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Simultaneous determination of (fluoro)quinolones antibacterials residues in bovine milk using ultra performance liquid chromatography–tandem mass spectrometry

Hong Zhang[a,*,1], Yiping Ren[b,1], Xiaoli Bao[a]

[a] College of Food Science and Biological Engineering, Zhejiang Gongshang University, Hangzhou 310035, PR China
[b] Zhejiang Center for Disease Prevention and Control, Hangzhou 310009, PR China

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ABSTRACT

The ultra performance liquid chromatography–tandem mass spectrometry (UPLC) method had been developed for 22 (fluoro)quinolone(QNs) antibacterials in milk with multiple reaction monitoring (MRM) as acquisition mode. The analytes were extracted from the sample using McIlvaine buffer by ultrasonic bath, and purified by solid-phase extraction (SPE) cartridge. The residue were dried under nitrogen and dissolved in mobile phase before UPLC–MS/MS final analysis. The calibration curve of six concentrations for 22 QNs showed good linearity and the good correlation coefficients ($r \geq 0.9851$) were achieved. The limit range of quantification was 0.008–0.339 μg/kg. The recovery range was 63.1–94.6% except flumequine, nalidixic acid and nadifloxacin. The method was precise: the relative standard deviations of the method for milk were not more than 13.12%. The accuracies and sensitivity of the method were good for simultaneous determination of 22 QNs.

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1. Introduction

Quinolones(QNs) were a potent group of synthetic antibacterial compounds used in the treatment of a variety of bacterial infections in human and animals. The antibacterials were widely used, and often misused, thus resulting in occurrence of veterinary residues drug in foodstuffs. Many countries have established Maximum Residue Limits (MRL) to monitoring the residues of the drugs in veterinary. Several quinolones were registered for use on food-producing animals. European Union (EU) Legislation has established the safe MRLs of the veterinary drugs in animal edible tissues [1]. There were five quinolones regulated in bovine milk by EU, 30 μg/kg for danofloxacin, 50 μg/kg for flumequime, 75 μg/kg for marbofloxacin, 100 μg/kg for enrofloxacin and ciprofloxacin.

Several techniques have been used for analysis of QNs in foodstuff, including immunoassay [2–4], luminescence analysis, thin-layer chromatography (TLC) [5], high performance capillary electrophoresis (HPCE) [6,7], gas chromatography (GC) [8] and high performance liquid chromatography (HPLC) [9–13] with fluorescence (FLD), ultraviolet (UV), diode array detectors (DAD) and mass spectrometric (MS) [14–21] detection. The determination of QNs residues was usually performed by HPLC with UV, FLD, MS or MS/MS. Some QNs have not high sensitivity and selectivity with UV, and LC method using FLD were restricted to a limited number of QNs. The two detectors were not as same as MS detector to confirm a sample. LC–MS/MS combines high specificity, selectivity and sensitivity, which could get more accurate qualitative and quantitative results. It was fit for the multiresidues determination of 22 QNs. Since the drug varieties were increasing in market, it was very necessary to establish a LC–MS/MS method for the multiresidue determination of QNs in bovine milk. In this study, there was a simple and sensitive method for the identification and determination of 22 QNs in milk by ultra performance liquid chromatography–tandem mass spectrometry (UPLC)–ESI–MS/MS. The optimization of the mass spectrometric parameters was performed in order to obtain the best signal and highest sensitivity for QNs. SPE was used to extract from the sample. Finally, satisfactory results were obtained.

2. Experimental

2.1. Materials and reagents

Methanol and acetonitrile for HPLC (99.9%) was from Merck (Darmstadt, Germany). Formic acid 99% from Ameisensaeure. Ethy-

* Corresponding author. Tel.: +86 571 88071024/8598; fax: +86 571 88905733.
E-mail address: hongzh1316@mail.zjgsu.edu.cn (H. Zhang).
[1] These two authors contributed equally to this paper.

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lacetate, citric acid, disodium hydrogen phosphate and disodium ethylenediamine tetracetate (EDTA-Na_2) were analytical-reagent grade from SCM in China. Ultrapure water was generated by a Milli-Q System (Millipore).

The QNs standards norfloxacin (NOR, 97.8%), ciprofloxacin (CIP, 99.0%), lomefloxacin (LOM, 90.0%), ofloxacin (OFL, 98.4%), fleroxacin (FLE, 99.1%), sparfloxacin (SPA, 99.8%), pefloxacin (PEF, 71.1%), enoxacin (ENO, 91.1%) were obtained from NICPBP (Beijing, China). Gatifloxacin (GAT), pazufloxacin (PAZ), moxifloxacin (MOX), nadifloxacin (NAD) were provided by Toronto Research Chemicals Inc. (Toronto, Canada). The purity are all more than 99.0%. Enrofloxacin (ENR, 98.0%), sarafloxacin (SAR, 93.5%), nalidixic acid (NAL, 99.5%), oxolinic acid (OXO, 98.0%), flumequine (FLU, 99.0%), danofloxacin (DAN, 96.0%), difloxacin (DIF, 99.0%), marbofloxacin (MAR, 99.0%) were obtained from Dr. Ehrenstorfer (GmbH, German). Cinoxacin (CIN, 99.0%) and pipemdic acid (PIP, 99.0%) were form Sigma–Aldrich (St. Louis, MO, USA).

2.2. Instrumentation and condition for UPLC–MS/MS

A centrifuge (Allegra™ X-22R, Beckman, USA), KQ-500E ultrasonic bath (Kunshan, CHINA) were used for sample extraction. Milford Massachusetts SPE equipment (Waters, USA) and Boud Elut Plexa SPE cartridges from VARIAN (3 mL, 60 mg) were used in sample purification.

The LC system consisted of a Waters ACQUITY™ UPLC (Waters, Milford, MA, USA). The MS instrument consisted of a Waters Micromass Quattro Micro™ triple-quadrupole system (Manchester, UK).

The chromatographic separation of QNs was achieved on Waters Acquity Shield RP 18 Column (100 mm × 2.1 mm, 1.7 μm). The LC separation was performed using a gradient elution. Mobile phase A was 0.2% formic acid aqueous at pH 3.0, mobile phase B was methanol–acetonitrile (40:60, v/v). The initial mobile phase consisted of 88% A and 12% B and stable for 4 min. From 4.1 min to 5 min, the percentage of B increased from 12% to 17%. From 5.1 min to 11.0 min, the percentage of B increased 48%, increasing 100% B from 11.1 min to 11.3 min, holding at it until 11.8 min, returning to 12% B by 12.0 min and holding at 88% B until 15 min. The flow rate of the mobile phase was 0.3 ml/min and injection volume was 10 μl. The column and sample temperature were 40 °C and 25 °C, respectively.

The ESI–MS/MS was operated in positive ion mode and other conditions were the followings: capillary voltage, 3.0 kV; source block, 120 °C; RF Lens 1, 40.0 V; RF Lens 2, 0.4 V; desolvation temperature, 350; desolvation gas (N_2) flows, 500 l/h; ion energy 1, 1.0 eV; the ion monitoring mode, multiple reaction monitoring (MRM). The dwell time was 150 ms. The collision energy for MRM acquisitions was presented in Table 1. The MS–MS transitions were monitored in five channels, the first with two, the second with six, the third with four, the fourth with six and the last was four channels. Two transition ions were followed for identification but only one was used for quantitation.

2.3. Preparation of standard working solutions

1 mg/ml standard stock solution was prepared for each QNs in methanol. The stock solution were protected from light and kept at −18 °C until they were used. The mixing working solutions of 1 μg/ml of 14 quinolones, 3 μg/ml of PIP, ENO, CIN and OXO, 5 μg/ml of PAZ and 0.5 μg/ml of SPA, OFL and GAT was prepared in acetonitrile–0.2% formic acid aqueous (12:88, v/v). The working solution was used for spiking blank milk sample and stored at the refrigerator temperature (4 °C) for 1 month.

2.4. Extraction and SPE procedure

2.00 g milk was transferred to a 50 ml centrifuge tube and 10 ml EDTA-Mcllvanie buffer solution (0.2 mol/l disodium hydrogen phosphate and 0.1 mol/l citric acid buffer solutions, pH 4.0) were added to the tube. The mixture was rotated for 30 s with the vortex and ultrasonic bath for 15 min. The tube was centrifuged for 10 min at 18,000 rpm and 0 °C. The supernatant liquid was removed. The residue was repeated with the process and two extraction solutions were merged.

The Blond elut plexa extraction cartridge was conditioned using 3 ml methanol, 3 ml water and 3 ml EDTA-Mcllvaine buffer solution. The cartridge was loaded with the extract and washed with 1 ml of 5% methanol in water. The elution of QNs was performed with 6 ml methanol. The eluate was evaporated to dryness at 40 °C under a stream of nitrogen and redissolved in 2 ml of initial mobile phase. This solution was filtered through a 0.22 μm water-film filter before UPLC–MS/MS analysis.

Table 1
The parameters for MS–MS detection of quinolones.

Quinolone	Precursor ion (m/z)	Cone voltage (V)	Transition ion[a] (collision energy) (*m/z*, eV)	
Pipemidic acid	304.3	40	217.4 (15)	*286.5 (15)*
Marbofloxacin	363.3	40	*320.4 (12)*	345.5 (18)
Fleroxacin	370.4	40	269.3 (25)	*326.4 (15)*
Enoxacin	321.3	40	232.4 (30)	*303.4 (15)*
Pazufloxacin	319.3	40	281.4 (20)	*301.4 (10)*
Ofloxacin	362.4	40	*318.4 (16)*	344.4 (18)
Pefloxacin	334.4	40	*290.4 (16)*	316.4 (18)
Norfloxacin	320.3	40	*276.3 (14)*	302.3 (18)
Ciprofloxacin	332.3	40	*288.3 (16)*	314.3 (18)
Danofloxacin	358.4	40	82.1 (32)	*340.3 (20)*
Lomefloxacin	352.4	40	*265.3 (20)*	308.3 (14)
Enrofloxacin	360.4	40	*316.4 (16)*	342.4 (18)
Difloxacin	400.4	40	299.4 (25)	*356.5 (15)*
Sarafloxain	386.4	40	*342.4 (16)*	368.4 (20)
Gatifloxacin	376.3	40	261.4 (25)	*332.4 (15)*
Sparfloxacin	393.4	40	*292.4 (20)*	349.5 (15)
Moxifloxacin	402.5	40	364.5 (24)	*384.5 (18)*
Cinoxacin	263.2	40	217.3 (12)	*245.3 (18)*
Oxolinic acid	262.2	40	216.3 (25)	*244.2 (15)*
Nalidixic acid	233.2	40	187.1 (24)	*215.2 (15)*
Flumequine	262.2	40	202.1 (26)	*244.2 (18)*
Nadifloxacin	361.3	40	283.3 (30)	*343.3(20)*

[a] The transition ion used for quantification is showed in italics.

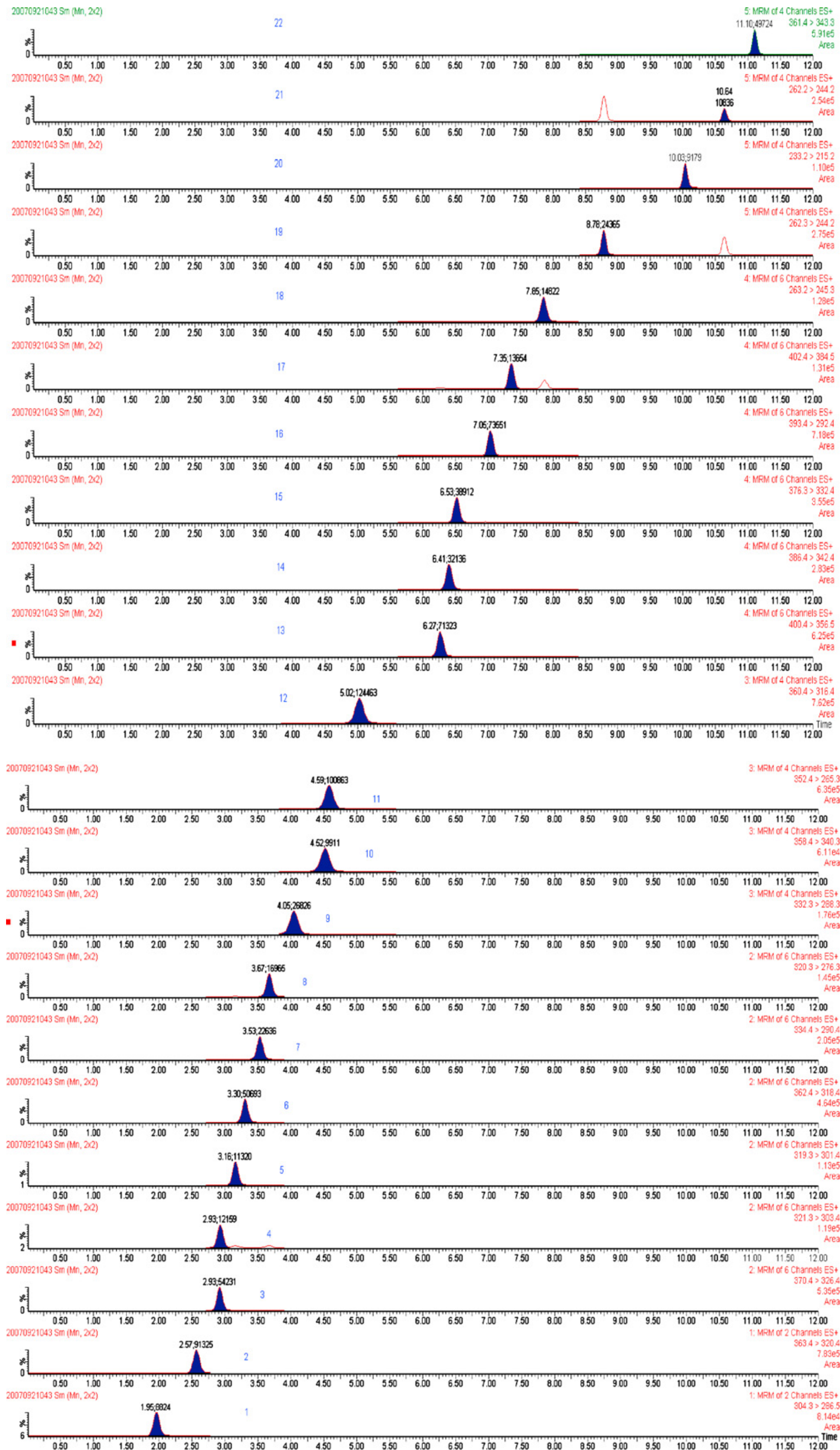

Fig. 1. UPLC–MS–MS chromatograms of standard at 100 ng/ml. Time scale in minutes.
1, PIP; 2, MBF; 3, FLE; 4, ENO; 5, PAZ; 6, OFL; 7, PEF; 8, NOR; 9, CIP; 10, DAN; 11, LOM; 12, ENR; 13, DIF; 14, SAR; 15, GAT; 16, SPA; 17, MOX; 18, CIN; 19, OXO; 20, NAL; 21, FLU; 22, NAD.

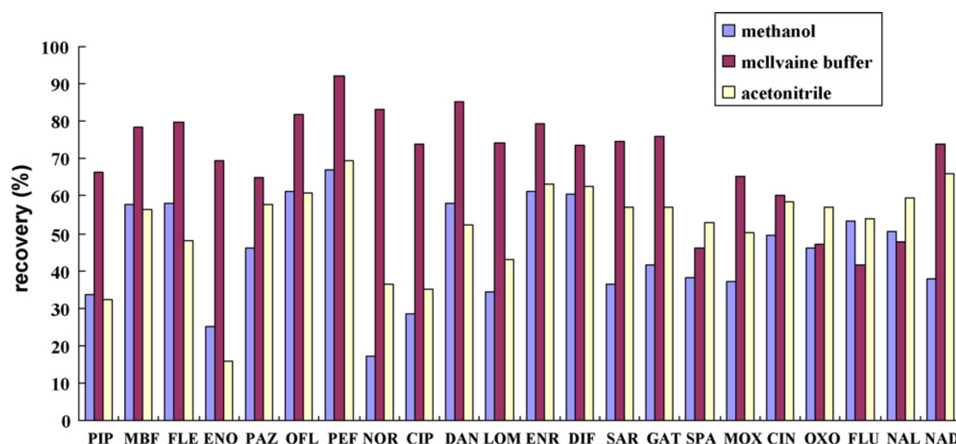

Fig. 2. The comparation of extracting effect for three extractants. Each compound was extracted in sample by 100 μl of standard solution (10 μg/ml of each) in 20 ml McIlvaine buffer (pH 4.0).

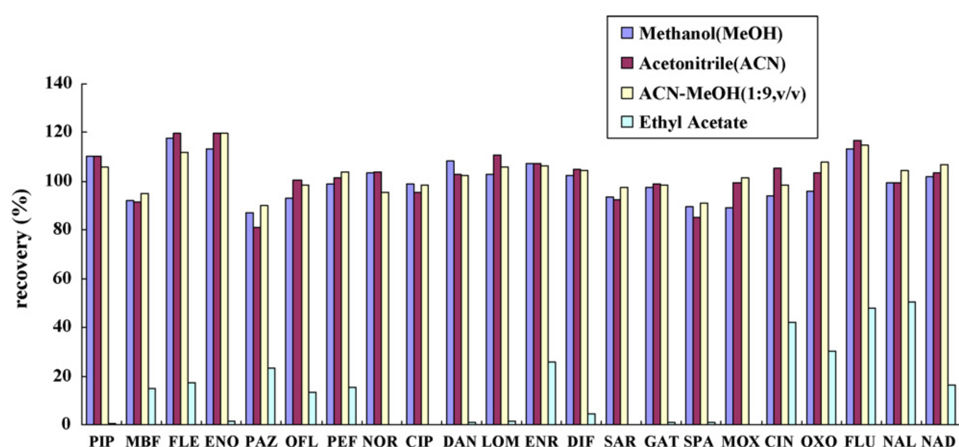

Fig. 3. The comparation of elution effect for four eluting solvents. Each compound was extracted by 100 μl of standard solution (10 μg/ml of each) in 20 ml McIlvaine buffer (pH 4.0). The volume of elution was 6 ml.

2.5. Recovery

The extraction recoveries of the analytes from milk was evaluated at the spiked three concentrations. The extraction recovery was calculated by comparing the peak area of extracted analyte to that of non-extracted analyte. Extracted analytes were prepared by the procedure described in Section 2.4. For the non-extracted sample, analytes were spiked after the extraction with McIlvaine buffer solution and SPE in the procedure.

2.6. Calibration standard

After extraction and SPE procedure described in Section 2.4, the different volumes of the working solution were added to blank matrix of milk. Blank matrix-matched calibration standards solutions were prepared at six concentration from 3 ng/ml to 300 ng/ml for PIP, ENO, CIN and OXO, from 5 ng/ml to 500 ng/ml for PAZ, from 0.5 ng/ml to 50 ng/ml for SPA, OFL and GAT, from 1 ng/ml to 100 ng/ml for other 14 quinolones.

3. Results and discussion

3.1. Optimization of LC and MS/MS condition

Four different reversed-phase chromatographic columns had been used in this study. The four columns were Waters ACQUITY

Table 2
Linear range, limits of detection (LOD) and quantification (LOQ) of 22 QNS.

QNs	Linear range (μg/kg)	LOQ (μg/kg)	LOD (μg/kg)
PIP	3–300	0.339	0.102
MBF	1–100	0.010	0.003
FLE	1–100	0.025	0.007
ENO	3–300	0.213	0.064
PAZ	5–500	0.323	0.097
OFL	0.5–50	0.014	0.004
PEF	1–100	0.047	0.014
NOR	1–100	0.215	0.064
CIP	1–100	0.046	0.014
DAN	1–100	0.026	0.008
LOM	1–100	0.043	0.013
ENR	1–100	0.024	0.007
DIF	1–100	0.022	0.006
SAR	1–100	0.041	0.012
GAT	0.5–50	0.008	0.002
SPA	0.5–50	0.008	0.002
MOX	1–100	0.031	0.009
CIN	3–300	0.319	0.409
OXO	3–300	0.078	0.023
FLU	1–100	0.037	0.012
NAL	1–100	0.040	0.012
NAD	1–100	0.117	0.035

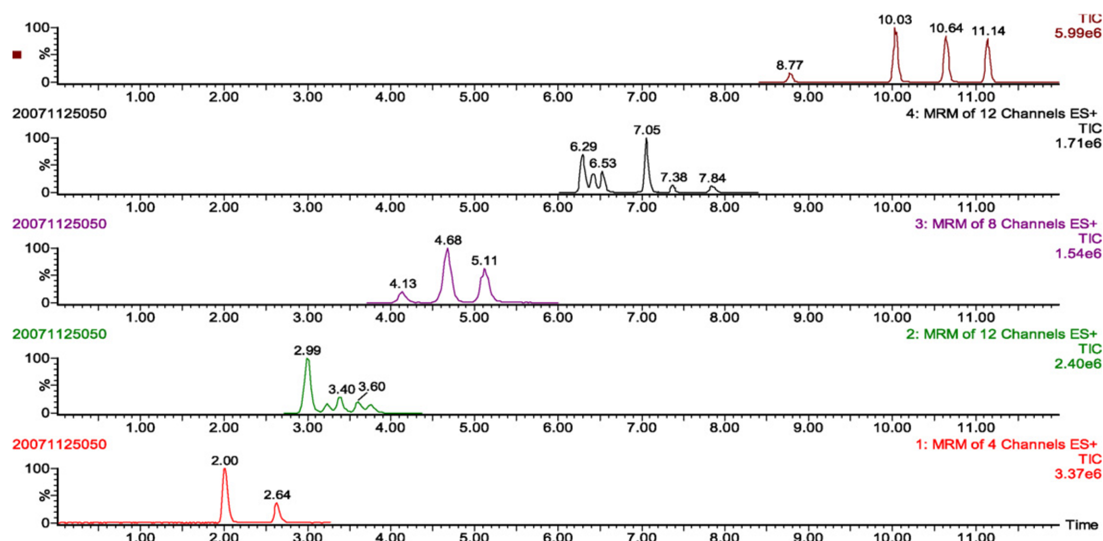

Fig. 4. Blank milk spiked with 22 QNs at 20 μg/kg with 22 QNs. The MS–MS transitions were monitored in five channels, the first with two, the second with six, the third with four, the fourth with six and the last was four channels.

UPLC HSS T3 Column (2.1 mm × 100 mm, 1.8 μm, column 1), Waters ACQUITY SHIELD RP18 Column (2.1 mm × 100 mm, 1.7 μm, column 2), Waters ACQUITY UPLC BEH C_{18} Column (2.1 mm × 100 mm, 1.7 μm, column 3), Waters ACQUITY UPLC BEH C_8 column (2.1 mm × 100 mm, 1.7 μm, column 4). The separation effect of column 3 was worst. Column 4 and Column 1 cannot separate six groups of QNs and four groups of QNs, respectively. Column 2 gives slightly better separation of a subgroup of 22 QNs, good peak shapes and high sensitivity. All analytes were eluted in less than 12 min the column 2 was applied in the research.

Table 3
The recoveries of 22 quinolones in milk spiked with different concentration.

QNs	Spiking level (μg/kg)	Recovery % (R.S.D.) ($n=3$)	QNs	Spiking level (μg/kg)	Recovery % ($n=3$)
	15	73.8 (1.3)		5.0	82.8 (3.5)
PIP	60	79.6 (3.7)	DAN	20	85.7 (1.4)
	150	82.3 (1.7)		50	83.7 (1.4)
	5.0	86.7 (2.8)		5.0	88.5 (0.5)
MBF	20	88.9 (2.4)	LOM	20	86.9 (0.5)
	50	86.3 (5.9)		50	85.4 (3.9)
	5.0	84.1 (5.6)		5.0	85.7 (2.9)
FLE	20	93.1 (3.1)	ENR	20	86.9 (0.3)
	50	85.8 (6.0)		50	85.6 (4.4)
	15	83.2 (3.9)		5.0	91.1 (3.8)
ENO	60	90.2 (0.6)	DIF	20	94.6 (1.6)
	150	88.9 (1.7)		50	92.9 (4.4)
	25	61.9 (6.8)		5.0	86.7 (3.1)
PAZ	100	63.2 (1.0)	SAR	20	87.9 (1.5)
	250	69.1 (7.9)		50	88.0 (3.4)
	2.5	82.4 (0.8)		2.5	84.2 (2.7)
OFL	10	84.1 (0.4)	GAT	10	87.4 (1.8)
	25	84.0 (3.5)		25	86.9 (5.0)
	5.0	86.7 (2.2)		2.5	85.8 (4.4)
PEF	20	83.4 (0.2)	SPA	10	82.3 (2.5)
	50	86.7 (1.8)		25	80.0 (4.1)
	5.0	85.0 (2.7)		5.0	85.6 (3.2)
NOR	20	85.6 (0.9)	MOX	20	81.0 (0.6)
	50	86.7 (4.3)		50	80.7 (6.4)
	5.0	81.0 (4.3)		15	74.3 (7.2)
CIP	20	86.6 (1.0)	CIN	60	81.9 (2.3)
	50	86.4 (2.1)		150	81.7 (6.4)
	15	70.8 (4.8)		5.0	76.8 (2.7)
OXO	60	63.1 (3.1)	NAL	20	51.8 (1.2)
	150	64.8 (0.4)		50	57.2 (5.3)
	5.0	67.2 (6.8)		5.0	51.8 (6.3)
FLU	20	51.8 (5.0)	NAD	20	41.3 (3.3)
	50	57.2 (2.8)		50	40.6 (3.3)

Table 4
The precision of intra-assay and inter-assay.

QNs name	Intra-day precision ($n=7$) R.S.D.%	Inter-day precision ($n=5$) R.S.D.%
PIP	4.89	6.01
MBF	4.34	1.41
FLE	5.21	2.56
ENO	4.20	2.85
PAZ	4.02	8.64
OFL	3.48	1.97
PEF	0.86	4.67
NOR	2.11	5.11
CIP	2.57	0.51
DAN	3.74	2.88
LOM	2.26	2.27
ENR	3.94	5.09
DIF	3.87	1.94
SAR	2.43	2.70
GAT	3.97	1.12
SPA	5.72	3.22
MOX	2.34	1.85
CIN	3.60	5.50
OXO	9.21	11.07

Formic acid aqueous solution used in solvent A could enhance ionization efficiency. The ionization of QNs is not enough for 0.1% formic acid, however, the ion inhibition will enhanced for more than 0.2% concentration. Indeed the use of 0.2% formic acid aqueous solution achieved the satisfying response. When 0.2% formic acid aqueous solution was compared with 10 mM ammonium acetate aqueous solution, the former showed a higher separation effect and good peak shape. The organic compositions of mobile phase B have methanol, acetonitrile and different proportion of methanol and acetonitrile. The experiment showed that the separation and peak shapes were not good with methanol and the early appearance of most QNs peaks resulted in difficult separation with acetonitrile. Finally, the acetonitrile–methanol (3:2, v/v) was the best solvent B.

For the MS/MS operation, ESI positive ion mode was the most effective for the ionization of 22 QNs. The ion $[M+H]^+$ was obtained by MS-Scan mode and chosen as precursor ion. Daughter ions were selected by Daughter-Scan. Parameters, including cone potential, collision energy, and collision cell exit potential and so on, were optimized by flow injection analysis (FIA). The multiple reaction monitoring mode was used to enhance the sensitivity and selectiv-

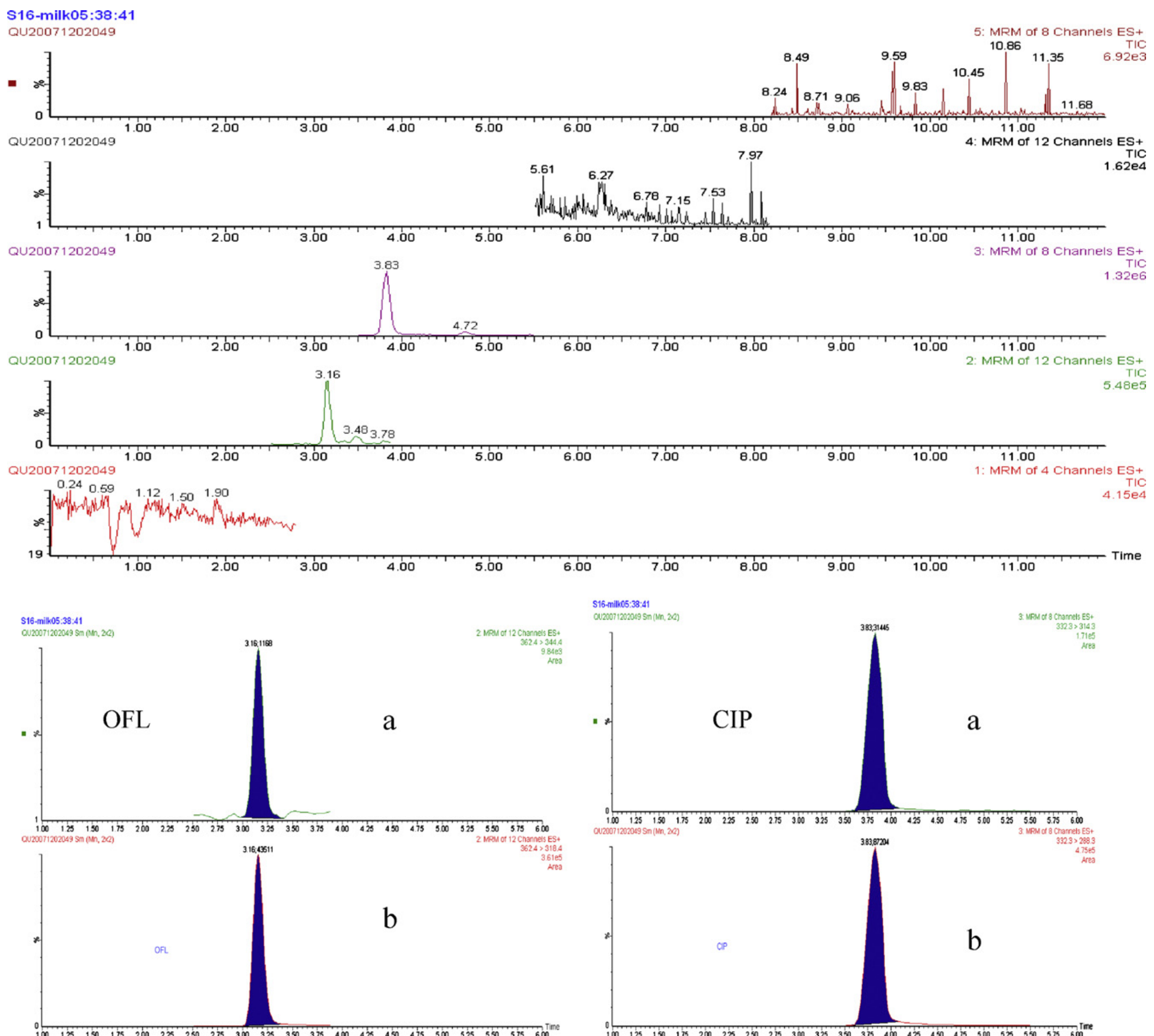

Fig. 5. The positive sample of bovine milk with the quantification transition (a) and the confirmation transition (b).

ity of the determination, to monitor for each QNs. Three transitions were chosen for each quinolones according to EU criteria, one precursor ion and two daughter ions. Table 1 shows the optimum MS/MS conditions of QNs. One of two daughter ions is the quantitative ion and another is the qualitative ion for each quinolone. Fig. 1 shows ion LC–MS–MS chromatogram of standard.

3.2. Optimization of extraction and the solid phase extraction condition

The extraction solvent must able to solubilize the analytes in sample, minimizing the co-extraction of other matrix components, considering the compatibility of the solvent with later analytical steps. Methanol, acetonitrile and McIlvaine buffer were tested as the extraction solvents. The result showed that the extraction efficiency of McIlvaine buffer is better than MeOH and ACN obviously, especially for FLE, FLU, OFL, DAN, MBF, GAT, DIF, CIP, in Fig. 2. McIlvaine buffer was used for extraction of QNs from milk.

In this study, two different SPE cartridges were tested, using Waters-HLB and Varian-Bond elut plexa (BEP) reversed phase cartridges. The elution of QNs with BEP was better than it with HLB for most quinolones. The BEP can decrease the interferences from the sample and improve the retention efficiency. Moreover, the price was satisfied. This solid phase cartridge was therefore selected for the clear-up step. An washing step was used after the sample loading. Different concentrations of methanol in water were tested. PIP had been eluted from SPE, when methanol concentration was more than 5%. Milli-Q water was used as washing solution without loss of any QNs, but it could not remove some interfering substances sufficiently. Hence 5% methanol with water was applied as washing solution. After loading and washing, the SPE cartridge was eluted with acetonitrile, methanol, ethyl acetate and ACN/MeOH (1:9, V/V), respectively. The result showed that the eluting effect of ethyl acetate was the worst from Fig. 3. There were less difference among other three eluting solvents. Finally 6 ml methanol was selected to elute QNs.

3.3. Method validation

3.3.1. Linearity

A sufficient number of standards should be used to adequately define the relationship between the corrected area and concentration. To test the linearity of the calibration curve, various concentrations of the 22 QNs, ranging from 3 ng/ml to 300 ng/ml for PIP, ENO, CIN and OXO, from 5 ng/ml to 500 ng/ml for PAZ, from 0.5 ng/ml to 50 ng/ml for SPA, OFL and GAT, from 1 ng/ml to 100 ng/ml for other 14 quinolones were analyzed. The linear relationship and related parameters are shown in Table 2. The correlation coefficients ranged from 0.9851 to 0.9997.

3.3.2. Limits of detection and quantification

The limit of quantification (LOQ) of each QNs was considered as the concentration giving a signal to noise ratio of 10. The limit of detection (LOD) was defined as a signal to noise ration of 3. The LOQ and LOD was presented in Table 2. The chromatogram of blank milk and milk spiked with 22 QNs at 20 μg/kg was shown in Fig. 4.

3.3.3. Recovery and precision

Under the instrumental conditions in Section 2, the sample spiked at three concentration levels were analyzed using the optimized analytical method. Recovery and repeatability data for milk were given in Table 3. The intra-day precision was obtained through repeating seven times a day and sample was spiked at 20 μg/kg for QNs (60 μg/l for PIP, ENO, CIN, OXO, 100 μg/kg for PAZ, 10 μg/kg for OFL, GAT, SPA). The inter-day precision was achieved though repeating 5 days. The precision data were given in Table 4. The recovery for NAL, FLU and NAD were lower than 60%, however, others were 61.9–94.6% with the R.S.D.s of 0.86–13.12%.

3.4. Application

19 milk samples from different markets were determinated with the analytic method. There were 10 positive samples which included four QNs. Eight of positive samples contained CIP. The CIP content was highest for one sample, containing 50.9 μg/kg. OFL, NOR and FLU had been found in three kinds of samples, respectively, containing 37.4 μg/kg, 9.1 μg/kg and 23.4 μg/kg. The levels were not above MRLs from (EU) 2377/90/EC. A positive sample was shown in Fig. 5.

4. Conclusion

The multiresidue determination of 22 QNs in milk could be successfully achieved using UPLC–MS/MS. The result show that: the linear range was from 1 μg/l to 100 μg/l for 14 QNs (3–300 μg/l for PIP, ENO, CIN, OXO, 5–500 μg/l for PAZ, 0.5–50 μg/l for OFL, GAT, SPA) and the good correlation coefficients ($r \geq 0.9851$) were achieved. The lower limit of quantification was 0.008–0.339 μg/kg. The recovery was 63.1–94.6% except FLU, NAL and NAD and the relative standard deviation was 0.86–13.12%. This method presented in this paper was developed in order to allow the simultaneous analysis of 22 QNs, which can usually hardly be determined using a single procedure. A common sample preparation for the QNs involves the liquid extraction of QNs in McIlvaine buffer and solid-phase extraction (SPE) using Boud elut plexa-RP extraction cartridges. The separation and detection of QNs are achieved using UPLC with ESI–MS/MS detection. The analytical time of UPLC is shortened by one time, namely 12 min. Good recoveries and fine reproducibility for 19 QNs are obtained, but the recoveries were lower than 60% for FLU, NAL and NAD. The LOQ are found to be low enough to determine quinolone residues in milk below the MRL by NO. 508/1999 of European Union.

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