Comparison of Extraction Efficiency of Tanshinones from *S. miltiorrhiza* by Solvent and Supercritical Carbon Dioxide

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Abstracts: *Salvia miltiorrhiza* (*S. miltiorrhiza*), also named as Danshen, is a traditional Chinese herbal medicine reported to possess anti-cancer and anti-inflammation activities, which can be attributed to presence of the major functional components tanshinones. The objectives of this study were to develop a high-performance liquid chromatography-mass spectrometry (HPLC-MS) method for determination of tanshinones in *S. miltiorrhiza* and comparison of extraction efficiency of solvent and supercritical CO_2. Results showed that a total of 6 tanshinones including 15,16-dihydrotanshinone I, 1,2,5,6-tetrahydrotanshinone I, cryptotanshinone, tanshinone I, 1,2-dihydrotanshinone I and tanshinone IIA could be separated within 18 min by employing a Metachem ODS-2 C18 column and a gradient mobile phase of 0.1% formic acid solution (A) and acetonitrile (B) with flow rate at 1 mL/min, detection wavelength at 280 nm and column temperature at 25°C. The highest yield of total tanshiones (2869.9 μg/g) extracted by supercritical CO_2 was attained at 70°C and 400 bar. For solvent extraction, the highest yield of total tanshinones was obtained by methanol or ethanol, which amounted to 3103.1 μg/g and 3021.6 μg/g, respectively. For future large production of tanshinones from *S. miltiorrhiza*, ethanol can be adopted to replace methanol and supercritical CO_2 amid its safety nature and higher yield than supercritical CO_2.

Keywords: *Salvia miltiorrhiza*, Tanshinones, HPLC-MS, Solvent, Supercritical CO_2.

1. INTRODUCTION

Salvia miltiorrhiza (*S. miltiorrhiza*), a vital Chinese medicinal herb also named as "Danshen" widely grown in Asian countries like China and Taiwan, are often used to treat chronic diseases such as hepatocyte injury [1] and osteoporosis [2]. In addition, *S. miltiorrhiza* extracts have been shown to be effective in anti-bacteria [3], anti-inflammation [4] and anti-cancer [5]. Many functional components including danshensu, protocatechu aldehydes, polysaccharides, phenolic acids and tanshinones are believed to be responsible for these biological activities. Among the various functional components, tanshinones are of particular importance due to its potentiality to be developed as drug [5]. The major tanshinones in *S. miltiorrhiza* include Tanshinone I, Tanshinone IIA, cryptotanshinone and dihydrotanshinone, with Tanshinone IIA present in the most abundant amount (0.3%), followed by Tanshinone I and cryptotanshinone (0.1% each) and dihydrotanshinone (<0.1%) [6]. In addition, some other tanshinone analogs such as Tanshinone IIB, isocryptotanshinone, hydroxytanshinone IIA, miltionone II and isotanshinones I & II are also present and found to possess anti-tumor activity [7].

Due to low-polarity nature of tanshinones, polar solvents such as methanol, ethanol or acetone are often used alone or in combination with low-polarity solvents for tanshinones extraction from *S. miltiorrhiza*. However, most organic solvents are toxic and can cause environmental pollution. Thus, in the past decades supercritical CO_2, also named as "green solvent" are often adopted to extract non-polar and low-polar bioactive compounds from Chinese medicinal herb based on the following reasons: (1) supercritical CO_2 has lower viscosity and higher diffusivity and thus it can penetrate into porous solid materials more effectively than liquid solvents to reduce extraction time substantially; (2) the solvation power of the fluid can be manipulated by changing pressure and/or temperature; (3) solutes dissolved in supercritical CO_2 can be easily separated by depressurization and thus the sample concentration process can be eliminated; (4) supercritical CO_2 can be conducted at low temperature and thus it can be used to study thermally labile compounds; (5) a much lower amount of sample is required than solvent extraction; (6) supercritical CO_2 is a safe solvent [8].For instance, Wang *et al*. [9] studied the effect of temperature on the yield of tanshinones and reported that the contents of dihydrotanshinone I, cryptotanshinone, tanshinone I and tanshinone IIA rose following an increase in temperature from 40-50°C, however, a declined trend was shown following a rise in temperature from 50-60°C. This phenomenon may be accounted for by the decreased solubility of tanshinones in supercritical CO_2 at an elevated temperature. Nevertheless, it has been well established that an increase in temperature can also increase yield of bioactive compounds caused by increase of solute vapor pressure [10]. Thus, both temperature and

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pressure should be taken into account during supercritical CO_2 extraction to attain a high yield of bioactives. Supercritical CO_2 finds wide application in the food industry for removal of fat, caffeine and alcohol from high-fat-containing foods such as potato chip, coffee bean or tea leaf, and wine, respectively [8-11]. Also, supercritical CO_2 can also be used to extract antioxidants or dietary supplements such as vitamin E from soybean cake, a waste obtained during soybean oil processing [11].

Following extraction, tanshinones in *S. miltiorrhiza* are often subjected to separation, identification and quantitation by HPLC or HPLC-MS [12-14]. However, the employment of HPLC without MS for identification of tanshinones should be inadequate because of possible matrix interference. Moreover, most published reports did not use internal standard for quantitation of tanshinones, which should decrease quantitation accuracy. Thus, the objectives of this work were to develop an HPLC-MS method for determination of tanshinones in *S. miltiorrhiza*. Also, the extraction efficiency of tanshinones from *S. miltiorrhiza* by solvents and supercritical CO_2 were compared.

2. MATERIAL AND METHODS

2.1. Materials

A total of 10 kg Danshen sample was provided by Taiwan Agriculture Experiment Institute and was cut into pieces for freeze-drying, followed by grinding into powder (<1 μm) using a coffee grinder and storing in a vacuum-sealed polyethylene Bag at -30°C prior to use.

Tanshinone standards including Tanshinone I, Tanshinone IIA and Cryptotanshinone were procured from Jiou-Din Biotech Co. (Taipei, Taiwan), while Dihydrotanshinone I was from Sigma (St. Louis, MO, USA), with the purities being 95%, ≥98%, ≥98% and ≥98%, respectively. The HPLC-grade solvents including methanol, ethanol, acetone, ethyl acetate, acetonitrile and dimethyl sulfoxide (DMSO) were from Merck Co. (Darmstadt, Germany). Deionized water was made using a Milli-Q water purification system from Millipore Co. (Bedford, MA, USA). Phosphoric acid was from Sigma-Aldrich Co. (Billerica, MA, USA).

2.2. Instrumentation

The HPLC system is composed of Rheodyne 7161 injector, Jasco PU980 and PU1980 pump, Jasco MD915 photodiode-array detector and Borwin software from Jasco Co. (Tokyo, Japan). A 6130 quadrupole LC/MS with multi-mode ion source (ESI and APCI) was from Agilent Technologies Co. (Palo Alto, CA, USA). A spe-ed SFE 7010 supercritical fluid extractor was from Applied Separation Co. (Allentown, PA, USA), which is composed of an extraction tank from Thermo Co. (Bellefonte, PA, USA). The FD24 freeze-dryer was from Chin-Ming Co. (Taipei, Taiwan). The Sorvall RC5C high-speed centrifuge was from DuPont Co. (Wilmington, Delaware, USA). The Eyela N-1 rotary evaporator was from Eyela Co. (Tokyo, Japan). The Firstek B402L low-temperature circulation water bath was from Li-Chen Instrument Co. (Taoyuan, Taiwan). The DC400H sonicator was from Chuan-Hua Co. (Taipei, Taiwan).

2.3. Extraction of Tanshinones by Solvent

A method based on Shi *et al*. [14] was modified for extraction of Tanshinones in *S. miltiorrhiza* by various solvents including methanol, ethanol, acetone and ethyl acetate for comparison of extraction efficiency. In brief, a 0.5-g Danshen sample was mixed with 25 mL of methanol, ethanol, acetone or ethyl acetate in a centrifuge tube, after which each mixture was sonicated for 40 min, followed by centrifuging at 4000 rpm for 10 min at 25°C. Then the supernatant was collected, evaporated to 10 mL, filtered through a 0.22-μm membrane filter, and 20 μL filtrate was injected into HPLC for quantitation of Tanshinones in *S. miltiorrhiza*.

2.4. Extraction of Tanshinones by Supercritical CO_2

A method based on Kao *et al*. [10]was modified to extract Tanshinones in *S. miltiorrhiza* by supercritical CO_2 at various temperature and pressure for comparison of extraction efficiency. Briefly, a 0.5 g Danshen sample was poured into a 10-mL extraction tank, with 0.25-g defatted cotton filling into both sides and then covered with a lid for extraction at various combinations of temperature (40, 50, 60 and 70°C) and pressure (200, 300 and 400 bar). After the desired temperature and pressure was reached, the static extraction was carried out for 10 min, followed by opening outlet valve, continuing dynamic extraction at a flow rate (5 mL/min) for 5 min for two circulations. After the most optimal temperature and pressure was attained, the co-solvent ethanol was added at 5% and 10% to study its effect on the yield of Tanshinones.

2.5. HPLC Analysis of Tanshinones

In most published reports the mobile phases used for separation of Tanshinones often include a mixture

of methanol and water or acetonitrile and water, with acetic acid, phosphoric acid or formic acid adding to mobile phase as modifier to prevent peak tailing or broadening [13,15].Thus, in our study various mobile phases composed of acetonitrile and water with formic acid (0.1%) or phosphoric acid (0.02%) as modifier was evaluated for separation efficiency of Tanshinones. In addition, 3 reversed-phase C18 columns were compared for separation efficiency including Vydac 201TP54 C18 (250x4.6 mm ID, particle size 5 μm) from Vydac Co. (Hesperia, CA, USA), ODS-80Ts (250x4.6 mm ID, particle size 5 μm) and ODS-2 C18 (250x4.6 mm ID, particle size 5 μm), both of which are from Metachem Co. (IL, USA). The separation efficiency was based on retention factor (k) and separation factor (α), with the former should be controlled between 2-10 and the latter >1 to attain a satisfactory separation [15].After numerous studies, a mobile phase of 0.1% formic acid solution (A) and acetonitrile (B) with flow rate at 1 mL/min, column temperature at 25°C, detection wavelength at 280 nm, and the following gradient elution was developed to separate Tanshinones in *S. miltiorrhiza*: 38% A and 62% B in the beginning, decreased to 32% A in 6 min, 31% A in 8 min, 30% A in 12 min, 20% A in 17 min, and returned to 38% A in 20 min.

2.6. Identification and Quantitation of Tanshinones

The various Tanshinones in *S. miltiorrhiza* samples were identified by comparing retention times, absorption spectra and mass spectra of unknown peaks with authentic standards. In addition, the MS detection was performed using ESI source with positive mode and the following conditions: scanning range 100-500, drying gas flow 7 mL/min, nebulizer pressure 60 psi, dry gas temperature 350°C, vaporizer temperature 250°C, capillary voltage 4000 V, charging voltage 2000 V and fragmentor voltage 300 V.

For quantitation, an internal standard butylated hydroxyanisole (BHA) was used and a concentration of 1000 μg/mL was prepared in methanol. Six concentrations of 0.5, 1, 5, 10, 15 and 20 μg/mL were prepared in methanol for 15, 16-dihydrotanshinone I, cryptotanshinone, tanshinone I and tanshinone IIA separately. Then each tanshinone standard was mixed with BHA whose final concentration was fixed at 10 μg/mL. Each concentration was injected into HPLC twice and the standard curves were prepared by plotting concentration ratio (tanshinone vs BHA) against area ratio (tanshinone vs BHA). The regression equation and coefficient of determination (R^2) of each standard curve was obtained with a software system automatically. The amount of each tanshinone in *S. miltiorrhiza* was calculated using the following formula:

W (μg/g)=(As/Aixa+b)xCixVx(times of dilution)x (1/recovery)x(1/sample weight)

where As: peak area of tanshinones in sample

- Ai: peak area of internal standard
- a: slope of standard curve
- b: intercept of standard curve
- Ci (μg/mL): concentration of internal standard
- V (mL): final extract volume
- W (μg/g): Tanshione concentration in sample

2.7. Method Validation

The intra-day and inter-day variability of tanshinones were determined based on a report issued by International Conference on Harmonization [16]. The former was carried out by determining tanshinones contents in *S. miltiorrhiza* samples in the morning, afternoon and evening three times each on the same day, while the latter was performed by analyzing tanshinones contents in *S. miltiorrhiza* on the 1st, 2nd and 3rd day three times each.

Both limit of detection (LOD) and limit of quantitation (LOQ) were determined by preparing three concentrations (0.1, 0.25 and 0.5 μg/mL) of each tanshinone standard and analyzing three times. The standard curve was prepared by plotting concentrations against peak height and the slope (s) was obtained from the regression equation, with LOD and LOQ being calculated by determining a constant factor δ through substitution of the maximum noise height (N_{p-p}) according to following equations:

$\delta = N_{p-p}/5$

$LOD = 3.3 \times \delta/S$

$LOQ = 3 \times LOD$

For recovery determination, two levels of each tanshinone standard (300 μg and 600 μg for 15,16-dihydrotanshinone I or tanshinone I; 1 mg and 2 mg for cryptotanshione; 400 μg and 800 μg for tanshinone IIA) was each mixed with 0.5-g*S. miltiorrhiza* sample for tanshinone extraction by solvent and then quantitation

by HPLC. The recovery of each tanshinone was calculated based on the ratio of tanshinone content after HPLC (amount of standard + tanshinone content in sample minus original tanshinone content in sample) relative to tanshinone content before HPLC (amount of standard added).

2.8. Statistical Analysis

All the data were subjected to analysis of variance and Duncan's multiple range test for significant comparison ($p<0.05$) using Statistical Analysis System [17].

3. RESULTS AND DISCUSSION

3.1. HPLC Analysis of Tanshinones

Initially three C18 columns as described in the method section were compared with respect to separation efficiency of tanshinones in *S. miltiorrhiza* extract. Due to difference in column characteristics such as carbon load, endcapping, polymerization, theoretical plate and porosity, the resolution of tanshinones can be varied for each column. After several experiments, Metachem ODS-2 C18 column was shown to provide better separation efficiency than the other two columns. For mobile phase selection, a gradient mobile phase of 0.1% formic acid solution and acetonitrile based on Liu *et al*. [18] was used. However, the separation time is lengthy and resolution remains inadequate. After various studies, the most appropriate separation condition was obtained: 38% of 0.1% formic acid solution (A) and 62% of acetonitrile (B) in the beginning, decreased to 32% A in 6 min, 31% A in 8 min, 30% A in 12 min, 20% A in 17 min, and returned to 38% A in 20 min. Figure **1** shows HPLC chromatograms of tanshinone standards (A) and extract (B) in *S. miltiorrhiza* extract. Four tanshinone standards including 15,16-dihydrotanshinone I, cryptotanshinone, tanshinone I and tanshinone IIA, as well as internal standard BHA were adequately resolved within 18 min (Figure **1A**), while in *S. miltiorrhiza* extract, two more tanshinones, 1,2,5,6-tetrahydrotanshinone I and 1,2-dihydrotanshinone I were present (Figure **1B**). Table **1** shows retention time, retention factor (k), separation factor (α) and peak purity of 6 tanshinones in *S. miltiorrhiza* extract, which were ranged from 8.74-17.82 min, 2.60-6.36, 1.01-1.39 and 97.7-99.8%, respectively,

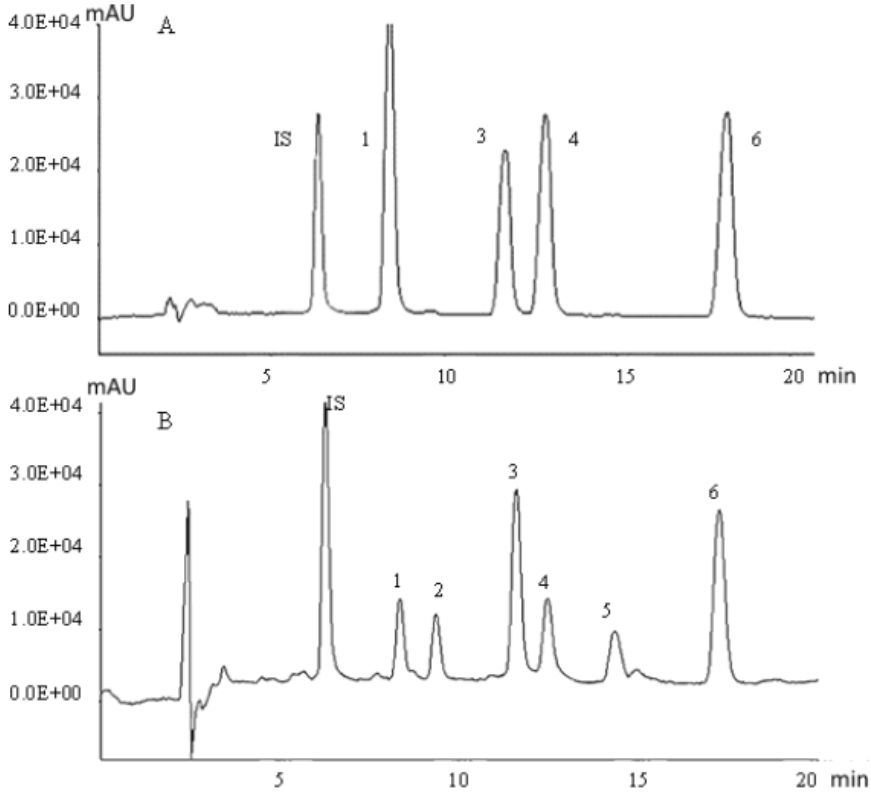

Figure 1: HPLC-DAD chromatogram of tanshinones standards (**A**) and extract (**B**) from *S. miltiorrhiza*.IS, butylated hydroxyanisole (BHA). Peaks: 1, 15,16-Dihydrotanshinone I; 2, 1,2,5,6-Tetrahydrotanshinone I; 3, Cryptotanshinone; 4, Tanshinone I; 5, 1,2-Dihydrotanshinone I; 6, Tanshinone IIA.

Table 1: Retention time (t_R), Retention Factor (k), Separation Factor (α), and Peak Purity of Tanshinones Extracted from *S. Miltiorrhiza*

Peak No.	Compound	t_R (min)	Retention Factor (k)	Separation Factor (α)	Peak Purity (%)
1	15,16-Dihydrotanshinone I	8.74	2.60	1.01 (1,2)[a]	99.7
2	1,2,5,6-Tetrahydrotanshinone I	9.33	2.85	1.01 (1,2)[a]	99.8
3	Cryptotanshinone	11.98	3.95	1.39 (2,3)[a]	97.9
4	Tanshinone I	13.13	4.42	1.12 (3,4)[a]	99.6
5	1,2-Dihydrotanshinone I	14.41	4.95	1.12 (4,5)[a]	99.8
6	Tanshinone IIA	17.82	6.36	1.28 (5,6)[a]	97.7

[a] Numbers in parentheses represent values between two neighboring peaks.

Both retention factor and separation factor were in the optimal range as suggested by Dolan [15], implying that a proper solvent strength of mobile phase and an appropriate selectivity of mobile phase to sample components was attained. In some other published reports Liu *et al*. [18] employed a gradient mobile phase of 0.02% phosphoric acid solution and acetonitrile as well as a C18 column (250x4.6 mm ID, 5 μm particle size) to separate 4 tanshinones within 37 min. Similarly, Shi *et al*. [14] used an isocratic mobile phase of methanol/tetrahydrofuran/water/acetic acid (20:35:44:1, v/v/v/v) and a Diamonsil C18 column (150x4.6 mm ID, 5 μm particle size) to separate 3 tanshinones within 18 min with flow rate at 1.0 mL/min and detection at 254 nm. Both methods suffer a major drawback that the number of tanshinones separated remains inadequate. In another study Liu *et al*. [13] developed a gradient HPLC mobile phase of water-phosphoric acid (100:0.026, v/v) and acetonitrile to separate various tanshinones and salvianolic acids in Danshen samples and a total of 23 standards were resolved within 76 min by an Agilent Zorbax Extend C18 column (250x4.6 mm ID, 5 μm particle size) with flow rate at 0.8 mL/min and detection wavelength at 280 nm. However, both 1,2-dihydrotanshinone I and 1,2,5,6-tetrahydrotanshinone I standards were not included for evaluation of separation efficiency. Likewise, Yang *et al*. [12] also developed an HPLC-MS method and a gradient mobile phase of formic acid solution (0.03%) and acetonitrile to separate 27 tanshinones in the roots of *S. miltiorrhiza* within 29 min by using the same Zorbax column shown above with flow rate at 1.0 mL/min, column temperature at 20°C and detection wavelength at 270 nm. Comparatively, the retention time in terms of the number of tanshinones separated in this study is short, however, peaks overlapping can occur [12].

3.2. Identification and Quantitation of Tanshinones

Table **2** shows identification data of tanshinones in *S. miltiorrhiza* extract. As mentioned above, a total of 6 tanshinones including 15,16-dihydrotanshinone I, 1,2,5,6-tetrahydrotanshione I, cryptotanshinone, tanshinone I, 1,2-dihydrotanshinone I and tanshinone IIA were identified based on comparison of absorption spectra and mass spectra of unknown peaks with reference standards and values reported in the literature.

For quantitation, an internal standard BHA as described in the method section was used and the regression equations of 15,16-dihydrotanshinone I, cryptotanshinone, tanshinone I, and tanshinone IIA were y=7.1278x-0.2929, y=5.584x-0.5896, y=6.6952x-0.566 and y=9.7561x-0.9297, respectively, with the coefficient of determination (R^2) being 0.9819, 0.9869, 0.9866 ad 0.9884.

Table **3** shows quality control data of tanshinones. The RSD(%) of intra-day variability for 15,16-dihydrotanshinone I, cryptotanshinone, tanshinone I and tanshinone IIA were 7.1, 3.2, 3.5 and 3.6%, respectively, whereas the inter-day variability were 3.4, 4.0, 3.5 and 2.6%. This outcome indicated that a high reproducibility was obtained with this method. The LOD of 15, 16-dihydrotanshinone I, cryptotanshinone, tanshinone I and tanshinone II A were 0.1, 0.15, 0.15 and 0.1 μg/g, respectively, whereas the LOQ were 0.3, 0.45, 0.45 and 0.3 μg/g. For recovery determination of tanshinones, the average recovery of 15,16-dihydrotanshinone I, cryptotanshinone, tanshinone I and tanshinone IIA was 105.8, 104, 109 and 109.3%, respectively, demonstrating a high accuracy of the method developed in our experiment.

Table 2: Identification Data of Tanshinones in *S. Miltiorrhiza* Extract

Peak No.	Compound	MW[a]	λ_{max} (nm)			[M+H]$^{+b}$		
			Extract	Standard	Reference	Extract	Standard	Reference
1	15,16-Dihydrotanshinone I	278	290, 334	289, 334	240, 292, 334[cd]	279	279	279[cde]
2	1,2,5,6-Tetrahydrotanshinone I	280	276	-	276[d]	281	-	281[d]
3	Cryptotanshinone	296	266, 360	266, 350	264, 299, 360[cd]	297	297	297[cdf]
4	Tanshinone I	276	242, 326	242, 326	246, 278, 324[cd]	277	277	277[cdef]
5	1,2-Dihydrotanshinone I	278	283	-	290[d]	279	-	279[d]
6	Tanshinone IIA	294	272, 350	272, 350	252, 270, 354[c]	295	295	295[cdf]

[a]Molecular weight. [b]Determined by LC-MS. [c]Based on a reference by Liu *et al*. (2007). [d] Based on a reference by Yang *et al*. (2006). [e] Based on a reference by Wang *et al*. (2010b).[f] Based on a reference by Hu *et al*. (2005).

Table 3: Quality Control Data of Tanshinones by HPLC-DAD

Compound	Intra-Day Variability[a]			Inter-Day Variability[a]		Linear range (μg/g)	LOD[c] (μg/g)	LOQ[d] (μg/g)
	Mean± SD (μg/g)	RSD[b] (%)		Mean ± SD (μg/g)	RSD[b] (%)			
15,16-Dihydrotanshinone I	300.0 ± 21.4	7.1		311.8 ± 10.6	3.4	0.5-20	0.1	0.3
Cryptotanshinone	892.5 ± 28.9	3.2		933.8 ± 37.2	4.0	0.5-20	0.15	0.45
Tanshinone I	301.4 ± 10.6	3.5		316.6 ± 13.9	3.5	0.5-20	0.15	0.45
Tanshinone IIA	563.9 ± 20.4	3.6		579.7 ± 15.3	2.6	0.5-20	0.1	0.3

[a] Mean of triplicate analyses ± standard deviation.[b]RSD, relative standard deviation. [c] LOD, limit of detection. [d] LOQ, limit of quantification.

Table 4: Contents of Tanshinones (μg/g)[a] in *S. Miltiorrhiza* as Affected by Various Solvents

Tanshinone	Methanol	Ethanol	Acetone	Ethyl Acetate
	Content (μg/g)[a]	Content (μg/g)[a]	Content (μg/g)[a]	Content (μg/g)[a]
15,16-Dihydrotanshinone I	331.3 ± 14.9 ^{A}	316.7 ± 11.0 ^{AB}	288.6 ± 6.3 ^{C}	304.4 ± 27.6 ^{ABC}
1,2,5,6-Tetrahydrotanshinone I	361.1 ± 17.8 ^{A}	317.4 ± 21.8 ^{A}	295.0 ± 29.7 ^{AB}	259.4 ± 6.5 ^{B}
Cryptotanshinone	980.7 ± 38.0 ^{A}	1010.4 ± 3.5 ^{A}	905.6 ± 24.7 ^{B}	907.1 ± 29.9 ^{B}
Tanshinone I	383.8 ± 5.4 ^{A}	377.1 ± 11.4 ^{A}	328.8 ± 10.9 ^{B}	353.0 ± 18.2 ^{B}
1,2-Dihydrotanshinone I	394.1 ± 9.8 ^{A}	320.3 ± 14.6 ^{B}	289.6 ± 17.7 ^{B}	255.0 ± 7.1 ^{C}
Tanshinone IIA	652.1 ± 18.7 ^{AB}	679.7 ± 23.1 ^{A}	576.7 ± 19.4 ^{C}	619.7 ± 15.5 ^{B}
Total	3103.1 ± 51.5^{A}	3021.6 ± 94.8 ^{A}	2684.3 ± 80.5 ^{B}	2698.6 ± 229.3 ^{B}

[a] Average of triplicate analyses ± standard deviation. Data with different letters (A-C) in the same row are significantly different at $p < 0.05$.

3.3. Extraction Efficiency of Tanshinones by Solvents

Table **4** shows extraction efficiency of tanshinones in *S. miltiorrhiza* by various solvents. Apparently methanol could produce the highest yield of tanshinones (3103.1 μg/g), followed by ethanol (3021.6 μg/g), ethyl acetate (2698.6 μg/g) and acetone (2684.3 μg/g) (Figure **2**). However, there was no significant difference ($p>0.05$) in the extraction yield between methanol and ethanol treatments, as well as between acetone and ethyl acetate treatments.

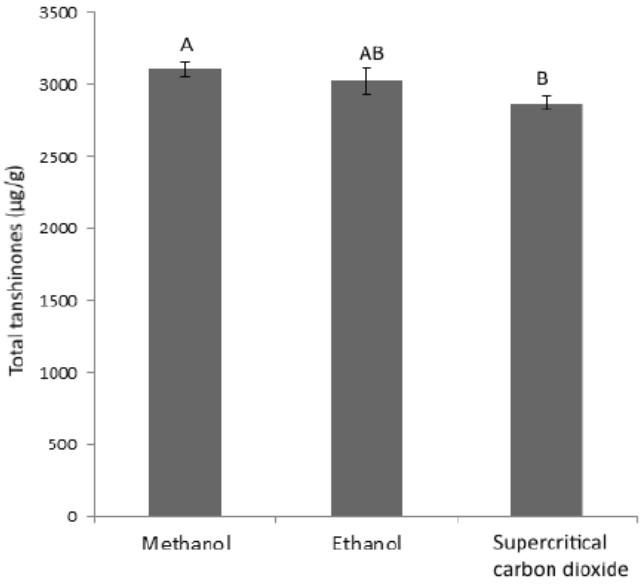

Figure 2: Comparison of the contents of total tanshinones (μg/g) by solvent extraction and supercritical carbon dioxide extraction. Results are presented as mean ± SD of triplicate determinations. Data with different letters (A-B) are significantly different at $p < 0.05$.

3.4. Extraction Efficiency of Tanshinones by Supercritical CO_2

Table **5** shows tanshinone contents in *S. miltiorrhiza* extracted by supercritical CO_2 at various pressures and temperatures. With temperature at 40°C, the highest yield of total tanshinones (2473.1 μg/g) was shown at 400 bar, followed by 300 bar (2455 μg/g) and 200 bar (2444.7 μg/g). However, there was no significant difference (p>0.05) in the contents of total tanshinones among various pressures at 40°C. Interestingly, with temperature at 50°C, the highest yield of total tanshinones (2447.5 μg/g) was shown at 400 bar, while the lowest yield (1724.4 μg/g) was found at 300 bar. Also, there was no significant difference (p>0.05) in the levels of total tanshinones between 400 bar and 200 bar (2437 μg/g). Similar trend was observed at 60°C, with the largest amount of total tanshinones (2447.2 μg/g) being shown at 400 bar, followed by 200 bar (2385.9 μg/g) and 300 bar (1788.5 μg/g). Likewise, following a rise in temperature to 70°C, the highest yield of total tanshinones was found at 400 bar (2869.9 μg/g), followed by 200 bar (2366.4 μg/g) and 300 bar (2337.4 μg/g). However, there was no significant difference (p>0.05) in total tanshinone contents between 200 bar and 300 bar. Comparatively, a better extraction yield of tanshinones could be attained at 70°C and 400 bar.

In several published reports Wang *et al*. [9] studied the effect of supercritical CO_2 at various pressures on the yield of dihydrotanshinone I, cryptotanshinone, tanshinone I and tanshinone IIA and found that at a constant temperature, the highest yield was generated at 40 MPa (400 bar), followed by 20 MPa (200 bar) and 30 MPa (300 bar). The increase in extraction efficiency at elevated pressure can be attributed to a rise in supercritical CO_2 density and thus the tanshinone solubility was greatly enhanced. Similarly, at a constant pressure, the vapor pressure of tanshinones could increase following a rise in temperature. It has been well established that the solubility of a target compound in a supercritical fluid is a major factor determining its extraction efficiency. The solubility is controlled by the volatility of the substance, a function of temperature and the solvation effect of the supercritical fluid, a function of fluid density [8]. Thus, a decrease of pressure at a constant temperature can cause a decrease of fluid density and solute solubility to reduce extraction efficiency. On the other hand, at a fixed pressure the increase of temperature can cause a decline of fluid density to reduce solute solubility and extraction efficiency. Nevertheless, the solubility of most organic compounds could be substantially improved as the solute's vapor pressure increases with increase in temperature [9]. In addition to pressure and temperature, CO_2 flow rate has to be carefully controlled as high flow rate can reduce the contact time between CO_2 and the solute, especially for the solute which has low solubility, while low flow rate can lower the mass transfer between CO_2 and the solute [9]. Also, both static extraction time and dynamic extraction time should be controlled to enhance penetration of the fluid into the matrix and solubility of solute in the fluid [9].

The effect of various temperatures on the yield of total tanshinones at a fixed pressure is also shown in Table **5**. The yield of total tanshinones followed a temperature-dependent decline at 200 bar, however, no significant difference (p>0.05) was observed among the various temperatures treatments. Interestingly, an inconsistent change was shown at 300 bar, with the largest yield being found at 40°C (2455 μg/g), followed by 70°C (2337.4 μg/g), 60°C (1788.5 μg/g) and 50°C (1724.4 μg/g). However, there was no significant difference (p>0.05) in total tanshinone contents between 50°C and 60°C treatments. Similarly, with pressure at 400 bar, the highest yield of total tanshinones was shown at 70°C (2869.9 μg/g), followed by 40°C (2473.1 μg/g), 50°C (2447.5 μg/g) and 60°C (2447.2 μg/g). However, there was no significant difference (p>0.05) among 40°C, 50°C and 60°C treatments. Taken together, with high pressure at

Table 5: Tanshinone Contents (μg/g)[a] in *S. Miltiorrhiza* Extracted by SCD[b] at Various Temperatures and Pressure

Tanshinone	Pressure		
	200 bar	300 bar	400 bar
At 40°C			
15,16-Dihydrotanshinone I	297.8±4.1^{A}	314.5±7.2^{A}	303.9±12.5^{A}
1,2,5,6-Tetrahydrotanshinone I	244.9±5.3^{B}	240.9±4.5^{B}	262.8±5.5^{A}
Cryptotanshinone	802.5±3.8^{A}	787.7±3.4^{A}	782.5±13.0^{A}
Tanshinone I	309.8±7.7^{A}	297.3±4.8^{A}	292.7±7.3^{A}
1,2-Dihydrotanshinone I	258.9±8.8^{AB}	255.8±7.2^{B}	279.7±2.6^{A}
Tanshinone IIA	530.8±3.3^{B}	558.8±1.9^{A}	551.5±6.8^{A}
Total	2444.7±26.3^{A}	2455.0±3.9^{A}	2473.1±31.5^{A}
At 50°C			
15,16-Dihydrotanshinone I	293.9±6.4^{B}	246.9±9.4^{C}	345.2±15.7^{A}
1,2,5,6-Tetrahydrotanshinone I	238.0±9.0^{B}	178.0±9.8^{C}	259.8±8.3^{A}
Cryptotanshinone	790.3±6.9^{A}	459.8±3.8^{C}	764.0±16.4^{B}
Tanshinone I	303.8±8.4^{A}	328.2±12.2^{A}	309.2±12.6^{A}
1,2-Dihydrotanshinone I	257.2±3.7^{A}	175.8±6.9^{B}	256.1±9.1^{A}
Tanshinone IIA	553.8±5.9^{A}	335.7±14.8^{C}	513.2±6.3^{B}
Total	2437.0±15.7^{A}	1724.4±56.9^{B}	2447.5±20.5^{A}
At 60°C			
15,16-Dihydrotanshinone I	290.9±12.4^{B}	242.9±13.3^{B}	314.0±6.7^{A}
1,2,5,6-Tetrahydrotanshinone I	219.3±1.3^{B}	173.3±6.3^{C}	260.6±3.2^{A}
Cryptotanshinone	784.7±4.5^{A}	508.3±8.3^{B}	783.7±22.5^{A}
Tanshinone I	291.7±8.3^{A}	303.7±6.0^{A}	252.1±3.5^{B}
1,2-Dihydrotanshinone I	239.9±9.1^{B}	159.8±6.3^{C}	285.9±6.7^{A}
Tanshinone IIA	559.4±10.5^{A}	400.5±2.3^{B}	550.9±11.6^{A}
Total	2385.9±28.0^{A}	1788.5±3.4^{B}	2447.2±40.7^{A}
At 70°C			
15,16-Dihydrotanshinone I	300.1±8.7^{A}	303.8±8.7^{A}	283.0±1.7^{A}
1,2,5,6-Tetrahydrotanshinone I	204.4±6.9^{B}	209.3±3.5^{B}	337.3±3.7^{A}
Cryptotanshinone	776.9±15.6^{B}	752.9±9.7^{B}	922.8±4.7^{A}
Tanshinone I	276.5±9.5^{B}	293.4±6.9^{B}	348.2±7.1^{A}
1,2-Dihydrotanshinone I	230.7±6.6^{B}	246.9±9.2^{B}	361.6±17.9^{A}
Tanshinone IIA	577.8±6.4^{B}	531.1±4.9^{C}	617.1±9.4^{A}
Total	2366.4±40.0^{B}	2337.4±33.2^{B}	2869.9±44.5^{A}

[a] Average of triplicate analyses ± standard deviation. [b] SCD, supercritical carbon dioxide. Data with different letters (A-C) in the same row are significantly different at $p<0.05$.

300 bar and 400 bar, a rise in temperature can affect the yield of total tanshinones, especially at 50°C and 60°C, as shown by a low yield of total tanshinones. Similar outcome was reported by Wang *et al*., [9] showing that the yield of total tanshinones rose pronouncedly following a rise in temperature from 40-50°C, but showed a declined trend from 50-60°C. It may be inferred that at low pressure, an increase in temperature can lower supercritical CO_2 density and in turn reduce solubility to decrease the yield of total tanshinones [19]. Conversely, the vapor pressure of supercritical CO_2 can be raised to enhance the yield of total tanshinones at an elevated temperature [19].

Table **6** shows the total tanshinone contents as affected by supercritical CO_2 extraction at 400 bar and 70°C in the presence of the cosolvent ethanol at 5% and 10%. Comparatively, the yield of total tanshinones was significantly lower ($p<0.05$) at 5% ethanol (2164.8 μg/g) than at 10% ethanol (2440.4 μg/g), implying the higher the level of ethanol, the larger the yield of total tanshinones. Nevertheless, the treatment (70°C and 400 bar) without ethanol was shown to produce a higher yield of total tanshinones (2869.9 μg/g) than that with 10% ethanol (2440.4 μg/g). This outcome indicated that the addition of ethanol could lower solubility of tanshinones in supercritical CO_2 and

resulted in a decline in extraction yield of total tanshinones. In a study dealing with extraction of polycyclic aromatic hydrocarbons (PAHs) from environmental samples by supercritical CO_2 at 80°C and 400 atm, Yang *et al*. [20] reported that the PAHs contents were higher for the treatment without cosolvent than that with cosolvent (toluene or ethylenediamine), Similarly, Sargenti and Lancas [21] found that the extraction yield of essential oil by supercritical CO_2 with cosolvent 10% acetone was higher than that with 20% acetone by 0.01%. By comparison, the yield of total tanshinones by methanol or ethanol was higher than that by supercritical CO_2 at 70°C and 400 bar. Thus, by taking the solvent safety issue into account, ethanol can be adopted instead of methanol and supercritical CO_2 for large production of tanshinones from *S. miltiorrhiza*.

Table 6: Tanshinone Contents (μg/g)[a] as Affected by SCD[b] at 400 Bar, 70°C and Different Co-Solvent Volumes

Tanshinone	5% Co-Solvent	10% Co-Solvent
15,16-Dihydrotanshinone I	209.9 ± 8.2 [B]	266.4 ± 5.1 [A]
1,2,5,6-Tetrahydrotanshinone I	208.9 ± 4.9 [B]	305.9 ± 6.8 [A]
Cryptotanshinone	744.9 ± 8.2 [B]	789.2 ± 9.7 [A]
Tanshinone I	280.5 ± 5.0 [B]	321.2 ± 6.8 [A]
1,2-Dihydrotanshinone I	212.8 ± 4.5 [B]	202.1 ± 0.7 [A]
Tanshinone IIA	507.9 ± 7.1 [B]	551.3 ± 9.5 [A]
Total	2164.8 ± 50.9 [B]	2440.4 ± 45.1 [A]

[a] Average of triplicate analyses ± standard deviation. [b] SCD: supercritical carbon dioxide. Data with different letters (A-B) in the same row are significantly different at $p < 0.05$.

4. CONCLUSIONS

In conclusion, an HPLC-MS method was developed to separate 6 tanshinones within 18 min by using a Metachem ODS-2 C18 column (250x4.6 mm ID, 5 μm particle size) and a gradient mobile phase of 0.1% formic acid solution and acetonitrile with flow rate at 1.0 mL/min, detection wavelength at 280 nm and column temperature at 25°C. After quantitation using the internal standard butylated hydroxyanisole, the yield of total tanshinones extracted by ethanol (3021.6 μg/g) or methanol (3103.1 μg/g) was found to be higher than that by supercritical CO_2 (2869.6) at 400 bar and 70°C. For future large production of tanshinones, ethanol should be the most appropriate agent amid its safety nature. Given the lack of both positive identification of tanshinones using MS and quantitation using an internal standard in several reported methods, the developed HPLC-MS method should provide a more precise identification and quantitation of tanshinones in traditional herbs, medicinal formulations and nutraceuticals.

REFERENCES

[1] Park EJ, Zhao YZ, Kim YC, Sohn DH. Preventive effects of a purified extract isolated from Salvia miltiorrhiza enriched with tanshinone I, tanshinone IIA and cryptotanshinone on hepatocyte injury *in vitro* and *in vivo*. Food Chem Toxicol 2009; 47: 2742-2748. http://dx.doi.org/10.1016/j.fct.2009.08.007

[2] Cui Y, Bhandary B, Marahatta A, Lee GH, Li B, Kim DS, Chae SW, Kim HR, Chae HJ. Characterization of Salvia miltiorrhiza ethanol extract as an anti-osteoporotic agent. BMC Compl Alt Med 2011; 11: 120. http://dx.doi.org/10.1186/1472-6882-11-120

[3] Zhao J, Lou J, Mou Y, Li P, Wu J, Zhou L. Diterpenoidtanshinones and phenolic acids from cultured hairy roots of Salvia miltiorrhiza Bunge and their antimicrobial activities. Molecules 2011; 16: 2259-2267. http://dx.doi.org/10.3390/molecules16032259

[4] Ding M, Ye TX, Zhao GR, Yuan YJ, Guo ZX. Aqueous extract of Salvia miltiorrhiza attenuated increased endothelial permeability induced by tumor necrosis factor-α. IntImmunopharmacol 2005; 5: 1641-1651. http://dx.doi.org/10.1016/j.intimp.2005.05.005

[5] Zhang Y, Jiang P, Ye M, Kim SH, Jiang C, Lu J. Tanshinones: sources, pharmacokinetics and anti-cancer activities. Int J Mol Sci 2012; 13: 13621-13666. http://dx.doi.org/10.3390/ijms131013621

[6] Zhang J, Wang L, Yuan C, Wang X, Yu Z. Simultaneous determination of four kinds of tanshinone in Salvia miltiorrhiza Radix by reversed phase high-performance liquid chromatography. Chinese J Anal Chem 2005; 33: 355-358.

[7] Dong Y, Natschk SLM, Lee KH. Biosynthesis, total syntheses, and antitumor activity of tanshinones and their analogs as potential therapeutic agents. Nat Prod Rep 2011; 28: 529-542. http://dx.doi.org/10.1039/c0np00035c

[8] Lang Q, Wai CM. Supercritical fluid extraction in herbal and natural product studies – a practical review. Talanta 2001; 53: 771-782. http://dx.doi.org/10.1016/S0039-9140(00)00557-9

[9] Wang L, Song Y, Cheng Y, Liu X. Orthogonal array design for the optimization of supercritical fluid extraction of tanshinones from Danshen. J Sep Sci 2008; 31: 321-328. http://dx.doi.org/10.1002/jssc.200700386

[10] Kao TH, Chien JT, Chen BH. Extraction yield of isoflavones from soybean cake as affected by solvent and supercritical carbon dioxide. Food Chem 2008; 107: 1728-1736. http://dx.doi.org/10.1016/j.foodchem.2007.10.015

[11] Brunner G. Supercritical fluids: technology and application to food processing. J Food Eng 2005; 67: 21-23. http://dx.doi.org/10.1016/j.jfoodeng.2004.05.060

[12] Yang M, Liu A, Guan S, Sun J, Xu M, Guo D. Characterization of tanshinones in the roots of Salvia miltiorrhiza (Dan-shen) by high-performance liquid chromatography with electrospray ionization tandem mass spectrometry. Rapid Commun Mass Spectro 2006; 20: 1266-1280. http://dx.doi.org/10.1002/rcm.2447

[13] Liu AH, Lin YH, Yang M, Guo H, Guan SH, Sun JH, Guo DA. Development of the fingerprints for the quality of the roots of Salvia miltiorrhiza and its related preparations by HPLC-DAD

and LC-MS(n). J Chromatogr B 2007; 846: 32-41. http://dx.doi.org/10.1016/j.jchromb.2006.08.002

[14] Shi Z, He J, Yao T, Chang W, Zhao M. Simultaneous determination of cryptotanshinon, tanshinone I, tanshinone IIA in traditional Chinese preparations containing Radix Salvia miltiorrhiza by HPLC. J Pharm Biomed 2005; 37: 481-486. http://dx.doi.org/10.1016/j.jpba.2004.11.015

[15] Dolan TW. Shortcuts for LC measurements. LC-GC 1990; 5: 1030-1032.

[16] International Conference on Harmonization (ICH). Guideline on the validation of analysis procedures: methodology Q2B, 2013.

[17] Statistical Analysis System (SAS). SAS procedures and SAS / graph user's guide. Version 6. Cary: SAS Institute, 2014. S, Lancas F. Supercritical fluid extraction of Cymbopogoncitratus (DC.) Stapf. Chromatographia 1997; 46: 285-290.

[18] Liu H, Wang X, Wang D, Zou Z, Liang Z. Effect of drought stress on growth and accumulation of active constituents in Salvia miltiorrhiza Bunge. Ind Crop Prod 2011; 33: 84-88. http://dx.doi.org/10.1016/j.indcrop.2010.09.006

[19] Palmer MV, Ting SST. Applications for supercritical fluid technology in food processing. Food Chem 1995; 52: 345-352. http://dx.doi.org/10.1016/0308-8146(95)93280-5

[20] Yang Y, Gharaibeh A, Hawthorne SB, Miller DJ. Combined temperature/modifier effects on supercritical CO_2 extraction efficiencies of polycyclic aromatic hydrocarbons from environmental samples. Anal Chem 1995; 67: 641-646. http://dx.doi.org/10.1021/ac00099a023

[21] Sargenti S, Lancas F. Supercritical fluid extraction of *Cymbopogoncitratus* (DC.) Stapf. Chromatographia 1997; 46: 285-290.

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http://dx.doi.org/10.15379/2408-9826.2016.03.01.01