Investigation on ultrasound-assisted extraction of salvianolic acid B from *Salvia miltiorrhiza* root

Juane Dong a,b,*, Yuanbai Liu c, Zongsuo Liang a,b, Weiling Wang a

a College of Life Sciences, Northwest A&F University, Yangling 712100, PR China
b Research Center of Soil and Water Conservation and Ecological Environment, Chinese Academy of Sciences and Ministry of Education, Yangling 712100, PR China
c College of Forestry, Northwest A&F University, Yangling 712100, PR China

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**A B S T R A C T**

It is reported that salvianolic acid B, a bioactive phenolic compound contained in the root of *Salvia miltiorrhiza*, exhibits a much stronger activity in free radical scavenging and antioxidance than those of vitamin E. When a conventional refluxing method is adopted to extract salvianolic acid B from the root, in which the materials are subjected to higher temperature and longer time, the yield of this phenolic compound is lower due to the possibility of its hydrolysis to tanshinol. However, a higher extraction yield can be achieved over a shorter time period and lower temperature when an ultrasound-assisted extraction method is used. This paper investigated the parameters influencing the extraction of salvianolic acid B. Factors such as extraction time, frequency of the ultrasound, the ratio of solvent to material, and types of extraction solvent were examined. A comparison was also conducted between conventional refluxing and ultrasound-assisted extraction. Results showed that the optimal parameters to extract salvianolic acid B from the root of *S. miltiorrhiza* were as follows: ultrasonic frequency: 45 Hz; solvent: 60% aqueous ethanol; extraction temperature: 30 °C; extraction time duration: 25 min.; ratio of solvent to material: 20:1 (v/w, ml/g). Under these conditions, the yield of salvianolic acid B was 5.17 mg/g (33.93 mg/g) higher than those with conventional refluxing method (28.76 mg/g), indicating that the efficiency and the yield of ultrasound-assisted extraction method are higher than reflux method, and the hydrolysis of salvianolic acid B to tanshinol is effectively avoided.

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1. Introduction

*Salvia miltiorrhiza* Bunge belongs to family Lamiaceae. Its root and rhizome have been traditional Chinese herbal drugs. The root of the plant is used for the treatment of thoracic-abdominal stinging syndrome, heat type arthralgia, ulcers, swelling and pain, insomnia, hepatosplenomegaly, and angina pectoris [1]. Phenolic compounds contained in the root of *S. miltiorrhiza*, such as salvianolic acid B, exhibits antithrombotic effect; it may prevent brain injury from cerebral ischemia-reperfusion [2]. Furthermore, salvianolic acid B also shows strong activities in free radical scavenging and antioxidance, stronger than those of vitamin E [3]. When a conventional refluxing method is adopted to extract salvianolic acid B from the root, in which the materials are subjected to higher temperature for a longer time, the yield of this phenolic compound is lower due to the possibility of its hydrolysis to tanshinol [4] (Fig. 1). Many investigations have reported that higher extraction yields could be achieved within shorter time period and/or lower temperature when an ultrasound-assisted extraction method was adopted on other plant species [5–11]. No reports were found on the extraction of salvianolic acid B by ultrasound-assisted method. This paper investigates the best technological parameters influencing the extraction of salvianolic acid B. Factors such as extraction time, frequency of the ultrasound, the ratio of solvent to material, and types of solvent were systematically examined. A comparison was also conducted between a conventional refluxing extraction method and ultrasound-assisted extraction.

Ultrasound-assisted extraction is a novel method to effectively extract chemical constituents from plant materials [5,6]. High-intensity shock waves generates intense pressures, shear forces and temperature gradient due to the bubble of cavitation inducing the majority of ultrasonic effects within a material, which can produce physical, chemical and mechanical effects [7], making the chemical constituents dissolve in the solvent without heating. Ultrasound can also facilitate the solvatation of plant materials by causing cell swelling and enlargement of the pores of the cell wall. Better swelling will improve the rate of mass transfer, and result in the increased extraction efficiency and/or reduced extraction time [8]. The main advantages of this new method are that the ultrasound
can increase the movement of the molecules enhancing the penetrability of the solvent and promoting a higher dissolution rate of plant constituents [9,10] in less time, which may decompose under higher temperatures used in reflux extraction, increasing the extraction yield [6,10,11]. This method has been applied in the extraction of many chemical constituents from different plant materials, such as tannins from myrobalan nuts [12], hesperidin from Citrus reticulata [13], chlorogenic acid from the leaves of Eucommia ulmoides [14,15], saikosaponins from Radix Bupleuri [16], saponins from ginseng [11], rutin from Euonymus alatus (Thunb.) Sieb [17], and carnosic acid from Rosmarinus officinalis [18], indicating that the method has potential in the extraction of thermal sensitive constituents used in foods, healthcare products, cosmetics, and pharmaceuticals.

In this study, we investigated the factors that influence the ultrasound-assisted extraction, such as extraction time, frequency of the ultrasound, the ratio of solvent to material, and types of solvent, to define optimal technological parameters for the extraction of salvianolic acid B from S. miltiorrhiza.

2. Experimental

2.1. Plant material

The roots of S. miltiorrhiza were collected in October 2006 from the bases for GAP medicinal material resources in Shangluo, Shaanxi Province. Samples were dried in vacuum oven (35 °C), then ground and sifted for homogenization (35 mesh) and stored in at −20 °C to avoid compound degradation. The extraction was accomplished within 1 month.

2.2. Reagents

All the solvents used were subjected to re-distillation and stored in brown bottles. Solvents used in the extraction, such as methanol, ethanol and n-butanol were all analytical reagent grade. Methanol used in the analysis of high performance liquid chromatography (HPLC) was purchased from TEDIA Company (Inc., 1000TEDIA WAY, FAIRFIELD, OH 45014, USA). Salvianolic acid B was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (No. 2 Tiantan Xili, Chongwen District, Beijing) with a purity of >99%.

2.3. Apparatus

The ultrasound-assisted extraction was carried out in an ultrasonic cleaner (Changzhou Nuoji Instrument Company, China; inner dimension: 230 mm × 140 mm × 130 mm) with an ultrasound power of 100 W, heating power of 800 W, and frequencies of 28 kHz, 45 kHz, and 100 kHz, equipped with digital timer and temperature controller.

2.4. Ultrasound-assisted extraction

A 0.5 g aliquot of the root powder of S. salvia was loaded into a 50 ml conical beaker. The selected solvent was added, and the beaker sealed. The extraction was conducted under certain conditions in the ultrasonic cleaner. After the extraction was finished, the extracted solution was clarified by filtration on a water pump, and was then concentrated in vacuo in a rotary evaporator below 40 °C. The concentrated extracts were diluted with 20 ml of distilled water and then acidified to pH 2. The obtained liquid was extracted with 10 ml of ethyl acetate by inversion mixing five times, allowing to stand for 10 min, and the supernatant collected. This extraction was repeated five times. The combined ethyl acetate extract supernatant was transferred into a flask and evaporated. Methanol was added to the volume of 10 ml and the solution was filtered through a millipore filter (0.45 μm). The filtrate was then analyzed by HPLC.

The parameters investigated include solvent types, such as water, methanol, ethanol and n-butanol, among them ethanol (diluted with water) was further examined with concentrations of 0%, 20%, 40%, 50%, 60%, 70%, 80% and 90%, the ratios of solvent to material, with the proportions of 5, 10, 20, and 30 (v/w, ml/g); extraction time with designed periods of 10, 15, 20, 25, 30, 35, and 40 min; extraction temperatures: 30, 50, and 60 °C; frequencies of ultrasound: 28, 45, and 100 kHz.

2.5. Conventional reflux extraction

A 0.5 g aliquot of accurately weighed root powder of S. salvia was loaded into a flask equipped with a water condenser tube. Aqueous ethanol with concentrations of 0%, 20%, 40%, 50%, 60%, 70%, 80%, and 90% was used as the extraction solvent, and the ratios of solvent to material were 5:1, 10:1, 20:1 and 30:1. The reflux extraction was conducted at temperatures of 40, 60, and 80 °C, and with the time periods of 10, 20, 40, 60, 80, 100 and 120 min. After the reflux extraction was finished, the same procedures were adopted as in the ultrasound-assisted extraction described in Section 2.4.

2.6. HPLC analysis

High performance liquid chromatograph (SHIMADZU, model SCL-10AVP), equipped with UV/Visible absorbance detector (SPD-10AVP) and infusion pump (LC-ATVP); quantitative HPLC analysis was conducted as the procedures described by Cheng [19], i.e., column: shim-pack VP-ODS (150 mm × 4.6 mm i.d. 5 μm); mobile phase: methanol-2% glacial acetic acid (40:60, v/v); flow rate: 1 ml/min; amount of injection: 5 μl; wavelength of detection: 281 nm; column temperature: 30 °C. The extraction yield of salvianolic acid B was calculated based on the integration of the chromatographic peak areas.

2.7. Data analysis

Statistical significance of extraction yields of salvianolic acid B under different extraction conditions was carried out by SAS software (SAS Institute, Cary, NC, USA).

3. Results and discussion

3.1. Extraction variables

The extraction follows the principle of “like dissolves like”. Low-polarity solvent yields more lipophilic components, while alcoholic solvent gives a larger spectrum of apolar and polar compounds [20]. In this study, water, methanol, ethanol and n-butanol were tested to extract salvianolic acid B from the roots of S. miltiorrhiza under sonication.

Fig. 2 shows the effects of different solvents on the extraction yield of salvianolic acid B from the roots powder of S. miltiorrhiza.
The extraction time was 30 min, extraction temperature: 30, 50, and 60 °C, ultrasound frequency: 45 kHz, and the ratio of solvent to material was 20:1. Four different solvents exhibited different effects on the extraction yield under same extraction conditions. Because salvianolic acid B is a polar compound solvents with high polarities such as methanol and ethanol are better for the extraction. n-butanol, with lower polarity than methanol and ethanol, exhibited a lower extraction yield. This result indicates that solvents show different extraction results on the objective constituents due to polarity differences between them.

Fig. 2 shows that the extraction in 50% aqueous ethanol is better than in pure ethanol. The effects of different concentrations of ethanol on the extraction were further examined. Fig. 3 shows the effects of different concentrations of aqueous ethanol on the extraction yield of salvianolic acid B. Other extraction conditions were fixed as: extraction time: 30 min, temperature: 30 °C, ultrasound frequency: 45 kHz, ratio of solvent to material 20:1. A 60% aqueous ethanol solution showed the highest extraction efficiency and was chosen as the optimal solvent for the following extraction experiments.

Solvent quantity is an important factor in the process of conventional reflux extraction. In general, a larger solvent volume can dissolve constituents more effectively, leading to an enhancement of the extraction yield [14]. However, this will lead to excess work in the concentration process, causing the waste of solvent. On the other hand, lower levels of solvent will result in the lower yield of the objective constituents and is not suitable for the ultrasound-assisted extraction [9]. The influence of the ratio of solvent to material on the extraction yield of salvianolic acid B in the roots of S. miltiorrhiza was evaluated.

Fig. 4 shows the effects of the ratio of solvent to material on the extraction yield of salvianolic acid B. The solvent used was 60% aqueous ethanol, time of extraction was 30 min, extraction temperatures were 30, 50, and 60 °C, and the frequency of the ultrasound was 45 kHz. It can be found that under the fixed conditions of other factors, such as extraction time, ultrasound frequency, and solvent, an increase of extraction efficiency of salvianolic acid B could be observed with the increase of the ratio of solvent volume to sample weight, especially when this ratio increased from ranges of 5:1 to 20:1. The ratio of solvent to sample of 20 was chosen in the following extraction experiments.

Time duration is another important factor in extraction procedures. Before the establishment of the equilibrium between the objective constituents in and out the plant cells, the yield of extraction increases with time. However, it will not increase with time after the establishment of the equilibrium [21]. In order to further understand the influence of extraction time on the extraction yield, different times were tested from 10 to 40 min. The other parameters were kept constant.

Fig. 5 shows the extraction results carried out under different time durations with fixed conditions of other factors, such as temperatures (30, 50, and 60 °C), ultrasound frequency (45 kHz), solvent (60% aqueous ethanol) and the ratio of solvent volume to material weight (20:1). The results showed that the dissolution...
of salvianolic acid B increased with increasing extraction time durations. The yield of extraction increased quickly within the initial 25 min, and reached the optimal extraction result. After 25 min, the extraction yield increased slowly under the temperature of 30 °C. Under the temperatures of 50–60 °C, it decreased along with time. These results can be explained as, on the one hand, salvianolic acid B is a thermal labile compound, and decomposed into tanshinol under higher temperature. On the other hand, the principle of ultrasound-assisted extraction is that ultrasound can induce special functions, such as acoustic cavitation, and rupture of plant cells. The cavitation will cause vacuum bubbles in the solvent within the plant cells. The rupture of the bubbles will promote the interpenetration of the solvent into the plant cells to dissolve objective constituents there [7]. Along with the increase of extraction time, all the plant cells will be completely cracked by acoustic cavitation effect, and extraction yield increases within a certain time duration. As the plant cells rupture, impurities such as insoluble substances, as well as cytosol and lipids suspend in the extraction liquid, resulting in the lower permeability of the solvent [16]. Dissolved constituents will also re-adsorb on the smashed plant particles due to their relatively large specific surface areas lowering yields of recovered compounds. Therefore, longer time of extraction is unnecessary after the maximum extraction yield is achieved. Hence, 25 min is suitable time duration for the extraction of salvianolic acid B in the roots of *S. miltiorrhiza*.

Temperature is also an important factor in the extraction of heat sensitive compounds. Along with the increase of temperature, the solvent viscosity and diffusion rate, as well as the mass transfer increase, result in the increase of dissolution of objective constituents. Meanwhile, the tendency of the dissolution of impurities will also increase, and some thermal labile constituents, such as salvianolic acid B will decompose [21,22]. In a short time duration, such as 10 min, extraction yields increased along with temperature. While in longer time durations, such as 30–40 min, extraction yields tended to decrease along with the increase of temperature (Fig. 5). As mentioned above, higher temperature will cause decomposition of salvianolic acid B into tanshinol. Therefore, the preferable temperature for extracting salvianolic acid B is 30 °C.

### 3.2. Effects of ultrasound frequency on the extraction yield of salvianolic acid B

Fig. 6 shows the extraction yields of salvianolic acid B under different ultrasound frequencies with different time durations. Variables were designed as follows: frequency, 28, 45, and 100 kHz; solvent 60% aqueous ethanol; extraction temperature 30 °C; ratio of solvent volume to material 20:1. Within the time duration of 10–40 min, the extraction yield under higher frequency (100 kHz) was lower than those under lower frequencies (28 and 45 kHz). This result conformed to the result of the extraction of hesperidins [13]. This result demonstrates that ultrasound with high frequency can soften the middle lamella in vegetal tissues, which becomes more malleable. On the other hand, the low frequency one vibration has a strong mechanical effect on the middle lamella and all cellular structures leading to an enhanced milling process [10], which promotes the dissolution of objective constituents into the solvent. In this study, 45 kHz was found to be the most suitable frequency for the ultrasound-assisted extraction of salvianolic acid B from the root powder of *S. miltiorrhiza*.

Considering the above results, the optimum experimental conditions for the ultrasound-assisted extraction of salvianolic acid B were as follows: solvent (60% aqueous ethanol); ratio of solvent volume to sample weight (20:1); an extraction time duration of 25 min; an extraction temperature of 30 °C and an ultrasound frequency of 45 kHz.

### 3.3. Comparison of the results between ultrasound-assisted extraction and conventional reflux extraction

In order to make the comparison, optimal conditions of reflux extraction were examined (Figs. 7 and 8). Aqueous ethanol with different concentrations was used as solvent to carry out the reflux extraction under different ratios of material to solvent (Fig. 7). Experimental results showed that the optimal extraction solvent was 60% aqueous ethanol and the ratio of solvent to material was 20:1. Under these conditions, the extraction yields under different temperature with different time durations were showed in Fig. 8. At higher extraction temperature (80 °C), the yield of salvianolic acid B increased to its maximum within short period of time duration (60 min), then decreased with time. The reason is that when the temperature is higher, the reflux rate of the solvent is increased. However, salvianolic acid B will decompose at higher temperature, resulting in the decrease of extraction yield [4] (Fig. 1). On the other hand, at lower temperature such as 30–60 °C, the yield of extraction increased with extraction time, however, the time duration was long (over 100 min) to reach the maximum yield. The optimal conditions of reflux extraction were as follows: the extraction solvent, 60% aqueous ethanol; the ratio of solvent to material 20:1, and the temperature, 80 °C with the time duration of 60 min.

![Graph showing the effects of ultrasound frequency on the extraction yield of salvianolic acid B](image)

**Fig. 6.** Effects of ultrasound frequency on the extraction yield of salvianolic acid B. Other conditions were fixed at: sonication time, 25 min; solvent, 60% aqueous ethanol; the ratio of the volume of solvent extractor to sample mass, 20:1; extraction temperature, 30 °C.

![Graph showing the effects of the ratio of solvent to sample mass on the extraction yield](image)

**Fig. 7.** Effects of the ratio of solvent to sample mass (v/w, ml/g) and the concentrations of aqueous ethanol on the extraction yield of salvianolic acid B by the conventional reflux extraction. Other conditions were fixed at: extraction temperature, 80 °C; extraction time, 60 min.
To understand the advantages of ultrasound-assisted extraction, the method was compared with the conventional reflux extraction method (Fig. 8). The extraction conditions for the ultrasonic extraction were: solvent, 60% aqueous ethanol; the ratio of solvent to sample mass, 20:1; for ultrasonic extraction: sonication temperature, 30 °C; frequency, 45 kHz; solvent, 60% aqueous ethanol, and the ratio of solvent to sample mass, 20:1.

Higher yield of extraction can be achieved under lower temperature with shorter period of time when ultrasound-assisted extraction method is adopted in the extraction of salvianolic acid B from the root of *S. miltiorrhiza*. Solvent, temperature, and time duration are important factors influencing the extraction. The influence of solvent mainly manifests on the polarity. Ethanol is a suitable solvent to extract salvianolic acid B among the solvents tested due to its high polarity. Higher temperature is not suitable to the extraction of salvianolic acid B. Its extraction yield is lower at higher temperature due to its thermal labile property. The yield did not increase with the extraction time after 30 min.

The extraction yield increased with the amount of solvent used. However, no significant difference was found between the solvent volumes to sample weight ratios above 20:1. Therefore, the suitable ratio of solvent to material for extracting salvianolic acid B is 20:1. Within the range tested, the yield of extraction with the ultrasonic frequency of 45 kHz was higher than those with 28 and 100 kHz. The experiment suggests that the optimal conditions for the extraction of salvianolic acid B are: frequency of the ultrasonic: 45 Hz; solvent: 60% aqueous ethanol; extraction temperature: 30 °C; extraction time duration: 25 min.; ratio of solvent to material: 20:1 (v/w, ml/g). Under these conditions, the yield of salvianolic acid B was 5.17 mg/ml (33.93 mg/g) higher than that with conventional reflux method (28.76 mg/g), indicating that the efficiency and the yield of ultrasound-assisted extraction method are higher than conventional one and the hydrolysis of salvianolic acid B to tanshinol is effectively avoided.

4. Conclusions

This study shows that an ultrasound-assisted extraction can be easily carried out within a short time period and possesses advantages such as low heating and higher yield efficiency. It is especially suited for extractions of thermally labile compounds such as salvianolic acid B. Both the extraction yield and efficiency of the ultrasonic method are higher than those of a conventional reflux extraction.

**References**