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Short communication

Multiresidue method for simultaneous determination of quinolone antibacterials in pig kidney samples by liquid chromatography with fluorescence detection

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Abstract

A new analytical method for simultaneous determination of eight quinolones namely, ciprofloxacin, danofloxacin, difloxacin, enrofloxacin, flumequine, marbofloxacin, oxolinic acid and sarafloxacin, in pig kidney samples was developed. The procedure involves the extraction of the quinolones from the samples by traditional extraction, a step for clean-up and preconcentration of the analytes by solid-phase extraction (SPE) and subsequent liquid chromatography separation with fluorescence detection (LC–FD). The mobile phase was composed of acetonitrile and 10 mM citrate buffer solution of pH 4.5, with an initial composition of acetonitrile–water 12:88 (v/v) and using linear gradient elution. Norfloxacin was used as internal standard. The limits of detection (1–8 $\mu g\,kg^{-1}$) and the limits of quantification (5–27 $\mu g\,kg^{-1}$) found were lower than the maximum residue limits regulated by the European Union for these compounds in pig kidney.

Keywords: Quinolones; Pig kidney; Liquid chromatography–fluorescence detection (LC–FD)

1. Introduction

Antibiotics are widely used in food-producing animals for treatment and prevention of diseases and as feed additives to increase the animal mass. Their misuse can leave residues in edible animal tissues, which may give rise to public health concern (toxic effects, development of resistant strains of bacteria, allergic hypersensitivity reactions, etc.) [1,2].

Quinolones constitute an important group of synthetic antibiotics developed in recent years which are used to treat various infections in both human and veterinary medicine. These compounds exhibit high activity against a broad spectrum of gram-negative and gram-positive bacteria through inhibition of their DNA-gyrase or topoisomerase II [3]. A significant increase in the use of quinolones in animal production was noted over the last decade. The European Union (EU) has established maximum residue limits (MRLs) for quinolone residues in animal tissues [4]. Thus, the establishment of sensitive methods for the analysis of residual amounts of these drugs is required for quality control of food products for consumers and for the evaluation of the correct application of withdrawal times.

Multiresidue analysis of quinolones in biological samples and animal tissues [5–9] involves liquid chromatography with ultraviolet (LC–UV) [10–15], fluorescence (LC–FD) [16–24] or mass spectrometric detection (LC–MS) [25–30], gas chromatography–mass spectrometry (GC–MS) [31,32], high-performance thin layer chromatography (HPTLC) [33,34] and capillary electrophoresis (CE) [35–41].

Only a few methods [22,25–28,32,38] have focused however on the determination of quinolone residues in pig kidney. Asami et al. [32] proposed a GC–MS method for the determination of four quinolones in porcine meat and kidney. Achieved recoveries were in the range 80–90% and limits of detection in the range 10–20 $\mu g\,kg^{-1}$.

Using nonaqueous capillary electrophoresis with diode array detection (CE–DAD), Hernández et al. [38] developed a method for the determination of seven quinolones in pig kidney samples. A solid-phase extraction was carried out using C_{18} cartridges.

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Table 1
EU MRL ($\mu g\,kg^{-1}$) for quinolones in pig kidney

Regulation no.	Date	Annex of Reg. 2377/90	Compound	MRL
2338/00	20/10/00	I	Marbofloxacin	150
1181/02	01/07/02	I	Danofloxacin	200
1181/02	01/07/02	I	Difloxacin	800
1181/02	01/07/02	I	Enrofloxacin + Ciprofloxacin	300
1181/02	01/07/02	I	Flumequine	1500
1358/05	18/08/05	I	Oxolinic acid	150

The recoveries achieved for all quinolones were over 80%; the limits of detection ranging from 57 $\mu g\,kg^{-1}$ for ciprofloxacin to 119 $\mu g\,kg^{-1}$ for enrofloxacin.

Toussaint et al. [25–28], using liquid chromatography–tandem mass spectrometry (LC–MS/MS) proposed, in several papers, a method for the multiresidue determination of 11 quinolones in pig kidney samples. The method involves a sample preparation by solid-phase extraction on SDB-RPS disk cartridges. Good recoveries were obtained (80–100%) and the limits of detection found were between 0.3 and 2.1 $\mu g\,kg^{-1}$.

Verdon et al. [22] developed a LC–FD method for the determination of 10 quinolone residues in multimatrix/multispecies animal tissues including porcine kidney. It involved extraction of residues from the biological tissues/fluids by acidic aqueous solution, centrifugation and filtration prior to injection on a C_{18} narrow-bore column, and detection through a three-step-mode fluorescence detector. The limits of detection found ranged from 4 to 11 $\mu g\,kg^{-1}$. For some compounds, achieved recoveries were low (29% for FLU, 30% for SAR and 32% for DIF and nalidixic acid).

In this work, we report a LC–FD method for simultaneous determination of the regulated quinolones in pig kidney by the EU: marbofloxacin (MAR), danofloxacin (DAN), difloxacin (DIF), enrofloxacin + ciprofloxacin (ENR + CIP), flumequine (FLU) and oxolinic acid (OXO) (Table 1), and sarafloxacin (SAR) which has not yet an assigned MRL but is the main metabolite of DIF. Norfloxacin (NOR) was the internal standard selected because this quinolone is forbidden in veterinary medicine.

The column used, a Zorbax Eclipse XDB-C_8, permits the separation of these nine quinolones with good resolution in less time. A systematic study on the optimisation of the mobile phase and peak resolution was made by using the linear solvation energy relationship (LSER) formalism [42]. Finally, the analytical performance of the optimised method was assessed.

2. Experimental

2.1. Chemicals and reagents

All reagents were analytical grade, unless stated otherwise. Water (18.2 $M\Omega\,cm^{-1}$) was purified by means of a Milli-Q plus system (Millipore, Bedford, MA, USA).

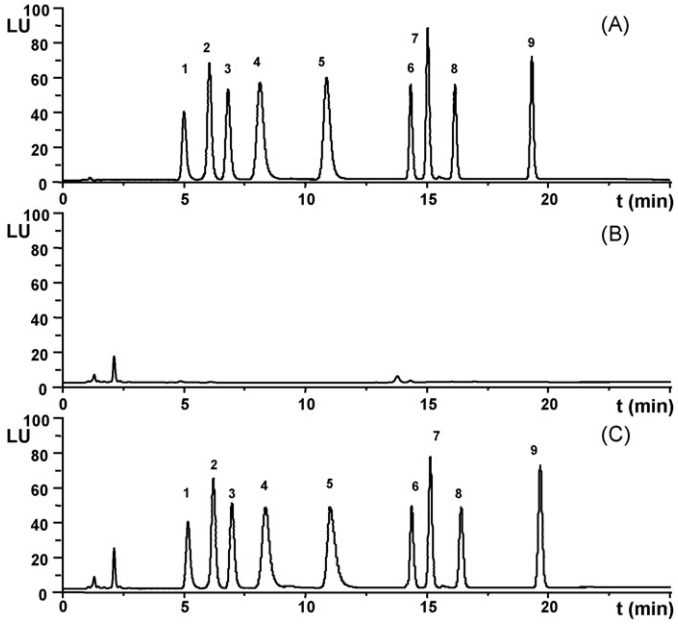

Fig. 1. Representative chromatogram of: (A) a standard mixture of the selected quinolones; (B) an unspiked pig kidney sample; (C) a spiked pig kidney sample Chromatographic conditions are described in the text. Peaks identifications: (1) marbofloxacin, 200 $\mu g\,kg^{-1}$; (2) norfloxacin (IS), 100 $\mu g\,kg^{-1}$; (3) ciprofloxacin, 100 $\mu g\,kg^{-1}$; (4) danofloxacin, 25 $\mu g\,kg^{-1}$; (5) enrofloxacin, 100 $\mu g\,kg^{-1}$; (6) sarafloxacin, 100 $\mu g\,kg^{-1}$; (7) difloxacin, 100 $\mu g\,kg^{-1}$; (8) oxolinic acid, 200 $\mu g\,kg^{-1}$; (9) flumequine, 200 $\mu g\,kg^{-1}$.

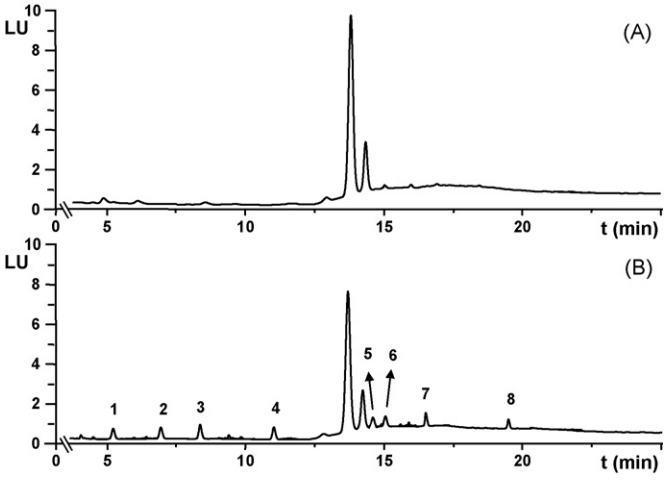

Fig. 2. Representative chromatogram of: (A) an unspiked pig kidney sample; (B) a spiked pig kidney sample at around LOQ levels. Chromatographic conditions are described in the text. Peaks identifications: (1) marbofloxacin, 25 $\mu g\,kg^{-1}$; (2) ciprofloxacin, 10 $\mu g\,kg^{-1}$; (3) danofloxacin, 5 $\mu g\,kg^{-1}$; (4) enrofloxacin, 10 $\mu g\,kg^{-1}$; (5) sarafloxacin, 30 $\mu g\,kg^{-1}$; (6) difloxacin, 5 $\mu g\,kg^{-1}$; (7) oxolinic acid, 15 $\mu g\,kg^{-1}$; (8) flumequine, 15 $\mu g\,kg^{-1}$.

Table 2
Analytical and statistical parameters

Parameter	MAR	CIP	DAN	ENR	SAR	DIF	OXO	FLU
Intercept (a)	0.0186	−0.0240	−0.4019	−0.0538	0.0108	0.0093	0.0056	0.0096
Intercept standard deviation (S_a)	0.0025	0.0029	0.0152	0.0060	0.0081	0.0025	0.0012	0.0023
Slope (b) (kg μg^{-1})	0.0020	0.0070	0.0615	0.0153	0.0058	0.0105	0.0020	0.0030
Slope standard deviation (S_b)	0.0001	0.0001	0.0002	0.0001	0.0001	0.0001	0.0001	0.0001
Regression standard deviation ($S_{y/x}$)	0.0049	0.0057	0.0303	0.0119	0.0162	0.0050	0.0023	0.0046
Correlation coefficient (r^2)	0.9990	0.9999	0.9999	0.9999	0.9981	0.9999	0.9997	0.9995
Lack-of-fit test (p-value)	0.46	0.15	0.21	0.11	0.68	0.26	0.36	0.40
Linear dynamic range ($\mu g\,kg^{-1}$)	24–250	8–250	5–125	8–250	27–250	5–250	12–250	15–250
Detection limit ($\mu g\,kg^{-1}$)	7	2	2	2	8	1	4	5
Quantification limit ($\mu g\,kg^{-1}$)	24	8	5	8	27	5	12	15

Quinolones were obtained from different pharmaceutical firms: CIP (Ipsen Pharma, Barcelona, Spain), DAN (Pfizer, Karlsruhe, Germany), DIF and SAR (Abbott, Madrid, Spain), ENR (Cenavisa, Tarragona, Spain), FLU, NOR and OXO (Sigma–Aldrich, Madrid, Spain) and MAR (Vetoquinol, Lure, France).

Individual stock solutions of CIP, DAN, DIF, ENR, MAR, NOR and SAR were prepared in ethanol (99.9%, v/v) at a concentration of 100 $\mu g\,mL^{-1}$. Individual stock solutions of FLU and OXO were prepared in acetonitrile at a concentration of 100 $\mu g\,mL^{-1}$. These solutions were stored at 4 °C in the dark for not longer than 2 months. Individual working solutions were prepared by diluting suitably with an acetonitrile–water mixture (12:88, v/v).

Acetonitrile (HPLC-gradient grade), *o*-phosphoric acid and citric acid were obtained from Panreac (Barcelona, Spain). Methanol, ethanol, formic acid, hexane, trifluoroacetic acid and ammonia were supplied by Merck (Darmstadt, Germany). *m*-Phosphoric acid was obtained from Sigma–Aldrich. The 10 mM citrate buffer solution of pH 4.5 was prepared from citric acid and ammonia.

All solutions prepared for LC were filtered through 0.22-μm PVDF filter membranes (Millipore) before use.

Isolute ENV + (200 mg/3 mL) solid-phase extraction (SPE) adsorbent cartridges were purchased from Isolute Sorbent Technologies (Mid Glamorgan, UK).

2.2. Apparatus and software

The chromatographic system consisted of an Agilent Technologies (Palo Alto, CA, USA) 1100 series high-performance liquid chromatograph equipped with a quaternary pump, an online degasser, an autosampler, an automatic injector with a loop of up to 100 μL, a thermostated column compartment and a fluorescence detector (flow-cell volume, 8 μL) connected online. ChemStation for LC 3D software (Agilent) was used for instrument control and for data acquisition and analysis.

All pH measurements were made with a Crison (Crison Instruments SA, Barcelona, Spain) combined glass-saturated calomel electrode using an earlier calibrated Crison 2000 digital pH-meter.

A model Bapi 600 homogeniser from Taurus (Lérida, Spain) and a vortex mixer model MS2 from IKA (Staufen, Germany) were used to mince, mix and homogenise kidney samples during pretreatment. A model Universal 32 centrifuge from Hettich Zentrifugen (Tuttligen, Germany) was used in order to perform the extractions.

SPE was performed on a Supelco (Madrid, Spain) vacuum manifold for 12 columns connected to a Supelco vacuum tank and to a vacuum pump.

Statgraphics software package [43] was used for statistical analysis of data and for regression analysis (linear model).

2.3. Preparation of fortified samples

Fortified kidney samples were prepared by spiking 1-g (accurately weighed) of minced blank kidney adding the adequate volumes of working solutions of studied quinolones and norfloxacin, used as internal standard.

Before sample treatment and analysis, all samples were allowed to stand in the dark for 30 min at room temperature to permit the total interaction between the antibiotics and pig kidney. All samples were prepared in duplicate.

The recovery (parameter considered in order to optimise all the parameters) was evaluated by comparison with samples of pig kidney that were spiked after the SPE procedure and that were considered the 100% extracted.

2.4. Sample treatment

In order to extract quinolones from pig kidney, two sequential volumes (25 and 10 mL) of 0.3% (w/v) *m*-phosphoric acid solution–acetonitrile mixture (75:25, v/v) were used. After shaking for 5 min and sonication for 10 min, the mixtures were centrifuged at 4000 rpm (2630 × g) for 10 min and subsequent filtered through a 0.22 μm PVDF membrane. To improve the retention of quinolones on the SPE cartridge, by decreasing the high concentration of acetonitrile, 75 mL of deionised water was added at each extract.

2.5. Solid-phase extraction

The isolute ENV + cartridges were conditioned with 2 mL of methanol, 2 mL of deionised water and 2 mL of 50 mM *o*-phosphoric acid at pH 3.0. After samples were passed for the system, the cartridge was cleaned with 2 mL of deionised water,

4 mL of 1% formic acid in water–methanol mixture (60:40, v/v) and 5 mL of hexane in order to defat extracts. The analytes were eluted with 5 mL of 2% trifluoroacetic acid in water–acetonitrile mixture (25:75, v/v), followed by 1 mL of acetonitrile. The samples were evaporated to dryness at 50 °C under a stream of nitrogen. One millilitre of mobile phase was added in order to resuspend the residue and the mixture was centrifuged at 18,000 rpm (23,900 × *g*) for 10 min. The supernatant obtained was injected into the LC system.

2.6. Chromatographic conditions

Chromatographic separation of the quinolones was performed on a Zorbax Eclipse XDB-C_8 column (150 mm × 4.6 mm i.d., 5 μm particle size) from Agilent. The column was protected with an Eclipse XDB-C_8 (Agilent) pre-column (12.5 mm × 4.6 mm i.d., 5 μm particle size).

The flow-rate was 1.5 mL min^{-1}, the injection volume 20 μL and the column temperature was maintained at 25 °C.

The initial mobile phase used was a mixture of 10 mM citrate buffer solution of pH 4.5 and acetonitrile (88:12, v/v). Good chromatographic separation of the quinolones can be achieved by using the following optimised linear gradient elution: from 0 to 10 min, the initial mobile phase contains a 12% acetonitrile; from 10 to 15 min, the percentage of organic modifier linearly increases to 30% acetonitrile; from 15 to 19 min, the acetonitrile percentage is maintained to 30%; from 19 to 20 min, the acetonitrile percentage decreases to 12% and finally, the acetonitrile percentage is maintained to 12% from 20 to 25 min. The separation of the eight quinolones studied and the internal standard was achieved in 25 min.

The excitation and emission wavelengths selected for the detection of CIP, ENR, DAN, DIF, NOR and SAR were 280 and 450 nm, respectively, for the detection of MAR 290 and 495 nm, respectively, and for the detection of FLU and OXO 325 and 365 nm, respectively.

3. Results and discussion

3.1. Optimisation of the LC conditions

As in other studies made by the authors [13,24], a Zorbax Eclipse XDB-C_8 column and a gradient elution were used for quinolones separation. To optimise the separation of the compounds of interest in this column the LSER methodology was applied in the same way that has been previously described [13,42]. Resolution between adjacent peaks (R_S) [44] was calculated at 10 different acetonitrile percentages (from 10 to 40%) to predict the optimum percentage of organic phase.

In relation with the pH optimisation, the retention factors [44] for the quinolones at different pH values of buffer solution (between 3.0 and 5.0) were determined from three different injections at every pH considered, and the R_S was calculated. The optimised LC conditions are described in Section 2.6. R_S values found under these conditions for the nine quinolones were in all cases higher than 2.0.

Table 3
Intra-day and inter-day recovery (%) and precision (R.S.D., %)

Compound		Concentration level (μg kg^{-1})[a]		
		50	100	200
MAR	Day 1	84 (3)	86 (3)	85 (2)
	Day 2	85 (2)	86 (3)	87 (3)
	Day 3	85 (2)	85 (2)	84 (3)
CIP	Day 1	85 (4)	84 (3)	83 (3)
	Day 2	83 (3)	82 (3)	85 (4)
	Day 3	84 (4)	84 (3)	82 (3)
DAN[a]	Day 1	74 (2)	76 (3)	74 (3)
	Day 2	76 (3)	77 (2)	75 (3)
	Day 3	77 (3)	77 (3)	75 (4)
ENR	Day 1	86 (3)	87 (3)	87 (3)
	Day 2	86 (3)	85 (3)	85 (3)
	Day 3	85 (4)	84 (4)	85 (3)
SAR	Day 1	89 (4)	87 (3)	88 (3)
	Day 2	87 (4)	86 (4)	86 (3)
	Day 3	88 (4)	85 (3)	86 (2)
DIF	Day 1	82 (3)	83 (3)	85 (4)
	Day 2	84 (4)	85 (2)	82 (3)
	Day 3	84 (4)	83 (2)	83 (3)
OXO	Day 1	87 (3)	88 (4)	91 (3)
	Day 2	89 (4)	90 (4)	89 (4)
	Day 3	89 (3)	91 (4)	90 (3)
FLU	Day 1	90 (3)	88 (3)	91 (3)
	Day 2	87 (4)	89 (3)	88 (4)
	Day 3	89 (3)	87 (2)	90 (4)

Data obtained for the determination of studied quinolones in pig kidney samples ($n = 6$).
[a] For DAN, the concentration levels were 20, 40 and 80 μg kg^{-1}.

Norfloxacin (used in human medicine) was selected as internal standard for LC quantification, because this quinolone was efficiently extracted from kidney (95 ± 3%) and did not coelute with any of the evaluated quinolones. However, owing to the possible illegal use of this compound in veterinary medicine, prior to its addition an analysis of samples should be carried out in all cases to detect norfloxacin in order to guarantee its adequate use as internal standard.

A typical chromatogram corresponding to a standard mixture of the selected antibiotics is shown in Fig. 1A. The separation of these nine quinolones was achieved in less than 25 min.

3.2. Optimisation of the extraction procedure

In a previous paper [13], about the determination of quinolones in chicken muscle, the extraction of the analytes was carried out using a double extraction with dichloromethane and a double re-extraction with sodium hydroxide followed by a SPE step. This method provides good recoveries, higher than 70% for all quinolones regulated by EU for chicken muscle, but the method is too long. In order to improve the method in relation with time of analysis, a reduction of the number of extractions is essential. Aqueous acidic solutions (trichloroacetic or *m*-phosphoric acids) in mixtures with acetonitrile were some of the extracting agents used in the literature for the separation of

Table 4
Results of recovery assays in pig kidney samples to check the accuracy of the proposed method

Compound	Spiked ($\mu g\,kg^{-1}$)	Found[a] ($\mu g\,kg^{-1}$) (Recovery, %)			
		Sample 1	Sample 2	Sample 3	Sample 4
MAR	50	49.7 ± 0.5 (99)	49.6 ± 0.8 (99)	50.3 ± 0.7 (101)	49.9 ± 0.6 (100)
	100	100.3 ± 1.4 (100)	101.0 ± 1.6 (101)	100.1 ± 1.1 (100)	100.4 ± 0.9 (100)
	200	201.3 ± 2.3 (101)	198.8 ± 1.9 (99)	198.5 ± 3.5 (99)	200.9 ± 1.5 (101)
CIP	50	49.8 ± 0.4 (100)	50.3 ± 0.5 (101)	49.9 ± 0.6 (100)	50.2 ± 0.8 (100)
	100	100.3 ± 1.1 (100)	99.6 ± 1.8 (100)	100.7 ± 1.8 (101)	99.5 ± 1.1 (100)
	200	200.8 ± 3.0 (100)	200.7 ± 2.3 (100)	198.5 ± 3.5 (99)	199.4 ± 2.3 (100)
DAN	20	19.9 ± 0.5 (100)	19.8 ± 0.3 (99)	20.2 ± 0.3 (101)	20.2 ± 0.4 (101)
	40	40.4 ± 0.6 (101)	39.7 ± 0.6 (99)	40.2 ± 0.8 (101)	39.8 ± 0.5 (100)
	80	79.6 ± 0.9 (100)	80.1 ± 0.5 (100)	80.5 ± 0.9 (101)	78.9 ± 1.7 (99)
ENR	50	50.3 ± 0.5 (101)	49.8 ± 0.4 (100)	50.1 ± 0.6 (100)	49.9 ± 0.6 (100)
	100	100.6 ± 1.0 (101)	99.8 ± 1.9 (100)	100.3 ± 0.6 (100)	100.2 ± 1.2 (100)
	200	199.6 ± 2.2 (100)	200.7 ± 2.3 (100)	200.9 ± 1.5 (101)	199.5 ± 1.9 (100)
SAR	50	50.1 ± 0.4 (100)	50.3 ± 0.7 (101)	49.8 ± 0.5 (100)	49.7 ± 0.5 (99)
	100	100.2 ± 0.6 (100)	100.4 ± 0.9 (100)	99.2 ± 1.2 (99)	99.6 ± 0.8 (100)
	200	198.6 ± 2.5 (99)	198.9 ± 2.0 (100)	200.3 ± 1.9 (100)	200.9 ± 1.5 (101)
DIF	50	49.6 ± 0.7 (99)	50.3 ± 0.7 (101)	49.9 ± 0.5 (100)	50.0 ± 0.7 (100)
	100	99.5 ± 0.6 (100)	100.3 ± 1.2 (100)	99.4 ± 1.5 (99)	100.2 ± 0.6 (100)
	200	198.8 ± 1.5 (99)	201.8 ± 2.2 (101)	200.8 ± 1.9 (100)	200.7 ± 2.1 (100)
OXO	50	50.2 ± 0.4 (100)	49.8 ± 0.6 (100)	49.7 ± 0.4 (100)	50.4 ± 0.6 (101)
	100	100.6 ± 1.8 (100)	100.4 ± 1.5 (100)	99.6 ± 0.9 (100)	99.7 ± 0.6 (100)
	200	198.9 ± 2.0 (100)	201.3 ± 2.6 (101)	200.9 ± 1.2 (100)	198.6 ± 2.5 (99)
FLU	50	50.3 ± 0.8 (101)	50.0 ± 0.7 (100)	50.1 ± 0.7 (100)	49.9 ± 0.6 (100)
	100	99.8 ± 1.2 (100)	100.3 ± 1.4 (100)	100.4 ± 0.9 (100)	99.3 ± 1.8 (99)
	200	198.9 ± 2.0 (100)	200.3 ± 1.1 (100)	201.3 ± 2.3 (101)	198.5 ± 2.6 (99)

[a] Mean value ± standard deviation of four determinations.

the quinolones from different matrices [7,45,46]. In relation with the acid added in a preliminary study using 20% of acetonitrile, the recoveries obtained with trichloroacetic acid were lower than those obtained by using *m*-phosphoric acid. In the optimisation of the acetonitrile concentration (from 10 to 40%, v/v) using a 0.2% (w/v) concentration of *m*-phosphoric acid, a 25% concentration of acetonitrile gives the best recoveries (higher than 85%) for all quinolones. When the percentage of *m*-phosphoric acid present in the aqueous fraction (from 0.2 to 0.5%, w/v) was optimised, the best results were obtained with 0.3% (w/v). Two sequential extractions were necessary with (25 + 10 mL) because the recoveries were 20–30% higher using two extractions. In order to defat the sample, a step using hexane was necessary. We considered two options: a liquid–liquid extraction after the extraction of the quinolones from the pig kidney or the addition of hexane to the SPE cartridge. The recoveries were better when hexane was used in the SPE step. The differences between recoveries ranged from 5% for MAR to 17% in the case of DIF and FLU.

3.3. Calibration and method performance

For the calibration, spiked standard samples at six concentration levels were extracted following the extraction procedure

Table 5
Results of recovery assays in pig kidney samples (at MRL level) to check the accuracy of the proposed method

Compound	Spiked ($\mu g\,kg^{-1}$)	Found[a] ($\mu g\,kg^{-1}$) (Recovery, %)			
		Sample 1	Sample 2	Sample 3	Sample 4
CIP[b]	300	298 ± 5 (99)	303 ± 5 (101)	299 ± 3 (100)	302 ± 4 (101)
DAN[c]	200	201 ± 4 (100)	198 ± 3 (99)	197 ± 4 (99)	202 ± 5 (101)
DIF[d]	800	796 ± 6 (99)	803 ± 7 (101)	799 ± 6 (100)	805 ± 8 (101)
ENR[b]	300	301 ± 6 (101)	297 ± 5 (99)	303 ± 3 (101)	298 ± 4 (99)
FLU[e]	1500	1507 ± 11 (100)	1501 ± 10 (100)	1504 ± 12 (100)	1493 ± 10 (100)

[a] Mean value ± standard deviation of four determinations.
[b] Dilution of extract: 1/2.
[c] Dilution of extract: 1/4.
[d] Dilution of extract: 1/8.
[e] Dilution of extract: 1/10.

previously explained (each level was prepared by duplicate, and each calibration sample was analysed twice—24 experimental data were used for each calibration curve). Calibration curves were constructed using analyte/internal standard peak area ratio *versus* concentration of analyte. The *lack-of-fit* test [47] was used to check the linearity of the calibration graphs according to the Analytical Methods Committee. The limits of detection (LOD) and quantification (LOQ) were calculated according with the IUPAC criterion [48]. Therefore, the analysis of 10 sample blanks was carried out in order to estimate the standard deviation (s_0). The *t*-Student parameter for α and $\beta = 0.05$ and $n - 1$ degrees of freedom is also needed to calculate LOD and LOQ.

Fig. 2 shows the comparison between the chromatograms obtained for an unspiked kidney sample (Fig. 2A) and a spiked kidney sample at around LOQ level for each studied quinolone (Fig. 2B).

The analytical and statistical parameters for each quinolone studied are summarised in Table 2.

In order to determine the intra-day and inter-day repeatability, blank pig kidney samples were spiked at three concentration levels (50, 100 and 200 $\mu g\,kg^{-1}$ for CIP, ENR, DIF, FLU, MAR, OXO and SAR and 20, 40 and 80 $\mu g\,kg^{-1}$ for DAN) and six analyses were performed on 3 days. Recoveries were achieved by comparing the analytical results for extracted standard samples of pig kidney at aforementioned concentrations with unextracted standards prepared at the same concentrations in blank extract representing 100% recovery. The results obtained, summarised in Table 3, fulfill the requirements defined by the European Union legislation [49].

3.4. Application and validation of the method

The proposed method was applied to the determination of possible quinolones in four different commercial pig kidney samples purchased in several supermarkets of Granada. In all cases, the studied quinolone contents in the assayed samples were smaller than the above-stated detection limits.

In Fig. 1(B and C), representative chromatograms of unspiked and spiked pig kidney samples, respectively, are shown

Kidney samples were spiked at different levels (50, 100 and 200 $\mu g\,kg^{-1}$ for CIP, ENR, DIF, FLU, MAR, OXO and SAR and 20, 40 and 80 $\mu g\,kg^{-1}$ for DAN).

The validation of the proposed method for these samples was tested by using a recovery test (Student *t*-test) [49,50]. As the *P*-values calculated in all cases are greater than 0.05, the null hypothesis appears to be valid, *i.e.*, recoveries are close to 100%. The obtained results are shown in Table 4.

As MRL's for CIP, DAN, DIF, ENR and FLU are not included in the linear dynamic ranges (LDR) for these compounds, kidney samples were also spiked at these levels in order to demonstrate that the proposed method is fit for the quantification of these levels for those quinolones. An appropriated dilution of the extracts obtained according to the procedure above-described (Sections 2.3–2.5) was required for their determination using the established calibration curves. The achieved results are shown in Table 5.

4. Conclusions

A sensitive liquid chromatographic with fluorescence detection method is described for the determination of the seven quinolones regulated by de European Union in pig kidney and sarafloxacin, which has not yet an assigned maximum residue limit but is the main metabolite of difloxacin. The separation of these compounds was successfully performed on a Zorbax Eclipse XDB-C_8 column, with a linear gradient composed of acetonitrile and 10 mM citrate buffer of pH 4.5. A solid-phase extraction procedure for clean-up and preconcentration of the analytes was used. The limits of detection and quantification were in all cases lower than the maximum residue limits regulated by the European Union for these compounds in pig kidney. The method was validated by a recovery assay with spiked samples.

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