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Molecularly imprinted solid-phase extraction of (−)-ephedrine from Chinese Ephedra

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Abstract

Method of molecularly imprinted solid phase extraction (MISPE) of (−)-ephedrine from Chinese Ephedra has been developed in the research. The molecularly imprinted polymer (MIP) with good selectivity and affinity for (−)-ephedrine was synthesized with (−)-ephedrine as the template, methacrylic acid as the functional monomer. The washing and elution conditions in MISPE were selected and optimized for efficient analyte extraction and sample clean-up. A clean analytical HPLC base line of ephedra extract was obtained after MISPE, which indicated that the sample pre-treatment was efficient. Good recovery and precision were obtained in the assessment for the MISPE–HPLC procedure, which demonstrated it is a reliable method and can be used for the determination of (−)-ephedrine in herbal ephedra.

Keywords: Molecularly imprinted solid phase extraction; (−)-Ephedrine; Chinese Ephedra; Sample clean-up

1. Introduction

Chinese Ephedra (Ephedra sinica Stapf) is one of herba ephedrae containing (−)-ephedrine, an adrenal pharmaceutical. As the major active component (about 80% among the total alkaloid in the ephedra), the (−)-ephedrine in Chinese Ephedra has to be quantified for the quality determination of the herb and some Chinese medicinal products containing herbal ephedra. Due to the complexity of the herb composition, tedious procedures involving several liquid–liquid extractions are generally performed in the traditional method for the determination of the ephedrine alkaloid including (−)-ephedrine in herba ephedrae [1,2]. Development of more efficient method for the analysis of (−)-ephedrine in Chinese Ephedra is desirable.

Molecular imprinting is a technology of making polymers with pre-determined selectivity and attracts much attention in recent years [3–6]. With recognition ability for the template molecule, molecularly imprinted polymer is able to selectively extract analyte from complex samples and has shown great application potential in solid-phase extraction [7–12]. (−)-Ephedrine imprinted polymer and its ability to separate (−)-ephedrine isomers have been studied by several research groups [13–16], but using MIP for (−)-ephedrine extraction from herba ephedrae has not been reported.

To develop a method for efficient sample clean-up in the determination of the (−)-ephedrine in herbal ephedra, solid-phase extraction material with good selectivity was synthesized with molecularly imprinting technology and used for (−)-ephedrine extraction. The MIP synthesis, evaluation and MISPE method development was presented in this article.

2. Experimental

2.1. Materials and chemicals

(−)-Ephedrine–HCl and (+)-pseudoephedrine–HCl were obtained from National Institute for the Control of Pharma-

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ceutical and Biological Product, China. (−)-Ephedrine–HCl was converted to (−)-ephedrine free base before the MIP synthesis. (+)-Ephedrine–HCl was purchased from Aldrich Chem. Co., USA. Methacrylic acid (MAA) was from Donghuan United Chemicals (Beijing, China) and distilled under vacuum. Ethylene glycol dimethacrylate (EDMA) was purchased from Anli Chemical Company (Suzhou, China). Azobis(isobutyronitrile) [AIBN] was from Special Reagent Labs of Affiliated School of Nankai University (Tianjin, China). Methanol (MeOH) and acetonitrile (MeCN) were HPLC grade. Trifluoroacetic acid (TFA), chloroform ($CHCl_3$) and *n*-hexane were analytical grade. Chinese Ephedra was provided by Tianjin Municipal Institute for Drug Control, China.

2.2. Preparation of the molecularly imprinted polymer

(−)-Ephedrine imprinted polymer was synthesized by non-covalent imprinting method. In the MIP synthesis, template [(−)-ephedrine, 3.5 mmol], functional monomer (MAA, 13.9 mmol), cross-linker (EDMA, 69.5 mmol) and initiator (AIBN, 1.0 mmol) were dissolved in 15 mL MeCN. After being shaken for homogeneity, the mixture was transferred into a glass tube and sparged with nitrogen for 10 min. The tube was flame sealed under vacuum. The polymerization was initiated with 366 nm UV light at ambient temperature. After 24 h, the bulk polymer was ground and particles were sieved. The particles with size less than 45 μm were collected. Fine particles were removed by repeated sedimentations in acetone. (−)-Ephedrine and unreacted reagents in the polymers were removed by extraction with alcohol and 10% HOAc in H_2O. The solution after the extraction were measured by UV spectrophotometry to make sure that template molecules has been cleaned-up from MIP. As a control, non-imprinted blank polymer (BP) was also synthesized with the same method except no template was used.

2.3. Equilibrium adsorption and chromatographic experiments for the MIP property evaluation

2.3.1. Equilibrium adsorption for the affinity evaluation

MIP particles (50 mg) were mixed with 5.0 mL MeCN solution containing (−)-ephedrine with different concentration (from 0.5 to 18 mmol L^{-1}). The mixtures were incubated for 16 h under the continuous shaking in a horizontal shaker at room temperature. After incubation the mixture was centrifuged. The concentration of unbound (−)-ephedrine in the supernatant was determined by UV absorption measurement at 220 nm. The amount of (−)-ephedrine bound to the polymers, *B*, was calculated by subtracting the amount of unbound (−)-ephedrine from the amount of (−)-ephedrine added in the mixture. Scatchard analysis was also used for binding affinity evaluation. The apparent maximum number of binding sites (B_{max}) and the equilibrium dissociation constant (K_D) were determined from the Scatchard equation: $B/[\text{ephe}] = (B_{max} - B)/K_D$, where [ephe] is the concentration of unbound (−)-ephedrine, while *B* is the amount of (−)-ephedrine bound to the polymers.

2.3.2. Chromatographic experiment for MIP selectivity evaluation

An Agilent 1100 HPLC system with a quaternary pump, a multiple-wavelength detector, and a manual injector was used in the experiment. MIP particles was slurry packed into a 150 mm × 4.6 mm HPLC stainless steel column. HPLC was performed with the MIP column using aqueous buffer (0.1 mol mL^{-1} HOAc/NaOAc)–MeOH (4:6, v/v) as the mobile phase while pH of the aqueous buffer was changed. The pH values of the aqueous buffers used in different experiment were 3.6, 4.6 and 5.6 respectively. The flow rate was 1.0 mL min^{-1}. The detection wavelength was 220 nm. The retention was calculated as $k = (t - t_0)/t_0$ where the t and t_0 are the retention time of the analyte and acetone, respectively. (−)-Ephedrine, (+)-ephedrine and (−)-pseudoephedrine were used for the selectivity evaluation.

2.4. SPE cartridge and Chinese Ephedra sample preparation

A lab made glass SPE cartridge dry packed with 500 mg MIP particles and secured by polyethylene frits was used for the solid-phase extraction. BP cartridge packed with 500 mg of non-imprinted polymer (BP) particles was prepared with the same method. The flow of the solution through the SPE cartridges was driven by the pressure of N_2 with a flow rate of 0.5 mL min^{-1}.

For Chinese Ephedra sample preparation, dry herbaceous stems of Chinese Ephedra were chopped with a food processor and sieved with a 50-mesh sieve. One gram of processed herb was weighed and then soaked with 3 mL of 20% sodium hydroxide solution and 50 mL chloroform. After soaking for 24 h, the mixture was sonicated for 45 min and then filtered under vacuum. The filtrate was dried under a stream of air. The residues were dissolved in 16.0 mL MeCN and used as sample in the MISPE experiment.

2.5. Selection of elution solution in MISPE

The eluting abilities of MeOH solvent containing different concentrations of TFA were compared for the elution procedure. After the MISPE cartridge was conditioned with 20 mL MeCN, 0.5 mL of MeCN containing (−)-ephedrine (1.0 mg mL^{-1}) was loaded on the cartridge. Aliquots of 1.0 mL of elution solvent were then applied. The eluates from the MISPE column were collected in each application and were dried with air stream. The residues from each collection were re-dissolved in 0.5 mL MeCN and used for the analytical RP–HPLC quantification. Total volumes of the elution solvent required to completely elute the (−)-ephedrine from MISPE column was determined.

2.6. Selection of washing solution in MISPE

The MISPE cartridge was conditioned with 20 mL MeCN first. After 0.5 mL Chinese Ephedra extract was loaded, the cartridge was washed with 5 mL washing solvent and then elute with 5 mL 5% TFA in MeOH. The eluates in the washing and elution steps were collected and dried with air stream. The residues were dissolved in 0.5 mL MeCN and analyzed with RP–HPLC.

2.7. Evaluation of the MIP cartridge capacity

After the cartridge was conditioned with MeCN, aliquots (1.0 mL for each application) of Chinese Ephedra extract were applied on the cartridges until release of (−)-ephedrine was detected. The cartridge was washed with 10 mL MeCN and then eluted with 15 mL of 5% TFA in MeOH. Eluates were collected at each application and then dried with air steam. The residues from each fraction collection were dissolved with 1.0 mL MeCN and analyzed by analytical RP–HPLC.

2.8. Solid-phase extraction

A volume of 0.5 mL Chinese Ephedra extract was applied on the MISPE cartridge after the cartridge was conditioned with 20 mL MeCN. The cartridge was then eluted with 10 mL washing solvent (MeCN) followed by 5 mL elution solvent (5% TFA in MeOH). Eluted fractions were collected and dried with air stream. The residues were dissolved with 0.5 mL MeCN and analyzed by analytical RP–HPLC.

2.9. HPLC analysis for the (−)-ephedrine quantification

RP–HPLC on a 250 mm × 4.6 mm Zorbax Bonus-RP C_{18} column was used for the quantitative determination of the (−)-ephedrine in the fractions from the MISPE process. The analysis was performed at 40 °C with an Agilent 1100 HPLC system. The phosphate buffer- triethylamine (TEA) mobile phase (pH 6.5) was prepared by mixing solution B (50 mM H_3PO_4–30 mM TEA) with solution A (50 mM KH_2PO_4–30 mM TEA) [17]. A gradient elution, in which the flow-rate was linearly increased from 1.5 to 2 mL min^{-1} in 10 min and remained unchanged for the rest of time, was used in the analysis. All compounds were detected with UV detector at 220 nm.

3. Results and discussion

Except (−)-ephedrine, Chinese Ephedra contains (+)-pseudoephedrine and other (−)-ephedrine structural analogs (Fig. 1).

Chinese Ephedra also contains flavonoids, tannin, proanthocyanidine and volatile oil such as benzylmethylamine. Many components in Chinese Ephedra can be co-extracted with organic solvents. To determine the (−)-ephedrine, labor consuming procedures were often needed for sample clean-up to prevent column contamination in HPLC analysis. Development of an efficient sample pre-treatment method for the analysis of (−)-ephedrine in Chinese Ephedra is necessary.

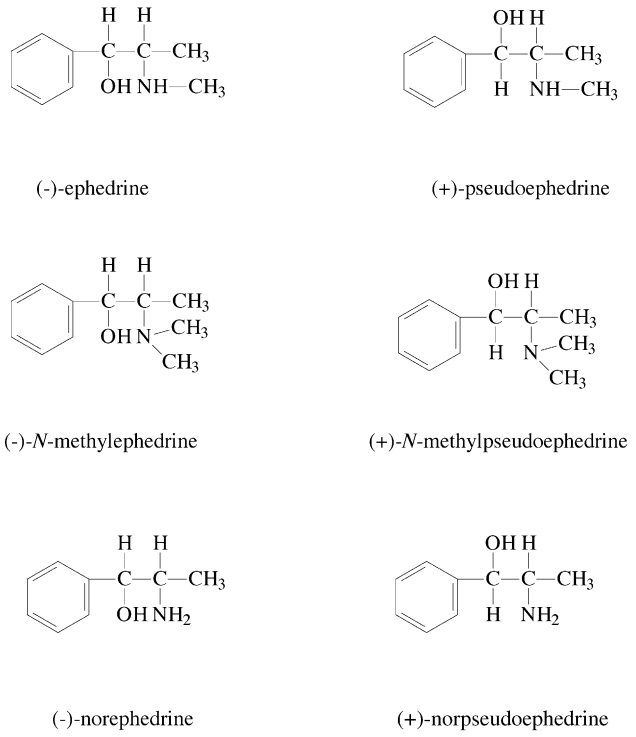

Fig. 1. The structures of alkaloids in Chinese Ephedra.

3.1. Molecularly imprinted polymer preparation and evaluation

In the (−)-ephedrine imprinting synthesis, MAA was chosen as the functional monomer. EDMA was used as the cross-linker. The ratio of template/monomer/cross-linker was 1:4:20 and amount of the monomers to porogen (MeCN) was 1:1 (g mL^{-1}). This synthetic condition can provide MIP with higher binding capacity based on our study of the correlations between the synthetic conditions and properties of the (−)-ephedrine imprinted MIPs.

After MIP synthesis, equilibrium adsorption experiment was performed with MIP and BP (non-imprinted polymer) particles to evaluate the binding affinity of the MIP. The result showed that MIP has higher binding capacity than the BP when the concentration of (−)-ephedrine was higher than 1.5 mmol L^{-1} (Fig. 2). Meanwhile, data in Scatchard plot for MIP can be separated into two groups. Each data group can be linearly fitted and two linear equations were obtained. The calculated dissociation constants (K_D) were 150 μmol L^{-1} (low concentration section) and 530 μmol L^{-1} (high concentration section) while apparent maximum numbers (B_{max}) were 602 and 715 μmol g^{-1} respectively. For

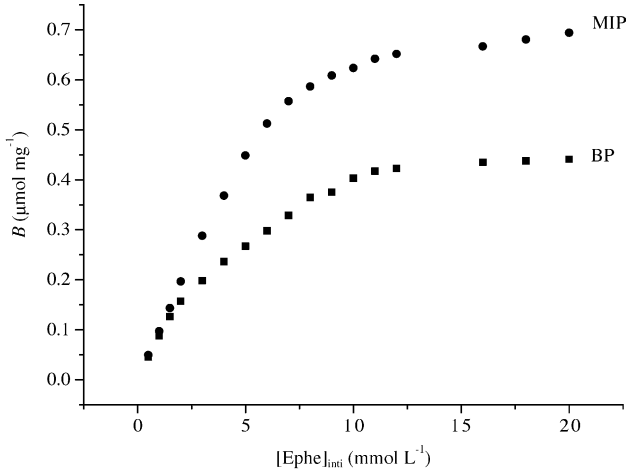

Fig. 2. Binding isotherms of (−)-ephedrine on BP (non-imprinted polymer) and MIP.

non-imprinted polymer (BP), the K_D was 212 μmol L^{-1} and B_{max} was 248 μmol g^{-1} in low concentration section. Results of Scatchard analysis demonstrated that MIP has high affinity and more binding sites (higher capacity) for (−)-ephedrine.

Selectivity of the MIP was studied with chromatographic method using MIP as the stationary phase. The effect of pH of eluent on the retention of the (−)-ephedrine was also investigated. The retention factors of (−)-ephedrine and (+)-pseudoephedrine in different mobile phases were listed in Table 1. Experimental result demonstrated that the MIP has good selectivity (Table 1). Meanwhile, the retention of (−)-ephedrine increased as the pH of the mobile phase was increased. The chiral separation ability of the MIP column [resolving (−)-ephedrine and (+)-ephedrine] was also evaluated using 0.04 mol L^{-1} HOAc–NaOAc/MeOH (3:7, v/v) as the mobile phase. (−)-Ephedrine and (+)-ephedrine can be well separated with α value 1.8. These experimental results demonstrated that the MIP is able to distinguish the (−)-ephedrine from its stereoisomers: (+)-pseudoephedrine and (+)-ephedrine.

3.2. Condition selection and optimization in the molecularly imprinted solid-phase extraction

After the MIP evaluation, MISPE cartridge was prepared for the solid-phase extraction (Section 2.4). MeCN, the porogen in the MIP synthesis and $CHCl_3$, the solvent for herb extraction were considered as solvent for SPE sample. Both MeCN and $CHCl_3$ have been used as the porogen for the (−)-ephedrine imprinted polymer synthesis [16] and can stabilize the interactions between the template and MIP. If $CHCl_3$ could be used as the SPE sample solvent then direct loading the extract of Chinese Ephedra on the SPE column was possible. Based on this consideration, $CHCl_3$ was used as the sample solvent in the MISPE experiment first. It was found that with $CHCl_3$ containing (−)-ephedrine as sample in SPE, the loading capacity of the MIP column was decreased after several SPE experiments. In addition, the swellings of the MIP, given as volume of swollen polymer per volume of dry polymer, were 1.55 in $CHCl_3$ and 1.28 in MeCN also indicated that the MIP structure could have more changes in $CHCl_3$. Because a stable column capacity was obtained when MeCN was used as sample solvent, MeCN was finally chosen as the SPE sample solvent. The method for preparation of the sample of Chinese Ephedra for MISPE is described in Section 2.4.

Table 1
Influence of mobile phase acidity on the retention of the (−)-ephedrine and (+)-pseudoephedrine on the MIP column[a]

pH of the aqueous buffer in the mobile phases	$k_{(-)\text{-ephe}}$	$k_{(+)\text{-pseu}}$	α
3.6	3.1	1.8	1.7
4.6	7.3	3.1	2.4
5.6	19.1	6.8	2.8

[a] In the mobile phase, ratio of buffer to MeOH was 40: 60 (v/v). The aqueous buffers were prepared with 0.1 mol L^{-1} HOAc–NaOAc.

3.2.1. Elution solvent selection and optimization

In the MISPE, the elution solvent, which is able to release the analyte from the column efficiently has to be selected. MeOH with TFA was used as elution solvent to disrupt the hydrogen bonding and ionic interactions between the MIP and the template. TFA was used in the elution solvent because it can compete with MIP for the interaction with (−)-ephedrine. Different concentrations of TFA in MeOH were compared in the experiment. The (−)-ephedrine eluted by every milliliter of elution solvent is depicted with Fig. 3. The result indicated that the volume of the elution solution required for the complete elution of the (−)-ephedrine decreased when the concentration of TFA increased (Table 2). Because using as

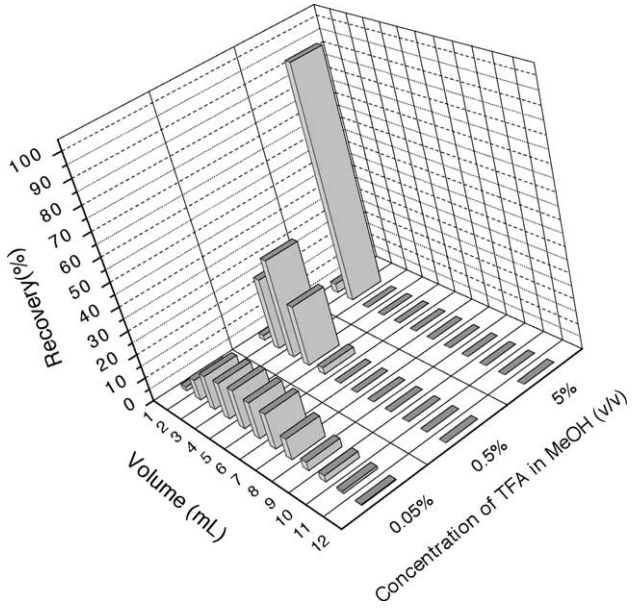

Fig. 3. Eluting ability of elution solutions with different TFA concentrations for (−)-ephedrine.

Table 2
Comparison of eluting ability of solvents with different TFA concentrations for (−)-ephedrine ($n = 3$)[a]

Elution solvent	Total volume of elution solvent required (mL)	Recovery of (−)-ephedrine, (%) (R.S.D., %)
0.05%TFA in MeOH	12	101.4 (11.8)
0.5%TFA in MeOH	5	98.3 (1.4)
5% TFA in MeOH	2	100.5 (0.6)

[a] 0.5 mL of (−)-ephedrine in MeCN (1.0 mg mL^{-1}) was loaded on the column for the test. The (−)-ephedrine in the eluates was determined with analytical RP–HPLC (Section 2.9).

small volume as possible of elution solution can reduce the experiment time and MeOH consumption, 5% TFA in MeOH was selected as the elution solvent for the further experiment. To assure completely removing of the ephedrine analogs from the SPE column, 5 mL 5% TFA in MeOH was used in the elution for the MISPE of the 0.5 mL of Chinese Ephedra extract [containing about 0.161 mg (−)-ephedrine].

3.2.2. Washing solvent selection

Due to the complexity of the Chinese Ephedra extract, the washing step is critical in the MISPE procedure. The MIP could have both polar and non-polar interactions through its carboxyl groups and chain skeleton with the matrix compounds possessing different polarities. The best washing solvent should be able to stabilize the binding of the analyte on column whereas disrupting the non-specific interactions between the matrix components and MIP. To find a proper washing solvent, four solvents (MeOH, MeCN, $CHCl_3$ and *n*-hexane) with different polarities were examined. The Chinese Ephedra extract in MeCN (0.5 mL containing 0.161 mg (−)-ephedrine determined by RP–HPLC after MISPE) was used as the sample for the experiment. The cleaning-up abilities of four solvents were compared by examining the HPLC profile of the extracts after the washing steps. The result demonstrated that MeOH, MeCN and $CHCl_3$ have similar ability of removing the interfering components from the MISPE column while *n*-Hexane could not elute the yellow matrix components from the MISEP column, indicating that non-polar solvent has weaker washing ability for some matrix compounds. Meanwhile, MeCN, $CHCl_3$ did not elute (−)-ephedrine in washing step while MeOH eluted 5.03 μg (−)-ephedrine from MIP cartridge which demonstrated it is not a proper washing solvent. Because the structure of the MIP was stable in MeCN, whereas in $CHCl_3$, MIP swelled and lost binding capacity gradually, MeCN was finally chosen as the washing solvent in the MISPE process.

The proper volume of the washing solvent (MeCN) used in the washing step was determined with experiments. In the experiment, 0.5 mL Chinese Ephedra extract was used as the sample while different washing volumes (3, 5, 7, 10, 12 and 15 mL respectively) were used in the washing step. After the washing step, 5 mL 5% TFA in MeOH was applied for elution. The eluates from the washing and elution steps were analyzed by RP–HPLC. The results showed that one matrix component having retention time of 70 min in RP–HPLC could not be cleaned-up from MISPE column when the washing volume was less than 10 mL. Meanwhile, (−)-ephedrine was eluted when the volume of MeCN reached 15 mL. A volume of 10 mL of MeCN was selected for the washing step.

3.2.3. Capacity of the SPE column

The binding capacity of the MIP cartridge was determined according to the steps in Section 2.7. Considering that co-extracted (−)-ephedrine analogs in the Chinese Ephedra extract can compete the binding sites on the MIP, that is the matrix effect can influence the capacity of the SPE column [18], Chinese Ephedra extracts instead of (−)-ephedrine standard solution were used as the sample for the SPE capacity test. The amount of (−)-ephedrine in 1.0 mL of Chinese Ephedra extract was 0.322 mg determined by the MISPE–HPLC method. Aliquots of 1.0 mL extract were applied to the cartridges until release was detected. Eluates from every step including sample loading, washing and elution were collected, dried and analyzed by analytical RP–HPLC. The experimental result showed that only trace amount of (−)-ephedrine was released from SPE column in the washing step. The total quantity of (−)-ephedrine (2.08 mg) eluted with 5% TFA in MeOH was taken as the capacity of the column.

Binding capacity of the BP column (packed with non-imprinted polymer) for the (−)-ephedrine was also measured for comparison. A volume of 0.5 mL of Chinese Ephedra extract was loaded on the column. The column was washed with 5 mL MeCN and then eluted with 5 mL of 5%TFA in MeOH. The results showed that MIP column has much higher binding capacity than the BP column (Table 3).

3.3. The accuracy and precision of the MISPE method

Considering that slow release of the template molecule from the MISPE column could affect the accuracy for the determination of the (−)-ephedrine in the Chinese Ephedra extraction step, possible bleeding of the MISPE column was examined. In the experiment after adding 0.5 mL of MeCN on the MISPE column, 5 mL of 5% TFA in MeOH was

Table 3
Comparison of binding capacity of the MIP and BP columns

Column		BP	MIP
Total volume of extracts loaded on the column		4.0	7.0
Amount of (−)-ephedrine loaded (mg)		1.288	2.254
Amount of recovered (−)-ephedrine (mg)	In loading step	0.27	0.07
	In washing step	0.13	0.08
	In elution step	0.84	2.08

The results are the average of two measurements. The amount of (−)-ephedrine in 1.0 mL of Chinese Ephedra extract was 0.322 mg. The quantities of (−)-ephedrine eluted from each step were determined by RP–HPLC (Section 2.9). The BP column was packed with non-imprinted polymer.

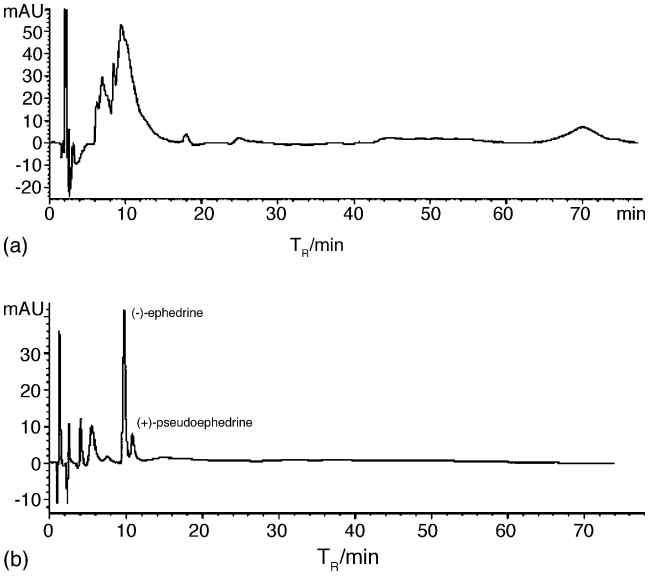

Fig. 4. The chromatogram of Chinese Ephedra extracts before and after MISPE. HPLC column: Zorbax Bonus-RP ODS; mobile phase: 50 mM phosphate buffer–30 mM TEA solution (pH 6.5). Flow rate: 1.0 mL min^{-1}. Detection wavelength: 220 nm. (a) The chromatogram of the Chinese Ephedra extract before the MISPE. (b) The chromatogram of the extract after the MISPE.

Table 4
Recoveries of (−)-ephedrine in the (−)-ephedrine spiked Chinese Ephedra extract ($n = 5$)

Amount of spiked (−)-ephedrine[a] (mg)	0.0145	0.0345	0.0500
Recovery, (%)	102.0	100.8	102.5
(R.S.D., %)	(3.1)	(2.7)	(3.3)

[a] Spiked amount is the amount in the 0.5 mL sample solutions. The amount of (−)-ephedrine in 0.5 mL of Chinese Ephedra extract before spiking was 0.161 mg. The MISPE process was performed as in Section 2.8. The HPLC quantification condition was in Section 2.9.

used for elution. The eluate from elution step was analyzed following the same steps in Sections 2.8 and 2.9. In the RP–HPLC analysis, no detectable amount of (−)-ephedrine was observed. Because the detection limit (3 times of the noise of the chromatogram) in the RP–HPLC analysis was 0.35 μg mL^{-1} while 0.5 mL is the volume of the final solution for HPLC analysis, we assume the possible amount of template molecule bleeding from MISPE column was less than 0.18 μg. This experiment indicated that the amount of possible bleeding was less than 0.1% of the (−)-ephedrine loaded for the extraction experiment (0.18 μg over 161 μg) and can be ignored.

After the MISPE process, the amount of (−)-ephedrine from elution step was determined by RP–HPLC. Compared with the Chinese Ephedra extract before the MISPE, a much cleaner HPLC profile was obtained (Fig. 4), which demonstrated that the sample was cleaned-up efficiently. Standard addition method was used to evaluate the accuracy and precision of the MISPE–HPLC process. Chinese Ephedra extract was spiked with (−)-ephedrine and processed by MISPE–HPLC procedure. The test samples were prepared by mixing 10.0 mL Chinese Ephedra extract with 1.0 mL of standard MeCN solution containing (−)-ephedrine. The recoveries of (−)-ephedrine in spiked Chinese Ephedra extracts was shown in Table 4.

4. Conclusions

The molecularly imprinted polymer was synthesized for the analyte extraction and sample clean-up for the determination of (−)-ephedrine from Chinese Ephedra. After washing and elution condition selection and optimization, the (−)-ephedrine extraction and matrix components removing were performed efficiently in the MISPE process. Good recovery and precision have demonstrated that the MISPE process is a suitable method for the sample pre-treatment for the determination of the (−)-ephedrine in herbal ephedra.

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