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Micelle-mediated extraction of tanshinones from *Salvia miltiorrhiza bunge* with analysis by high-performance liquid chromatography

Zhihong Shi, Jiantao He, Wenbao Chang*

Key Laboratory of Bio-organic Chemistry and Molecular Engineering of Ministry of Education, College of Chemistry and Molecular Engineering, Peking University, Beijing 100871, PR China

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Abstract

The feasibility of employing non-ionic surfactant oligoethylene glycol monoalkyl ether (Genapol X-080) as an alternative and effective solvent for the extraction of tanshinones from *Salvia miltiorrhiza bunge* was studied for the first time. Various experimental conditions were investigated to optimize the extraction. Under optimum conditions, i.e. 10% Genapol X-080 (w/v), liquid/solid ratio of 20:1 (ml g^{-1}), ultrasonic-assisted extraction for 45 min, the extraction recovery of the tanshinones reached the highest value. When compared with commonly used solvents, 10% Genapol X-080 yielded almost the same extraction efficiency as methanol and dichloromethane–methanol (1:4). For the pre-concentration of tanshinones by cloud-point extraction (CPE), sodium chloride was added to the solution to facilitate the phase separation and increase the pre-concentration factor by reducing the volume of the surfactant-rich phase.

Keywords: Tanshinones; Micelle-mediated extraction; Cloud-point extraction; *Salvia miltiorrhiza bunge*; HPLC

1. Introduction

The root and rhizoma of *Salvia miltiorrhiza bunge* (Chinese name: Danshen) has been widely used in traditional Chinese medicine for promoting blood circulation to remove blood stasis, clearing away heat, relieving vexation, nourishing blood, tranquilizing the mind, cooling the blood to relieve carbuncles, treating hemorrhages, menstrual disorders, and miscarriages [1,2]. Dihydrotanshinone, cryptotanshinone, tanshinone I and tanshinone IIA are the main abietane-type diterpenes contained in Danshen (molecular structures are shown in Fig. 1). It is reported that tanshinones can dilate coronary arteries, increase coronary flow, modulate mutagenic activity, and protect the myocardium against ischaemia. They also have sedative and tranquilizing effects, and some of them have been used to treat neurasthenic insomnia [3,4]. In addition, tanshinones have attracted particular attention because they exhibit significant antibacterial [5,6], anti-dermatophytic [6], antioxidant [7,8], anti-inflammatory [6,9], anti-neoplastic [10], and anti-platelet aggregation [11] activities.

Extraction, using organic solvents, of tanshinones from Danshen has been reported by several workers with subsequent analysis by high-performance liquid chromatography [12–14] and spectrophotometry [15]. Conventional extraction methods not only involve the use of large amounts of toxic organic solvents but also need a long time for the pre-concentration of the extracts. In order to eliminate or at least minimize the use of organic solvents and simplify the operating procedure, other extraction methods should be developed.

The micelle-mediated extraction method offers a convenient alternative to the conventional extraction systems. The micelle-mediated extraction process can be divided into two parts: the first step is to solubilize and purify tanshinones from solid herbal materials into the aqueous surfactant solution and the second step is to pre-concentrate the tanshinones based on phase separation by the cloud-point methodology. The micelle-mediated extraction shows advantages in the following aspects: good capacity to solubilize solutes of different types and nature, ability to concentrate solutes with high recoveries, safety and cost benefits, very small amounts of the relatively non-flammable, and non-volatile surfactants are required, easy disposal of the surfactant (it is reportedly easily incinerated in the presence of waste acetone or ethanol) [16]; compatibility with micellar or hydroorganic

* Corresponding author. Tel.: +86-10-6275-4905; fax: +86-10-6275-8153.
E-mail address: changwb@chem.pku.edu.cn (W. Chang).

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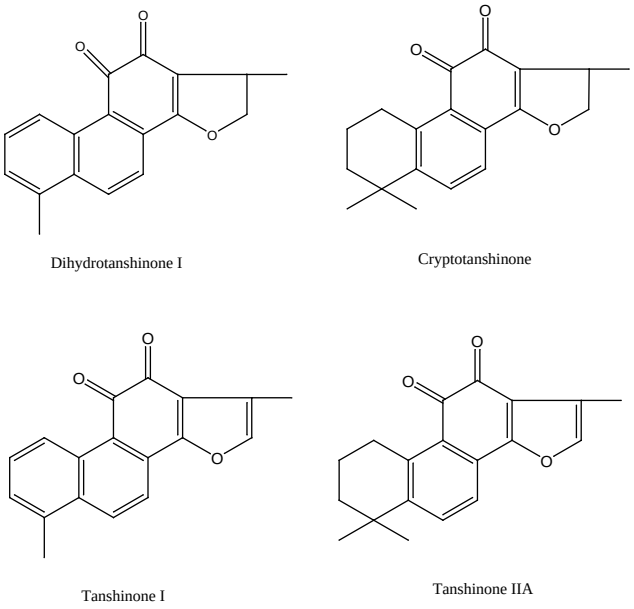

Fig. 1. Molecular structure of dihydrotanshinone, cryptotanshinone, tanshinone I, and tanshinone IIA.

mobile phase; preclusion of analyte losses during the evaporation of solvents used in traditional liquid–liquid extraction techniques and the inhibition by the surfactants of adsorption of non-polar analytes to glass surfaces [17], etc.

The analytical potential of cloud-point extraction (CPE) has been demonstrated in many studies concerning the extraction and pre-concentration of solutes from water [18–20], human serum [21], soil [22] and dried sewage sludge [23]. Whereas, the use of aqueous surfactant solutions as alternative solvent systems in the extraction of chemical constituents from plants or herbal materials has rarely been reported in the literature. To the best of our knowledge, there are only two papers concerning the use of surfactant solution as solvent for the extraction of chemical constituents from herbal products [24,25]. Up till now, no work has been reported on the use of aqueous surfactant solution as solvent for the extraction of tanshinones from herbal materials. The purpose of this work therefore, was to study the feasibility of employing non-ionic surfactant solution as an alternative and effective solvent for the extraction of tanshinones from *Salvia miltiorrhiza bunge*.

2. Experimental

2.1. Plant materials

Dried root of *Salvia miltiorrhiza bunge* (Chinese name: Danshen) was purchased from Tongrentang pharmaceutical store, which had already been cut into pieces. The plant materials were pulverized and sieved to produce samples with partical sizes up to 70 mesh. The dried Danshen powder was stored in a moisture-controlled cabinet.

2.2. Chemicals and reagents

Authentic standards of dihydrotanshinone, cryptotanshinone, tanshinone I and tanshinone IIA were obtained from National Institute for Control of Pharmaceuticals and Biological Products (Beijing, China). Non-ionic surfactant oligoethylene glycol monoalkyl ether (Genapol X-080) was obtained from Fluka (USA) and used as received without further purification. Various concentrations (w/v) of aqueous surfactant solutions were prepared by weighing appropriate amounts of the surfactant and by directly dissolving the surfactant in water. All other reagents were of analytical grade. Water was double-distilled before use.

2.3. Apparatus

All analyses were performed on an HP 1100 liquid chromatograph (Hewlett-Packard, USA) which consisted of a quaternary pump, an on-line de-gasser, a column thermostat, a model 0497 injection valve (sample loop 20 μl) and a multi-wavelength detector. The chromatographic data were recorded and processed with an HP chemstation software. The analytical column was a Diamonsil C_{18} (150 mm × 4.6 mm i.d., 5 μm) column connected with an Agilent Zorbax extend-C_{18} guard column (12.5 mm × 2.1 mm i.d., 5 μm). The column temperature was controlled at 25 °C.

A versatile plant pulverizer (Tianjin, China) was used to make the plant materials into powder. An SB 3200 ultrasonic generator (50 kHz, 120 W) from the Shanghai Branson Ultrasonics Co. Ltd. (Shanghai, China) was used to extract tanshinones from the samples. An Avanti J-25 high-performance refrigerated centrifuge (Beckman, USA) was employed to centrifuge the sample solutions. Cloud-point extraction was carried out in a water bath with a thermostat.

2.4. Procedures for the extraction and pre-concentration of tanshinones

Danshen (0.5 g) was accurately weighed and placed in a 50 ml centrifuge tube, 10 ml Genapol X-080 solution of various concentrations was added. The tube was then capped and placed in the ultrasonic cleaning bath, employing the ultrasound energy to extract tanshinones. After ultrasonic-assisted extraction (UAE), the Danshen extracts were centrifuged at 11424 RCF($\times g$) for 10 min, the supernatant was filtered through 0.45 μm membrane and then injected into the HPLC system.

For experiments which compared the extraction efficiency of different types of extractants (10% Genapol X-080, dichloromethane–methanol(1:4, v/v), acetone, methanol, ethanol, hexane, cyclohexane, and 50% methanol), identical experimental conditions were used: sample amount: 0.5 g, extractant volume: 10 ml, and extraction time: 45 min.

In studying the effect of extraction mode on the micelle-mediated extraction of tanshinones, the following two

extraction methods were also employed: one was to extract the sample statically at ambient temperature for 24 h; the other was to extract the sample in a temperature-controlled shaking culture tank, keeping the rotate speed at 160 rpm, and controlling the temperature at 40 °C for 4 h. The sample amount was 0.5 g and the extractant was 10% Genapol X-080.

To study the pre-concentration of the extracted tanshinones by phase separation of the aqueous surfactant solution, 0.1 g Danshen was extracted with 25 ml 10% Genapol X-080 in ultrasonic bath for 45 min. After centrifugation, the supernatant was transferred into a 50 ml centrifuge tube. An appropriate amount of sodium chloride was added to the sample solution and vortex dissolved for 2 min. The sample solution was then kept in a thermostatic water bath at 50 °C until the solution completely separated into two distinct phases—the upper phase was the small volume of surfactant-rich phase and the lower phase was the large volume of aqueous phase. After centrifugation at 11424 RCF($\times g$) for 10 min, the aqueous phase was sucked out using a syringe with long pinhead, and the sticky surfactant-rich phase was left in the tube. Methanol was added into the tube to lower the viscosity of the surfactant-rich phase. After filtration through a 0.45 μm nylon membrane, 20 μl of the solution was injected into the HPLC system for analysis.

2.5. HPLC analysis

The HPLC mobile phase was a mixture of methanol–tetrahydrofuran–water–glacial acetic acid (23:32:44:1, v/v). The flow rate was 1.0 ml min^{-1}, the detection wavelength was set at 254 nm. Peaks in the chromatograms were identified by comparison with retention times and UV spectra of the tanshinone standards. Peak area was used for quantification.

Percentage of recovery (R%) was determined by comparing the amounts of the tanshinones obtained from a single extraction using either aqueous surfactant solution or other extractants with those obtained from multiple extractions using dichloromethane–methanol (1:4, v/v) as the extractant. Experimental conditions for multiple extractions were as follows: sample amount: 2.5 g, number of extraction: 5, volume of dichloromethane–methanol (1:4, v/v) per extraction: 10 ml, and time duration per extraction: 45 min. The amounts of tanshinones obtained from multiple extractions were considered as reference (i.e., 100% efficiency).

3. Results and discussion

3.1. Selection of the surfactant

At the beginning of this work, Triton X-100, Triton X-114, Triton X-45, and Genapol X-080 were all tried as extraction solvents. However, the Triton X series showed high UV absorbance and gave very broad peaks in the HPLC chromatogram, and thus, interfered severely with the determination of the tanshinones. As shown in Fig. 2A, when the surfactant-rich phase was injected, a large signal, which can be attributed to the surfactant, appeared after a few seconds and lasted for at least 15–20 min, effectively masking the tanshinones, all of which appear within this time interval.

Genapol X-080 is a polyoxyethylene glycol monoether-type surfactant that has eight oxyethylene units and tridecyl alkyl moieties. Possessing no aromatic moiety, Genapol X-080 does not absorb above 210 nm, thus it will not interfere with the determination of the tanshinones (as shown in Fig. 2B). In this case, Genapol X-080 was chosen as the CPE surfactant for further study. Its critical micellar concentration (CMC) is 0.05 mmol l^{-1} (0.028%, w/v) and cloud-point is 42 °C in pure water.

3.2. Analysis of tanshinones by HPLC and UV absorbance detection

The four tanshinones were completely separated from each other as shown in Fig. 2C. It can be seen from Fig. 2C that the elution of the surfactant did not interfere with the detection of the tanshinones. The detector response was linear from 0.4 to 12.0 μg ml^{-1} for dihydrotanshninone ($y = -14.33 + 92.89x$, $r = 0.9998$, $n = 6$), 0.4 to 25.3 μg ml^{-1} for cryptotanshinone ($y = 14.28 + 70.62x$, $r = 1.0000$, $n = 6$), 0.6 to 19.2 μg ml^{-1} for tanshinone I ($y = 5.42 + 122.64x$, $r = 0.9996$, $n = 6$) and 2.1 to 62.4 μg ml^{-1} for tanshinone IIA ($y = -4.60 + 72.73x$, $r = 0.9998$, $n = 6$). This method is sensitive and accurate with good reproducibility. The analytical operation can be completed within 20 min.

3.3. Optimization of the micelle-mediated extraction conditions

3.3.1. Effect of the surfactant concentration on the percentage extraction of tanshinones

Fig. 3 shows that the extraction recovery of tanshinones from the root of *Salvia miltiorrhiza bunge* was increased with the increase of surfactant concentration in the concentration range from 0.1 to 10%. When the surfactant concentration increased from 10 to 20%, the extraction recovery of each tanshinone remained fairly constant. It is known that tanshinones are a kind of hydrophobic compounds, and they can not be extracted by pure water. It was demonstrated in our experiment that tanshinones can be extracted by surfactant solution with certain concentration. The ability of the aqueous non-ionic Genapol X-080 solution in extracting tanshinones may be related to the solubility-enhancement effect of the surfactant micelles. For example, certain surfactants are known to increase the mass-transfer coefficient during the desorption of pollutants from soil to water, presumably due to better swelling of the soil organic matters and more complete diffusion of the solvent into the solid matrix [26]. As maximum extraction

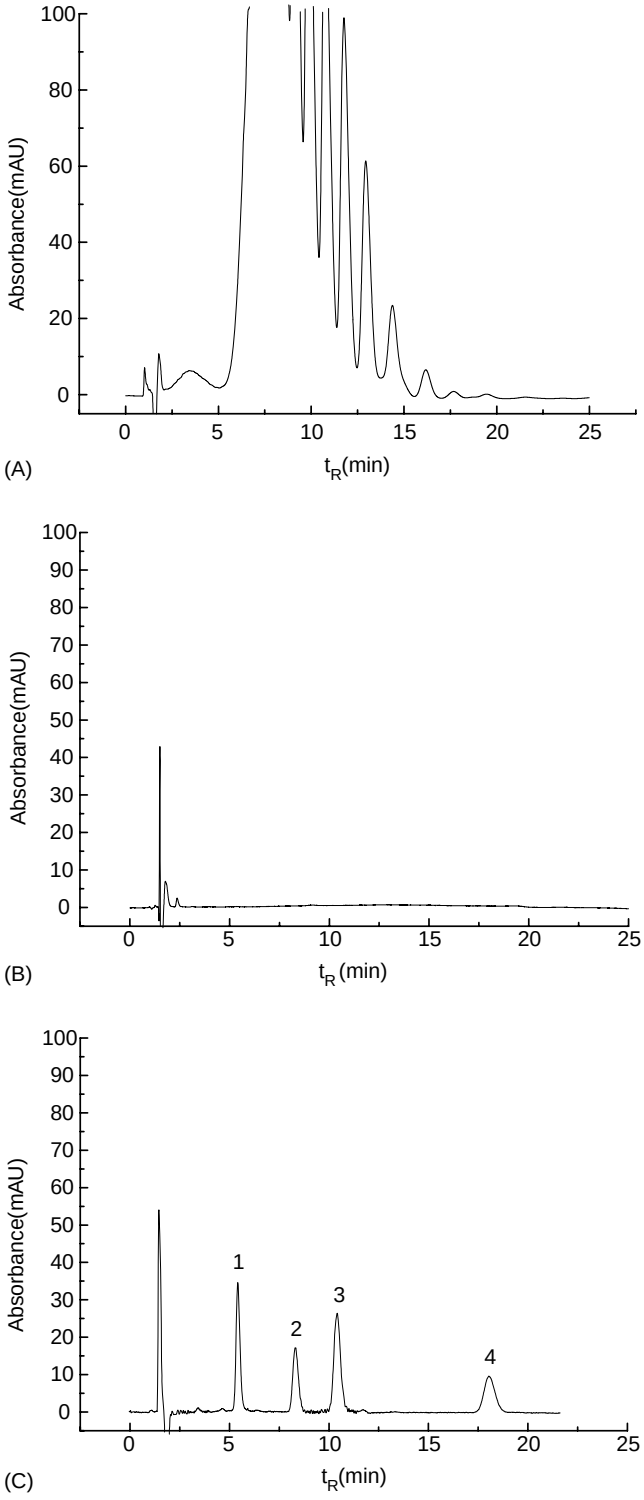

Fig. 2. HPLC chromatograms of surfactants and tanshinones. HPLC conditions: Diamonsil C_{18} column (150 mm × 4.6 mm i.d., 5 μm) connected with an Agilent Zorbax extend-C_{18} guard column (12.5 mm × 2.1 mm i.d., 5 μm) controlled at 25 °C; a mixture of methanol–tetrahydrofuran–water–glacial acetic acid (23:32:44:1, v/v) as mobile phase at a flow rate of 1.0 ml min^{-1}; the detection wavelength was set at 254 nm. (A) Triton X-100 (10%, w/v); (B) Genapol X-080 (10%, w/v); and (C) authentic tanshinones—1, Dihydrotanshinone; 2, Cryptotanshinone; 3, Tanshinone I; and 4, Tanshinone IIA.

recovery can be obtained at surfactant concentrations above 10%, 10% Genapol X-080 was selected for further study.

3.3.2. Effect of extraction time on the extraction recovery of tanshinones

The effect of extraction time on the extraction recovery of tanshinones was studied by varying the extraction time from 5 to 60 min. The results indicated that the recovery of tanshinones was increased with the increase of extraction time and reached the highest value for each compound when extracted for 45 min. When the extraction time was longer than 45 min, the recovery of cryptotanshinone was decreased, while the values of the other compounds kept almost constant. This is because cryptotanshinone is easy to decompose when being treated at higher temperature for a long period of time [27]. So, in the following experiments, 45 min was selected for the extraction of the tanshinones.

3.3.3. Effect of liquid/solid ratios on the extraction recovery of tanshinones

The liquid/solid ratio is one of the factors influencing the extraction recovery of tanshinones. The experimental results show that the liquid/solid ratio of 20:1 (ml g^{-1}) was sufficient for each compound to reach the highest recovery, so it was employed in the following experiments.

3.4. Comparison of genapol X-080 with conventional extraction solvents

The extraction recovery results were compared between 10% Genapol X-080 and various polar and non-polar organic solvents. Among the organic solvents, dichloromethane–methanol (1:4, v/v) is the most efficient extractant, and dichloromethane–methanol (1:4, v/v) was used to prepare the extract of multiple extraction. Fig. 4 shows that 10% Genapol X-080 has almost the same high extraction efficiency as dichloromethane–methanol (1:4) and methanol, then comes acetone and ethanol. Non-polar solvent hexane and cyclohexane exhibit lower extraction efficiency, and the extraction efficacy of 50 % methanol is the lowest. The reason why the extraction efficacy of 10% Genapol X-080 is higher than that of most polar and non-polar solvents may be due to the more complete diffusion of surfactant solution into the particles of the herbal material and the solubility-enhancing effect of the surfactant micelles.

3.5. Comparison of UAE with other extraction methods

Compared with shake flask extraction method (40 °C, 4 h) and static extraction method (room temperature, 24 h), the UAE method had the highest extraction efficacy. This result is not surprising because the cavitation effect of ultrasonic wave facilitates the interaction between the surfactant

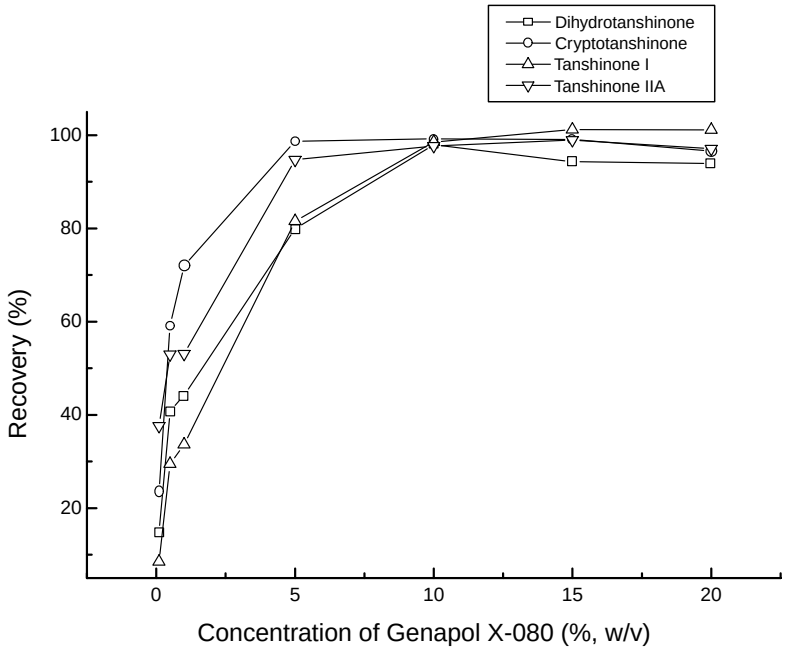

Fig. 3. Effect of concentration of Genapol X-080 (%, w/v) on extraction recovery of tanshinones. Root of *Salvia miltiorrhiza bunge*: 0.5 g; solvent volume: 10 ml; liquid/solid ratio: 20:1 (ml g^{-1}); ultrasonic-assisted extraction time: 45 min.

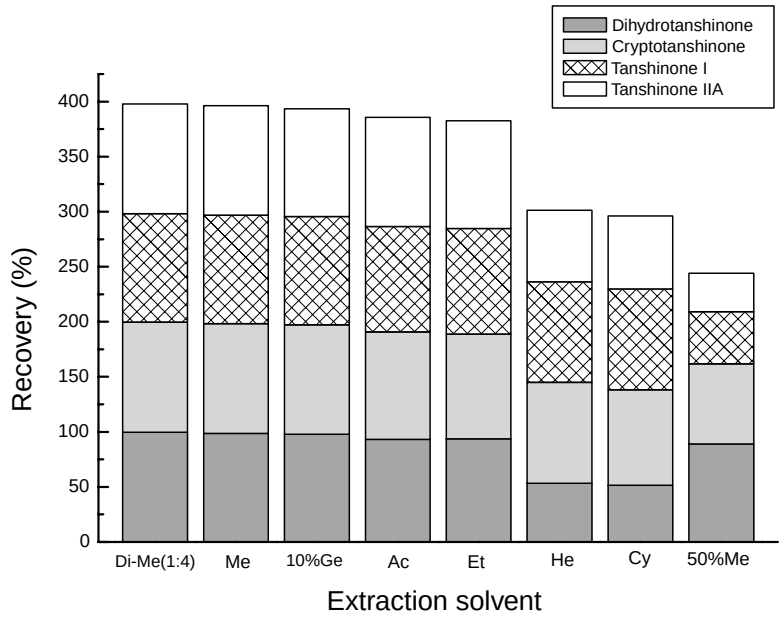

Fig. 4. Comparison of the recovery of extraction using different solvents. Solvent volume: 10 ml; root of *Salvia Miltiorrhiza bunge*: 0.5 g; ultrasonic-assisted extraction time: 45 min. Di–Me(1:4): dichloroform–methanol (1:4, v/v); Me: Methanol; 10% Ge: 10% Genapol; Ac: acetone; Et: ethanol; He: hexane; Cy: cyclohexane; 50% Me: 50% Methanol.

solution and the tanshinones in herbal material, so UAE can reach higher extraction efficacy in shorter extraction time.

3.6. Pre-concentration of tanshinones by cloud-point extraction

After the first part of the micelle-mediated extraction process (i.e., the use of aqueous Genapol X-080 solution for the solubilization and purification of tanshinones), we now study on the pre-concentration of the tanshinones by cloud-point extraction. The most commonly used method to induce CPE is by raising the temperature of the sample solution above the cloud-point temperature of the surfactant. In our study, we first tried the temperature-induced CPE at 50 °C, but the time for complete separation of the two phases was longer than 2 h. It has been reported that the addition of

electrolytes may facilitate the separation of the two phases since it increases the density of the bulk aqueous phase [28] Electrolytes, which have ever been used in CPE, include urea, sodium chloride, sodium azide, and potassium chloride [29]. In this paper, sodium chloride was the modifier of choice because it is both cost-effective and environmentally friendly. The influence of the content of NaCl on the volume of the surfactant-rich phase was studied by adding different concentration of NaCl into the solution, and by heating the solution in thermostatic water bath at 50 °C for 30 min. It is clearly demonstrated that the volume of the surfactant-rich phase decreased significantly as a result of increasing the amount of salt added. Fang et al. [25] described the same phenomenon when they employed NaCl to induce cloud-point extraction. When the amount of salt added exceeded 5 g, the volume of the surfactant-rich phase appeared to exhibit a minimum value of 3.5 ml for the original surfactant solution of 25 ml. The pre-concentration factor is about seven. To reduce the viscosity of the surfactant-rich phase and facilitate the injection to the HPLC system, methanol was added to the surfactant-rich phase and the final volume was fixed to 5 ml. Fig. 5B shows a chromatogram of pre-concentrated tanshinones present in the surfactant-rich phase. For comparison, Fig. 5A shows a chromatogram of the aqueous Genapol X-080 solution containing the tanshinones prior to CPE. The pre-concentration effect of the CPE is clearly demonstrated in Fig. 5A and B, showing an average pre-concentration factor for the tanshinones of about five, using 10% aqueous Genapol X-080 solution as the extractant. Although the pre-concentration factor obtained using 10% Genapol X-080 was not very impressive, it is a good indication that CPE can be used to extract and pre-concentrate tanshinones from *Salvia miltiorrhiza bunge*. To improve the pre-concentration factor, lower surfactant concentrations should be used. In this case, the extraction efficiency will inevitably be sacrificed. For example, if 5% Genapol X-080 is employed, the pre-concentration factor will be increased to about 18, but the extraction recovery of dihydrotanshinone and tanshinone I will be decreased to 80%. How to improve the extraction efficiency of lower concentration surfactant is a question deserves further study. One of the solutions is to employ the microwave-assisted extraction, and we will conduct the related experiments in the future.

3.7. Repeatability of the HPLC profile and reproducibility of the established CPE method

The repeatability of the HPLC profile was determined by injecting the same CPE processed sample five times on the same day. The RSD of retention time was 0.22% for dihydrotanshinone, 0.24% for cryptotanshinone, 0.15% for tanshinone I, and 0.14% for tanshinone IIA, respectively. The RSD of peak area was 1.82% for dihydrotanshinone, 1.31% for cryptotanshinone, 1.20% for tanshinone I, and 1.68% for tanshinone IIA, respectively.

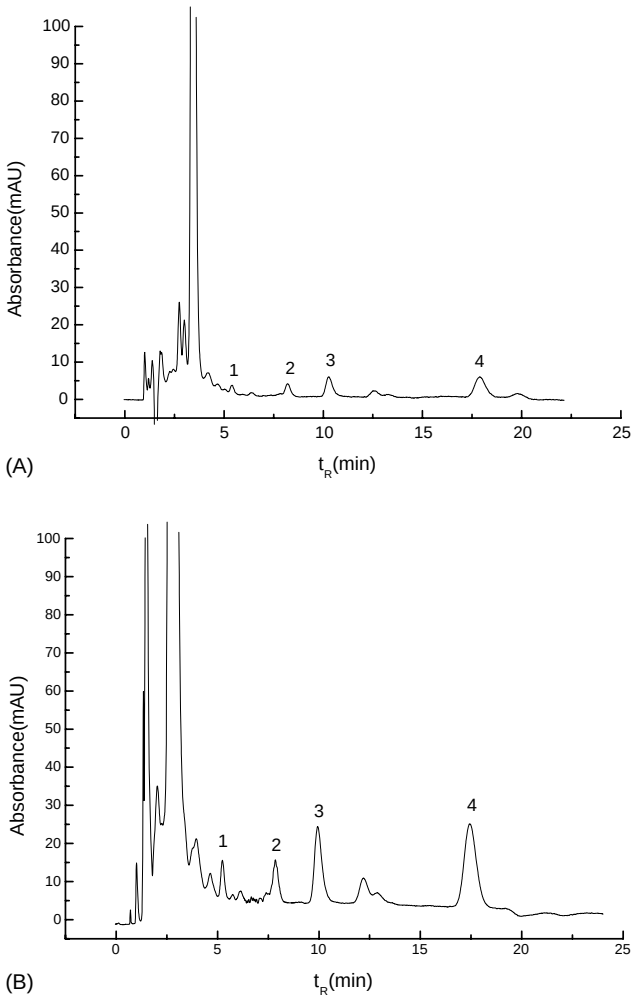

Fig. 5. HPLC chromatograms of the tanshinones extracted from the root of *Salvia Miltiorrhiza bunge*. Chromatographic conditions as in Fig. 2. (A) Chromatogram of the extracts prior to cloud-point extraction (non-pre-concentrated sample); (B) chromatogram of the surfactant-rich phase after cloud-point extraction. Root of *Salvia miltiorrhiza bunge*: 0.1 g; the original sample solution: 25 ml; the concentration of Genapol X-080: 10%; equilibration temperature: 50 °C; equilibration time 10 min; the amount of salt added: 5 g.

The reproducibility of the CPE method was determined by processing five replicates of *Salvia miltiorrhiza bunge*. The RSD of peak area was 6.33% for dihydrotanshinone, 6.00% for cryptotanshinone, 1.49% for tanshinone I, and 3.73% for tanshinone IIA, respectively.

4. Conclusions

The optimized method described in this work gives satisfactory results for the extraction and pre-concentration of tanshinones from root of *Salvia miltiorrhiza bunge* without needing to use expensive and potentially toxic organic solvents. The present work demonstrated that micelle-mediated extraction/CPE is a potentially powerful tool for the solubilization, purification, and pre-concentration of active

ingredients from herbal medicines. This capability should be highly valuable in the large-scale extraction and purification of active ingredients from herbal materials. It should be noted that a key step in the purification process would likely to be surfactant removal, which can be carried out by various methods based on exploiting the differences in size, charge, and hydrophobicity between the surfactant and extracted compounds [30]. A popular method of removing non-ionic surfactants is via hydrophobic adsorption of the surfactants with polystyrene resins [31,32] The resins are usually added batch-wise to the preparation and removed, together with the bound surfactants, simply by centrifugation.

Acknowledgements

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