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Determination of Tetracycline Residues After Ionic Liquid Assisted Dispersive Liquid Liquid Microextraction in Dairy Foods

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Keywords
Tetracyclines, ionic liquid, imidazolium, milk, honey, egg, dispersive liquid-liquid microextraction

Abstract: In this study, ionic liquid based dispersive liquid-liquid microextraction method was developed for preconcentration of the six tetracycline residues (minocycline, oxytetracycline, tetracycline, chlortetracycline, methacycline , doxycycline) before high performance liquid chromatographic determination in milk, egg and honey samples. For extraction, 1-hexyl-3-methylimidazolium bis[(trifluoromethane)sulfonyl] imide ionic liquid was used as an extractant. To enhance the extraction efficiency, pH, volume of ionic liquid, type and volume of dispersive solvent, extraction time and centrifugation time were optimized. The enrichment factors of the studied tetracycline antibiotics were calculated in the range of 15 to 105 at optimized conditions. No residue of tetracyclines were found in the studied real samples. For the accuracy of this method, the 100 and 250 μg L^{-1} of standard tetracycline mixture solutions were spiked to the milk, honey and egg samples and the percentage recoveries were obtained between 50 and 95

Gıdalardaki Tetrasiklin Kalıntılarının İyonik Sıvı Destekli Dispersif Sıvı Sıvı Mikroekstraksiyonu Sonrası HPLC ile Tayini

Anahtar Kelimeler
Tetrasiklinler, iyonik sıvı, imidazolyum, süt, bal, yumurta, dispersif sıvı-sıvı mikroekstraksiyon

Özet: Bu çalışmada, süt, yumurta ve bal numunelerinde yüksek performanslı sıvı kromatografisi tayininden önce altı tetrasiklin kalıntısının (minosiklin, oksitetrasiklin, tetrasiklin, klortetrasiklin, metasiklin, doksisiklin) zenginleştirilmesi için iyonik sıvı bazlı dispersif sıvı-sıvı mikro ekstraksiyon yöntemi geliştirilmiştir. Çalışmada ekstraktant olarak 1-hegzil-3-metilimidazolyum bis[(trifloremetan)sülfonil]imid iyonik sıvısı kullanılmıştır. Ekstraksiyon verimliliğini arttırmak için, pH, iyonik sıvı hacmi, dispersif çözücü türü ve hacmi, ekstraksiyon ve santrifüj süresi optimize edilmiştir. İncelenen tetrasiklin antibiyotiklerinin zenginleştirme faktörleri, optimize koşullar altında 15 ile 105 aralığında hesaplanmıştır. Çalışılan gerçek numunelerde herhangi bir tetrasiklin kalıntısına

rastlanmamıştır. Yöntemin doğruluğu için, süt, bal ve yumurta örneklerine 100 ve 250 µg L^{-1} derişimde standart tetrasiklin karışımı eklenmiş ve geri kazanım değerleri 50 ile 95 arasında elde edilmiştir.

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1. Introduction

The amphoteric tetracycline antibiotics are the primary antibiotics used worldwide since 1950 for the control of bacterial infections in humans, animals and agricultural activities [1, 2]. When tetracycline antibiotics are taken less amount, they are metabolized or absorbed in the living organism, most of them (about 70-90%) are excreted from the urine or feces without metabolism in humans and animals. Tetracycline antibiotics used in aquaculture and veterinary medicine residues cause resistant microorganisms and pose a threat to human health by increasing infection [3]. The most common tetracyclines include minocycline, oxytetracycline, chlortetracycline, methacycline, and doxycycline. The maximum residue limits of tetracycline antibiotics are determined (µg/kg) as 100 for milk, 200 for honey and 10–50 for eggs in the European Union [4].

Dispersive liquid-liquid microextraction (DLLME) method developed by Rezaee and co-workers in 2006 [5], has been used in preconcentration studies in analytical studies due to its easy operation and usage of less amount of organic solvents. In this method, polar disperser solvent and nonpolar extractant solvent are traditionally injected to aqueous solution and the final mixture is stirred and centrifuged for phase separation. Recently, adopting of various stirring or cooling techniques, usage of ionic liquids, low density solvents and magnetic nanoparticles have improved this method and caused some advantages as high enrichment factor and extraction recoveries [6, 7]. Nowadays, ionic liquids, having low melting point, less toxicity, less volatility and also environmentally friendly solutions, are proposed as an alternative extractant to the DLLME method [8, 9].

In some recent studies, trace level of antibiotics in different water and food samples were determined by liquid chromatography and spectrofluorimetry coupled to ionic liquid based DLLME [10-14]. In only a few studies, a 1-butyl-3-methyl-imidazolium hexafluorophosphate as hydrophobic ionic liquid was employed in the determination of several tetracyclines in environmental water and eggs samples [12-14]. The lanthanium(III) salt for complex formation was used in these studies to improve extraction.

In this study, we investigated the enrichment of six tetracycline antibiotic residues (minocycline (MiC), oxytetracycline (OTC), tetracycline (TC), chlortetracycline (CTC), methacycline (MC), doxycycline (DC)) in different food samples (milk, egg and honey) using ionic liquid assisted DLLME before chromatographic determination. In this proposed DLLME method, the experimental parameters as pH, volume of ionic liquid, type and volume of dispersive solvent, extraction time and centrifugation time were examined.

2. Material and Method

2.1 Reagents and standards

Standards of minocycline hydrochloride, oxytetracycline hydrochloride (≥ 95.0%), tetracycline hydrochloride, chlortetracycline hydrochloride (80%),

methacycline hydrochloride (99.2%) and doxycycline hyclate (≥ 98%), -hexyl-3-methylimidazolium bis[(trifluoromethane)sulfonyl]imide were obtained from Sigma-Aldrich. HPLC grade of methanol (MeOH, 99.0%), acetonitrile (ACN, 99.0%), acetone (99.0%), formic acid, trichloroacetic acid (TCA) and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich and Merck.

Stock standard solution of each tetracycline antibiotics (1000 mg L^{-1}) was prepared in methanol and stored at -18°C, individually. The stock tetracycline mix solution at 100 mg L^{-1} was prepared by mixing of six standards in methanol, weekly, and the working tetracycline mix solutions were prepared by dilution of this solution with water, daily. Buffer solution (pH 3) was prepared with 0.01 mol L^{-1} TFA and 5 mol L^{-1} NaOH solution and used in mobile phase for chromatographic separation. For all dilutions ultrapure water was obtained from Milli-Q system (Millipore, USA).

2.2 HPLC

Chromatographic separation and quantification were achieved using Thermo Scientific Dionex Ultimate 3000 High Performance Liquid Chromatography (HPLC) equipped with diode array detector (DAD). The Thermo Syncronics C18 column (150 × 4.6 mm i.d., 3 µm particle size) was used. The injection volume was 10 µL and the detection wavelength was 360 nm. Chromatographic elution was achieved using gradient program. Mobile phase was composed of ACN (A) and 0.01 M TFA (pH 3.0) (B). The gradient elution was started from 20 % A to 30 % A in 15 min and then decreased to 20 % A in 2 min at a flow rate of 1.0 mL min^{-1} [14]. The retention times of the studied tetracyclines (MiC, OTC, TC, CTC, MC, DC) were: 1.50, 2.00, 2.80, 6.00, 6.70 and 7.50, respectively.

2.3 DLLME procedure

A 5 mL aqueous sample solution containing 250 µg L^{-1} of tetracycline antibiotics was adjusted to pH 3 with 1 mol L^{-1} formic acid. Then, 50 µL of h-MIM-NTF_2 and 750 µL of ACN were rapidly added to the aqueous sample solution. This mixture was first stirred for 1.5 min by vortex and then shaked on a shaker for 8.5 min to form a cloudy solution. Finally, the cloudy mixture was centrifuged at 4000 rpm for 10 min. A 45 µL of the lower phase containing tetracyclines was removed by micropipette, diluted with ACN to 100 µL and tetracyclines were analyzed by HPLC.

2.4 Samples

The developed ionic liquid assisted DLLME method was applied to milk, honey and egg samples which were supplied from a local market in Izmir.

Milk: An aliquot of 10 mL homogenized milk sample was transferred to 15 mL of polyethylene tube and vortexed for 1.5 min. Then it was stayed at 4 °C for 1 hour. Later, 1 mL of TCA solution (15 %, w/v) was added and the final sample mixture was centrifuged at 3000 rpm for 10 min for protein denaturation [15].

Honey: One g of honey sample was homogenized with 5 mL of ultrapure water and stirred for 1 h in glass vials, magnetically [16].

Egg: Two grams of whole egg sample was mixed with 2 mL of water for 1 h in glass vials. After that 10 mL of acetic acid (1 %, v/v) prepared in ACN was added and the mixture was vortexed for 2 min. After protein precipitated by centrifugation at 3000 rpm for 5 min, the supernatant was

filtered through 0.45 filter, and the ACN was evaporated at 40 °C in an evaporator. Then the remaining part was diluted to 5 mL with water at pH 3 and stored at 4 °C until analysis. [14].

3. Results

3.1 Optimization of DLLME

In this study, the extraction mixture combining h-MIM-NTf_2 and ACN was added to tetracycline antibiotics spiked aqueous test solution. After the mixture was vortexed and shaken on an orbital shaker, the cloudy solution was formed. Finally, the cloudy mixture was centrifuged. The lower phase containing tetracyclines was diluted with ACN and the tetracyclines were analyzed by HPLC. To optimize the extraction method pH, volume of extractant (h-MIM-NTf_2) and volume of dispersive (ACN) solvent, extraction time and centrifuge time were optimized. The optimized conditions were examined using enrichment factor (EF) calculated as follows;

$$EF = \frac{c_{IL}}{c_{aq}}$$

where, c_{IL} and c_{aq} are the concentrations of analyte in the ionic liquid phase and sample solution, respectively.

3.1.1 Effect of pH

The extraction efficiency could be varied by adjusting the pH value of the aqueous solution containing the analytes. In this study, the pH values of the aqueous solutions containing the tetracyclines were adjusted between 3 and 5 with formic acid (1 mol L^{-1}). As seen in Figure 1, the best enrichment factor was calculated at pH 3. Above pH 3, the enrichment factors of tetracyclines were decreased gradually. The reason maybe the chemical structure of these compounds. They are amphoteric compounds that have methylamino, phenolic hydroxy and enol base groups at different positions in their heterocyclic structures. Depending on these groups, they have three pK_a values. Many studies have been focused on the effect of pH on the extraction of efficiency of tetracyclines at medium acidic conditions [12, 17, 18].

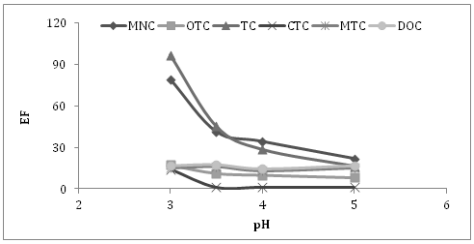

Figure 1. Effect of pH. 5 mL working solution. 50 µL of *h*-MIM-NTf_2; 0.5 mL of MeOH; extraction time, 90 s; centrifuge, 4000 rpm for 10 min.

3.1.2 Effect of volume of ionic liquid

The volume of ionic liquid affects the extraction efficiency. In the DLLME method, extraction solvent used should be denser than water, the solubility of the water should be less and the solubility of analyte should be high in it. In this study, when the volume of h-MIM-NTf_2 was increased from 25 to 100 µL, the efficiency of extraction increased as seen in Figure 2. But the peaks of tetracyclines were expanded above 50 µL of ionic liquid. For this reason, the optimum volume was selected as 50 µL for the following studies.

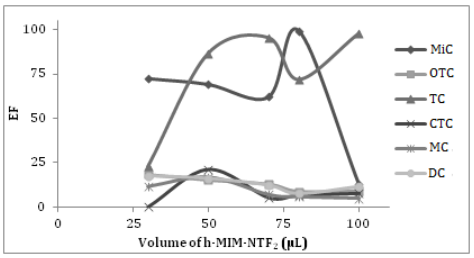

Figure 2. Effect of volume of ionic liquid. 5 mL working solution; pH=3; volume of MeOH; 0.5 mL.

3.1.3 Effect of dispersive solvent type and its volume

Determination of dispersive solvent is a key factor for DLLME because of the achievement of the analyte transition between aqueous and extractant phase within cloudy solution. Methanol, acetonitrile and acetone was used for extraction of tetracyclines. As seen in Figure 3, when ACN was used, the enrichment factor was highest for all. So further studies, ACN was preferred for dispersive solvent.

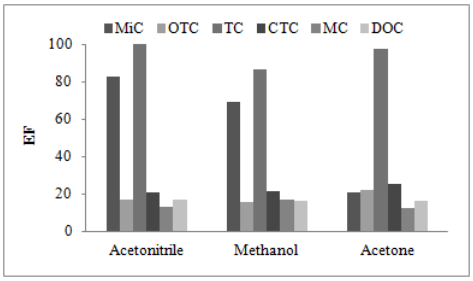

Figure 3. Effect of dispersive solvent type. 5 mL working solution; pH=3, 50 µL of *h*-MIM-NTf_2

To obtain the best recovery of extraction depending on the dispersive solvent volume, ACN volume was tested in the range of 200 to 1000 µL (Figure 4). Differentiation of dispersive solvent volume effects the cloudy solution formation, dispersion of ionic liquid in aqueous upper phase and preconcentrated lower phase, directly [19].

While volume of ACN increased, the enrichment factors of OTC, CTC, MC, DC were not significantly affected. But while the volume of ACN was increased to 750 µL, the enrichment factor increased for MiC and TC. After that point, their enrichment factors decreased. The reason might be the decrease in phase transfer. So, 750 µL volume of ACN was used for further studies.

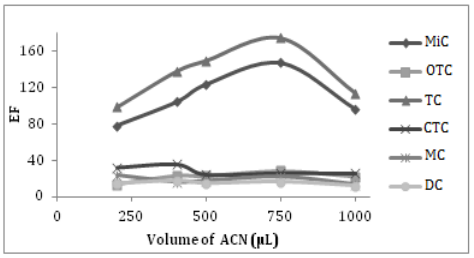

Figure 4. Effect of dispersive solvent volume. 5 mL working solution; pH=3; 50 µL *h*-MIM-NTf_2.

3.1.4 Extraction time and centrifuge time

The extraction time includes the formation of the cloudy solution and the pre-centrifugation interval [11]. Different time intervals were used to determine optimum extraction recovery. As shown in Figure 5, the highest enrichment recovery was obtained at 10 min for almost all tetracyclines, except MC and DC. For this reason, the extraction was continued for 10 min in subsequent studies.

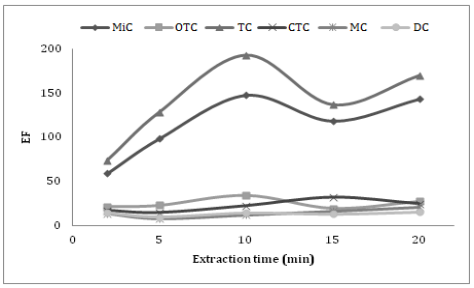

Figure 5. Effect of extraction time. 5 mL working solution; pH=3, 50 µL of *h*-MIM-NTf_2; 750 µL of ACN.

Centrifugation plays an important role to collect the enriched ionic liquid phase at the bottom of the test tube. To survey the effect of the centrifugation time, the cloudy solutions were centrifuged at 4000 rpm at different time intervals. As given in Figure 6, the best yield was obtained for 10 min. During the DLLME procedure, the centrifugation time was maintained as 10 min.

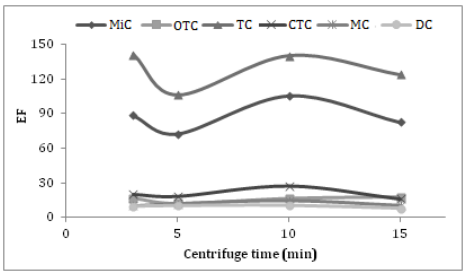

Figure 6. Effect of centrifugation time. 5 mL working solution; pH=3, 50 µL of *h*-MIM-NTf_2; 750 µL of ACN.

3.2 Analytical Figures of Merit

Calibration data of all six tetracyclines (TCs) were obtained using DLLME-HPLC-DAD system within the range of 50-1000 µg L^{-1} by the correlation coefficients (0.9798-0.9988). The within-day and between-day precision of the proposed method were determined as relative standard deviation percentage (RSD %) by performing for 100 and 250 µg L^{-1} of tetracyclines. The limit of detection (LOD) was calculated as the ratio of three times of standard deviation of blank to the slope of the method calibration curve. The obtained results were summarized in Table 1. The enrichment factors of MiC, OTC, TC, CTC, MC, and DC were found as 105, 25, 98, 32, 15 and 25, respectively.

Table 1. Analytical parameters of the method

Parameters	TCs					
	MiC	OTC	TC	CTC	MC	DC
LOD (µg L^{-1})	3.93	9.06	4.53	1.60	16.3	8.82
RSD (%, within day)						
100 µg L^{-1}	7.14	1.54	1.43	7.24	1.59	0.83
250 µg L^{-1}	3.98	7.66	4.50	1.31	5.70	0.48
RSD (%, between day)						
100 µg L^{-1}	2.46	3.51	6.95	3.63	6.07	1.56
250 µg L^{-1}	9.96	1.67	10.12	2.01	1.83	5.44

3.3 Sample Analysis

The real samples -milk, honey and eggs - were bought from the markets in Izmir. Sample solutions were prepared according to the methods in Section 2.4 and analyzed according to the optimization conditions of the ionic liquid assisted DLLME method. No TC residues were found in all samples. For accuracy o the method, 100 and 250 µg L^{-1} of tetracycline standards were added to sample solutions. The HPLC chromatograms of milk, egg and honey samples were given in Figures 7-9. The calculated recoveries were varied between 50 % and 95 % in all spiked samples (Table 2).

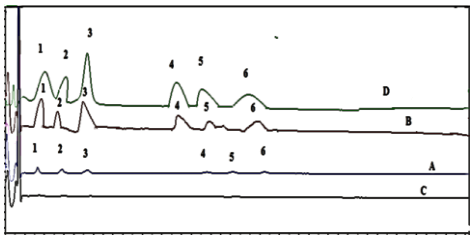

Figure 7. Chromatograms of standard mixture of TCs at 250 µg L^{-1} (A) , after IL based DLLME of standard mixture of TCs at 250 µg L^{-1} (B), blank milk sample (C), after added TCs mix standard at 250 µg L^{-1} to milk sample (D) (1=MiC, 2=OTC, 3=TC, 4=CTC, 5=MC, 5=DC).

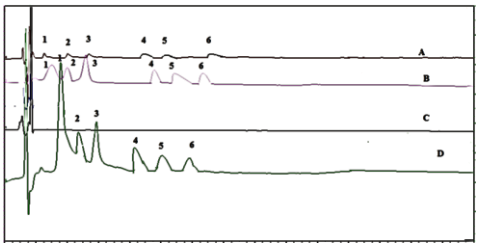

Figure 8. Chromatograms of standard mixture of TCs at 250 µg L^{-1} (A) , after IL based DLLME of standard mixture of TCs at 250 µg L^{-1} (B), blank egg sample (C), after added TCs mix standard at 250 µg L^{-1} to egg sample (D) (1=MiC, 2=OTC, 3=TC, 4=CTC, 5=MC, 5=DC) .

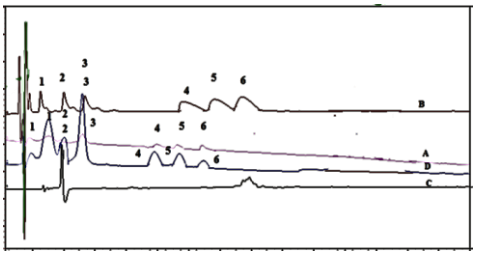

Figure 9. Chromatograms of standard mixture of TCs at 250 µg L^{-1} (A) , after IL based DLLME of standard mixture of TCs at 250 µg L^{-1} (B), blank honey sample (C), after added TCs mix standard at 250 µg L^{-1} to honey sample (D) (1=MiC, 2=OTC, 3=TC, 4=CTC, 5=MC, 5=DC) .

Table 2 Accuracy of the method for tetracyclines in spiked food samples (n = 3)

TCs	Added (µg L^{-1})	Milk		Egg		Honey	
		Found (µg L^{-1})	Recovery (%)	Found (µg L^{-1})	Recovery (%)	Found (µg L^{-1})	Recovery (%)
MiC	100	95.5±2.1	95.5±6.2	93.3±3.2	93.3±3.5	90.6±4.8	90.6±5.5
	250	240±3	96.1±2.4	235±3	94.0±2.5	230±7	92.1±2.4
OTC	100	51.3±2.1	51.3±2.2	57.2±6.4	57.2±8.2	51.3±3.1	51.3±2.2
	250	144±4	57.6±3.4	126±5	50.3±4.6	144±5	57.6±3.4
TC	100	95.9±3.9	95.9±4.7	89.1±4.1	89.1±4.4	85.9±5.2	85.9±4.9
	250	241±6	96.4±7.2	233±5	93.3±6.2	224±8	89.4±6.6
CTC	100	56.6±4.8	56.6±5.8	58.5±2.9	58.5±3.4	57.6±3.5	57.6±4.7
	250	143±4	57.2±5.7	148±7	59.2±6.2	146±9	58.2±7.9
MC	100	75.6±2.8	75.6±3.2	52.4±4.8	52.4±4.2	87.1±4.8	87.1±5.9
	250	192±5	76.8±5.2	141±6	56.3±3.9	209±10	83.8±7.6
DC	100	62.8±3.4	62.8±3.5	60.2±4.6	60.2±8.7	74.7±4.6	74.7±5.3
	250	146±6	58.4±4.5	169±9	67.6±7.2	171±8	68.4±6.2

4. Conclusion

In this study, for determination of tetracyclines residues in milk, honey and egg samples ionic liquid assisted DLLME method has been developed as separation and enrichment of these analytes before HPLC determination.

For the ionic liquid assisted DLLME method, 1-hexyl-3-methylimidazolium bis[(trifluoromethane)sulfonyl]imide was used as an extractant and the optimization steps of the method such as pH, volumes of extraction solvent and dispersive solvent, extraction and centrifugation time were improved. The optimized conditions were summarized as pH 3, 50 µL *h*-MIM-NTf_2, 750 µL acetonitrile, 10 min extraction time and 5 min centrifugation time.

The proposed method has been successfully applied to milk, honey and eggs samples. Tetracycline residues were not found in food samples. This extraction technique was environmentally friendly because of ionic liquid. The results of this method have been comparable with the recent ionic liquid-based microextraction studies in

literature by enrichment factor of our study (15-105).

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