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Carrier mediated hollow fiber liquid phase microextraction combined with HPLC–UV for preconcentration and determination of some tetracycline antibiotics

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ABSTRACT

In the present study, a simple and efficient preconcentration method was developed using carrier mediated three phase liquid phase microextraction prior to HPLC–UV for simultaneous extraction and determination of trace amounts of highly hydrophilic tetracycline antibiotics including tetracycline (TCN), oxytetracycline (OTCN) and doxycycline (DCN) in bovine milk, human plasma and water samples. For extraction, 11.0 mL of the aqueous sample containing TCNs and 0.05 M Na_2HPO_4 ($9.1 \leq pH \leq 9.5$) was filled into a 12.0-mL vial. The solution of 1-octanol (containing 10% (w/v) of Aliquat-336 as carrier) was immobilized in the pores of a porous polypropylene hollow fiber. Aqueous receiving phase (RP, 24 μL of 0.1 M H_3PO_4 and 1.0 M NaCl with pH = 1.6) was located inside the lumen of hollow fiber and the fiber was transferred into the aqueous sample. After the extraction period, the receiving phase was directly injected into HPLC. In order to obtain high extraction efficiency, the parameters affecting the liquid phase microextraction were evaluated and optimized. Under the optimized conditions, the calibration curves were linear in the range of 0.5–1000 μg L^{-1} for TCN and OTCN, and in the range of 5–1000 μg L^{-1} for DCN with good linearity ($r^2 > 0.995$). Finally, applicability of the proposed method was successfully confirmed by extraction and determination of the drugs in water and plasma samples as well as in bovine milk samples with low and high fat contents. Comparing to the traditional methods, the proposed method exhibits high sensitivity and high preconcentration factors as well as good precision. The extraction setup is simple and due to active transport of analytes, high cleanup effect and good selectivity are obtained in extraction process.

1. Introduction

Tetracyclines (TCNs) are some of the most commonly used antibacterial compounds having broad spectrum of activity against gram-positive and gram-negative bacteria. They are actively transported into the cells of susceptible bacteria and exert a bacteriostatic effect by inhibiting of protein biosynthesis [1]. TCNs are widely used in human therapy or for veterinary purposes in husbandry and cattle as well as in aquaculture for prevention and treatment of disease and also as growth additives in animal food [1–5]. They are regularly applied for the treatment of mastitis and metritis in cows. This may result in TCNs residues in market milk if they are improperly administered or if the withdrawal time for the treated cows is not observed. The widespread use of TCNs could lead to find their residues in surface waters and animal based foods [3,6]. There is a growing concern that consumption of water or animal foods containing antibiotics' residues for long periods can cause problems such as producing drug-resistant microorganisms [7]. Large molecules of tetracycline antibiotics are neutral or negatively charged at pH of environmental waters, reducing the removal of these pharmaceuticals by conventional techniques such as sand filtration, sedimentation, flocculation, coagulation, chlorination and activated carbon [8]. Accordingly, the maximum residue limit (MRL) of TCNs in milk has been established by EU, FAO/WHO and FDA as 0.1 μg L^{-1} [9–11]. Due to the potential adverse effects of TCNs on environment and humans, it is critical to develop reliable analytical methods for routine monitoring of TCNs that are rapid, precise, economical in cost and time, and harmless to the environment as well [4].

Eight TCNs are now commercially available, of which oxytetracycline (OTCN), tetracycline (TCN), chlorotetracycline (CTCN) and doxycycline (DCN) are commonly applied to food-producing animals [1]. The structure of TCNs investigated in the present study and their physicochemical parameters ($\log P$, pK_a) are shown in Fig. 1. These compounds are highly polar and may exist with different charges depending on the pH of sample. As shown in Fig. 1, their partition coefficients into organic solvents are extremely low which are related to the existence of highly hydrophilic groups in their structure.

Detection of TCN residues in complex matrices such as milk is difficult because the sample cannot be directly measured by analytical instruments. Therefore, stringent sample cleanup is required

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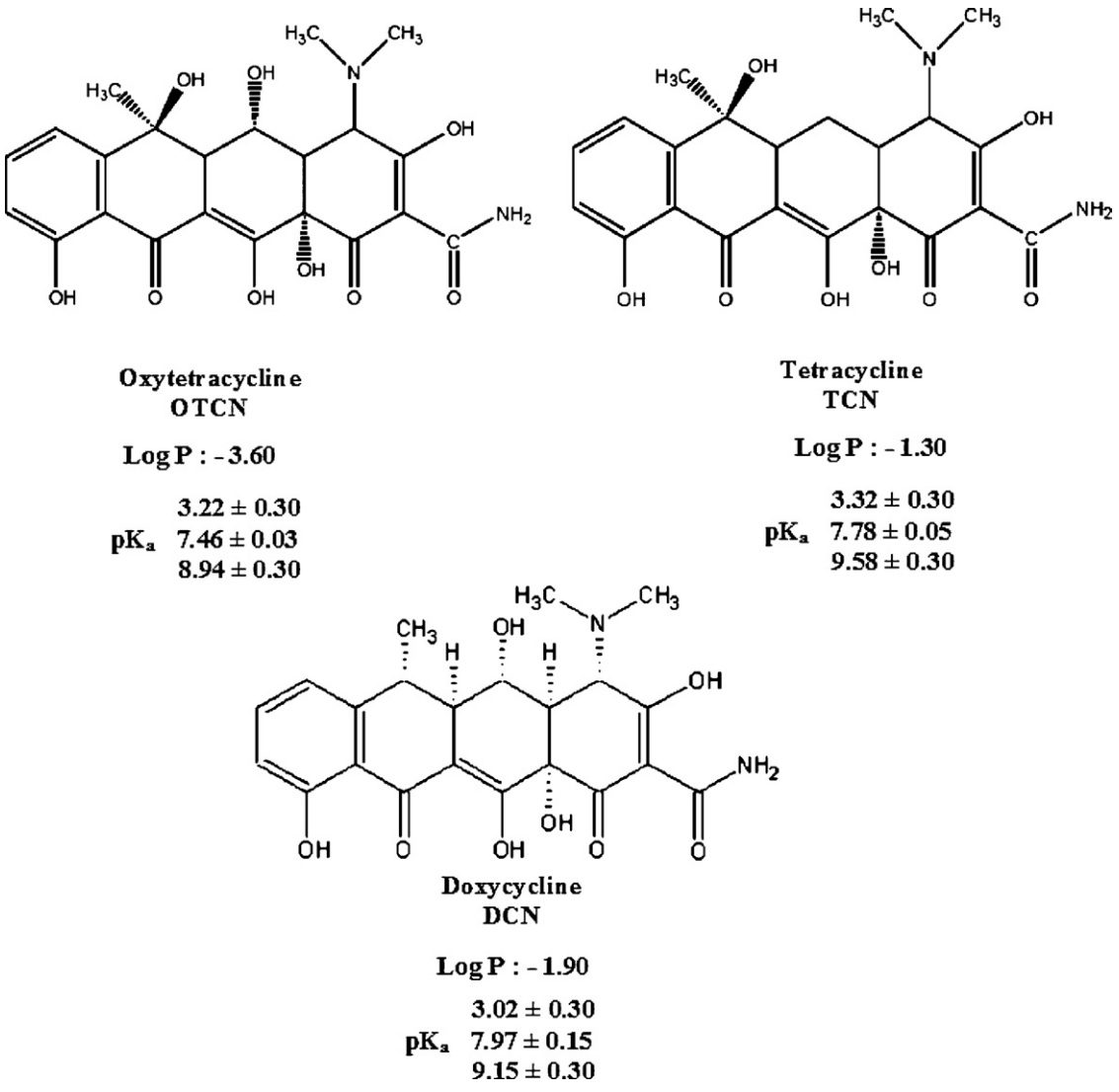

Fig. 1. Structure and physical properties of the selected TCNs.

to remove most of the matrix. For residual analysis, due to low concentration of TCNs, it is important to further preconcentrate the analytes to obtain maximum measurement sensitivity. Solid phase extraction (SPE) has been the most commonly used technique for the extraction of TCNs from food substrates and environmental waters. Different SPE methods using C_8, C_{18}, ion-exchange, and graphitized carbon black cartridges have been developed for TCNs in milk [4,12], animal tissues [13,14] and water samples [7,15–17]. However, SPE requires relatively high sample volume and involves multiple steps. Also, it consumes some toxic solvents as eluent. In recent years, hollow fiber based liquid phase microextraction (HF-LPME) was introduced by Pedersen-Bjergaard and Rasmussen to minimize organic solvent usage and to overcome the problems of conventional preconcentration methods [18]. Three phase LPME based on porous-walled hollow fiber involves extraction of ionizable or polar analytes from an aqueous sample into an organic phase immobilized in the pores of hollow fiber (membrane phase), followed by back extraction of the analytes into a secondary aqueous phase inserted into the lumen of the hollow fiber (receiving phase; RP). This method has been successfully utilized for the extraction of different analytes from various samples [19–30]. Recently, in order to enhance the extraction of hydrophilic drugs, carrier mediated LPME has been reported as an active transport mode of LPME in which a carrier is added to the sample solution [26,27,31,32] or is dissolved in the impregnation solvent in the pores of hollow fiber [24]. Carrier mediated LPME has been applied for extraction and determination of the analytes from complex matrices such as biological samples [24,31,32].

Following the extraction and isolation of TCNs from matrix, their identification and determination can be performed using HPLC equipped with diode-array, single UV or fluorimetric detection. Some methods have applied mass spectrometry detection, but they require complex and expensive instrumentation which may not be suitable for routine monitoring of TCNs [33].

In the present study, a simple and efficient microextraction method was developed by utilizing carrier mediated three phase liquid phase microextraction prior to HPLC–UV for simultaneous extraction and determination of trace amounts of TCN, OTCN and DCN in bovine milk, human plasma and water samples.

2. Experimental

2.1. Reagents and materials

Tetracycline (TCN), oxytetracycline (OTCN) and doxycycline (DCN) were obtained from Sigma (St. Louis, MO, USA). Three caprylil methyl ammonium chloride (Aliquat-336) was obtained from Fluka (Buchs, Switzerland). HPLC grade methanol and ace-

tonitril were purchased from Caledon (Georgetown, Ont., Canada). Benzyl alcohol, octanol, hexanol, n-dodecane and other reagents with analytical grade were purchased from Merck (Darmstadt, Germany). Deionized water was prepared using a Milli-Q ultra pure water purification system from Millipore (Milford, MA, USA).

2.2. Preparation of standards and real samples

Stock standard solutions of each drug (1000 mg L^{-1}) was prepared using HPLC grade methanol. Mixed standard solutions of TCNs (5 and 20 mg L^{-1} respect to each drug) were prepared by diluting the stock solutions with deionized water. Then, the solutions were cooled and stored at 4 °C. Mixed working standard solutions containing μg L^{-1} ranges of the TCNs were daily prepared by addition of Na_2HPO_4 to the solution (final solution containing 0.05 M Na_2HPO_4 with pH = 9.1–9.5). Also, solution of Aliquat-336, as carrier (10%, w/v), was prepared in octanol.

2.3. HPLC system

Separation and determination of the drugs were performed on a Varian 9012 high performance liquid chromatography (HPLC) system (Mulgrave Victoria, Australia) equipped with a Varian 9050 variable wavelength UV/Vis detector. All injections were performed manually with a Rheodyne 7725 manual injector (Cotati, CA, USA) with a 20 μL sample loop. Chromatographic data were recorded and analysed using a Waters 746 data module integrator and GC solution software. Chromatographic separations were carried out using a C_{18} analytical column (Supelcosil™, 150 mm × 4.6 mm I.D., 3 μm) from Supelco (Bellefonte, PA, USA). All the separations were performed at room temperature and under gradient elution conditions. The mobile phase (with the composition of oxalic acid (0.005 M, pH = 2.4), acetonitrile and methanol (71:16:13, v/v/v)) was passed through the column for 0–6 min. After 6 min, the composition of mobile phase changed to oxalic acid (0.005 M, pH = 2.4), acetonitrile and methanol (50:25:25, v/v/v) and passed through the column up to 15 min. Then, it returned to its initial composition. The oxalic acid solution was prepared daily with deionized water, filtered through 0.45 μm of cellulose acetate filter and degassed in ultrasonic bath before use. The mobile phase flow rate was 1 mL min^{-1} and the detection wavelength was set at 360 nm. The injection volume was 24 μL and all of the drugs were eluted within 11 min.

2.4. HF-LPME procedure

All of the extractions were carried out using an Accurel Q3/2 polypropylene hollow fiber membrane (0.2 μm pore size, 600 μm internal diameter and 200 μm wall thickness) from Membrana GmbH (Wuppertal, Germany). Under the optimized conditions, each hollow fiber was cut carefully into 8.8 cm length pieces and the lumen of the hollow fiber was filled with the receiving phase. Each piece was used only once to decrease the memory effect. Before use, they were sonicated for 5 min in acetone to remove any possible contaminants. They were then removed from acetone and the solvent was allowed to evaporate completely. The internal volume of the hollow fibers was approximately 24 μL. The end of hollow fibers was connected to the needle of a model 702 NR, 25 μL Hamilton microsyringe (Bonaduz, Switzerland). The microsyringe was employed to introduce the RP into the lumen of hollow fiber, to suspend the hollow fiber in the solution and also to inject the extracted drugs into the HPLC injector.

For each experiment, 11 mL of the aqueous sample containing the drugs and 0.05 M Na_2HPO_4 (pH between 9.1 and 9.5) was poured into a 12-mL sample vial having a 4 mm × 14 mm magnetic stirring bar. The sample vial was placed on an IKA multi-station magnetic stirrer (Staufen, Germany). For extraction, 25 μL of the RP (0.1 M H_3PO_4, 1.0 M NaCl with pH = 1.6) was withdrawn into the 25 μL microsyringe and its needle was inserted into the channel of the hollow fiber (approximately 1.5 mm). The hollow fiber was immersed in the organic solvent (10% (w/v) of Aliquat-336 in octanol) for 10 s to impregnate its pores with organic solvent and then the hollow fiber was inserted into the water for 10 s to wash excessive organic solvent from the surface of the hollow fiber. Finally, the RP was introduced into the hollow fiber with slow pushing of the microsyringe plunger. Next, the end of the hollow fiber was sealed by a piece of aluminum foil. The hollow fiber was then introduced into the aqueous sample at U-shape configuration and the top of the vial was covered via parafilm (Fig. 2). Extraction was done during the prescribed period of time (35 min). Then, the microsyringe containing the hollow fiber was removed from the aqueous sample, the end of the hollow fiber was opened and the RP was withdrawn into the microsyringe (24 μL). Finally, the RP was injected into the HPLC system.

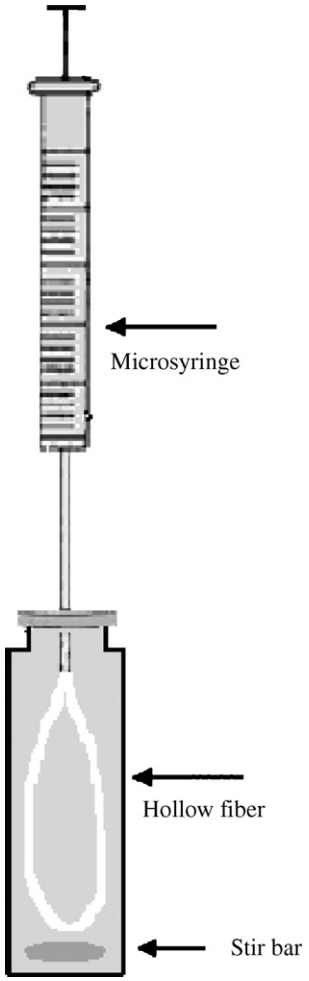

Fig. 2. A schematic diagram of extraction setup.

3. Results and discussion

3.1. Optimization of separation conditions

TCNs are highly polar molecules. They can form complexes with metal ions and strongly react with residual silanol groups on the HPLC column which often results in severe peak tailing [15,34–36]. In order to avoid this problem, reverse phase-HPLC with mobile phases containing various acids (phosphoric, acetic, tartaric acids and EDTA) and also ion-pair chromatography were applied to sep-

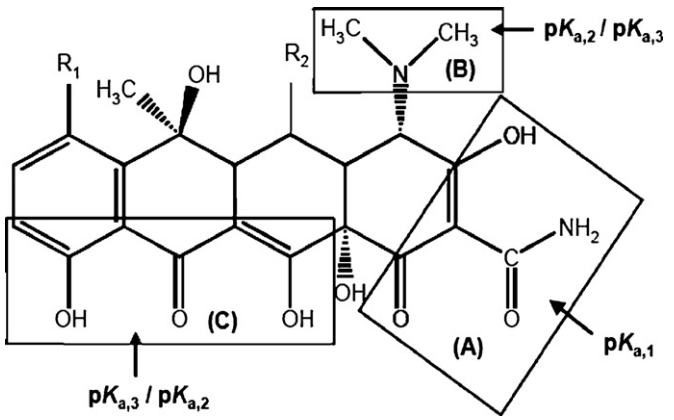

Fig. 3. The functional groups corresponding to the pK_a values.

arate TCNs [37–41]. However, TCNs still showed extreme tailing on the RP-HPLC columns. Only, the mobile phase containing oxalic acid appeared to produce symmetric peaks without tailing [1,15]. Therefore, in the present study, to avoid tailing, oxalic acid was used in the mobile phase composition. The variation of oxalic acid concentration in the range of 0.001–0.05 M showed that by utilizing 0.005 M oxalic acid (pH = 2.4), a proper separation with good peak shape was obtained. A gradient elution allowed the optimum separation of OTCN, TCN and DCN in 4, 5 and 10 min, respectively.

The study of UV–vis spectrum of the compounds showed that TCNs have maximum absorption at 275 and 360 nm. It was also found that under gradient elution conditions, baseline drift occurred at λ = 275 nm. The phenomenon was serious at 275 nm but not at 360 nm. Therefore, λ = 360 nm was selected as detection wavelength for further works.

3.2. Optimization of extraction conditions

TCNs are practically challenging drugs because they are hydrophilic compounds with high solubility in aqueous media. They have acidic and basic functionalities. Depending on solution pH, they can exist at various forms and their ionization is controlled by the solution pH and their acidic dissociation constants (pK_a values). The pK_a values of TCNs are approximately 3–4, 7–8 and 9–10 [6,7]. Researchers assigned p$K_{a,1}$ value to the tricarbonyl group, p$K_{a,2}$ and p$K_{a,3}$ to the dimethyl amine group and β-diketone group [6,42,43]. Fig. 3 shows the functional groups corresponding to the pK_a values [6]. According to the pK_a values, these antibiotics are positively charged below pH 2–3 ($TCNs^+$). As the pH increases, they become uncharged as zwitterionic form (TCNs) and then changed to anionic forms ($TCNs^-$, $TCNs^{2-}$) [6]. Therefore, the direct passive transport of TCNs from aqueous sample to RP using organic impregnated solvents is difficult. In preliminary experiments, TCNs were extracted in uncharged form by passive diffusion from the aqueous sample with the pH in the range of 4–5, adjusted with 0.05 M Na_2HPO_4, through the organic solvents immobilized in the pores of the hollow fiber into the receiving phases both acidic (0.01 M HCl, pH < 4) and alkaline (0.001 M NaOH, pH > 8). Under these conditions, the maximum preconcentration factor (PF) of two was achieved for passive transport of TCNs. The results of preliminary experiments showed that passive transport of TCNs in the absence of carrier is difficult. Because of existence of TCNs as zwitterionic forms (at the studied conditions) in solution they have very small tendency to pass through the impregnated organic solvent.

According to the pK_a values at pH 7–11, some percents of TCNs exist as anionic form ($TCNs^-$). Therefore, by utilizing a cationic carrier in membrane solvent, it is possible to actively transport $TCNs^-$ from the aqueous sample into the RP. According to our previous experience [24] three caprylil methyl ammonium chloride (Aliquat-336, $R_3NCH_3{}^+Cl^-$) was chosen as cationic carrier and was dissolved in the membrane solvent. A unique advantage of Aliquat-336 is that it stays at cationic form in all pH range [44]. The extraction mechanism of TCNs in the presence of Aliquat-336 occurs via ion-exchange reaction between anionic functional groups of the analytes with chloride ions of carrier. The experiments showed that presence of an anion such as Cl^- as a competitor ion in the RP can promote the back extraction of $TCNs^-$ into the RP. A simplified mechanism for transport of TCNs across the supported hollow fiber membrane containing Aliquat-336, as carrier, is shown schematically in Fig. 4. In the aqueous sample–membrane interface, $TCNs^-$ form a neutral ion-pair with Aliquat-336 as $(R_3NCH_3)^+TCN^-$, while releasing a Cl^- anion. Due to concentration gradient, the ion-pair diffuses across the membrane. At the RP-membrane interface, $TCNs^-$ are released from the organic phase while Cl^- is given back to the carrier. In this process, the driving force for transport of TCNs is the gradient of pH and concentration of Cl^- between the aqueous sample and RP.

In the proposed method, to achieve maximum extraction efficiency, various parameters affecting the extraction efficiency were studied and optimized. According to the preliminary trials, octanol containing 10% (w/v) Aliquat-336 was utilized as organic solvent. Concentration of TCNs in optimization experiments was 100 μg L^{-1} and each extraction was repeated at least three times. Then, the mean of the obtained results was plotted for each parameter. The chromatograms of TCNs after extraction from the standard solutions (in the range of 0.5–5 μg L^{-1}) using the proposed method are shown in Fig. 5.

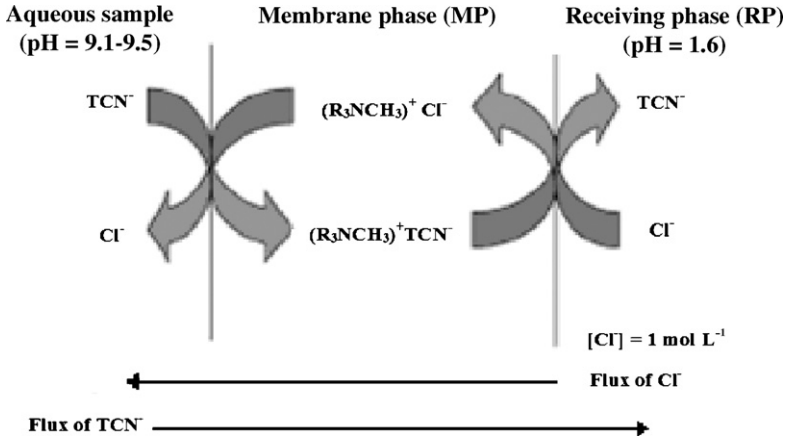

Fig. 4. The proposed mechanism for the extraction of TCNs by the proposed three phase LPME.

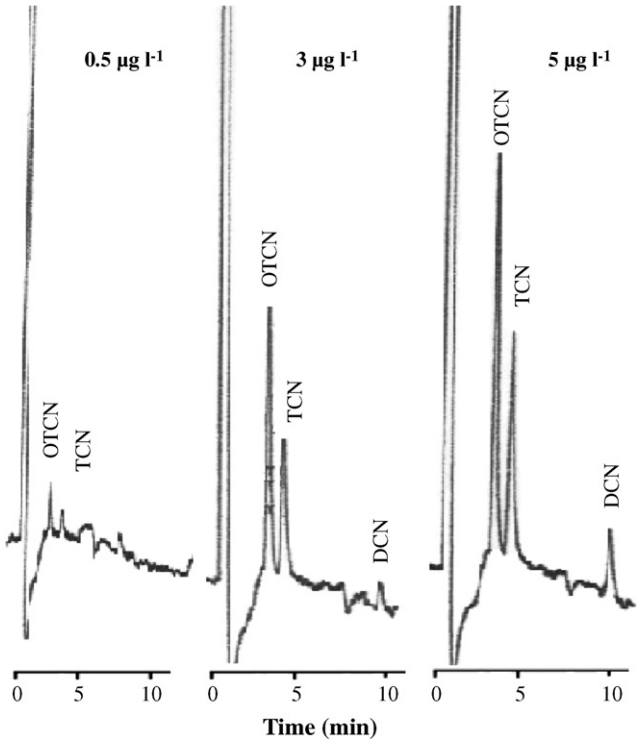

Fig. 5. HPLC chromatogram of the standard solutions of TCNs after extraction with the proposed method.

3.2.1. *Effect of compositions of the aqueous sample and RP*

At the present study, using Aliquat-336 as a cationic carrier, transport of TCNs in anionic form, $TCNs^{-}$, was accomplished. In this aspect, optimization of pH of the aqueous sample is essential to assure that all of the drugs are changed to $TCNs^{-}$ forms. According to the pK_a values of the drugs at the pH range of 7–11, most of the TCNs exist in $TCNs^{-}$ form. In order to investigate the effect of pH of the aqueous sample on the extraction efficiency of TCNs, solutions of 100 μg L^{-1} drugs in 0.05 M Na_2HPO_4 with various pH in the range of 6.9–11.0 were prepared and transport of TCNs occurred. Fig. 6 shows that the best extraction efficiency occurred at pH around 9. In order to detect the best range of pH for the aqueous sample, the pH range of 8.1–10 was studied again. It was found that the highest transport was obtained at the pH range of 9.1–9.5. At this range of pH, the main portion of the drugs exists in $TCNs^{-}$ form. At higher pH values, extraction efficiency decreased due to the formation of $TCNs^{2-}$.

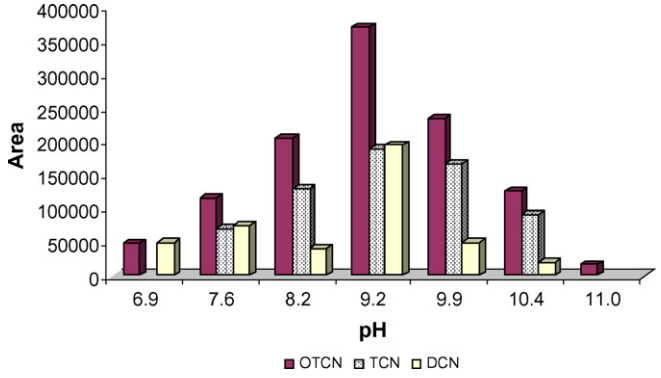

Fig. 6. Effect of the aqueous sample pH on extraction efficiency. Aqueous sample: 11 mL of 100 μg L^{-1} TCNs in the solution (0.05 M Na_2HPO_4), RP: 0.1 M HCl (pH < 1.0), organic membrane: 10% (w/v) Aliquat-336 in octanol, stirring rate: 500 rpm and extraction time: 30 min.

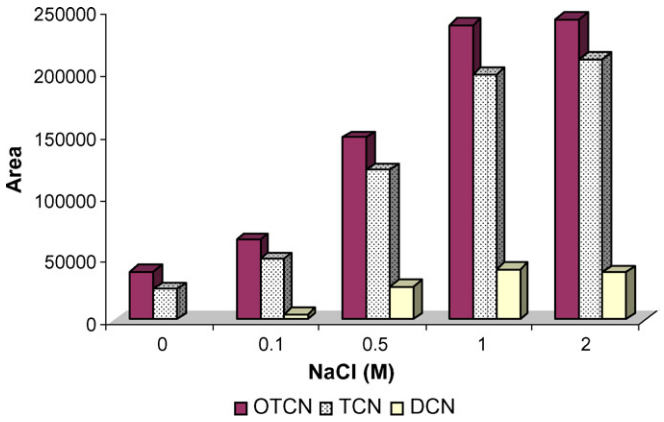

Fig. 7. Effect of Cl^{-} concentration in RP on extraction efficiency. Aqueous sample: 11 mL of 100 μg L^{-1} drugs in the solution (0.05 M Na_2HPO_4, pH = 9.3), RP: 0.1 M H_3PO_4 (pH = 2.0), organic membrane: 10% (w/v) Aliquat-336 in octanol, stirring rate: 500 rpm and extraction time: 30 min.

In order to investigate the effect of concentration of phosphate buffer on the extraction efficiency, aqueous solutions with various concentrations of Na_2HPO_4 (0.01, 0.05, 0.15 and 0.5 M) were prepared and the extraction procedure was conducted. The results showed that the best extraction efficiency was obtained at 0.05 M concentration of Na_2HPO_4. Therefore, further experiments were done using the aqueous solutions containing 0.05 M Na_2HPO_4 with the pH controlled in the range of 9.1–9.5. To study the effect of the composition of the RP on extraction efficiency, HCl solutions with various concentrations from 2×10^{-4} M (pH = 3.5) to 0.5 M (pH < 0.5) were used as RP. According to the results, no extraction occurred at HCl concentrations below 0.02 M. On the other hand, to investigate the effect of Cl^{-} concentration in the RP on extraction efficiency of TCNs, the solutions containing 0.1 M H_3PO_4 (pH = 2.0) and various concentrations of NaCl in the range of 0–2 M were used as RP. Fig. 7 indicates that by increasing of NaCl concentration in the RP, the more extraction occurs. The maximum extraction was observed at the NaCl concentration of 1.0 M or higher. In subsequent experiments, solutions containing 1.0 M NaCl and 0.1 M H_3PO_4 (pH < 2.0) were used as the RP.

3.2.2. *Effect of the type of impregnated organic solvent and Aliquat-336 concentration*

The membrane solvent should be easily immobilized in the hollow fiber pores. It should be immiscible with water to serve as a barrier between two phases and should have high capacity for dissolving the carrier and allow ion-pair formation between the drugs

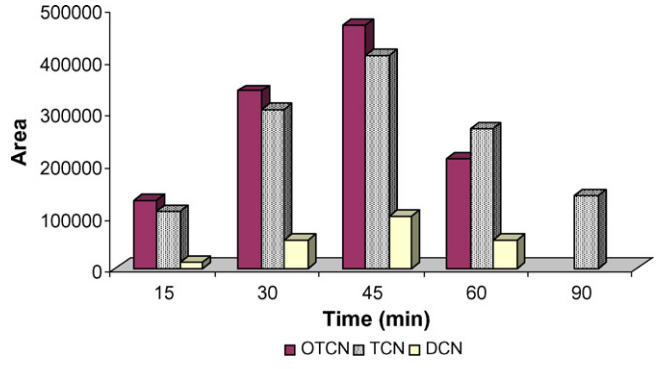

Fig. 8. Effect of extraction time on extraction efficiency. Aqueous sample: 11 mL of 100 μg L^{-1} drugs in the solution (0.05 M Na_2HPO_4, pH = 9.3), RP: 0.1 M H_3PO_4 (pH = 1.6) containing 1.0 M NaCl, organic membrane: 10% (w/v) Aliquat-336 in octanol and stirring rate: 900 rpm.

Table 1
Analytical characteristics of the proposed three phase LPME method.

Drug	LOQ ($\mu g\,L^{-1}$)	DLR ($\mu g\,L^{-1}$)	r^2	RSD% ($n=3$, 50 $\mu g\,L^{-1}$)	PF (at 10 $\mu g\,L^{-1}$)	Enhancement factor	*R*%
OTCN	0.5	0.5–500	0.9980	4.4	180	187	42.5
TCN	0.5	1–1000	0.9980	4.3	175	196	44.5
DCN	1.0	3–1000	0.9948	8.9	125	109	24.8

LOQ: limit of quantification; DLR: dynamic linear range; PF: preconcentration factor; *R*%: recovery percent.

and carrier. Also, it should have low volatility to prevent solvent loss during the extraction and low viscosity to promote drug transfer between the aqueous sample and RP. At the present work, the effect of five different solvents including benzyl alcohol, hexanol, octanol, dodecane and methyl isobutyl ketone on extraction efficiency was investigated. Only, octanol containing 10% (w/v) Aliquat-336 showed considerable preconcentration factor. Therefore, octanol was selected as the organic solvent in future experiments.

For studying the influence of Aliquat-336 concentration in the organic phase on extraction efficiency, the solutions of octanol containing various concentrations of carrier in the range of 3–20% (w/v) were prepared and used as the impregnated organic solvent. According to the results, 10% (w/v) Aliquat-336 in octanol showed the best extraction efficiency. At higher concentrations of Aliquat-336, the flux of drugs from the aqueous sample to RP reduced due to an increase in the viscosity of the organic phase.

3.2.3. Effect of stirring rate

Stirring of aqueous sample reduces the extraction time by increasing the diffusion rates of the drugs from the aqueous sample to RP. The results showed that extraction efficiency was improved by increasing of the stirring rate up to 1000 rpm. However, very high stirring rates would produce excessive air bubbles and lose solvent that could affect the precision. Therefore, 900 rpm was chosen as a suitable stirring rate for future experiments.

3.2.4. Effect of extraction time

The influence of extraction time on extraction efficiency was studied in the range of 15–90 min. The amount of the extracted drugs was found to increase by increasing of the extraction time in the range of 15–45 min (Fig. 8). At the extraction times higher than 45 min, the extraction efficiency reduced. The study of the properties of TCNs in the literature showed that their stability is weak under strong acidic or alkaline conditions. They form reversible epimers, 4-epi-TCN, anhydro-TCN and iso-TCN under weakly acidic (pH 3), strongly acidic (below pH 2) and alkaline conditions, respectively [1,45]. At the extraction times higher than 40 min, new peaks appeared in the HPLC chromatogram of RP solution and the peaks of TCNs became smaller. This can be related to the increase in residence time of TCNs in acidic RP solution that causes degradation of them. Therefore, in order to obtain high sensitivity and prevent TCNs degradation, the extraction time of 35 min was chosen as the optimum time for the subsequent experiments.

3.3. Evaluation of method performance

Quantitative parameters of the proposed three phase LPME method were calculated under the optimized conditions described in the previous sections (aqueous sample: 11 mL of 0.05 M Na_2HPO_4 with the pH range of 9.1–9.5, membrane phase: 10% (w/v) Aliquat-336 in octanol, RP: 24 μL of the aqueous sample containing 0.1 M H_3PO_4 and 1.0 M NaCl with pH < 2.0, stirring rate: 900 rpm and extraction time: 35 min). The calculated figures of merit are summarized in Table 1. The calibration curves were plotted using 11 spiked levels in the range of 0.5–1000 $\mu g\,L^{-1}$. Each standard sample was extracted by the proposed method under the optimized conditions. For each level, three replicate extractions were performed. The calibration curves were obtained by plotting the peak areas of

Table 2
Comparison of the proposed method with other published methods for the extraction and determination of TCNs.

Tetracyclines	Sample	Method	Detection	Detection limit	Ref.
OTCN, TCN, DCN	Milk, plasma, water	HF-LPME	HPLC–UV	0.5–1.0 ($\mu g\,L^{-1}$)	Proposed method
OTCN, TCN, CTCN, DCN	Milk, serum, urine	Metal chelate affinity column	CE-UV	1.4–5.3 ($\mu g\,L^{-1}$)	[12]
OTCN, TCN, CTCN	Urine, plasma	LLE as calcium complex	HPLC–UV	1–1.5 ($mg\,L^{-1}$)	[26]
OTCN, TCN, CTCN	Urine, plasma	LLE as calcium complex	HPLC–UV	0.25–0.5 ($mg\,L^{-1}$)	[46]
TCN	Plasma	Ion-pair extraction with TBA[a]	HPLC–UV	0.2 ($mg\,L^{-1}$)	[47]
OTCN, TCN	Milk	Extraction with TBA into CH_2Cl_2	HPLC–UV	10 ($\mu g\,L^{-1}$)	[48]
OTCN, TCN, CTCN	Milk	Matrix solid phase dispersion	HPLC–UV	0.1 ($mg\,L^{-1}$)	[49]
OTCN, TCN, CTCN	Milk	C_{18} cartridge	HPLC–particle beam MS	0.1 ($mg\,L^{-1}$)	[50]
OTCN, TCN, CTCN	Milk	Extraction with 1 M HCl and CH_3CN	HPLC–UV	2–4 ($\mu g\,L^{-1}$)	[51]
OTCN, TCN, CTCN	Urine	Addition of 0.2 M KH_2PO_4	ESI-MS-MS	10 ($\mu g\,L^{-1}$)	[52]
OTCN, TCN, CTCN, DCN	Water	SPE	HPLC–ESI-MS	4–6 ($ng\,L^{-1}$) (for 1000 mL sample)	[16]
OTCN, TCN, CTCN, DCN, ...	Water	On-line SPE	LC–MS	0.09 ($ng\,L^{-1}$)	[7]

[a] Tetrabutyl-ammonium bromide.

TCNs against the concentration of the drugs in the aqueous sample. A broad dynamic linear range (DLR) with good determination coefficient ($r^2 > 0.99$) was obtained. The limit of quantification (LOQ) was calculated based on:

$$\mathrm{LOQ} = \frac{10 S_b}{m}$$

where, S_b is the standard deviation for ten blank measurements and m is the slope of calibration curve. The preconcentration factors were calculated as the ratio of the drugs concentration in the RP to their initial concentration (10 μg L^{-1}) in the aqueous sample. Also, the enhancement factors (EF), that had a better value for a wide range of the analyte concentration were calculated by dividing the slope of the calibration curve after preconcentration (in the RP) to that obtained without preconcentration (in the aqueous sample). Precision of the recommended method was determined by performing three replicate extractions on the samples with 50 μg L^{-1} of TCNs and RSD% values in the range of 4.3–8.9% were obtained (Table 1). The extraction percent (R%) was calculated as follows:

$$R\% = 100 \frac{C_{r,f}\, V_r}{C_{s,i}\, V_s} = 100\, \frac{V_r}{V_s}\, \mathrm{EF}$$

where, $C_{s,i}$ is the initial analyte concentration in the aqueous sample and $C_{r,f}$ is the final analyte concentration in the RP. Also, V_s and V_r are the volumes of aqueous sample and RP, respectively. The extraction percents in the range of 24.8–44.5% were obtained for the TCNs.

A comparison between the LODs of the proposed method and those of the published methods (Table 2) shows that this method has higher sensitivity and better precision. Also, the obtained LODs of the proposed method are better than those obtained by the most presented methods [46–53]. It is clear that by utilizing mass spectrometer as detection system, the sensitivity and performance of the proposed method can be improved [7,16].

3.4. Analysis of real samples

Applicability of the proposed LPME method to real samples was evaluated by extraction and determination of TCNs from bovine milk (1.5 and 3.0% fat), tap water and human plasma.

To determine the concentration of TCNs in plasma and water samples, human plasma was obtained from Dr. Shariati Hospital (Tehran, Iran) and diluted at 1:3 ratio with deionized water. Also, tap water was obtained from our laboratory (Tarbiat Modares University). After the addition of 0.05 M Na_2HPO_4 and adjustment of the pH of samples at 9.3, each sample was extracted via the proposed method under the optimum conditions. Since the TCNs were not detected in the plasma and water samples, thus μg L^{-1} amounts of TCNs were added into the real samples and the extraction and determination procedures were repeated again. Table 3 shows that the results of three replicate analysis of each real sample obtained by the proposed method are in satisfactory agreement with the spiking amounts.

In order to determine the amounts of TCNs in bovine milk, three milk samples from different companies were purchased from the market. Each milk sample was diluted at 1:1 ratio with deionized water after the addition of Na_2HPO_4 (0.05 M) into the solution. Also, to avoid complex formation between TCNs and Ca^{2+} in the milk sample, 0.04 M EDTA was added to the solutions and the pH of the samples was adjusted at pH > 10. The samples were centrifuged at 4000 × g for 20 min. Then, the fat settled as a thin layer on the top of solution and was removed by spatula. Subsequently, the pH of the samples was reduced to 9.1–9.5. Finally, the samples were extracted by applying the proposed method to evaluate their TCNs contents. According to the obtained results (Table 3), all of the milk samples contained TCNs in the range of 6.0–27.4 μg L^{-1} that was below the MRL obtained by FAO. Fig. 9 shows the chromatograms of the non-spiked and the spiked milk sample with the TCNs (at the concentration levels of 5, 10 and 20 μg L^{-1}) under the optimum conditions.

Table 3
Analysis of the real samples.

Sample		OTCN	TCN	DCN
Milk 1[a] (1:1)[b]	Determined (μg L^{-1})	13.7	–	–
	Added (μg L^{-1})	5.0	5.0	5.0
	Found (μg L^{-1})[c]	18.3 ± 0.2	4.5 ± 0.8	4.7 ± 0.3
	Relative recovery (%)	92.0	90.0	94.0
Milk 2[d] (1:1)	Added (μg L^{-1})	20.0	20.0	20.0
	Found (μg L^{-1})[c]	37.0 ± 2.2	18.5 ± 0.9	16.5 ± 0.7
	Relative recovery (%)	116.3	92.5	82.5
	Determined (μg L^{-1})[c]	7.6 ± 1.0	–	–
	Added (μg L^{-1})	10.0	10.0	10.0
	Found (μg L^{-1})[c]	16.3 ± 1.5	9.2 ± 0.8	9.2 ± 2.1
	Relative recovery (%)	87.0	92.0	92.0
Milk 3[e] (1:1)	Determined (μg L^{-1})[c]	3.0 ± 0.2	–	–
	Added (μg L^{-1})	20.0	20.0	20.0
	Found (μg L^{-1})[c]	24.3 ± 1.5	18.8 ± 1.4	16.9 ± 0.8
	Relative recovery (%)	106.5	94.0	84.5
Plasma[f] (1:3)	Determined (μg L^{-1})	–	–	–
	Added (μg L^{-1})	20.0	20.0	20.0
	Found (μg L^{-1})	18.9	19.4	18.0
	Relative recovery (%)	94.5	97.0	90.0
Tap water[g]	Determined (μg L^{-1})	–	–	–
	Added (μg L^{-1})	5.0	5.0	5.0
	Found (μg L^{-1})	5.1	5.0	5.1
	Relative recovery (%)	102.0	100.0	102.0

[a] Milk 1 (1.5% fat) was prepared from the market (Pak Company).
[b] Dilution ratio.
[c] Mean of three replicates ± standard deviation.
[d] Milk 2 (1.5% fat) was prepared from the market (Khazar Shir Company).
[e] Milk 3 (high fat, 3.0% fat) was prepared from the market (Choopan Company).
[f] Plasma sample was collected from Dr. Shariati Hospital (Tehran).
[g] Tap water was collected from Tehran.

Table 3 shows that the results of three replicate analysis of each real sample, obtained by the proposed method, are in satisfactory agreement with the spiking amounts. Also the results showed that combination of the proposed LPME method with

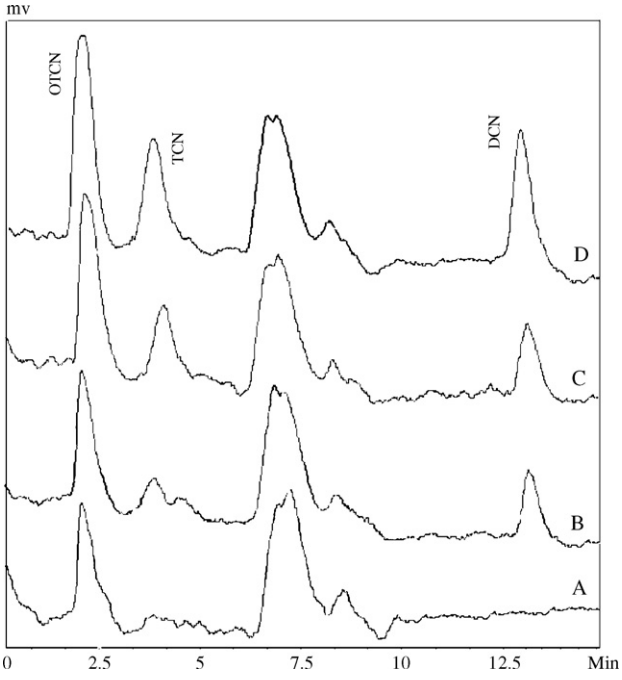

Fig. 9. HPLC chromatogram of the milk 1 sample after the extraction under the optimized conditions by the proposed method. (A) Non-spiked milk sample, (B) 5 μg L^{-1} spiked, (C) 10 μg L^{-1} spiked and (D) 20 μg L^{-1} spiked of TCNs.

HPLC introduced a powerful method to analyze TCNs in complex matrices.

4. Conclusion

The present study developed carrier based HF-LPME method for the analysis of trace amounts of polar amphoteric antibiotics in complex matrixes such as milk and biological fluids. The proposed method provides an interesting alternative to traditional sample preparation techniques for TCNs. Comparing to the traditional methods; this method exhibits a very low limit of detection with higher preconcentration factor. It has a good precision with wide dynamic linear range. The extraction setup is simple and due to low cost of hollow fiber, the hollow fiber can be discarded after each extraction to avoid cross-contamination. Utilizing a fresh acceptor phase and hollow fiber for each extraction serves to maintain the high reproducibility and repeatability of the method. Also, in the present method, the consumption of organic solvent is low and since the acceptor phase is protected by hollow fiber, the whole operation is very convenient to handle. Each extraction may last 40 min and due to the simplicity of the extraction process; several extractions can be processed in parallel using a multi-stirrer. On the other hand, the recommended procedure is compatible with a broad range of samples such as milk and biological fluids. It is possible to obtain a better limit of detection by extraction of TCNs from larger volumes of samples or by using mass detection. In addition, extraction of other members of TCNs such as CTCN is possible without any changes in the optimum conditions. The method was also verified by determination of the drugs in the real samples and satisfactory results were obtained.

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