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Simple and sensitive determination of five quinolones in food by liquid chromatography with fluorescence detection

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Abstract

A simple and sensitive high-performance liquid chromatographic (HPLC) method has been developed for the determination of five different quinolones: enrofloxacin, ciprofloxacin, sarafloxacin, oxolinic acid and flumequine in pork and salmon muscle. The method includes one extraction and clean-up step for the five quinolones together which are detected in two separated HPLC runs by means of their fluorescence. The proposed analytical method involves homogenizing of the tissue sample with 0.05 *M* phosphate buffer, pH 7.4 and clean-up by Discovery DS-18 cartridges. For chromatographic separation a Symmetry C_{18} column is used in two different runs: (1) ciprofloxacin, enrofloxacin and sarafloxacin with acetonitrile–0.02 *M* phosphate buffer pH 3.0 (18:82) as mobile phase and the detector at excitation wavelength: 280 nm and emission wavelength 450 nm; and (2) oxolinic acid and flumequine with acetonitrile–0.02 *M* phosphate buffer pH 3.0 (34:66) as mobile phase and excitation wavelength: 312 nm and emission wavelength: 366 nm. Detection limit was as low as 5 ng g^{-1}, except for sarafloxacin which had a limit of 10 ng g^{-1}. Standard curves using blank muscle tissues spiked at different levels showed a good linear correlation coefficient, r^2 higher than 0.999 for all quinolones.

Keywords: Quinolones

1. Introduction

Fluoroquinolones are a group of chemotherapeutic agents highly active against a wide range of Gram negative and Gram positive bacteria, including those resistant to beta-lactam antibiotics and sulphonamides. Quinolones represented by flumequine and oxolonic acid are effective antibacterial drugs widely used against various important diseases of farmed fish [1]. However, since 1990 other quinolones as sarafloxacin and enrofloxacin were studied and they appeared to have an excelent efficiency against fish and chicken pathogens. These fluorquinolones also have a satisfactory effect in the treatment of severe intestinal and respiratory infections in turkeys, pigs, calves and poultry [2]. They are absorbed well after oral administration and distributed extensively in the tissue. They are highly active against Gram positive and negative bacteria including those resistant to beta-lactam antibiotics and sulfonamides. These characteristics make these drugs suitable for the therapy of a high number of infections in livestock and fish farm.

These practices imply that drug residues may persist in edible tissues derived from treated animals

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and therefore maximum permitted levels have been set for most of them, and accordingly, their residues need to be controlled.

The European Residue Plan for the Surveillance in Food of animal origin includes the control of quinolone residues in different species. Generally, after a positive result of the microbiological four plates inhibition test, identification and quantification of the quinolone residue is requiered, as different quinolones and different species or kind of tissue have different maximum residue limits (MRL). So the importance of one multiresidue method is clear.

Generally, analytical methods described in the literature show a wide range of different extraction and clean-up procedures depending on the kind of quinolone and the class of matrix. Sample treatment includes an extraction step with medium to high polarity organic solvents or hydro-organic mixtures in acid or basic media followed by several liquid–liquid or solid-phase extractions as clean-up procedures [3–5]. Different chromatographic methods based on HPLC with fluorescence detection have been published, generally for one or two quinolones in fish or eggs [6–8] and by means of ion-pair chromatography followed by UV detection for four quinolones but they do not include flumequine and oxolinic acid and have rather high detection limits [9]

For the analysis of more than one quinolone also non-common used techniques have been applied, as terbium(III)-sensitised luminescence for enrofloxacin and ciprofloxacin [10,11] or capillary isotachophoresis [12–14].

But, generally, these techniques are not available in most of the rutine laboratories within the National Residue Monitoring Plan. Till the appearance of the last generation of LC–MS(-MS) equipments, no sensitive methds for multiresidue analysis of quinolones in different matrixes have been published [15,16]. This technique allows the multiresidue determination of quinolones in different matrixes with the possibilty to confirm the presence of these compounds by means of fragment abundace ratios at rather low concentration levels. The equipment is very expensive and actually only few laboratories can afford the acquisition of these instruments. However, in future, LC–MS(-MS) will be the technique of choice for the determination of almost all the veterinary drug residue analysis, as it reach high sensitivity with high specificity.

Many different HPLC conditions for the determination of quinolones are described in the literature, especially concerning the mobile phases, with variations in ionic strenght or acidity, or adding modifiers such as citric acid, perchloric acid or tertiary amine salts [17,18].

The aim of this work was the development of a single extraction and clean-up procedure for all quinolones: the fluoroquinolones: enrofloxacin, ciprofloxacin and sarafloxacin, as well as the other acidic drugs: oxolinic acid and flumequine (Fig. 1). The final extract may be used for the determination of both groups in two different HPLC separation and detection conditions.

After the comparison of different columns (Symmetry and Supelcosil ABZ Plus), different mobile phase compositions without contra-ions or organic modifiers and different pH values, the best column for the separation of ciprofloxacin and enrofloxacin was selected. Then three more quinolones were

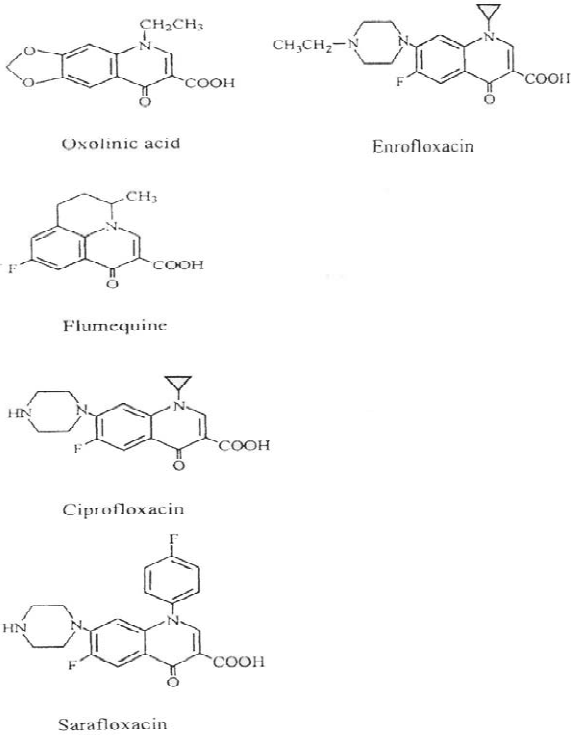

Fig. 1. Chemical structures of the studied quinolones.

included in the development of the analytical method for the determination of a total of five quinolones in food samples.

2. Experimental

2.1. Materials

Ciprofloxacin (CF) and enrofloxacin (EF) from Bayer AG (Wuppertal, Germany) and sarafloxacin (SF) from Cyanamid (Princeton, NJ, USA), oxolonic acid (Oxo) and flumequine (Flu) from Sigma (St. Louis, MO, USA) were donations of the European Reference Laboratory, AFSSA, Fougères (France).

Potassium dihydrophosphate, disodium-hydrogenphosphate, sodium hydroxide (HPLC grade), and 85% phosphoric acid were from Merck (Darmstadt, Germany). Then 34% ammonium hydroxide was supplied by Panreac (Barcelona, Spain). Methanol and acetonitrile were purchased from Lab-Scan (Dublin, Ireland)

De-ionized water (Alpha-Q, Millipore, Milford, MA, USA) was used to prepare all aqueous solutions. Nylon Millex filters (0.8 μm and 25 mm) (Millipore) were used for filtration of sample extracts.

Discovery DSC-18 solid-phase extraction cartridges were obtained from Supelco (Bellafonte, USA).

An ultrasonic bath and a Macrotonic centrifuge (Selecta, Madrid, Spain), a sample concentrator with nitrogen (Techne LD, Cambridge, UK), a Moulinex homogenizer (Moulinex, Bilbao, Spain) and a pH-meter from Beckman (Fullerton, USA) were used in the sample extraction and clean-up procedures.

2.2. Chromatographic systems and conditions for

2.2.1. Separation study

For the study of the chromatographic separation conditions, a Diode Array detector Model 990 (Waters), set at 278 nm, with a 990 Printer Plotter (Waters) were used.

The analytical columns tested for the optimization of the separation of enrofloxacin and ciprofloxacin, were a Symmetry C_{18}, 5 μ (4.6×150) column (Waters) and a Supelcosil ABZ Plus (4.6×150) column (Supelco). They were tested with different mobile phases, based on different mixtures of acetonitrile and phosphate buffer and the influence of variations in ionic strenght (0.01, 0.02 and 0.05 *M*) and pH values (2.5; 3.0; 3.5; 4.0 and 5.0) was studied. Flow rates were 1 ml min^{-1}.

2.2.2. Validation study

Chromatographic analysis was performed using Waters instruments: an HPLC pump Model 515, an automatic injector Model 717 and a Fluorescence Detector, Model 474 with Millenium software. The analytical column used in the analysis of sample extracts was a Symmetry C_{18}, 5 μm (4.6×150) column (Waters) with a Symmetry precolumn.

Quinolones were analyzed in two different groups:

(A) Ciprofloxacin, enrofloxacin and sarafloxacin: Mobile phase: acetonitrile–0.02 *M* phosphate buffer pH 3.0 (18:82), flow-rate: 1 ml min^{-1}. Fluorescence detector: Excitation wavelength: 280 nm, Emission wavelength: 450 nm.

(B) Oxolinic acid and flumequine. Mobile phase: acetonitrile–0.02 *M* phosphate buffer pH 3.0 (34:66), flow-rate: 1 ml min^{-1}. Fluorescence detector: Excitation wavelength: 312 nm, Emission wavelength: 366 nm

2.3. Standard solutions

Standard solutions of each of the quinolones were prepared dissolving 100 mg in 100 ml metanol+0.02 *M* sodium hydroxide (1 mg ml^{-1}) and stored in the dark at 0–4 °C. Working solutions of these solutions were made by dilution in 0.05 *M* potassium phosphate buffer of pH 7.4, obtaining working solutions of concentrations ranging from 0.02 till 1.2 μg ml^{-1}.

2.4. Sample preparation

Fish and pork tissues were kept at −20 °C, till analysis. After thawing, fish muscle was separated from skin and bones and pork muscle was cleaned from fat and homogenized in the Moulinex homogenizer.

2.5. Clean up procedure

Subsequently, 2.0 g of homogenized sample are transferred to a polypropylene tube and thoroughly mixed during 10 min with 10 ml 0.05 *M* phosphate buffer pH 7.4, using an Ultrasonic bath. After centrifugation (10 min, 4000×*g*), the supernatant is transferred to a clean second tube and the extraction is repeated. The joint extracts are filtered through 0.8 μm 25 mm Millex filters and 15 ml of the filtrate is applied to a previously activated (3 ml methanol and 3 ml water) Discovery DSC-18 cartridge, avoiding dryness. After sample transfer, the cartridge is washed with 3 ml of water and the quinolones are eluted, in previously weighed reagent tubes, with 5 ml of a mixture of methanol and 30% ammonium hydroxide (75:25, v/v) The eluate is evaporated under a stream of nitrogen at 40 °C, avoiding dryness. Then, 0.05 *M* phosphate buffer pH 7.4 is added to the wet residue till a total content of 1 g. The suspension is mixed well and filtered through a 0.45 μm 25 mm nylon Millex filter and 20 μl of this solution is injected in the HPLC system.

2.6. Validation

Calibration curves and recovery studies were performed and evaluated to validate the whole procedure. Detection capability was calculated from the obtained results.

3. Results and discussion

3.1. Optimization of HPLC conditions

A general problem of HPLC analysis of basic and polar substances, is the severe peak broadening and tailing on reversed-phase columns, due to specific interactions of the bases with the support. The two best-known antimicrobial fluoroquinolones are enrofloxacin and its main metabolite ciprofloxacin. They are ampholytic compounds, and as generally is well-known, this kind of substances may give tailing peaks in reversed-phase chromatography. Residual sylanol groups and metal impurities in traditional phase columns, as Novapak C_{18} (Waters) or Hypersil ODS (TracerAnalysis) column materials are known to be the cause of this tailing in reversed-phase liquid chromatography, which makes it impossible to achieve chromatographic separations suitable for residue analysis with fulfilment of requirements as short retention times, adequate resolution and peak shapes. This behaviour can be reduced by means of mobile phases with high acidity and ionic strength and/or using ion-paired techniques, which may rapidly deteriorate the packing [19] and as can be seen in Fig. 2, where variation of sodium dodecylsulfate concentration in the mobile phase yields quite different peak shapes on C_{18} columns. Only rather high concentrations of SDS allow chromatograms with acceptable peaks.

Highly purified Silica with a low content of metal impurities has recently been developed as packing material for HPLC columns, especially designed for the chromatography of polar and basic compounds. Some of these ''base deactivated'' or ''high purity'' columns, as the Symmetry C_{18} and the Supelcosil ABZ Plus columns, can improve peak shape, de-

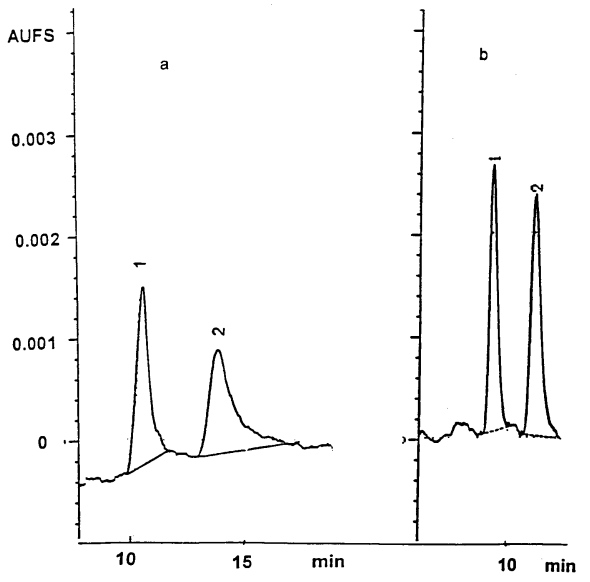

Fig. 2. Influence of variation of the counter-ion (SDS) concentration in the mobile phase on peak shapes of enrofloxacin (1) and ciprofloxacin (2). Column: Novapak C_{18}5μ. (a) Mobile phase: acetonitrile – 0.01 M Sodium dodecylsulfate (50:50), pH 3.5. (b) Mobile phase: acetonitrile – 0.04 M Sodium dodecylsulfate (50:50), pH 3.5.

Table 1
Comparison of the influence of pH and ionic strength variation in the mobile phase on retention times of enrofloxacin and ciprofloxacin on two different analytical columns: a Symmetry C_{18}, 5 μ (4.6×150) column (Waters) and a Supelcosil ABZ Plus (4.6×150) column (Supelco)

Parameter	Symmetry C_{18}				Supelcosil ABZ-Plus		
	pH	Minutes			Minutes		Diff
		RT Cipro	RT Enro	Diff.	RT Cipro	RT Enro	
	2.5	6.4	9.0	2.6	8.2	9.6	1.4
Buffer pH	3.0	6.3	8.9	2.6	8.4	9.8	1.4
Constant ionic strength	3.5	7.1	10.3	3.2	9.1	10.8	1.7
of 20 m*M*	4.0	7.6	11.5	3.9	8.9	11.0	2.1
	5.0	8.1	17.2	9.1	9.6	13.6	4.0
	m*M*						
m*M* Ionic strength	10	5.4	7.5	2.1	6.1	7.0	0.9
and constant pH 3.0	20	6.0	8.4	2.4	7.5	8.8	1.3
	50	6.1	8.7	2.6	8.6	10.4	1.8
	100	6.0	8.8	2.8	8.8	11.0	2.2

Mobile phase: acetonitrile–phosphate buffer (15:85).

pending on mobile phase composition. Since pH is one of the most powerful tools for optimization of the separation of analyte mixtures, columns delivering the same peak shape across a broad pH range (2.0 till 7.0) could be very useful to perform solvent optimization. Therefore, the chromatographic behaviour of ciprofloxacin and enrofloxacin on both columns was studied and compared.

The effect of variations in pH values and ionic strength of the aqueous component, phosphate buffer, with a constant percentage of the organic modifier (15%) of the mobile phase, is presented in Table 1, where the retention times of both compounds and their differences on both columns are shown.

When increasing the pH of the aqueous phase at constant ionic strength and buffer/acetonitrile ratio,

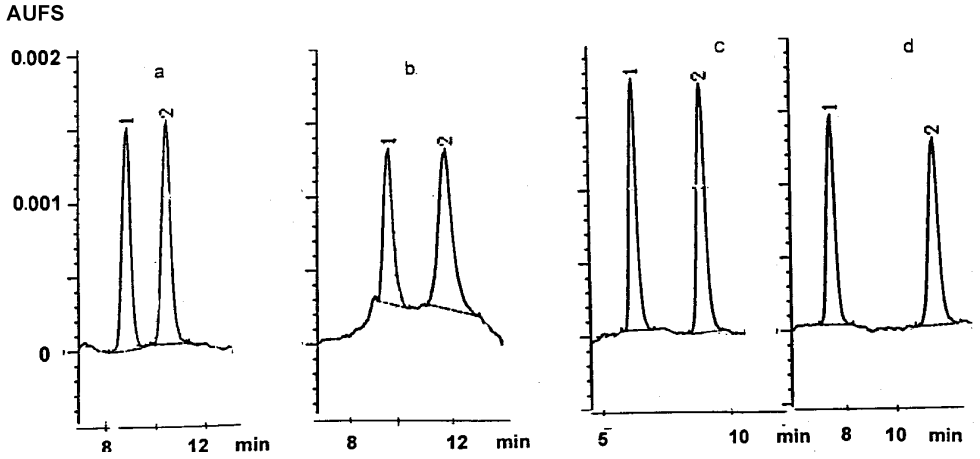

Fig. 3. Typical chromatograms obtained after injection of enrofloxacin (1) and ciprofloxacin (2). Influence of different pH values and different chromatographic columns: (a) and (b) pH 3 and pH 4, with a Supelcosil ABZ Plus (4.6×150) column. (c) and (d) pH 3 and pH 4, with a Symmetry C_{18}, 5 m (4.6×150) column. Mobile phase: acetonitrile – 0.02 M phosphate buffer (15:85).

enrofloxacin was more strongly retained than ciprofloxacin. This effect was quite clear in the Symmetry column where enrofloxacin in these conditions of pH 5.0 eluted at 17.2 min.

Variation of pH has less influence on peak shapes on a Symmetry column than on the Supelcosil column: no-tailing peaks were obtained over the whole range of tested pH values on the Symmetry column; however on the Supelcosil column enrofloxacin tails already at pH 3.5 (Fig. 3).

On the contrary, ionic strength of the buffer has little or no influence on retention times, nor on the resolution between the two compounds. Table 1 schematically presents the results obtained in this separation study.

The effect of differences in percentage of acetonitrile as organic modifier with constant ionic strength of phosphate buffer (20 m*M*, pH 3) as the aqueous component of the mobile phase upon separation was studied. At constant pH and ionic strength, retention time of both quinolones appeared to be very sensitive to slight variations of the acetonitrile content. Results obtained with both columns are presented in Fig. 4a and b. Fig. 4a shows the effect of the proportion of acetonitrile in the mobile phase on the retention time of ciprofloxacin and enrofloxacin standards on a Supelcosil ABZ Plus column. At organic modifier concentrations higher than 20%, the drugs eluted at very low retention times: lower than 4 min.

In sample extracts, the front together with matrix interferences may also elute till 4 min. However, proportions of acetonitrile lower than 10% in the mobile phase yielded analysis times of more than 33 min. Best separations were achieved with acetonitrile and 0.02 *M* phosphate buffer at pH 3.0 in a 15/85 (v/v) mixture, with retention times for ciprofloxacin between 7.9 and 8.3 and for enrofloxacin between 9.4 and 11.0 min.

The same experiment was performed with a Symmetry C_{18} column and results are presented in Fig. 4b. At constant pH and ionic strength, retention times of quinolones appeared to be very sensitive to slight variations of the acetonitrile content; similar behaviour occurs on the ABZ Plus column. As can be observed comparing both figures, resolution is better with the Symmetry column (see also Table 1). Even at a low concentration of acetonitrile (10%) in the mobile phase and rather high retention times (23 and 38 min), these quinolones eluted with sharp peak shapes on this column.

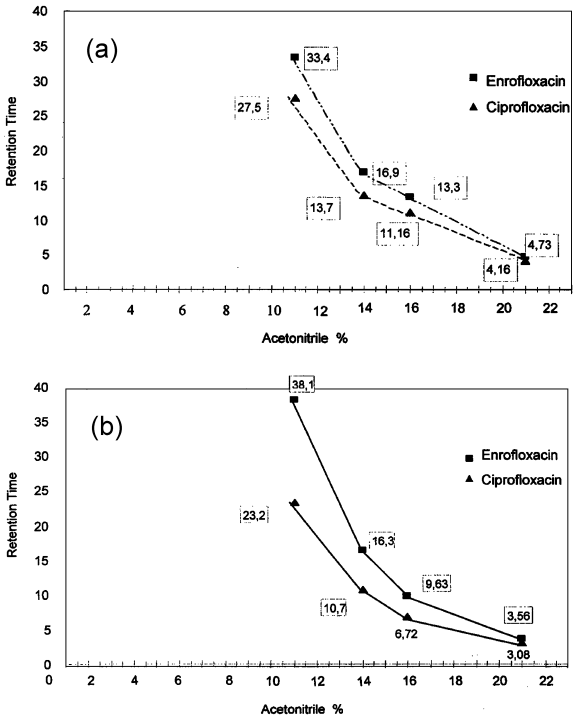

Fig. 4. (a) Effect of the proportion of acetonitrile in the mobile phase (0.02 M phosphate buffer pH 3) on the retention time of Ciprofloxacin (▲) and Enrofloxacin (■) with Supelcosil ABZ+ Plus Column. (150×46 mm, 5 μm) at a flow rate of 1 ml/min and detection at 278 nm. (b) Effect of the proportion of acetonitrile in the mobile phase (0.02 M phosphate buffer pH 3) on the retention time of Ciprofloxacin (▲) and Enrofloxacin (■) with a symmetry C_{18} (150×46 mm, 5 μm) at a flow rate of 1 ml/min and detection at 278 nm.

As conclusion of these results, best separation of enrofloxacin and ciprofloxacin can be achieved with the following selected conditions:

- Symmetry C_{18} column with acetonitrile–0.02 *M* phosphate buffer pH 3.5 (15/85), although any concentration higher than 0.01 *M* and pH values between 2.5 and 4.0 may be used. The Supelcosil column was not used in further studies.

Once a good separation of the most difficult compounds was achieved, optimization of the chromatographic conditions and detection mode for all

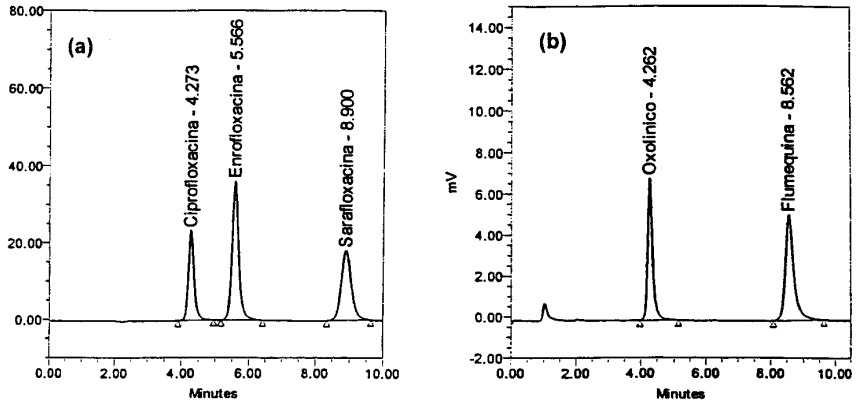

Fig. 5. Typical chromatograms of the five quinolones in the two different chromatographic systems. (a) Ciprofloxacin, enrofloxacin and sarafloxacin: Mobile phase: acetonitrile – 0.02 M phosphate buffer pH 3.0 (18:82). Flow rate: 1 ml min^{-1}. Fluorescence detector: Excitation wavelength: 280 nm, Emission wavelength: 450 nm. (b) Oxolinic acid and flumequine: Mobile phase: acetonitrile – 0.02 M phosphate buffer pH 3.0 (34:66). Flow rate: 1 ml min^{-1}. Fluorescence detector: Excitation wavelength: 312 nm, Emission wavelength: 366 nm.

five quinolones was performed. The best system appeared to be the one described in Section 2.2.

The corresponding chromatograms of both systems are presented in Fig. 5: ciprofloxacin, enrofloxacin and sarafloxacin standards in Fig. 5a and oxolinic acid and flumequin standards in Fig. 5b.

Typical chromatograms of blank salmon tissue (Fig. 6a) and tissue spiked (Fig. 6b) with oxolinic acid (60 ng g^{-1}) and flumequine (60 ng g^{-1}) and of blank pork tissue (Fig. 7a) and pork tissue spiked (Fig. 7b) with enrofloxacin (20 ng g^{-1}), ciprofloxacin (20 ng g^{-1}) and sarafloxacin (40 ng g^{-1}) are shown in Figs. 6 and 7, respectively.

3.2. Validation results

The five quinolones studied had good linear fluorescence response within the range of 0.4 to 12 ng injected amount: correlation coefficients (r) were higher than 0.9993 for all of them. Different extraction and solid-phase clean-up combinations were assayed to develop this part of the analytical method

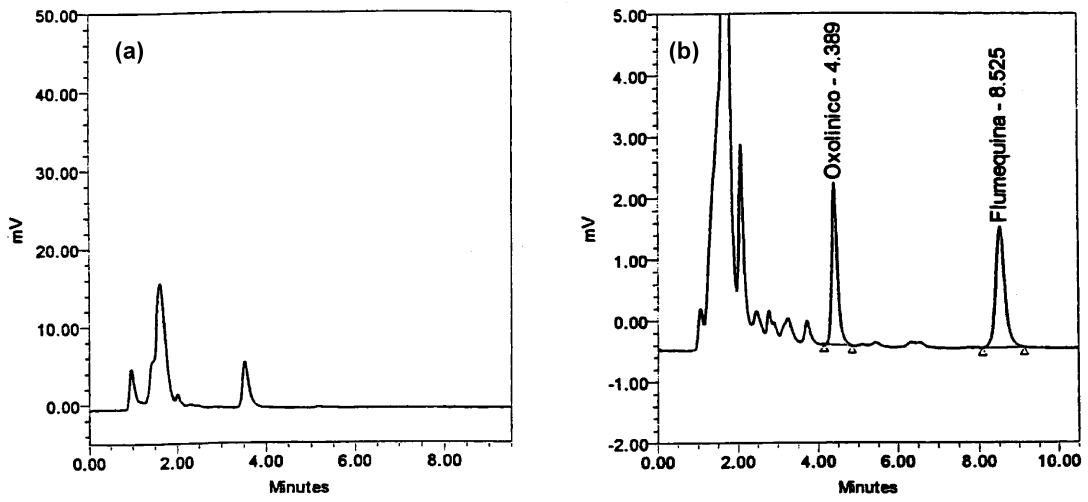

Fig. 6. Typical chromatograms of blank salmon muscle tissue (a) and salmon muscle tissue spiked (b) with oxolinic acid (60 ng g^{-1}) and flumequine (60 ng g^{-1}).

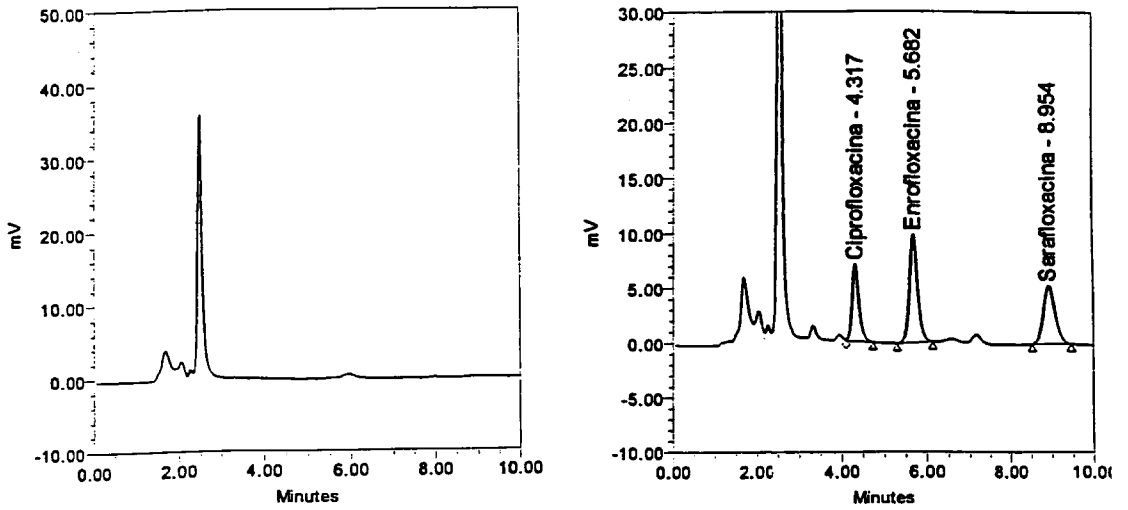

Fig. 7. Typical chromatograms of blank pork muscle tissue (a) and pork muscle tissue spiked (b) with enrofloxacin (20 ng g^{-1}), ciprofloxacin (20 ng g^{-1}) and sarafloxacin (40 ng g^{-1}).

Table 2
Recoveries of oxolinic acid and flumequine in salmon muscle tissue at different spiked levels, after application of the proposed analytical method

Spiked level	Mean recoveries (n=4, each spiked level)			
	Oxolinic acid (%)	C.V. (%)	Flumequine (%)	C.V. (%)
50 ppb	92.8	4.5	95.2	5.5
150 ppb	90.1	4.4	92.7	6.0
250 ppb	85.5	3.6	86.9	3.6
500 ppb	89.2	4.9	90.1	4.8

and the best recoveries were obtained with the described (2.5) extraction and clean-up mode. Results of these assays are presented in Tables 2 and 3, where the recoveries, standard deviations and coefficients of variation (C.V.) obtained after validation of the analytical method in salmon tissue and pork muscle are summarized.

As a conclusion of the obtained results, the limit

Table 3
Mean recoveries of enrofloxacin, ciprofloxacin and sarafloxacin at different spiked levels in pork muscle tissue after application of the proposed analytical method (n=6)

Spiked level (ppb)	Ciprofloxacin	C.V. (%)	Enrofloxacin	C.V. (%)	Oxolinic acid	C.V. (%)	Flumequine	C.V. (%)	Spiked level (ppb)	Sarafloxacin	C.V. (%)
20	83.8	8.3	83.0	10.6	75.4	14.1	86.4	8.8	40	78.4	5.6
50	84.0	7.0	81.5	12.1	78.0	4.8	84.9	5.2	100	84.4	7.6
150	81.8	3.7	81.2	4.3	73.4	4.0	79.4	4.1	300	82.8	4.1
300	84.6	3.4	84.2	4.6	76.4	1.3	81.1	1.7	600	83.4	2.3

of quantification of these quinolones, expressed as the lowest tested level with acceptable C.V. was 20 ng g^{-1} for all quinolones, except for sarafloxacin, which yielded a 40 ng g^{-1} limit of quantification.

4. Conclusions

The analytical method proposed in this work includes a single extraction and clean-up procedure for five different quinolones. Chromatographic conditions as a selected combination of column and mobile phase avoids the use of complexing agents or high salt concentrations, which could rapidly deteriorate the packing of HPLC columns.

The procedure is rapid and allows the determination of the residues of these compounds in different matrixes and with high sensitivity.

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