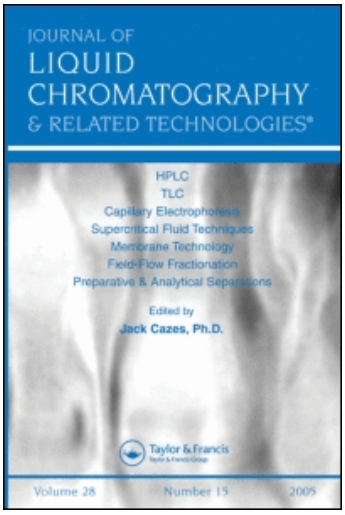

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Shi, Zhihong , Wang, Yu and Zhang, Hongyi(2009) 'Combination of Microwave Assisted Micellar Extraction and Liquid Chromatography for Determination of Cryptotanshinone, Tanshinone I, and Tanshinone IIA in *Salvia Miltiorrhiza* Bunge', Journal of Liquid Chromatography & Related Technologies, 32: 5, 698 — 711
To link to this Article: DOI: 10.1080/10826070802711170
URL: http://dx.doi.org/10.1080/10826070802711170

Journal of Liquid Chromatography & Related Technologies®, 32: 698–711, 2009

ISSN: 1082-6076 print/1520-572X online
DOI: 10.1080/10826070802711170

Combination of Microwave Assisted Micellar Extraction and Liquid Chromatography for Determination of Cryptotanshinone, Tanshinone I, and Tanshinone IIA in *Salvia Miltiorrhiza* Bunge

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Abstract: A new method based on the microwave assisted micellar extraction technique (MAME) has been optimized for the extraction of tanshinones from the traditional Chinese medicine-*Salvia miltiorrhiza* Bunge. Non-ionic surfactant Genapol X-080 solution was employed as extractant. Under optimum conditions, i.e., 10% Genapol X-080 (w/v), liquid/solid ratio 80:1 ($mL \cdot g^{-1}$), microwave assisted extraction time 7 min (4 min + 3 min), microwave power 260 W, particle size of *Salvia miltiorrhiza* Bunge <105 μm, the extraction efficiency of tanshinones reached the highest value. Compared with static extraction and ultrasonic assisted extraction techniques, MAME has the highest extraction yield of tanshinones and shortest extraction time. Furthermore, MAME has similar extraction efficiency with the heating reflux method recommended by Chinese Pharmacopoeia.

Keywords: Cryptotanshinone, HPLC, Microwave assisted micellar extraction, *Salvia miltiorrhiza* Bunge, Tanshinone I, Tanshinone IIA

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INTRODUCTION

As one of the most popular Chinese herbs, the rhizome of *Salvia miltiorrhiza* Bunge (Chinese name: Danshen) has been widely used for promoting blood circulation to remove blood stasis, relieving vexation, nourishing the blood, tranquilizing the mind, and cooling the blood to relieve carbuncles.[1] It is effective for various microcirculatory disturbance related diseases, such as cardiovascular disease, cerebrovascular disease, liver dysfunction, renal deficiency, and diabetic vascular complication.[2,3] In addition, *Salvia miltiorrhiza* Bunge also exhibits significant antioxidant,[4] antibacterial,[5] antiallergic,[6,7] anti-HIV,[8] anti-inflammatory,[9] anti-immunological,[10] and antitumor[11,12] activities.

The main lipophilic bioactive ingredients in *Salvia miltiorrhiza* Bunge are reported to be abietane-type diterpenes such as cryptotanshinone, tanshinone I, and tanshinone IIA.[13] Figure 1 shows the chemical structure of the above tanshinones. Several methods including HPLC,[14–17] HPCE,[18] LC/MS,[19] and LC-MS/MS[20] have been employed for the determination of tanshinones in *Salvia miltiorrhiza* Bunge. In all the above mentioned methods, toxic and inflammable organic solvents are used to extract tanshinones from *Salvia miltiorrhiza* Bunge, such as methanol, acetonitrile, ethanol, etc. In order to eliminate or at

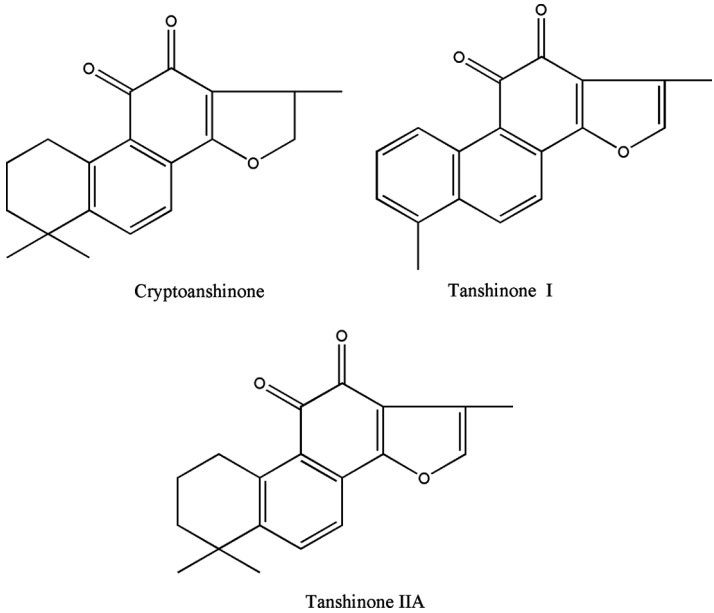

Figure 1. Chemical structures of the tanshinones.

least minimize the use of organic solvents and simplify the operating procedure, other extraction methods should be developed. In our previous work, nonionic surfactant oligoethylene glycol monoalkyl ether (Genapol X-080) solution was employed as extractant for the ultrasonic assisted extraction of tanshinones.[21] In this paper, the microwave-assisted extraction (MAE) technique was employed in order to further reduce the extraction time. To the best of our knowledge, there is no report on the combination of the MAE technique with the use of micellar media, which makes the extraction of tanshinones to be a simple, fast, and non-toxic procedure.

EXPERIMENTAL

Plant Materials

Salvia miltiorrhiza Bunge, which had already been cut into pieces, was purchased from a local pharmaceutical store (Baoding, China). The dried plant materials were pulverized and sieved to produce samples with particle sizes of $<105\,\mu m$, 105–150 μm, 150–180 μm, 180–250 μm, 250–420 μm, and $>420\,\mu m$.

Chemicals and Reagents

Authentic standards of tanshinones were purchased from National Institute for the Control of Pharmaceutical 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 doubly distilled water. All other reagents used in this work were of analytical grade.

Apparatus

All analyses were performed on an LC-10ATvp plus liquid chromatograph (Shimadzu, Japan) which consisted of an LC-10ATvp plus pump, a Rheodyne model 7725i injection valve (sample loop 20 μL) and an SPD-10Avp plus multi-wavelength detector. The chromatographic data were recorded and processed with CBM-10Avp plus LC Solution Lite software. The analytical column was a Diamonsil C_{18} (150 mm × 4.6 mm i.d., 5 μm) column.

A versatile plant pulverizer (Foshan, Guangdong, China) was used to make the plant materials into powder. A WBFY-201 microwave chemical

reactor (650 W, Gongyi, China) was used to perform the MAME of tanshinones from *Salvia miltiorrhiza* Bunge. A KQ-250 ultrasonic generator from the Kunshan Company (Jiangsu, China) was also used to extract tanshinones from the samples in contrast. Sieves (40 mesh/420 μm, 60 mesh/250 μm, 80 mesh/180 μm, 100 mesh/150 μm, 140 mesh/105 μm) (Zhejiang, China) were used to sieve the *Salvia miltiorrhiza* Bunge powder. A high speed centrifuge was employed to centrifuge the sample solutions (LG 10–2.4A, Beijing, China).

Experimental Procedure

Preparation of Standard Solutions

Standard stock solutions of cryptotanshinone and tanshinone IIA were directly prepared in methanol. Tanshinone I was first dissolved in a small volume of chloroform and then diluted to the mark with methanol. The standard stock solutions were stored at 4°C. Working standard solutions containing each of the three compounds were prepared by diluting the stock solutions with methanol to proper volumes. The standard stock solutions and working solutions were all prepared in dark brown calibrated flasks.

Preparation of Surfactant Solution

Various concentrations (w/v) of aqueous surfactant solutions (20%, 15%, 10%, 5%, 1%, and 0.5%) were prepared by weighing appropriate amounts of the surfactant and by directly dissolving the surfactant in doubly distilled water.

Extraction Procedure

Salvia miltiorrhiza Bunge particle was accurately weighed and placed in a round bottom flask and Genapol X-080 solution was added. The flask was capped and blended adequately, and then it was placed in the microwave chemical reactor to extract tanshinones. After the extraction procedure, the extracting solution was transferred into a centrifugal tube and the tanshinone extract was centrifuged for 15 min ($4000\,r \cdot min^{-1}$). The supernatant was filtered with a 0.45 μm membrane filter and 10 μL was injected into the HPLC for the analysis of tanshinones.

HPLC Analysis

The analytical column was a Diamonsil C_{18} (150 mm × 4.6 mm i.d., 5 μm) column. The HPLC mobile phase was a mixture of methanol,

tetrahydrofuran, water, and glacial acetic acid (20:35:44:1, v/v/v/v). The flow rate was $0.8\,mL \cdot min^{-1}$; the detection wavelength was set at 254 nm. In this report, peaks in the chromatograms were identified by comparing the retention times and UV spectra with those of the authentic tanshinones. Peak area was used for quantification. To avoid the potential influence of Genapol X-080 to the separation of tanshinones, the column was flushed with methanol to completely elute Genapol X-080 after each day's work.

RESULTS AND DISCUSSION

Optimization of the Microwave-Assisted Extraction Conditions

To optimize the microwave-assisted micellar extraction of tanshinones from the solid herbal materials, a number of experiments under different conditions were performed. The extraction time, microwave power, concentration of the surfactant solution, liquid/solid ratio, granularity of the plant powder were all investigated and evaluated via the yield of tanshinones (%, w/w):

$$\text{yield of tanshinone}(\%, \text{w/w}) = \frac{\text{amount of tanshinone extracted}}{\text{amount of herbal material}} \times 100\%$$

Effect of the Surfactant Concentration on the Extraction Efficiency of Tanshinones

Tanshinones are known to be hydrophobic. The ability of the aqueous nonionic Genapol X-080 solution in extracting tanshinones from *Salvia miltiorrhiza* Bunge may be related to the solubility enhancement effect of the surfactant micelles. The influence of surfactant concentration on the extraction efficiency of tanshinones was studied by varying the surfactant concentration between 0.5% and 20%. As shown in Figure 2, the extraction efficiency for tanshinones rapidly increased when the surfactant concentration increased from 0.5% to 5%. Then fairly constant extraction efficiency was obtained for cryptotanshinone with the surfactant concentration rising from 5% to 15%, while for tanshinone IIA, the extraction efficiency was a little bit decreased at surfactant concentration of 10% and an increase was observed at surfactant concentration up to 15%. On the contrary, tanshinone I showed an opposite tendency. Taking into account the extraction efficiency of the three tanshinones, 10% was chosen as the optimum surfactant concentration for the extraction of tanshinones for further studies.

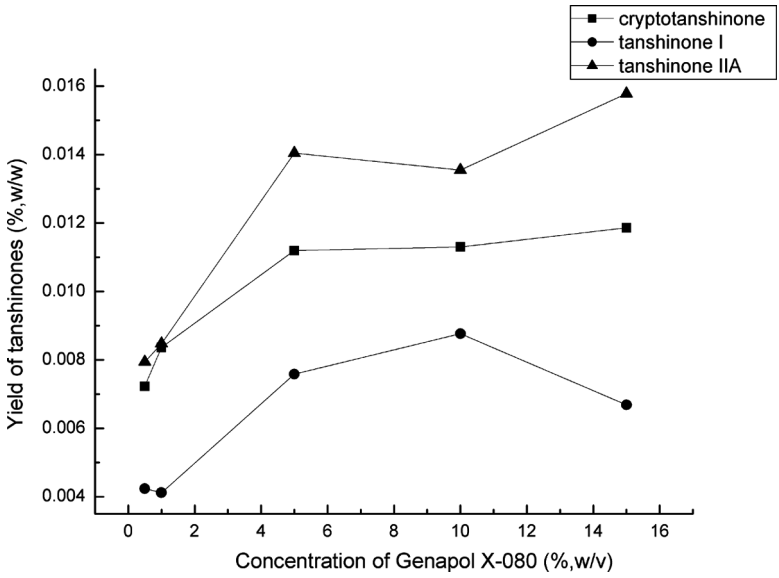

Figure 2. Effect of concentration of Genapol X-080 on the extraction efficiency of tanshinones. Microwave power: 260 W, granularity: 250–420 μm, microwave-assisted extraction time: 3 min, liquid/solid ratio (mL/g): 20.

Effect of Microwave Power on the Extraction Efficiency of Tanshinones

Microwave power is one of the important influential factors for the extraction efficiency of tanshinones. In this experiment, microwave power 65 W, 130 W, 195 W, 260 W, 325 W were evaluated to optimize the extraction procedure. It could be seen from Figure 3, that the extraction efficiency for tanshinones significantly increased when the microwave power increased from 65 W to 130 W, and a steady increase was observed from 130 W to 260 W. When the microwave power was above 260 W, the extraction efficiency decreased. The possible reason for this phenomenon lies in that tanshinones decomposes at high temperature induced by high microwave power during the extraction. Finally, 260 W was chosen as the optimum microwave power for the extraction of tanshinones.

Effect of Extraction Time on the Extraction Efficiency of Tanshinones

Parallel samples were extracted for a total extraction time of 1, 3, 5, 7 (4 + 3), 10 (4 + 3 + 3) and 15 (4 + 4 + 4 + 3) min, respectively. As shown in Figure 4, the extraction efficiency for all the three tanshinones was sharply increased when the extraction time rose from 3 min to 7 min.

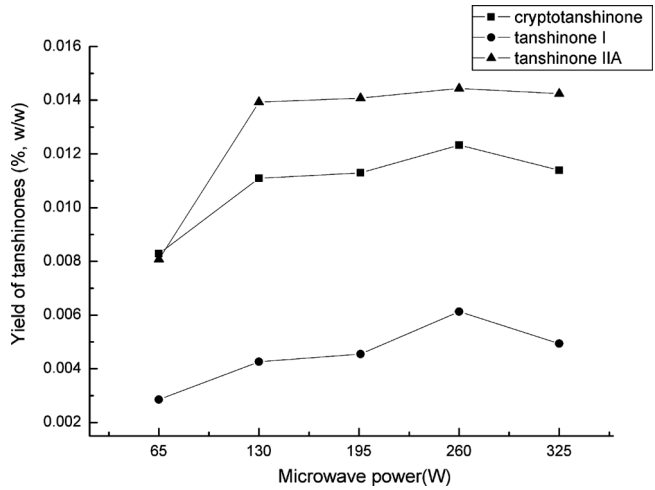

Figure 3. Effect of microwave power on the extraction efficiency of tanshinones. Concentration of Genapol X-080: 10% (m/v), granularity: 250–420 μm, microwave-assisted extraction time: 3 min, liquid/solid ratio (mL/g): 20.

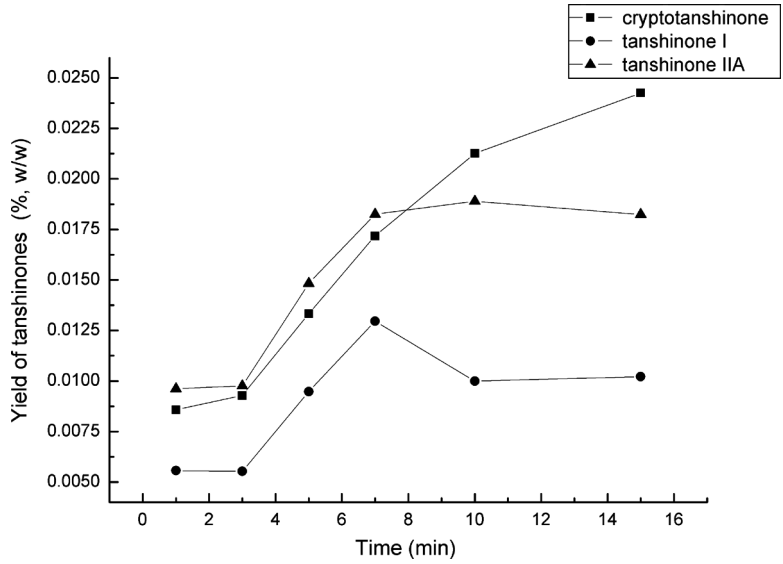

Figure 4. Effect of microwave-assisted extraction time on the extraction efficiency of tanshinones. Concentration of Genapol X-080: 10% (m/v), granularity: 250–420 μm, microwave power: 260 W, liquid/solid ratio (mL/g): 20.

After 7 min, extraction efficiency of cryptotanshinone was still increased, and tanshinone IIA kept a constant value. As for tanshinone I, the extraction efficiency declined after 7 min. For simultaneous extraction of the three tanshinones, 7 min was selected for further study.

Effect of Liquid/Solid Ratio on the Extraction Efficiency of Tanshinones

The liquid/solid ratio is the proportion of the extractant volume to the mass of herbal material. It is one of the factors influencing the extraction efficiency of tanshinones. As shown in Figure 5, liquid/solid ratios (mL/g) of 5, 10, 20, 25, 50, 80, 100 were tested to optimize the extraction procedure. When the liquid/solid ratio increased from 5 to 80, the yield of extracts of tanshinones all increased continuously, but at the point of 100, there was little difference: cryptotanshinone, tanshinone IIA, got the maximum value and tanshinone I reached the minimum. This presumably might be that larger liquid/solid ratio could ensure *Salvia miltiorrhiza* Bunge particles and the Genapol X-080 solution in sufficient contact with each other. It could be seen from Figure 5, that the liquid/solid ratio of 80:1 (mL/g) was sufficient for tanshinones to reach the highest extraction efficiency, therefore, it was employed in the following experiments.

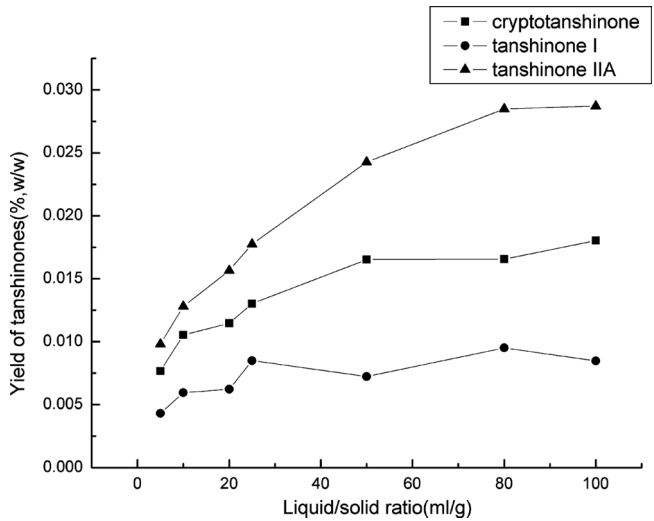

Figure 5. Effect of liquid/solid ratio on the extraction efficiency of tanshinones. Concentration of Genapol X-080: 10% (m/v), microwave power 260 W, granularity: 250–420 μm, microwave-assisted extraction time: 3 min.

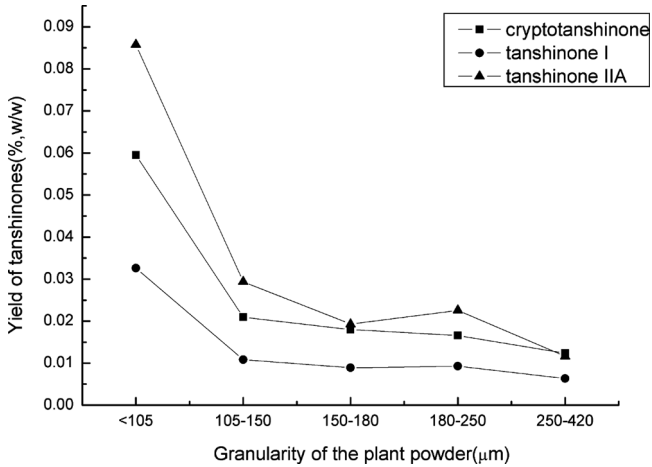

Figure 6. Effect of granularity on the extraction efficiency of tanshinones. Concentration of Genapol X-080: 10% (m/v), microwave power 260 W, microwave-assisted extraction time: 3 min, liquid/solid ratio (mL/g): 20.

Effect of Granularity of the Plant Powder on the Extraction Efficiency of Tanshinones

Granularity is another important factor influencing the extraction efficiency. In our experiment, a series of granularity (250–420 μm, 180–250 μm, 150–180 μm, 105–150 μm, <105 μm) was compared. It could be seen from Figure 6 that the extraction efficiency reached the highest value when plant powders of <105 μm were used. The reason for this result lies in that the plant powders of smaller granularity own larger effective contact area. So plant powders of <105 μm were chosen for further studies.

Comparison of Microwave-Assisted Extraction with Conventional Extraction Techniques

Extraction efficiency was compared between MAME and conventional extraction techniques, such as ultrasonic assisted extraction (UAE), static extraction at room temperature (SERT). For the comparison experiment, identical experimental conditions were used except the extraction time: sample amount 1.0 g, 10% Genapol X-080, liquid/solid ratio of 80:1 ($mL \cdot g^{-1}$), particle size of *Salvia miltiorrhiza* Bunge <105 μm. The extraction time for UAE was 45 min, MAME 7 min and SERT 24 h. Figure 7 shows that MAME has the highest extraction efficiency among the three extraction techniques, especially for tanshinone I and tanshinone

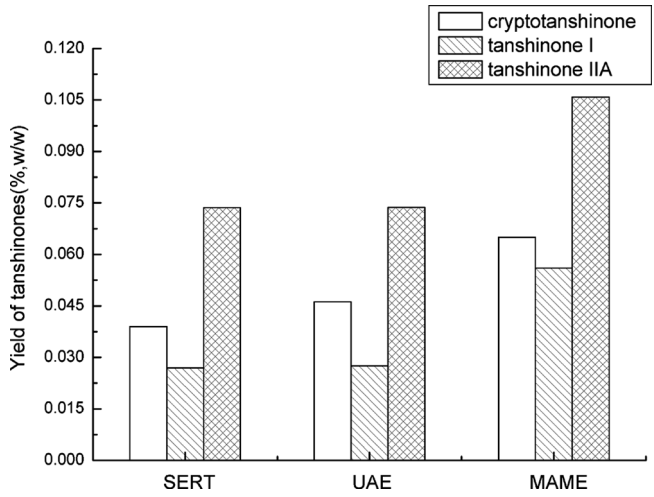

Figure 7. Comparison of microwave-assisted extraction and conventional extraction techniques (experimental conditions: Genapol X-080: 10% (m/v), *Salvia miltiorrhiza* Bunge: 0.125 g, liquid/solid ratio: 80:1, SERT: 24 h, UAE: 45 min, MAME: 7 min).

IIA. In addition, compared with the other two extraction methods, MAME not only achieves high extraction efficiency, but also spends a shorter extraction time and is less labor intensive.

Comparison of the MAME Method with the Chinese Pharmacopoeia Method

Extraction efficiency was compared between the established method and the heating reflux method recommended by Chinese pharmacopoeia. According to Chinese pharmacopoeia (part I, 2005 edition),[22] 0.125 g of *Salvia miltiorrhiza* Bunge powder was extracted with 50 mL methanol for 1 h by the heating reflux extraction method. From Figure 8, it could be seen that the established method owned similar extraction efficiency with the CPEM method, especially for tanshinone I and tanshinone IIA. Therefore, MAME is an alternative extraction technique for fast and environment friendly extraction of tanshinones from *Salvia miltiorrhiza* Bunge.

Analysis of Tanshinones by HPLC

As shown in Figure 9, tanshinones in *Salvia miltiorrhiza* Bunge were completely separated from each other, and other coexisting components in the extracts did not interfere with the detection of the tanshinones. As

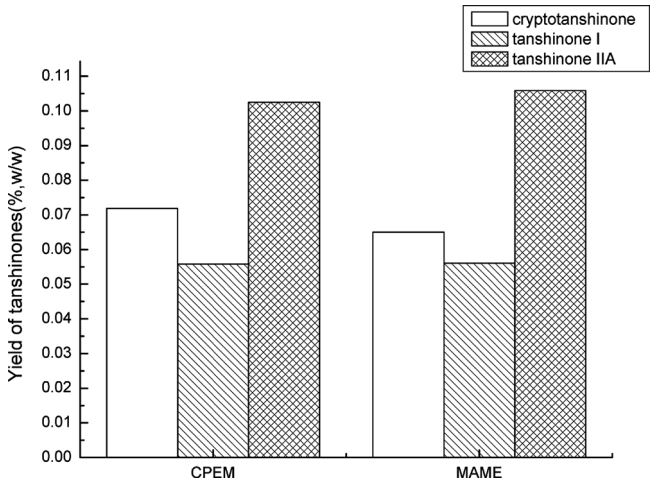

Figure 8. Comparison of extraction efficiency of tanshinones between Chinese pharmacopoeia extraction method (CPEM) and MAME (CPEM conditions: methanol 50 mL, *Salvia miltiorrhiza* Bunge: 0.125 g, heating reflux extraction: 1 h).

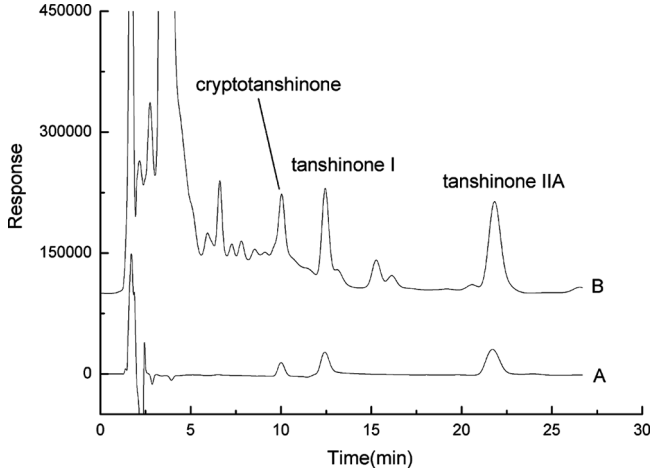

Figure 9. HPLC chromatograms of authentic tanshinones as well as extracts from *Salvia miltiorrhiza* Bunge. Chromatographic conditions: Diamonsil C_{18} (150 mm × 4.6 mm i.d., 5 μm) column, mobile phase: a mixture of methanol, tetrahydrofuran, water and glacial acetic acid (20:35:44:1, v/v/v/v), flow rate: 0.8 mL · min^{-1}, detection wavelength: 254 nm. (A) The chromatogram of standard solution of tanshinones; (B) The chromatogram of tanshinones extracted from *Salvia miltiorrhiza* Bunge by MAME.

Table 1. HPLC data for the calibration graphs and limit of detection

Compounds	Linear regression	Linear range ($\mu g \cdot mL^{-1}$)	R	LOD ($\mu g \cdot mL^{-1}$)
Cryptotanshinone	$Y = 3250.92786 + 30155.83837X$	$0.84 - 16.8$	1.00000	0.0566
Tanshinone I	$Y = 594.36776 + 72316.74809X$	$0.80 - 15.9$	0.99990	0.0327
Tanshinone IIA	$Y = 12083.71061 + 47753.05368X$	$1.68 - 33.6$	0.99997	0.0653

X denotes concentration ($\mu g \cdot mL^{-1}$) of the tanshinones, Y denotes peak area, $n = 5$.

genapol X-080 shows no absorption above 210 nm, it does not interfere with the determination of tanshinones.

Method Validation

All calibration graphs were plotted based on linear regression analysis of the integrated peak areas (Y) versus concentrations ($\mu g \cdot mL^{-1}$, X) of the tanshinones in the standard solution at five different concentrations (each concentration injected three times). The regression equations, correlation coefficients, and linear ranges for the analysis of the tanshinones are shown in Table 1. The limit of detection (LOD) was calculated as the amount of the injected sample, which gave a signal-to-noise ratio of 3. The LOD values of the method for the three components are also listed in Table 1.

The reproducibility test was carried out by treating three duplicate *Salvia miltiorrhiza* Bunge samples with the established method. The relative standard deviations (R.S.D.) of the integrated peak area are 1.16% for cryptotanshinone, 2.30% for tanshinone I, and 1.46% for tanshinone IIA, respectively.

CONCLUSIONS

In this paper, microwave–assisted micellar extraction was developed for the determination of tanshinones from *Salvia miltiorrhiza* Bunge by HPLC. Compared with conventional extraction techniques, the proposed MAME method obtained higher extraction efficiency with shorter extraction time. At the same time, compared with the method recommended in Chinese Pharmacopoeia Part I (2005 edition), MAME owned similar extraction efficiency especially for tanshinone I and tanshinone IIA, saving a lot of pretreatment time which is a more important influential

factor in routine analysis of herbal materials and pharmaceutical preparations. Additionally, this environmental friendly method employed nonionic surfactant solution as extracting agent, totally avoiding the use of toxic organic solvents.

ACKNOWLEDGMENTS

Financial support from the National Natural Science Foundation of China (20575016) and the Natural Science Foundation of Hebei Province China (B2006000953) are gratefully acknowledged. The authors also thank the financial support from 'the introduction of talents' programme of Hebei University (project no. 036).

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Received September 2, 2008
Accepted October 23, 2008
Manuscript 6413