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Ultrasound-assisted surfactant/ionic liquid aqueous two-phase system extraction prior to high performance liquid chromatography for the determination of tetracyclines in milk and honey samples

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Abstract: In this work, an ultrasonic-assisted surfactant/ionic liquid aqueous two-phase system (ATPS) extraction method was developed to extract six tetracycline antibiotics from food samples before their chromatographic determination using high performance liquid chromatography. The ATPS was formed with 1-allyl-3-methyl-imidazolium bromide, Triton X-100, and dipotassium hydrogen phosphate. The parameters including type and amount of surfactant, ionic liquid and salt, pH of sample solution, and sonication time were optimized. Under the optimized conditions, linear calibration curves of the six tetracyclines were obtained in the range of 10–500 μg L^{-1} with $> r^2 = 0.990$ (n = 9). The proposed green analytical extraction method was applied to the analysis of tetracycline antibiotics in milk and honey samples with recovery of 50%–110% and 68%–117%, respectively.

Key words: Aqueous two-phase system, tetracycline, HPLC, ionic liquid, surfactant

1. Introduction

Drugs in veterinary medicine have been used for preventing animal diseases, changing behaviors, accelerating growth, feed utilization, and increasing yield. Honey and cow's milk are directly consumed natural products. These are important foods for humans, especially for babies. The residues of agricultural drugs, antibiotics, negatively affect honeybees and cows. At the same time, these antibiotics, such as sulfonamides, quinolones, penicillins, and tetracylines, can be a risk factor for human health because of their accumulation in animal products like honey and milk.[1–3]

The regulations for drugs used in animal raising and controlling of antibiotic residues in animal food products have been legislated by the European Union.[4] The maximum residue limits (MRLs) for veterinary products in food has been established by European Commission Regulation. The recommended MRL in milk is 100 μg L^{-1} by the European Committee. However, no MRL is given for honey.[5]

Tetracycline antibiotics have been successfully analyzed by reversed-phase high performance liquid chromatography (HPLC) with diode array (DAD), fluorescence, and mass spectrometer detector.[6–8] Before chromatographic analysis, separation and preconcentration of tetracyclines in food samples have been accomplished by different extraction techniques like liquid–liquid extraction[9] and solid-phase extraction.[10] Besides the advantages of these techniques, use of organic solvent and long analysis time are their basic disadvantages. In recent

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years, the aqueous two-phase system, a novel extraction method, has been used for extraction of metal ions[11–13] such as Cu(II), Au(III), cadmium(II), and zinc(II), and biomolecules[14–16] such as proteins, antibiotics, and enzymes because of its short analysis time and fast phase separation, and because it is more environmentally friendly and economical. ATPS is generally formed by combination of two different polymers, one polymer with inorganic salt or ionic liquid with inorganic salt at high concentration. Nowadays, a new ATPS extraction method is performed using surfactant with ionic liquid.[17–21] The ionic liquid-based ATPS extraction systems such as ionic liquid/sodium carbonate,[22] 1-octyl-3-methylimidazolium bromide/sodium dodecyl sulfate,[15] 1-methyl-3-octylimidazolium tetrafluoroborate/sodium dihydrogen phosphate,[23] 1-butyl-3-methylimidazolium tetrafluoroborate/ammonium sulfate,[24] 1-butyl-3-methylimidazolium tetrafluoroborate/sodium dihydrogen phosphate,[25] and 1-butyl-3-methylimidazolium halide ([Bmim] X (X = Cl, Br))/di potassium hydrogen phosphate[26] were used for the separation and preconcentration of tetracylines before their analysis.

In this work, an ultrasonic-assisted surfactant/ionic liquid based aqueous two-phase system is proposed for the determination of six tetracyclines by HPLC-DAD in milk and honey samples. Target tetracycline compounds were tetracycline (TC), oxytetracycline (OTC), chlorotetracycline (CTC), doxytetracycline (DC), minocycline (MITC), and metacycline (MTC). The effect of some parameters such as type and amount of surfactant, ionic liquid and salt, pH of sample solution and sonication time were studied.

2. Results and discussion

2.1. Characterization of ionic liquids

The structures of synthesized ionic liquids was characterized with FTIR and ^{1}H NMR spectra. In the FTIR spectra, the peaks of wave numbers of 3100–3050 cm^{-1} and 2850–2950 cm^{-1} were the aromatic =C–H stretching vibrations and aliphatic symmetric and asymmetric –C–H stretching vibrations. Peaks at wave numbers 1450–1460 cm^{-1} and 1560–1570 cm^{-1} were due to C=C and C=N stretching vibrations. The aliphatic –C–H bending vibrations and aliphatic –C–C stretching vibrations were at wave numbers 1280–1330 cm^{-1} and 1050–1168 cm^{-1}, respectively. The peaks at wave numbers 719–847 cm^{-1} were due to aromatic =C–H out-of-plane bending vibrations.

^{1}H NMR spectra were reported as ppm (δ) with internal standard (TMS). The D_2O and $CDCl_3$ were used as solvent. The results of analysis are given for the synthesized ionic liquids as follows:

AMIM-BF_4; ^{1}H NMR (D_2O, ppm): 8.92 (s, 1H, NC*H*N) 7.67 (d, 2H, NCH_2CHCH_2), 6.20 (m, 1H, NCH_2C*H*CH_2), 5.61 (m, 2 × CH, NC*H*C*H*N), 4.99 (d, 2H, NCH_2CHCH_2), 4.11 (s, 3H, NCH_3)

BMIM-BF_4; ^{1}H NMR (D_2O ppm): 8.81 (s, 1H, NC*H*N), 7.54 (d, 1H, NC*H*CHN), 4.27 (t, 2H, NCH_2CH_2), 3.99 (s, 3H, NCH_3), 1.91 (m, 2H, NCH_2CH_2CH_2CH_3), 1.36 (h, 2H, NCH_2CH_2CH_2CH_3), 0.96 (t, 3H, CH_2CH_3)

BeMIM-Br; ^{1}H NMR ($CDCl_3$ ppm): 9.75 (s, 1H, NC*H*N), 7.33 (d, 1H, NC*H*CHN), 7.24 (d, 1H, NCHC*H*N), 7.14 (d, 2H, –ArH), 6.92 (m, 3H, –ArH), 3.62 (s, 3H, NCH_3)

BeMIM-BF_4; ^{1}H NMR ($CDCl_3$ ppm): 9.26 (s, 1H, NC*H*N), 7.24–7.52 (m, 2H, NC*H*C*H*N; 2H, ArH), 7.02–6.99 (m, 3H, –ArH), 3.61 (s, 3H, –NCH_3)

2.2. Optimization of extraction parameters

In order to get high extraction efficiency, type and amount of surfactant, ionic liquid and salt, pH of sample solution, and sonication time as experimental parameters were optimized.

2.2.1. Type of surfactant

Presence of surfactant in ATPS is useful for better phase separation because of getting dense aqueous solution.[27] In this proposed method, sodium dodecyl sulfate (SDS) as anionic, cetyl trimethylammonium bromide (CTAB) as cationic, and Triton X-100 as nonionic surfactant were used to investigate their effects on the extraction efficiency of tetracyclines. To a polyethylene test tube, ionic liquids (AMIM-Br, BMIM-Br, BeMIM-Br, AMIM-BF_4, BMIM-BF_4, BeMIM-BF_4) whose final concentration was 0.12 mol L^{-1}, 1 g of K_2HPO_4, and 1 mL of 5% (w/v) each surfactant were added. Finally, the test solution (adjusted to pH 5 with acetate buffer) having 100 μg L^{-1} (initial concentration) of each tetracycline was diluted to 10 mL with deionized water. After sonication of the last mixture for 5 min, it was centrifuged. Some white particles were formed at the bottom or suspended in solution when SDS and CTAB were used as surfactant. With Triton X-100, the ionic liquid rich upper phase and inorganic salt rich bottom phase were obtained. Therefore, Triton X-100 was selected as surfactant for further studies.

2.2.2. Type of ionic liquid

Ionic liquids having different cation and anion groups can affect the phase behavior of solution.[28] For this manner, two different hydrophilic anion groups like bromide and tetrafluoroborate and imidazolium cation group having three different organic group like butyl, allyl, and benzyl were studied to investigate the extraction efficiency of six tetracycline compounds using Triton X-100 concentration. The six tetracycline compounds were extracted using all ionic liquids studied except MITC in BeMIM-BF_4 and MITC and CTC in BeMIM-Br. Therefore, AMIM-Br, AMIM-BF_4, BMIM-Br, and BMIM-BF_4 ionic liquids were used for other optimization parameters.

2.2.3. Amount of surfactant

To get the optimum amount of surfactant, the concentration of Triton X-100 in the final solution was studied from 0.25% (w/v) to 1.5% (w/v) for ionic liquids containing alkyl groups as butyl- and allyl-. The phase separation was not obtained when the concentration of Triton X-100 was 0.25% (w/v). As seen in Figure 1, the extraction yield increased up to 1% (w/v) and afterward decreased gradually by increasing to 1.5 (w/v). For further studies, the concentration of Triton X-100 in the final solution was selected as 1% (w/v).

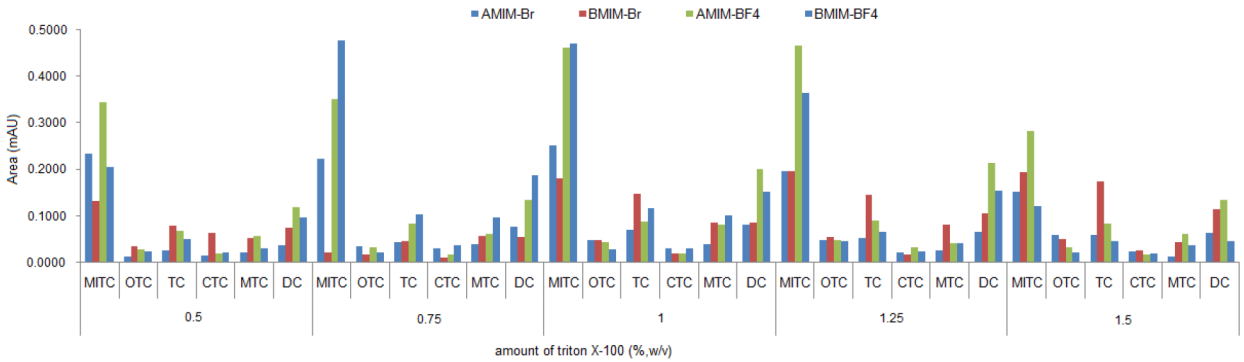

Figure 1. The optimization of amount of Triton X-100 for extraction of tetracyclines (concentration and volume of sample: 100 μg L^{-1}, 4 mL; pH: 5; concentration of ionic liquid: 0.12 mol L^{-1}; type and amount of salt: K_2HPO_4, 1 g; sonication time: 5 min).

2.2.4. pH of the system

The extraction degree of analyte can be affected by the pH of sample solution.[29] Tetracyclines have different dissociation constants, $pKa_1 = 3.3$, $pKa_2 = 7.5$, and $pKa_3 = 9$. The influence of pH on the extraction of tetracyclines from the sample solution was tested in the pH range of 3 to 9. The peak areas of the six tetracycline compounds were high at pH 3 and pH 5 (Figure 2). The tetracyclines are in the protonated or zwitterion ion form at pH 3 and pH 5, respectively. At higher pH values, it could be concluded that the extraction of these compounds was low. The interaction between tetracyclines and ionic liquid could be defined as a hydrophobic and $\pi-\pi$ interaction between aromatic rings of tetracycline compounds and imidazolium ring, especially containing allyl groups, and also possible electrostatic interactions between ionic groups' presence in the aqueous media in the ATPS method. Afterwards, pH of test solutions was adjusted to 3 because of slightly higher peak areas of tetracyclines.

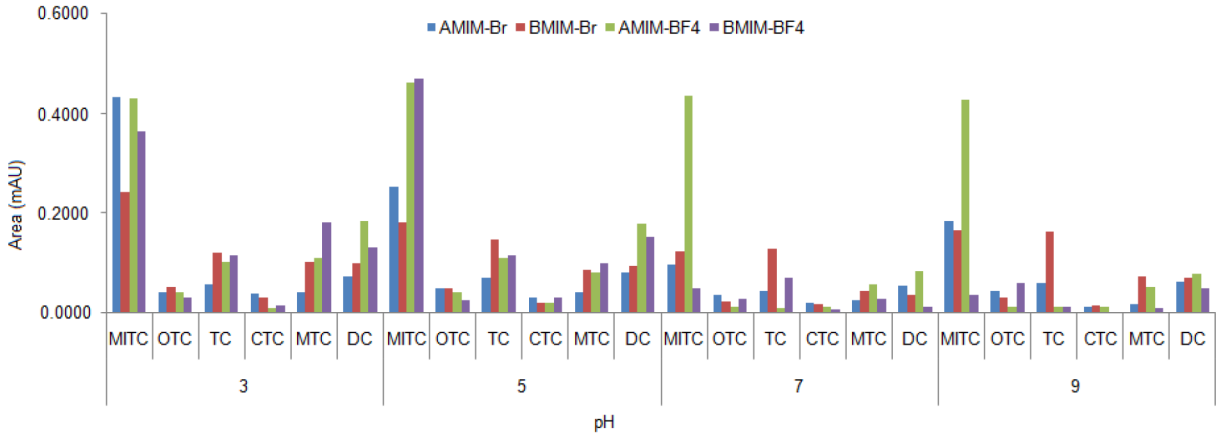

Figure 2. The optimization of pH for extraction of tetracyclines (concentration and volume of sample: 100 μg L^{-1}, 4 mL; concentration of ionic liquid: 0.12 mol L^{-1}; type and amount of surfactant: Triton X-100, 1% (w/v); type and amount of salt: K_2HPO_4, 1 g; sonication time: 5 min).

2.2.5. Amount of ionic liquid

The concentration of ionic liquid directly affects the formation of phase separation in ATPS.[30] For this purpose, a series of test solutions having final ionic liquid concentration from 0.06 to 0.18 mol L^{-1} were prepared. When ATPS was applied to these solutions, it was observed that the higher extraction efficiency was achieved with 0.09 mol L^{-1} BMIM-Br or AMIM-Br and 0.06 mol L^{-1} BMIM-BF_4 or AMIM-BF_4 (Figure 3). Therefore, further studies were carried out with these concentrations of ionic liquids.

2.2.6. Type and amount of salt

The optimization of salt is important parameter in the transition of the target analyte to organic phase.[31] To optimize the type, extraction was carried out with 1 g of K_2HPO_4, KH_2PO_4, $(NH_4)_2SO_4$, and K_2SO_4. The highest preconcentration was obtained with K_2HPO_4.

The amount of K_2HPO_4 was changed from 0.5 to 1.5 g under the same experimental conditions in test solutions to achieve effective extraction. The highest extraction efficiency was obtained with 1 g of K_2HPO_4. Thus, the further studies were carried out with 1 g of K_2HPO_4.

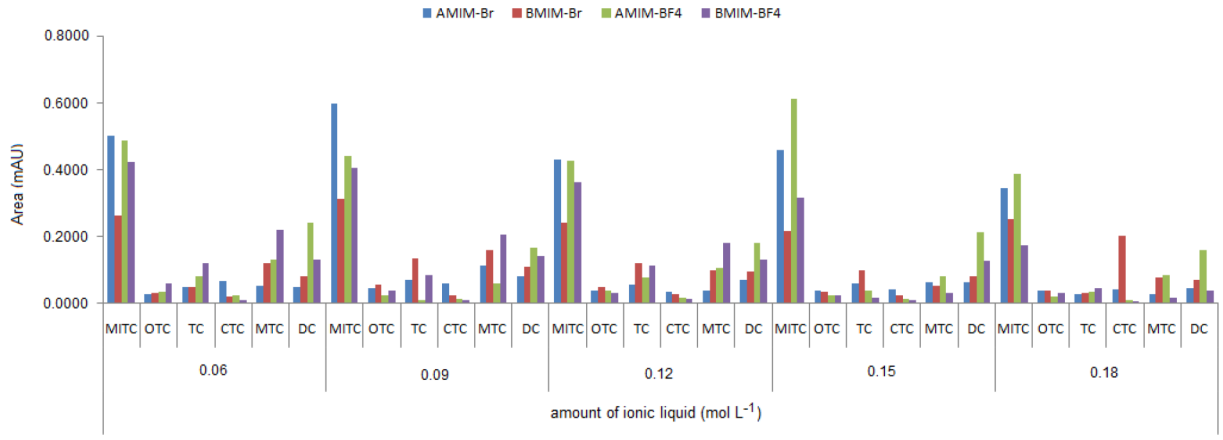

Figure 3. The optimization of amount of ionic liquid for extraction of tetracyclines (concentration and volume of sample: 100 μg L^{-1}, 4 mL; pH: 3; type and amount of surfactant: Triton X-100, 1% (w/v); type and amount of salt: K_2HPO_4, 1 g; sonication time: 5 min).

2.2.7. Sonication time

To determine the best extraction time for the highest yields of tetracyclines by ATPS, the sonication time was investigated. For this, the method was carried out for 30, 60, 120, and 180 s. The extraction yield was increased when the sonication time was 60 s for all tetracyclines in the four ionic liquids studied (Figure 4). Thus, 60 s was selected as the optimum sonication time for maximum extraction efficiency.

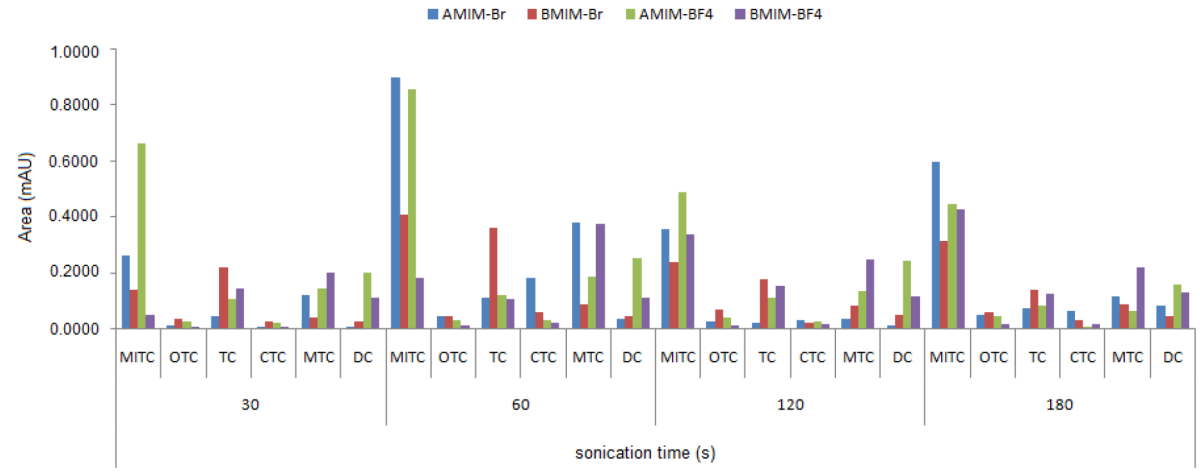

Figure 4. The optimization of sonication time for extraction of tetracyclines (concentration and volume of sample: 100 μg L^{-1}, 4 mL; pH: 3; concentration of ionic liquid: 0.09 mol L^{-1} AMIM-Br, BMIM-Br, 0.06 mol L^{-1} AMIM-BF_4, BMIM-BF_4; type and amount of surfactant: Triton X-100, 1% (w/v); type and amount of salt: K_2HPO_4, 1 g).

2.2.8. Analytical performance

According to the obtained optimization data (Figure 4, 60 s) the best results were obtained with AMIM-Br for MITC, OTC, CTC, and MTC antibiotics. Thus, the evaluation of the developed method was carried out by this ionic liquid. The linearity, precision, and limit of detection (LOD) were investigated as analytical parameters under optimized conditions. The precision of ATPS was defined as relative standard deviation percentage (RSD

%) and determined by measuring the interday and intraday for 100 μg L^{-1} and 25 μg L^{-1}, respectively. The calibration curves showed a linear peak area in the concentration of 10–500, 20–500, and 40–500 μg L^{-1} for MITC, CTC, MTC, DC and TC and OTC, respectively, and the satisfactory coefficient of determination (R^2) ranging from 0.990 to 0.998 were obtained. LOD was calculated by ten blank measurements as a signal to noise ratio of 3 and all the results are given in Table 1.

Table 1. The analytical parameters of the developed method for extraction of tetracycline compounds.

Tetracycline	Linear range (μg L^{-1})	R^2	LOD (3 × S/N) (μg L^{-1})	RSD % (interday, n = 5) (25 μg L^{-1})	RSD % (interday, n = 5) (100 μg L^{-1})
MITC	10–500	0.996	2.60	2.62	6.62
OTC	40–500	0.997	11.98	2.01*	1.16
TC	20–500	0.998	1.37	2.46	6.49
CTC	25–500	0.997	7.72	0.75	5.77
MTC	10–500	0.992	3.24	1.27	0.29
DC	10–500	0.990	3.19	4.28	9.97

*for 50 μg L^{-1}

2.2.9. Application of the ATPS method to real samples

The developed ionic liquid-based ATPS method was applied to milk and honey samples with AMIM-Br under optimal conditions. MITC, OTC, TC, CTC, MTC, and DC tetracyclines were not observed in the milk samples. The observed tetracycline residues in the honey samples were under maximum residue limits of tetracyclines. Recovery was calculated by spiking tetracycline standard mixture at 50 μg L^{-1} and 100 μg L^{-1} concentrations in final solution to the milk and honey samples. It was observed that the recoveries of tetracyclines were 50%–110% and 68%–117% milk and honey samples, respectively (Table 2). The chromatograms of spiked and nonspiked real samples are given in Figures 5a–5c and Figures 6a–6c. The recoveries indicated that the developed ionic liquid-based ATPS method was successfully applicable to milk and honey samples.

2.2.10. Comparison of the developed method with the other ionic liquid-based ATPS methods

The performance of the developed method was evaluated with the other extraction methods for tetracyclines. As shown in Table 3, the developed method for separation and preconcentration of tetracyclines before their analysis offered a short analysis time and a wide linear range. Moreover, the method had small LOD values, in comparison with other extraction methods for tetracyclines.

3. Conclusions

In this study, a new ionic liquid-based aqueous two-phase system was developed for the separation and preconcentration of tetracyclines from an analytical matrix before their HPLC determination. For this purpose, imidazolium-based ionic liquids containing butyl, allyl, and benzyl as alkyl groups and bromide and tetrafluoroborate as anion groups were used. The parameters such as type and amount of surfactant, ionic liquid and salt, pH of sample solution, and sonication time were optimized with all ionic liquids. The highest extraction efficiency was obtained with AMIM-Br at the optimum conditions for MITC, OTC, CTC, and MTC antibiotics. Thus, the analytical performance and application of this method to real samples was carried out with this ionic liquid. For accuracy of this method, 50 μg L^{-1} and 100 μg L^{-1} standard tetracycline mixture were added to milk and honey samples and the recovery was $\geq$50% and 68%, respectively.

Table 2. Results of recoveries of tetracyclines in milk and honey samples.

	Added	MITC		OTC		TC		CTC		MTC		DC	
	(μg L^{-1})	Found (μg L^{-1})	Recovery (%)	Found (μg L^{-1})	Recovery (%)	Found (μg L^{-1})	Recovery (%)	Found (μg L^{-1})	Recovery (%)	Found (μg L^{-1})	Recovery (%)	Found (μg L^{-1})	Recovery (%)
Honey	-	29 ± 1	-	40 ± 1		44 ± 2		50 ± 2		13 ± 1		20 ± 1	
	50	75 ± 3	94 ± 19	85 ± 8	103 ± 9	90 ± 6	106 ± 3	95 ± 6	102 ± 8	60 ± 3	117 ± 2	64 ± 2	114 ± 9
	100	122 ± 3	97 ± 7	137 ± 9	112 ± 11	141 ± 7	68 ± 7	143 ± 7	113 ± 5	115 ± 6	109 ± 1	115 ± 6	74 ± 11
Milk	-	-		-		-		-		-		-	
	50	48 ± 1	74 ± 8	51 ± 5	74 ± 3	45 ± 4	50 ± 2	46 ± 3	101 ± 3	44 ± 2	89 ± 2	47 ± 3	110 ± 9
	100	101 ± 5	79 ± 6	103 ± 8	65 ± 3	92 ± 6	80 ± 8	93 ± 5	89 ± 4	98 ± 4	109 ± 1	94 ± 5	81 ± 9

Concentrations of 50 and 100 μg L^{-1} added to the sample correspond to 48 and 96 μg kg^{-1} for milk and 35 and 70 μg kg^{-1} for honey, respectively.

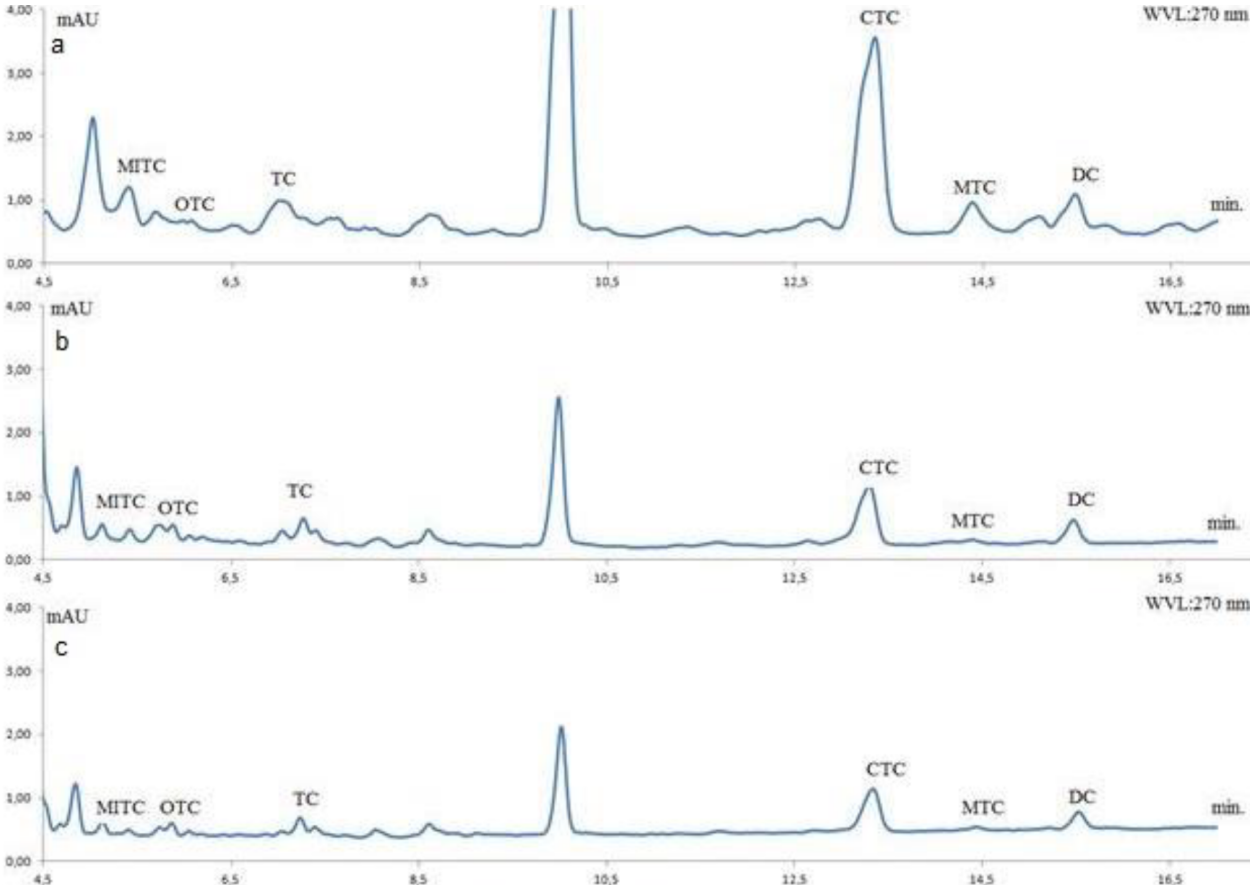

Figure 5. HPLC chromatograms of honey sample spiked with 100 μg L^{-1} ((70 μg kg^{-1}) tetracycline standard (a); honey sample spiked with 50 μg L^{-1} (35 μg kg^{-1}) tetracycline standard (b); unspiked honey sample (c) after ATPS method, (retention time (min): MITC: 5.4, OTC: 6.1, TC: 7.2, CTC: 13.4, MTC: 14.4, DC: 15.6.

The developed method was simple, had good repeatability, was rapid, and was applicable to food products. It might also be applicable to other antibiotic groups after optimization of the proposed ATPS method.

4. Experimental

4.1. Chemicals

The standards of OTC, TC, CTC, MITC, MTC, and DC (purity 97%–99.7%) were obtained from Sigma-Aldrich and Fluka. The ionic liquids [1-butyl-3-methyl imidazolium bromide (BMIM-Br) and 1-allyl-3-methyl imidazolium bromide (AMIM-Br)] were purchased from Sigma-Aldrich. 1-Methylimidazole, benzyl bromide, ammonium tetrafluoroborate, formic acid, trifluoroacetic acid (TFA), trichloroacetic acid (TCA), citric acid, sodium citrate, toluene, dichloromethane, HPLC grade acetonitrile (ACN), and methanol were purchased from Sigma-Aldrich, Alfa Easer, or Merck. All other chemicals used were of analytical grade. Deionized water was obtained with a Milli-Q water purification system. Deuterium oxide (D_2O) and deuterated chloroform ($CDCl_3$) were used for ^{1}H NMR spectra and chemical shift values were recorded as ppm.

The individual stock solution of each tetracycline compound (1000 μg L^{-1}) was prepared by methanol and stored in a refrigerator at –18 °C. The mixed stock solution containing six tetracyclines was prepared from

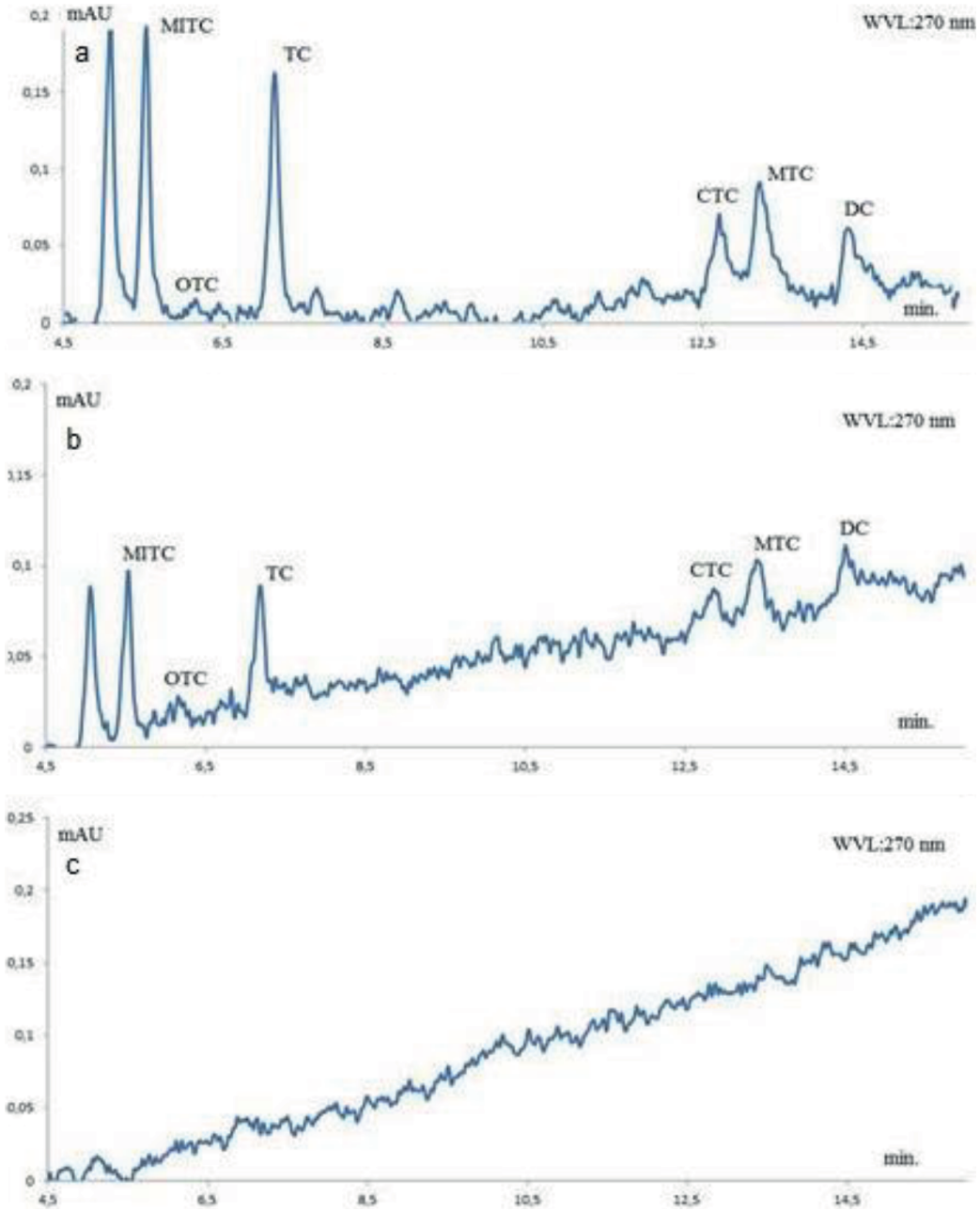

Figure 6. HPLC chromatograms of milk sample spiked with 100 μg L^{-1} (96 μg kg^{-1}) tetracycline standard (a); milk sample spiked with 50 μg L^{-1} (48 μg kg^{-1}) tetracycline standard (b); unspiked milk sample (c) after ATPS method, retention time (min): MITC: 5.4, OTC: 6.1, TC: 7.2, CTC: 12.8, MTC: 13.2, DC: 14.4.

individual stock solution by diluting with methanol and stored at 4 °C in dark conditions. The mixed working solutions were prepared daily.

Ionic liquids [1-benzyl-3-methyl imidazolium bromide (BeMIM-Br), 1-butyl-3-methyl imidazolium tetrafluoroborate (BMIM-BF_4), 1-allyl-3-methyl imidazolium tetrafluoroborate (AMIM-BF_4), 1-benzyl-3-methyl imidazolium tetrafluoroborate (BeMIM-BF_4)] were prepared according to the literature.[36] For the synthesis of BeMIM-Br, N-methyl imidazole and benzyl bromide were refluxed at 70 °C for 10 h. Then the BeMIM-Br

Table 3. Comparison of the proposed method with other extraction method for tetracyclines

Method	Extr. time (min)	Linear range (μg L^{-1})	LOD (μg L^{-1})	Spiked recovery (%)	Ref.
Ionic liquid-based ATPS	1	20.1–303.6 (μg kg^{-1})	5.8–8.2 (μg kg^{-1})	85.5–109.6 (honey)	15
Ionic liquid-based DLLME	0.5	5–1000	2–12	60.8–95.3 (egg)	32
Ultrasound-assisted DLLME	4	30–600	6.4–11.1	85.7–96.4 (egg)	33
Ionic liquid-based DLLME	5	0.1–200	0.031–0.079	62.6–96.3 (river water) 58.9–94.5 (fishpond water) 55.1–86.1 (hog leachate)	34
SPE		100–1000	20–40 (ng g^{-1})	77.5–93 (milk) 74–90.2 (egg) 76.4–92.3 (pork)	35
Ionic liquid-based ATPS	1	10–500	1.37–5.98	68–117 (honey) 50–110 (milk)	This work

SPE: solid phase extraction
DLLME: dispersive liquid–liquid microextraction

collected in the lower phase was taken and washed with ethyl ether solution. The AMIM-BF_4, BMIM-BF_4, and BeMIM-BF_4 were also synthesized by mixing ammonium tetrafluoroborate and AMIM-Br, BMIM-Br, or BeMIM-Br, respectively, in dichloromethane at room temperature for 24 h.

4.2. Instruments

A Buchi rotary evaporator for synthesis of ionic liquids, Selecta 2001 pH meter for pH adjustment of aqueous solutions, Nuve ultrasonic bath and JP Selecta Ivymen system Cy-500 ultrasonic homogenizer system (500 W, 20 kHz) for sonication, and Spectrum 100 model Fourier transform infrared spectrometer with attenuated total reflectance (ATR) probe for characterization of ionic liquids were used. The spectra were obtained with the sum of 25 scans in the range of wave number from 4000 to 400 cm^{-1}. The ^1H NMR spectra of the synthesized ionic liquids were obtained at Ege University, Science Technology Research and Application Center in İzmir, Turkey.

The HPLC-DAD measurements were carried out by a Dionex Ultimate 3000 at 270 nm. Data acquisitions were performed by software. Chromatographic separation of tetracyclines was achieved using a Thermo Syncronics C_{18} (150 mm × 4.6 mm, 3 μm) analytical column. The mobile phase was composed of ACN (A) and TFA (pH 3, B). The injection volume was 10 μL. The flow rate was 1 mL min^{-1} with a linear gradient at the following conditions: 0 min: 20% A; 0–15 min: 30% A; 15–17 min: 20% A.[32]

4.3. Aqueous two-phase system

The sample solution or test solution having mixed tetracycline standards as 4 mL from solution adjusted to pH 3 with citrate buffer was put into a 15-mL centrifuge tube. Then 0.098 g of 1-allyl-3-methyl-imidazolium

bromide of final concentration 0.09 mol L^{-1}, 1 mL of 5% (w/v) Triton X-100 solution, and 1 g of dipotassium hydrogen phosphate (K_2HPO_4) were added to tube. This mixture was sonicated at 37% amplitude at room temperature for 1 min. Then the phase separation was achieved by centrifuging at 5000 rpm for 5 min. Next, 200 μL of the upper phase was transferred to a vial using a microsyringe and diluted with 100 μL of ACN. The resulting solution was filtered with a 0.45-μm PTFE filter membrane and subjected to HPLC analysis.

4.4. Sample preparation

Milk and honey samples were purchased from a local market in İzmir, Turkey. First, 10 g of milk sample (blank or fortified sample) was put in a 15-mL centrifuge tube and vortexed for 1 min. After 1 h in the refrigerator, 1 mL of 15% (w/v) TCA was added and the final mixture was centrifuged at 5000 rpm for 10 min to precipitate the protein part of the milk. The supernatant part was filtered using a 0.45-μm PTFE filter membrane, transferred to another tube, and kept in the refrigerator at 4 °C up to analysis.[37]

First, 1 g of honey sample (blank or fortified sample) was diluted with 5 mL of deionized water and homogenized for 1 h by stirring with a stirring bar. After that, this sample mixture was filtered using a 0.45-μm PTFE filter membrane and kept in the refrigerator at 4 °C until analysis.[38]

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