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Simultaneous Determination of Fluoroquinolone Antibiotic Residues in Milk Sample by Solid-Phase Extraction−Liquid Chromatography−Tandem Mass Spectrometry

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A simple, sensitive, and reliable high-performance liquid chromatographic (HPLC) method coupled with tandem mass spectrometry via electrospray ionization (ESI) source (LC-MS/MS) has been developed and validated for the simultaneous determination of five fluoroquinolone residues in milk. The studied fluoroquinolones were norfloxacin, ciprofloxacin, ofloxacin, enrofloxacin, and rufloxacin. The method involved a single solid-phase extraction (SPE) on C_{18} followed by the analysis of all fluoroquinolones in a single chromatographic run using LC-ESI-MS/MS. Lomefloxacin was employed as the internal standard (IS). The limit of quantification (LOQ) was 0.5 ng/g in milk, much lower than the maximum residue limit (MRL) of 100 ng/g of enrofloxacin established by the Ministry of Agriculture of China. Standard curves were linear ($r > 0.99$) over the concentration range of 0.5−200 ng/g with good accuracy and precision. The method has been successfully applied to the analysis of 22 different brands of cow's milk on the Chinese market.

KEYWORDS: Fluoroquinolones; cow's milk; LC-MS/MS

INTRODUCTION

Quinolones are a group of structurally related antibacterial agents used in human and veterinary medicine. The 6-fluorinated piperazinyl derivatives, fluoroquinolones, have a wide range of antibacterial activities and have been increasingly use in veterinary medicine because of their effectiveness in treating bacterial infections (*1*). In fact, they have been used successfully to treat infections caused by Gram-positive and Gram-negative bacteria and micoplasma such as pulmonary infections, urinary infections, and digestive infections. The mechanism of their effect consists of a specific inhibition of DNA gyrase, whereby synthesis of bacterial DNA and, consequently, bacterial multiplication are blocked.

The widespread use of fluoroquinolones in agriculture has resulted in the potential presence of these compounds' residues in foodstuffs of animal origin. The use of fluoroquinolones in lactating breeding animals may leave residues in the milk and tissues. Therefore, animal food may be a potential hazard for consumers, causing allergic reaction and also leading to the emergence of drug-resistant bacteria. To ensure safety, the Chinese Ministry of Agriculture established maximum residue limits (MRLs) in foodstuffs of animal origin. Ciprofloxacin and enrofloxacin are allowed at concentrations below their MRLs, which is 100 μg/kg for the sum of ciprofloxacin and enrofloxacin (ciprofloxacin is a metabolite of enrofloxacin) (*2*).

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Earlier methods of fluoroquinolone analysis in biological matrices are based on liquid chromatography (LC), mainly with fluoresence (FL), ultraviolet (UV), or mass spectrometric (MS) detection. A rapid and sensitive colloidal gold immunochromatography test strip based on one monoclonal antibody with broad specificity, which can detect 12 fluoroquinolones in chicken muscle and liver, was developed by Zhu et al. (*3*). Only a few methods have focused on the determination of fluoroquinolone multiresidues in milk. Rodriguez et al. (*4*) developed a SPE-LC-FL method for the determination of residues of ciprofloxacin, enrofloxacin, danofloxacin. and sarafloxacin in powdered infant formulas with the limit of quantification (LOQ) of 6−80 μg/kg. A multiresidue determination of fluoroquinolones in milk by column liquid chromatography with fluorescence and ultraviolet absorbance detection was developed by Marazuela et al. (*5*) with the LOQ pf 2.4 ng/mL. Liquid chromatography with fluorescent detection is commonly used as the determination method. However, false positives have been reported (*6*) due to the complex matrix. Cinquina et al. (*7*) determined enrofloxacin and its metabolite ciprofloxacin in goat's milk by LC−diode array detection (DAD),; the quantification limits were 20 ng/mL for both analytes. Holtzapple et al. (*8*) used online immunoaffinity extraction for sample cleanup and preconcentration and reported the contamination of the analytical column after 15−20 milk sample injections. The procedure of Roybal et al. (*9*) required a complex milk extraction method and the need for periodic column regeneration due to the reaction of the milk proteins with the stationary phase. Adrian et al. (*10*) used a semiquantitative multianalyte ELISA for immunochemical screening of

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sulfonamide, fluoroquinolone, and β-lactam antibiotics in milk samples using class-selective bioreceptors. Because LC with MS detection has been used for confirmatory analysis. A LC-MS/MS method for the simultaneous quantification of eight quinolones in bovine muscle, milk, and aquacultured products was developed by Van Hoof et al. (*11*) with detection limits of 42−120 μg/kg. Hermo et al. (*12*) developed a LC-MS/MS method for the simultaneous quantification of quinolones in milk with LOQ = 2.0 ng/g.

This paper describes a SPE-LC-MS/MS multiresidue method for the determination of norfloxacin, ciprofloxacin, ofloxacin, enrofloxacin, and rufloxacin in milk. All fluoroquinolones were extracted by a single solid-phase extraction and analyzed in a single chromatographic run using LC-ESI-MS/MS. The limits of quantification of these five fluoroquinolones were 0.5 ng/g, which is $^{1}/_{200}$ of the MRL of enrofloxacin. The LOQs obtained were much lower than the results of the earlier LC-UV, LC-FD, LC-MS, and LC-MS/MS methods. The method was successfully applied to the analysis of these five fluoroquinolones in milk.

MATERIALS AND METHODS

Chemicals and Reagents. Norfloxacin, enrofloxacin, and ciprofloxacin reference standards were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China); ofloxacin reference standards was supplied by Chengdu Tianyin Pharmaceutical Co. Ltd. (Chengdu, China). Rufloxacin hydrochloride reference standards was supplied by Spring Pharmaceutical Co. Ltd. (Beijing, China). Lomefloxacin hydrochloride reference standards was supplied by Huanghe Medical (Yancheng, China). The structures of these six fluoroquinolones are shown in **Figure 1**. Methanol and acetonitrile were from Merck (Darmstadt, Germany), *n*-hexane was from Caledon (Goeregetwon, ON, Canaida), and all were of HPLC grade. All other chemicals were of analytical grade. The mobile phase was filtered with a 0.22 μm cellulose membrane before use. The water was distillated twice before use.

Equipment. LC-MS/MS analysis was performed using a Finnigan TSQ Quantum Discovery MAX system consisting of Finnigan Surveyor LC pump, a Finnigan Surveyor autosampler, and a vacuum degasser and combined with a triple-quadrupole TSQ Quantum mass spectrometer (Thermo Electron Corp.), run by Xcalibur 2.0 software (Thermo Electron Corp.).

Samples were separated on a Shimadzu Shim-pak VP-ODS C_{18} column, which was maintained at 40 °C. The mobile phase consisted of 25% of methanol and 75% (v/v) of aqueous ammonium acetate solution (10 mM, pH 2.5) at a flow rate of 0.2 mL/min.

Ionization was performed in the positive mode, and MS/MS was operated at unit resolution in multiple reaction monitoring (MRM) mode. Source conditions were optimized as follows: spray voltage. 5000 V; transfer capillary temperature, 350 °C; sheath gas and auxiliary gas (nitrogen) pressure, 30 and 5 arbitrary units (set by the LCQ software, Thermo Electron Corp.), respectively. Argon was used as collision gas at a pressure of 1.5 mTorr. Transition ions *m*/*z* and the collision energy of each fluoroquinolone are listed in **Table 1**. The scan width for SRM was *m*/*z* 0.01, and scan time was 0.01 s.

Preparation of Stock Solutions. Stock solutions of norfloxacin, ciprofloxacin, and enrofloxacin (1 mg/mL) were prepared in 0.1 M hydrochloric acid aqueous solution. Stock solutions of ofloxacin (1 mg/mL) was prepared in a methanol/water mixture (30:70; v/v). Stock solution of rufloxacin (1 mg/mL) was prepared by dissolving the substance in water. Lomefloxacin, used as internal standard (IS) was prepared by dissolving the substance in 0.1 M sodium hydroxide aqueous solution. Working solutions of each fluoroquinolone for calibration and quality control (QC) were prepared by appropriate dilution in methanol and aqueous ammonium acetate solution (10 mM, pH 2.5) (25:75, v/v) to final concentrations of 0.01, 0.1, 1, and 10 μg/mL. The stock solution of lomefloxacin was further diluted with methanol and aqueous ammonium acetate solution (10 mM, pH 2.5) (25:75, v/v) to prepare the working internal standard solution containing 1 μg/mL of lomefloxacin. All of the solutions were stored at 4 °C.

Calibration Standards and Quality Control Samples. Calibration curves were prepared on five different days by spiking blank milk with the proper volume of one of the working solutions mentioned above to produce the standard curve points equivalent to 0.5, 1, 5, 10, 20, 50, 100, 150, and 200 ng/g of the five fluoroquinolones. Blank milk samples (without IS) were also analyzed. Calibration curves were prepared by determining the best fit of peak area ratios (peak area of analyte/peak area of internal standard) versus concentration and fitted to the equation $R = aC + b$ by weighted least-squares regression ($1/C$).

Quality control (QC) samples were prepared by spiking blank milk with the proper volume of one of the working solutions mentioned above to produce a final concentration equivalent to 1 ng/g (low level), 10 ng/g (middle level), or 100 ng/g (high level) of the five fluoroquinolones. The subsequent procedures were the same as described above. The limit of detection (LOD) and the lower limit of quantification (LLOQ) were determined as the concentrations with signal-to-noise ratios of 3 and 10, respectively.

Preparation of Sample. To 1 g of milk sample was added 10 μL of IS (10 μg/mL lomefloxacin). To precipitate the proteins in the milk, 2 mL of acetic acid (5% in acetonitrile) was added. The mixture was vortex-mixed for 2.0 min and then centrifuged at 2951*g* for 10 min. After evaporation to approximately 1−2 mL at 40 °C under a gentle stream of nitrogen, 5 mL of *n*-hexane was added to remove fat. After 2 min of vortex mixing, the upper layer was discarded. The lower layer was further purified by SPE using a C_{18} cartridge that had been previously been conditioned with 2 mL of methanol and 2 mL of water. After the extract had passed through the cartridge, it was rinsed with 2 mL of water. The fluoroquinolones were eluted from the column with 2 mL of 1 M aqueous ammonia (25% in methanol).

The eluate was evaporated to dryness at 40 °C under a gentle stream of nitrogen. The residue was resuspended in 300 μL of mobile phase under vortex and centrifuged at 13772*g* for 8.0 min. Ten microliter aliquots of the supernatant were injected into the LC-MS/MS system.

Method Validation. Calibration curves were generated by using the ratios of the analytes peak area to the IS peak area versus concentration and were fitted to the equation $R = aC + b$ by weighted least-squares linearity regression. Blank milk samples used for testing specificity of the method were obtained from six different origins. The visible interferences were tested with blank milk samples and milk samples with the analytes concentrations close to the LLOQ. The potential matrix effect on the ionization of the analytes was evaluated by comparing the peak area of the analytes dissolved in the supernatant of the processed blank milk to that of standard solutions at the same concentration. Three different concentration levels of the analytes (1, 10, and 100 ng/g) were evaluated by analyzing five samples at each set. The matrix effect of internal standard (10 ng/g in milk) was evaluated using the same method.

The interbatch precision and accuracy were determined by analyzing 15 sets of spiked milk samples of the analytes at each QC level (1, 10, and 100 ng/g) in a day. The intrabatch precision and accuracy were determined by analyzing five sets of spiked milk samples of the analytes at each QC level (1, 10, and 100 ng/g) on three consecutive days. The concentration of each sample was calculated using a standard curve prepared and analyzed on the same day.

The absolute extraction recovery of the analytes was assessed by comparing the analytes to the IS peak area ratios obtained from extracted milk samples with those from the standard solutions at the same concentration. This procedure was repeated ($n = 5$) at each QC level (1, 10, and 100 ng/g). Similarly, the absolute recovery of the IS was assessed by comparing the IS mean peak areas obtained from extracted QC milk samples ($n = 5$) to those from the standard solutions at the concentration of 10 ng/mL.

Application of the Assay. To apply the proposed method to the analysis of the selected antibiotics in milk samples, a screening study was developed. For this purpose, 22 different brands of cow's milk samples bought from supermarkets were analyzed in our study.

RESULTS AND DISCUSSION

LC and MS-MS Method Development. For several years LC with MS detection has been used for confirmatory analysis because this detection method is more sensitive and selective,

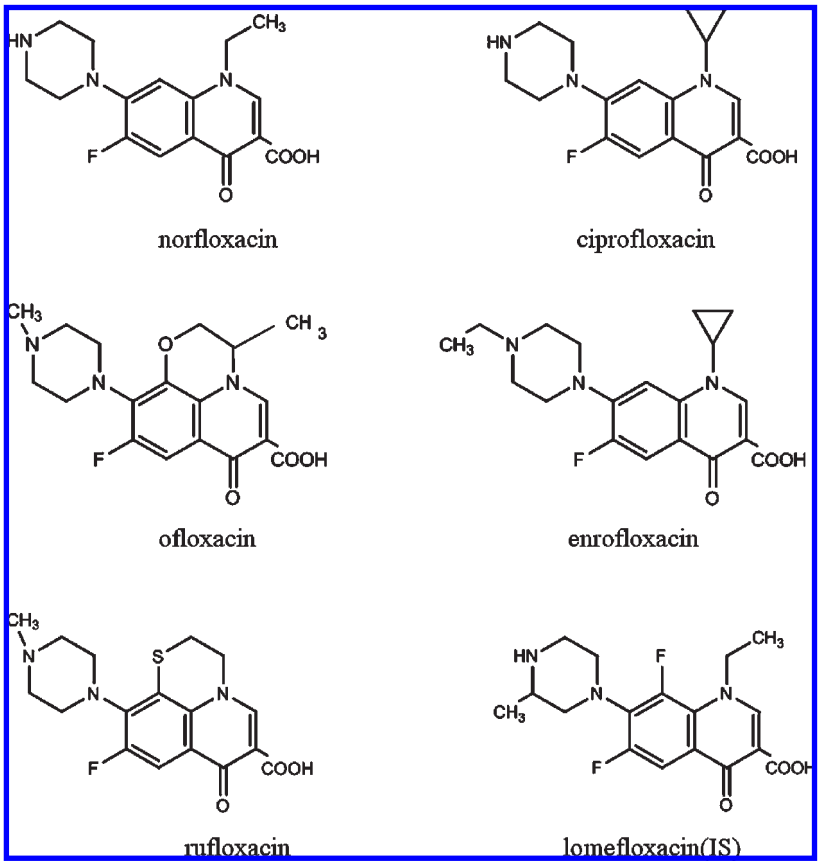

Figure 1. Chemical structures of fluoroquinolones.

Table 1. Conditions for the MS/MS Determinations of Fluoroquinolones

compound	precursor ion (*m*/*z*)	product ion (*m*/*z*)	collision energy (V)
norfloxacin	320.00	302.10	21
		276.10	17
ciprofloxacin	332.05	288.10	17
		231.00	31
enrofloxacin	360.05	316.10	20
		245.10	25
ofloxacin	362.00	318.10	18
		261.00	23
rufloxacin	364.25	320.10	18
		262.98	27
lomefloxacin (IS)	352.10	265.05	26
		308.07	17

allows rapid and multiresidue determination in complex matrices, and gives structural information. In our study, a LC-ESI-MS/MS multiresidue method was developed for the detection of five fluoroquinolones.

It is generally known that fluoroquinolones give severely tailing peaks in reversed-phase chromatography, but this can be reduced by using mobile phases which have a high ionic strength or high acidity. During LC method development, the LC mobile phases investigated were methanol with aqueous acetic acid and acetonitrile with aqueous formic acid (both suitable for electrospray ionization) (*13*). The separations for the analytes and IS on the C_{18} column eluting by the above two mobile phase were compared. The analytes were eluted quickly and with some overlap among the six peaks when eluted by acetonitrile system. A methanol/aqueous ammonium acetate solution (pH 2.5) system on a Shim-pak VP-ODS C_{18} column gave satisfactory separation of the analytes.

MS method development was partially automated with the aid of the Xcalibur 2.0 software supplied by Thermo Electron Corp. Strict European Union (EU) guidelines for confirmatory techniques state that LC-MS/MS monitoring of two or three transition product ions gives sufficient data to confirm the identity of a residue (*14*). Therefore, two transition product ions were selected for quantitative analysis. The transition product ions of each analyte selected are given in **Table 1**.

Optimization of the Milk Extraction Procedure. Traditional extraction strategies for antibiotics in milk involve precipitation of proteins with organic solvents (e.g., methanol, acetonitrile), extracting fat with diethyl ether or *n*-hexane (*15*). Methanol, acetonitrile, methanol/acetic acid (95:5, v/v), acetonitrile/acetic acid (95:5, v/v), methanol/1 M aqueous ammonia (95:5, v/v), and acetonitrile/1 M aqueous ammonia (95:5, v/v) were compared for precipitating the proteins present in milk. Acetonitrile/acetic acid (95:5, v/v) was finally adapted for the clear supernatant of mixer after mixing and centrifugation. For safety reason, *n*-hexane was used to remove fat in the milk.

The lower layer after extraction with *n*-hexane was further purified by SPE using a C_{18} cartridge. The parameters evaluated in our study for the optimization of the SPE procedure were the pH of the sample, composition and volume of the eluting solution, and the breakthrough volume of the SPE cartridges. After extraction with acid acetonitrile and *n*-hexane, the milk samples spiked with 100 ng/g enrofloxacin were passed through a C_{18} cartridge directly or alkalized. The different eluting solutions used and the recoveries and standard deviations (SD) are shown in **Table 2**. The procedure finally chosen was as below: After the extract had passed through the cartridge, it were rinsed with 2 mL of water. The fluoroquinolones were eluted from the column with 2 mL of 1 M aqueous ammonia (25% in methanol).

Table 2. Mean Recoveries of Enrofloxacin and the Standard Deviations (SD) for Incurred Raw Milk Samples, Spiked at the Concentration of 100 ng/g, after the Application of the Different Eluting Solutions of SPE (n = 5)

	recovery (%, mean ± SD) with		
	methanol/water (75:25) eluting solution	methanol/1% acetic acid (75/25) eluting solution	methanol/1 M ammonia (75:25) eluting solution
acidic sample	30.15 ± 4.25		74.08 ± 4.85
basic sample	26.52 ± 5.26	50.36 ± 4.09	

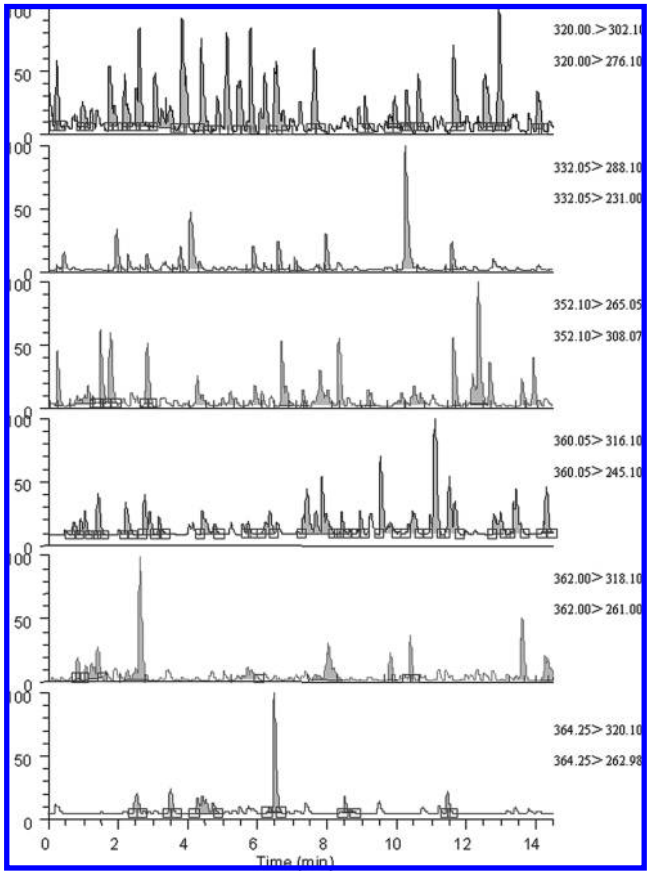

Figure 2. Chromatograms for milk samples with no fluoroquinolones above the LOQ.

Assay Selectivity and Matrix Effect. No analyte-interfering peaks were observed due to the high selectivity of SRM. Six kinds of drug-free milk were mixed and extracted according to the developed method. **Figure 2** shows the representative HPLC-MS/MS chromatograms for a drug-free milk sample, indicating that no endogenous peaks are present at the retention times (t_R) of the five analytes or of the IS. All of the ratios of the peak area of the analytes dissolved in the supernatant of the processed blank milk compared to that of standard solutions at the same concentration were between 85 and 115%. The results showed that there was no significant difference in peak areas. This indicated that no coeluting "invisible" compounds significantly influenced the ionization of the five analytes and IS.

Linearity of Calibration Curves and Specificity. The weighted regression calibrations were linear over the concentration range of 0.5−200 ng/g in milk. The equations of linearity were as follows: norfloxacin, R = 0.1085C − 0.0002713, r = 0.9972; ciprofloxacin, R = 0.07112C + 0.0004978, r = 0.9985; enrofloxacin, R = 0.2193C + 0.0009627, r = 0.9978; ofloxacin, R = 0.2134C − 0.001110, r = 0.9996; rufloxacin, R = 0.1222C + 0.01041, r = 0.9940. The use of the weighted regression resulted in <15% deviation between the nominal and experimental concentrations calculated from the equations.

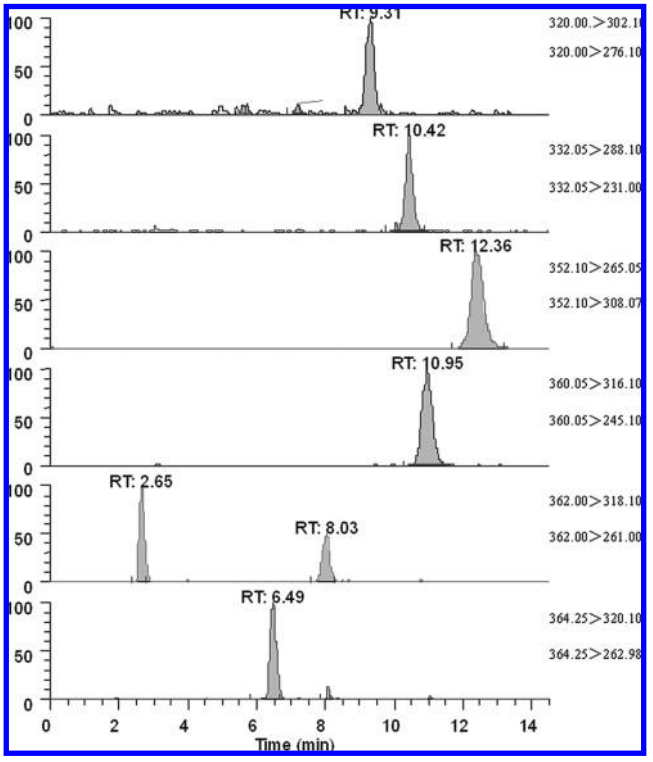

Figure 3. Representative HPLC-MS/MS chromatograms for a milk sample with five analyte concentrations at LLOQ. $t_{R,norfloxacin}$ = 9.31 min, $C_{norfloxacin}$ = 0.5 ng/g; $t_{R,ciprofloxacin}$ = 10.42 min, $C_{ciprofloxacin}$ = 0.5 ng/g; $t_{R,enrofloxacin}$ = 10.95 min, $C_{enrofloxacin}$ = 0.5 ng/g; $t_{R,ofloxacin}$ = 8.03 min, $C_{ofloxacin}$ = 0.5 ng/g; $t_{R,rufloxacin}$ = 6.49 min, $C_{rufloxacin}$ = 0.5 ng/g; $t_{R,IS}$ = 12.36 min, C_{IS} = 10 ng/g.

Ion chromatograms from milk with five analyte concentrations at the LLOQ gave a signal-to-noise ratio 10, and ion chromatograms from milk with five analyte concentrations at 0.2 ng/g gave a signal-to-noise ratio 3. Therefore, the LLOQ for five analytes was proved to be 0.5 ng/g in milk, and the LOD was 0.2 ng/g. **Figure 3** shows the representative HPLC-MS/MS chromatograms for a milk sample with five analyte concentrations at LLOQ.

Precision and Accuracy. An assessment of intrabatch and interbatch precision was conducted by analyzing quality control (QC) samples at three levels. Data of precision are presented in **Table 3**. The precision of the assay was <15%, and the accuracy deviation values for intrabatch and interbatch are all within 100 ± 15% of the actual values at each QC level. The results revealed good precision and accuracy.

Quantitative Analysis of Milk Samples. The described assay was successfully applied to the analysis of fluoroquinolones in 22 different brands of cow's milk samples. **Table 4** compiles the information of the different samples (a total of 22) as well as the results obtained concerning their contents in the different antibiotics. In all cases, ciprofloxacin of the selected antibiotics was found in 4 of the 22 samples to exceed the MRL; other fluoroquinolones were below the MRL in all samples.

In this investigation, the newly developed SPE-LC-MS/MS method has proven to be an efficient and sensitive method to determine norfloxacin, ciprofloxacin, ofloxacin, enrofloxacin, and rufloxacin simultaneously in milk samples. The limits of quantification of these five fluoroquinolones were 0.5 ng/g, which are much lower than the results of the earlier LC-UV, LC-FD,

Table 3. Intra- and Interbatch Precision of the Method for Determination of the Five Fluoroquinolones (Intrabatch, $n = 5$; Interbatch, $n = 15$)

		1 ng/g			10 ng/g			100 ng/g		
		detected concn (mean, ng/g)	mean accuracy(%)	RSD (%)	detected concn (mean, ng/g)	mean accuracy (%)	RSD (%)	detected concn (mean, ng/g)	mean accuracy (%)	RSD (%)
intrabatch ($n = 5$)	norfloxacin	0.9746	97.46	8.66	9.633	96.33	5.82	99.40	99.40	3.04
	ciprofloxacin	0.9884	98.84	7.54	10.14	101.36	5.54	93.88	93.88	4.04
	enrofloxacin	0.9283	92.83	7.55	9.195	91.95	5.90	91.75	91.75	3.79
	ofloxacin	0.9364	93.64	6.30	9.159	91.59	5.16	92.95	92.95	2.27
	rufloxacin	0.8922	89.22	8.76	8.766	87.66	6.76	85.58	85.58	4.31
interbatch ($n = 15$)	norfloxacin	0.9531	95.31	7.48	9.578	95.78	5.16	98.21	98.21	3.48
	ciprofloxacin	0.9797	97.97	7.42	1.028	102.84	6.22	96.52	96.52	5.78
	enrofloxacin	0.9213	92.13	8.83	9.419	94.19	5.06	92.98	92.98	3.98
	ofloxacin	0.9341	93.41	8.34	9.348	93.48	4.03	94.83	94.83	3.88
	rufloxacin	0.8960	89.60	8.84	8.941	89.41	5.57	87.40	87.40	4.27

Table 4. Results of the Determination of the 22 Different Brands of Milk

	concn (ng/g)				
sample	ciprofloxacin	enrofloxacin	ofloxacin	norfloxacin	rufloxacin
2008-01				85.30	
2008-02	46.06	6.89	19.78		
2008-03	333.41[a]	2.49		3.09	
2008-04	242.48[a]				
2008-05	209.60[a]				
2008-06					
2008-07	326.24[a]		17.03		
2008-08			3.46	2.41	
2008-09	12.88		2.32	2.35	
2008-10			4.28		
2008-11					
2008-12	19.79	5.40		2.44	
2008-13	2.28	6.89			
2008-14	1.25	7.82			
2008-15	5.68	4.08		0.72	
2008-16	0.46	3.02		0.34	
2008-17	21.42				
2008-18		8.59			
2008-19				65.02	
2008-20	5.57	3.81			
2008-21	10.01	2.20		5.07	
2008-22	0.95	7.13			

[a] Exceeded the MRL (100 ng/g).

LC-MS, and LC-MS/MS methods. Twenty-two different brands of cow's milk bought from supermarkets were analyzed by using the validated method. The result was important for the residue monitoring and residue determination.

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