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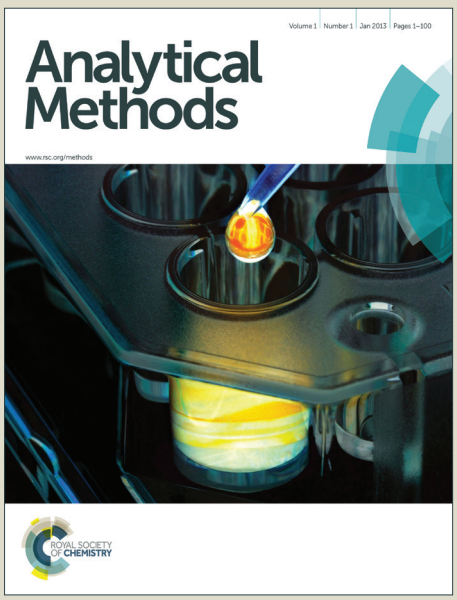

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Determination of fluoroquinolone drugs in meat by ionic liquid dispersive liquid-liquid microextraction-high performance liquid chromatography

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In this study, ionic liquid 1-butyl-3-methylimidazolium hexafluorophosphate ([C_4MIM][PF_6]) was used to develop a dispersive liquid-liquid microextraction method for the extraction of four fluoroquinolone drugs in meat followed by determination with high performance liquid chromatography. During the experiments, some important parameters (ionic liquid and its volume, disperser solvent and its volume, extraction and centrifuge time, pH and salt addition) were investigated and optimized. Under the optimal conditions, the method achieved different enrichment factors (11-42 folds) for four fluoroquinolones (norfloxacin, ciprofloxacin, lomefloxacin, enrofloxacin). Results showed that the limits of detection for the four drugs were in the range of 0.5-1.1 ng mL^{-1} and their recoveries from the standards fortified blank meat (chicken, pork and fish) were in the range of 60.4%-96.3% with coefficients of variation lower than 11.5%. Among 60 real meat samples, seven samples were determined to contain the

residues of norfloxacin, ciprofloxacin and enrofloxacin, but the residual levels were lower than their maximum residue levels (100 ng g^{-1}).

1. Introduction

Fluoroquinolone drugs (FQs) are a class of synthetic drugs that are usually used to treat various bacterial infections in animal, aquaculture, and human being. The broad use of FQs in animals may produce their residues in animal derived foods that are dangerous to the consumers. Therefore, the Europe Union has established different maximum residue levels (MRLs) for various FQs in different animal derived foods: enrofloxacin and ciprofloxacin, 100 ng g^{-1} in pork, chicken and fish.[1] Therefore, it is very important to monitor the residues of FQs in animal derived foods.

Up to now, many methods, such as high performance liquid chromatography (HPLC), [2-5] liquid chromatography tandem mass spectrometry, [6-9] surface plasmon resonance biosensor [10] and immunoassay, [11-14] have been reported to determine the residues of FQs in different foods of animal origin. For determination of the residual FQs in foods of animal origin, the first step is to extract the analytes from the samples. In those reported methods, liquid-liquid extraction (LLE), solid phase extraction (SPE), molecularly imprinted polymer (MIP) and immunoaffinity chromatography (IAC) were usually used as the extraction and purification methods. LLE and SPE are the conventional extraction methods, but LLE requires large volume of organic solvents and SPE cartridge is easily interfered by the substances in the samples. IAC is able to differentially adsorb and purify the target analyte, but the specific antibody has to be prepared in advance. MIP is only able to extract and purify the specific analyte, i.e. lack of generality. Therefore, all the previously used extraction methods were not the ideal sample preparation techniques.

In a recent report, a dispersive liquid-liquid microextraction technique (DLLME) was developed to extract organic compounds in water. [15] This extraction method was consisted of

three steps. First step, an extraction solvent and a dispersive solvent were injected into an aqueous solution to form a cloudy system. Second step, the analytes in the aqueous solution were rapidly transferred into the fine droplets of the extraction solvent. Third step, the analytes enriched in the extraction solvent were sedimented by centrifugation for analysis. Because DLLME method is simple, rapid, and shows high enrichment effect for the analyte, it has been widely used to extract and enrich various analytes in environmental samples and animal derived samples. [15-21] In the past few years, ionic liquids (ILs), as a class of novel solvents, were used more and more as the extraction solvents to extract various analytes. [22, 23] Therefore, IL and DLLME procedure were combined to generate a novel sample preparation method (IL-DLLME). In a previous report, IL-DLLME was first reported to extract polycyclic aromatic hydrocarbons in water, and the satisfactory results were obtained.[24] Thereafter, many ILs based microextraction methods, such as temperature controlled IL-DLLME, [25, 26] ultrasound-assisted IL-DLLME, [27] IL based homogenous liquid-liquid microextraction, [28] and IL-DLLME, [29-36] were developed to extract various analytes from water and complex samples. All these ILs based microextraction methods achieved high sensitivities and high enrichment effects, but IL-DLLME was simpler and rapider than others.

However, there have been only several articles reporting the use of DLLME method or IL for the extraction of FQs residues. In three recent reports, the authors used different organic solvents to develop the DLLME procedures for the extraction of FQs in animal derived samples.[18-20] In a recent report, the authors used ionic liquid 1-hexyl-3-methylimidazolium tetrafluoroborate ($[C_6MIM][BF_4]$) to develop a homogeneous liquid phase microextraction for the extraction of four FQs in milk. [28] This means that there has been no article reporting the development of an IL-DLLME procedure for extraction of FQs in animal derived foods so far.

In the present study, ionic liquid 1-butyl-3-methylimidazolium hexafluorophosphate ([C_4MIM][PF_6]) was used to develop an IL-DLLME method for the extraction of four FQs (norfloxacin, ciprofloxacin, lomefloxacin and enrofloxacin) in meat followed by determination with HPLC. Results showed that the developed IL-DLLME-HPLC method could be used as simple, rapid and accurate tool to determine the residues of the four FQs in meat.

2. Experimental

2.1. Reagents and chemicals

Ciprofloxacin (CIP), norfloxacin (NOR), lomefloxacin (LOM) and enrofloxacin (ENR) were obtained from the China Institute of Veterinary Drug Control (Beijing, China). Ionic liquids 1-butyl-3-methylimidazolium hexafluorophosphate [C_4MIM][PF_6], 1-hexyl-3-methylimidazolium hexafluorophosphate [C_6MIM][PF_6], and 1-octyl-3-methylimidazolium hexafluorophosphate ([C_8MIM][PF_6]) were purchased from Acros Organics (Morris Plains, NJ, USA). Liquid chromatographic grade acetonitrile were purchased from Dikma (Richmond Hill, USA). Other chemical reagents were all analytical grade or better from Beijing Chemical Company (Beijing, China). Standard stock solutions of the four FQs were prepared with methanol (10 μg mL^{-1}) and their working solutions with series concentrations (0.1-1000 ng mL^{-1}) were diluted from the stock solutions with methanol. Phosphoric acid/triethylamine buffer (0.05%, pH 3.0) was prepared by diluting 3.4 mL of phosphoric acid to 1000 mL with water and adjusting the pH to 3.0 with triethylamine.

2.2 HPLC equipments and conditions

HPLC system was consisted of Waters 1525 liquid chromatography, Waters 2998 DAD detector (Waters, USA) and a Diamonsil C18 column (150×4.6 mm, 5μm). The mobile phase was consisted of acetonitrile and phosphoric acid/ triethylamine buffer (13:87, v/v) with

isocratic elution mode at a flow rate of 1.0 mL min^{-1}. The injection volume was 20 μL and the detection wavelengths were 278 and 289 nm. HPLC qualitative analysis was performed by comparing the retention times of chromatogram peaks of the samples with those of the standards. Quantification was calculated according the chromatogram peak area of each analyte.

2.3 Sample preparation

2.3.1 Isolation of FQs from meat

The extraction of FQs from meat (chicken, pork, fish) was according to a previous report. [4] Briefly, 2 g homogenized meat sample and 10 mL of acetonitrile were added into a 20 mL polypropylene centrifuge tube. Then, the mixture was mixed vigorously on a vortex mixer for 5 min and sonicated for 5 min. After the tube was centrifuged at 5000 rpm for 10 min, the supernatants were collected and concentrated to nearly dry (about 0.1 mL) on a rotary evaporator at 40 ℃. The left solution was diluted to 5.0 mL with water and filtered through a 0.22 μm Millipore filter prior to IL-DLLME procedure.

2.3.2 IL-DLLME procedure

The IL-DLLME procedure was performed as follows. Briefly, the 5 mL of sample extract was transferred into a 10 mL conical flask, and 0.3 mL of acetonitrile containing 50 μL of ionic liquid was quickly injected into the sample extract. The flask was shaken immediately for 20 s, laid for 30 s, and centrifuged at 4000 rpm for 5 min. Then, the fine droplets of ionic liquid were settled down to the bottom of the conical flask. The upper aqueous phase was discarded and 20 μL of the settled ionic liquid phase was injected into HPLC system for analysis.

Some meat samples (chicken, pork and fish) from the controlled slaughterhouses and fisheries were used as the blank samples to evaluate the extraction efficiency and recovery.

During the experiments, the standards of the four FQs were diluted with blank extracts respectively to prepare the 25 ng mL^{-1} solutions that were used to evaluate the enrichment efficiency of the IL-DLLME procedure. Enrichment factor (EF) was calculated as following: $EF = C_{IL}/C_0$, where C_{IL} is the analyte concentration in the settled IL phase after IL-DLLME and C_0 is 25 ng mL^{-1}.

2.4 Real meat samples

Twenty chicken samples (chicken thigh), 20 pork samples (hindquarter) and 20 fishes (carp) were purchased from different supermarkets in China at different times. Each of these samples was put in individual reclosable bag and kept at -20 ^{o}C until use. All these samples were analyzed by the developed IL-DLLME-HPLC method.

3. Results and discussions

3.1 Isolation of FQs from meat

In the previous reports, IL-DLLME procedure was usually used to extract the target analytes from water samples directly. [24-27, 29, 31-33, 35] In several other reports, the analytes in milk, [28] banana, [30] flour and maize steamed bread, [34] and vegetable samples [36] were first transferred into an aqueous phase that was then used to perform the IL-DLLME procedure or IL based homogeneous liquid-liquid microextraction. This meant that an aqueous phase is required for the development of an IL-DLLME method. Therefore, the first step in the present study was to transfer the residual FQs in meat into an aqueous phase. In the previous reports, the residues of FQs in animal derived samples were usually extracted with different organic solvents [2, 3, 6-9] and aqueous buffers. [5, 11, 14] However, the use of the reported aqueous buffers as extraction solvents was inappropriate in the present study because the salt ions in those buffers maybe influenced the subsequent IL-DLLME procedure. Therefore, acetonitrile was used to transfer

the residual FQs in meat into a liquid phase according to the previous report, [4] and then the acetonitrile phase was evaporated to a small volume and diluted to 5.0 mL with water for IL-DLLME procedure.

3.2 Optimization of IL-DLLME procedure

This is the first study reporting an IL-DLLME method for the extraction of FQs in meat. In the present study, ionic liquid and its volume, disperser solvent and its volume, extraction and centrifuge time, pH and salt addition were investigated respectively as described below. During the experiments, the enrichment factors (EFs) of the four FQs were used to optimize the above mentioned parameters.

3.2.1 Selection of ionic liquid

In an IL-DLLME method, an appropriate IL is critical. In two reviews, the authors compiled the previous ILs based extraction methods and showed that the extraction efficiency of the imidazolium-ILs improved with the increase of alkyl chain length of imidazole ions, [22,23] so ILs $[C_6MIM][PF_6]$ and $[C_8MIM][PF_6]$ were usually used as the extraction solvents in the previous IL-DLLME methods. [29-32, 34-36] In the present study, three ILs $[C_4MIM][PF_6]$, $[C_6MIM][PF_6]$ and $[C_8MIM][PF_6]$ were used to optimize the best extraction solvent. As shown in Fig. 1, the three ILs showed different enrichment effects for the four FQs, and the EFs when using $[C_4MIM][PF_6]$ were higher than that when using $[C_6MIM][PF_6]$ and $[C_8MIM][PF_6]$, so $[C_4MIM][PF_6]$ was used for the subsequent experiments in the present study. This result was different from that of the previous reports in which the performance of $[C_4MIM][PF_6]$ was worse than that of $[C_6MIM][PF_6]$ and $[C_8MIM][PF_6]$. [29-32, 34-36] Maybe the chemical and physical properties of the FQs interfered with the enrichment of $[C_6MIM][PF_6]$ and

$[C_8MIM][PF_6]$ for the four FQs, but the actual reason was unknown and remained to be studied .

3.2.2 Selection of disperser solvent

In an IL-DLLME method, an appropriate disperser solvent can help to form a cloudy solution. In the previous IL-DLLME methods, methanol and acetonitrile were the mostly used disperser solvents.[29-36] In the present study, methanol, ethanol, acetone and acetonitrile were used to optimize the best disperser solvent. The experiments were carried out by mixing the FQs fortified blank extracts (25 ng mL^{-1}) with 0.5 mL of each solvent containing 60 μL of $[C_4MIM][PF_6]$ to calculate the EFs. When using ethanol as the disperser solvent, the cloudy solution was not separated to two phases after centrifugation, so ethanol was omitted. When using other three solvents as the disperser solvents, the cloudy solutions were formed and the IL phase was settled down after centrifugation. As shown in Fig. 2, the EFs of the four FQs when using acetonitrile were higher than that when using methanol and acetone, so acetonitrile was used as the best disperser solvent for the subsequent experiments.

3.2.3 Selection of volume of $[C_4MIM][PF_6]$

In an IL-DLLME procedure, the IL volume is also a critical factor. Within a certain limit, the larger the volume of IL is used, the more volume of IL can be sedimented and the larger enrichment effect can be obtained; but when the IL volume reaches a certain level, the enrichment effect will turn bad because the analyte concentration in the sedimented IL phase decreases along with the increase of the sedimented IL volume. In the previous IL-DLLME methods, the IL volumes ranged from 35 to 60 μL. [29-36] In the present study, the EFs of the four FQs were calculated by using 0.5 mL of acetonitrile containing different volumes of $[C_4MIM][PF_6]$ (30, 40, 50, 60, 70, 80 μL). Results showed that the EFs of the four FQs all

increased when IL volume increased from 30 μL to 50 μL, and then decreased when IL volume exceeded 50 μL. Therefore, the use of 50 μL $[C_4MIM][PF_6]$ was selected for the subsequent experiments.

3.2.4 Selection of volume of acetonitrile

In an IL-DLLME method, the volume of disperser solvent is also an important factor. When a small volume of disperser solvent is used, the cloudy solution can not be well formed; when a large volume of disperser solvent is used, the IL can be dissolved in the disperser solvent, resulting in low volume of sedimented IL phase or no sedimented IL phase after centrifugation. Furthermore, a previous report has showed that the volume of dispersive solvent directly influenced the dispersion degree of the IL in an aqueous phase, consequently the volume of sedimented IL phase. [33] In the present study, different volumes of acetonitrile (0.2, 0.3, 0.5, 0.6, 0.8 mL) containing 50 μL of $[C_4MIM][PF_6]$ were used to determine the EFs of the four FQs. Results showed that the EFs of the four FQs all increased when acetonitrile volume increased from 0.2 to 0.3 mL, and then decreased to zero rapidly when acetonitrile volume increased from 0.3 to 0.8 mL. Therefore, the use of 0.3 mL acetonitrile was selected for the subsequent experiments.

3.2.5 Selection of extraction and centrifugal time

In an IL-DLLME procedure, the maximum quantity of analyte is transferred into the IL phase when the extraction equilibrium is obtained. In the present study, the formed cloudy solutions were laid for different times (10, 20, 30, 40, 50, 60 seconds) to evaluate the optimum extraction time. Results showed that the EFs of the four FQs reached balance when the extraction time reached 20 seconds. To ensure the maximum extraction efficiency, the cloudy solution was laid for 50 seconds before centrifugation in this study.

The IL droplets in the cloudy solution can be settled down by centrifugation, so the centrifugal time affects the settled IL volume and the analyte concentration in the IL phase. In the present study, the cloudy solutions were centrifuged for different times (3, 5, 8, 10, and 15 min) at 4000 rpm after extraction. Results showed that the EFs of the four FQs reached balance when the centrifugal time reached 5 min. Therefore, 5 min of centrifugation for the formed cloudy solution was selected in this study.

3.2.6 Salt addition

The salt addition (e.g. NaCl) in an extraction system can improve the extraction efficiency due to salting out effect, but the high ionic strengthen in an IL-DLLME system can enhance the solubility of an IL in aqueous phase, consequently decrease the extraction performance. In the previous IL-DLLME procedures, salt addition was not used because the extraction efficiency for the analytes decreased with the increase of the salt concentration. [29-36] In the present study, various amount of NaCl (0%, 0.5%, 1%, 2%, 5%, 10%, w/v) were added in the FQs fortified blank extracts prior to IL-DLLME procedure. As shown in Fig. 3, the EFs of the four FQs increased when NaCl concentrations were at 0.5% and 1%, but decreased rapidly when NaCl concentrations exceeded 1%. Therefore, the addition of 0.5% NaCl was used for the subsequent experiments.

3.2.7 pH

FQs are a class of synthetic amphoteric compounds that usually have two dissociation constants: carboxylic acid group, 5.5-6.4; nitrogen on piperazinyl ring, 7.9-8.8. For evaluation of the influence of pH variation on enrichment effect, the pH values of the FQs fortified blank extracts were adjusted to 3.0-11.0 with hydrochloric acid or ammonium hydroxide prior to IL-DLLME procedure. As shown in Fig. 4, the EFs of the four FQs were stable when the pH

values were in the range of 3.0-7.0, but decreased to zero when the pH value reached 9.0. When the pH values were in the range of 3.0-7.0, the solubility of FQs in aqueous phase was stable, so the EFs were also stable. When the pH value increased to 9.0 or higher, the solubility of FQs in aqueous phase decreased dramatically, so the amount of FQs transferred into the IL phase was little; thus the EFs of the four FQs decreased to zero. Therefore, the pH adjustment was not used in this study.

3.3 Analytical features of the IL-DLLME-HPLC method

This study for the first time developed an IL-DLLME-HPLC method to determine the residues of four FQs in meat. The chromatograms of the four FQs standards before and after IL-DLLME procedure are shown in Fig. 5. Under the optimal conditions, the determination parameters of the IL-DLLME-HPLC method for the four FQs were shown in Table 1: linear relationship, 1-500 ng mL^{-1}; correlation coefficients (r^2), 0.9984-0.9996; relative standard deviation (RSD), 2.1-3.5%; LODs (signal/noise of 3), 0.5-1.1 ng mL^{-1}; LOQs (signal/noise of 10), 1.5-3.8 ng mL^{-1}; EFs, 11-42. Finally, the four FQs were fortified into the blank meat samples (chicken, pork, and fish) respectively at concentrations of 5-100 ng g^{-1} to evaluate the method recovery. The mean recoveries were in the range of 60.4%-96.3% and the coefficients of variation (CV, on six successive days) were in the range of 4.6%-11.5% (Table 2). The representative chromatograms of blank pork and the four FQs fortified blank pork are shown in Fig. 5.

In the previous reports for determination of FQs in animal derived samples, the commonly used methods were HPLC and liquid chromatography mass spectrometry. [2-9, 18, 20] Among these methods, the LODs when using LLE, SPE, IAC and MIP as the extraction methods were in the range of 0.1-10 ng g^{-1}/mL^{-1}. [2-9] In two recent reports, the DLLME procedure (without use of

ionic liquid) was used to extract FQs in milk and chicken liver, and the LODs were in the range of 4.1-19 ng g^{-1}. [18, 20] There was only one paper in which $[C_6MIM][BF_4]$ based homogeneous liquid-liquid microextraction was used to extract FQs in milk, and the LODs were in the range of 4.0-15.8 ng mL^{-1}.[28] In comparison, the sensitivity of the IL-DLLME-HPLC method in this study was higher than that of the HPLC methods [2-5, 18, 28] and similar to that of the mass spectrometry methods. [6-9, 20] Furthermore, the IL-DLLME-HPLC method was simple and rapid, and used low volume of organic solvent.

3.4 Analysis of real samples

Finally, the unknown 60 meat samples (20 chicken, 20 pork and 20 fish) were determined by using the IL-DLLME-HPLC method. Result showed that 2 chicken samples and 5 fish samples contained the residues of CIP, ENR and NOR, but the residue levels were lower than their MRL levels (Table 3).

4. Conclusion

As a class of novel solvents, ionic liquids were used as more and more as the extraction solvents to extract various analytes from different samples. In the present study, ionic liquid $[C_4MIM][PF_6]$ was used to develop a dispersion liquid-liquid microextraction method combining HPLC for the determination of four fluoroquinolone drugs in meat. Results showed the method achieved high extraction efficiency, enrichment performance and sensitivity. From the analysis of fortified blank meat and unknown meat samples, the developed method could be used as a sensitive and accurate method to monitor the residues of the four fluoroquinolone drugs in meat.

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Table 1. Detection parameters of the IL-DLLME-HPLC for the four FQs (*n*=6).

Analyte	Linearity range (ng mL^{-1})	r^2	RSD (%)	LOD (ng g^{-1})	LOQ (ng g^{-1})	Enrichment factor
NOR	2-500	0.9984	2.4	0.7	2.0	15
	(20-1000) [a]	(0.9996)	(0.9)	(10)	(30)	--
CIP	2-500	0.9991	3.5	0.9	3.2	11
	(20-1000)	(0.9997)	(1.8)	(10)	(30)	--
LOM	2-500	0.9979	2.1	1.1	3.8	18
	(50-1000)	(0.9993)	(0.8)	(20)	(80)	--
ENR	1-500	0.9996	2.6	0.5	1.5	42
	(50-1000)	(0.9999)	(0.7)	(20)	(60)	--

[a] The results in parentheses were obtained from direct HPLC method (without IL-DLLME).

Table 2. Recoveries of the four fluoroquinolones from blank meat samples (*n*=6).

Analyte	Added (ng g^{-1})	Chicken		pork		fish	
		Recovery (%)	CV (%)	Recovery (%)	CV (%)	Recovery (%)	CV (%)
NOR	5	72.5	9.7	60.4	10.6	68.7	11.5
	25	88.3	6.3	87.6	6.8	89.7	7.2
	50	92.1	5.4	88.3	6.4	91.5	4.7
	100	90.6	5.8	94.5	5.2	96.3	6.3
CIP	5	71.6	8.5	72.1	7.6	72.4	7.3
	25	74.3	7.4	68.4	6.7	72.3	8.7
	50	78.6	8.2	73.2	8.5	76.9	8.5
	100	70.9	8.0	77.0	8.6	81.2	8.0
LOM	5	67.4	11.8	60.5	9.6	70.3	9.3
	25	65.8	8.5	69.8	8.7	70.6	7.3
	50	69.8	9.3	72.3	7.3	72.5	7.8
	100	72.1	8.6	68.5	9.0	74.3	6.9
ENR	5	73.5	6.3	76.3	7.2	79.0	7.0
	25	83.6	5.8	76.1	5.2	80.1	6.0
	50	82.7	4.6	77.2	5.4	82.0	6.2
	100	86.4	6.0	82.9	5.8	77.5	5.1

Table 3. Residue levels the four FQs in real meat samples.

Analyte	Fish 1	Fish 2	Fish 3	Fish 4	Fish 5	Chicken 1	Chicken 2	MRL	
								Chicken	Fish
CIP		32		60				100	100
ENR	71		18		53		60	100	100
NOR		13				16		- [a]	50
LOM								- [b]	- [b]

The unit of the numbers was ng g^{-1}.

[a] No MRL. [b] No authorization in veterinary medicine.

Fig. 1. Effect of ionic liquid on enrichment factors of the four FQs (blank extracts, 5.0 mL; ionic liquid, 60 μL; disperser solvent (acetonitrile), 0.5 mL; extraction time, 1 min; centrifugal time, 5 min).

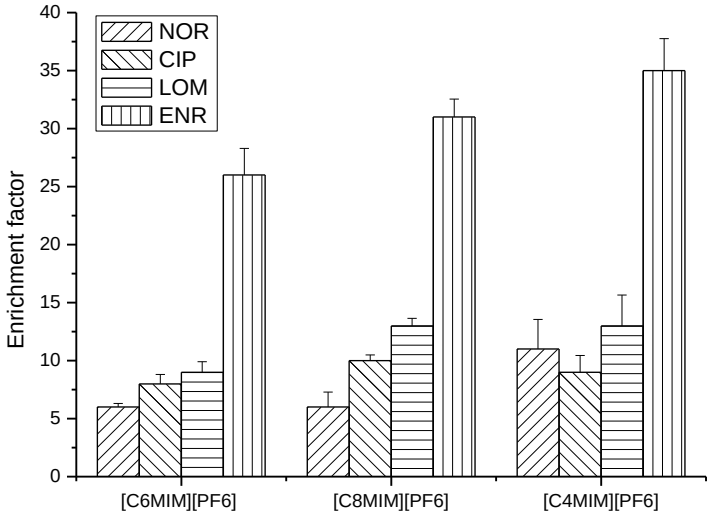


Fig. 2. Effect of disperser solvent on enrichment factors of the four FQs (blank extracts, 5.0 mL; $[C_4MIM][PF_6]$, 60 μL; disperser solvent, 0.5 mL; extraction time, 1 min; centrifugal time, 5 min).

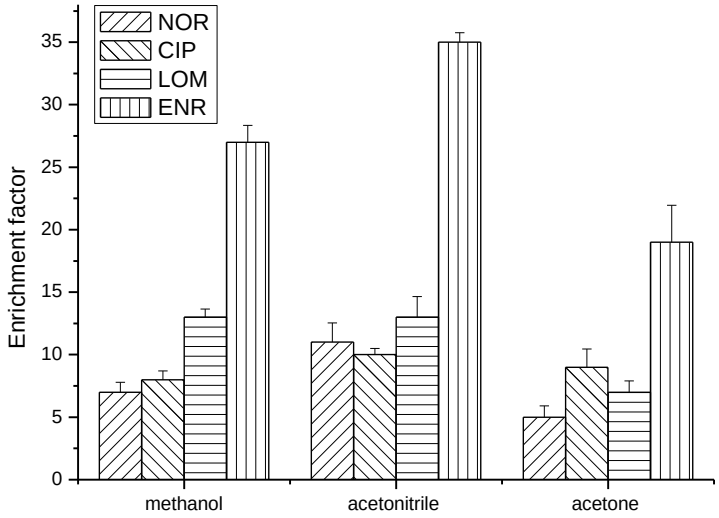


Fig.3. Effect of NaCl concentration on enrichment factors of the four FQs (blank extracts, 5.0 mL; $[C_4MIM][PF_6]$, 50 μL; acetonitrile, 0.3 mL; extraction time, 50 s; centrifugal time, 5 min).

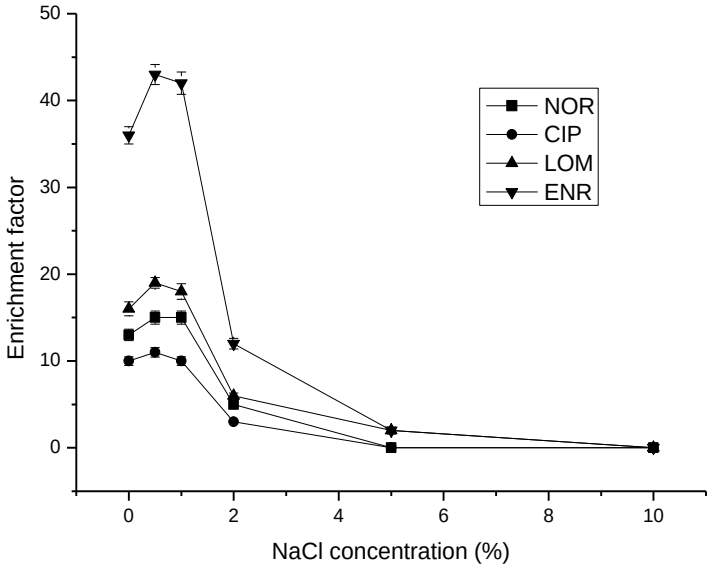


Fig. 4. Effect of extract pH on enrichment factors of the four FQs (blank extracts, 5.0 mL; $[C_4MIM][PF_6]$, 50 μL; acetonitrile, 0.3 mL; extraction time, 50 s; centrifugal time, 5 min; NaCl, 0.5%).

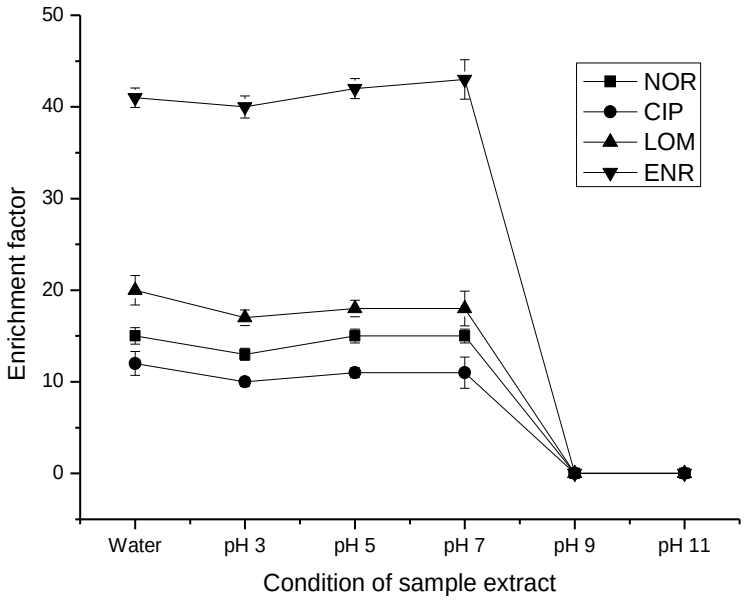
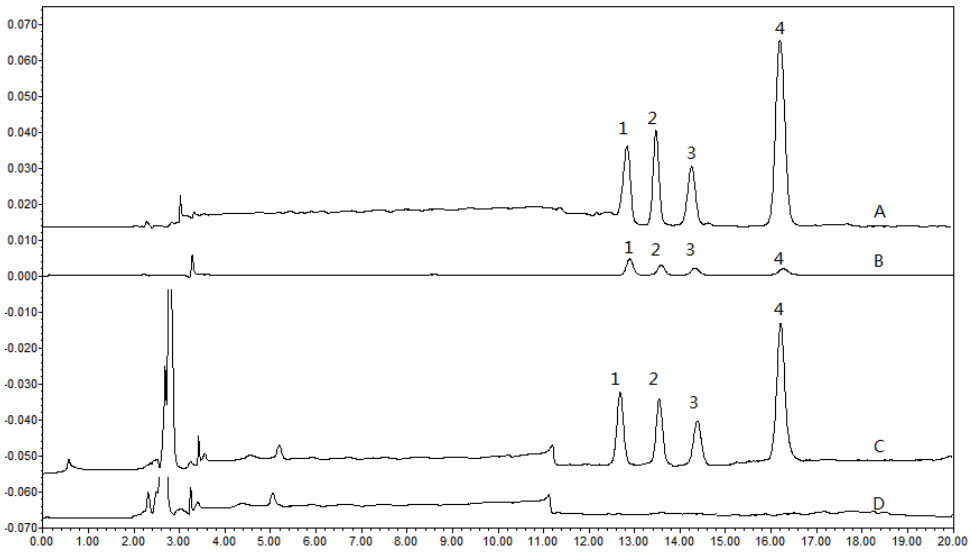


Fig. 5. Chromatograms of (A) the four FQs after IL-DLLME, (B) the four FQs before IL-DLLME, (C) the four FQs fortified blank pork, and (D) the blank pork (1 =NOR, 2 = CIP, 3 = LOM, 4 = ENR; 50 ng mL^{-1}).



Ionic liquid dispersive liquid-liquid microextraction combining high performance liquid chromatography was used to determine four fluoroquinolone drugs in meat.