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Journal of Chromatography A, 1034 (2004) 25–32
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Multiresidue determination of fluoroquinolones in milk by column liquid chromatography with fluorescence and ultraviolet absorbance detection

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Received 7 October 2003; received in revised form 3 February 2004; accepted 3 February 2004

Abstract

Column liquid chromatography with fluorescence (FLD) and UV-diode array detection (UV-DAD) was used for the simultaneous determination of ciprofloxacin (CIPRO), enrofloxacin (ENRO), marbofloxacin (MARBO), danofloxacin (DANO) and sarafloxacin (SARA) residues in milk, using norfloxacin (NOR) as internal standard. Two solid-phase extraction (SPE) cartridges, were evaluated for sample clean-up and preconcentration, Strata X, based on a modified styrene–divinylbenzene polymer, and Strata Screen A, a mixed anion exchanger/C_8 reversed-phase sorbent. The fluoroquinolones (FQs) were separated on a polar endcapped column (AQUA^{TM} C_{18}). The recoveries for raw milk spiked with the antibiotics at three concentrations close to the maximum residue limit (MRL), were 80–103% for ENRO, CIPRO and DANO, with relative standard deviations (R.S.D.) lower than 6.6%. SARA recoveries were 70% (R.S.D. = 7%) and values in the order of 95% (R.S.D. = 1.5%) were obtained for MARBO at the MRL level. The quantification limits ranged from 2.4 to 10 ng ml^{-1} and are below the MRL established for these drugs by the European Union. The method was successfully applied to the analysis of ENRO and its metabolite CIPRO in an incurred milk sample.

Keywords: Solid-phase extraction; Milk; Food analysis; Antibiotics; Fluoroquinolones

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 [1–4]. The misuse of these pharmaceuticals may give rise to public health (e.g. allergic reactions, antibiotic resistance, etc.), environmental and industrial (e.g. cheese or yoghurt production, etc.) problems and, in agreement with the Council Directive 96/23/EC, the European Union (EU) countries must monitor the presence of these drugs and other veterinary residues in live animals and animal products [5]. Fluoroquinolones (FQs) are synthetic antibiotics with a broad-spectrum antibacterial activity [6,7]. They were introduced for human use in Europe and the US in the mid-1980s and approved for livestock treatment in the mid-1990s [8]. Several FQs (e.g. enrofloxacin (ENRO), danofloxacin (DANO), marbofloxacin (MARBO) and sarafloxacin (SARA)) have been specifically developed for veterinarian applications [9], while others like ciprofloxacin (CIPRO) and norfloxacin (NOR) are restricted to human treatment. DANO, CIPRO, SARA, ENRO and MARBO have been included in the EU Council Regulation 2377/90, which establishes maximum residue limits (MRLs) of veterinary medicinal products in foodstuffs of animal origin [10].

Current methods of FQ analysis in biological matrices are based on liquid chromatography (LC), mainly with fluorescence [11–13], ultraviolet (UV) [14,15] or mass spectrometric (MS) detection [16,17]. Various types of stationary phases (reversed-phase [15,18], polymer or phenyl columns [12,13,19,20]) and mobile phases (changes in ionic strength, acidity and/or presence of modifiers such as citric acid, perchloric acid or tertiary amines) have been used. Limitations include the analysis of only one or two FQs, relatively low recoveries, tedious sample preparation, and/or the incomplete separation of all FQs in one run [21,22]. Other non-routine techniques such as terbium(III)-sensitised luminescence [23,24], capillary isotachophoresis [25,26] or immunoaffinity chromatography [27] have also been applied. The analysis of the antimicrobials in bovine, porcine and

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doi:10.1016/j.chroma.2004.02.022

poultry tissue and fish has been studied [17,20,22–24,28]. However, only a few methods have focused on the determination of FQ residues in milk [14,19,20]. Cinquina et al. [14] determined ENRO and its metabolite CIPRO, in goat milk by LC–UV detection, combined with LC–MS for confirmation; the quantification limits were 20 ng ml^{-1} for both analytes. Holtzapple et al. [19] used on-line immunoaffinity extraction for sample clean-up and preconcentration, and reported the contamination of the analytical column after 15–20 milk sample injections. The procedure of Roybal et al. [20] required a complex milk extraction method and the need of periodic column regeneration due to the reaction of the milk proteins with the stationary phase.

This paper reports the development of a selective and sensitive LC method with fluorescent (FLD) and UV-diode array detection (UV-DAD) for the analysis of FQ residues in milk. We have focused on the analysis of MARBO, DANO and ENRO, which are administered to milk-producing animals, and CIPRO and NOR which are exclusively applied in human medicine. A new polar endcapped column (AQUA^{TM}) was used to separate the FQs. Several sample pretreatment procedures and newly marketed solid-phase extraction (SPE) cartridges have been evaluated for milk clean-up and preconcentration. The method was successfully applied to the analysis of ENRO and its metabolite CIPRO in an incurred milk sample.

2. Experimental

2.1. Reagents and materials

Acetonitrile and methanol (HPLC-grade) were provided by SDS (Peypin, France) and orthophosphoric acid (HPLC-grade, 85%) from Fluka (Buchs, Switzerland). Trichloroacetic acid (TCA) from Riedel de Haën (Seelze, Germany), ammonium chloride and sodium hydroxide from Merck (Darmstadt, Germany), glacial acetic acid from Panreac (Barcelona, Spain), sodium monohydrogenphosphate and sodium dihydrogenphosphate monohydrate from Aldrich (Steinheim, Germany) were all analytical grade reagents. Ciprofloxacin hydrochloride (99.8%) and enrofloxacin (99.7%) standards were a gift of Bayer AG (Leverkusen, Germany), sarafloxacin hydrochloride (90%) was a gift from Fort Dodge Veterinaria S.A. (Girona, Spain), marbofloxacin (98%) from Vétoquinol (Madrid, Spain) and danofloxacin methanesulphonate (75.1%) was supplied by Pfizer S.A. (Groton, CT, USA). Three compounds were tested as possible internal standards: tosufloxacin tosilate (TOSU, 99.4%) from Abbott Laboratories S.A. (Madrid, Spain), norfloxacin and lomefloxacin hydrochloride (LOME) from Sigma (St. Louis, MO, USA). Chemical structures of the FQs included in this study are shown in Fig. 1.

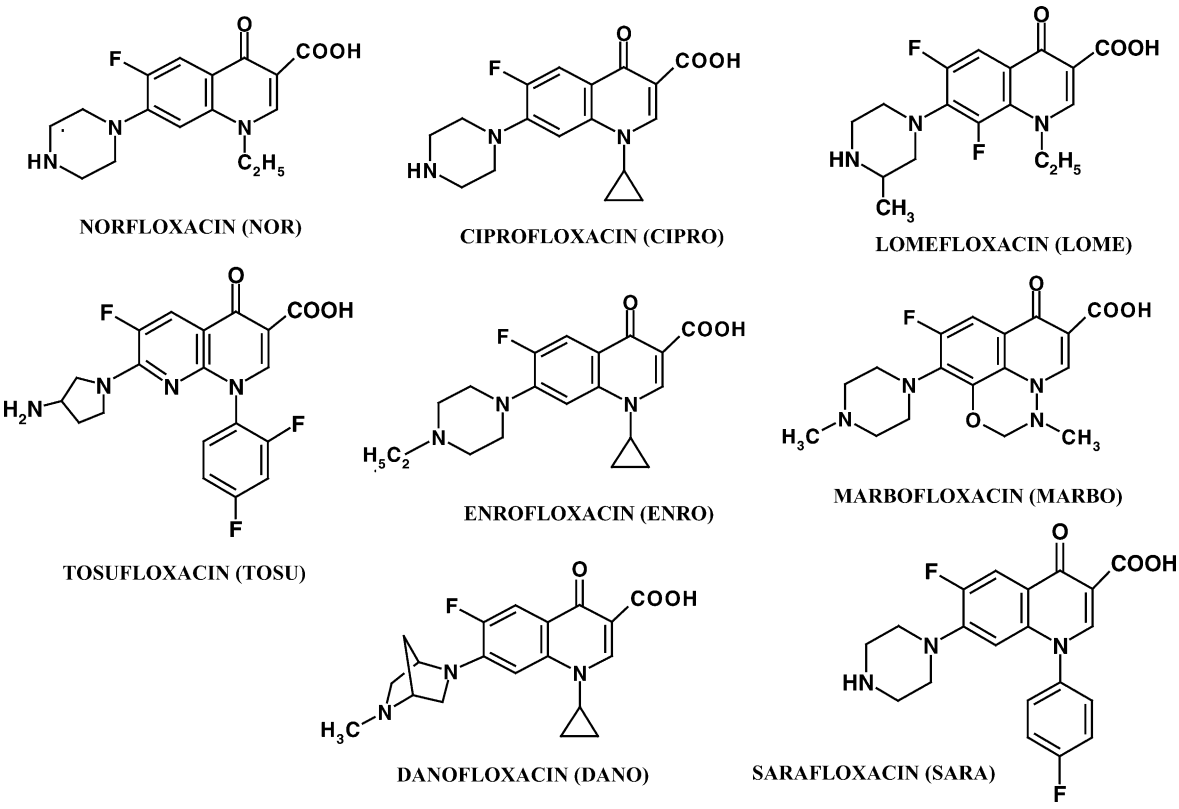

Fig. 1. Chemical structure and acronyms of the studied fluoroquinolones (human use: norfloxacin (NOR), ciprofloxacin (CIPRO), lomefloxacin (LOME) and tosufloxacin (TOSU); veterinarian use: enrofloxacin (ENRO), marbofloxacin (MARBO), danofloxacin (DANO) and sarafloxacin (SARA)). NOR has been used as internal standard.

Water was purified with a Milli-Q system (Millipore, Bedford, MA, USA). All solutions prepared for HPLC were passed through a 0.45 μm nylon filter before use.

Two different SPE disposable cartridges were tested: Strata X (surface-modified styrene–divinylbenzene polymer, 200 mg, 6 ml) and Strata Screen A (mixed C_8/anion exchange, 200 mg, 3 ml) from Phenomenex (Torrance, CA, USA). Centricon Plus-20® (polyethersulphone, 5 kDa MWCO membrane) and Amicon Ultra-4® (regenerated cellulose, 10 kDa MWCO membrane) centrifugal filter devices were kindly provided by Millipore.

2.2. Instrumentation

A vortex mixer IKA®-MS2 Minishaker (IKA Labortechnik, Staufen, Germany) was used to mix and homogenise milk samples during pretreatment. For milk defatting and protein removal an ultracentrifuge with cooling system (Eppendorf-5804R, Hamburg, Germany) was employed. The pH of the buffer solutions and samples was adjusted with an ORION 710A pH/ISE meter (Beverly, MA, USA). Solid-phase extraction was carried out on a VacElut vacuum manifold for 20 cartridges (Varian, Palo Alto, CA, USA). The chromatographic system consisted of a HP-1100 series high performance liquid chromatograph from Agilent Technologies (Palo Alto, CA, USA) equipped with a quaternary pump, on-line degasser, autosampler, automatic injector, column heater, and diode array and fluorescence detectors connected on-line.

2.3. Chromatographic conditions

Chromatographic separation of the FQs was performed on an AQUA™ C_{18} (polar endcapped) (250 mm × 4.6 mm, 5 μm) column protected by a RP18 guard column (4.0 mm × 3.0 mm, 5 μm), both from Phenomenex. A gradient programme was used with the mobile phase, combining solvent A (25 mM orthophosphoric acid adjusted to pH 3.0 with NaOH) and solvent B (acetonitrile) as follows: 18% B (4 min), 18–37% B (8 min), 37% B (1 min), 37–18% B (2 min), 18% B (3 min). The flow rate was 1.0 ml min^{-1}, the injection volume 25 μl and the column temperature was maintained at 25 °C. The absorbance detector was set at 298 nm for marbofloxacin detection, while the fluorescence excitation/emission wavelengths were 280/440 nm for the analysis of the remaining FQs.

2.4. Standard solutions

Fluoroquinolone individual stock solutions were prepared in methanol at a concentration of 100 μg ml^{-1}, taking into account the purity of the standards. These solutions were stored at 4 °C in the dark for not longer than 2 months. Working 5 μg ml^{-1} solutions were prepared daily using methanol as diluent. For quantitation, matrix-matched calibration standards were prepared in triplicate at six concentrations, 2–200 ng ml^{-1} for ENRO, CIPRO and SARA, 2–130 ng ml^{-1} for DANO, and 10–200 ng ml^{-1} for MARBO. NOR at a concentration of 150 ng ml^{-1} was used as internal standard. For the recovery experiments, control milk aliquots (5 g) were spiked with increasing amounts of the selected FQ standard solutions. The fortification levels were: 50–150 mg kg^{-1} of milk for ENRO, CIPRO and SARA; 15–50 mg kg^{-1} for DANO; 50–125 mg kg^{-1} for MARBO.

2.5. Sample preparation

Two different procedures were tested:

(a) *Milk extraction/deproteinisation using trichloroacetic acid*: Aliquots (5 g) of pasteurised whole milk were accurately weighed into 50 ml polypropylene centrifuge tubes and fortified with the FQs. Samples were shaken on a vortex mixer for 30 s and then allowed to stand at 4 °C in the dark, for at least 30 min, to enable sufficient equilibration with the milk matrix. After addition of 2.5 ml of trichloroacetic acid (20% in methanol), the samples were shaken again for 30 s and centrifuged at 13,800 × *g* for 30 min at 10 °C. Ammonium chloride buffer (12.5 ml, 50 mM, pH 9.0) was added and the mixture centrifuged again at 13,800 × *g* for 30 min at 10 °C. The clear supernatant was carefully removed with the aid of a Pasteur pipette and further purified by solid-phase extraction, using Strata X or Strata Screen A cartridges, previously conditioned with methanol (twice with 3 ml) and ammonium chloride buffer (twice with 3 ml, 50 mM, pH 9.0). Prior to elution of the FQs, the cartridges were rinsed with 3 ml of 5% methanol in water and 2 ml of 5% methanol in ammonium chloride buffer (50 mM, pH 9.0), respectively. The antibiotics were eluted from both columns using twice 1 ml of 4% orthophosphoric acid in methanol, and 25 μl of the eluates were injected into the HPLC system.

(b) *Milk extraction/deproteinisation by ultrafiltration*: Portions of milk (4 g) were weighed into 50 ml polypropylene centrifuge tubes and fortified with the desired amount of FQs. Samples were vortex-mixed for 30 s, stored in the dark at 4 °C for around 30 min and finally centrifuged at 13,800 × *g* for 30 min at 10 °C for fat removal. The supernatant was carefully removed with a Pasteur pipette and transferred into Centricon Plus-20® or Amicon Ultra-4® centrifugal filter devices. Centrifugation was performed following the manufacturer's recommendations at 4000 × *g* for 60 min at 10 °C. Finally, the ultrafiltrates (∼2 ml) were set to a final volume of 5 ml with Milli-Q water and 25 μl were directly injected into the HPLC.

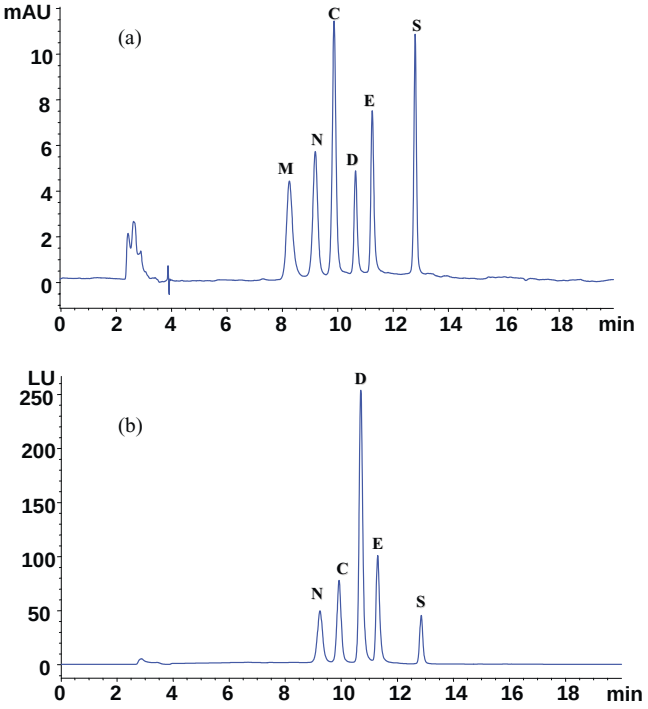

Fig. 2. Typical chromatograms of a six FQs standard mixture with: (a) UV detection; (b) fluorescence detection. M: marbofloxacin; N: norfloxacin; C: ciprofloxacin; D: danofloxacin; E: enrofloxacin; S: sarafloxacin. Column: AQUA^{TM} C_{18}. Mobile phase: 25 mM orthophosphoric acid (pH 3.0)–acetonitrile (gradient elution). Flow rate: 1 ml min^{-1}. Fluorescence detection: excitation at 280 nm, emission at 440 nm. The other chromatographic parameters are described in Section 2.

3. Results and discussion

3.1. Optimisation of the HPLC conditions

A new AQUA^{TM} C_{18} polar-endcapped column and gradient elution was used for FQ separation. Typical chromatograms corresponding to a standard mixture of the selected antimicrobials, using both fluorescence and/or UV detection, are shown in Fig. 2. The optimised HPLC conditions are described in Section 2.3. The R_s values [29] for the six FQs on the AQUA^{TM} column were in all cases higher than 2.0. Unlike conventional C_{18} columns, AQUA^{TM} endcapped with hydrophilic (polar) functional groups enable separation of polar compounds in highly aqueous mobile phases, allowing a fast column equilibration for the gradient analysis. A good sensitivity was observed using fluorescence detection for all the FQs, except for MARBO that was better monitored by its absorbance at 298 nm.

Three different FQs have been tested as potential internal standards for HPLC quantification: NOR, LOME and TOSU (see Fig. 1). The best results were obtained with NOR, which was efficiently extracted from milk (82 ± 1%). Moreover, this antibiotic can be detected with higher sensitivity than LOME and TOSU and does not coelute with any of the evaluated FQs.

Table 1
Recovery values (%) obtained after different milk extraction procedures

Compound	Treatment 1[a]	Treatment 2[b]	Treatment 3[c]
MARBO	77	49	42
NOR	86	36	43
CIPRO	78	33	42
DANO	90	28	38
ENRO	82	23	26
SARA	72	33	31

The total spiked amount of each fluoroquinolone was 2.5 μg.
[a] TCA (20%) in MeOH. Sample amount 5 g ($n = 4$, R.S.D. <8%).
[b] Ultrafiltration (regenerated cellulose membranes, 10 kDa MWCO). Sample amount 4 g ($n = 3$, R.S.D. <7%).
[c] Ultrafiltration (polyethersulphone membranes, 5 kDa MWCO). Sample amount 4 g ($n = 3$, R.S.D. <7%).

3.2. Selection of the milk extraction procedure

Traditional extraction strategies for antibiotics in milk involve precipitation of proteins with organic solvents (e.g. methanol, acetonitrile), alone or in combination with strong inorganic or trichloroacetic acids, as well as ultrafiltration with molecular mass cut-off filters [30,31]. We have compared two extraction procedures: precipitation with 20% trichloroacetic acid in methanol and ultrafiltration with molecular mass cut-off filters, of regenerated cellulose or polyethersulphone. Recoveries were calculated by comparison with standards prepared in control matrix extracts spiked with the adequate amount of the FQs (2.5 μg for each FQ). Two different milk brands were tested in combination with the TCA treatment. As it is shown in Table 1, precipitation with TCA led to significantly higher recovery than those obtained with the molecular mass cut-off filters. This finding could be attributed to the binding of the FQs (20–76% [32]) to the milk proteins and/or to the partial adsorption of the FQs onto the filter membranes. When aqueous standard solutions of the FQs were passed through the filters (see Section 2.5 for details) very poor recoveries were obtained in all cases, ranging from 2.0 to 54 and 9.0 to 50% for regenerated cellulose and polyethersulphone membranes, respectively. The high adsorption of the antibiotics onto the filter material prevents their use for this application and the procedure using trichloroacetic acid was selected for method development.

3.3. SPE optimisation

Two different SPE cartridges have been tested for sample clean-up and preconcentration: (a) Strata X, a modified styrene–divinylbenzene polymer suitable for a wide range of basic, neutral and acidic compounds; and (b) Strata Screen A, a mixed mode silica based sorbent (C_8 and anion exchanger), recommended for the extraction of acidic compounds. The parameters evaluated for the optimisation of the SPE procedure were: pH of the sample, composition and volume of the eluting solution, and the breakthrough volume of the SPE cartridges.

Table 2
Extraction recoveries for different sample volumes using Strata Screen A and Strata X cartridges ($n = 3$)

Compound	Recovery (%)							
	50 ml		100 ml		250 ml		500 ml	
	Strata Screen A[a]	Strata X[b]	Strata Screen A[c]	Strata X[d]	Strata Screen A[e]	Strata X[f]	Strata Screen A[g]	Strata X[h]
MARBO	105	106	111	105	95	102	46	90
NOR	99	94	95	95	62	97	35	94
CIPRO	102	113	118	100	76	100	39	105
ENRO	107	98	116	103	111	107	92	103
SARA	105	90	113	99	107	86	64	90

[a] R.S.D. = 1–4%.
[b] R.S.D. = 2–5%.
[c] R.S.D. = 1–7%.
[d] R.S.D. = 1–4%.
[e] R.S.D. = 1–8%.
[f] R.S.D. = 1–9%.
[g] R.S.D. = 2–6%.
[h] R.S.D. = 2–6%.

As was indicated previously, FQs can be present in aqueous solution as cationic, anionic or intermediate forms due to the presence of carboxylic group and the charged amino group of the piperazine moiety and their extraction will be pH dependent. The pK_{a1} values for the carboxylic function of FQs range from 5.5 to 6.0 and the pK_{a2} corresponding to the amino group, from 7.5 to 8.5 [33]. Buffered standard solutions (4.5–9.0) spiked with 2.5 μg of each FQ were passed through the cartridges, to evaluate the influence of the pH on the extraction efficiency. The Strata X cartridges showed recovery of 86–106%, regardless of the sample pH. However, for the Strata Screen A cartridges the best recoveries (85–107%) were obtained at pH 9.0. Under these conditions [33] the FQs are present in their basic form allowing both anion exchange and hydrophobic interactions with the sorbent. Several eluting mixtures containing acetic or phosphoric acids (2–5%) in methanol were evaluated. The amount and type of acid modifier did not affect the final recovery rates, but the antibiotics were eluted faster with increasing acidic strength, probably due to a better pH adjustment. Therefore, a 4% phosphoric acid solution in methanol was selected as the extraction eluent. Two elution volumes were assayed, 1 and 2 ml, and the latter gave better recoveries.

The breakthrough volume of the cartridges was calculated by extracting 50–500 ml of aqueous solutions spiked with 2.5 μg of each FQ. The recoveries and their R.S.D. are shown in Table 2. The Strata Screen A cartridges allowed good recoveries for sample volumes up to 100 ml, while for Strata X the breakthrough volume was not reached even when 500 ml were applied. This finding can be of great interest for the preconcentration of these compounds from waste water samples where they may be present at much lower concentrations than in milk.

3.4. Calibration and method performance

Six-point matrix-matched calibration curves were constructed by fortification of control milk extracts with each of the five FQs. The linear regression data and limits of detection (LODs) are given in Table 3. All values are below the MRLs for these antibiotics in EU Council Regulation 2377/90.

To evaluate the intra-day repeatability of the method blank milk samples, spiked at three concentrations (0.5 × MRL, MRL and 1.5 × MRL) were analysed. The results are summarised in Table 4. As no MRL has been set for SARA in milk, 100 μg kg^{-1} was selected as a reference value for this compound. Recoveries of 80–103% were obtained for ENRO, CIPRO and DANO at all fortification levels with R.S.D. lower than 6.6%. Mean recoveries of the IS at the spiked level (150 ng ml^{-1}) were 82 ± 1%. These results fulfil the requirements defined by EU legislation [34]. SARA was not so efficiently extracted (70 ± 7%). The lower polarity of this fluoroquinolone (see Fig. 1) affects its extraction

Table 3
Analytical characteristics of HPLC–FLD–UV of five FQs in milk

Compound	Tested range (ng ml^{-1})	Calibration equation[a]	R^2	LOD (ng ml^{-1})
MARBO	8.2–200	$(104 \pm 4) \times 10^{-5}C + (0.007 \pm 0.004)$	0.981	3
CIPRO	5.6–200	$(667 \pm 1) \times 10^{-5}C + (0.020 \pm 0.010)$	0.996	2
DANO	2.4–130	$(0.072 \pm 0.001)C - (0.18 \pm 0.09)$	0.996	0.5
ENRO	8.3–150	$(160 \pm 3) \times 10^{-4}C + (0.040 \pm 0.024)$	0.995	3
SARA	10.0–200	$(430 \pm 8) \times 10^{-5}C + (0.007 \pm 0.009)$	0.994	3

[a] Uncertainty values calculated at a 95% confidence limit; six data points ($n = 3$); C: FQ concentration (ng ml^{-1}).

Table 4
Comparison of intra-day recovery (%) and precision (R.S.D., %) obtained for the analysed FQs after solid-phase extraction of spiked milk samples, using different types of SPE cartridges ($n = 5$)

Compound	0.5 × MRL[a]		MRL[a]		1.5 × MRL[a]	
	STX[b]	STA[c]	STX[b]	STA[c]	STX[b]	STA[c]
MARBO	–	65 (4)	–	95 (5)	96 (5)	94 (2)
CIPRO	88 (3)	102 (2)	86 (6)	80 (7)	80 (6)	90 (2)
DANO	97 (3)	103 (5)	82 (2)	86 (5)	79 (3)	88 (3)
ENRO	86 (3)	82 (3)	83 (5)	81 (4)	76 (2)	88 (3)
SARA	72 (3)	61 (4)	66 (5)	71 (8)	71 (3)	76 (2)

NOR at 150 ng ml^{-1} has been applied as internal standard as indicated in the text.
[a] MRL: maximum residue level.
[b] Strata X cartridges.
[c] Strata Screen A cartridges.

with 20% TCA in methanol (Table 1). Good recoveries (95.0 ± 1.5%) were obtained for MARBO, at the MRL and 1.5 × MRL fortification levels. However, reliable quantitation was not possible at 0.5 × MRL, especially with the Strata X cartridges (Table 4), due to partial coelution of a matrix interference peak This interference was detected in different commercial fresh milk brands but no attempt at identification has been made.

To determine the inter-day repeatability using Strata Screen A cartridges, blank milk samples were spiked with the FQs at the corresponding MRLs and five analyses were performed on 3 days (Table 5). Recoveries of 71–95%, with R.S.D. of 2.0–8.0%, were obtained which demonstrate the good repeatability and precision of the optimised method.

3.5. Analysis of incurred milk samples

A milk sample from a cow with clinical mastitis was collected 24 h after the intramuscular administration of 40 ml of a solution containing 2 g of ENRO. The cow was being treated at the Veterinary Hospital (Faculty of Veterinary, Complutense University, Madrid) and the total collected milk amount was 40 ml.

Table 5
Inter-day recovery (%) and precision (R.S.D., %) data for the determination of FQs in milk samples ($n = 5$)

Compound	Day 1	Day 2	Day 3
MARBO	77 (7)	90 (5)	95 (5)
NOR (IS)	82 (3)	81 (2)	82 (2)
CIPRO	80 (3)	80 (6)	80 (7)
DANO	82 (2)	75 (6)	86 (5)
ENRO	87 (4)	79 (6)	81 (4)
SARA	78 (3)	71 (8)	73 (5)

Matrix-matched calibration curves were prepared using raw milk (from a non-treated healthy cow) fortified with ENRO and CIPRO at different concentration levels. Fig. 3 shows the chromatograms (fluorescent traces) of an extract of raw milk fortified with ENRO (100 μg kg^{-1}) and CIPRO (500 μg kg^{-1}) and an extract of the incurred milk sample. The incurred milk chromatogram showed several non-identified peaks at different retention times to those of the analytes. These peaks were not present in the raw milk extract and could be due to the low milk volume obtained for the treated cow. Analysis of the incurred milk sample, rendered concentrations of 57 μg kg^{-1} (R.S.D. = 5.6%, $n = 5$) and 705 μg kg^{-1} (R.S.D. = 1.0%, $n = 5$) for ENRO and CIPRO, respectively. These results show that 24 h after the treatment with ENRO the compound has been almost completely metabolised to CIPRO. This agrees with literature reports on the fast deethylation of ENRO to CIPRO in the living cow, and the longer elimination time of the latter antibiotic form the milk [35,36]. For validation purposes, aliquots (3 g, $n = 3$) of the incurred milk sample were fortified with

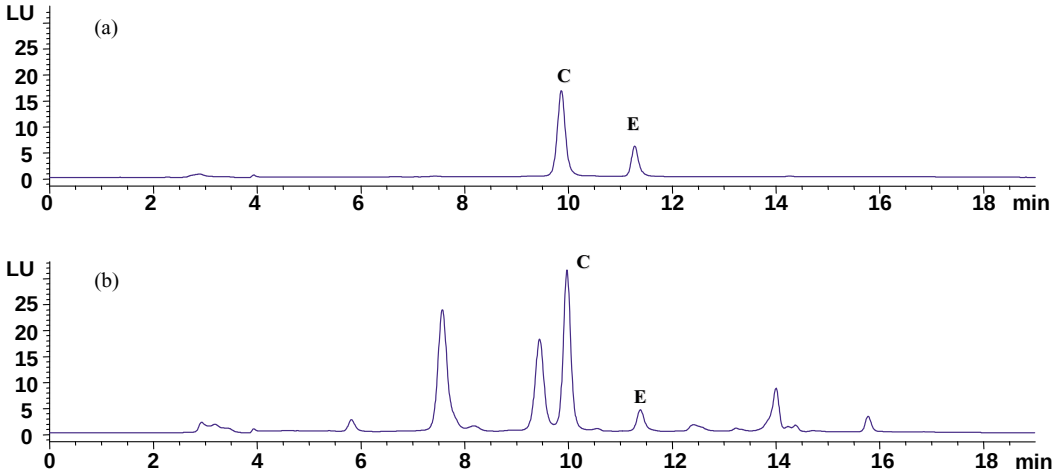

Fig. 3. Chromatograms with fluorescence detection of: (a) an extract of raw milk fortified with enrofloxacin (E) (100 μg kg^{-1}) and ciprofloxacin (C) (500 μg kg^{-1}); (b) an extract of an incurred milk sample. Mobile phase: 25 mM orthophosphoric acid (pH 3.0)–acetonitrile (gradient elution). Flow rate: 1 ml min^{-1}. Fluorescence detection: excitation at 280 nm, emission at 440 nm. The other chromatographic parameters are described in Section 2.

Table 6
Mean recoveries (%) of ENRO and CIPRO and relative standard deviations (R.S.D., %) for incurred raw milk samples, spiked at two different concentration levels ($n = 3$), after the application of the proposed analytical method

Compound	Added ($\mu g\,g^{-1}$)	Found ($\mu g\,g^{-1}$)	R.S.D. (%)	Recovery (%)
ENRO	0	0.06[a]	5.0	–
	0.20	0.25	7.3	96
	0.40	0.47	3.0	102
CIPRO	0	0.70[a]	1.0	–
	1.0	1.50	1.4	88
	2.0	2.60	1.0	97

[a] Mean of five determinations.

ENRO and CIPRO at two concentration levels (Table 6). According to the EU legislation the accuracy of a confirmatory method should be 80–110% for samples spiked at levels above 10 $\mu g\,kg^{-1}$ [28,37]. Recoveries were 88–102%, with R.S.D. of 1–7% which fulfils the legislation requirements.

4. Conclusions

This work describes a simple LC methodology for the quantification of five FQ antibiotics in milk, using preconcentration on novel mixed-phase extraction cartridges. The use of a polar endcapped C_{18} column enables separation of six FQs in a short time (13 min) without the need of ion-pair reagents. No column blocking problems were observed as described by other authors after the analysis of FQs in milk. The method is sensitive enough for milk analysis, with limits of quantification lower than the MRLs imposed by the EU Legislation. Excellent levels of accuracy and precision were obtained for ENRO, CIPRO, DANO and SARA, whereas MARBO was determined at concentrations in the order of its MRL value. The method was successfully applied to the analysis of ENRO and its metabolite CIPRO in an incurred milk sample. The optimised procedure could also be useful for the preconcentration of FQs in wastewater where they can be found at much lower concentrations. Further work is in progress to extend the developed methodology to other food matrices and water samples.

Acknowledgements

The authors are gratefully acknowledged to the Spanish Ministry of Science and Technology (Project No. BQU2002-04515-C02-01) for financial support. M.D.M. also thanks to the Spanish Ministry of Science and Technology for a Ramón y Cajal contract. We would like to acknowledge Bayer AG (Leverkusen, Germany), Pfizer S.A. (Groton, CT, USA), Abbott Laboratories S.A. (Madrid, Spain), Vétoquinol (Madrid, Spain) and Fort Dodge Veterinaria (Girona, Spain) for providing the reference compounds. We also thank Dr. Javier Blanco from the Faculty of Veterinary (U.C.M.) for providing us the incurred raw milk samples, Dr. Ma Eugenia de León González for helpful discussions and Dr. G. Orellana for reviewing the manuscript.

References

[1] The White Paper on Food Safety, European Commission, Brussels, 2000, http://europa.eu.in/comm/dgs/health_consumer/library/pub/pb06_en.pdf.
[2] D.C. Hooper, J.S. Wolfson, Quinolone Antimicrobial Agents, 2nd ed., American Society for Microbiology, Washington, DC, 1993.
[3] G. Pascal, H. Reichenbach, K. Jones, P. Courvalin, in: Proceedings of the Conférence Internationale sur la Résistence aux Substances Antimicrobiennes, European Commission, Brussels, 1999.
[4] H. Oka, H. Nakazawa, K. Harada, J.D. MacNeil (Eds.), Chemical Analysis for Antibiotics Used in Agriculture, AOAC International, Arlington, VA, 1995.
[5] Council Directive 96/23/EC on use of antibiotics in agriculture, Off. J. Eur. Commun. OJ L125, 23 April 1996.
[6] V.T. Andriole, The Quinolones, Academic Press, San Diego, CA, 1998.
[7] J. Kuhlmann, A. Dalhoff, H. Zeiler, Quinolone Antibacterials, Springer, Berlin, 1997.
[8] S.A. Anderson, R.W. Yeaton Woo, L.M. Crawford, Food Control 12 (2001) 13.
[9] L. Kaartinen, M. Salonen, L. Allí, S. Pyörälä, J. Vet. Pharmacol. Ther. 18 (1995) 357.
[10] Diario Oficial de las Comunidades Europeas (DOCE), Council Regulation No. 2377/90 L224, 991, 2601, 18 August 1990.
[11] P.S. Chu, R.C. Wang, H.V. Chu, J. Agric. Food Chem. 50 (2002) 4452.
[12] J.C. Yorke, P. Froc, J. Chromatogr. A 882 (2000) 63.
[13] G.Y. Eng, R.J. Maxwell, E. Cohen, E.G. Piotrowski, W. Fiddler, J. Chromatogr. A 799 (1998) 349.
[14] A.L. Cinquina, P. Roberti, L. Giannetti, F. Longo, R. Draisci, A. Fagiolo, N.R. Brizioli, J. Chromatogr. A 987 (2003) 221.
[15] P.G. Gigosos, P.R. Revesado, O. Cadahía, C.A. Fente, B.I. Vázquez, C.M. Franco, A. Cepeda, J. Chromatogr. A 871 (2000) 31.
[16] D.G. Kennedy, R.J. McCraken, A. Cannavan, S.A. Hewitt, J. Chromatogr. A 812 (1998) 77.
[17] B. Toussaint, G. Bordin, A. Janosi, A.R. Rodríguez, J. Chromatogr. A 976 (2002) 195.
[18] G. Carlucci, J. Chromatogr. A 812 (1998) 343.
[19] C.K. Holtzapple, S.A. Buckley, L.H. Stanker, J. AOAC Int. 82 (3) (1999) 607.
[20] J.E. Roybal, A.P. Pfenning, S.B. Turnipseed, C.C. Walker, J.A. Hurlbut, J. AOAC Int. 80 (5) (1997) 982.
[21] D.H. Wright, V.K. Herman, F.N. Konstantinides, J.C. Rotschafer, J. Chromatogr. B 709 (1998) 97.
[22] M.J. Schneider, J. Chromatogr. Sci. 39 (2001) 351.
[23] J.A. Hernández-Arteros, R. Compañó, M.D. Prat, Analyst 123 (1998) 2729.
[24] J.A. Hernández-Arteros, R. Compañó, R. Ferrer, M.D. Prat, Analyst 125 (2000) 1155.
[25] D. Barron, E. Jiménez-Lozano, S. Bailac, J. Barbosa, J. Chromatogr. B 767 (2002) 313.
[26] A. Hernández, C. Aguilar, F. Borrull, M. Calull, J. Chromatogr. B 772 (2002) 163.
[27] C.K. Holtzapple, S.A. Buckley, L.H. Stanker, J. Agric. Food Chem. 47 (1999) 2963.
[28] G. van Vyncht, A. Janosi, G. Bordin, B. Toussaint, G. Maghuin-Rogister, E.D. Pauw, A.R. Rodríguez, J. Chromatogr. A 952 (2002) 121.
[29] L.R. Snyder, J.J. Kirkland, J.L. Glajch (Eds.), Practical HPLC Method Development, Wiley/Interscience, New York, 1998.

[30] W.A. Moats, J. AOAC Int. 73 (3) (1990) 343.
[31] F.J. Schenck, P.S. Callery, J. Chromatogr. A 812 (1998) 99.
[32] H. Liang, M.B. Kays, K.M. Sowinski, J. Chromatogr. B 772 (2002) 53.
[33] D.A. Volmer, B. Mansoori, S.J. Locke, Anal. Chem. 69 (1997) 4143.
[34] Commission of the European Communities, Draft SANCO/1805/2000 Rev. 1, Laying down performance criteria thereof in live animals and animal products according to Council Directive 96/23/EC.
[35] L. Kaartinen, M. Salonen, L. Älli, S. Pyörälä, J. Vet. Pharmacol. Ther. 18 (1995) 357.
[36] C. Mellgren, A. Sternesjö, J. AOAC Int. 81 (2) (1998) 394.
[37] Commission Decision 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC, Concerning the performance of analytical methods and the interpretation of results, European Commission, Brussels.