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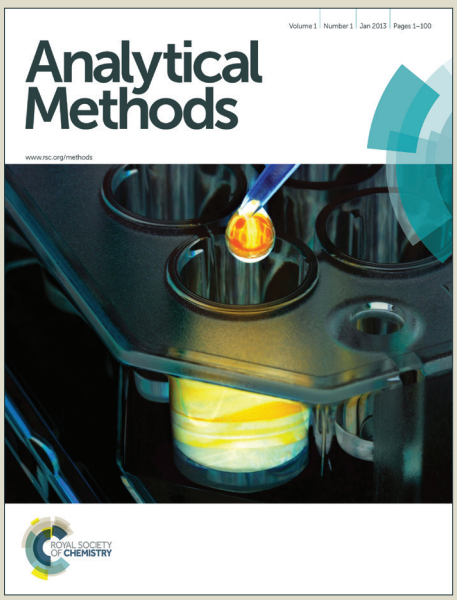

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Ionic liquid dispersive liquid-liquid microextraction combining high performance liquid chromatography for determination of tetracycline drugs in egg

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Ionic liquids are used more and more as a class of novel extraction solvents to extract various compounds from simple and complex samples. The objective of this study was to develop an ionic liquid-based dispersive liquid-liquid microextraction procedure combining with high performance liquid chromatography for determination of tetracycline drugs in egg. Ionic liquid 1-butyl-3-methylimidazolium hexafluorophosphate was proven to show the best performance among four tested ionic liquids. Several parameters possible influencing the extraction efficiency (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, this method showed different enrichment factors (12-44 folds) for four tetracycline drugs (tetracycline, oxytetracycline, doxycycline and chlortetracycline). The limits of detection for the four analytes in egg were in the range of 2.0-12 ng g^{-1} and the recoveries from the standards fortified blank egg were in the range of

58.6%-95.3% with coefficients of variation lower than 6.2%. Therefore, this method can be used as a simple and sensitive tool to determine the residues of the four tetracycline drugs in egg.

1. Introduction

Tetracyclines (TCs) are a class of broad antibacterial drugs that are widely used to treat various bacterial induced diseases in animals, and tetracycline (TC), oxytetracycline (OTC), doxycycline (DC) and chlortetracycline (CTC) are four commonly used TCs in China. However, the wide use of TCs in farm animals may produce their residues in animal derived foods that can cause allergic reactions to the consumers [1] and accelerate the spreading of antimicrobial resistance.[2, 3] For protection of consumer health, the Ministry of Agriculture of China has established different maximum residue levels (MRLs) for TCs in different animal derived foods, e.g. 100 ng mL^{-1} for TC, OTC and CTC in milk; 200 ng g^{-1} for TC, OTC and CTC in egg. Because DC has been banned for use in milk cows and laying hens in China, there is no MRL level for DC. Therefore, it is very important to monitor the residues of TCs in animal derived foods.

By now, many methods, such as high performance liquid chromatography (HPLC),[4-9] liquid chromatography mass spectrometry [5, 10-13] and immunoassay, [14] have been reported to determine the residual TCs in animal derived foods. In these methods, the first thing was to extract TCs from the samples, and the commonly used extraction methods were liquid-liquid extraction (LLE) and solid phase extraction (SPE).[4-11, 13, 14] Furthermore, molecularly imprinted polymer (MIP) was also used as the extraction method.[12] However, these sample preparation methods all had their respective disadvantages. As a conventional extraction method, LLE is tedious and time consuming, and requires large volume of organic solvents. SPE is also a conventional extraction method that is simple and rapid, but the SPE column is easily interfered by the sample matrices. MIP is a special SPE method that is prepared for a specific analyte, so it is a kind of differential extraction method.

In a previous report, a novel extraction method, dispersive liquid-liquid microextraction (DLLME), was developed.[15] In this method, the extraction solvent and the dispersive solvent were rapidly injected into an aqueous sample to form a cloudy solution. During this process, the analytes in the aqueous sample were rapidly transferred into the fine droplets of the extraction solvent. Then the cloudy mixture was separated by centrifugation, and the analytes enriched in the extraction solvent (sediment) were determined by chromatography or spectrometry method. DLLME method has the advantages of rapidity, low cost, simple, high recovery and high enrichment factor, so this technique has been used to extract organic pollutants and metal ions in environmental samples.[15-17]

Ionic liquids (ILs) are a class of melting salts that are composed of organic cations and organic or inorganic anions. ILs have been used in organic synthesis and catalysis for their advantages of low vapor pressure, viscosity and the miscibility with water and other organic solvents. In the last few years, ILs were used more and more as the extraction solvents to extract and concentrate various organic compounds and metal ions.[18-25] In a previous report, an IL based dispersive liquid-liquid microextraction method (IL-DLLME) was developed for extraction of polycyclic aromatic hydrocarbons, and the results were very satisfactory.[26] Thereafter, many ILs based liquid-liquid microextraction (LLME) methods were developed for extraction of metal ions, pesticides, organic pollutants and pharmaceuticals from water samples [27-32] and various complex samples.[33-36] These methods included IL-DLLME, [29-31, 33-35] temperature controlled IL-DLLME, [27, 28] ultrasound-assisted IL-DLLME [32] and IL based homogenous LLME.[36] Results showed that these ILs based LLME methods all achieved satisfactory results with high sensitivities and high enrichment effects, but IL-DLLME was simpler and rapider than others.

However, the report in which IL-DLLME technique was used to extract veterinary drugs from animal derived foods is rare, and there has been no paper reporting the use of this method for extraction of TCs from animal derived foods so far. In the present study, an IL-DLLME method was developed to extract and enrich four TCs (TC, OTC, DC, and CTC) from egg, and some important parameters related with the extraction method were optimized. Then the IL-DLLME method coupled with HPLC was used to determine the residues of the four TCs in egg.

2. Experimental

2.1. Reagents and chemicals

The standards of tetracycline (TC), oxytetracycline (OTC), chlortetracycline (CTC) and doxycycline (DC) were purchased from Sigma (St. Louis, MO, USA). Three 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 was 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 these TCs were prepared with acetonitrile (10.0 μg mL^{-1}) and their working solutions with series concentrations were diluted from the stock solutions with the HPLC mobile phase described below. All the standard solutions were stored at 4 ^{o}C to be stable for 6 weeks.

2.2. HPLC equipments and conditions

HPLC system was consisted of a Waters 1525 liquid chromatography, a Waters 2998 DAD detector and a C18 column (150×4.6 mm, 5μm) (Waters, USA). The mobile phase was consisted of (A) acetonitrile and (B) 0.01 mol L^{-1} trifluoroacetic acid (pH 3.0) with binary gradient elution at a flow rate of 1.0 mL min^{-1}. The gradient elution started with 10% (A), linearly increased to 20% (A) in 5.0 min and further linearly increased to 30% (A) in 7.0 min, then linearly decreased to 20% (A) in 3.0 min, brought back to 10% (A) in 5.0 min and maintained for 2.0 min with a total running time of 22 min. The injection volume was 20 μL and the detection wavelength was 350 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. Synthesis of new ionic liquid

About 5 mL of 1-methyl imidazole was added into a round-bottom flask, and 8.1 mL of 1-bromo-3-methyl-2-butene was added dropwise into the above flask under stirring within 1 hour. Then the mixture was stirred for 4 hours under the protection of nitrogen. After the reaction was stopped, the mixture was washed with 5 mL of ethyl acetate for several times until the refractive index of ethyl acetate was not changed any more. Then the mixture was evaporated on a rotary evaporator at 40 ℃ for 1 hour to remove the excess ethyl acetate. About 10.4 g of the intermediate product and 10.0 g of KPF_6 were dissolved in 20 mL of water to be stirred for 1 hour at room temperature, and then the mixture was laid until the mixture was separated to two phases. The supernatant phase was discarded and the lower phase was washed with water until bromide ion was not detected by using of $AgNO_3$ test solution. Finally, the product was concentrated on a rotary evaporator at 50 ℃ for 2 hours

to obtain the new IL 1-isopentene-3-methylimidazolium hexafluorophosphate ([IMIM][PF_6]) (^{1}H NMR ($CDCl_3$, 600 MHz) δ: 1.67 (s, 6H, CH_3), 3.62 (s, 3H, CH_3), 4.35 (d, J =4.8 Hz,2H, CH_2), 5.21 (t, J = 4.8 Hz,1H, C=CH), 6.92~7.45 (m, 3H); IR(KBr): 3280~3310, 3075, 2900， 2870, 1650, 1560, 1435, 1415, 1180 cm^{-1}).

2.4. Sample preparation

Some eggs obtained from the controlled farms were used as the blank samples. About 2.0 g of homogenized egg sample, 5 mL of acetonitrile and 1 mL of 0.01 mol L^{-1} trifluoroacetic acid (pH 3.0) were added into a 20 mL polypropylene centrifuge tube to be stirred vigorously on a vortex mixer for 3 min. Then the tube was centrifuged at 10,000 rpm for 5 min. The supernatant phase was collected and evaporated to about 1 mL on a rotary evaporator at 40 ℃, and the left solution was diluted to 5.0 mL with water and filtered with a 0.22 μm Millipore filter prior to IL-DLLME procedure. The IL-DLLME procedure was performed as follows. Briefly, the obtained 5 mL aqueous solution was put into a 10 mL conical flask, and the pH of the solution was adjusted to about 3.0 by using 1.0 mol L^{-1} formic acid. Then 400 μL of acetonitrile containing 50 μL of IL was quickly injected into the solution. The flask was shaken immediately for 30 s, laid for 60 s, and centrifuged at 4000 rpm for 5 min. Then the IL phase was settled down to the bottom of the conical flask. The upper aqueous phase was removed with a syringe, and 20 μL of the settled IL was injected into HPLC system for analysis.

In the present study, the newly synthesized IL ([IMIM][PF_6]) and three commercial ILs ([C_4MIM][PF_6], [C_6MIM][PF_6] and [C_8MIM][PF_6]) were used to optimize the best IL. During the experiments, the solutions of the four TCs prepared with blank extracts (50 ng mL^{-1}) 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 50 ng mL^{-1}.

2.5. Real egg samples

Thirty unknown eggs purchased from the local supermarkets of China were analyzed by the developed IL-DLLME-HPLC method.

3. Results and discussion

3.1. Isolation of TCs from egg

In many previous IL-DLLME methods, the analytes were usually extracted from water samples directly.[26-32] In several other reports, the analytes in different complex samples were firstly transferred into an aqueous phase, and the aqueous extracts were then treated by using IL-DLLME [33-35] or IL based homogeneous LLME. [36] Therefore, the first thing for development of an IL-DLLME method for the residual TCs in egg was to transfer the analytes from egg into an aqueous phase. In the previous reports for determination of TCs in animal derived samples, different acidic aqueous solvents (oxalate buffer, citrate buffer or EDTA-Mcllvaine buffer; pH range of 3.0-4.5) were usually used as the extraction solvents.[4-14, 37] However, the different salt ions in those buffers possibly influenced the extraction efficiency of the subsequent IL-DLLME method. Therefore, 5 mL of acetonitrile and 1 mL of trifluoroacetic acid (0.01 mol L^{-1}, pH 3.0) were used to transfer TCs from egg into a liquid phase in the present study, i.e. the mixed extraction solvent was in acidic condition and free of salt ion. Then acetonitrile was evaporated and the left solution was diluted to 5.0 mL with water, i.e. the residual TCs in egg were transferred into an aqueous phase prior to IL-DLLME procedure.

3.2. Optimization of IL-DLLME procedure

In order to obtain the maximum extraction efficiency, some important parameters possible influencing the enrichment performance were investigated by using a series of experiments. In this study, the enrichment factors (EFs) for the four TCs were used to evaluate the best condition of each parameter.

3.2.1. Selection of ionic liquid

The development of an IL-DLLME procedure requires a suitable IL. In the previous reports, imidazolium-ILs containing $[PF_n]^{n-}$ and hydrophobic alkyl side chain were usually used as the extraction solvents,[26-36] and the commonly used ILs were $[C_6MIM][PF_6]$ and $[C_8MIM][PF_6]$ due to their high hydrophobicity.[26-29, 31-35] The original objective of the present study was to synthesize a new hydrophobic IL and develop an IL-DLLME procedure for TCs. During the experiments, the newly synthesized IL ($[IMIM][PF_6]$) and other three ILs ($[C_4MIM][PF_6]$, $[C_6MIM][PF_6]$ and $[C_8MIM][PF_6]$) were used to optimize the best IL.

As shown in Fig. 1, $[C_6MIM][PF_6]$ showed no enrichment effect for CTC, and $[C_8MIM][PF_6]$ showed no enrichment effect for OTC. $[C_4MIM][PF_6]$ and the newly synthesized $[IMIM][PF_6]$ showed enrichment effects for the four TCs, and the EFs for the four TCs when using $[C_4MIM][PF_6]$ were higher than that when using $[IMIM][PF_6]$. This meant that $[C_4MIM][PF_6]$ showed the best performance among the four ILs in this study. In a previous report, when $[C_4MIM][PF_6]$ was used in a DLLME procedure, the cloudy solution was not formed and the settled ionic liquid was not obtained because of its high water solubility, [33] so $[C_4MIM][PF_6]$ showed the worst performance in many previous reports.[26-29, 31, 32, 34, 35] In those reports, the aqueous solutions were all at neutral condition when performing the IL-DLLME, but it was found during our experiments that only if the

aqueous solution was in a medium acidic condition (pH 3.0-5.0) could the four TCs be transferred into the IL phase (see section of 3.2.6). Maybe the medium acidic condition helped [C_4MIM][PF_6] to extract the four TCs but interfered with other ILs' extraction for the four TCs. However, the actual reason was unknown and remained to be studied. In the present study, [C_4MIM][PF_6] was used for the subsequent experiments.

3.2.2. Selection of disperser solvent

For development of an IL-DLLME method, the disperser solvent is very important, because it must have the appropriate miscibility in both IL phase and aqueous phase to form a cloudy solution. In the previous ILs based LLME methods, methanol and acetonitrile were usually used as the disperser solvents.[26-36] In the present study, four organic solvents (methanol, ethanol, acetone and acetonitrile) were used to optimize the best disperser solvent. When using ethanol as the disperser solvent, the mixture was not separated to two phases after centrifugation, i.e. the settled IL phase was not obtained, so ethanol was eliminated. As shown in Fig. 2, when using methanol, acetonitrile and acetone as the disperser solvents, the IL-DLLME method showed enrichment effects for the four TCs, but the EFs when using acetonitrile were higher than that when using acetone and methanol. Therefore, acetonitrile was selected as the optimal disperser solvent for the subsequent experiments.

3.2.3. Selection of volume of IL

In an IL-DLLME procedure, the IL volume is a critical factor for obtaining high enrichment efficiency and high volume of sedimented IL phase. When small volume of IL is used, it is difficult to operate; when IL volume reaches a certain level, the enrichment performance will turn bad because the analyte concentration in the sedimented IL phase

will decrease with the increase of IL volume. Therefore, a previous report showed that within a certain limit the larger the volume of IL was used, the more amount of IL was sedimented down and the larger enrichment performance was obtained.[26] In the previous ILs based LLME methods, the ILs volumes ranged from 35 μL to 150 μL.[26-36] In the present study, the EFs for the four TCs were determined by using 0.5 mL acetonitrile containing different volumes of $[C_4MIM][PF_6]$ (30, 40, 50, 60, and 70 μL). The curves of the EFs versus the IL volumes are shown in Fig. 3. As shown in Fig. 3, the EFs of the four TCs increased slowly when the IL volume increased from 30 μL to 50 μL, and then decreased when the IL volume reached 60 μL. Therefore, the use of 50 μL $[C_4MIM][PF_6]$ was selected as the optimal IL volume.

3.2.4. Selection of volume of disperser solvent

A previous report showed the organic solvent could help enhance the extraction of the analyte due to reduction of the adsorption of the analyte onto the tube wall.[25] Moreover, the volume of dispersive solvent directly affected the formation of cloudy solution and the dispersion degree of IL in aqueous phase, consequently influenced the volume of the sedimented IL phase and the enrichment performance.[29] In the present study, various volumes of acetonitrile (0.3, 0.4, 0.5, 0.6, 0.7, 1.0 mL) containing 50 μL $[C_4MIM][PF_6]$ were tested. The curves of the EFs versus the acetonitrile volumes are shown in Fig. 4. As shown in Fig. 4, the EFs of the four TCs increased firstly and then decreased with the increasing of acetonitrile volume from 0.3 to 1.0 mL. At low volume (0.3 mL), the cloudy dispersion system was not well formed, leading to low volume of sedimented IL phase that was difficult to operate. When acetonitrile volume increased from 0.4 to 0.7 mL, the EFs of the four TCs decreased constantly. When acetonitrile volume increased to 1.0 mL, no IL

phase was settled down at the bottom of the conical flask after centrifugation. Therefore, the use of 0.4 mL acetonitrile was selected as the optimal disperser solvent volume.

3.2.5. Selection of extraction and centrifugal time

Extraction time is the interval between the formation of cloudy solution and before centrifugation. To evaluate the optimum extraction time, the IL-DLLME procedure was carried out at different time intervals. As shown in Fig. 5, the EFs of the four TCs reached balance when the extraction time reached 30 seconds, and longer extraction time did not affect the extraction efficiency any more. To ensure the maximum extraction efficiency, the cloudy solution was laid for 60 seconds before centrifugation in this study.

Centrifugation plays an important role in separation of the IL phase from the aqueous phase, and during this process the IL phase is settled down to the bottom of the conical flask. In order to evaluate the influence of centrifugal time, the cloudy solutions were centrifuged for 3, 5, 8, 10, and 15 min respectively at 4000 rpm. As shown in Fig. 6, the EFs of the four TCs increased with the increasing of centrifugal time from 3 to 5 min. When it exceeded 5 min, the EFs had no further increase. Therefore, 5 min was selected as the optimal centrifugal time.

3.2.6. pH

The extraction efficiency for an organic compound can be changed by adjusting the pH of the aqueous solution, because the existing form of the analyte is dependent on it.[30] In the previous reports, different acidic buffers (pH 3.0-4.5) were usually used to extract the residues of TCs from animal derived samples because of the polar nature of TCs.[4-14, 37] In the present study, it was also found that only if the aqueous extraction solution was in a medium acidic condition could TCs be transferred into IL phase. During the experiments,

the pH values of the sample extraction solutions were adjusted to 3.0-7.0 by adding appropriate volume of 1.0 mol L^{-1} formic acid before IL-DLLME. As shown in Fig. 7, the EFs of the four TCs decreased slowly when pH value increased from 3.0 to 5.0, and decreased to zero when pH value reached 6.0. This maybe was because of the chemical properties of TCs. TCs are a class of amphoteric compounds that have several dissociation constants: the acidic hydroxyl group at 3-position, pKa 2.8-3.3; the dimethylamino group at 4-position, pKa 7.3-8.3; the hydroxyl group at 12-position, pKa 9.3-10.2.[37] When the pH values increased from 3.0 to 7.0, the solubility of TCs in aqueous phase decreased, so the amount of TCs transferred into the IL phase also decreased; thus the EFs for the four TCs decreased. Therefore, pH 3.0 was selected as the optimal pH condition in the present study.

3.2.7. Salt addition

Ionic strength has dual influences on the extraction efficiency of IL. The increase of ionic strengthen can decrease the solubility of IL in aqueous phase to improve the extraction performance, but the occurrence of ion exchange procedure can enhance the solubility of IL in aqueous phase and decrease the extraction performance. In many previous IL-DLLME methods, salt addition was not used because the extraction efficiency decreased with the increase of the salt concentration.[26-32] However, there was a reverse report in which 15% NaCl was added to improve the enrichment effect due to salting out effect.[36] In the present study, various amount of NaCl (0, 1%, 2%, 5%, 10%, 15%, w/v) were added into the TCs fortified blank extracts prior to IL-DLLME procedure. As shown in Fig. 8, the EFs of the four TCs decreased rapidly with the increasing of NaCl concentration from 0 to 15%, so salt addition was not used in this study.

3.3. Analytical features of the IL-DLLME-HPLC method

This is the first study reporting the extraction of TCs residues in egg by using IL-DLLME procedure followed by determination with HPLC method. The chromatograms of the four TCs standards before and after IL-DLLME procedure are shown in Fig. 9. Under the optimal IL-DLLME conditions, the EFs for OTC, TC, CTC and DC were 12, 29, 38 and 44 respectively. The determination parameters of the IL-DLLME-HPLC method for the four TCs are shown in Table 1. It was found that there was good linear relationship over the concentration range of 5-1000 ng mL^{-1} for these TCs with the correlation coefficients (r^2) higher than 0.9992. The relative standard deviations (RSD) based on the peak areas of replicate injections were in the range of 2.65%-3.50%. The limits of detection (LODs, defined as signal/noise of 3) for the four TCs in egg were in the range of 2.0-12 ng g^{-1}, and the limits of quantification (LOQs, defined as signal/noise of 10) were in the range of 5.0-20 ng g^{-1}. In the previous instrumental methods for the residues of TCs in egg, the limits of detection ranged from 20 to 125 ng g^{-1}, [4, 10, 11] so the IL-DLLME-HPLC method developed in this study was more sensitive than those methods. Then the four TCs were fortified into blank egg samples respectively to evaluate the method recovery. Intra- and inter-day recoveries were in the range of 58.6%-95.3% with coefficients of variation (CV) lower than 6.2% (Table 2). The chromatograms of blank egg and the TCs fortified blank egg are shown in Fig. 9.

3.4. Analysis of real samples

For evaluation of the detect capability of the method, the 30 unknown eggs were determined by using the IL-DLLME-HPLC method. Among these eggs, 4 eggs contained the residue of TC, but the residue levels were far lower than its MRL level.

4. Conclusion

As a class of new extraction solvents, ionic liquids have been widely used to extract many organic compounds and metal ions from environmental samples and complex samples. This study first reported the use of $[C_4MIM][PF_6]$ for development of a dispersion liquid-liquid microextraction procedure combining HPLC method for determination of tetracycline drugs in egg. Results showed the method achieved high extraction efficiency, enrichment performance and sensitivity, though the performance of $[C_4MIM][PF_6]$ was different from some previous reports. From the analysis of fortified blank egg and unknown egg samples, the developed method could be used as a rapid, sensitive and accurate method to monitor the residues of the four tetracycline drugs in egg.

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Table 1. Determination parameters of the IL-DLLME-HPLC method for the four TCs.

Analyte	Linearity range (ng mL^{-1})	r^2	RSD (%)	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	Enrichment factor
OTC	20-1000	0.9992	3.16	12	20	12
	(50-2000)[a]	(0.9999)	(1.70)	(30)	(50)	--
TC	10-1000	0.9997	2.65	4.0	10	29
	(50-2000)	(0.9998)	(1.24)	(30)	(50)	--
CTC	10-1000	0.9993	3.50	6.0	10	38
	(100-2000)	(0.9996)	(1.15)	(50)	(100)	--
DC	5-1000	0.9997	2.68	2.0	5.0	44
	(100-2000)	(0.9999)	(1.80)	(60)	(100)	--

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

Table 2. Recoveries of the four TCs from blank egg sample.

Analyte	Added ($ng\ g^{-1}$)	Intra-day		Inter-day	
		Recovery (%)	CV (%)	Recovery (%)	CV (%)
TC	20	62.5	5.3	58.6	6.2
	200	60.8	6.1	64.9	5.8
OTC	20	82.1	4.2	84.6	4.8
	200	75.3	3.4	81.0	4.2
CTC	20	77.5	5.1	82.3	4.9
	200	73.2	3.6	76.9	5.7
DC	20	95.3	2.6	92.5	3.6
	200	94.6	2.8	91.3	3.2

Fig. 1. Effect of ionic liquid on enrichment factors of the four TCs (blank extracts, 5.0 mL; pH, 4.0; ionic liquid volume, 60 μL; disperser solvent (acetonitrile), 0.5 mL; extraction time, 1 min; centrifugal time, 5 min).

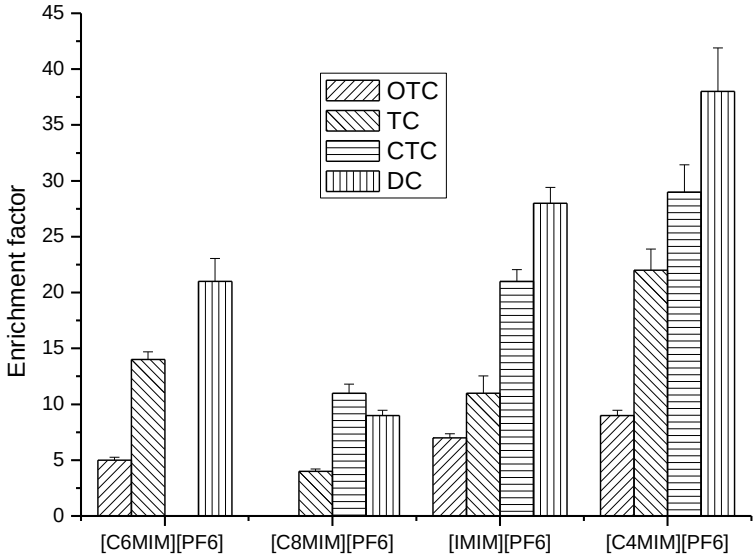

Fig. 2. Effect of disperser solvent on enrichment factors of the four TCs (blank extracts, 5.0 mL; pH, 4.0; $[C_4MIM][PF_6]$, 60 μL; disperser solvent, 0.5 mL; extraction time, 1 min; centrifugal time, 5 min).

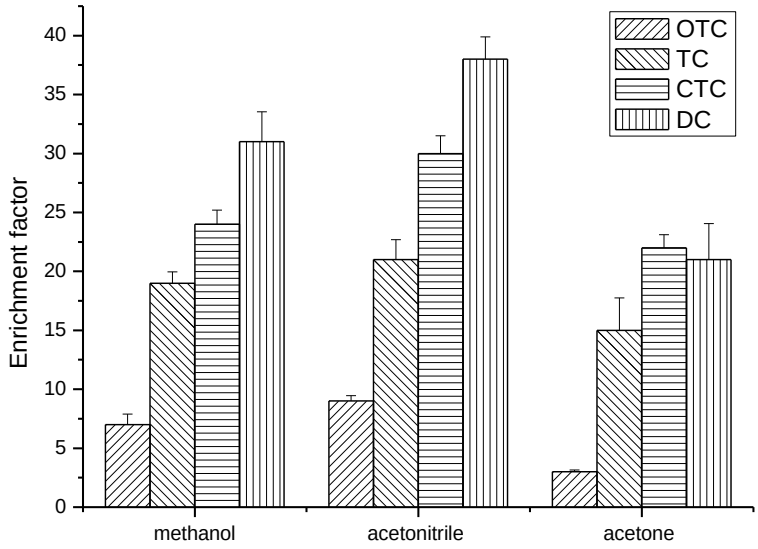

Fig. 3. Effect of the volume of $[C_4MIM][PF_6]$ on enrichment factors of the four TCs (blank extracts, 5.0 mL; pH, 4.0; acetonitrile, 0.5 mL; extraction time, 1 min; centrifugal time, 5 min).

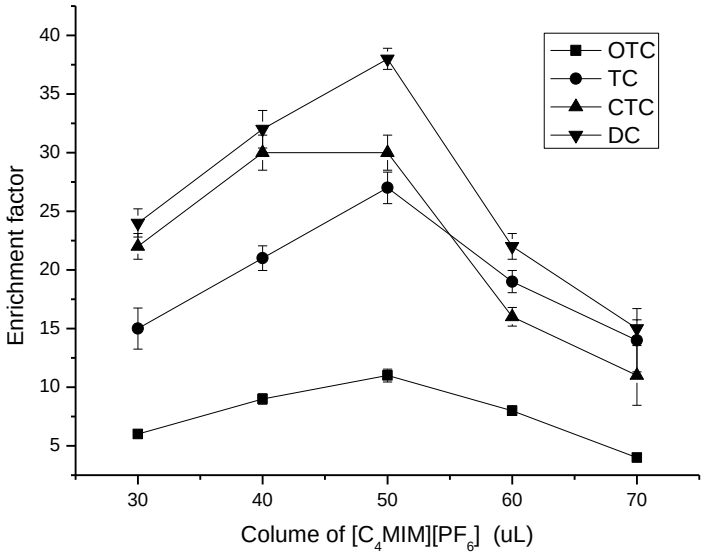

Fig. 4. Effect of acetonitrile volume on enrichment factors of the four TCs (blank extracts, 5.0 mL; pH, 4.0; $[C_4MIM][PF_6]$, 50 μL; extraction time, 1 min; centrifugal time, 5 min).

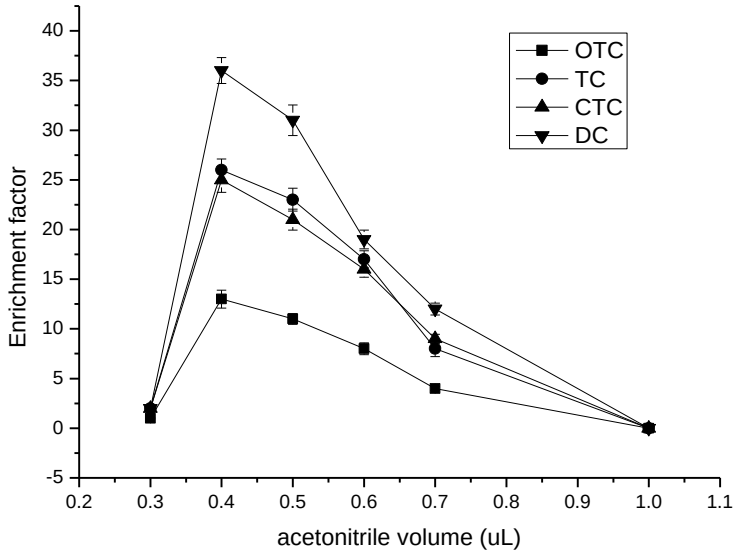

Fig. 5. Effect of extraction time on enrichment factors of the four TCs (blank extracts, 5.0 mL; pH, 4.0; $[C_4MIM][PF_6]$, 50 μL; acetonitrile, 0.4 mL; centrifugal time, 5 min).

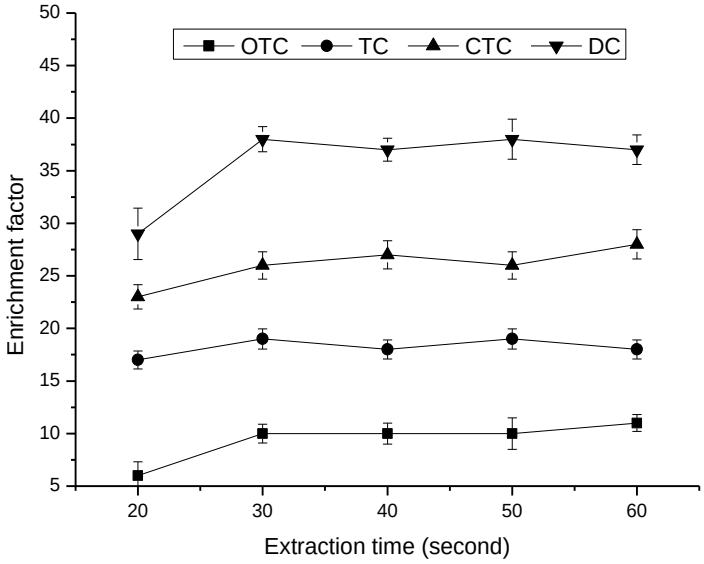

Fig. 6. Effect of centrifugal time on enrichment factors of the four TCs (blank extracts, 5.0 mL; pH, 4.0; $[C_4MIM][PF_6]$, 50 μL; acetonitrile, 0.4 mL; extraction time, 60 s).

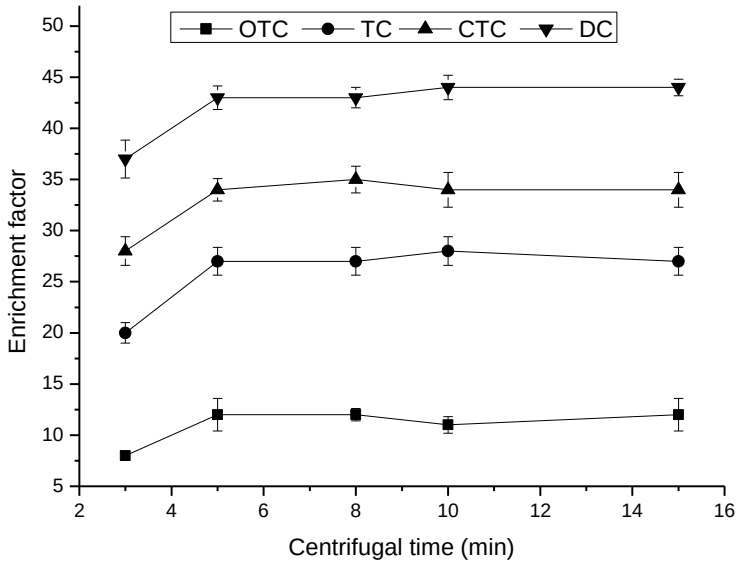

Fig. 7. Effect of extracts pH on enrichment factors of the four TCs (blank extracts, 5.0 mL; $[C_4MIM][PF_6]$, 50 μL; acetonitrile, 0.4 mL; extraction time, 60 s; centrifugal time, 5 min).

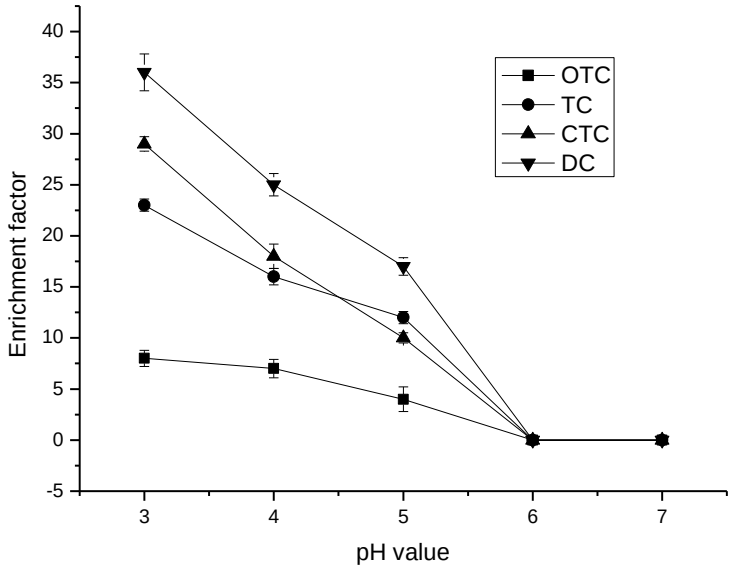

Fig. 8. Effect of the concentration of NaCl on enrichment factors of the four TCs (blank extracts, 5.0 mL; pH, 3.0; $[C_4MIM][PF_6]$, 50 μL; acetonitrile, 0.4 mL; extraction time, 60 s; centrifugal time, 5 min).

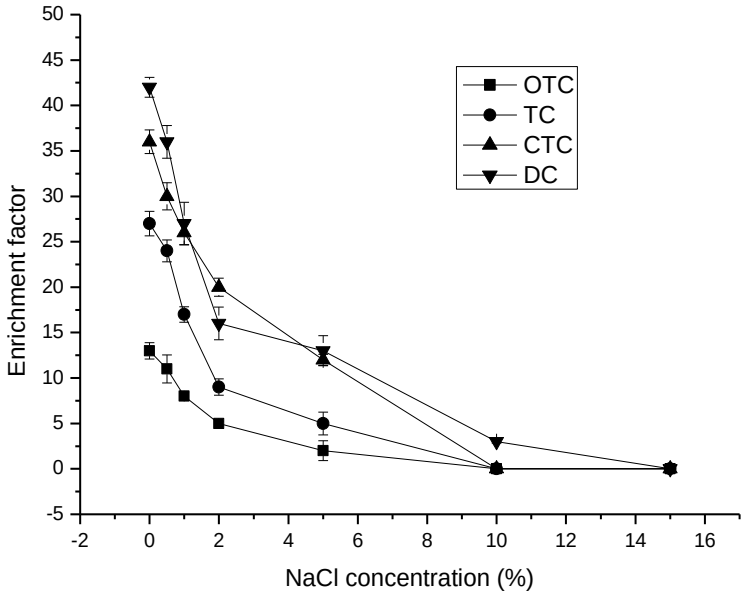

Fig. 9. Chromatograms of the four TCs (A) before and (B) after IL-DLLME, (C) the blank egg, and (D) the four TCs fortified blank egg (1 = OTC, 2 = TC, 3 = CTC, 4 = DC).