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Determination and on-line clean-up of (fluoro)quinolones in bovine milk using column-switching liquid chromatography fluorescence detection

Clare Ho, Della W.M. Sin, Hubert P.O. Tang*, Lucy P.K. Chung, Sylvia M.P. Siu

Analytical and Advisory Services Division, Government Laboratory, 88 Chung Hau Street, Ho Man Tin, Kowloon, Hong Kong SAR, China

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Abstract

A simple, cost-effective, and high throughput method using on-line column-switching liquid chromatography fluorescence detection was developed and validated for analysing five (fluoro)quinolones (FQs)—enrofloxacin (ENRO), ciprofloxacin (CIPR), sarafloxacin (SARA), oxolinic acid (OXOL), and flumequine (FLUM) in bovine milk. Norfloxacin (NORF) and nalixidic acid (NALI) were used as internal standards. After simple deproteination of milk sample with 5% (w/v) metaphosphoric acid, the supernatant was subject to on-line column clean-up and direct analysis by LC–FLD. The extraction cartridge was prepared in-house by slurry packing with hydrophilic–lipophilic polymer sorbent. The accuracy of measurement for each (fluoro)quinolone at different maximum residue limits (MRL) was 101–103% (ENRO), 92.8–97.4% (CIPR), 89.8–92.8% (SARA), 116–121% (OXOL), and 81.3–85.5% (FLUM), whilst the precision was 2.9–6.1% (ENRO), 2.5–5.1% (CIPR), 2.3–5.0% (SARA), 3.1–5.9% (OXOL), and 5.6–6.5% (FLUM). The decision limits, detection capabilities, specificity and analytes stability during storage were also investigated.
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Keywords: Enrofloxacin; Ciprofloxacin; Sarafloxacin; Oxolinic acid; Flumequine; Fluoroquinolones; Quinolones; Milk; Column-switching

1. Introduction

Quinolones (e.g. oxolinic acid) have been demonstrated to be very effective antibiotics in treating various diseases in animal husbandry and aquaculture [1,2]. Their second generation drugs, fluoroquinolones, have been increasingly used in veterinary applications owing to enhancing antibacterial activities against Gram-positive and -negative organisms [3–5]. Due to their possible abuse or misuse that may lead to problems to public health, maximum residue limit (MRL) is set for enrofloxacin and ciprofloxacin in milk under the food-related regulations [6,7] in the Hong Kong Special Administrative Region (HKSAR).

Conventional methods for detecting (fluoro)quinolones in milk include liquid chromatography, with fluorescence detection [8–10,12], diode array detection combined with LC–MS technique [11], and ultra-violet detection [12]. Roybal et al. [8] used cation exchange solid phase extraction for sample clean-up of four fluoroquinolones in milk. Further development was done by Holtzapple et al. [9] with an automated on-line immunoaffinity extraction for these drugs. However, a decrease in resolution in chromatographic separation of some FQs was reported after 15–20 sample injections and column flushing was necessary to restore the column efficiency. Idowu et al. [10] described the procedure using liquid–liquid extraction for analysing enrofloxacin and ciprofloxacin in bovine milk. Similarly, Cinquina et al. [11] validated a method for determining enrofloxacin and ciprofloxacin in goat milk with off-line C18 cartridge clean-up. Marazuela et al. [12] used a modified styrene-divinylbenzene polymer sorbent for clean-up of five FQs in milk. Neither of these methods was fast nor robust enough for routine screening of large numbers of samples.

This paper reports the development of a fast and economical LC method with fluorescence detection using on-line extraction cartridge clean-up. In this approach, (fluoro)quinolones including enrofloxacin, ciprofloxacin,

* Corresponding author. Tel.: +852 2762 3749; fax: +852 2714 4083.
E-mail address: potang@govtlab.gov.hk (H.P.O. Tang).

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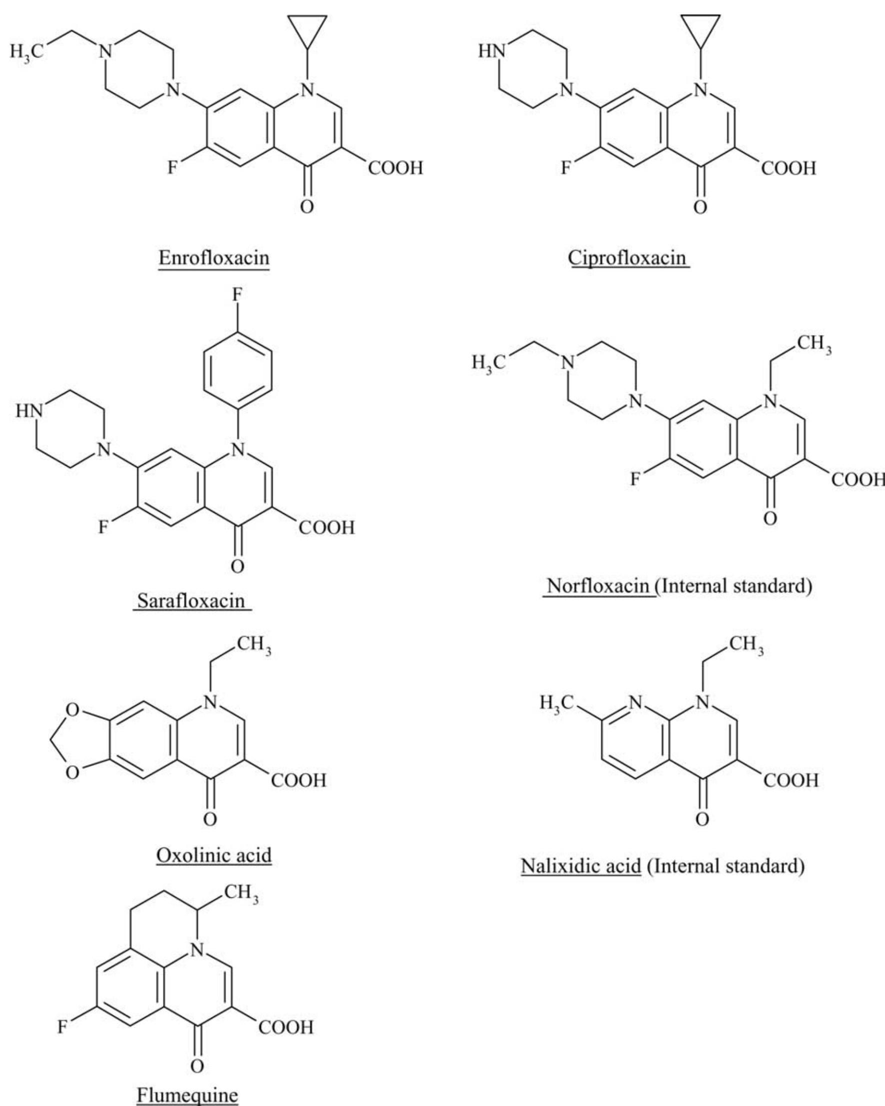


Fig. 1. Structures of (fluoro)quinolones under study.

sarafloxacin, oxolinic acid, and flumequine (Fig. 1) are trapped in an extraction cartridge packed with hydrophilic–lipophilic polymer sorbent. After removal of the matrix, the analytes are desorbed from the cartridge directly to a second analytical column for chromatographic separation. Method validation in terms of specificity, accuracy, precision, decision limit, detection capability, linearity, and short-term analytes stability during sample storage is presented.

2. Experimental

2.1. Chemicals and reagents

Two different sources of enrofloxacin (RIVM, Bilthoven, The Netherlands; Fluka, Buchs, Switzerland), ciprofloxacin (ICN, Irvine, CA; US Pharmacopeia, Rockville, MD), sarafloxacin (B. Dent Global, Lower Hurt, New Zealand; Sequoia Research Products, Oxford, UK), norfloxacin (Sigma, St. Louis, MO; ICN, Irvine, CA), flumequine (RDH, Seelze, Germany; European Pharmacopeia, Strasbourg Cedex, France), and oxolinic acid (Sigma; European Pharmacopeia, Strasbourg Cedex, France) were purchased. Purities of the standards were verified or cross-checked to be of over 95%. Nalixidic acid was purchased from Sigma (St. Louis, MO). Metaphosphoric acid was obtained from Wako Chemical (Osaka, Japan). Disodium salt of EDTA and 2-amino-2-(hydroxymethyl)-1,3-propanediol (TRIS) were acquired from Riedel-de Haën (Seelze, Germany). Acetonitrile and formic acid (min. 98%) were purchased from Labscan (Bangkok, Thailand) and BDH (Poole, England), respectively. Other antibiotics used for specificity tests were obtained from commercial sources.

Table 1
Solvent program for on-line LC–LC/FLD analysis of (fluoro)quinolones

Step	Time (min)	Binary pump			Quaternary pump					Valve position
		Flow rate (mL/min)	A (%)	B (%)	Flow rate (mL/min)	C (%)	D (%)	E (%)	F (%)	
1	0.00	1.0	85	15	4.0	0	100	0	0	1
2	2.00	1.0	85	15	4.0	0	100	0	0	1
3	3.00	–	–	–	1.0	0	100	0	0	2
4	7.50	–	–	–	1.0	0	100	0	0	2
5	8.50	–	–	–	4.0	30	50	0	20	1
6	11.00	1.0	82	18	4.0	30	50	0	20	1
7	11.50	–	–	–	4.0	30	50	0	20	1
8	12.50	–	–	–	2.0	0	100	0	0	1
9	13.00	1.0	60	40	2.0	0	90	10	0	1
10	15.00	1.0	50	50	2.0	0	90	10	0	1
11	16.00	1.0	50	50	1.0	0	100	0	0	1
12	18.00	1.0	50	50	4.0	0	100	0	0	1

A: 1% (v/v) formic acid in water; B: acetonitrile; C: acetonitrile; D: 0.1% (v/v) formic acid in water; E: 10 mM EDTA in water; F: methanol.

2.2. Columns and mobile phases

An empty extraction cartridge, which was a metal-free PEEK-lined cartridge with dimensions of 30 mm × 2.0 mm (Alltech, Deerfield, IL), was slurry-packed with HLB sorbent obtained from Oasis HLB cartridge (60 mg, 3 mL; Waters Corp., Milford, MA). The sorbent was of particle size of about 30 μm. The on-line column-switching mobile phase during initial sample loading was of 0.1% (v/v) formic acid in water.

Separation was performed on a phenyl reversed-phase column: Xterra phenyl 150 mm × 4.6 mm i.d. with 5 μm particle size (Waters Corp., Milford, MA) and a guard column 20 mm × 4.6 mm i.d. of similar material and maintained at 30 °C. The analytical mobile phase was a mixture of 1% (v/v) formic acid in water–acetonitrile. The flow rates were 4 and 1 mL/min for the extraction and the analytical runs, respectively. The solvent programs for both extraction and chromatographic separation are depicted in Table 1.

2.3. Instrumentation and description of the column-switching system

The HPLC system consisted of an Agilent 1100 liquid chromatograph system (Agilent Technologies, Atlanta, GA) with a binary pump, a quaternary pump, an autosampler, a thermostated column compartment with built-in six-port switching valve and a fluorescence detector set at λ_{ex} 280 nm and λ_{em} 450 nm for detecting enrofloxacin, ciprofloxacin, sarafloxacin, and norfloxacin, and at λ_{ex} 312 nm and λ_{em} 366 nm for detecting oxolinic acid, nalixidic acid, and flumequine. As illustrated in the schematic diagram of the column-switching HPLC system (Fig. 2), the mobile phase delivered by the quaternary pump loads the sample onto the extraction cartridge while simultaneously directs the unretained matrix to waste during the extraction process. After complete elution of the unretained components, the valve rotates automatically by 60° where the extraction cartridge is coupled to an analytical column. The analytical mobile phase desorbs the analytes

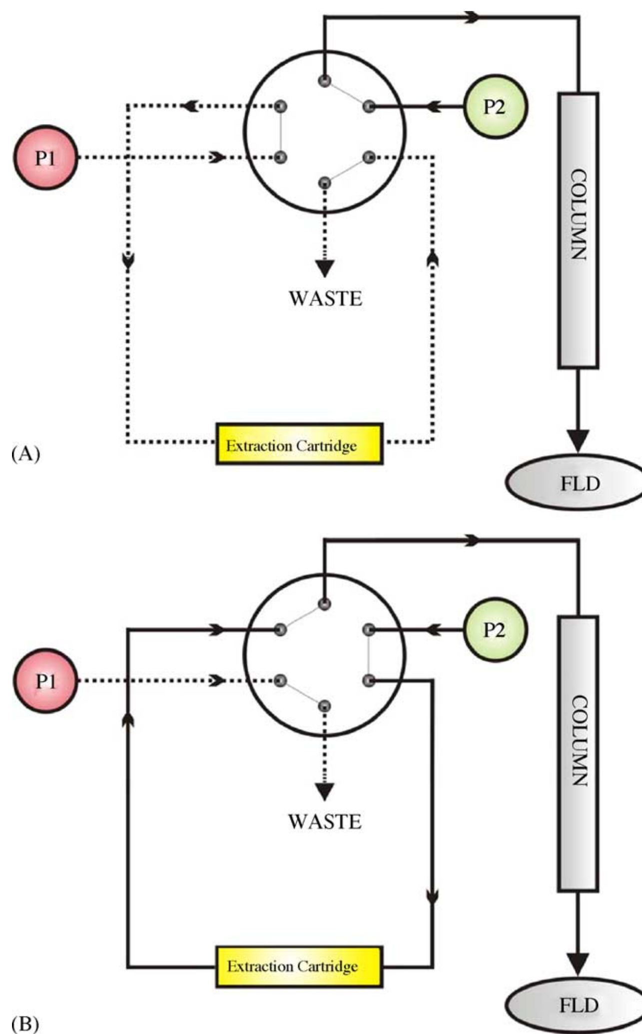


Fig. 2. Schematic diagram of the column-switching HPLC system: (A) flow path during sample loading and clean-up (valve position 1) and (B) flow path during elution (valve position 2). P1: quaternary pump; P2: binary pump. Flow direction is indicated by arrows.

from the extraction cartridge by backflushing which focuses the analytes onto the analytical column.

Subsequent to the transfer of analytes onto the analytical column, the valve returns to its initial position. The extraction cartridge is then cleaned by serial flushings with acetonitrile, methanol, and 10% of 10 mM EDTA solution. Prior to the loading of another sample, the cartridge is conditioned with 0.1% (v/v) formic acid.

2.4. Sample preparation

2.4.1. Calibration standard solutions of (fluoro)quinolones

Stock standard solutions of enrofloxacin, ciprofloxacin, sarafloxacin, flumequine, nalixidic acid were prepared by dissolution of each compound in methanol to obtain a concentration of about 0.1 mg/L. A small amount of dilute sodium hydroxide solution should be added to the methanol when prepared the standard solution of oxolinic acid. These solutions were kept at −20 °C and were stable for at least 3 months. Five-point calibration curve for each analyte was established by dilution of appropriate stock standard solutions in pH 9 Tris buffer to obtain enrofloxacin, ciprofloxacin, sarafloxacin, oxolinic acid and flumequine solutions at concentrations of 0–200 μg/L. Each of the solution contained 100 μg/L of norfloxacin and nalixidic acid as internal standards.

2.4.2. Extraction of milk samples

Milk samples were firstly homogenized on a high-speed vortex mixer. Samples (1 mL) were then added into 15 mL polypropylene centrifuge tubes. Appropriate amounts of norfloxacin and nalixidic acid (100 μg/L in final sample solution) were spiked into the samples as internal standards. Samples were deproteinated by mixing vigorously with 0.25 mL of 5% (w/v) metaphosphoric acid on a vortex mixer for at least 1 min. Sample mixtures were allowed to stand for another 15 min in the dark. The mixtures were then centrifuged and the supernatants were filtered through 0.45 μm syringe filter (Alltech) into 2-mL vials for direct analysis.

2.5. Method validation

Method robustness was evaluated with the following performance indices: specificity, accuracy, precision, decision limit, detection capability, linearity and short-term stability of analyte during sample storage.

2.5.1. Specificity

Specificity was demonstrated by analyzing different milk products such as fresh milk (pasteurized milk), processed milk (UHT milk), and flavoured milk (chocolate milk). Besides, known amounts of macrolides, aminoglycosides, sulphonamides, nitroimidazoles, beta-agonists, and tetracyclines were spiked into blank milk samples to evaluate possible interferences encountered in the method.

2.5.2. Accuracy

Three sets, each of six, of blank milk samples were fortified with 0.5, 1 and 1.5 times the permitted limit (i.e. MRL) of enrofloxacin and ciprofloxacin (100 μg/L), respectively and analysed.

Since no MRL was set for sarafloxacin, oxolinic acid, and flumequine, the same concentration levels as enrofloxacin/ciprofloxacin were used. The fortified samples were analysed and percentage recovery was calculated as 100 times the measured amount divided by the fortification level.

2.5.3. Precision

Three sets, each of six, of blank milk samples were fortified with 0.5, 1 and 1.5 times the MRL of enrofloxacin and ciprofloxacin respectively. Similar approach was applied for sarafloxacin, oxolinic acid, and flumequine. They were analysed on different days with the same instrument and different operators. The overall standard deviation and coefficient of variation (%) were calculated.

2.5.4. Linearity

A five-point calibration curve for each analyte was established in every batch of analysis to evaluate the instrument robustness on different days.

2.5.5. Decision limit and detection capability

Milk samples ($n = 3$) fortified at 0, 25, 50, 75, 100, 125 and 150 μg/L of FQs, respectively were analysed. The decision limit and detection capability were determined in accordance with ISO 11843 [13].

2.5.6. Short-term analytes stability in bovine milk during storage

The experimental design by Taguchi orthogonal array technique was established for investigating the stability of FQs in different milk matrices during storage. Four parameters, viz. concentration, storage temperature, type of milk, and storage period were tested for their significance toward analytes stability during storage. Each parameter containing three levels, was distributed in a balanced orthogonal array of $L_9(3^4)$ (Table 2) leading to a total of nine experiments. The average recovery in each experiment was determined as per Section 2.5.2.

3. Results and discussion

3.1. Extraction procedure

The objective of the present study was to develop a simple and efficient method for fast throughput analysis of FQs in milk products. Regardless of the detection methods, traditional procedures involved laborious sample preparation for deproteination of milk samples to release protein bound drug residues, followed by appropriate off-line solid phase

Table 2
Study on analytes stability during sample storage for (fluoro)quinolones in bovine milk arranged in $L_9(3^4)$ orthogonal array

Test parameters					Average recovery (%)				
Test no.	Concentration (μg/L)	Type[a]	Temperature (°C)	Time (days)	CIPR	ENRO	SARA	OXOL	FLUM
1	0.5 MRL	Milk A	4	14	98	101	101	110	98
2	0.5 MRL	Milk B	−20	21	80	78	75	115	61
3	0.5 MRL	Milk C	20	5	105	98	104	115	114
4	1 MRL	Milk A	−20	5	94	93	95	114	103
5	1 MRL	Milk B	20	14	107	105	105	95	103
6	1 MRL	Milk C	4	21	80	28	61	54	54
7	1.5 MRL	Milk A	20	21	97	67	76	84	54
8	1.5 MRL	Milk B	4	5	92	67	66	129	72
9	1.5 MRL	Milk C	−20	14	107	91	111	96	110

[a] Milk A, B, and C represent UHT pure milk, pasteurized high-calcium, low-fat milk product, and UHT chocolate milk product, respectively.

extraction clean-up with different sorbents to remove interfering matrices [8,10,12]. Although on-line immuno-affinity column clean-up [9] was reported, the column packing involving complex preparation of selective monoclonal antibody would require specific facilities that many analytical laboratories might not have.

Tandem extraction column and analytical reversed phase (RP) column were employed in this method in an attempt to minimize sample preparation and clean-up process. Prior to analysis, milk protein in the sample was simply precipitated with acid, and removed by filtration through a 0.45 μm filter. The supernatant was then analysed directly for the FQs. This significantly improves the sample throughput when handling a large number of samples.

In choosing the extraction column, two factors were considered: the extraction/trapping efficiency for FQs and matrix clean-up efficiency. As FQs are hydrophilic and comprise of amphoteric and acidic moieties (Fig. 1), simple RP columns may not have a good affinity towards extracting these compounds. Firstly, restricted access media (RAM) columns with alkyl-diol silica (ADS) packing bonded with C4, C8, and C18 (Merck, Darmstadt, Germany) were tested. These media have pore sizes of approximately 6 nm providing a physical diffusion barrier to exclude macromolecules such as proteins. The adsorption sites, covered by reversed stationary phases (C4, C8 or C18) and locates in the inner surface of these porous particles, are freely accessible for analytes of low molecular weight. It was (data not shown) discovered that an increase of carbon loading of the media led to an increase of peak areas of hydrophilic/amphoteric FQs, but no such change was observed for acidic quinolones. Thus, increasing the carbon loading of the RP media provided better affinities of charged amphoteric fluoroquinolones and C4 RP media was adequate in trapping acidic quinolones in low pH mobile phase. However, these RAM columns caused peak tailing for amphoteric FQs, probably due to secondary interaction of the amino moiety of the FQs with the residual silanol groups on the column packing. To eliminate this problem and to cater for the broad spectra of hydrophilicity of FQs, an Oasis HLB on-line extraction column (Waters Corp., Milford, MA) was thus evaluated. This extraction column was packed with a polymeric sorbent (divinylbenzene-*N*-vinylpyrrolidone copolymer) with particle size of 30–50 μm. Applying a high mobile phase flow rate resulted in rapid percolation of macromolecules such as protein and other hydrophilic endogenous substances through the extraction support while small analytes were retained by means of hydrophobic interactions. With this column, a significant improvement of peak areas and shapes of all FQs were noted. In order to reduce analytical cost further, it was attempted to prepare an in-house extraction cartridge by packing polymer sorbent obtained from Oasis HLB cartridges (60 mg, 3 mL). The sorbent has the same functionality and similar particle size as that in the HLB on-line extraction column. In preparing an in-house extraction cartridge, polymer sorbent was first suspended in distilled water and wetted with a small amount of methanol. Large amount of organic solvents were not used as they would swell the polymer and led to decrease in packed bed density. To pack a cartridge, sorbent slurry was added slowly to one end of an empty cartridge whilst the other end was connected to a suction pump. The polymer sorbent inside the cartridge was then packed or compressed further by applying a high flow rate of distilled water through an LC pump. Any void volume present thereafter in the cartridge was again filled up with sorbent. In general, the extraction cartridge should have a column pressure of 20–30 bars with aqueous mobile phase of ca. 0.1% (v/v) formic acid in water at 4 mL/min to check for optimal packed bed density and uniformity. No noticeable difference in extraction efficiency of FQs was observed between the in-house prepared extraction cartridge and the commercially available HLB on-line extraction column. As expenses for preparing this extraction cartridge was 50–100 times less than that for the commercially available on-line column and the preparation did not require any special technique and equipment, testing cost was largely reduced.

Carryover is another major problem to address in on-line column clean-up process. To this end, the solvent program was designed to allow organic solvents such as acetonitrile and methanol flushing the cartridge to remove residual milk matrix. About 1 mM EDTA solution was added as chelating reagent in the final washing step to remove any metallic ions such as Ca^{2+} adsorbed and accumulated in the cartridge;

or peak tailing would occur. With this solvent flushing program, no carryover was then noticed and performance of the extraction cartridge was found consistent after at least 180 injections.

3.2. Method validation

The method performance was investigated with respect to various parameters such as specificity, accuracy, precision, decision limit, detection capability, and analytes stability during sample storage. With regard to specificity, fresh milk, processed milk (UHT) milk, and flavored milk (chocolate milk) together with blank milk spiked with about 1 mg/L of chloramphenicol, dimetridazole, metronidazole, dihydrostreptomycin, erythromycin, gentamicin, josamycin, kitasamycin, neomycin, spectinomycin, tylosin, clenbuterol, salbutamol, dapsone, sulfacetamide, sulfachlorpyridazine, sulfadiazine, sulfadimethoxine, sulfamerazine, sulfamethazine, sulfamethiazole, sulfamethoxazole, sulfamethoxypyridazine, sulfanitran, sulfapyridine, sulfaquinoxaoline, sulfathiazole, sulfisoxazole, demeclocycline, doxycycline, oxytetracycline, tetracycline, and chlortetracycline were analysed. No interference or interfering peaks were observed in the retention windows of FQs in all chromatograms (Fig. 3). On the other hand, danofloxacin co-eluted with the matrix interfering peak next to ciprofloxacin and thus could not be analysed by this method. The accuracy (calculated as spike recovery) and precision as within-lab reproducibility at 0.5–1.5 MRL were shown in Table 3. The calibration curves also showed good linearity (for example, $y = 1.505 \pm 0.001x - 0.008 \pm 0.001$, $r^2 > 0.999$ for ENRO) within the concentration range of 0–200 μg/L.

3.3. Decision limit and detection capability

In the Commission Decision 2002/657/EC under the Council Directive 96/23/EC [14], the decision limit (CCα) means that the limit at and above which it can be concluded with an error probability of α that a sample is non-compliant. Likewise, detection capability (CCβ) is the concentration at which the method is able to detect permitted concentrations with a statistical certainty of 1–β. To evaluate the CCα and CCβ values for the method, fortified milk samples at equidistant concentrations at 0, 1/4, 1/2, 3/4, 1, 5/4, and 3/2 of the MRL, in triplicate, were prepared. Critical values and minimum detection values were calculated in accordance with ISO 11843 with $\alpha = \beta = 0.05$ [13]. Borremans et al. [15] described the procedures briefly for calculating CCα and CCβ for preservatives in cosmetics. According to their equations in calculating CCα and CCβ for preservatives in cosmetics, the CCα and CCβ values of the method were determined for each (fluoro)quinolone and the results are given in Table 4.

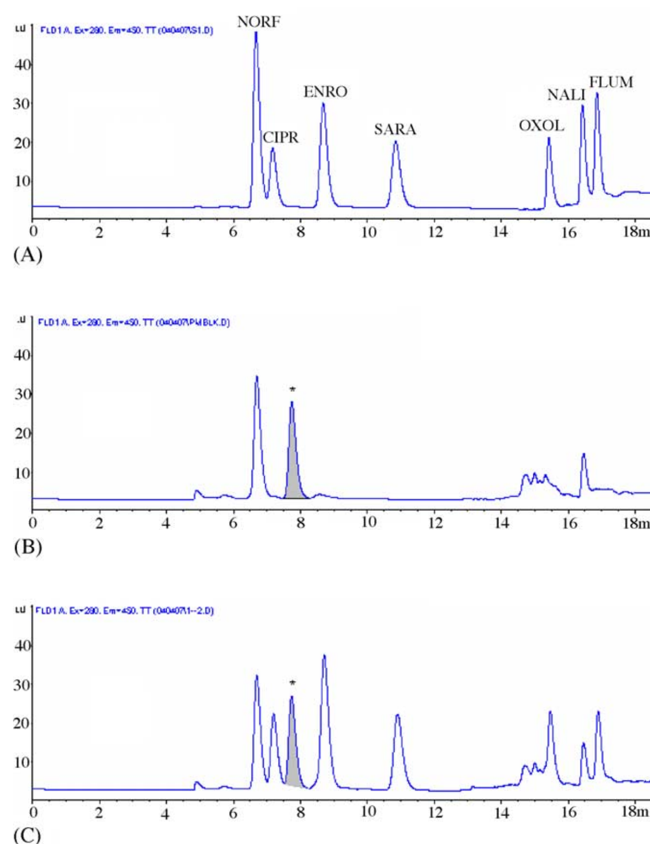


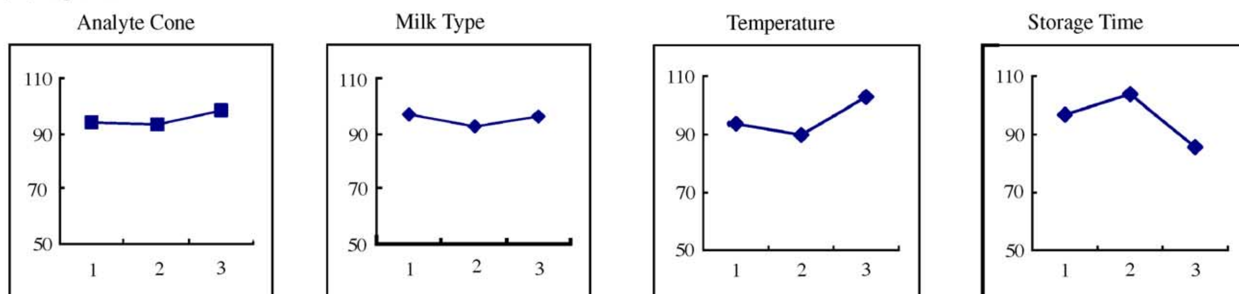
Fig. 3. Representative chromatograms of: (A) calibration standard solution at 50 μg/L; (B) a blank milk; and (C) a blank milk fortified with MRL of (fluoro)quinolones. NORF: norfloxacin; NALI: nalixidic acid; CIPR: ciprofloxacin; ENRO: enrofloxacin; SARA: sarafloxacin; OXOL: oxolinic acid; FLUM: flumequine. The peak marked with asterisk (*) indicates the matrix interferent from milk sample.

Table 3
Average recovery of (fluoro)quinolones in fortified bovine milk at different MRL

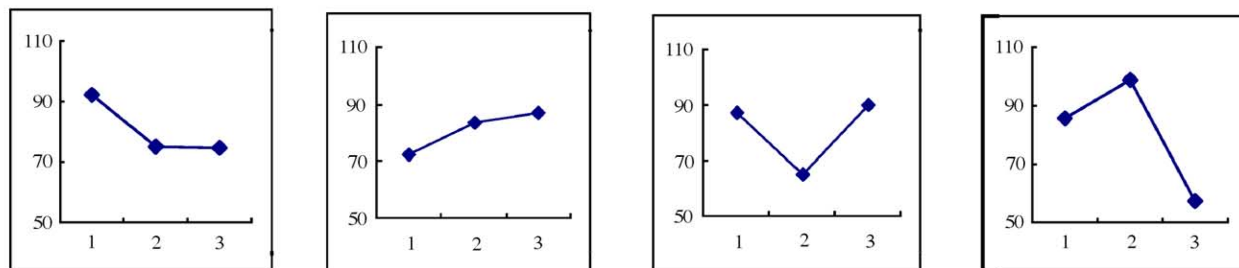
Recovery (%) at indicated fortification level[a]									
(Fluoro)quinolones	50 μg/L			100 μg/L			150 μg/L		
	Mean	S.D.	CV	Mean	S.D.	CV	Mean	S.D.	CV
Ciprofloxacin	92.8	2.2	5.1	94.4	2.2	2.5	97.4	6.7	4.9
Enrofloxacin	101	3.1	6.1	102	3.0	2.9	103	7.8	5.0
Sarafloxacin	89.8	2.2	5.0	90.8	2.1	2.3	92.8	5.7	4.2
Oxolinic acid	121	3.7	5.9	116	3.9	3.1	117	10	5.4
Flumequine	81.3	2.9	6.5	82.1	5.0	5.6	85.5	8.8	6.4

[a] Each mean value is the average of three separate batches performed by different operators on different days. For each batch, six samples were analysed for each (fluoro)quinolone at 0.5, 1, and 1.5 MRL, respectively.

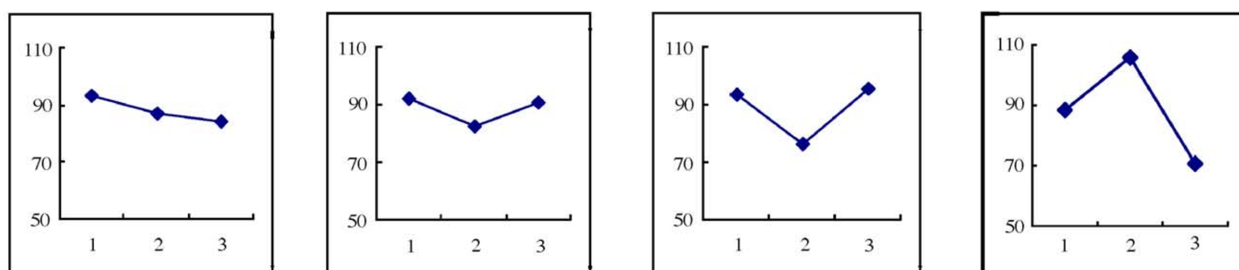
(A) Ciprofloxacin



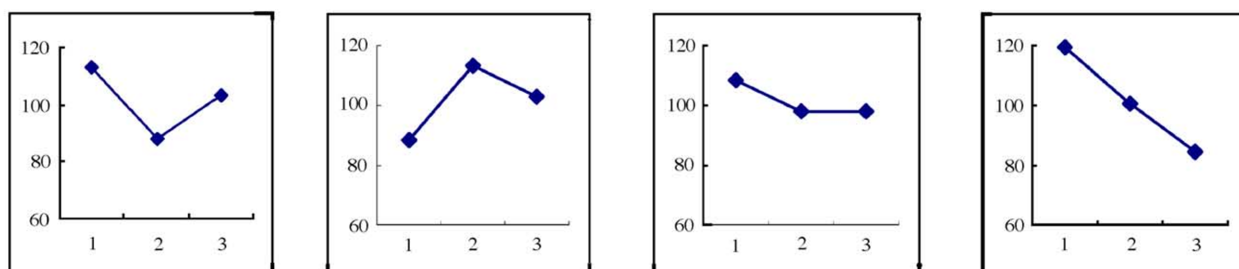
(B) Enrofloxacin



(C) Sarafloxacin



(D) Oxolinic acid



(E) Flumequine

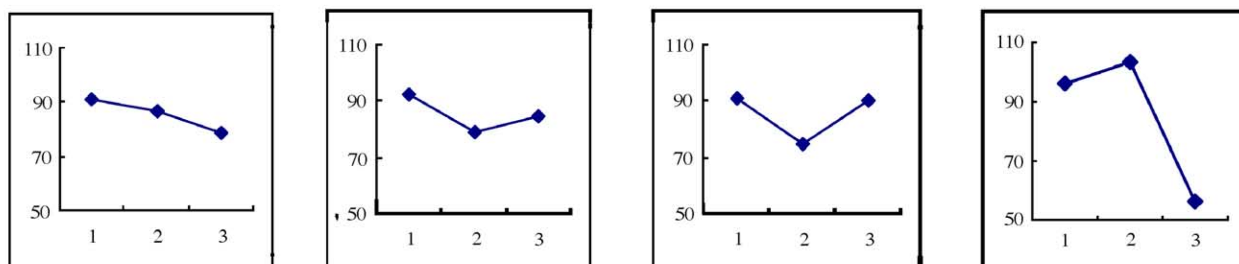


Fig. 4. Response graphs of four test parameters in $L_9(3^4)$ orthogonal array for analytes stability test in bovine milk. For 1, 2, and 3 indicated on *X*-axis represent, respectively: analyte conc: 0.5, 1, and 1.5 MRL; milk type: chocolate milk, high-calcium, low-fat milk, and UHT pure milk; temperature: 20, 4, and −20 °C; and storage time: 5, 14, and 21 days, respectively. *Y*-axis in each graph indicates the percentage recovery of the analyte under study.

Table 4
Critical values, minimum detectable values, decision limits (CCα) and detection capability (CCβ) for (fluoro)quinolones under study in bovine milk

(Fluoro)quinolones	Critical value[a] (μg/L)	Minimum detectable value[a] (μg/L)	Decision limit[b] (CCα) (μg/L)	Detection capability[b] (CCβ) (μg/L)
Ciprofloxacin	8.7	17	108	116
Enrofloxacin	9.1	18	109	118
Sarafloxacin	8.2	16	108	116
Oxolinic acid	14	27	113	126
Flumequine	12	24	112	124

[a] The critical value and minimum detectable value are calculated as in ISO 11843 with $K=1$, $I=7$, and $J=3$.
[b] The MRLs for all (fluoro)quinolones in bovine milk are set at 100 μg/L.

Table 5
One-way ANOVA test for studying stability of (fluoro)quinolones in different bovine milk during storage

Test parameters[a]	$F_{(\text{calculated})}$[b]					F ($P=0.05$)	Significance[c]
	CIPR	ENRO	SARA	OXOL	FLUM		
Analytes concentration	0.14	0.43	0.14	1.01	0.15	5.143	No
Milk type	0.11	0.24	0.20	0.90	0.19	5.143	No
Storage temperature	1.37	0.91	0.94	0.18	0.36	5.143	No
Storage time	4.47	3.94	5.62	2.57	11.0	5.143	SARA, FLUM

[a] The levels of each test parameters were listed in Table 2.
[b] A one-way ANOVA was used to evaluate the between-level variance. F_{calc} was determined by the ratio of mean square of between-level to that of within-level.
[c] If the calculated F-value is greater than the critical value of $F_{2,5}$ ($P=0.05$) then the test parameter indicates to be a significant influencing factor.

3.4. Short-term stability of (fluoro)quinolones in bovine milk during storage

Stability of FQs during sample storage was tested by a design of experiment approach using the Taguchi orthogonal array technique [16]. This statistical technique gives a systematic methodology for improving cost effectiveness in quality decisions. Detailed procedures were described elsewhere [17]. Under this investigation, four critical parameters, namely concentration of analytes, milk type, storage temperature, and storage time that may affect analytes stability during sample storage were selected and arranged orthogonally in a $L_9(3^4)$ array. A total of nine experiments were analysed in duplicate and their average recoveries were calculated. By combining the associated recovery at each level for every factor (for example, the mean recovery for the analyte's concentration at 0.5 MRL was determined by averaging the recoveries at 1st, 2nd, and 3rd runs), the effects of each factor could be visualized in their respective response graphs as shown in Fig. 4. The significance of the levels in each factor could also be assessed statistically using one-way analysis of variance (ANOVA). According to the results shown in Table 5, it was found that the calculated F-values of storage time for sarafloxacin and flumequine were larger than the critical value $F_{2,5}$ ($P=0.05$) from one-tailed F-test at the probability of 0.05 (or 5% level). In other words, the storage time was a statistically significant factor on the stability of sarafloxacin and flumequine in bovine milk (UHT pure milk, chocolate milk, or high-calcium, low-fat milk) at the temperature range of −20 to 20 °C. As shown in the response graphs, other FQs also exhibited a tendency of degradation in bovine milk against storage time, albeit their calculated F-values were less than the critical one. This may denote that a more drastic storage condition (e.g. −70 °C) might be necessary for prolonged storage of bovine milk for FQs analysis. Other factors (analyte concentration, temperature, and milk type) were seemingly less influencing the analyte stability in bovine milk during storage ($F_{(\text{calculated})} < F_{(\text{critical})}$) (Table 5).

4. Summary

A simple and economical on-line column clean-up method was developed for the analysis of enrofloxacin, ciprofloxacin, sarafloxacin, oxolinic acid, and flumequine in bovine milk. The on-line technique was found beneficial over other traditional off-line procedures by minimizing tedious sample preparation and increasing productivity. The method has been extensively validated and was found applicable for use in food surveillance programs.

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