Evaluation of Pressurized Liquid Extraction and Pressurized Hot Water Extraction for Tanshinone I and IIA in *Salvia miltiorrhiza* Using LC and LC–ESI-MS

Eng Shi Ong* and **Shea Mei Len**
Centre for Analytical Science, Health Sciences Authority, 11 Outram Road, Singapore 169078, Republic of Singapore

Abstract

Pressurized liquid extraction (PLE) and pressurized hot water extraction (PHWE) using a laboratory-made system are applied for the extraction of thermally labile components such as tanshinone I and IIA in *Salvia miltiorrhiza*. PLE and PHWE are carried out dynamically at a flow of 1 mL/min, temperature between 95–140°C, applied pressure of 10–20 bars, and extraction times of 20 and 40 min, respectively. Effects of ethanol added into the water used in PHWE are explored. PLE is found to give comparable or higher extraction efficiencies compared with PHWE with reference to Soxhlet extraction for tanshinone I and IIA in *Salvia miltiorrhiza*. The tanshinone I and IIA present in the various medicinal plant extracts are determined by liquid chromatography and liquid chromatography–mass spectrometry.

Introduction

Dan-shen, the dried roots of *Salvia miltiorrhiza*, has been commonly used in traditional Chinese medicine for promoting blood circulation to remove blood stasis, clearing away heat, relieving vexation, nourishing blood and tranquillizing the mind, cooling the blood to relieve carbuncles treating hemorrhages, menstrual disorders, and miscarriages. The major active constitutes of this herb are tanshinones, including tanshinone IIA, cryptotanshinones, dihydrotanshinone I, and tanshinone I (Figure 1) (1,2).

Studies on the chemical composition of Dan-shen have been extensively reported in the literature. Modern studies in recent years have confirmed many of its traditional properties and also uncovered some additional properties including anticoagulant and antibacterial activities and beneficial effects in patients with chronic renal failure. Components in Dan-shen can be grouped into two major classes: lipid and water soluble. The lipid solubles, normally obtained by extraction with alcohol solvents, are rich in abietanoids and diterpene quinone pigments. The three representative bioactive components in this fraction are tanshinone I, tanshinone IIA, and cryptotanshinone. The major active ingredients in the water solubles include many plant phenolic acids, which are mostly caffeic acid derivatives. Monographs on *Salvia miltiorrhiza* can be found in the Chinese Pharmacopoeia (1–3).

For botanical drugs and herbal preparations, there is a need to approach scientific proof and clinical validation with chemical standardization, biological assays, animal models, and clinical trials. Quality assurance of botanical drugs and herbal preparations are the prerequisites of credible clinical trials. In recent studies, high-speed counter-current chromatography (HSCCC) was successfully used for isolation and purification of tanshinones from *Salvia miltiorrhiza* using stepwise elution (4–6).

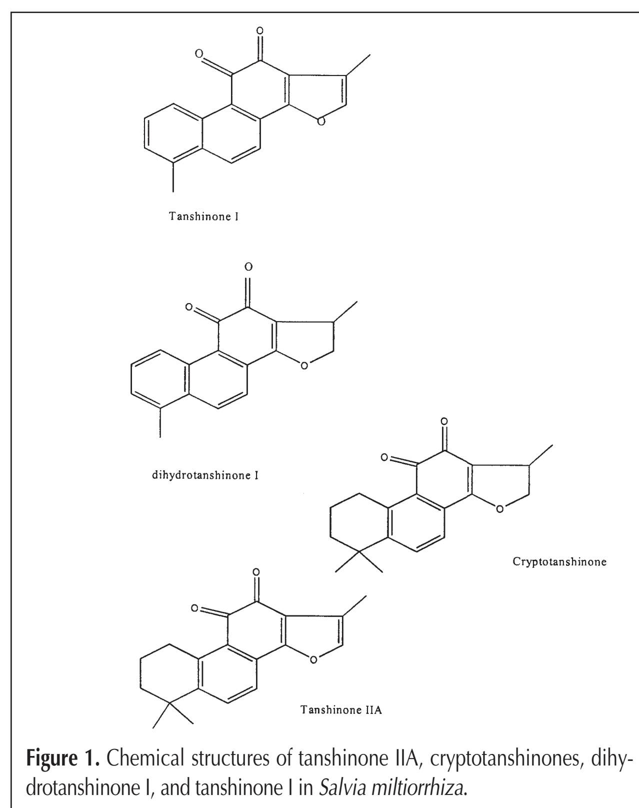

Figure 1. Chemical structures of tanshinone IIA, cryptotanshinones, dihydrotanshinone I, and tanshinone I in *Salvia miltiorrhiza*.

* Author to whom correspondence should be addressed: Eng Shi Ong, Applied Science School, Temasek Polytechnic, 21 Tampines Avenue 1, Singapore 529757. Email: ong_eng_shi@hsa.gov.sg.

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Supercritical carbon dioxide with and without a methanol modifier has been compared with a new portable cold extraction technique, phytosol solvent extraction (PSE), for the extraction of tanshinone IIA from *Salvia miltiorrhiza* (7). A novel microwave-assisted extraction (MAE) method had been developed for the extraction and determination of tanshinones (tanshinone IIA, cryptotanshinone, and tanshinone I) from the root of *Salvia miltiorrhiza* with analysis by high-performance liquid chromatography (HPLC) (8,9).

In the interest of reducing the usage of organic solvent in analytical methods, subcritical water extraction had been developed for the extraction of organic pollutants from environmental solids (10–12). An approach using static–dynamic subcritical water extraction of essential oil components from plant materials was developed (13,14). Subcritical water under pressure and between 125–175°C had been used to rapidly extract oxygenated fragrance and flavor compounds from rosemary (15). A laboratory-made system using subcritical water was developed for the extraction of iridoid glycosides in plant matrix with final determination by micellar electrokinetic capillary chromatography (16). Subcritical water between 100–175°C with an applied pressure of 50 bar was used for the extraction of lactones from a kava root with final determination by gas chromatography (GC) (17). In our laboratory, pressurized hot water extraction (PHWE) using a laboratory-made system was applied for the extraction of thermally labile and reasonably polar components such as berberine in *Coptidis rhizoma*, glycyrrhizin in *Radix glycyrrhizae* (liquorice), and baicalein in *Scutellariae radix* (22).

The aim of this work was to develop methods for the rapid analysis of active or marker compounds in medicinal plants using PLE and PHWE with *Salvia miltiorrhiza* as the test sample. As reference substances for the proposed botanical were not readily available, a method was developed to isolate the marker compounds such as tanshinone I and tanshinone IIA in *Salvia miltiorrhiza*. The extract was analyzed by gradient elution HPLC and liquid chromatography–electrospray ionization-mass spectrometry (LC–ESI-MS). The extraction efficiencies of the proposed methods using PLE and PHWE were compared with Soxhlet extraction.

Experimental

Materials and reagents

Ethanol, diethyl ether, and methanol of analytical-reagent and HPLC grade were purchased from APS (Chipping Norton, NSW, Australia). Hexane was purchased from EM Science (Gibbstown, NJ). Propan-1-ol was purchased from AJAX Chemicals (Seven Hills, NSW, Australia). Sand purified by acid (~ 40–100 mesh) was purchased from BDH Chemical (Poole, England). Pure water was obtained from a Millipore Alpha-Q water system (Bedford, MA).

Preparation of medicinal plant

To prepare a homogenous sample, different batches of the root samples of *Salvia miltiorrhiza* were ground using an IKA MF10 microfine grinder (Staufen, Germany) with a sieve insert of hole size 0.5 mm.

For extraction using PHWE, 0.2 g *Salvia miltiorrhiza* sample was weighted directly into a glass tube and mixed thoroughly with a high proportion of sand. The sand and plant material mixture was transferred into the extraction cell for PHWE, as mentioned in the following. The extraction cells were finally filled with sand to avoid any voids. One of the problems encountered using PHWE was that plant materials had a strong tendency to adsorb water in the course of extraction. Hence, the ground plant materials must be dispensed evenly with sand to prevent any blockage of the system. However, this step was not required for PLE.

For extraction by PLE, 0.2 g sample was weighed directly into the extraction cell as mentioned in the PLE instrumentation. For all experiments, the sample powder was sandwiched between the sand. The extraction cells were finally filled with sand to avoid any voids.

Isolation of marker compounds

The active compounds were isolated from *Salvia miltiorrhiza* using Soxhlet extraction with methanol as the solvent. The amount of methanol in the extract was reduced to one tenth of its original volume, and an equal volume of water was added. The aqueous portion was extracted three times with hexane, diethyl ether, and propan-1-ol, respectively. The fraction was evaporated to dryness and redissolved in 10–15 mL methanol–water (70:30). The methanol–water portion was prefractioned on a LiChroprep RP-18 reversed-phase column (240- × 10-mm i.d., 40–63 µm) (Merck, Darmstadt, Germany) using methanol–water (70:30) as the mobile phase. Fractions were collected every 5 min. After 16 fractions, the mobile phase was changed to 100% methanol, and a further 10–16 fractions were collected.

The target fraction was finally subjected to semipreparative HPLC with a Shimadzu LC 10 series (Kyoto, Japan) equipped with a binary gradient pump, autosampler, column oven, diode array detector (DAD), and fraction collector. The gradient elution consisted of a mobile phase of (A) water and (B) methanol. The initial condition was set at 30% B and then gradient up to 100% B in 15 min before returning to the initial condition for 10 min. Detection was at 254 nm. The oven temperature was set at 40°C, and the flow rate was set at 2.0 mL/min. For all experiments, 100 µL sample extract was injected. The column used for separation was a Hypersil C_{18} (250- × 10.0-mm i.d., 10 µm) (Runcorn, Cheshire, England). The purity of the marker compounds isolated was determined by analytical HPLC using the conditions described following.

PLE/PHWE system

The instrumentation used for PLE was custom made, and details were given in earlier reports (18–22). Briefly, the stainless steel tubings used were 0.16-inch o.d. and 0.18-mm i.d. The back pressure was generated using a back pressure regulator by VICI Jour Research (Onsala, Sweden). The stainless steel extraction cells measured 100- and 10-mm i.d. (7.85 mL). The extraction cell was heated in an HP5890 gas chromatograph oven (Hewlett-Packard, Palo Alto, CA). The pump used was a Shimadzu LC 10AT pump. The pump flow was set at 1.0 mL/min, and the oven temperature was set at 120°C. The pressure in the system indicated by the HPLC pump was between 10–20 bars. The extraction cell was prefilled with methanol to check for possible leakage before setting the temperature of the oven to the required value. For PLE,

extraction with methanol was carried out for a period of 20 min, and 20–25 mL solvent was collected in a 25-mL volumetric flask. In between runs, the system was washed with methanol for 5 min. All extracts were filtered through a 0.45-µm membrane filter (Whatman, Kent, U.K.) before analysis by HPLC.

The instrumentation used for PHWE was the same as that used for PLE. For PHWE, a different percentage of ethanol–water was used as the extraction liquid. The pump flow was set at 1.0 mL/min, and the oven temperature was set at 80°C. The pressure in the system indicated by the HPLC pump was between 10–20 bars. The extraction cell was prefilled with methanol to check for possible leakage before setting the temperature of the oven to the required value. Extraction with ethanol–water was carried out for a period of 40 min, and 40–45 mL of liquid was collected. In between runs, the system was washed with the extraction liquid for 5 min.

For PHWE, the aqueous extract obtained was cleaned up on a 1000-mg Strata (Phenomenex, Torrence, CA) C_{18} SPE column conditioned with 10 mL of methanol and water, respectively. The loaded solid-phase extraction columns were washed with 5 mL water before eluting with 10 mL methanol. Excess solvent was evaporated under a stream of nitrogen. The sample extracts were diluted to 10 mL before final analysis by HPLC. All extracts were filtered through a 0.45-µm membrane filter before analysis by HPLC.

HPLC conditions

For all experiments, a Shimadzu LC 10 series equipped with a binary gradient pump, autosampler, column oven, and DAD was used. The gradient elution used a mobile phase consisting of (A) water and (B) methanol. The initial condition was set at 20% B, gradient up to 100% in 15 min, and then returned to the initial condition for 12 min. The detection was at 254 nm. The oven temperature was set at 40°C, and the flow rate was set at 1.0 mL/min. For all experiments, 20 µL standard and sample extract were injected. The column used for the separation was a reversed-phase C_{18} Luna (150 × 4.6 mm, 5 µm) (Phenomenex).

For LC–MS, a Shimadzu LC 10 series equipped with a binary gradient pump, autosampler, column oven, and DAD was coupled with a Shimadzu LCMS-2010A quadruple mass spectrometer. The gradient elution used a mobile phase consisting of (A) water and (B) methanol. The initial condition was set at 20% B, gradient up to 100% in 15 min, and then returned to the initial condition for 6 min. The detection was at 254 nm. The oven temperature was set at 40°C, and the flow rate was set at 0.2 mL/min. For all experiments, 20 µL standard and sample extract were injected. The column used for the separation was a reversed-phase C_{18} Luna (150 × 2.0 mm, 5 µm). The ESI-MS was acquired in the positive ion mode. The probe voltage was set at 4.5 kV and the curve desolvation line temperature at 230°C. The scanning mass range was 250–700.

Soxhlet extraction

A 2-g sample was weighed into the thimble. Ethanol–water (95:5) was selected as the solvent, based on earlier reports (8,9). The coextract gave an orange-red color with the extraction solvent. The red color turned lighter and lighter through the course of the extraction. Hence, after extraction with 120–150 mL ethanol–water (95:5) for 4–5 h, the extraction solvent was essentially colorless. The excess solvent was evaporated with the rotary evaporator and filtered through Whatman No. 1 filter paper into a 50-mL volumetric flask.

Preparation of reference standards

For tanshinones I and IIA, the working solutions were prepared in the range 0–60 mg/L in methanol. Linearity of tanshinones I and IIA was established between 0–60 mg/L, with a correlation coefficient $r^2 > 0.99$. For the quantitation of marker compounds, a three-point calibration based on the linearity established was used. The system precision [relative standard deviation (RSD), $n = 6$) for tanshinones I and IIA was found to be less than 2% on different days using the lowest concentration working standard prepared.

Results and Discussion

Isolation and characterization of compounds from *Salvia miltiorrhiza*

In *Salvia miltiorrhiza*, the marker compounds were reported to be tanshinone I, tanshinone IIA, and cryptotanshinone. Isolation and characterization of compounds in *Salvia miltiorrhiza* had been well reported (23–26). Preparative isolation and purification of components from *Salvia miltiorrhiza* by CCC had been reported (4–6). Here, we reported a method for the isolation of tanshinone I and tanshinone IIA from *Salvia miltiorrhiza* using semipreparative HPLC.

Tanshinone I had been reported in independent works to elute earlier than tanshinone IIA using different C_{18} reversed-phase HPLC columns with methanol–acetonitrile–water as the mobile phase (4,6,8). The ESI-MS detection of active ingredients in Salvia miltiorrhiza in the ethanol extract was found to give unique $[M+H]^+$ and $[M+Na]^+$ masses for the marker compounds present. ESI-MS was reported to be able to detect the presence of the prominent marker compounds in *Salvia miltiorrhiza* without column separation. It was reported that tanshinones I was observed to give $[M+H]^+$ and $[M+Na]^+$ at 277.1 and 299.1, respectively. For tanshinones IIA, $[M+H]^+$ and $[M+Na]^+$ were observed at 295.1 and 317.1 (28).

In our studies, the mass spectra from the ESI-MS for tanshinone I and IIA isolated from *Salvia miltiorrhiza* are showed in Figure 2A and 2B. Our data showed that $[M+Na]^+$ was observed to be the prominent peak. Tanshinones I and IIA were observed to give $[M+Na]^+$ at 299.0 and 317.0, respectively. By lowering the ESI probe voltage to 2 kV, more ions such as $[M+H]^+$, $[M+Na]^+$, and dimmers of $[M+M+Na]^+$ (Figure 2C and 2D) were observed for tanshinone I and IIA, respectively. It was observed that the ions for $[M+H]^+$ and $[M+Na]^+$ obtained for tanshinone I and IIA were in close agreement with another report (28). With a single-quadruple mass spectrometer, it was found that characteristic ions for tanshinone I and IIA were produced by varying the probe voltage. Hence, tanshinone I and IIA isolated from *Salvia miltiorrhiza* were identified by comparing the elution order by reversed-phase HPLC, UV spectra, and mass spectra from ESI-MS obtained.

PLE and PHWE of compounds in *Salvia miltiorrhiza*

For PLE, the parameters that had a significant effect on the extraction efficiencies of marker compounds in botanicals and herbal preparations were the applied temperature and solvent used. The time for extraction was set at 20 min, as it was found that a significant portion of the target analytes would be extracted. The pressure was reported to have little effect on the extraction efficiency, as it was applied to keep the solvent in the liquid phase (18–22,27). Methanol was selected as the solvent in PLE, as methanol and ethanol were commonly used as the extraction solvent in other reports (4,8,24,25).

The effects of temperature from 80–140°C on the extraction efficiencies of tanshinone I and IIA from *Salvia miltiorrhiza* by PLE are shown in Figure 3. The data obtained shows that there was a slight increase in the extraction efficiencies in the range of temperatures studied, and tanshinone I and IIA were rather stable at temperatures up to 140°C. Hence, 120°C was selected as the optimum temperature for the extraction of tanshinone I and tanshinone IIA from *Salvia miltiorrhiza*.

Our earlier works show that comparable or higher extraction efficiencies were obtained using the proposed PLE system for berberine, aristolochic acids, strychnine, ginsenosides, glycyrrhizin, and baicalein in medicinal plants or herbal preparations with reference to sonication or Soxhlet extraction (18–22,27). For tanshinone I and tanshinone IIA from *Salvia miltiorrhiza*, the data in Table I show that the extraction efficiencies of PLE in 20 min were comparable or higher to those obtained by Soxhlet extraction in 3–4 h. The method precision was found to vary from 1.4–6.3% (n = 6) for different batches of medicinal plants on different days. The comparable performance of PLE with reference to Soxhlet extraction was because of the higher diffusion rate and solubility of analytes in the solvent as a result of the higher temperature.

To reduce the use of organic solvent, PHWE was used for the extraction of thermally labile components such as baicalein in *Scutellariae radix*, berberine in *Coptidis rhizoma*, and glycyrrhizin in *Radix glycyrrhizae* (liquorice). PHWE was observed

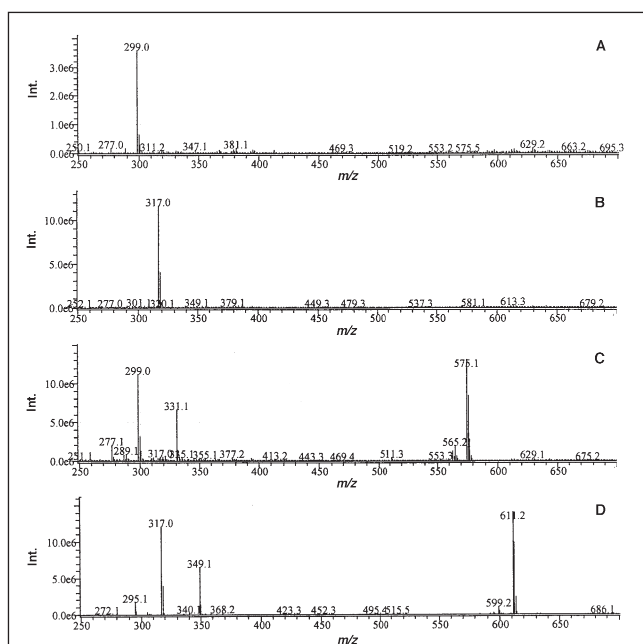

Figure 2. ESI-MS spectra of tanshinone I and IIA with the probe voltage set at 4.5 kV (A and B) and ESI-MS spectra of tanshinone I and IIA with the probe voltage set at 2.0 kV (C and D).

Table I. Comparison of Amount of Tanshinones I and IIA in *Salvia miltiorrhiza* by PLE at 120°C with Soxhlet Extraction

	Tanshinone I (mg/g)	Tanshinone IIA (mg/g)
Amount extracted from *Salvia miltiorrhiza* I by PLE	2.36 ± 0.15 (n = 6, RSD = 6.3%)	5.84 ± 0.36 (n = 6, RSD = 6.2%)
Amount extracted from *Salvia miltiorrhiza* I by Soxhlet extraction	2.24 (n = 2)	5.70 (n = 2)
Amount extracted from *Salvia miltiorrhiza* II by PLE	3.26 ± 0.20 (n = 6, RSD = 6.2%)	8.13 ± 0.42 (n = 6, RSD = 5.1%)
Amount extracted from *Salvia miltiorrhiza* II by Soxhlet extraction	3.23 (n = 2)	8.04 (n = 2)
Amount extracted from *Salvia miltiorrhiza* III by PLE	3.99 ± 0.99 (n = 6, RSD = 2.2%)	9.12 ± 0.13 (n = 6, RSD = 1.4%)
Amount extracted from *Salvia miltiorrhiza* III by Soxhlet extraction	3.85 (n = 2)	8.56 (n = 2)

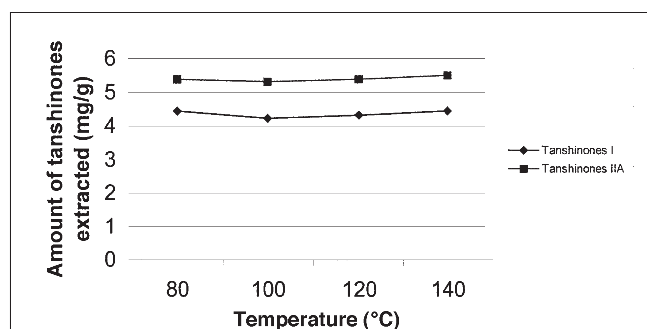

Figure 3. Effects of temperature on the amount of tanshinones I and IIA extracted from medicinal plants by PLE. PLE conditions: flow, 1 mL/min; time for extraction, 20–25 min; and the solvent, methanol (n = 2).

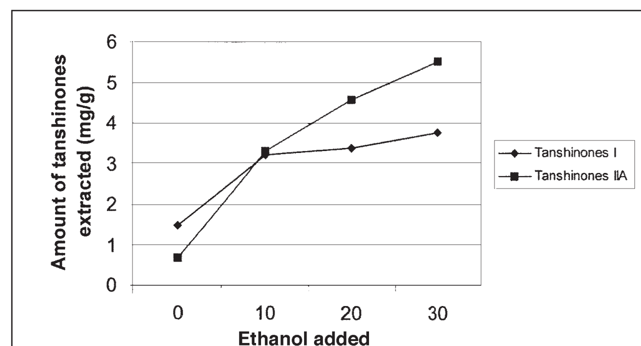

Figure 4. Effects of ethanol added in the water on the amount of tanshinones I and IIA extracted from medicinal plant by PHWE at 95°C. PHWE conditions: flow, 1 mL/min and time for extraction, 40 min (replicates, n = 2).

to give a similar chromatographic profile as compared with other methods of extraction using organic solvent (22). The parameters that had a significant effect on the extraction efficiencies of marker compounds in botanicals using PHWE were the applied temperature and percentage of organic modifiers (10,12,22). The nonsignificant effect of the pressure was well reported (10,12). The time for extraction was set at 40 min, as it was observed to give extraction efficiencies comparable to sonication and Soxhlet extraction (22).

Table II. Comparison of Amount of Tanshinones I and IIA in *Salvia miltiorrhiza* by PHWE at 95°C with 30% Ethanol as Modifier with Soxhlet Extraction

	Tanshinone I (mg/g)	Tanshinone IIA (mg/g)
Amount extracted from *Salvia miltiorrhiza* I by PHWE	2.31 ± 0.21 (*n* = 6, RSD = 9.0%)	6.09 ± 0.28 (*n* = 6, RSD = 4.6%)
Amount extracted from *Salvia miltiorrhiza* I by Soxhlet extraction	2.49 (*n* = 2)	7.20 (*n* = 2)
Amount extracted from *Salvia miltiorrhiza* II by PHWE	3.05 ± 0.18 (*n* = 6, RSD = 6.1%)	7.44 ± 0.42 (*n* = 6, RSD = 6.3%)
Amount extracted from *Salvia miltiorrhiza* II by Soxhlet extraction	2.70 (*n* = 2)	7.68 (*n* = 2)
Amount extracted from *Salvia miltiorrhiza* III by PHWE	2.79 ± 0.07 (*n* = 6, RSD = 2.4%)	6.74 ± 0.21 (*n* = 6, RSD = 3.1%)
Amount extracted from *Salvia miltiorrhiza* III by Soxhlet extraction	3.18 (*n* = 2)	7.88 (*n* = 2)

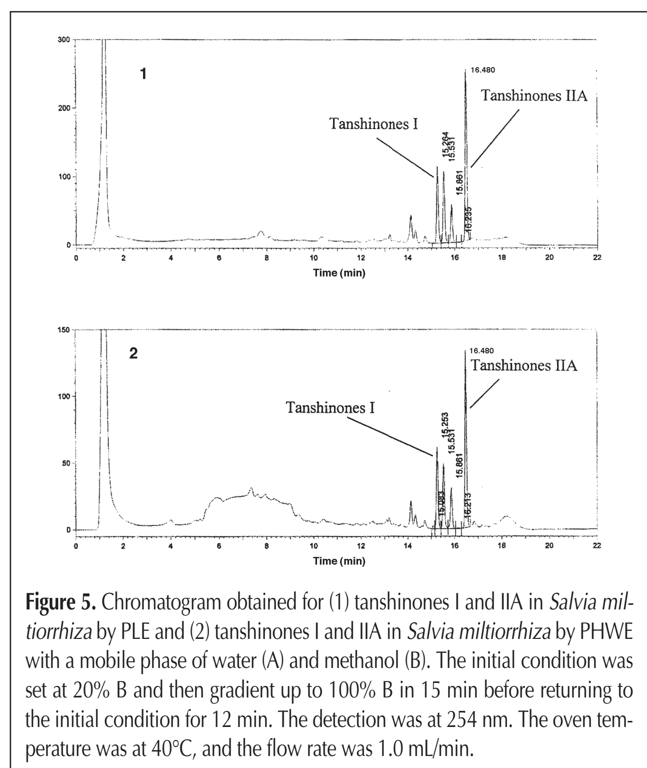

Figure 5. Chromatogram obtained for (1) tanshinones I and IIA in *Salvia miltiorrhiza* by PLE and (2) tanshinones I and IIA in *Salvia miltiorrhiza* by PHWE with a mobile phase of water (A) and methanol (B). The initial condition was set at 20% B and then gradient up to 100% B in 15 min before returning to the initial condition for 12 min. The detection was at 254 nm. The oven temperature was at 40°C, and the flow rate was 1.0 mL/min.

The effects of ethanol (0–30%) at an applied temperature of 95ºC are shown in Figure 4. The data in Figure 4 show that the extraction efficiencies of tanshinone I and IIA in *Salvia miltiorrhiza* increased with the increasing percentage of ethanol added in the extraction liquid. Hence, 30% ethanol was added in the extraction liquid. However, increasing the applied temperature from 80ºC to 140ºC in PHWE did not affect the extraction efficiency of tanshinone I and IIA. It was observed that above 120ºC, the coextracts often resulted in blockage of the PHWE system. The objective of the present work was to study the extraction efficiencies of marker compounds from plant materials using a temperature below the boiling point of the liquid used. Hence, for the extraction of tanshinone I and IIA in botanicals, a temperature of 95ºC with 30% ethanol added were selected for comparison studies.

The extraction efficiencies of tanshinone I in different batches of *Salvia miltiorrhiza* by PHWE were found to be comparable to Soxhlet extraction, as shown in Table II. For tanshinone IIA, the amount of target analytes extracted by PHWE was observed to be lower than Soxhlet extraction. It was suggested that PHWE modified with 30% ethanol was not as efficient as PLE using organic solvent to extract naturally occurring and reasonably more hydrophobic substances such as tanshinone IIA in botanicals. The method precision (RSD) was found to vary between 2.4–9.0%. With the premixing step with sand that was required for the sample preparation in PHWE, the method precision observed was comparable to that obtained in PLE.

Conclusion

The continuing drive to optimize health in today's society has created a huge market for dietary supplements for which there is a growing need to validate methods of analysis in botanicals and herbal preparation (29). Hence, methods that are simple, rapid, and reduce the use of organic solvent are preferred. The study showed that thermally labile components such as tanshinone I and IIA in medicinal plants could be extracted using PLE and PHWE at a temperature below the boiling point and lower applied pressure. The chromatograms for the various plant extracts from PLE and PHWE are shown in Figure 5A and 5B, respectively, and similar profiles were observed. The data obtained showed that for botanicals, the marker compounds were present naturally where significant analytes–matrix interactions were present. Spiking of target analytes into the plant matrix was not able to mimic the analytes–matrix interaction present naturally. A high recovery obtained in the spiking experiments did not imply that the method was accurate. PLE was found to give comparable or higher extraction efficiencies compared with PHWE with reference to Soxhlet extraction for tanshinone I and IIA in *Salvia miltiorrhiza*.

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