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Determination of Fluoroquinolones in Milk by High-Performance Liquid Chromatography Using Mixed-Templates Imprinted Polymer Extraction

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A high-performance liquid chromatography-diode array detection method (HPLC-DAD) for the determination of fluoroquinolone antibiotics in milk has been developed using molecularly imprinted polymer solid phase extraction (MISPE). The molecularly imprinted polymer (MIP) has been prepared using levofloxacin and ciprofloxacin as the templates. The imprinted polymer has been characterized by equilibrium binding experiment to investigate the binding ability to template molecule. Various parameters affecting the extraction efficiency of the polymer in the MISPE procedure have been evaluated to achieve optimal preconcentration. The fluoroquinolones are extracted from milk with 2 % acetic acid in acetonitrile, purified on the MISPE cartridge and then analyzed using HPLC-DAD set at 281 nm. The method has been validated by analyzing spiked milk samples at two levels (100 and 200 μg kg^{-1}), the mean recoveries of the method range from 84.1 to 104.7 % with relative standard deviation no more than 4.3 % (n = 3) for fleroxacin, enoxacin, pefloxacin, norfloxacin, ciprofloxacin, levofloxacin, lomefloxacin, enrofloxacin, gatifloxacin and sparfloxacin. The method detection limits range between 1.34 and 7.35 μg kg^{-1}. The method was demonstrated to be suitable for the sensitive and accurate quantification and confirmation analysis of fluoroquinolones.

Key Words: Milk, Mixed-templates imprinted polymer, Solid phase extraction, Fluoroquinolones, Antibiotics.

INTRODUCTION

Fluoroquinolones (FQs) are derivatives of quinolones (QNs) that have a common quinolone skeleton with a fluorine atom and a piperazinyl group placed at positions 6 and 7[1], respectively. The chemical structures of the antibiotics are shown in Fig. 1. They are used as inhibitor for the synthesis of bacterial DNA and their primary targets are bacterial DNA gyrase and topoisomerase IV enzymes which are very important for DNA replication and transcription[2]. As synthetic broad-spectrum antibiotics widely used in human and veterinary medicine over the last two decades, FQs have several characteristics such as efficient, low toxicity, strong tissue penetration and low price. Due to the extensive application of FQs in human and edible animal, the residue of the antibiotics is easy to cause the appearance of drug resistant strains, so FQs become the key monitoring objects[3-5] of many countries.

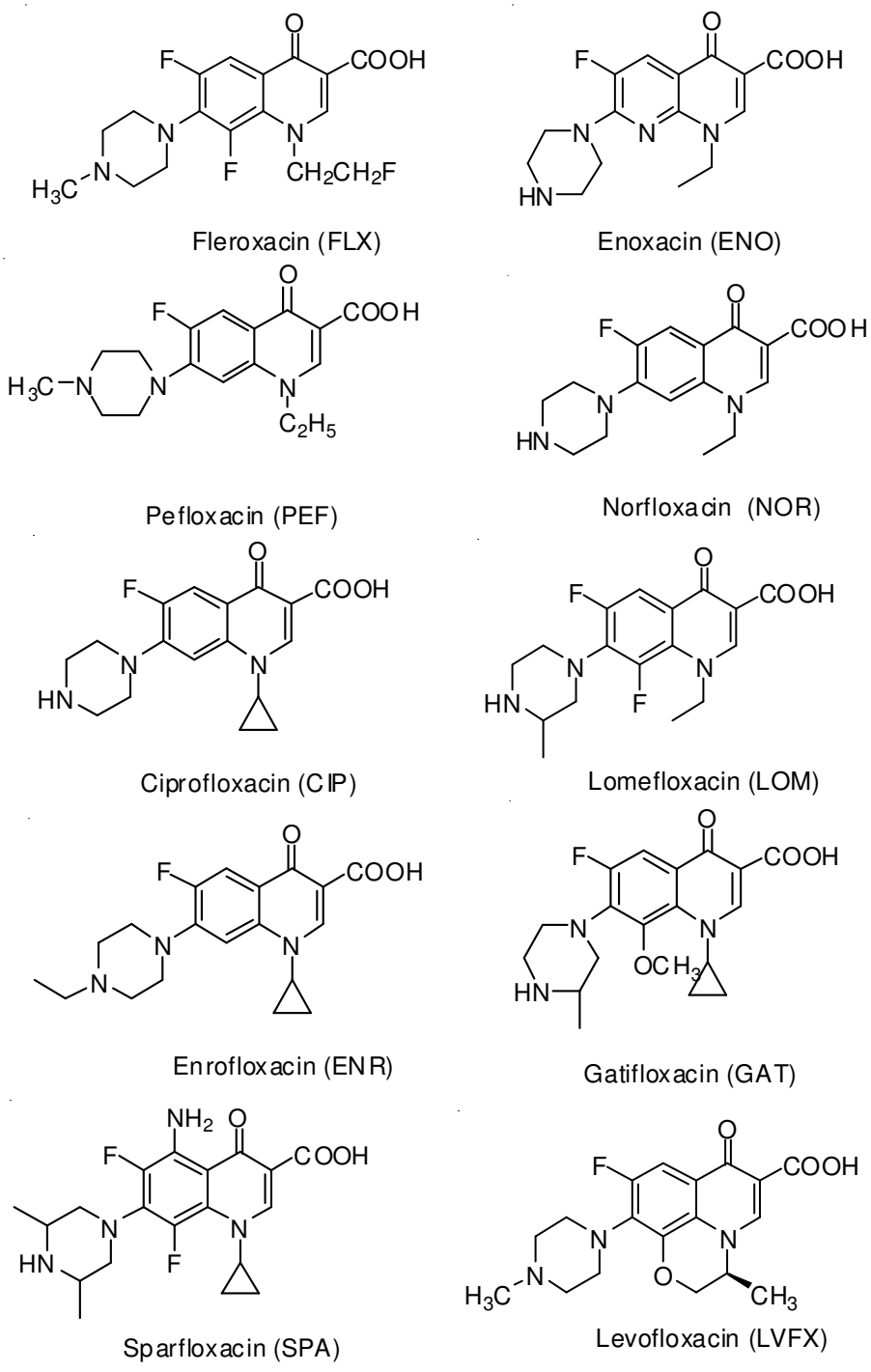

Fig. 1. Molecular structures of different fluoroquinolones

Several methods have been reported for the analysis of FQs in biological samples[6,7], animal derived food[8,9] and environmental samples[10,11]. Most of them have generally taken advantage of high performance liquid chromatography (HPLC) equipped with diode array[12], ultraviolet[13], fluorescence[14] and MS detector[15]. The complexity of matrix and impurities can interfere with qualitative and quantitative severely, hence it is necessary for samples to make further cleanup before analysis. Some sample treatment methods have been used recently including pressurized liquid extraction (PLE)[11], supercritical fluid extraction (SFE)[16], solid phase micro-extraction (SPME)[10] and conventional solid phase extraction (SPE)[17]. Among them, some demand special instrumentation (PLE or SFE) not obtained in every laboratory, the high expense of SPME makes it difficult to extend. Compared to better purification effect of commercial SPE cartridges, one substantial shortcoming of commercial SPE sorbents is their low adsorption selectivity towards target analytes. Therefore, it is a perfect preparation method for purification and separation of FQs in complex matrix to purify the samples using FQs imprinted polymers.

Molecularly imprinted solid phase extraction (MISPE)[18] which has recently become a new extraction technique is the combination of molecularly imprinted technique (MIT) and SPE. Molecularly imprinted technique is a technology to prepare polymers (molecularly imprinted polymer, MIP) that can recognize a target or a class of target molecules. At first, a template molecules and functional monomers form a complex *via* non-covalent interactions. Then these interaction sites are fixed through polymerization with a crosslinker added. Before obtaining binding cavities capable of recognizing the template molecules, the template must be eliminated with organic solvent to create the imprinted cavities that are complementary to the structure of template. Using imprinted polymers as SPE sorbents allows not only selective separation of the target analytes but also preconcentration and cleanup of the samples, which is important particularly when complex matrix disturb the analysis. Molecularly imprinted solid phase extraction has been employed for the analysis of FQs in several matrices, such as environmental samples (soil[19], river water[20]), biological samples (urine[21,22]) and animal derived food[23]. Single-template imprinted polymers are used as sorbents in reports above mentioned, but the preparation and characterization of the FQs mixed-template imprinted polymers has not been reported yet.

Mixed-template or multi-template imprinted polymers[24-28] refer to the preparation of the polymers using more than one compound as templates. Since the selectivity and affinity of polymers is connected with the rigidity and steric hindrance of the template, compounds with similar structure can be used together as templates to get stronger selectivity and affinity of the polymers towards allied compounds. Consequently, this property can be applied to multi-residue analysis. In this paper mixed-template imprinted polymers are prepared with ciprofloxacin and levofloxacin as templates using a procedure reported previously[29]. The polymers show excellent recognition properties and high affinity for templates and were applied successfully to the extraction of FQs from milk samples. The prepared polymers are able to bind with all ten FQs. The method is validated by spiked milk samples and demonstrates good recoveries (84.1-104.7 %) and reproducibility (RSD < 4.3 %).

EXPERIMENTAL

Ciprofloxacin (CIP), levofloxacin (LVFX), fleroxacin (FLX), enoxacin (ENO), pefloxacin (PEF), norfloxacin (NOR), lomefloxacin (LOM), enrofloxacin (ENR), gatifloxacin (GAT), sparfloxacin (SPA) were supplied by Beijing Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). α-Methylacrylic acid (MAA), methylbenzene, methanol, acetic acid, all analytically pure, were obtained from Tianjin. Azobisisobutyronitrile (AIBN) was purchased from Shanghai No. 4 Reagent and H.V. Chemical Co., Ltd (Shanghai, China). Trimethylolpropane trimethylacrylate (TRIM) was provided by Tianjin Tianjiao Chemical Co., Ltd (Tianjin, China). Hexane was from Tianin Reagent Chemicals Co., Ltd (Tianjin, China). Ammonia and triethylamine were supplied by Beijing Chemical Works (Beijing, China). Sodium dihydrogen phosphate was purchased by Qinhuangdao Chemical Reagent Factory (Qinhuangdao, China).

HPLC-grade methanol (MeOH) and acetonitrile (ACN) were obtained from Tianjin Kermel Chemical Reagent Co., Ltd. (Tianjin, China) and water was purified with a RZ-93 automatic double pure water distiller (Shanghai Ya Rong Biochemistry Instrument Factory, Shanghai, China). All solutions prepared for HPLC were passed through a 0.45 μm nylon filter before use. A phosphate buffer solution (0.02 mol L^{-1}), pH 2.8, was prepared by dissolving 1.560 g sodium dihydrogen phosphate, ammonia and triethylamine in 1 L of double distilled water.

Chromatographic analysis was carried out with a LC-10A HPLC from Shimadzu Corporation (Kyoto, Japan) equipped with two LC-10AT pumps, a DGU-12A degasser, a SIL-HTA auto-sampler, a CTO-10AS column oven and a SPD-M10A diode array detector (DAD). The pH of the buffer solution was adjusted with a PHS-2C Precision pH/mV Meter (Shanghai LIDA Instrument Factory). LG10-2.4A Centrifuge (Beijing Jingli Centrifuge Co., Ltd.), THZ-82 Constant-temperature Shaker (Changzhou Guohua Electric Appliance Co., Ltd), KQ-250B Ultrasonic Cleaner (Kunshan Ultrasonic Instrument Co., Ltd), BF-2000M Nitrogen Evaprator (Beiing Bafang Century Technology Co., Ltd), WH-861 Vortex Shaker (Taicang Hua Li Da Laboratory Equipment Co., Ltd).

Chromatographic separation of the fluoroquinolones was performed on an Agilent HC-C18 (250 mm × 4.6 mm I.D., 5 μm) HPLC column protected by a Shim-pack GVP-ODS column(10 mm × 4.6 mm I.D., 5 μm). The mobile phase included solvent A (MeOH) and solvent B (phosphate buffer solution). Analyses were performed at a flow rate of 1.0 mL min^{-1} and the column temperature was kept at 40 ºC. The injection volume was 10 μL and the detection wavelength of DAD was programmed at 281 nm. The mobile phase ratio varied with the FQs: For FLX, LVFX, ENO, NOR, CIP, ENR and LOM, the ratio comprised with 22 % A and 78 % B. For PEF, GAT and SPA, the ratio comprised with 28 % A and 72 % B. All the compounds eluted within 0.5 h and chromatograms for standard solutions of ten FQs can be seen in Fig. 2.

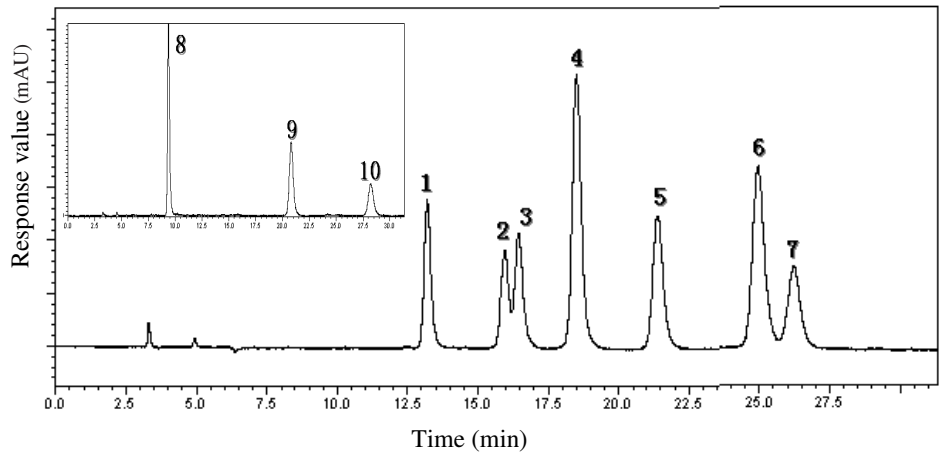

Fig. 2. Chromatograms of standard solutions (1 μg mL^{-1}) for 10 fluoroquinolone antibiotics. Mobile phase (A/B) = 22/78: (1) FLX; (2) LVFX; (3) ENO; (4) NOR; (5) CIP; (6) ENR; (7)LOM. Inset: mobile phase (A/B)=28/72: (8) PEF; (9) GAT; (10) SPA

Standard solutions: Standard stock solutions (250 μg mL^{-1}) were prepared in methanol and stored at -18 ºC for no longer than 3 months. Intermediate and working standard solutions were prepared daily by diluting with the mobile phase.

Calibration standard solutions were prepared at concentrations of 0.05, 0.1, 0.2, 0.5, 1.0 and 2.0 μg mL^{-1} for each fluoroquinolone and injected in replicates of three.

Preparation of the mixed-template imprinted polymer: The mixed-template imprinted polymer was prepared as follows: the functional monomers MAA (517.4 mg, 6 mmol) and the template molecules comprised with CIP (165.6 mg, 0.5 mmol) and LVFX (180.6 mg, 0.5 mmol) were weighed into a flask, dissolved in 6 mL toluene and then ultrasonicated to ensure complete association. TRIM (5.408 g, 16 mmol) and AIBN (56 mg) were added to the flask and the mixture was subjected to further ultrasonication. The flask was degassed with nitrogen for 10 min and sealed immediately before polymerization at 60 ºC for 24 h in a water bath. After this period, the flask was broken and the monolith obtained was crushed, ground and then filter through sieves with mesh sizes of 200-300 to get polymer particles with diameters between 54 and 74 μm. The material was washed with methanol-acetic acid (8/2 v/v) solution under ultrasonication to eliminate the template molecules. After ultrasonication, the mixture was centrifuged at 6000 rpm and the supernatant was removed. The washing procedures were repeated until no template molecule in the supernatant could be detected *via* HPLC-DAD. After that, methanol was used to wash the particles until pH 7. The mixed-template imprinted polymer (MIP) was obtained after drying at room temperature.

A non-imprinted polymer (NIP) was prepared in the same way except for the absence of CIP and LVFX.

Equilibrium binding experiment: The polymer particles (50 mg) were mixed with a 5 mL water solution containing different amounts of CIP and LVFX (0.1-4 mmol L^{-1}) in a 25 mL colorimetric tube. The tube was put in a constant-temperature shaker and vibrated at 25 ºC for 16 h. After vibration, the supernatant was collected and analyzed by HPLC. The calculation of the adsorption capacity of the polymer was based on the concentration change before and after the adsorption. The binding experiments were carried out by triplicate.

Optimized extraction procedure of FQs using MIP cartridges: Solid phase extraction cartridges (Supelco, Bellefonte, PA, USA) with a 3 mL volume, were packed with 200 mg imprinted polymer. The cartridges were equilibrated with 3 mL methanol and 3 mL water. The FQs mixture dissolved in the mobile phase was percolated at a constant flow rate of 0.5 mL min^{-1}. The cartridges were rinsed with 3 mL water to wash out the impurities bound to imprinted polymer. At last, the FQs were eluted with 3 mL methanol with 4 % ammonia. The eluents from the MISPE cartridges were evaporated to dryness at 45 ºC under a stream of nitrogen. The residues were dissolved in 1mL mobile phase subsequently. The mixture was filtered through a 0.22 μm filter for HPLC analysis.

Sample preparation: For every fluoroquinolone, blank milk samples as control and milk samples with spiked levels at 100 and 200 μg kg^{-1} have been analyzed. The samples (5 g) were weighed into a 50 mL polypropylene centrifuge tube and fortified with FQs. Samples were homogenized firstly on a vortex shaker for 1 min and then left in the dark for 20 min to enable sufficient equilibration with the milk matrix. After addition of 7 mL acetonitrile with 2 % acetic acid, the samples were shaken again for 1 min, ultrasonicated for 10 min and centrifuged at 10000 rpm for 10 min to precipitate the protein, the supernatant was decanted into another 50 mL polypropylene centrifuge tube. The above procedure was repeated with 5 mL acetonitrile with 2 % acetic acid and the supernatant was combined. 6 mL of the supernatant was pipetted on to a 15 mL polypropylene centrifuge tube. After being evaporated to 2 mL at 60 ºC under a stream of nitrogen, the supernatant was defatted with hexane. The upper, hexane layer was removed and the underlayer continued to be evaporated to 1 mL for MISPE procedure described in "optimized extraction procedure of FQs using MIP cartridges".

RESULTS AND DISCUSSION

Equilibrium adsorption experiment: The static equilibrium adsorption experiments for the imprinted and non-imprinted polymer were carried out by varying the initial concentration of CIP and LVFX in the range of 0.1-4 mmol L^{-1}. The adsorption isotherms were shown in Fig. 3.

It can be concluded from the curve in Fig. 3 that, the adsorbance of MIP increased with the increment of the initial concentration and the adsorbance of NIP reached saturation when the initial concentration of CIP and LVFX was beyond 1 mmol L^{-1}. Obviously, the adsorbance of MIP was larger than that of NIP, which

demonstrated that the cavities formed on MIP by selective bonding and the active binding sites in cavities determined that high affinity and specific recognition of MIP on the template were much larger than the non-selective bonding interaction. In reports on molecule imprinting, the Scatchard Model was often used to evaluate the binding characteristics of MIP and the Scatchard equation (eqn. 1) can be described as[30]:

$$Q/C = (Q_{max}-Q)/K_d \quad (1)$$

where K_d is the dissociation constant of the binding site, Q_{max} is the maximum binding capacity of the binding site and C is the equilibrium concentration of the substrate in the supernatant.

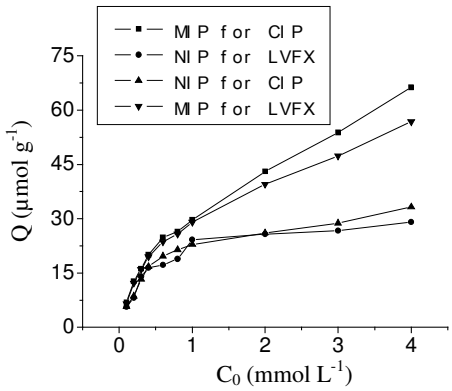

Fig. 3. Adsorption equilibrium isotherm of MIPs and NIP for CIP or LVFX

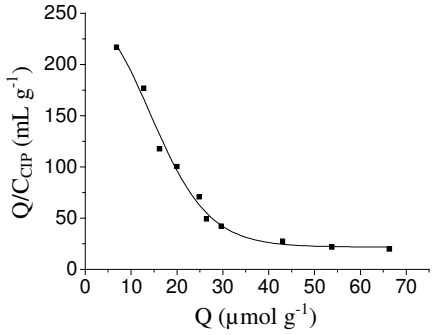

Fig. 4. Scatchard plot of MIPs for CIP

Q/C_{CIP} and Q/Q_{LVFX} were plotted *versus* Q in Figs. 4 and 5, respectively. From Figs. 4 and 5, it can be seen that Q/C *versus* Q shows an apparent non-linear relationship. It illustrated that the binding sites of MIP for CIP and LVFX were heterogeneous, but there were good linear relationships at both ends of the graph. According to this, it can be concluded that there existed two classes of binding sites with different affinities in the range of the different concentration. It is probably because that there were various interactions between the functional monomers and imprinted molecules and the interactions formed many kinds of complexes with different components. Various complexes have binding sites with different properties after polymerization. The two sections of the linear relationship in Figs. 4 and 5 were fitted respectively to get the fitting linear equations. According to eqn. 1, K_d and Q_{max} can be calculated from the slope and intercept of the linear equations (eqns. 2-5), shown in Table-1. The data obtained were listed in Table-1.

For the MIP with different binding sites, there were errors in the analysis with the Scatchard Model. Thus, a Multi-Point Model was used to get the data fitted according to the Multi-Point Model formula (eqn. 6)[31]:

$$Q = Q_{max,1}C/(K_{d1} + C) + Q_{max,2}C/(K_{d2} + C) \quad (6)$$

TABLE-1
FITTING LINEAR EQUATIONS, CORRELATION COEFFICIENTS (R), THE DISSOCIATION CONSTANT OF BINDING SITE (K_d) AND THE MAXIMUM BINDING CAPACITY OF BINDING SITE (Q_{max}) OF MIP

Polymer	Adsorption fitting of CIP	Adsorption fitting of LVFX
Higher affinity binding site	Q/C=235.799-7.264Q (2) (R = 0.9952)	Q/C=273.3-8.471Q (4) (R = 0.9867)
Lower affinity binding site	Q/C=63.063-0.877Q (3) (R = 0.9375)	Q/C=57.048-0.605Q (5) (R = 0.9362)
K_{d1} (mol L^{-1})	1.181×10^{-4}	1.377×10^{-4}
$Q_{max,1}$ (μmol g^{-1})	32.3	32.5
K_{d2} (mol L^{-1})	1.652×10^{-3}	1.141×10^{-3}
$Q_{max,2}$ (μmol g^{-1})	94.2	71.9

By substitution of the dissociation constant of the Scatchard Model into the Multi-Point Model formula (eqn. 6), curve fitting was done and the result was shown in Fig. 6. The obtained fitting curve fitted well with the experimental results.

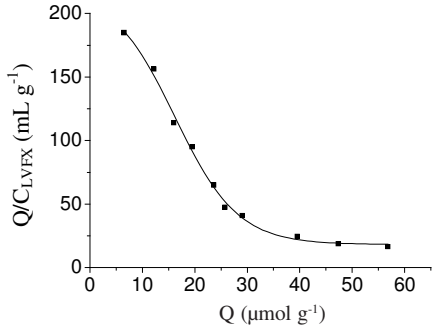

Fig. 5. Scatchard plot of MIP for LVFX

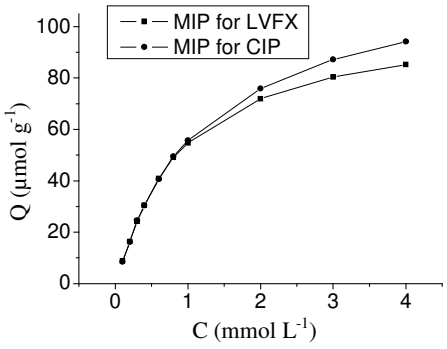

Fig. 6. Non-linear fitting for the multi-binding model of MIP for CIP and MIP for LVFX

MISPE procedure

Optimization of the sample loading flow rate: To evaluate the effect of the sample loading flow rate on FQs recoveries, 5 mL of a solution of the FQs (0.5 μg mL^{-1}, in mobile phase) were loaded into the MIP cartridges at flow rates ranged from 0.2 to 2.0 mL min^{-1}. Recoveries over 90 % were obtained at a flow rate of 0.5 mL min^{-1}. The recoveries decreased while the flow rate improved continuously. This can be interpreted as that the analyte did not have sufficient time to interact with the binding sites of polymer. Therefore, 0.5 mL min^{-1} was chosen for optimized flow rate.

Evaluation of elution solvent: To optimize the elution solvent, 1 mL of samples (1 μg mL^{-1}), dissolved in mobile phase, were percolated through the MIP cartridges

and 3 mL (1 + 1 + 1 mL) of different solvents. Methanol and acetonitrile were investigated because of their strong polarity compared to dichloromethane and acetone. The results showed that methanol was more suitable for elution solvent and then methanol with 2 % ammonia and 2 % acetic acid were selected for further evaluation considering the acid base amphoteric property of FQs. As shown in Table-2, the recoveries (90.6-100.7 %) for methanol with 2 % ammonia were higher than that (74.3-88.1 %) of methanol with 2 % acetic acid. After that, the ratio of ammonia in methanol was improved for further observation. It can be seen in Table-2 that good recoveries (90.6-102.6 %) were obtained when the ratio of ammonia reached 4 %. Finally, The best conditions found to quantitatively elute and recover all the retained analytes was to use 3 mL methanol with 4 % ammonia.

TABLE-2
EXTRACTION RECOVERIES (%) OBTAINED ON THE MIP FOR TEN FLUOROQUINOLONES AFTER PERCOLATION OF 1 mL STANDARD SOLUTION (1 μg mL^{-1}, IN MOBILE PHASE) USING ELUTION SOLVENTS WITH MeOH, MeOH WITH 2 % ACETIC ACID, MeOH WITH 2 % AMMONIA, 3 % AMMONIA, 4 % AMMONIA AND 5 % AMMONIA

Analyte	Recoveries (%) (Elution solvent)					
	MeOH	MeOH with 2 % acetic acid	MeOH with 2 % ammonia	MeOH with 3 % ammonia	MeOH with 4 % ammonia	MeOH with 5 % ammonia
FLX	7.8	81.7	100.7	100.7	102.6	98.0
LVFX	30.8	77.9	93.9	96.1	99.5	98.8
ENO	0	80.8	87.3	93.8	98.5	96.8
NOR	2.7	74.6	88.4	89.3	90.6	91.8
CIP	25.2	74.3	90.1	91.2	95.0	92.8
ENR	13.1	80.7	91.3	93.4	93.6	94.2
LOM	9.7	88.1	95.7	97.2	97.2	97.1
PEF	7.4	82.4	89.8	91.6	97.0	97.5
GAT	5.9	73.2	86.5	93.7	95.7	94.6
SPA	2.6	70.2	82.3	88.9	92.1	93.2

Optimization for volume of washing solvent: To determine the optimum washing volume with water as washing solvent, 1 mL standard solution of FQs (1 μg mL^{-1}, in mobile phase) were loaded into the MIP cartridges and different volumes (3, 4, 5 and 6 mL) of water were applied to the washing step. Then the FQs were eluted with 3 mL methanol with 4 % ammonia and the extract was analyzed by HPLC-DAD. The results were listed in Table-3.

Between 3 and 6 mL, the recoveries obtained were higher than 90 % in the MIP cartridges for all the FQs tested while the washing step washed out the impurities without causing the leakage of the MIP cartridges. Given for the consumption of washing solvent, the optium volume chose 3 mL.

TABLE-3
EXTRACTION RECOVERIES (%) OBTAINED ON THE MIP FOR TEN FLUOROQUINOLONES AFTER PERCOLATION OF 1 mL STANDARD SOLUTION (1 μg mL^{-1}, IN MOBILE PHASE) USING A WASHING STEP WITH 3, 4, 5 AND 6 mL OF WATER

Analyte	Recoveries (%) (Washing volume)			
	3 mL	4 mL	5 mL	6 mL
FLX	97.9	98.4	95.6	98.7
LVFX	98.4	97.7	97.5	96.9
ENO	97.6	98.3	97.7	96.8
NOR	91.4	92.0	90.7	91.5
CIP	94.8	92.7	90.4	93.2
ENR	93.2	92.8	91.8	92.5
LOM	97.3	93.1	95.3	94.5
PEF	96.6	94.9	92.7	93.1
GAT	95.4	96.1	94.2	93.8
SPA	91.5	92.2	90.6	90.1

Reproducibility and precision for enrichment and desorption of FQs in MISPE cartridges: To validate the adsorption and desorption properties of MISPE cartridges for FQs, 1 mL standard solution of FQs (1 μg mL^{-1}, in mobile phase) were loaded into the MIP cartridges. The extract was analyzed by HPLC-DAD and the results were collected in Table-4. Good recoveries (90.9-102.3 %) and reproducibilities (RSD < 4.5 %) were achieved for all the FQs tested and this proved that it is feasible for MISPE cartridges to be applied in residue analysis of FQs in real samples.

TABLE-4
RECOVERIES (%), AVERAGE RECOVERIES (AVE) AND RELATIVE STANDARD DEVIATIONS (RSD, %, n = 3) OBTAINED AFTER SOLID PHASE EXTRACTION OF 1 mL STANDARD SOLUTION (1 μg mL^{-1}, IN MOBILE PHASE) OF TEN FLUOROQUINOLONE ANTIBIOTICS

Analyte	Recoveries (%)				RSD (%)
	1	2	3	Average	
FLX	96.1	98.6	99.3	98.0	1.7
LVFX	101.3	100.4	99.5	100.4	0.9
ENO	100.3	95.0	98.5	97.9	2.6
NOR	92.2.	92.7	90.2	91.7	1.4
CIP	91.1	90.5	95.0	92.2	2.4
ENR	94.0	93.9	93.6	93.8	0.2
LOM	105.2	104.6	97.2	102.3	4.5
PEF	97.0	94.3	98.3	96.5	2.1
GAT	95.2	94.9	94.7	95.1	0.2
SPA	92.1	90.5	90.2	90.9	1.1

Selection of extraction solvent: Fluoroquinolones were soluble in acid or base media because of the existence of a carboxyl group and a piperazinyl group in FQs. According to the reports[32], the main extraction solvents included methanol, acetonitrile and dichloromethane. The matrix of milk samples is relatively complicated and it consists of a certain amount of protein and fat. The extraction should be able to precipitate protein effectively. There were many impurities in extraction solution of methanol and dichloromethane had a poor ability for removing protein in spite of its good degreasing effect. The extraction effect of acetonitrile, acetonitrile with acetic acid and acetonitrile with ammonia had been evaluated. After the comparation, it had been found that acetonitrile with acetic acid gives the highest efficiency. Acetonitrile with 2 % acetic acid was selected for the extraction solvent after the experiments. In Fig. 7, it can be found that the impurity peaks hardly interfere the quantification of the FQs tested.

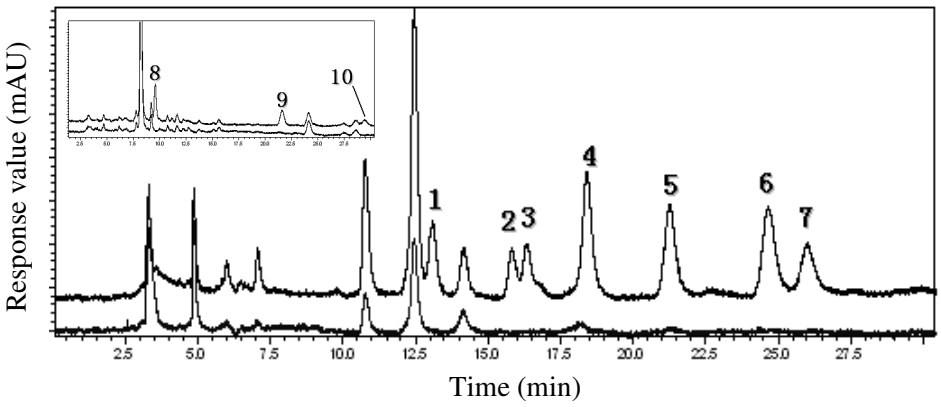

Fig. 7. Chromatograms of blank milk samples and milk samples fortified with 200 μg kg^{-1} of ten fluoroquinolone antibiotics, black line for spiked samples, purple line for blank samples. Mobile phase (A/B) = 22/78: (1) FLX; (2) LVFX; (3) ENO; (4) NOR; (5) CIP; (6) ENR; (7) LOM. Inset: mobile phase (A/B) = 28/72: (8) PEF; (9) GAT; (10) SPA

Linearity: The calibration curve was calculated by line regression of the measured peak areas and the corresponding concentrations of the calibration standard solutions described in standard solutions. In the range of 0.05-2 μg mL^{-1} (the range of SPA was 0.1-2 μg mL^{-1}), the calibration curve of each fluoroquinolone showed good linearity with correlation coefficient (R) more than 0.9996. The instrument detection limits for FQs were calculated three times of the signal noise ratio according to the lowest concentration point in standard curve and shown in Table-5.

Spiked milk samples: The fortified milk samples with spiked levels at 100 and 200 μg kg^{-1} had been analyzed by HPLC-DAD in triplication. According to the spiked level of 100 μg kg^{-1}, the limits of detection (LODs) and the limits of quantification (LOQs) for the method were calculated three times and ten times of the

TABLE-5
LINEAR EQUATIONS, CORRELATION COEFFICIENTS (R) AND INSTRUMENT DETECTION LIMITS (μg L^{-1}) FOR FQs

Analyte	Linear equation	R	Instrument detection limits (μg L^{-1})
FLX	$Y=5.37\times10^{5}X-1.77$	0.99989	4.29
LVFX	$Y=4.10\times10^{5}X+6.54$	0.99967	5.96
ENO	$Y=5.01\times10^{5}X+59.87$	0.99988	6.72
NOR	$Y=1.40\times10^{6}X-389.45$	0.99999	2.56
CIP	$Y=7.29\times10^{5}X-165.98$	0.99996	4.71
ENR	$Y=1.19\times10^{6}X+231.76$	0.99985	3.52
LOM	$Y=4.62\times10^{5}X+72.90$	0.99996	6.48
PEF	$Y=5.58\times10^{5}X-90.57$	0.99996	5.86
GAT	$Y=4.03\times10^{5}X-44.87$	0.99999	9.67
SPA	$Y=2.05\times10^{5}X-103.37$	0.99999	15.79

signal noise ratio, respectively. The fortified recoveries were 84.1-104.7 % with RSD no more than 4.3 %. The LODs and LOQs were 1.34-7.35 μg kg^{-1} and 4.45-24.49 μg kg^{-1}, respectively (Table-6). The results compared favourably with those obtained using commercial cartridges for the same type of samples. For instance, Cinquina *et al.*[33] reported recoveries of 84 % for ENR, 88 % for CIP and LOD of 15 μg kg^{-1} while in the proposed method the recoveries of 96.7 % for ENR, 94.6 % for CIP, the LODs of 2.88 μg kg^{-1} for ENR and 3.97 μg kg^{-1} for CIP. The MISPE cartridges can be reused more than 20 times without losing their concentration efficiency, which is important particularly when the analysis of samples need to be performed in the same cartridge. The results for spiked samples demonstrated the applicability of the extraction of FQs from milk samples using MISPE cartridges. The presence of the matrix components in milk samples did not interfere the preconcentration and separation efficiency of FQs on mixed-template imprinted polymer (Fig. 7) and the recoveries for all the FQs tested were excellent (Table-6).

Conclusion

This study demonstrates the applicability of mixed-template imprinted polymer for the preconcentration of ten fluoroquinolones in milk samples. The optimized procedure was mainly based on a extraction and a MISPE step followed by HPLC-DAD. Using the mixed-template imprinted polymer as SPE sorbent resulted a appropriate method for extracting FQs from animal derived samples with that good recoveries and reproducibility of the method were guaranteed. The MISPE cartridges can be reused more than 20 times without losing their concentration efficiency. The high extraction efficiency of MISPE showed that unlike the recognition ability of the single-template imprinted polymer for only one compound, mixed-template imprinted polymer can be used to selective extract a class of compounds. This extended the application range of MIP and provided a new idea for the study

TABLE-6
RECOVERIES (%), AVERAGE RECOVERIES (AVE), RELATIVE STANDARD DEVIATIONS (RSD, %, n =3), LIMITS OF DETECTION (LOD) AND LIMITS OF QUANTIFICATION (LOQ) OBTAINED AFTER SOLID PHASE EXTRACTION OF MILK SAMPLES WITH SPIKED LEVELS AT 100 AND 200 μg kg^{-1}

Analyte	Spiked level (μg kg^{-1})	Recoveries (%)				RSD (%)	LOD (μg kg^{-1})	LOQ (μg kg^{-1}
		1	2	3	Average			
FLX	200	96.3	94.8	99.2	96.8	2.3	3.41	11.37
	100	98.1	96.8	98.8	97.9	1.0		
LVFX	200	88.4	87.3	91.0	88.9	2.1	5.22	17.39
	100	90.0	90.5	87.1	89.2	2.1		
ENO	200	93.9	92.5	93.4	93.3	0.8	4.70	15.67
	100	87.6	84.1	85.8	85.8	2.0		
NOR	200	89.8	92.6	91.9	91.4	1.6	1.95	6.51
	100	93.3	88.7	91.2	90.1	2.5		
CIP	200	93.1	91.1	91.2	91.8	1.2	3.97	13.23
	100	98.3	96.3	97.2	97.3	1.0		
ENR	200	94.3	97.6	96.7	96.2	1.8	2.88	9.60
	100	95.4	97.2	98.6	97.1	1.7		
LOM	200	99.2	104.7	101.2	101.7	2.7	5.00	16.67
	100	98.9	103.8	98.5	100.4	2.9		
PEF	200	90.7	89.8	91.6	90.7	1.0	1.34	4.45
	100	96.1	94.0	95.1	95.1	1.1		
GAT	200	98.8	97.9	96.4	97.7	1.2	3.25	10.83
	100	93.8	98.3	94.9	95.7	2.5		
SPA	200	91.6	94.7	94.9	93.7	1.9	7.35	24.49
	100	95.3	102.3	103.1	100.2	4.3		

and application of MIP. Thus, the mixed-template imprinted polymer was more suitable for the residue analysis of antibiotics in animal derived food.

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