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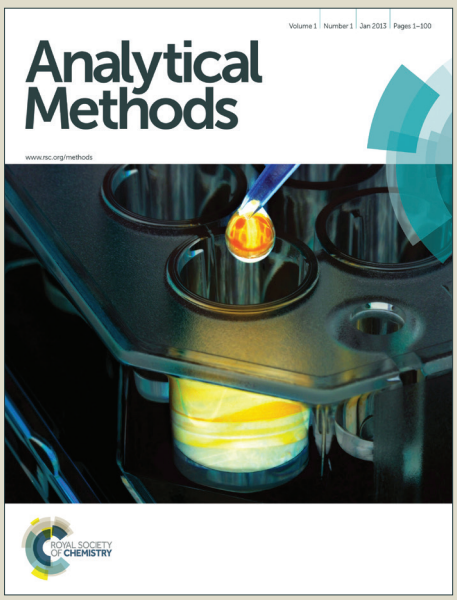

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Determination of tetracycline residues in lake water by on-line coupling of molecularly imprinted solid-phase extraction with high performance liquid chromatography

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Abstract

A new method has been developed for the determination of tetracycline residues in water samples by on-line solid-phase extraction-HPLC analysis. Tetracycline-imprinted microspheres, synthesized by reversible addition-fragmentation chain transfer precipitation polymerization, were applied for the extraction of the tetracyclines. Tetracycline residues were selectively extracted from 10.0 mL lake water using the molecularly imprinted solid-phase extraction (MISPE) method. After the sample clean-up, tetracyclines were back-flushed into a reversed-phase C_{18} analytical column and oxytetracycline, tetracycline and doxycycline were separated for quantifications. The recoveries of three tetracyclines in the spiked lake water were from 83.2 % to 111.0% with relative standard deviations (RSDs) < 5.3%, demonstrated that the established MISPE-HPLC method has good accuracy and precisions. Linearity in the range of 1-200 μg L^{-1} was obtained. The detection limits and quantification limits for the analytes were 0.08-1.02 μg L^{-1} and 0.41-3.56 μg L^{-1}, respectively. The enhancement factors, ranged from 376 to 528, indicated the MISPE has ability of enriching trace tetracycline residues. The results demonstrated that this method can be used in the determination of tetracyclines in the environmental water samples with good sensitivity and efficiency.

Keywords

On-line solid-phase extraction; Molecularly imprinted microspheres; HPLC determination of tetracyclines; Water samples

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1. Introduction

Tetracyclines (TCs) are antibiotics that are widely used as veterinary drugs and feed additives in the livestock husbandry due to their broad spectrum property and economic costs. However, the release of animal excrement with TCs and the discharge of waste water from animal farms have led to the environmental pollution. The accumulation of tetracyclines in the environment results in many health hazards for human being, such as toxic effects, allergies, yellow teeth, liver damage and gastrointestinal disorders. Therefore, monitoring and control of TCs residues in environment are very important. Various methods for the TC determinations in environmental samples by HPLC analysis have been reported [1-3].

In the trace TCs determinations, sample pre-concentrations are generally required because the concentrations of TCs in environmental samples are too low to be determined directly by general analytical methods. Solid phase extraction (SPE) is a technique with advantages of separation and enrichment for sample pre-treatment. Different SPE materials have been synthesized for the determination of TCs, such as silica-based magnetic support [4], sulfobetaine-type polymer resin [5], molecularly imprinted monolithic column [6] and molecularly imprinted particles [7]. Among these materials, molecularly imprinted polymers (MIPs) have received increasing attention in the complex sample analysis. Due to the merit such as high selectivity and stability in harsh chemical environment, the MIPs have become ideal materials in solid-phase extractions.

Despite of advantages in the separation and extraction in the complex sample analysis, the MIPs reported in the literatures for the TC analysis still have some shortages. For molecularly imprinted monolithic column, the reproducibility of preparation is not easy to be achieved and industrial scale fabrication may be difficult. Aggregated [7] or irregular shaped [8] MIP particles were used for on-line extraction for the TCs determination. To prepare spherical polymers for the column separation, TC-imprinted polymer with desirable selectivity and affinity to TCs were synthesized by reversible addition-fragmentation chain transfer (RAFT) precipitation polymerization by our group [9].

Automation is the trend in sample pre-treatment in the analytical field [10]. Generally, the off-line sample pre-treatments are tedious and time-consuming. On the contrary, the on-line determinations

facilitate the automation process and save time and labor. In this study, the MIP microspheres synthesized by RAFT precipitation polymerization were used as sorbent for on-line SPE and coupled with HPLC for the determination of trace TCs in lake water. The parameters affecting the performance of on-line operation were studied and optimized. Compared with some on-line SPE methods reported in literature [2, 11, 12], the established method has better enrichment ability, lower detection limits and reasonable linear ranges by using of on-line MISPE-HPLC. The developed method can be applied for the determination of TCs in lake water samples.

2. Experimental

2.1. Materials and reagents

TC and humic acid sodium salt were purchased from Heowns Biochem Technologies Co., Ltd. (Tianjin, China). Oxytetracycline (OTC) and doxycycline (DC) were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Methacrylic acid (MAA) and 2, 2′-azobisisobutyronitrile (AIBN) were obtained from Tianjin Chemical Reagent Company (Tianjin, China). Ethylene dimethacrylate (EDMA) was purchased from Beijing Hengye Zhongyuan Chemical Co., Ltd. (Beijing, China). Polyethylene glycols (PEGs) were purchased from Experimental Chemical Plant of Tianjin University (Tianjin, China). Methanol (MeOH), acetonitrile (ACN) and acetic acid (AcOH) were analytical grade and obtained from Concord Technology (Tianjin) Co. Ltd. (Tianjin, China). The tetracyclines stock solutions (500 μg L^{-1}) were prepared in ACN. The standard solutions were prepared by dilution of the stock solution with sample loading mobile phase. All solutions were stored at 4 ^{o}C in dark.

The phosphate buffers were prepared with Na_2HPO_4/citric acid according to the Handbook of Biochemistry and Molecular Biology [13].

2.2. Preparation of tetracycline imprinted microspheres

The TC-imprinted polymer microspheres were prepared by reversible addition-fragmentation chain transfer precipitation polymerization based on our previous work [9]. In the imprinting

polymerization, AIBN was used as the initiator and cumyl dithiobenzoate (CDB) was the chain transfer agent. The molar ratio of TC (template)/MAA (functional monomer)/EDMA (cross-linker) was 1/8/40. The (MAA+EDMA)/AIBN/CDB was 150/2/4 (molar ratio) and the total monomer concentration in ACN was 2% (w/v), respectively. Polyethylene glycol-12000 (PEG-12000) was used as co-porogen. Briefly, the TC, MAA, EDMA, AIBN, CDB and PEG-12000 were dissolved in ACN and the mixture was deoxygenated. The polymerization temperature was raised from 15 to 60 ^{o}C in 45 min and kept at 60 ^{o}C for 24 h. After polymerization, the particles were Soxhlet-extracted by MeOH/AcOH (8/2, v/v) and MeOH successively and then dried at 60 ^{o}C under vacuum.

2.3. Selection of MISPE-HPLC conditions

The influence of mobile phase property on the retention of TC on the TC-imprinted column was evaluated in order to choose the MISPE-HPLC conditions**.** Mobile phases with various pH or different ratios of buffer/organic solvent were used in the study. The retention factor (k) was calculated by $k = (t_r - t_0)/t_0$, where t_r is the retention time of the analyte and t_0 is the dead time determined by potassium dichromate.

The binding capacity of the TC on MISPE column was measured by frontal chromatography according to the method described in the literatures [14-16]. The samples with certain concentration of TC (C_{TC}) were prepared by dissolving TC in aqueous solution (pH 3.0 adjusted by citric acid). The sample was pumped into the MIP column continuously at 1.0 mL min^{-1} flow rate. TC eluted from the column was monitored by UV detection at 355 nm wavelength. The amount of saturated adsorption (m_{TC}) on the column was calculated by $m_{TC} = C_{TC}$ (V_R-V_D), where V_R is the retention volume of TC and V_D is the dead volume, respectively. The V_R and V_D were determined from the breakthrough curve.

2.4. Sample collection and preparation

Water samples were collected from a local lake. After filtered through a filter paper, samples were

frozen at -18 °C and then thawed and centrifuged to remove the colloid. The sample pH was adjusted to 3.0 with citric acid and was filtered through a 0.22 μm membrane before use. The TCs standard compounds were weighed and dissolved into the water samples to prepare the spiked lake water samples with various TCs concentrations.

2.5. On-line MISPE-HPLC procedures

A schematic diagram of on-line MISPE-HPLC for the determination of TC antibiotic residues is shown in Fig. 1. TC-imprinted microspheres (0.40 g) were dry-packed into a stainless steel HPLC column (30 × 4.6 mm) for on-line SPE processes. The HPLC system (Shimadzu, Japan), consisted of three LC-20AD pumps, a SPD-M20A UV detector, was used in the analysis. A 7725i valve with a 10.0 mL sample loop was used for the sample injection. A 10-port switching valve (Valco instruments Co. Inc., USA) controlled by the Vacom software was employed for column switching. The mobile phase A was aqueous solution, pH 3.0 adjusted by citric acid. The mobile phase B was 0.05 M Na_2HPO_4 aqueous solution (pH 6.0 adjusted by citric acid) and ACN was used as mobile phase C. In the MISPE-HPLC, SPE column was conditioned with mobile phase A for 10 min at 1.0 mL min^{-1} first. Then, the sample was injected into the SPE column at 2.0 mL min^{-1} flow rate while the 10-port switching valve was in the position A. After the cleaning-up process, the 10-port valve was switched to the position B and analytes retained on the SPE column were back-flushed into a Luna C_{18} (2) column (250 × 4.6 mm, Phenomenex, USA). The separation was performed by step gradient elution with mixture of mobile phases B and C at 1.0 mL min^{-1}. The gradient elution conditions were: 7% C at 0-7 min, and then changed to 47 % in 20 min. The TCs were detected by UV detection at 355 nm. Finally, the 10-port switching valve was turned to the position A for next sample pre-treatment.

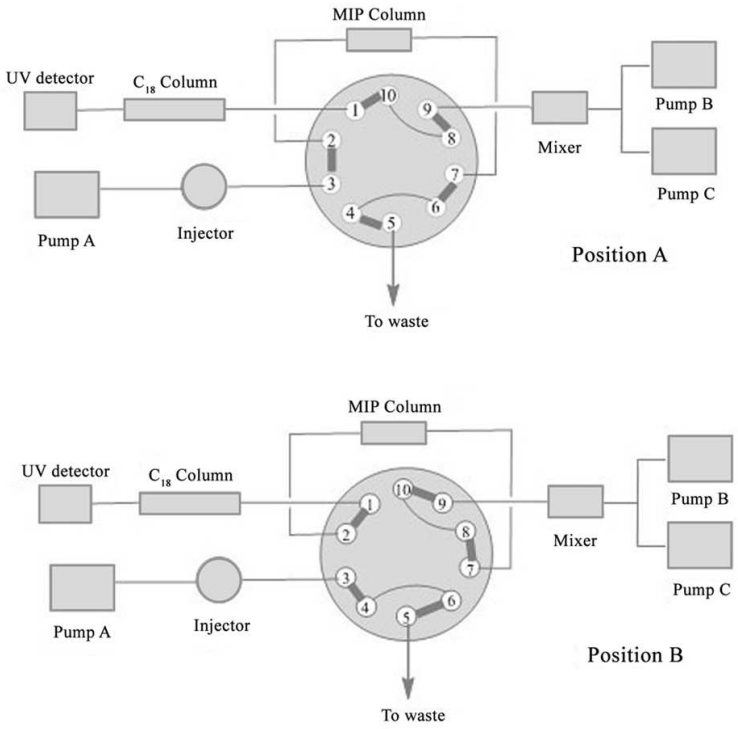

Fig. 1. Schematic diagram of the on-line MISPE-HPLC for the determination of trace tetracyclines in water samples. Switching valve position A: sample pre-treatment position; Position B: analytical position.

3. Results and discussion

3.1. Selection of the MISPE conditions

Monodispersed tetracycline-imprinted polymer microspheres with size of 4.8 μm were synthesized for the selective extraction of TC. In the MIP microsphere synthesis, MAA was used as the functional group and EDMA was the cross-linking agent to form the MIP framework. After the template removal, the remaining cavities with recognition property were formed, which exhibited specific affinities to TC compounds. The hydrogen-bonding and ionic attractions are presumably the determinant interactions between the TCs and MIP binding sites. To select mobile phase in the MIPSPE-HPLC analysis, the influence of the mobile phase compositions on the affinity of TCs on the MIP were evaluated first. The MISPE conditions for the sample extraction and clean-up were

investigated and optimized.

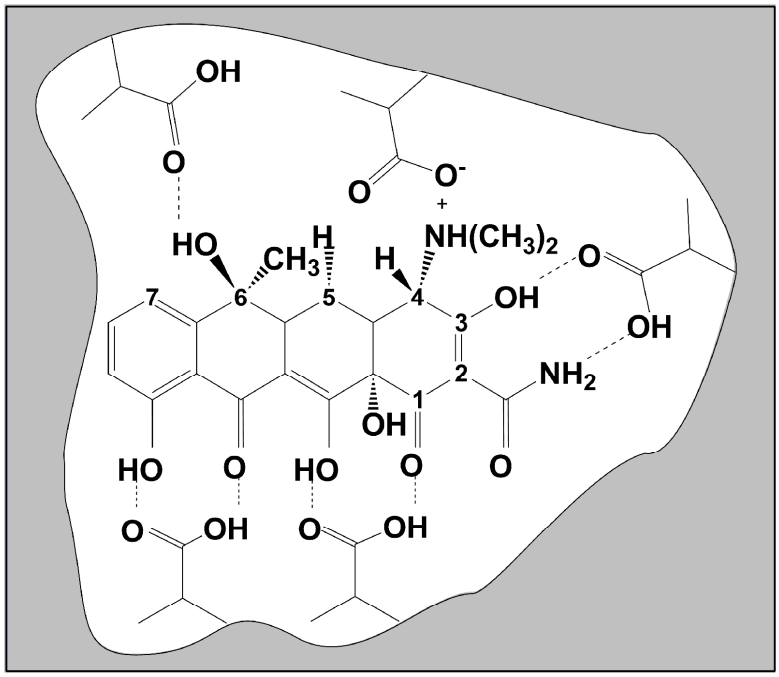

Fig. 2. Schematic presentation of proposed interactions between tetracycline and functional groups in the recognition cavity of MIP microsphere

Effect of buffer pH in mobile phase on the retention of TC on the MIP column

The influence of the pH of mobile phase on the retention of TC was studied. The Na_2HPO_4 buffer solution (0.05 M, pH 2 to 8 adjusted by 0.025 M citric acid)/MeOH (60/40, v/v) was used in the study. The results indicated that the retention of TC on the MIP column was weakened with the increase of buffer pH (Fig. 3A). It is assumed that the rising of pH resulted in more ionization of the MAA carboxyl groups, which decreased the hydrogen bonding interaction between the MIP and TC and resulted in a decreased retention. However, the stability of TCs is poor under strong acidic conditions as reported in the literature [17]. To insure the stability of TCs, pH 3.0 condition was chosen as the mobile phase condition in extraction process.

Effect of buffer salt concentration in mobile phase on the retention of TC on the MIP column

The mobile phases of Na_2HPO_4 buffer (pH 3.0 adjusted by citric acid)/ MeOH (60/40, v/v) with different Na_2HPO_4 concentrations were used to investigate the influence of salt concentration on the retention of TC. The result has showed that the retention of TC on the MIP column was reduced with the increasing of Na_2HPO_4 concentration in the mobile phase buffer (Fig. 3B). This phenomenon demonstrated that the electrostatic interaction also contribute to the binding of TC on the MIP sorbent. In the acidic aqueous solution (pH 3.0), TC is positively charged since the tertiary amine in its structure is protonated. The electrostatic interaction existed between the carboxylic acid groups of MAA and the positively charged group of TC. When the salt concentration of buffer in the mobile phase was raised, there are more competitions of cations in solution with TC for the binding sites, which led to a reduced retention of TC compound.

Effect of organic solvent proportion in mobile phase on the retention of TC on the MIP column

The influence of organic solvent concentration on the retention of TC was studied by employing different ratio of Na_2HPO_4 buffer (0.05 M, pH 3.0)/MeOH for the mobile phase (Fig. 3C). We found that the TC cannot be eluted from the MIP column in 40 min when the proportion of MeOH is lower than 20%. The retention factor of TC was decreased as the ratio of MeOH in mobile phase increased. It demonstrated that a reversed-phase retention mechanism existed in the TC retention. High content of water in the mobile phase can enhance the retention of TC on the MIP, which is conducive to the pre-concentration of analytes on the MIP column.

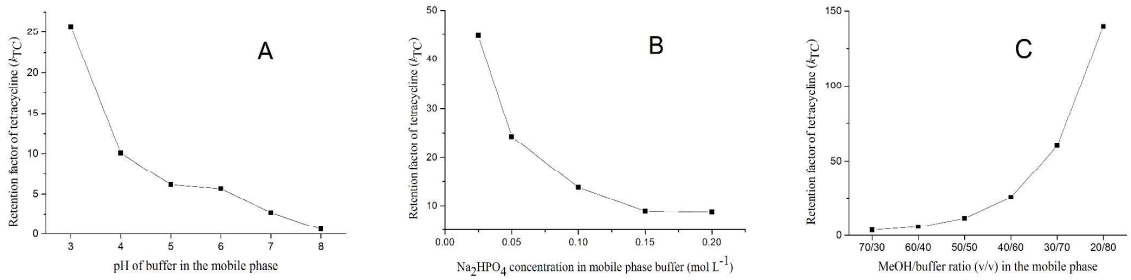

Fig. 3. The influence of buffer pH (A), buffer salt concentration (B) and organic solvent proportion

(C) in the mobile phase on the retention of tetracycline. The flow rate was 1.0 mL min^{-1}. The column size was 30 × 4.6 mm and the detection wavelength was 355 nm.

In the on-line MISPE-HPLC, the MIP column was used for the pre-concentration of TCs and the sample purifications. Based on the experimental results in this section, mobile phase A (aqueous solution, pH 3.0 adjusted by citric acid) was selected as conditioning and loading solvent in the subsequent experiments.

3.2. Determination of the extraction capacity of the MISPE column

The frontal chromatographic analysis was carried out to determine the adsorption capacity of the MIP column in this work. In the experiment, the sample solution containing TC was pumped into the MIP column continually. Because the general concentration range for the TCs determination in environmental water samples is from ng L^{-1} to μg L^{-1} [18-21], TC solution (0.1 μg mL^{-1}) was used first to find out the column extraction capacity in this concentration. No elution of TC peak was detected in 40 min, indicating that no less than 4 μg of TC can be extracted by the column. In order to calculate the amount of saturated adsorption, C_{TC} was increased to obtain breakthrough curves, and m_{TC} of 5.18 mg was determined with the method described in section 2.3.

To have high extraction enrichment, a 10.0 mL sample loop was used for the quantitative sample injection. The frontal chromatographic analysis was employed to estimate whether the injection volume is appropriate for the on-line determination. When C_{TC} was 373 μg mL^{-1}, the breakthrough volume (V_B) was 10.8 mL (Fig. 4), which indicated that the TC could be quantitatively retained by the MIP column when the sample volume was 10 mL. This result demonstrated that the 10.0 mL injection volume is suitable for the on-line concentration of environmental samples in which the concentration of analyte is much lower than the solution used for the experiment.

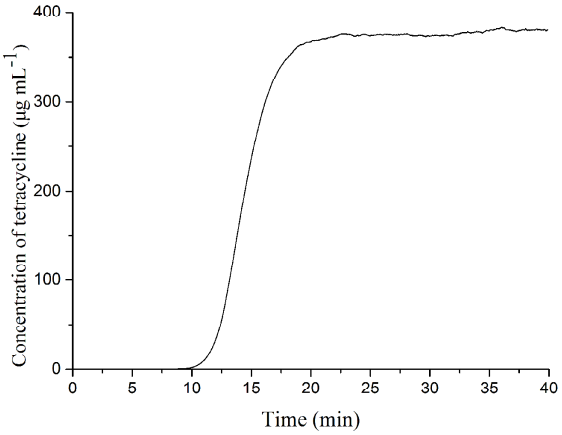

Fig. 4. Breakthrough curve for tetracycline solution (373 μg mL^{-1}) on MIP-packed column at a flow rate of 1.0 mL min^{-1}. The column size was 30 × 4.6 mm and the detection wavelength was 355 nm.

3.3. Selection of washing condition

In this study, 10.0 mL was selected as sample volume and comparatively large amount of sample matrix has to be removed by washing mobile phase in the pre-concentration step. Humic acids, naturally occurring organic substances, are the major matrix components in soil, mud and environmental water. Therefore, humic acids were used as the interference model compounds and spiked in water samples to search for the washing condition. The results indicated that the humic acids can be completely washed out from the MIP column in 1.5 min by aqueous solution (pH 3.0 adjusted by citric acid) /MeOH at 2 mL min^{-1}, while the MeOH ratio (from 0 to 20%) in mobile phase had little influence on their retention. On the other hand, the amount of TCs retained on the MIPSE column decreased when the MeOH ratio was increased. Thus, mobile phase A was employed as washing solution. Meanwhile, TC can be totally retained when the washing time was less than 1.5 min, thus 1.5 min was chosen as the washing period.

3.4. Optimization of on-line MISPE-HPLC procedure

It is known that the low flow rate is in favor of the binding equilibrium of the analytes on the

stationary phase. On the other hand, longer loading time may result in broad injection band and longer analytical time. The experimental results have shown that there is no obvious difference in the TC chromatograph peak areas indicating complete binding of the injected analytes when the loading flow rate were from 1.0 to 2.0 mL min^{-1} and concentration of TCs were from 1 to 200 μg L^{-1}. Meanwhile, the backpressure was in an acceptable level. With overall consideration of pre-concentration efficiency, backpressure and analytical time, 2.0 mL min^{-1} was selected as the injection flow rate.

The eluting conditions were also selected based on the result of section 3.1. A back-flush mode and a strong mobile phase are generally preferred to elute the TCs from the SPE column to make a narrow injection band on the analytical column. However, stronger eluting mobile phase for TC-imprinted column also resulted in weaker TCs retention on the reversed-phase C_{18} column, which led to poorer HPLC separations. In this study, a gradient elution was employed to satisfy both the requirements of elution from the MISPE column and separation on the analytical column. Because the elution strength of ACN is stronger than MeOH for the MIP column, ACN was chosen as the organic solvent in elution mobile phase. The optimized gradient elution solvents for the TCs separation on Gemini C_{18} analytical column consisted of mobile phase B (aqueous solution, pH 3.0 adjusted by citric acid) and mobile phase C (ACN).

3.5. Method validation

The calibration curves of three TCs (OTC, TC and DC) were established by using 10.0 mL of standard solutions with different TCs concentrations. The limits of detection (LOD) (S/N=3), limits of quantitation (LOQ) (S/N=10), enhancement factor (EF) and RSD of the analysis are shown in the Table 1. The EF was calculated by the slope ratio of the calibration curve after extraction to that before the extraction [22]. The satisfactory EF results demonstrated the method has good ability of concentration. The RSDs for five replicate extractions of the standard mixture of TCs (50 μg L^{-1} for each) were in the range of 1.1-2.5 %, showing the high precision for the TCs determination.

Table 1 Performance of the on-line MISPE-HPLC method for the determination of tetracyclines [a]

Tetracycline antibiotics	Determination coefficient (R^2)	LOD (μg L^{-1})	LOQ (μg L^{-1})	EF	RSD (%) (n=5)
Oxytetracycline	0.9987	0.08	0.41	528	1.1
Tetracycline	0.9996	0.10	0.75	442	1.1
Doxycycline	0.9990	1.02	3.56	376	2.5

[a] The R^2 was determined in the linear range of 1 to 200 μg L^{-1} for OTC and TC, 5 to 200μg L^{-1} for DC. The RSD was determined with solution containing 50 μg L^{-1} TCs.

The lake water samples were used for the study of MISPE-HPLC method on the environmental analysis. The recoveries for the spiked samples with three TCs concentrations were from 83.2 % to 111.0 % (Table 2), indicating the method has good accuracy. The RSDs < 5.26 % were obtained, showing that the precision of the method is acceptable. The Fig. 5 shows the chromatographs obtained by direct injection of a lake water sample, purification of a spiked lake water sample and a spiked standard solution.

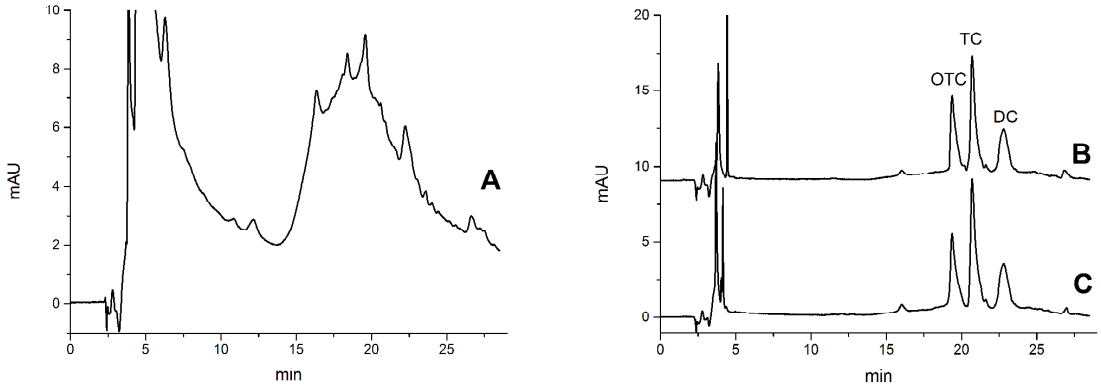

Fig. 5. Chromatograms obtained by direct injection of a lake water sample (A), purification of a spiked lake water sample (10 μg L^{-1}) with online MISPE (B) and purification of a spiked standard solution (10 μg L^{-1}) with online MISPE (C). The MISPE-HPLC condition is in the section 2.5.

Table 2 The accuracy and precision of the on-line MISPE-HPLC method determined by tetracyclines spiked lake water samples (n=5)

Spiked concentration ($\mu g\ L^{-1}$)	OTC	TC	DC
	Recovery (RSD) %	Recovery (RSD) %	Recovery (RSD) %
10	111.0 (1.72)	85.8 (5.26)	86.9 (4.51)
50	101.5 (3.24)	94.4 (1.42)	90.5 (3.14)
100	100.9 (1.08)	83.2 (0.87)	92.6 (1.92)

Table 3 compared the linear range and LOD in TCs determination from some published results and result from this work. Every method has its advantages and limitations in terms of accuracy, sensitivity and analytical time. Compared with the other methods listed in Table 3, the method established in this research has lower LOD and desirable linear range. Meanwhile, the procedure of sample pre-treatment and analysis is simple and automated. It is demonstrated that the method can be used for the determination of trace TCs in environmental water samples with good efficiency.

Table 3 Comparison of different methods for the determination of tetracyclines in complex samples

Pre-concentration method	**Detection**	**Linear range ($\mu g\ L^{-1}$)**	**LOD ($\mu g\ L^{-1}$)**	**Samples**	**Reference**
Off-line dispersive-SPME using PSA as sorbent	HPLC-DAD	2-50	0.7-3.5	Water, milk	Tsai et al. [23]
Off-line coprecipitation with magnesium hydroxide	HPLC-DAD	0.75-8	0.13-0.51	Water	Tsai et al. [3]
Off-line LPME using a hollow fiber membrane	HPLC-UV	3-1000	0.5-1.0	Water, plasma, milk	Shariati et al [24]
On-line SPE using ZIF-8 as sorbent	HPLC-PAD	5-1000	1.5-8.0	Water, milk	Yang et al. [2]
On-line SPE using an Oasis HLB cartridge	LC-MS	0.05-0.5	0.03	Water	Choi et al [25]
On-line SPE using MIP as sorbent	HPLC-UV	1-200	0.08-1.02	Water	This work

4. Conclusion

A new on-line MISPE-HPLC method for trace TC residues determination in water samples has been established. The method has good sensitivity and accuracy as well as high concentration enhancement factors. The result indicates that TC-imprinted microspheres are good for extraction of trace TCs from complex samples. The simultaneous analysis of oxytetracycline, tetracycline and doxycycline residues in complex environmental water samples can be performed by the method established in this work.

Acknowledgements

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Figure captions

Fig. 1. Schematic diagram of the on-line MISPE-HPLC for the determination of trace tetracyclines in water samples. Switching valve position A: sample pre-treatment position; Position B: analytical position.

Fig. 2. Schematic presentation of proposed interactions between tetracycline and functional groups in the recognition cavity of MIP microsphere

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