apparatus

The HPLC system was an Alliance 2695 equipped with an autosampler, degasser and heater column purchased by Waters (Mildford, Massachussetts, USA). The mass spectrometer system was a ZQ 2000 single quadrupole from Waters-Micromass (Manchester, UK). Data was collected by MassLynx 4.0 software in a personal computer. An Atlantis dC_{18} 150 mm × 2.00 mm i.d. 5 μm (Waters) was used for all separations. The C_{18} column was equilibrated at 30 °C with a mobile phase consisting of 90% of eluent A (0.1% aqueous solution of formic acid) and 10% of eluent B (methanol) at a flow rate of 0.3 mL/min, using the following gradient profile: 90% eluent A for 3 min; then, the percentage of eluent A was decreased linearly to 45% in 9 min, maintained at this composition for 3 min; later, eluent A was decreased again linearly to 10% in 3 min, keeping this composition for 5 min; finally, eluent A was restored to 90% in 2 min and maintained at this composition for 5 min.

Analytes were detected with ESI in positive mode. The source and desolvation temperatures were 120 and 350 °C, respectively, and the flow rates for desolvation and cone gas were 350 and 50 L/h, respectively, from a generator N_2 Flo purchased from Claind (Lenno, Italy). Capillary voltage was set to 3.5 kV.

All pH measurements were made with a Crison Basic 20 pH-meter (Insulab, Valencia, Spain) equipped with a combined AgCl–glass electrode assembly. A high-speed homogenizer Polytron PT2100 (Kinematica A.G., Littan/Luzern, Switzerland), a P-selecta Centromix mod S-549 centrifuge (Selecta, Barcelona, Spain), a kitchen blender Braun MX32 (Barcelona, Spain) and a rotary evaporator R-114 (Büchi, Flawil, Switzerland) were used to process samples. An analytical balance AB204-S from Mettler Toledo (Greifensee, Switzerland) was also used.

2.3. Extraction procedure

The sample was edible muscle and skin from gilthead seabream (*sparus aurata*). The tissue samples were minced and homogenized using a kitchen blender. To 1 g of sample, 10 mL of acetonitrile, 1 mL of 1 M citric acid (pH 4.0) and 0.5 mL of 0.5 M EDTA–Na_2 solution were added and homogenised using Polytron for 3 min. The sample was centrifuged at 3000 rpm for

10 min and the supernatant was evaporated under a stream of nitrogen and taken up in 1 mL of mobile phase, injecting 20 μL into the LC system.

2.4. Method validation

Linearity was evaluated using matrix-matched calibration, spiking blank extracts at four different concentration levels (from 25 to 500 μg/kg). Precision and accuracy of the method were evaluated by spiking blank samples. Thus repeatability (intraday precision) was studied at three concentration levels (25, 250 and 500 μg/kg), using five replicates per concentration level. Interday precision (reproducibility) was studied spiking blank samples at the same concentration levels, and they were analysed at five different days. Recovery was tested by analysing blank samples, which was fortified at three concentration levels (25, 250 and 500 μg/kg). Limits of detection (LOD) were calculated according to IUPAC recommendations [34], based on the concept of hypothesis testing, with default probabilities of 0.05 for false positives and negatives. Limit of quantification (LOQ) was obtained as the lowest concentration tested for which an acceptable recovery is obtained (70–110%) and precision lower than 20% [35].

2.5. Sample collection and preparation

Samples were collected from a fish farm and they were immediately frozen to reduce degradation of the antibiotics during transport and they were kept at −20 °C not more than 2 days till sample treatment and analysis. Before analysis, fish were partially thawed at room temperature and muscle tissue plus skin were taken for analysis. Known antibiotic-free tissue samples (muscle and skin) were obtained from fishes, which had not been on medication. Tissue samples with antibiotic residues were obtained from fishes, which were on medication with a commercial formulation of oxytetracycline. Fishes were on medication during 10 days and they were slaughtered in four groups of five samples at different time points after cessation of medication. In this sense, gilthead seabream were sampled 1, 7, 20 and 34 days after the last administration of medicated feed.

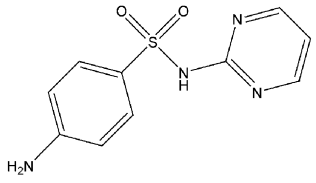
Finally, 20 samples were purchased from local grocery stores and were treated as unknown samples, and the muscle and skin were analysed.

3. Results and discussion

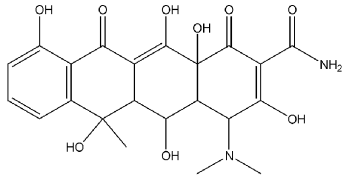
Several drugs from different classes of antibiotics, such as flumequine, oxolinic acid, oxytetracycline, sulfadiazine and trimethoprim were evaluated in this study, showing in Fig. 1 the structure of the target compounds.

3.1. LC–MS analysis

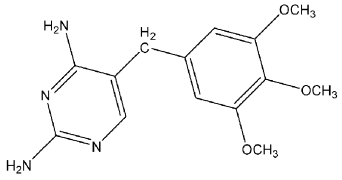
ESI-MS conditions were first optimised by direct infusion of a standard solution of 15 mg/L of each target compound at a flow rate of 0.01 mL/min. Solutions were prepared in a mixture of 50% water and 50% methanol, and injected into the ESI source in positive mode at different cone voltages. Under the experimental conditions, full scan provides mainly protonated ions ($[M+H]^+$) for oxytetracycline, trimethoprim and sulfadiazine and sodiated species ($[M+Na]^+$) for, flumequine and oxolinic acid. Table 1 shows the MS conditions selected for identification and quantification for the selected compounds. As it can be observed, for sulfadiazine and trimethoprim, their molecular ions $[M+H]^+$ and sodium adducts ($[M+Na]^+$) were obtained, selecting the first one for quantification purposes for both compounds. Besides, two typical fragment ions of sulphonamides, with *m/z* of 156 and 92, were also selected. For trimethoprim, the fragment ion at *m/z* 230 ($[M+H–2CH_3O]^+$) was also monitored. For oxytetracycline, despite of molecular ion, *m/z* 461, the ions at *m/z* 443 and 426, were monitored. Finally, the same ions were obtained (*m/z* 284 and 244) for oxolinic acid and flumequine, corresponding with the sodium adduct and the loss of water. The



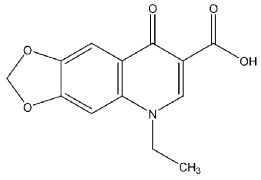
Sulfadiazine (MW: 250.3)



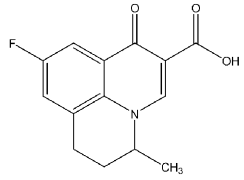
Oxytetracycline (MW: 460.4)



Trimethoprim (MW: 290.3)



Oxolinic acid (MW: 261.2)



Flumequine (MW: 261.2)

Fig. 1. Chemical structures and molecular weight of selected antibiotics.

Table 1
Maximum residue limits and LC–MS parameters for the selected antibiotics

Analyte	MRL (μg/kg)[a]	RTW (min)	*m/z*[b]
Sulfadiazine	100	7.50–8.10	273 (25); **251** (**25**); 156 (30); 92 (40)
Trimethoprim	50	9.72–10.25	313 (30); **291** (**35**); 230 (70)
Oxytetracycline	100	11.60–12.00	461 (20); 443 (30); **426** (**40**)
Oxolinic acid	100	15.80–16.05	284 (40); **244** (**40**)
Flumequine	600	18.04–18.42	284 (20); **244** (**40**)

[a] MRL for fish related to muscle and skin in natural portions accordance with European Union regulation (EEC) No. 2377/90 and updates up to 01.02.2007 (http://www.emea.eu.int/htms/vet/mrls/a-zmrl.htm#).

[b] Fragment ions in bold were used for quantification. Cone voltages are given in brackets.

second one was used for quantification purposes and the sodium adduct for confirmation.

Other parameters, such as desolvation and cone gas flow, source and desolvation temperature and capillary voltages were studied, selecting the optimum conditions indicated in Section 2.

Secondly, chromatographic conditions were studied in order to provide an overall optimum peak shape and resolution. Thus the mobile phase composition was investigated to maximise the method sensitivity and resolution, obtaining the best results when methanol was used as organic modifier and an aqueous solution of formic acid 0.1% was employed. Bearing in mind that the two quinolones evaluated in this study (flumequine and oxolinic acid) can interfere each other in case of coelution several gradient profiles were tested to obtain the best separation. This interference could not be avoided using different ions, because the two same ions were observed for both compounds, sodium molecular ion and the loss of water. A good separation was obtained using the gradient profile indicated in Section 2, which was applied in further experiments. In order to ensure the elution of these two compounds, they were injected alone to be sure of their retention time, observing that oxolinic acid eluted at 15.92 min and flumequine at 18.23 min, so they are well resolved with the optimised conditions and they do not interfere each other.

3.2. Optimization of extraction procedure

The critical step in the multiresidue antibiotic methods is the extraction and clean-up procedure. Different solvents, buffer systems and pHs were tested for the optimisation of the extraction procedure. Thus, methanol and acetonitrile were studied as well as aqueous buffer solutions containing sodium citrate with or without EDTA, obtaining the results shown in Fig. 2. First, it can be observed that the highest recoveries were obtained at pH 4, noting that recovery decreased when extraction pH increases. Besides, acetonitrile provides better results than methanol as organic solvent extractant. Furthermore, the addition of EDTA was studied in order to check if its addition increases the sensitivity of the oxytetracycline, avoiding the complexation of this analyte with cations [31]. As it can be observed, oxytetracycline shows better recoveries rates when EDTA was added, whereas the other antibiotics are slightly affected, presenting similar or higher recoveries values.

Finally, the need of an additional clean-up process was evaluated. Thus, SPE were tested to clean up the extracts obtained previously, using two types of cartridges, C_{18} and Oasis HLB. For that purpose the extracts were diluted with Milli-Q water to an acetonitrile content below 10% prior SPE extraction. The cartridges were preconditioned with 3 mL of methanol and 3 mL of water. After the diluted extracts passed through the cartridge, they were dried and the analytes were eluted with 3 mL of methanol. The eluate was evaporated to dryness and the residue was redissolved in 1 mL of mobile phase prior chromatographic analysis. No better chromatograms were achieved (data not shown), so SPE was not used for clean up the extracts obtained after acetonitrile extraction, reducing cost and analysis time.

Fig. 3 shows a typical chromatogram of a blank fish sample spiked with 100 μg/kg of the target antibiotics after the extraction procedure was applied. It can be observed the optimised extraction procedure provides a clean chromatogram without interferences and oxolinic acid and flumequine are well resolved. Fig. 3b shows the chromatogram obtained for oxytetracycline when the other two ions are selected to confirm the results. It can be noted that good responses without interferences are also obtained at *m/z* 461 and 443.

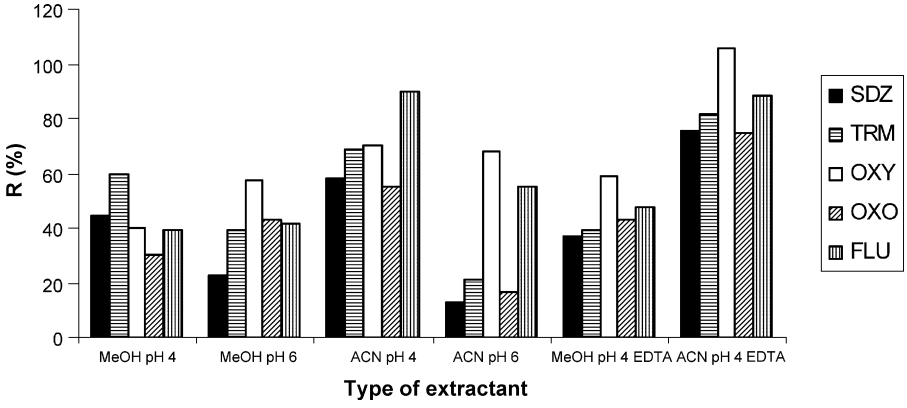


Fig. 2. Effect of different type of extractants on extraction recovery of antibiotics from fish muscle and skin. Abbreviations: SDZ: sulfadiazine; TRM: trimethoprim; OXY: oxytetracycline; OXO: oxolinic acid; FLU: flumequine; MeOH: methanol; ACN: acetonitrile.

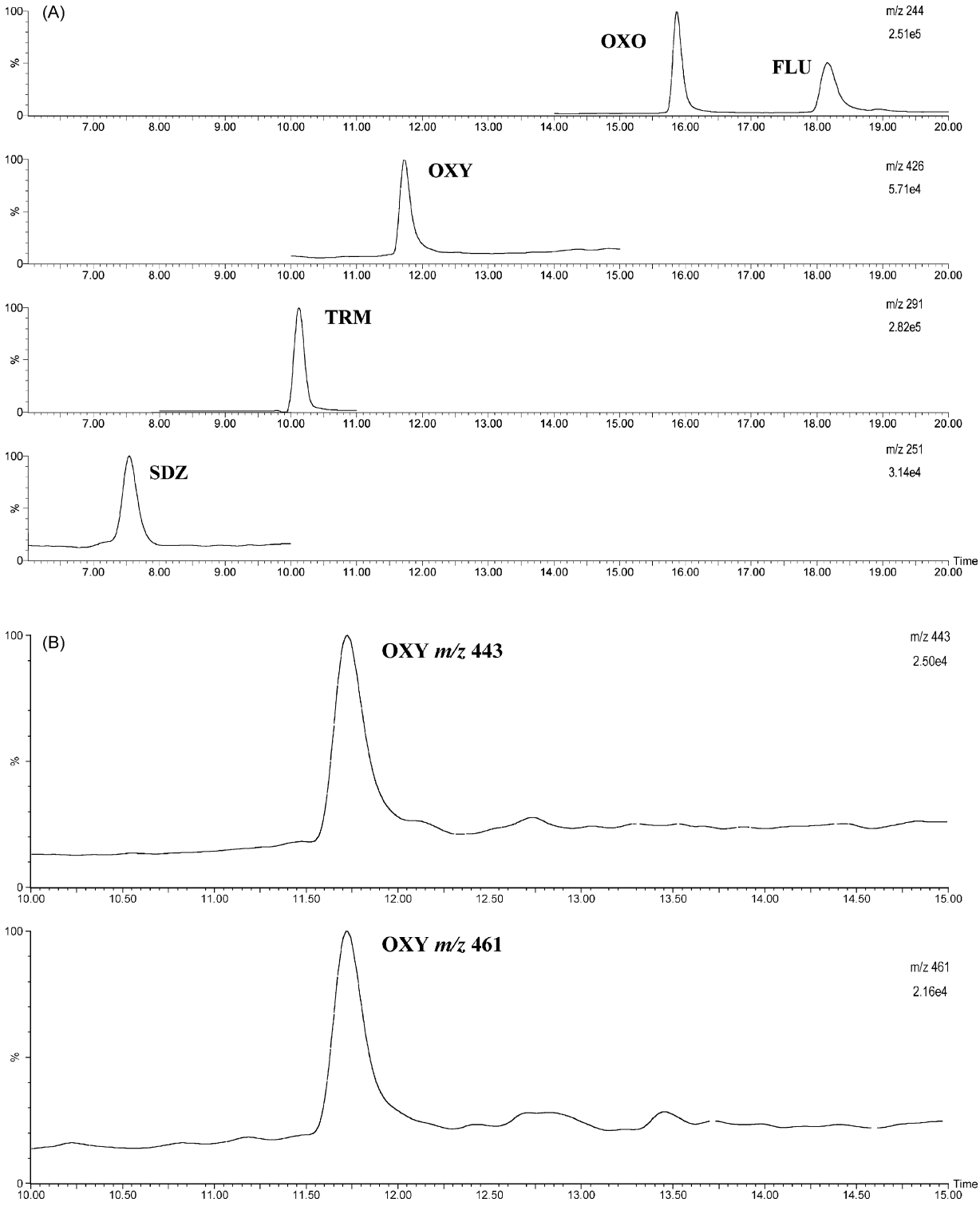


Fig. 3. (a) SIM chromatograms of extracts obtained from blank muscle and skin spiked with 25 μg/kg of antibiotics (b) SIM chromatograms obtained for the ions selected for monitoring oxytetracycline. Abbreviations as in Fig. 2. Chromatographic and MS conditions indicated in Section 2.

3.3. Matrix effects

One significant drawback, especially when using electrospray ionisation, is the presence of matrix components that may affect the ionisation of the target analytes. Matrix effects can both reduce or enhance the response when compared to standards in neat solvents. For assessment of matrix effect, signal suppression/enhancement was studied by analysing standard solutions of the selected antibiotics in solvent at 50 μg/L and extract of gilthead sea bream spiked with the same analytes and concentration level. MS responses (peak area) of spiked extracts were compared with those obtained from pure standard solutions, and

Table 2
Validation parameter of the proposed LC–MS method

Antibiotic	R^2	LOD (μg/kg)	LOD (μg/kg)	Recovery (%)			Precision[a]		
				25 μg/kg	250 μg/kg	500 μg/kg	25 μg/kg	250 μg/kg	500 μg/kg
Sulfadiazine	0.990	6	14	89.5	86.4	84.3	14.9 (17.8)	1.7 (5.4)	1.6 (4.9)
Trimethoprim	0.989	4	11	79.8	83.9	80.3	9.8 (12.9)	1.0 (8.2)	0.8 (6.7)
Oxytetracycline	0.993	4	10	90.2	86.4	100.2	12.4 (18.0)	3.2 (6.5)	2.8 (7.2)
Flumequine	0.991	8	20	95.0	80.9	88.4	10.9 (13.4)	2.3 (5.9)	2.0 (4.9)
Oxolinic acid	0.988	7	16	71.3	83.8	103.5	12.0 (15.6)	2.7 (8.0)	3.0 (7.7)

[a] Repeatability expressed as relative standard deviation (%). Interday precision is given in brackets.

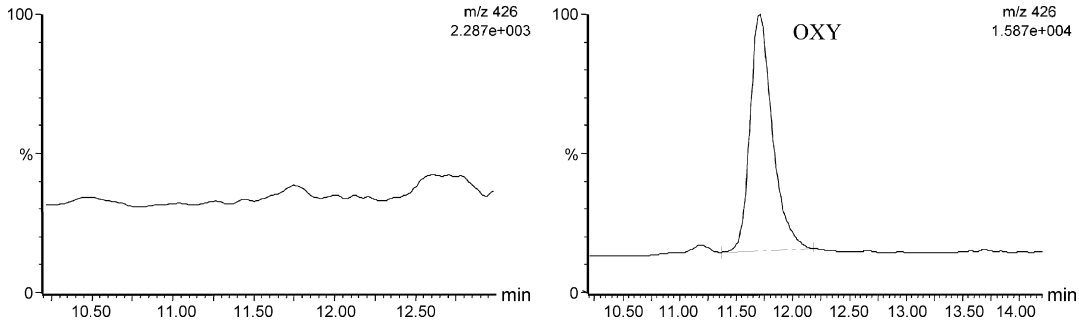


Fig. 4. Representative chromatogram of oxytetracycline (OXY): (A) extract from control fish muscle sample and (B) extract from a fish sampled 7 days post-treatment.

the relative responses (response matrix/response solvent) were obtained. Relative responses ranged from 0.79 for sulfadiazine and 0.97 for oxytetracycline and oxolinic acid, showing minimal matrix suppression for all the analytes. Although no significant matrix effects were observed, matrix matched calibration was selected for quantification purposes to avoid slight variations in the MS signal for the analytes and unexpected enhancement or signal suppressions in real samples.

3.4. Validation of the proposed method

Performance characteristics of the optimised method were established by a validation procedure with spiked fish muscle samples, studying linearity, selectivity, sensitivity, accuracy, LOD, LOQ, repeatability and interday precision. Calibration was performed by use of matrix-matched calibration standards prepared as described in Section 2 and results are summarized in Table 2. Peak area was selected as response and good linearity was found for all the antibiotics assayed within the tested interval, with coefficients of determination higher than 98.8%. Intra and interday precision values, expressed as relative standard deviations, were lower than 18% for all the analytes through the three concentrations assayed. LODs and LOQs were below the MRLs established by European Union. Thus, LODs ranged from 4 to 8 μg/kg and LOQs from 10 to 20 μg/kg. Accuracy was estimated through recovery studies. Thus, three different fortification levels were evaluated. Satisfactory results were found, with recoveries between 70 and 103% for the selected levels (Table 2). Selectivity was evaluated extracting and analysing blank tissue samples. The absence of any signal at the same elution time as the selected antibiotics indicates that there were no matrix interferences.

3.5. Sample analysis

The developed method was applied for the determination of antibiotic residues in muscle and skin tissues of gilthead seabream with "winter ulcer disease". In this sense, fishes medicated with oxytetracycline, were analysed just after treatment and up to 34 days after treatment, evaluating the depletion of this antibiotic in this type of fish, taking into account that the water temperature was 16 °C. First, control tissue samples were analysed and no apparent residues higher or equal to the limits of detection estimated previously were found. Fig. 4 shows two chromatograms of a control and treated tissue sample with oxytetracycline. Mean residue level obtained from five replicates are indicated in Fig. 5 for the treated fishes. It can be observed that the concentration of oxytetracycline is higher than the MRL established by the European Union at one and seven days post-treatment, presenting significant values at 20

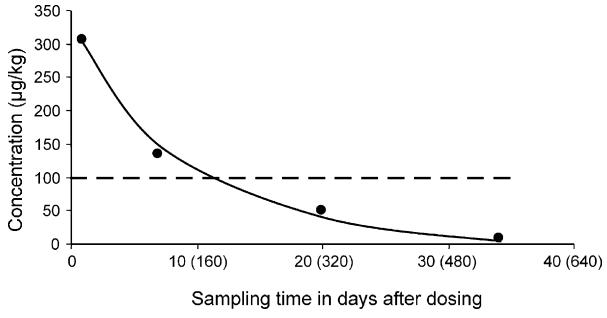


Fig. 5. Oxytetracycline depletion in muscle tissue plus skin of gilthead seabream fish. Time parameter expressed as degree-days is given in brackets (water temperature 16 °C). Dash line (— —) indicates MRL established by European Union for oxytetracycline in fish.

days post-treatment, although below than the MRL. Finally, this antibiotic was detected below the established LOQ at 34 days post-treatment.

Considering the influence of water temperature on fish metabolism and, consequently, on the pharmacokinetics of drugs, time parameter can also be expressed as degree-days, calculated by multiplying the mean daily water temperature (in degrees Celsius) by the total number of measured days. Using the data showed in Fig. 5, it was noted that 200 °C-days could be used as withdrawal time for oxytetracycline. It can be indicated that the general value recommended by the European Union (500 °C-day) [36] for off-label drug use in aquaculture would be too conservative, although more studies are required.

Finally, twenty gilthead seabream samples purchased from local groceries were extracted and analysed according to the optimised method, to determine the presence of oxytetracycline, flumequine, oxolinic acid, sulfadiazine and trimethoprim in muscle and skin. No antibiotic residues were found in the samples, although traces of oxytetracycline were detected in three of the samples analysed, presenting a concentration lower than the LOQ, which is below the MRL established by the European Union.

4. Conclusions

A multiresidue method was developed for rapid and simultaneous determination of flumequine, oxolinic acid, oxytetracycline, sulfadiazine and trimethoprim in fish by LC–ESI-MS. The extraction of the antibiotics is based on a simple extraction step, and no clean-up step is necessary. The method is simple and gives quantitative results for the assayed antibiotics, providing good validation parameters in terms of linearity, limits of detection and quantification and precision. This methodology has been used for the quantitative analysis of treated fishes with oxytetracycline, resulting from residue depletion studies after administration of antibiotic via feed. Bearing in mind the wide range of physico-chemical properties of the studied compounds, the method would be expected to be applicable to most of the antibiotics present simultaneously in fish.

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