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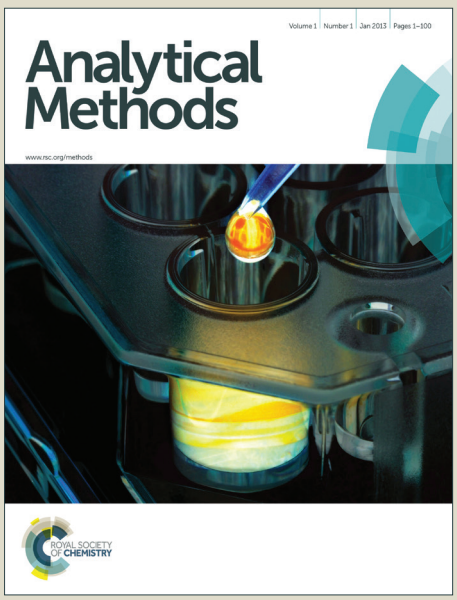
Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

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Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

ARTICLE TYPE

Simultaneous determination of six fluoroquinolones by validated QuEChERS-DLLME HPLC-FLD in milk

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Abstract

Fluoroquinolones (FQs) in milk are not destroyed at high temperature and the stability of them increases the risk of human exposure and may cause food-borne illness. The present study reports the use of QuEChERS- Dispersive liquid-liquid micro extraction (DLLME) for extraction and determination of six FQs (marbofloxacin, norfloxacin, ciprofloxacin, difloxacin, enrofloxacin and danofloxacin) in whole milk sample. Linearity was obtained over a concentration range of 0.25, 0.5, 1.0, 2.0 and 4 maximum residue limits (MRLs) with regression coefficients ranging from 0.9916 to 0.9993. Intra and inter-day (n=3) repeatability expressed as percent of the relative standard deviation (RSD%) were between 2.1-11.1% and 1.6-6.5 %. The LOQs were below than 2.5 µg/kg for danofloxacin and 15 µg/kg for other FQs. The recoveries in spiked milk samples at 0.5, 1 and 2 MRLs ranged from 69.2 to 104.8%. The validated method was successfully applied for the determination of FQs in milk samples, and the mean contamination of samples was lower than European legal limits.

1. Introduction

Antibiotics used widespread as a veterinary medicine and alternatively as a growth promoters in farm animals [1, 2]. Fluoroquinolones (FQs) the synthetic group of antibiotics are highly potent, that due to their activity against a wide range of Gram-negative and Gram-positive bacteria, widely used in treating of human diseases [3], also used in food producing animals for treatment of the respiratory diseases and enteric bacterial infections [4]. Residue of veterinary drugs in animal-based food could cause allergic reactions or antibiotic resistance in humans. Maximum residue limits (MRLs) have been established for eight FQs: marbofloxacin: MAR; ciprofloxacin: CIP; danofloxacin: DAN; enrofloxacin: ENR; sarafloxacin: SAR; difloxacin: DIF; flumequine: FLUME; and oxolinic acid: OXO by European Union (EU) [5] . EU has been set maximum residue limits (MRLs) for four FQs in milk, 30 µg/kg for DAN, 75 µg/kg for MAR and 100 µg/kg for the sum of ENR and CIP. Norfloxacin (NOR) and difloxacin (DIF) have been reported used in treatment of milk producer animals and residue of these compounds represent a potential hazard for human health [6]. Roca et al., showed that the residues of FQs in milk were not destroyed at high temperature and when heated to 120 ºC for 20 min, maximum loss was12.71% for CIP and 12.01% for NOR [7], also results of another research revealed the stability of NOR residue in chicken muscle against the cooking procedures [8]. The stability of FQs and high consumption of milk, more than 300 kg of milk/ year in some Europe countries [9], led to improve the determination methods for trace level of FQs in milk. Enzyme-linked immunosorbent assay (ELISA)[10], High performance liquid chromatography (HPLC)[11] or ultra-high performance liquid chromatography (UPLC), also capillary electrophoresis (CE) in combination with different detectors have been used for the determination of FQs in milk [12, 13]. The high fluorescence property of the most FQs, make fluorescence detector as a suitable detection method for quantification of FQs in food residue [14]. Sample preparation step has a critical role in analysis of residues in milk, because analytes are present at low concentrations in an aqueous matrix that containing highly concentrated lipids, proteins, vitamins [15, 16]. Liquid-liquid extraction, solid phase extraction (SPE) and recently, new methodologies, so-called Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS), have been proposed for the extraction of veterinary drug residues[17-25]. Dispersive liquid-liquid micro extraction (DLLME) as a fast extraction method which successfully used in food analysis [26, 27], and Yan et al used DLLME for analysis of four FQs in pharmaceutical waste water [28]. Moema et al analyzed 8 chicken liver for determination of six FQs residue by DLLME-DAD [29]. Geo et al applied DLLME for analysis of enoxacin, pefloxacin, NOR, ENR, sulfamethoxazole and sulfadimethoxine in milk samples [30]. The present study reports the use of QuEChERS-DLLME for extraction and determination of six FQs in whole milk sample, and SAR was used as internal standard (IS).

2. Experimental

2.1 Chemicals

Analytical standards (pestanal quality) of MAR, NOR, CIP,

DAN, ENR, SAR, and DIF were purchased from Sigma–Aldrich (Germany). The HPLC-grade acetonitrile, methanol, and all analytical grade extraction solvents were purchased from Merck (Darmstadt, Germany). Deionized water was prepared from a Milli-Q water purification system at 18.2 MΩ cm (Bedford, MA, USA). Citric acid monohydrate, trisodium citrate dihydrate, sodium chloride, sodium sulfate, and magnesium sulfate were purchased from Merck (Darmstadt, Germany). Individual 100.0 µg/mL stock standards of MAR, NOR, CIP, DAN, ENR, SAR and DIF were prepared by dissolving of each FQs in Methanol [31]. These solutions were stored at – 20 ° C. A 10.0 µg/mL of SAR as internal standard [32], 10.0 µg mL $^{-1}$ mixtures of NOR, CIP, DAN, and ENR and a 2.5 µg/mL MAR and DAN was prepared by appropriate combination and dilution of stock standards solutions with deionized water. These standards were kept at 5 ° C for no more than 10 days.

2.2 Instrumentation

HPLC analysis was carried out on a Waters Alliance 2695 separations Module coupled to a Waters 474 scanning fluorescence detector (Waters Corp, Milford, Massachusetts). The LC column was Zorbax Eclipse XDB-C18 (150× 4.6 mm, 5 µm) (Agilent Technologies). The mobile phase, consisting of citric acid/citrate buffer 10 mmol/L of pH 4.5, acetonitrile and methanol, in the gradient mode, is described in Table 1. Separation was carried out at 20 ° C, and the fluorescence detection was performed at the following λ ex. and λ em.: from zero to 8 min 294 and 514 nm (MAR), from 8 to 30 min was 278 and 466 nm (NOR, CIP, DAN, ENR, SAR and DIF) respectively [33].

Table 1. Gradient program used for the separation of quinolones

Time (min)	Citrate buffer (%)	Methanol (%)	Acetonitrile (%)
0	79	14	7
21	79	14	7
28	64	14	15
28.1	79	14	7

2.4. Sample treatment

In this study, we developed QuEChERS –DLLME technique for extraction of quinolones from whole cow milk samples.

2.4.1. QuEChERS

Two gram milk was placed into 50 mL centrifuge tube, spiked at MRLs and 300 ng IS was added, spiked sample stand in dark place for 10 min and two different procedures was performed:

In first method (Fig.1a), 8 mL of 30 mM NaH_2PO_4 buffer pH 7.0 was added to spiked sample and shaken for 30 seconds. Ten mL of 5% formic acid in acetonitrile was added to the tube, shaking by hand for 30 s, then a mix of 2 g $MgSO_4$ and 1.5 g CH_3COONa was added to the tube then vigorously was shaken for 1 min [21].

In the second method (Fig. 1b), the spiked milk sample was diluted by 4 mL water and mixed by vortexing for 1 min, then 10 mL 1% acetic acid in acetonitrile was added, the extraction was performed by shaking the mixture for 1 min. Other QuEChERS mix salts (4 g Na_2SO_4, 1 g NaCl) was added and vigorously shaken for 1 min[34].

After this step, samples of two methods were centrifuged for 5 min at 9000 rpm, 3 mL of supernatant transfer to two different tubes and dried under nitrogen stream at 30 ºC. The residue was resolved in 500 µL mobile phase then filtered through a 0.45 µm membrane, and 20 µL of this sample was injected to HPLC. The results showed that, the co-extracted interferences with MAR in second method were lower than first method, so this QuEChERS method was used for QuEChERS-DLLME method validation. The clean-up step with PSA and $MgSO_4$ significantly decreased the area under curve so this step was pretermitted in our research.

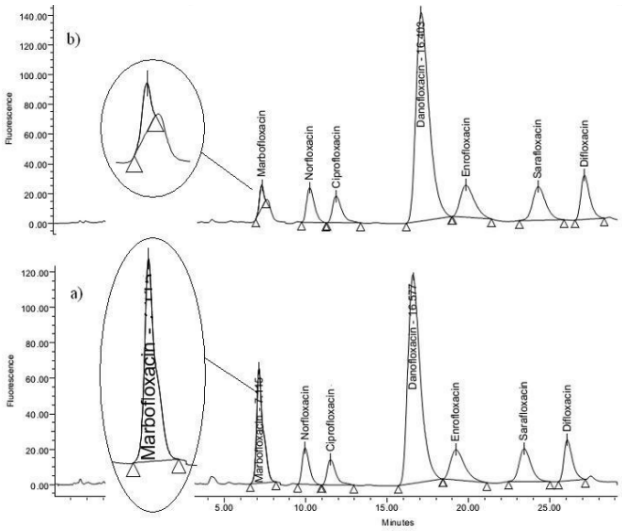

Fig 1. Chromatogram of milk spiked sample at MRLs and effect of interferences in MAR retention time when samples extracted via, a) first QuEChERS method and b) second QuEChERS method

2.4.2 QuEChERS -DLLME

The dried supernatant in the QuEChERS procedure was resolved in 1000 µL acetonitrile- acetic acid 10%, and 200 µL chloroform and then was shaken for 30 s; this solution was dispersed quickly into the 4 mL deionized water via a 1 mL Hamilton syringe and a cloudy state was formed in the test tube. After centrifugation at 4500 rpm for 5 min, the dispersed fine droplets of chloroform were sediment in the bottom of the conical test tube. The upper phase was removed and organic phase was dried under nitrogen stream at 30 ºC. The residue was resolved in 500 µL mobile phase and filtered through a 0.45 µm membrane before HPLC analysis.

3. Results and discussion

3.1 Optimization of DLLME

In order to reach optimum experimental conditions for quantitative extraction of FQs by QuEChERS -DLLME, the effect of different parameters such as type and volume of the extraction, disperser solvent and effect of acetic acid were evaluated by spiking the blank samples with 75 µg/kg for MAR, 30 µg/kg for DAN and 100 µg/kg for NOR, CIP, ENR and DIF. Each step of evaluation and optimization of the method was repeated for 3 times.

3.1.1 Type and volume of the extraction solvent

The extraction solvent for DLLME should have three important properties (higher-density than water, because of rapid accumulation at the bottom of the test tube; Low solubility in aqueous phase and high extraction capacity for the target analyte). By considerations of these parameters, carbon

tetrachloride (CCl_4, 1.59 kg/L), chloroform ($CHCl_3$, 1.47 kg/L), and carbon disulfide (CS_2, 1.2 kg/L) were selected as extraction solvents and the efficacy of extraction by these solvents were studied. The residue of QuEChERS method was resolved in 1000 µL acetonitrile, and 100 µL of extraction solvent was added and then subjected to the DLLME procedure, a stable cloudy solution for all of the extraction solvents were formed. Based on the obtained results (Fig. 2) $CHCl_3$ was selected as the best extraction solvent.

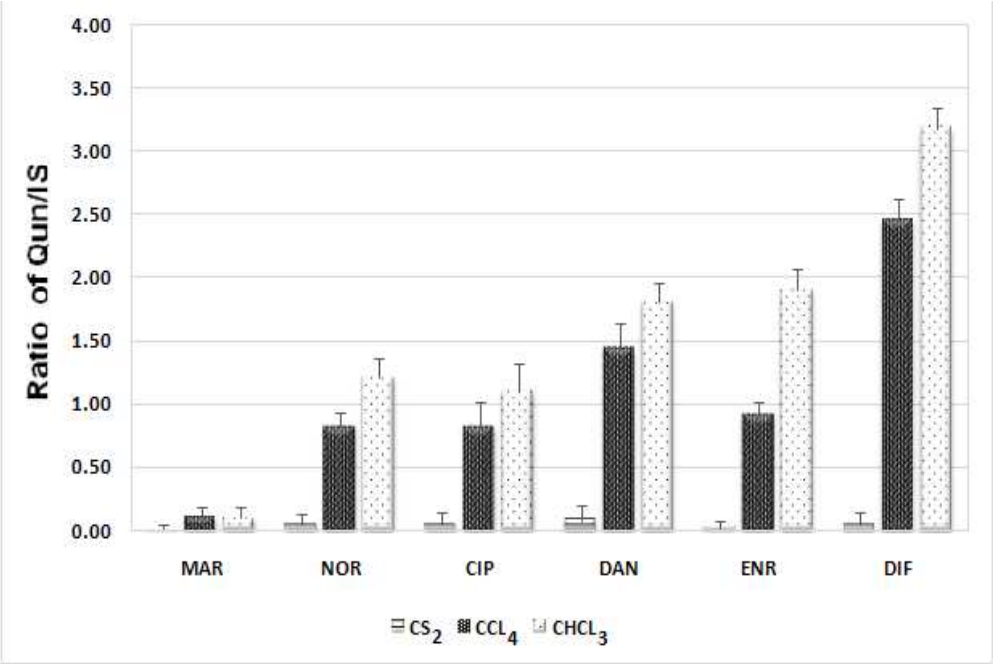

Fig 2. Effect of different extraction solvents on FQs extraction efficiencies

In order to evaluate the effect of extraction solvent volume, different volumes of $CHCl_3$ (100, 150, 200, 250 and 300 µL) were added to1000 µL acetonitrile and the efficacy of extraction was evaluated (Fig. 3). The efficiency of extraction by 200 µL of $CHCl_3$ was better than the other volumes, so this volume of $CHCl_3$ was selected for subsequent experiments.

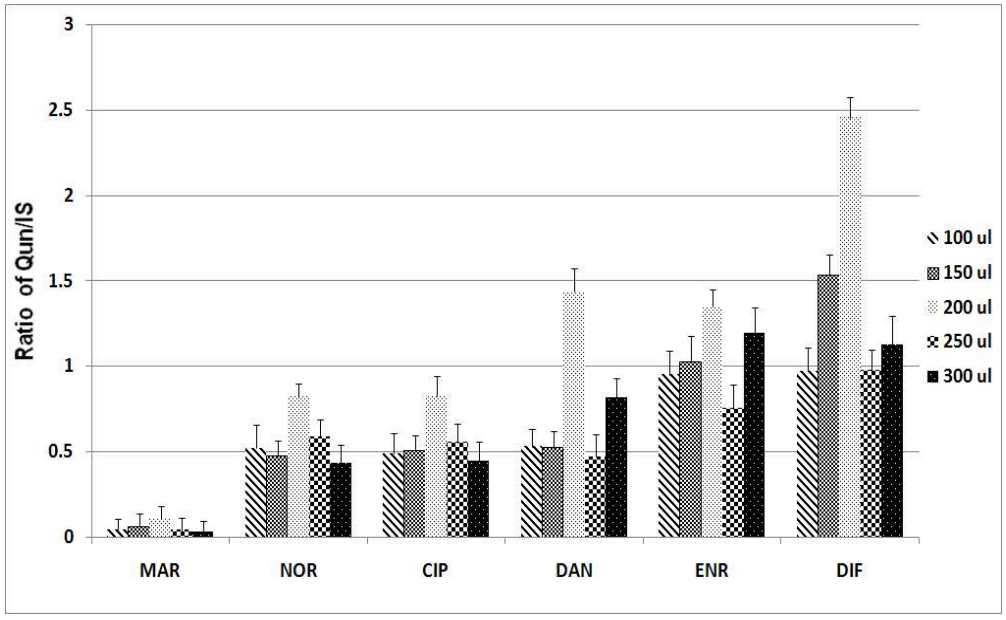

Fig 3. Effect of different volumes of extraction solvent on FQs extraction efficiencies

3.1.2 Type and volume of the dispersive solvent

The most important criterion for choosing a disperser solvent is

its miscibility in the organic phase and aqueous sample. At this study the good solubility of the residues of FQs after QuEChERS step in dispersive solvent is other important parameter. By considering of these factors only methanol (MeOH) and acetonitrile (MeCN) were selected as disperser solvents. The dried residue of QuEChERS extraction step was separately dissolved in 1000 µL of MeOH and MeCN. DLLME procedure was applied by rapidly injection of a mixture of dispersive solvents that contain 200 µL $CHCl_3$. Better results were achieved by using MeCN, so this solvent was selected as disperser in DLLME procedure (fig. 4).

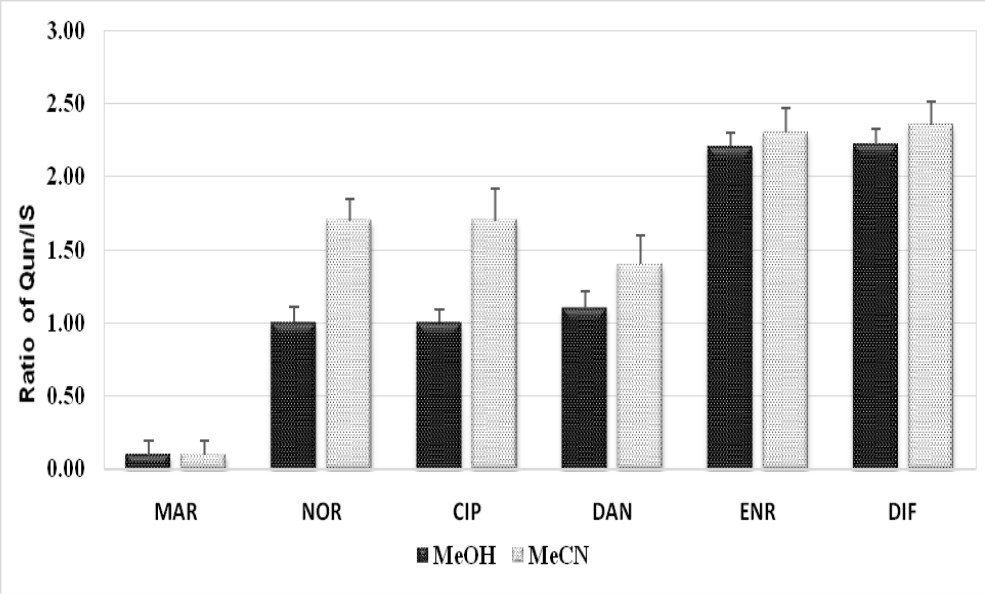

Fig 4. Effect of disperser solvents on FQs extraction efficiencies

The dispersive solvent volume is one of the significant factors in extraction efficiency. When volume of disperser was high, the solubility of FQs in aqueous phase increased which caused lowering the analyte partition with extractant droplets and decreased the extraction efficiency. The lower volumes of the disperser form the tiny droplets with low ability of extraction, so cause a decrease in the extraction efficiency. To determine the optimal MeCN volume, a series of MeCN volumes (500, 1000, 1500 and 2000 µL) that contain 200 µL $CHCl_3$ were examined. It was observed that with 1000 µL of MeCN extraction was more efficient than other volumes (Fig. 5).

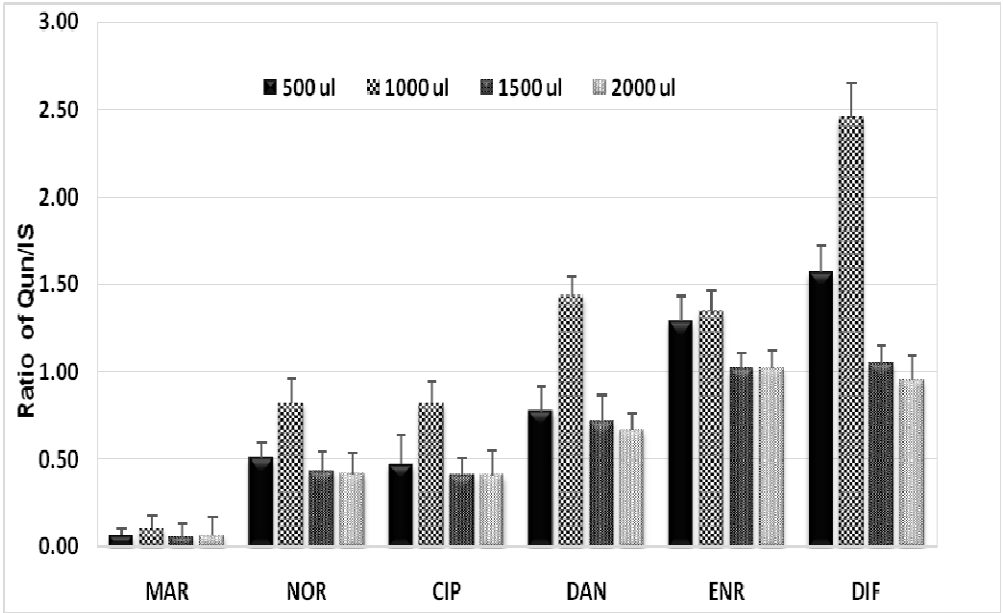

Fig 5. Effect of different volumes of disperser solvent on FQs extraction efficiencies

3.1.3 Effect of acetic acid in disperser solvent

FQs have two p*K*a values; therefore, their molecular structure will be considerably affected by pH. To investigate the effect of pH of solvent for extraction of the FQs, the pH of the disperser

solvent was changed by adding different amounts of acetic acid. As Fig. 6 shown, the efficiency of the extraction increased as the amount of acetic acid increased until 10%, but higher than 10%, FQs being poorly recovered. This phenomena may be linked to the change in the ionic form of the FQs [35]. Also results showed that DAN, ENR and DIF are more sensitive to amount of acetic acid.

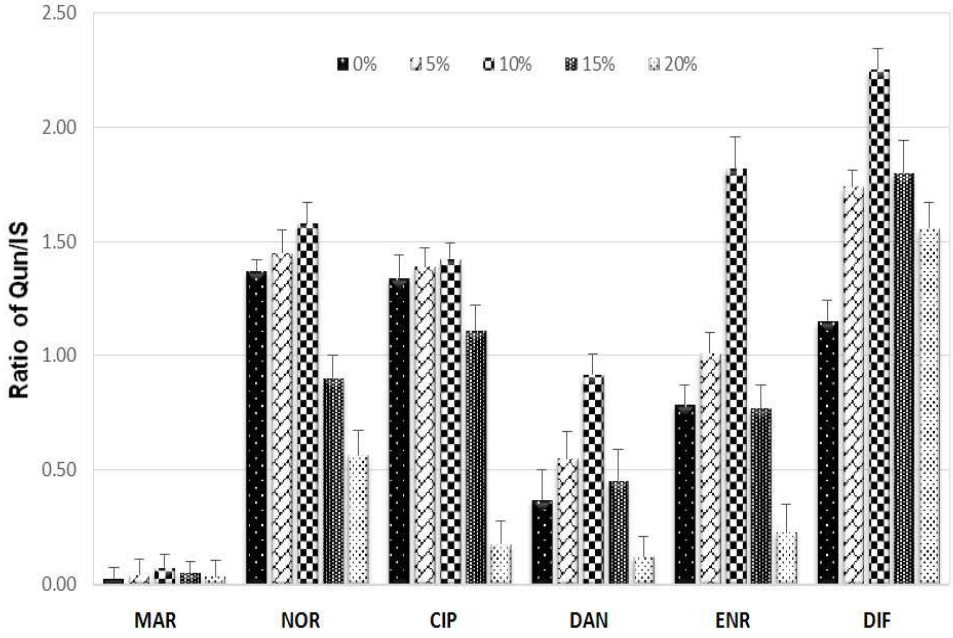

Fig 6. Effect of different concentrations of acetic acid on FQs extraction efficiencies

4. Method validation

4.1 Precision

Precision was determined by intra-day assay (within-day repeatability) and inter-day assay (between-day repeatability). Three levels (0.5, 1 and 2 MRL) were prepared by spiking the blank milk samples and extracted by QuEChERS-DLLME method. The procedure was repeated in three different days; the recovery and percentage of relative standard deviation (RSDs %) were calculated (Tab.2).

Table 2. Recovery of QuEChERS -DLLME method for different levels of spiked samples

	Intra-day (n=3) Recovery ±RSD %			Inter-day (n=3) Recovery ± RSD %		
	0.5 MRL	1 MRL	2 MRL	0.5 MRL	1 MRL	2 MRL
MAR	74.3±2.1	101.2±7.4	99.3±3.7	80.2±4.8	104.8±5.3	96.8±4.9
NOR	79.4±5.1	92.3±7.0	90.4±5.6	77.3±2.6	93.8±2.2	88.8±2.3
CIP	82.1±8.5	83.7±5.3	89.8±4.1	85.6±4.3	84.6±2.8	94.4±2.9
DAN	69.2±3.4	75.1±6.8	78.2±9.9	70.2±6.4	78.6±3.6	74.1±6.2
ENR	91.0±11.1	97.1±5.2	90.5±7.3	75.3±7.1	93.0±4.4	97.4±5.5
DIF	84.7±7.9	93.1±6.6	95.4±4.6	81.2±3.9	99.7±1.6	101.4±6.5

4.2 Calibration curve

Because of differences between area under curve (A.U.C) in real standard solutions and in spiked samples at same concentrations, we have used matrix match calibration curves [36]. Based on MRLs, different ranges of matrix match calibration curve of FQs were constructed over a concentration range of 0.25, 0.5, 1.0, 2.0 and 4 MRL. The calibration curve points were prepared by spiking of 2.0 g blank milk sample at mentioned concentrations and adding 300 ng IS. The spiked samples were analyzed by using the validated QuEChERS-DLLME-HPLC-FLD method. Peak area ratios (antibiotic/IS) were plotted against the equivalent antibiotic nominal concentrations. Least-squares linear regression analysis of the calibration data was performed. The limits of detection (LODs) and limits of quantification (LOQs) were obtained by decreasing the spike levels and diluting up to 2.5 µg/kg for DAN and 15 µg/kg for other FQs until the peak height of analytes was 3 and 10 times of the back ground noise. The

LODs and LOQs data were shown at table three [37].

4.3 Analysis of cow milk samples

The validated method was applied for the quantitative determination of FQs residues in 17 milk samples obtained from retail store in November 2013. The whole milk samples were analyzed by QuEChERS-DLLME. Fig.7 shows the chromatograms of blank and spiked samples at MRLs that extracted by QuEChERS-DLLME method. Between all samples only one of them was contaminated to DAN; 9.2 µg/kg.

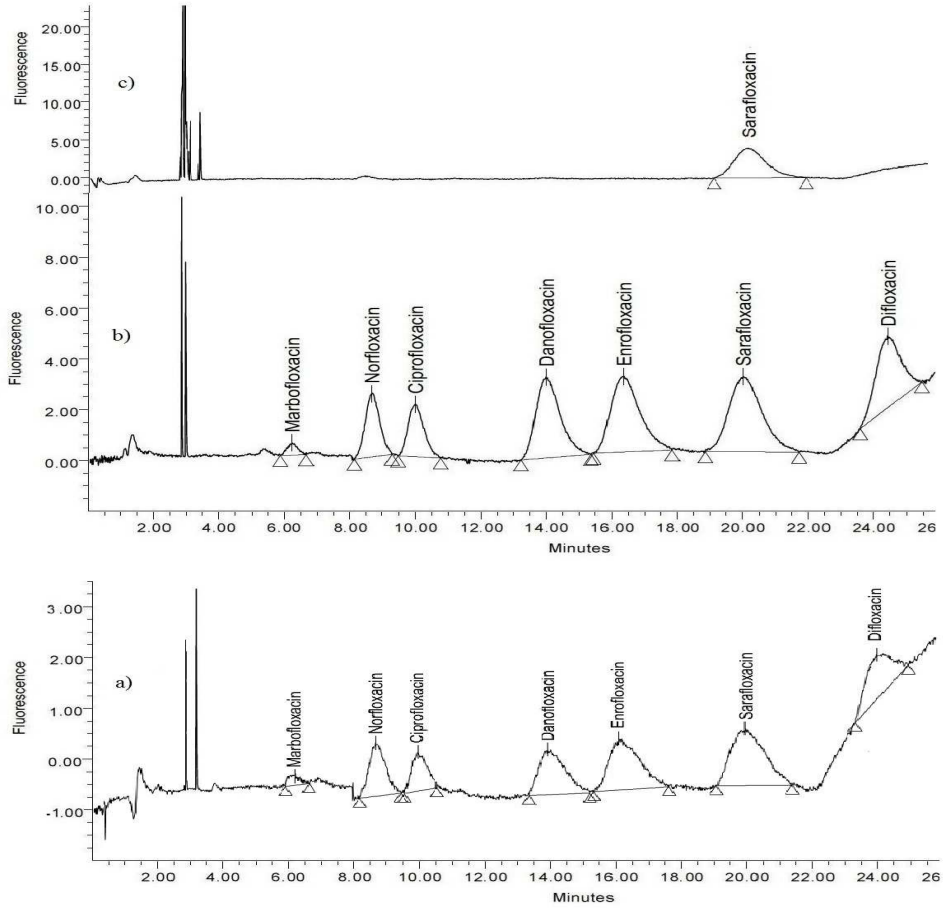

Fig 7. The chromatograms of, a) The LOQs of method, b) A spiked milk sample at MRLs of FQs, extracted by QuEChERS-DLLME method, c) A blank milk sample, extracted by QuEChERS-DLLME method

Table 3.Calibration data, LODs, LOQs and R^2 of QuEChERS and QuEChERS-DLLME for FQs in milk

	MRL (µg/Kg)	Range of Concentration (µg/Kg)	R^2	LOD (ug/kg)	LOQ (ug/kg)
MAR	75	18.0-300	0.9976	5.0	15.0
NOR	-	25.0- 400	0.9961	5.0	15.0
CIP	100	25.0- 400	0.9969	5.0	15.0
DAN	30	5.0- 100	0.9916	0.8	2.5
ENR	100	25.0- 400	0.9979	5.0	15.0
DIF	-	25.0- 400	0.9993	5.0	15.0

5. Conclusions

This study has developed the novel, simple, fast and environmentally friendly application of QuEChERS–DLLME-HPLC-FLD method for determination of the multiple FQs in milk samples. Acceptable recoveries were obtained (69.2-104.8%), and the method validation parameters such as LODs, LOQs, also good linearity over the investigated concentration range, revealed

the applicability of method for quantitative determination of FQs. The LOQs were below than 2.5 µg/kg for DAN and 15 µg/kg for other FQs. In our research MeCN was a good disperser solvent and in classical liquid-liquid extraction, MeCN as extraction solvent decreased co-extraction of lipids and provides high extraction recoveries[24]. Co-extracted interferences in QuEChERS method was the main problem for detection of MAR in milk sample [38] and this problem was resolved by coupling the QuEChERS and DLLME methods. Moema et al, developed DLLME for six FQs in chicken liver; same as our results, they showed that acidification of MeCN could improve the extraction efficiency [29]. The RSDs% of intra-day and inter day indicated that this method have good repeatability and reproducibility. The validated method was successfully applied for the determination of FQs in milk samples, and contamination over the EU MRL was not detected.

6. Acknowledgements

The authors gratefully acknowledge the financial support extended for this project (Project no.6609) by the Shiraz University of Medical Sciences, Iran.

Notes and references

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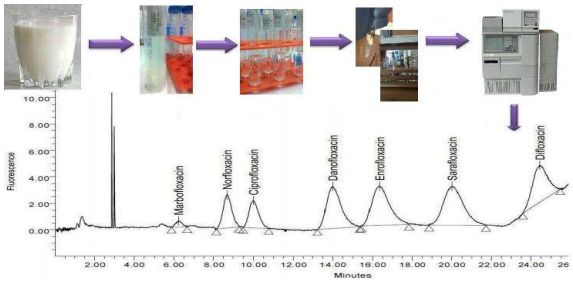

We developed a novel, simple, fast and environmentally friendly extraction method (QuEChERS–DLLME) for determination of fluoroquinolones in milk samples.