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Multiresidue determination of veterinary drugs in aquaculture fish samples by ultra high performance liquid chromatography coupled to tandem mass spectrometry

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ABSTRACT

A simple, selective and fast multiresidue method was developed for the determination of 32 veterinary drug residues belonging to several families, in gilthead sea bream (*Sparus aurata*) by ultra high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC–MS/MS). The extraction was based on modified QuEChERS (quick, easy, cheap, effective, rugged and safe) procedure, using as extraction solution a mixture of acetonitrile and methanol (75:25, v/v), and it reduces sample handling, increasing sample throughput in relation to current methodologies. The developed method was validated and mean recovery ranged from 69% to 125% (at 10, 25, 50 and 100 μg/kg). Intra and interday precision, estimated as the same levels and expressed as relative standard deviation, RSD, were lower than 20% and 30%, respectively. Limits of detection (LODs) and quantification (LOQs) were lower than 7.5 and 25 μg/kg, respectively, except for danofloxacin, oxytetracycline and tetracycline (LOD and LOQ of 15.0 and 50 μg/kg, respectively). Decision limit (CC_α) and detection capability (CC_β) were also calculated and ranged from 16.7 μg/kg (levamisole) to 605.0 (flumequine) μg/kg and from 23.5 μg/kg (levamisole) to 611.5 μg/kg (flumequine), respectively. The expanded uncertainty, *U*, was also evaluated ant it was below 25% at 100 μg/kg level, except for tetracycline (28%). Finally, the method was applied to ten samples obtained from local supermarkets in Almería (Spain) and traces of some compounds were detected.

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1. Introduction

Aquaculture is the production of marine or freshwater food fish under controlled conditions [1]. Most of the aquaculture systems in the world are based on intensive cultivation methods. These methods are characterized by high stock density and volume, use of formulated feeds containing antibiotics, antifungal and other pharmaceuticals, as well as the application of pesticides and disinfectants [1–3]. These factors can cause serious health problems in consumers, as allergic reactions in hypersensitive individuals and bacterial resistance [4,5]. To limit human exposure, many organizations, such as the Codex Alimentarius [6], European Union (EU) [7] and US Food and Drug Administration (FDA) [8], have established maximum residue limits (MRLs) for veterinary medicinal products in foodstuffs from animal origin.

Therefore to ensure food safety, sensitive and specific analytical methods are necessary for the determination of veterinary drugs in food matrices [9]. Some analytical techniques such as microbiological, i.e. enzymatic assay [10] or immunochemical procedures such as enzyme-linked immunosorbent assay (ELISA) [11,12], have been used because they are simple and very cost-effective. Nevertheless, they present poor selectivity and they are not able to differentiate among several types of drugs [13,14], providing only semi quantitative measures, which sometimes give rise to false positives.

However, in recent years, mass spectrometry (MS) has been selected as the most suitable technique for detection of veterinary drug residues in foodstuffs according to Public Health Agencies from many countries, because it provides an unambiguous identification and a reliable confirmation [15] as well as it reduces chromatographic interferences, especially when multiple reaction

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monitoring (MRM) mode is used [16,17]. Thus, high performance liquid chromatography (HPLC) coupled to tandem mass spectrometry (MS/MS) has become the predominant technique, combining analyte separation with structural information [18], for monitoring chemical residues in food matrices, such as fish, since this technique requires simple sample pretreatments and increases sample throughput [19–22]. In recent years, ultra high performance liquid chromatography (UHPLC) shows a variety of advantages in relation to HPLC, such as high selectivity and sensitivity can be achieved with minimal analysis time, increasing valuable characteristics for routine laboratories, such as reduction of the required time in the chromatographic separation [23].

Despite the advantages on the detection techniques, when dealing with real complex samples (e.g. foods, edible tissues from animals), these matrices usually require an appropriate sample preparation in order to decrease interferences and avoid possible matrix effects. Several procedures such as liquid–liquid extraction (LLE) [24], solid phase extraction (SPE) [25], matrix solid phase dispersion (MSPD) [26], supercritical fluid extraction (SFE) [27] and liquid–liquid extraction with fast partition at very low temperature (LLE–FPVLT) [28] have been carried out to improve the extraction of veterinary drugs. However, some of these methods are long and tedious, and they usually require a clean-up step [29]. In this sense, the QuEChERS (quick, easy, cheap, effective, rugged and safe) methodology presents a large number of advantages since it constitutes a fast and inexpensive procedure which requires few steps, and simplifies and minimizes the time taken to complete the extraction and clean-up processes [30], as well as it reduces the sample size and quantities of laboratory glassware [31]. This method has frequently been used for the extraction of pesticides from vegetables [32] or from fish [33,34], but there are few works related to veterinary drugs determination in edible tissue matrices [35], and it has not been applied for the extraction of this type of compounds from fish samples.

In this paper, a simple, robust and effective multiresidue method has been developed and validated for the simultaneous determination of 32 veterinary drugs belonging to different classes (i.e. macrolides, penicillins, quinolones, sulfonamides and tetracyclines) in gilthead sea bream (*Sparus aurata*). The developed method involves a QuEChERS procedure, which implies a simple extraction and subsequent analytical determination by UHPLC–MS/MS.

2. Materials and methods

2.1. Chemicals and reagents

All the standards were of high purity grade (>99.0%). Oxfendazole, oxolinic acid, fenbendazole, thiabendazole, trimethoprim and sulfadimidine were supplied by Sigma–Aldrich (Madrid, Spain). Albendazole was supplied by LGC Standards (Barcelona, Spain). Emamectin benzoate, mebendazole, levamisole hydrochloride, sulfachlorpyridazine, sulfadimethoxine, sulfaquinoxaline, chlorotetracycline hydrochloride, tetracycline hydrochloride, danofloxacin, enrofloxacin, marbofloxacin and sarafloxacin were obtained from Riedel de Haën (Seelze, Germany). Sulfathiazole, josamycin, ampicillin trihydrate, oxytetracycline hydrochloride, doxycycline hyclate, cloxacillin sodium salt monohydrate, penicillin G potassium salt, penicillin V potassium salt, dicloxacillin sodium salt hydrate, oxacillin sodium salt hydrate and erythromycin were supplied by Fluka (Steinem, Germany). Finally, sulfadiazine and flumequine were purchased from Dr. Ehrenstorfer (Augsburg, Germany).

Stock standard solutions of individual compounds (with concentrations between 200 and 300 mg/L) were prepared in acetoniltrile:water (1:1, v/v) for penicillins and tetracyclines or in methanol for the other families of veterinary drugs. Stock standard solutions were stored at −20 °C in the dark. Penicillins and tetracyclines were stored for 1 month, whereas the other families of veterinary drugs were stored for 4 months. A multicompound working standard solution of the selected compounds (4 mg/L) was prepared by appropriate dilution of the stock solution with acetonitrile and it was stored under refrigeration ($T < 5$ °C) and it was renewed weekly. All reagents were of analytical grade. HPLC-grade acetonitrile and methanol were supplied by Sigma–Aldrich. Formic acid (>98%) was purchased from Fluka. Anhydrous magnesium sulfate was supplied by Panreac (Barcelona, Spain). Anhydrous sodium acetate was obtained from J.T. Baker (Deventer, Holland). Na_2EDTA was purchased from Merck (Darmstadt, Germany). Ultrapure water was obtained from a Millipore Milli-Q system (Milford, MA, USA). Purified samples were filtered through Millex-GN nylon filters (0.20 μm, Millipore, Carrightwohill, Ireland).

2.2. Samples and sample preparation

Gilthead sea bream (*S. aurata*) samples were obtained from local supermarkets (Almeria, Spain). Before analysis, fish samples were partially thawed at room temperature and muscle tissue plus skin were taken for analysis. Then, they were homogenized and stored at −30 °C until analysis. Blank samples were fortified with the target compounds during the optimization and validation of the developed procedure. Veterinary drugs were extracted from fish using an extraction procedure based on QuEChERS methodology. The procedure was as follows: 5.0 g of the sample was weighed in a polypropylene tube followed by the addition of 2.0 mL of water and 10.0 mL of a mixture of acetonitrile:metanol (75:25, v/v) solution. Then, the mixture was stirred in a rotator shaker for 15 min. Afterwards, 4.0 g of anhydrous magnesium sulfate and 1.0 g of sodium acetate were added and the tubes were shaken for 15 min again. After centrifugation at 5000 rpm (4136 × *g*) for 5 min, 4.0 mL of the organic layer was evaporated to dryness at 40 ± 1 °C under a nitrogen stream. Then, 1.0 mL of a mixture 1:1 (v/v) of eluents A and B (see below) was added to the residue. The solution was filter through a Millex-GN nylon filter and 5 μL were injected into the UHPLC–MS/MS system.

2.3. UHPLC–MS/MS

Chromatographic analyses were performed using an Acquity UHPLC system (Waters, Milford, MA, USA) and separations were achieved using an Acquity UHPLC BEH C18 column (100 mm × 2.1 mm, 1.7 μm particle size) from Waters. Chromatographic separation was carried out with a mobile phase consisting of 0.1% formic acid in acetonitrile (eluent A) and 0.1% formic acid in water (eluent B) at a flow rate of 0.3 mL/min. The elution started at 10% of eluent A for 0.5 min and then it was linearly increased up to 100% of eluent A in 5 min, keeping constant for 1.5 min before being returned to the initial conditions in 1.5 min. Finally, the total run time, including the cleaning and pre-equilibration step, was 8.5 min. Injection volume was 5 μL and column temperature was set at 30 °C.

Mass spectrometry analysis was carried out using a Waters Acquity TQD tandem quadrupole mass spectrometer (Waters, Manchester, UK). The instrument was operated using electrospray ionization (ESI) in positive ion mode. The data acquisition was performed using MassLynx 4.1 software with QuanLynx program (Waters). The ionization source parameters were: capillary voltage 3.0 kV, extractor voltage 2 V, source temperature 120 °C, desolvation temperature 350 °C, cone gas flow 80 L/h and desolvation gas flow 600 L/h (both gases were nitrogen). Collision-induced dissociation was performed using argon as the collision gas at the pressure

Table 1
Retention time windows (RTWs) and MS/MS conditions of the selected compounds.

Analyte	RTW (min)	Voltage Cone (V)	Quantification transition[a]	Confirmation transition[a]	Ion ratio (%)
Albendazole	3.33–3.38	32	266.0 > 234.2 (20)	266.0 > 191.1 (35)	70
Ampicillin	2.13–2.18	30	350.0 > 106.2 (30)	350.0 > 160.1 (10)	44
Chlorotetracycline	2.55–2.87	35	479.3 > 444.3 (20)	479.3 > 462.3 (18)	76
Cloxacillin	3.80–3.89	25	436.2 > 160.1 (15)	436.2 > 277.2 (15)	77
Danofloxacin	2.06–2.37	38	358.3 > 340.3 (32)	358.3 > 255.2 (38)	24
Dicloxacillin	3.82–4.18	20	470.2 > 160.1 (20)	470.2 > 311.1 (15)	46
Doxycycline	2.81–2.88	30	445.3 > 428.3 (18)	445.3 > 154.2 (28)	17
Emamectin	4.72–4.89	60	886.6 > 158.2 (30)	886.6.3 > 82.2 (30)	6
Enrofloxacin	2.20–2.48	38	360.3 > 342.3 (20)	360.3 > 316.3 (20)	7
Erythromycin	3.22–3.69	35	717.1 > 158.2 (30)	717.1 > 116.2 (45)	11
Fenbendazole	3.74–3.78	32	300.0 > 268.2 (20)	300.0 > 159.1 (35)	89
Flumequine	3.55–3.65	20	262.3 > 244.3 (20)	262.3 > 202.2 (20)	6
Josamycin	3.68–3.75	55	829.3 > 174.2 (32)	829.3 > 109.1 (40)	86
Levamisole	2.07–2.14	36	205.0 > 123.1 (29)	205.0 > 117.2 (27)	51
Marbofloxacin	2.05–2.30	35	363.1 > 320.4 (15)	363.1 > 345.4 (20)	16
Mebendazole	3.29–3.33	37	296.2 > 264.2 (25)	296.2 > 77.1 (46)	85
Oxacillin	3.56–3.81	20	402.3 > 160.1 (15)	402.3 > 243.1 (15)	74
Oxfendazole	2.80–2.85	35	315.9 > 191.3 (22)	315.9 > 159.2 (35)	31
Oxytetracycline	2.12–2.43	28	461.4 > 443.3 (13)	461.4 > 426.3 (10)	26
Oxolinic acid	3.01–3.07	25	262.3 > 244.3 (20)	262.3 > 216.2 (34)	9
Penicillin G	3.30–3.62	25	335.2 > 160.1 (15)	335.2 > 176.3 (15)	28
Penicillin V	3.50–3.62	15	351.3 > 160.1 (15)	351.3 > 114.3 (35)	51
Sarafloxacin	2.46–2.76	45	386.2 > 368.4 (22)	386.2 > 348.4 (30)	10
Sulfachlorpyridazine	2.79–2.86	32	285.1 > 156.2 (15)	285.1 > 80.2 (50)	9
Sulfadiazine	1.96–2.09	20	251.0 > 156.0 (17)	251.0 > 92.0 (25)	75
Sulfadimethoxine	3.19–3.32	60	311.1 > 156.2 (20)	311.1 > 245.3 (18)	9
Sulfadimidine	2.39–2.55	35	279.1 > 92.1 (30)	279.1 > 124.2 (20)	45
Sulfaquinoxaline	3.12–3.29	32	301.2 > 156.1 (35)	301.2 > 108.1 (30)	59
Sulfathiazole	2.07–2.24	30	256.2 > 156.1 (15)	256.2 > 92.2 (25)	4
Tetracycline	2.38–2.60	28	445.4 > 410.2 (20)	445.4 > 427.3 (13)	87
Thiabendazole	2.04–2.11	30	201.8 > 175.2 (27)	201.8 > 131.2 (32)	42
Trimethoprim	2.17–2.26	20	291.4 > 261.3 (25)	291.4 > 230.2 (25)	61

[a] Collision energy (eV) is given in parentheses.

of 4×10^{-3} mbar in the collision cell. The specific MS/MS parameters for each compound are shown in Table 1.

2.4. Validation procedure

A validation protocol was carried out in order to establish the performance characteristics of the method, ensuring the adequate identification, confirmation and quantification of the target compounds. Analytical characteristics were sensitivity, trueness through recovery studies, intra and interday precision, uncertainty, limits of detection (LODs) and quantification (LOQs), decision limit (CC_{α}) and detection capability (CC_{β}), and selectivity. Linearity was evaluated using matrix-matched calibration (MMC), spiking blank extracts at five concentration levels, ranged from 10 μg/kg to 150 μg/kg. LODs and LOQs were estimated by fortifying blank fish samples with veterinary drugs (1, 3, 5, 10, 25 and 50) μg/kg and the extraction procedure was applied prior to chromatographic determination. LODs and LOQs were determined as the concentration of compound for which signal-to-noise ratio (*S*/*N*) were higher than 3 and 10, respectively. CC_{α} and CC_{β} parameters were calculated based on a linear regression model analyzing spiked blank samples at five concentration levels, according to BS ISO 11843-2 [36]. Recovery and intraday precision (repeatability) were performed spiking blank fish samples at four concentration levels (10, 25, 50 and 100 μg/kg), using five replicates for each concentration level in 1 day. To evaluate interday precision (reproducibility), the same concentration levels were studied, spiking blanks during five consecutive days. Finally, the estimation of the uncertainty (*U*) was carried out using the data derived from the validation of the method [37].

3. Results and discussion

3.1. UHPLC–MS/MS analysis

The optimization of MS parameters was performed by direct infusion of a standard solution of each compound (20 mg/L) in the MS. The electrospray source used in positive ion mode provided the highest signals and two transitions per each compound were selected, which are shown in Table 1, as well as other MS/MS parameters such as cone and collision voltages. Other parameters, such as desolvation and cone gas flow, source desolvation temperature, and capillary voltages were studied, selecting the optimum conditions indicated in Section 2.3. Then, the chromatographic conditions were studied in order to provide overall optimum peak shape and resolution. Thus, the mobile phase composition was investigated to maximize the method sensitivity and resolution. First, several experiments were performed testing different mobile phases consisting on methanol or acetonitrile (as organic phase) and water, both with different concentrations of formic acid (0.01% and 0.1%, v/v). Acetonitrile provided better sensitivity than methanol. Moreover the highest concentration of formic acid (0.1%, v/v) in acetonitrile provided the best overall sensitivity. Furthermore, the gradient was optimized in order to provide a good separation of the selected compounds in less than 9 min. Other parameters such as column temperature, flow rate and injection volume were tested in order to get a fast and reliable separation, obtaining the best results with the conditions described in Section 2.3. Using these conditions, the analytes were distributed in nine overlapping acquisition functions, using a maximum of eight compounds (16 transitions) per function. Good peak shape and enough points per peak were obtained when 0.025 s was used as dwell

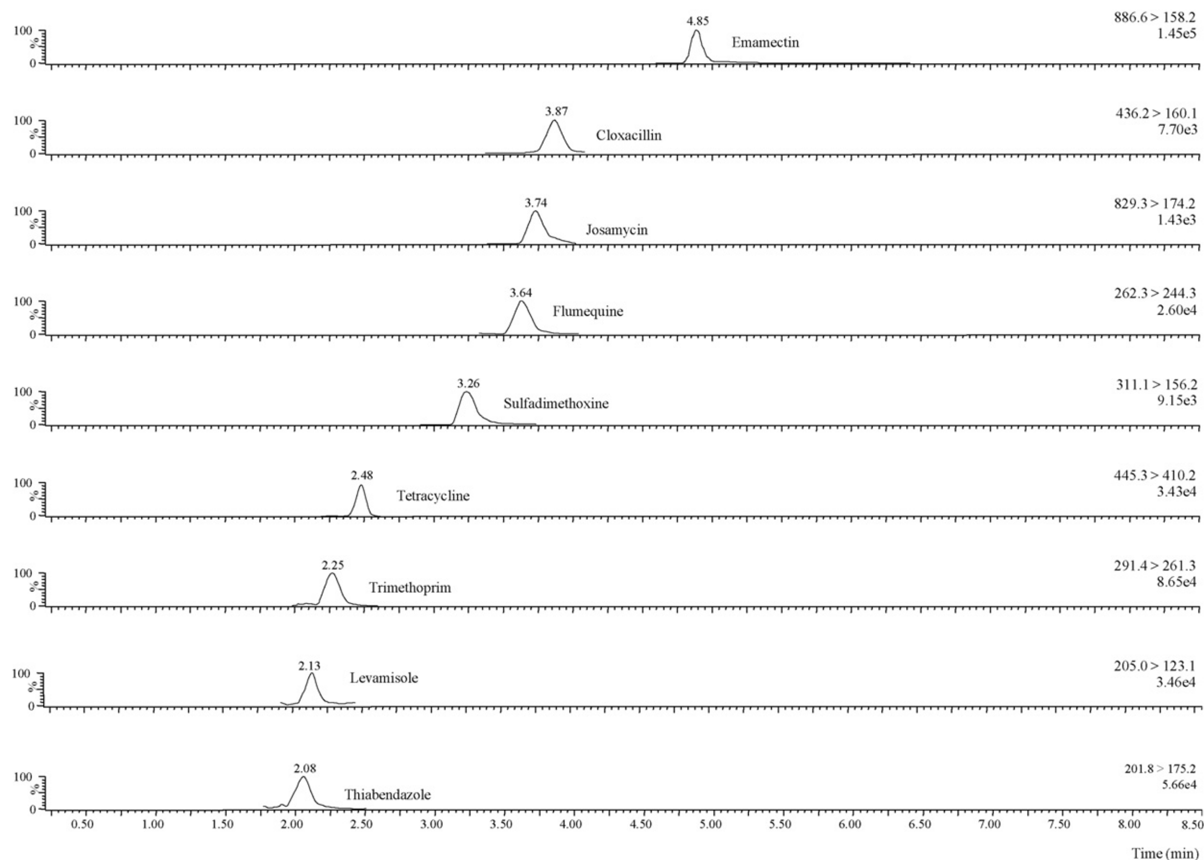

Fig. 1. Extracted-ion chromatograms obtained from a standard solution at 100 μg/kg with the following analytes (one of each class): thiabendazole (benzimidazoles), levamisole (imidazolthiazole), trimethoprim (diamino pyrimidine derivates), tetracycline (tetracyclines), sulfadimethoxine (sulfonamides), flumequine (quinolones), josamycin (macrolides), cloxacillin (penicillins), and emamectin (avermectins).

time, except for flumequine, fenbendazole and josamycin, which were monitored using a dwell time of 0.05 s. Finally, Fig. 1 shows the extracted ion chromatogram (XIC) from representative compounds for each class of veterinary drugs (quantification transition was shown), injecting 5 μL of a standard solution of 100 μg/L.

3.2. Extraction method

The original QuEChERS method contains two steps, a salting-out extraction and a dispersive SPE (dSPE) clean up [30]. However, many works described that the dSPE clean up is not always necessary [38], and therefore, it was not applied in this work. However, there are other critical factors, such as the best extraction solution and salts for elimination of the water and matrix compounds soluble in this phase, and the influence of several variables on the extraction of the selected compounds was evaluated, spiking blank fish samples at 100 μg/kg.

First, the influence of the extraction media was evaluated. Thus the addition of EDTA was studied in order to check if its addition increases the extraction capability of the tetracyclines, avoiding the complexation of these compounds with cations that may be present in the matrix [9,39]. Furthermore, the addition of acetic acid to the extraction solvent was evaluated, considering that it can improve the extraction of certain compounds [30] and finally, the type of salts to induce the partition was evaluated, bearing in mind that the official methods used in Europe [40] and USA [41] used different salts, such as citrate and acetate buffer, respectively. In order to evaluate simultaneously the three factors, experimental design was applied and a factorial design 2^3 was selected, studying three variables: *variable 1*: (+) addition of 2 mL of EDTA-Na_2 0.15 M and (−) without the addition of this solution; *variable 2*: (+) addition of 10 mL of a solution of 1% acetic acid in acetonitrile and (−) addition of 10 mL of acetonitrile; and *variable 3*: (+) use of citrate buffer and (−) use of acetate buffer. Statistical analysis was done using Excel spreadsheets [42]. The obtained results are shown in Table 2 and it can be observed that most of the compounds did not show significant effect for these variables, except oxolinic acid, which showed significant and positive effects for variables 2 and 3 and sulfadiazine that showed significant and negative effect for variables 2 and 3 (Table 2). Bearing in mind these results it was considered that it would be better to use the low level (−) of the variables for all compounds, since most of them showed no significance for the variables studied and the cost of the analysis would be reduced due to lower consumption of reagents. Furthermore, it must be highlighted that compounds belonging to quinolones (marbofloxacin, enrofloxacin, danofloxacin and sarafloxacin) and tetracyclines (tetracycline, chlortetracycline, oxytetracycline and doxycycline) were not extractable.

Then, a new extraction solution was investigated and therefore, a new factorial design, 2^2 was selected, varying the extraction solutions and the amount of water added in the procedure in order to extract quinolones and tetracyclines. Methanol was included in the extraction solution considering that quinolones are more soluble in methanol. The levels for the variables were for *variable 1*: (+) addition of 5 mL of water and (−) addition of 2 mL of water and for *variable 2*: (+) addition of 10 mL of acetonitrile:methanol solution (50:50, v/v) and (−) addition of 10 mL of acetonitrile. The levels were chosen according to the compound solubility, e.g. the tetracyclines are very soluble in water. The obtained results are shown in Table 2 and it can be observed that quinolones

Table 2
Results of the factorial designs during the optimization of the extraction procedure.

Analyte	First design: 2^3			Second design: 2^2	
	Variable 1[a]	Variable 2[b]	Variable 3[c]	Variable 1[d]	Variable 2[e]
Albendazole	NO[f]	NO	NO	NO	NO
Ampicillin	NO	NO	NO	SG (−)[g]	NO
Chlorotetracycline	Not extracted	Not extracted	Not extracted	NO	SG (+)[h]
Cloxacillin	NO	NO	NO	NO	SG (−)
Danofloxacin	Not extracted	Not extracted	Not extracted	SG (+)	NO
Dicloxacillin	NO	NO	NO	NO	SG (−)
Doxycycline	Not extracted	Not extracted	Not extracted	NO	SG (+)
Emamectin	NO	NO	NO	NO	SG (−)
Enrofloxacin	Not extracted	Not extracted	Not extracted	NO	SG (+)
Erythromycin	NO	NO	NO	NO	SG (−)
Fenbendazole	NO	NO	NO	NO	SG (−)
Flumequine	NO	NO	NO	NO	NO
Josamycin	NO	NO	NO	NO	SG (−)
Levamisole	NO	NO	NO	NO	SG (−)
Marbofloxacin	Not extracted	Not extracted	Not extracted	NO	SG (+)
Mebendazole	NO	NO	NO	NO	SG (−)
Oxacillin	NO	NO	NO	NO	NO
Oxfendazole	NO	NO	NO	NO	SG (−)
Oxolinic acid	NO	SG (+)	SG (+)	NO	NO
Oxytetracycline	Not extracted	Not extracted	Not extracted	NO	SG (+)
Penicillin G	NO	NO	NO	NO	SG (−)
Penicillin V	NO	NO	NO	NO	SG (−)
Sarafloxacin	Not extracted	Not extracted	Not extracted	NO	SG (+)
Sulfachlorpyridazine	NO	NO	NO	NO	SG (−)
Sulfadiazine	NO	SG (−)	SG (−)	NO	SG (−)
Sulfadimethoxine	NO	NO	NO	NO	SG (−)
Sulfadimidine	NO	NO	NO	NO	SG (−)
Sulfaquinoxaline	NO	NO	NO	NO	SG (−)
Sulfathiazole	NO	NO	NO	NO	SG (−)
Tetracycline	Not extracted	Not extracted	Not extracted	NO	SG (+)
Thiabendazole	NO	NO	NO	NO	SG (−)
Trimethoprim	NO	NO	NO	NO	SG (−)

[a] (+) EDTA addition; (−) without EDTA.
[b] (+) Addition of acetic acid; (−) without acetic acid.
[c] (+) Addition of buffer citrate; (−) addition of acetate buffer.
[d] (+) Addition of 5 mL of the water; (−) addition of 2 mL of the water.
[e] (+) Addition of acetonitrile:methanol (50:50, v/v); (+) addition of acetonitrile:methanol (100:0, v/v).
[f] Non-significant effect.
[g] Significant and negative effect, SG (−).
[h] Significant and positive effect, SG (+).

and tetracyclines showed significant positive effect for the second variable (composition of the extractant solution), indicating that the presence of methanol increases the extraction of these compounds. However, 18 compounds have shown a significant and negative effect for the second variable, indicating that the use of methanol decreased the extraction of these compounds. Nevertheless it must be pointed out that the use of this solvent in the extraction phase composition did not reduce the recovery of these compounds at levels lower than 70%. However, the extracts were very dirty, because methanol extracts contained many matrix compounds. Then, in order to get cleaner extract, several percentages of methanol were evaluated. The concentrations studied were acetonitrile:methanol (100:0, v/v), acetonitrile:methanol (75:25, v/v) and acetonitrile:methanol (50:50, v/v), showing the obtained results in Fig. 2. It can be observed that if only acetonitrile was used, quinolones and tetracyclines were not extractable. However, the addition of methanol increased the analytes recovery, but the greater the amount of methanol, dirtier extracts were obtained. Thus, an acetonitrile:methanol (75:25, v/v) solution was selected considering it provided satisfactory recoveries for all the compounds and cleaner extracts.

3.3. Method validation

The selectivity of the method was evaluated injecting extracted blanks samples, and the absence of signal, above a signal-to-noise ratio of 3, at the retention times of the target compounds showed that the method is free of interferences. Confirmation was carried out by comparison of the signal intensity ratios of the two transitions (quantification and confirmation) with those obtained using fortified blank fish samples. Confirmation was considered reliable if the ratio was within the criteria laid down in the European Commission Decision [15], showing in Table 1 the obtained ion ratios. Furthermore, another parameter that can influence the selectivity of the method is the suppression of the signal due to the coelution of interferents, and matrix effect should be also evaluated. For that purpose, the MMC curves for each compound were built spiking blank extracted samples ($n = 5$) at five concentration levels between 10 μg/kg and 150 μg/kg, except for danofloxacin, oxytetracycline and tetracycline whose range was between 50 and 150 μg/kg. The least-squares linear regression analysis was carried out by plotting the analyte peak area versus the concentrations. The calibration parameters showed good linearity and determination coefficients (r^2) were higher than 0.98 for all the analytes, and deviations of the individual points from the calibration curve were lower than 20%. Therefore, to evaluate matrix effect, the slopes obtained in the calibration with MMC were compared with those obtained with solvent standards at the same range described above. Then, slope ratios matrix/solvent were obtained for each compound (Fig. 3) considering a tolerable signal enhancement or suppression effect if the slope ratio ranged from 0.8 to 1.2, whereas higher values than 1.2 or lower than 0.8 implies a strong matrix effect. It can be observed that a significant matrix effect was noticed for most of the included compounds, except for

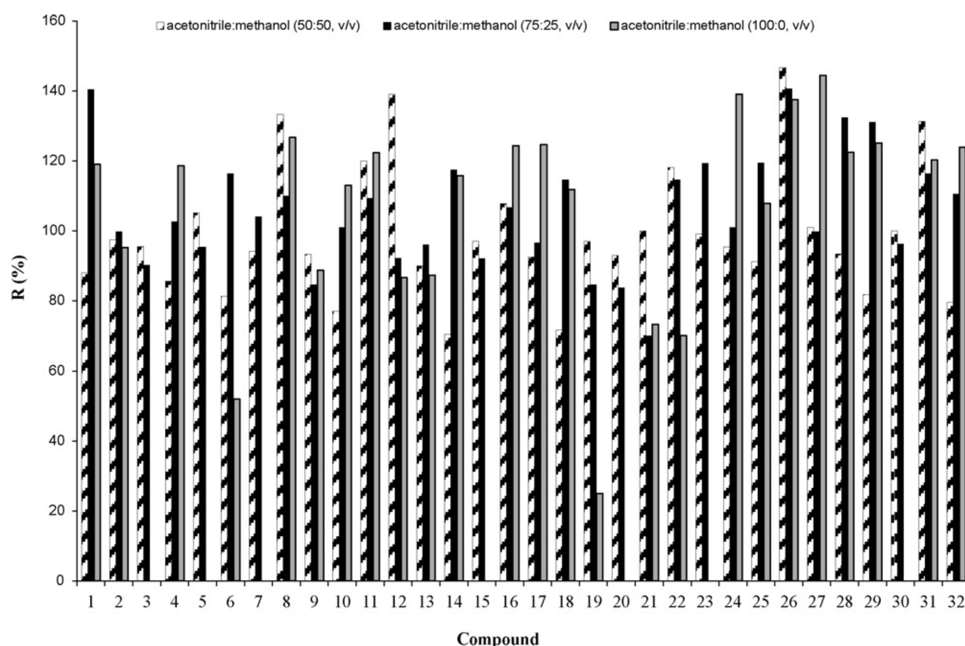

Fig. 2. Effect of the extraction solvent on the recovery of the target compounds. Veterinary drugs code: (1) albendazole; (2) ampicillin; (3) chlorotetracycline; (4) cloxacillin; (5) danofloxacin; (6) dicloxacillin; (7) doxycycline; (8) emamectin; (9) enrofloxacin; (10) erythromycin; (11) fenbendazole; (12) flumequine; (13) josamycin; (14) levamisole; (15) marbofloxacin; (16) mebendazole; (17) oxacillin; (18) oxfendazole; (19) oxolinic acid; (20) oxytetracycline; (21) penicillin G; (22) penicillin V; (23) sarafloxacin; (24) sulfachlorpyridazine; (25) sulfadiazine; (26) sulfadimethoxin; (27) sulfadimidine; (28) sulfaquinoxaline; (29) sulfathiazole; (30) tetracycline; (31) thiabendazole; (32) trimethoprim.

Table 3
Validation parameters of the optimized method.

Analyte	Recovery (%)[a]				Interday precision (RSD %)[b]				*U* (%)[c]
	10 (μg/kg)	25 (μg/kg)	50 (μg/kg)	100 (μg/kg)	10 (μg/kg)	25 (μg/kg)	50 (μg/kg)	100 (μg/kg)	
Albendazole	106 (14)	95 (8)	94 (9)	70 (7)	21	10	11	10	16
Ampicillin	NQ[d]	106 (8)	118 (8)	93 (11)	NQ	14	19	16	19
Chlorotetracycline	NQ	97 (10)	125 (11)	117 (10)	NQ	21	17	14	21
Cloxacillin	NQ	105 (12)	109 (10)	104 (10)	NQ	15	13	17	20
Danofloxacin	NQ	NQ	111 (14)	115 (2)	NQ	NQ	15	7	11
Dicloxacillin	NQ	110 (11)	98 (7)	105 (8)	NQ	15	13	12	14
Doxycycline	NQ	85 (14)	94 (6)	89 (3)	NQ	18	12	9	11
Emamectin	78 (20)	103 (20)	80 (10)	112 (6)	22	19	16	14	15
Enrofloxacin	112 (16)	81 (15)	109 (10)	109 (8)	17	12	12	11	13
Erythromycin	NQ	124 (8)	112 (9)	101 (11)	NQ	24	22	18	20
Fenbendazole	109 (16)	71 (13)	96 (10)	91 (10)	19	16	11	10	17
Flumequine	NQ	88 (8)	101 (10)	111 (4)	NQ	20	13	9	18
Josamycin	NQ	95 (16)	109 (7)	96 (3)	NQ	16	11	4	7
Levamisole	76 (19)	87 (4)	100 (4)	89 (6)	20	12	11	6	14
Marbofloxacin	NQ	100 (10)	112 (6)	118 (5)	NQ	30	27	16	25
Mebendazole	110 (5)	78 (5)	86 (6)	69 (5)	19	18	11	7	9
Oxacillin	NQ	109 (14)	114 (12)	111 (6)	NQ	17	16	12	15
Oxfendazole	93 (11)	108 (8)	97 (6)	94 (5)	19	16	15	13	17
Oxolinic acid	NQ	90 (8)	109 (6)	116 (4)	NQ	23	15	10	11
Oxytetracycline	NQ	NQ	111 (6)	92 (5)	NQ	NQ	20	14	18
Penicillin G	NQ	100 (8)	76 (7)	74 (6)	NQ	20	14	7	10
Penicillin V	NQ	123 (10)	93 (11)	110 (7)	NQ	19	20	13	21
Sarafloxacin	NQ	NQ	71 (20)	102 (17)	NQ	NQ	22	18	24
Sulfachlorpyridazine	96 (7)	92 (5)	93 (2)	94 (2)	17	16	14	6	12
Sulfadiazine	77 (10)	92 (3)	103 (3)	87 (1)	18	17	17	12	16
Sulfadimethoxine	NQ	76 (12)	76 (12)	108 (5)	NQ	13	17	8	21
Sulfadimidine	91 (12)	93 (4)	98 (7)	97 (4)	23	13	13	9	20
Sulfaquinoxaline	NQ	86 (13)	96 (11)	89 (2)	NQ	23	16	7	15
Sulfathiazole	82 (10)	94 (6)	92 (6)	96 (4)	21	12	7	7	14
Tetracycline	NQ	NQ	95 (7)	114 (5)	NQ	NQ	11	16	28
Thiabendazole	97 (17)	95 (8)	97 (8)	76 (6)	30	18	8	7	17
Trimethoprim	116 (6)	100 (6)	115 (6)	100 (6)	26	16	13	9	18

[a] Intraday precision is given in brackets as relative standard deviation ($n = 5$).
[b] Number of replicates = 5.
[c] Expanded uncertainty ($k = 2$) estimated at 100 μg/kg.
[d] Not quantifiable.

Table 4
Maximum residue limit (MRL) set by the EU in fish, limit of detection (LOD), limit of quantification (LOQ), decision limit (CC_α) and detection capability (CC_β) of the selected compounds.

Compound	MRL (μg/kg)	LOD (μg/kg)	LOQ (μg/kg)	CC_α (μg/kg)	CC_β (μg/kg)
Albendazole	100	3.0	10.0	106.5	112.9
Ampicillin	50	3.0	10.0	59.9	69.8
Chlortetracycline	100	7.5	25.0	103.5	107.0
Cloxacillin	300	7.5	25.0	308.0	315.9
Danofloxacin	100	15.0	50.0	107.8	115.6
Dicloxacillin	300	7.5	25.0	313.3	326.5
Doxycycline	100	7.5	25.0	109.3	118.6
Emamectin	100	3.0	10.0	108.4	116.7
Enrofloxacin	100	3.0	10.0	110.5	120.9
Erythromycin	200	7.5	25.0	210.7	221.3
Fenbendazole	50	3.0	10.0	61.2	72.5
Flumequine	600	7.5	25.0	605.7	611.5
Josamycin	200	7.5	25.0	215.3	230.7
Levamisole	10	3.0	10.0	16.7	23.5
Marbofloxacin	150	7.5	25.0	150.8	151.5
Mebendazole	60	3.0	10.0	67.0	73.9
Oxacillin	300	7.5	25.0	310.6	321.2
Oxfendazole	50	3.0	10.0	64.9	79.8
Oxolinic acid	100	7.5	25.0	109.0	118.0
Oxytetracycline	100	15.0	50.0	110.0	120.0
Penicillin G	50	7.5	25.0	63.2	76.4
Penicillin V	25	7.5	25.0	31.8	38.6
Sarafloxacin	30	10.0	25.0	37.5	45.0
Sulfachlorpyridazine	100	3.0	10.0	106.9	113.9
Sulfadiazine	100	3.0	10.0	102.4	104.8
Sulfadimethoxine	100	7.5	25.0	123.7	147.3
Sulfadimidina	100	3.0	10.0	110.4	120.8
Sulfaquinoxaline	100	7.5	25.0	115.1	130.2
Sulfathiazole	100	3.0	10.0	107.4	114.8
Tetracycline	100	15.0	50.0	111.1	122.1
Thiabendazole	100	3.0	10.0	108.7	117.4
Trimethoprim	50	3.0	10.0	54.8	59.5

chlorotetracycline, cloxacillin, danofloxacin, doxycycline, enrofloxacin, josamycin, oxfendazole, oxolinic acid, sarafloxacin, sulfadimidine, sulfathiazole and tetracycline. Therefore, MMC curves were used for quantification purposes.

Recovery and precision studies were performed at four levels: 10 μg/kg for 13 analytes (albendazole, emamectin, enrofloxacin, fenbendazole, levamisole, mebendazole, oxfendazole, sulfachloropyridazine, sulfadiazine, sulfadimidine, sulfathiazole, thiabendazole and trimethoprim); 25 μg/kg for 29 analytes (except for danofloxacin, oxytetracycline and tetracycline); 50 and 100 μg/kg for all analytes as described above (Section 2.4). The obtained results are shown in Table 3. For 10 μg/kg level, the

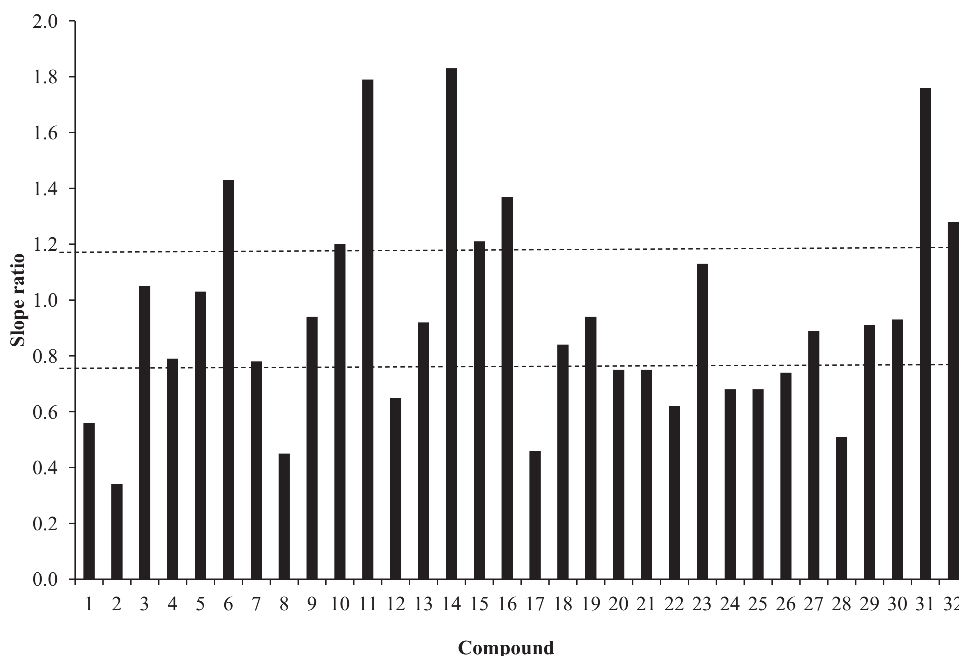

Fig. 3. Slope ratios between matrix-matched and solvent calibration. Compliance interval covering the range between 0.8 and 1.2 for tolerable matrix effect has been plotted. Veterinary drug codes are indicated in Fig. 2.

Table 5
Concentration of veterinary drug residues (μg/kg) found in real samples.

Compound	S_1	S_2	S_3	S_4	S_5	S_6	S_7	S_8	S_9	S_{10}
Albendazole							<LOQ			
Enrofloxacin			<LOQ							
Fenbendazole									<LOQ	
Mebendazole				<LOQ						
Oxfendazole			<LOQ					<LOQ		
Oxolinic acid						<LOQ	<LOQ			
Thiabendazole				<LOQ						

recoveries ranged from 76% (levamisole) to 116% (trimethoprim), and intraday precision was lower than 20%. It must be indicated that there were 18 analytes, which did not have enough sensitivity at this concentration level. At 25 μg/kg, intraday precision was lower than 20% and recoveries ranged from 71% (fenbendazole) to 110% (dicloxacillin), except for penicillin V (123%) and erythromycin (124%). At these levels there were four analytes which did not present satisfactorily sensitivity. Excellent results were obtained for all analytes at 50 and 100 μg/kg levels. Recoveries were between 69% (mebendazole at 100 μg/kg level) and 118% (ampicillin at 50 μg/kg level), except for chlorotetracycline (125%, respectively, at 50 μg/kg), and intraday precision was lower or equal than 20% for all analytes. Interday precision was lower than 30% (thiabendazole and marbofloxacin) for all analytes at the assayed concentration levels (Table 3). Furthermore, uncertainty (*U*) was evaluated at 100 μg/kg level, and the obtained results are shown in Table 3. It can be observed that *U* was below 25% for the assayed compounds, except for tetracycline (28%).

The estimated LODs and LOQs are shown in Table 4. It can be seen that LODs and LOQs were below 7.5 and 25.0 μg/kg, respectively, except for danofloxacin, oxytetracycline and tetracycline. For these compounds LOD and LOQ were 15.0 and 50 μg/kg, respectively, which were similar or lower than those described by other proposed methods [2,42–45]. Furthermore, it is important to note that the LOQs were always equal or lower than the MRL established by EU [6]. CC_α and CC_β parameters were calculated as described in Section 2.4, from MRL established for each analyte and they are shown in Table 4. It can be observed that CC_α ranged from 16.7 to 605.0 μg/kg and CC_β 23.5 and 611.5 μg/kg for levamisole and flumequine, respectively.

3.4. *Sample analysis*

The developed method was applied to the determination of veterinary drug residues in ten gilthead sea bream (*S. aurata*) samples obtained from local supermarkets in Almeria (Spain). In order to ensure the quality of the results when the proposed method was applied, an internal quality control was carried out in every batch of samples. This quality control implies a matrix-matched calibration, a reagent blank and a blank sample, which were spiked at 50 μg/kg before the extraction procedure, in order to evaluate the reliability of the proposed method. Furthermore, the retention time, quantification and confirmation transitions and relative ion intensities of the detected ions in retail samples were compared to those of corresponding calibration standards in the same batch to confirm the identity of the detected analytes using the criteria established by Decision Commission 657/2002/EC [15], obtaining the results indicated in Table 5. Only traces of veterinary drugs as albendazole, enrofloxacin, fenbendazole, mebendazole, oxfendazole, oxolinic acid and thiabendazole (<LOQ) were observed in 6 samples. It can be indicated that in four samples, traces of more than one compound were observed. The obtained results are in agreement with other studies [2,43,45], were few compounds were detected in the incurred samples.

4. Conclusions

A simple, fast, reproducible and sensitive method was developed for the quantification of 32 veterinary drug compounds (from nine different classes) in gilthead sea bream (*S. aurata*) samples. The method was based on QuEChERs extraction method in order to extract a wide range of analytes with different physic-chemical properties and experimental design has been applied in order to evaluate the influence of several factors involved in the extraction process. The addition of methanol to the extraction solution improves the extraction of tetracyclines and quinolones. The best composition was investigated and it was concluded that the acetonitile:methanol (75:25, v/v) showed better results. After that, the method was validated and good results were obtained in terms of linearity, trueness, precision, LODs, LOQs, CC_α and CC_β. When the proposed method is used, 15 samples can be extracted in less than 1 h and half, and the extracts can be analyzed in 2 h. Bearing in mind that many compounds are determined from a single extraction, the proposed method could be applied in routine analysis.

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