Indian Journal of Natural Products and Resources
Vol. 12(1), March 2021, pp. 74-83

Simultaneous quantitative analyses of Tanshinone I, Cryptotanshinone, and Tanshinone IIA in Danshen (*Salvia miltiorrhiza* Bunge) cultivated in Vietnam using LC-MS/MS

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Received 11 February 2020; Revised 16 December 2020

By using chromatography methods, the principal compounds tanshinon I, cryptotanshinone, tanshinone IIA were isolated from danshen (*Salvia miltiorrhiza* Bunge). Based on the spectroscopic data (^{1}H-NMR, ^{13}C-NMR and ESI-MS mass spectra), the structures were determined. The compound was purified (purity > 99.8%) by Agilent 218 purification system, which was used as the standard for analyzing tanshinon I, cryptotanshinone, tanshinone IIA in six samples. In this study, one LC-MS/MS method was developed for the simultaneous quantitative determination of three bioactive principles, tanshinone I, cryptotanshinone, and tanshinone IIA in Radix Salviae miltiorrhizae (RSM, the root of *S. miltiorrhiza*). The quantification of these diterpenoids is based on the fragments of $[M+H]^+$ under collision-activated conditions and in selected reaction monitoring (SRM) mode. The quantitative method is validated by determining the mean recovery from fortified samples of tanshinone I, cryptotanshinone, and tanshinone IIA as higher than 98%. The established method is successfully applied to the quality assessment of six batches of RSM samples collected from different regions of Vietnam. The results show that Lam Dong sample has the highest tanshinone I content (4.4286±0.0009 µg/mg), meanwhile Muong Long sample has the lowest (1.2717±0.0013µg/mg). Lam Dong sample has the highest cryptotanshinone content (8.1589±0.0006 µg/mg), whereas Guangxi-China sample has the lowest (2.8630±0.0008 µg/mg). Ha Giang sample has the highest tanshinone IIA content (13.0252±0.0004 µg/mg), whereas Muong Long sample has the lowest (3.8278±0.0003 µg/mg).

Keywords: Cryptotanshinone, LC-MS/MS, *Salvia miltiorrhiza*, Tanshinone I, Tanshinone IIA.

IPC code; Int. cl. (2015.01)- A61K 36/00, A61K 36/53, A61K 125/00

Introduction

Salvia miltiorrhiza Bunge, commonly known as red sage or danshen, is a perennial herb belonging to the Lamiaceae family[1-3]. Native to China and Japan, *S. miltiorrhiza* is also widely cultivated in other countries including Vietnam and Australia to yield typical red roots[4-7], which are highly valuable and have traditionally been used for cardiovascular diseases[2,7-9]. *S. miltiorrhiza* may offer a new strategy for the prevention and treatment of diabetes and its complications[10,11]. More than 200 individual compounds have been isolated and characterized from S. miltiorrhiza, which exhibited various pharmacological activities targeting different pathways for the treatment of cardiovascular diseases in various animal and cell models[12]. Phytochemically, nonpolar abietane-type diterpenes such as tanshinones I, IIA, and cryptotanshinone and polar phenolic acid including salvianolic acids A-C are considered as the bioactive principles of the danshen roots[2,3,6-8,13,14]. It becomes evident that the contents of tanshinone I, cryptotanshinone, and tanshinone IIA are indexed for the quality control of danshen and its conventional products, so then several analytical methods including TLC and HPLC have been developed for the determination of tanshinones[5]. Recently, the LC-MS/MS methodology with such advantage of high selectivity and sensitivity has been applied in the investigation on bioactive components including

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ginsenosides in ginseng and tanshinones in Danshen[2,3,5,7,8,14-18]. In our research on the roots of *S. miltiorrhiza* in Vietnam, the various bioactive components including several unique triterpenoids were reported by our group[4,19,20]. In respect to evaluate the danshen quality cultivated in Vietnam and compare these data with those from Australia, China, Korea, and Japan, it is necessary to develop an analytical method for the principal bioactive compounds. Therefore, in this study one feasible LC-MS/MS method has been established and validated for the quantitative determination of three major compounds, including tanshinone I, cryptotanshinone, and tanshinone IIA (Fig. 1) in *S. miltiorrhiza* samples cultivated in Vietnam.

Materials and Methods

Chemicals and reagents

Samples of *S. miltiorrhiza* were collected in different areas of Vietnam (Sa Pa, Moc Chau, Muong Long, Ha Giang, Lam Dong) and purchased from China (Guang Xi origin). LC–MS-grade solvents (acetonitrile, methanol, and formic acid) were purchased from Merck (Darmstadt, Germany) and used throughout the LC–MS study. Ultra-pure water was produced by Milli-Q Advantage system (Millipore, Milford, MA, USA). Solvents used in the extraction and isolation including *n*-hexane (Hex), methanol (MeOH), chloroform ($CHCl_3$), ethyl acetate (EtOAc), and butanol (BuOH) are all industrially standardized and re-distilled before using. Solvents used for analysis are methanol (Merck, Germany), acetonitrile (Merck, Germany), distilled water, and formic acid (Merck). Column chromatography (CC) utilizes silica gel (Kieselgel 60, 70-230 mesh and 20-400 mesh, Merck), and thin-layer chromatography (TLC) was performed on silica gel 60 F_{254} (1.05554, Merck). Substances were detected by using ultraviolet light at two wavelengths of 254 nm and 365 nm or using an I_2 vapour reagent.

Extraction and isolation

S. miltiorrhiza roots (500 g) were washed thoroughly, dried, and chopped then carefully soaked in ethanol (1.5 L) thrice using ultrasonic extraction at 40 °C for 4 hours. The resulting ethanol extracts were filtered through filter paper, collected, and concentrated under reduced pressure to afford 122 g ethanol extracts. About 100 g of extracts was dissolved in distilled water (500 mL) and extracted with Hex, EtOAc, and BuOH (three times with each solvent, 500 mL each). The fractional extracts were distilled off under reduced pressure to obtain the corresponding fractions Hex (6.1 g), EtOAc (26.4 g) and BuOH (17.2 g). In the obtained segments, the Hex fraction with a strong inhibitory effect on the development of HL-60 blood cancer cells was chromatographed using silica gel column (50 mm × 300 mm) eluted with the solvent mixture of Hex-EtOAc (5:1, v/v, 2500 mL) to yield seven subfractions ($F_1 \sim F_7$).

Subfraction F_2 (530 mg) was purified by silica gel column chromatography (30 mm × 300 mm) eluted with Hex-CH_2Cl_2 (1:1, v/v, 1500 mL), and further separated by C18 gel column chromatography (20 mm × 400 mm) eluted by MeOH-H_2O (4:1, v/v, 500 mL) solvent system. Tanshinone IIA (red powder, 75 mg) was collected from the eluted minor fractions by recrystallization. Similarly, cryptotanshinone (red powder, 45 mg) was isolated from F_6 stage (150 mg) by C18 reverse column chromatography (20 mm × 400 mm) with a 3:1 mobile phase of MeOH-H_2O, v/v, 400 mL). Finally, tanshinone I (reddish-brown, 31 mg) was purified from the F_4 subfraction (275 mg) by C18 reverse column chromatography (20 mm × 400 mm) with the mobile phase of MeOH-H_2O (4:1, v/v, 450 mL) combined with recrystallization. These compounds were checked by Agilent 218 HPLC purification system, equipped with ZORBAX SB-C18 (100 × 21.2 mm, 5.0 μm) eluted with the solvent

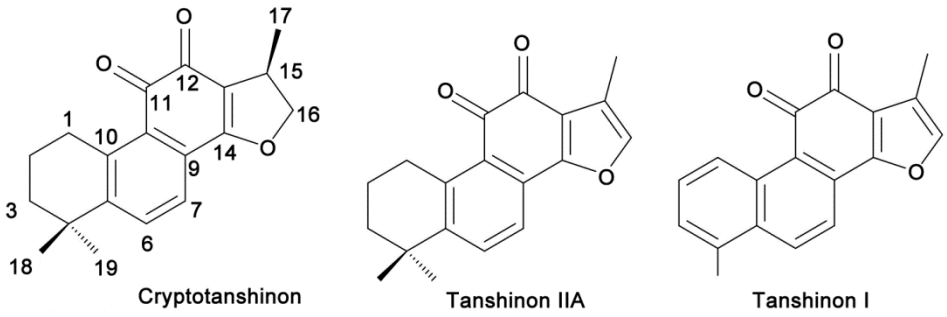

Fig. 1 — Chemical structures of the three main tanshinon compounds of Danshen (*S. miltiorrhiza*).

mixture of MeOH (A) - water (B) in 5 minutes at a rate of 7:3 (v/v), flow rate of 20.0 mL/min, and injection volume of 100.0 µL. All isolated standard compounds showed purity > 99.5%.

Instrumentation and LC–MS/MS conditions

Based on the literature[21], we had developed the chromatographic program using LC-MS/MS Agilent Technologies 6420 Triple Quad as follows: static column EC-C18 (100 x 2.1 mm, 2.7 µm), mobile phase: MeOH (A) - water containing 10 mM ammonium acetate and 0.1% HCOOH (B): 32 (v/v), flow rate: 0.25 mL/min; sample volume: 1.0 µL; column chamber temperature: 35 °C; analysis time 20 minutes; sample solvent: MeOH:H_2O - 70:30 (v/v). Mass spectral conditions: ESI ion source, positive ion, ionization source temperature of 300 °C.

Quantification was performed using multiple reaction monitoring (MRM) at transitions of *m/z* 277.2 →248.9 for tanshinone I, *m/z* 296.8→253.9 for cryptotanshinone, and *m/z* 295.0→248.8 for tanshinone IIA, respectively. Parameters for MRM were automatically optimized with a 100 ms dwell time. The optimal MS detection parameters were set as follows: interface voltage 4.5 kV, detector voltage 1.76 kV, desolvation line temperature 250 °C, and the heat block temperature 400 °C. Nitrogen was used as nebulizing gas and drying gas with a flow rate of 3.0 and 11.0 L/min, respectively. Data acquisition and quantitative analysis were carried out on the Agilent MassHunter Quantitative Analysis software (Agilent, USA).

Sample preparation

Linearity was tested for each of the reference standards at concentrations of 0.05-50 µg/mL with the correlation coefficients larger than 0.999. The intraday precisions were evaluated using the results of five replicate injections of the standard solutions containing the three tanshinones at a concentration of 1.0 mg/mL. The repeatability of the quantitative procedure was based on the results of five analyses of one batch of the RSM sample. The relative standard deviations (RSD) of precision and repeatability of three diterpenoids were both less than 3%. *S. miltiorrhiza* roots (0.02-0.04 g) were accurately weighed, and 20 mL of MeOH were added to extract at 40 °C for 2 hours with an ultrasonicator and then filtered through filter papers. Repeated three times and concentrated in a vacuum on a Buchi R-300 vacuum centrifuge system, and the final volume was set in a 100 mL volumetric flask with MeOH:H_2O 70:30. Subsequently, the filtration through the membrane 0.22 µm was pumped into the LC-MS/MS system for further analysis[14,17,18,22,23].

Results and Discussion

Identification of the standard compounds

Compounds **1-3** were isolated and structures were characterized by the comparison of their NMR and MS spectral data with the published data[7,19]. The purities of analytical standards were checked by the total peak area method carried out chromatography according to the above scheme to analyze the secondary peaks (impurities) in the chromatogram and calculate the percentages. Results showed that tanshinone I, cryptotanshinone, tanshinone IIA were purified with high purity (>99.5%) and the secondary peaks (impurities) were clearly separated from the analyzed peak.

Compound 1:red powder; mp 182-183°C; $[\alpha]_D^{25}$ = 90° (c 1,0, $CHCl_3$); ESI-MS: *m/z* 297 $[M+H]^+$; ^{1}H-NMR (400 MHz, CD_3OD): δ 7,75 (1H, d, *J* = 8,0 Hz, H-6), 7,46 (1H, *d*, *J* = 7,6 Hz, H-7), 4,89 (1H, *J* = 9,6 Hz, H-16a), 4,35 (1H, *dd*, *J* = 9,2, 6,4 Hz, H-16b), 3,49 (1H, *dd*,*J* = 9,6, 6,4 Hz, H-15), 3,40 (1H, *m*, H-1a), 3,13 (1H, *m*, H-1b), 2,16 (1H, *m*, H-3a), 1,76 (1H, *m*, H-2a), 1,74 (1H, *m*, H-3b), 1,68 (1H, *m*, H-2b), 1,30 (3H, *s*, H-18), 1,32 (3H, *s*, H-19), 1,24 (3H, *d*, J = 6,8 Hz, H-17); ^{13}C-NMR (100 Hz, CD_3OD): δ 29,7 (C-1), 19,1 (C-2), 37,8 (C-3), 34,9 (C-4), 143,7 (C-5), 132,6 (C-6), 122,5 (C-7), 128,4 (C-8), 126,3 (C-9), 152,4 (C-10), 184,3 (C-11), 175,7 (C-12), 118,3 (C-13), 170,8 (C-14), 34,6 (C-15), 81,5 (C-16), 18,9 (C-17), 31,9 (C-18), 32,0 (C-19).

Compound 1 was isolated as a reddish-orange powder. ^{1}H-NMR spectrum of **1** was characterized by a titanium triethane tanshinone diterpene compound. Of which, two signals of the proton and the 02 protons of the oxymethylene group (-CH_2O- were determined by the resonant signals at δ 7,75 (1H, d, *J* = 8,0 Hz, H-6), 7,46 (1H, *d*, *J* = 7,6 Hz, H-7), 4,89 (1H, *J* = 9,6 Hz, H-16a) and 4,35 (1H, *dd*, *J* = 9,2, 6,4 Hz, H-16b). The resonant signals at δ 1,30 (3H, *s*, H-18), 1,32 (3H, *s*, H-19), 1,24 (3H, *d*, *J* = 6,8 Hz, H-17) confirmed the existence of two methyl group germinal groups and one methyl group. The ^{13}C-NMR and DEPT spectra of **1** appeared for signals of 19 carbon. Among them, an oxymethylene group and two oxo groups (> C = O) were confirmed by resonant signals at δ 81,5 (C-16), 184,3 (C-11) và 175,7 (C-12). (C -twelfth). In addition, ^{13}C-NMR and DEPT spectra

indicate 8 resonance signals of the olefinic carbon and especially 3 methyl groups characterized by resonant signals at δ 18,9 (C-17), 31,9 (C-18) và 32,0 (C-19). Mass spectra of ESI-MS of **1** appeared at *m/z* 297 signal consistent with [M + H] + molecular ion of $C_{19}H_{20}O_3$ (M = 296). With all of the above analysis, complete matching of NMR data of **1** with the corresponding figures published[7,19] allowed to determine the chemical structure of cryptotanshinone.

Compound 2*:* red powder*;* mp 202-204^oC*;* ESI-MS: *m/z* 295 $[M+H]^+$; ^{1}H-NMR (400 MHz, CD_3OD): δ 7,63 (1H, *d*, J = 8,0 Hz, H-6), 7,56 (1H, *d*, J = 8,0 Hz, H-7), 7,23 (1H, *d*, J = 1,2 Hz, H-16), 3,19 (2H, *t*, J = 6,4 Hz, H-1), 2,27 (3H, *d*, J = 1,2 Hz, H3-17), 1,79 (2H, *m*, H-2), 1,66 (2H, *m*, H-3), 1,31 (6H, *s*, H3-18,19); ^{13}C-NMR (100 Hz, CD_3OD): δ 29,9 (C-1), 19,2 (C-2), 37,9 (C-3), 34,7 (C-4), 150,2 (C-5), 133,5 (C-6), 120,3 (C-7), 127,5 (C-8), 126,5 (C-9), 144,5 (C-10), 183,7 (C-11), 175,8 (C-12), 121,2 (C-13), 161,8 (C-14), 141,3 (C-15), 120,3 (C-16), 8,8 (C-17), 31,8 (C-18), 31,8 (C-19).

Compound 2 was collected in the form of a red powder and is the main substance of Danshen's sample as observed by TLC analysis. NMR data of the two varieties with substance **1** (cryptotanshinone) except furan ring signals (ring D) with low signal presence suggesting dehydrogenation with double bonds at C-15/C-16. This is additionally demonstrated by the ESI-MS mass spectrometry with the appearance of molecular peak ion at *m/z* 295 [M + H] + in accordance with the molecular formula $C_{19}H_{18}O_3$ (M=294). Based on the above analysis with the tanshinon IIA NMR spectra matching in the references[7,19] it is possible to confirm that substance **2** is tansinin IIA.

Compound 3: reddish brown powder; Mp 232-234 ^oC; ESI-MS: *m/z* 277 $[M + H]^+$; ^{1}H NMR (400 MHz, CD_3OD): δ9.26 (1H, d, *J* = 9.2 Hz, H-1), 8.31 (1H, d, *J* = 8.8 Hz, H-6), 7.82 (1H, d, *J* = 8.8 Hz, H-7), 7.54 (1H, dd, *J* = 9.2, 7.2 Hz, H-2), 7.35 (1H, d, *J* = 7.2 Hz, H-4), 7.30 (1H, br s, H-16), 2.71 (3H, s, H-18), 2.29 (3H, d, *J* = 1.2 Hz, H-17); ^{13}C NMR (100 Hz, CD_3OD): δ 124.7 (C-1), 130.7 (C-2), 128.4 (C-3), 135.2 (C-4), 133.7 (C-5), 133.0 (C-6), 118.7 (C-7), 129.7 (C-8), 123.2 (C-9), 132.8 (C-10), 183.6 (C-11), 175.7 (C-12), 121.8 (C-13), 161.3 (C-14), 142.0 (C-15), 120.5 (C-16), 8.8 (C-17), 19.9 (C-18).

Compound 3 is isolated as a red powder. Analysis of ^{1}H-NMR spectrum and ^{13}C-NMR of **3** found that this was also a diterpene tanshinone characteristic of Danshen (S. *miltiorrhiza*). The ESI-MS mass spectra exhibited an ionic signal at *m/z* 277 [M + H] + consistent with the molecular ion of tanshinone I, $C_{18}H_{14}O_3$ (M=276). According to the above analysis, ^{1}H-NMR and ^{13}C-NMR spectra with published data[7,19] help to confirm that substance **3** is tanshinon I.

Evaluation of the analytical method

Specificity

All blank solutions and samples of tanshinone I, cryptotanshinone, tanshinone IIA according to the above-optimized program were analyzed as illustrated chromatograms, retention times and UV spectra. The results show that no other significant peaks are observed in the chromatogram obtained with tanshinone I (t_R = 7.73 min), cryptotanshinone (t_R = 8.65 min) and tanshinone IIA (t_R = 15.17 min) (Fig. 2). The tanshinone I, cryptotanshinone, and tanshinone IIA have been characterized by high specificity.

The suitability of the system

The standard solutions of tanshinone I, cryptotanshinone, tanshinone IIA are analyzed repeatedly for six times and the respective retention time, and peak area are identified. The results show that each relative standard deviation of the parameter is lower than 1%. This indicates that the chromatographic conditions selected and the LC-MS/MS chromatographic system used are stable, suitable for qualitative, quantitative analysis of tanshinone I, cryptotanshinone, tanshinone IIA in *S. miltiorrhiza* samples.

Linearity

Standard solutions are prepared at the concentrations of 0.05-30 μg/mL (ppm) by diluting from the original stock solution with different dilution factors, followed by analyzed each in triplicate using the optimized program. The linear regression equations of concentration vs peak area are determined by the least squares method and the results obtained for tanshinone I, cryptotanshinone, tanshinone IIA have very high correlation coefficients (Fig. 2). The linear regression equations are as follows:

$$y = - 96.429 + 2.276 \times C_{Cry}, r^2 = 0.999 \quad \dots (1)$$

$$y = - 781.061 + 15.643 \times C_{Tan\text{-}1}, r^2 = 0.999 \quad \dots (2)$$

$$y = - 135.941 + 4.162 \times C_{Tan\text{-}2}, r^2 = 0.999 \quad \dots (3)$$

Limitation of detection (LOD) and limitation of quantitation (LOQ)

The standard solutions of tanshinone I, cryptotanshinone, and tanshinone IIA are diluted and

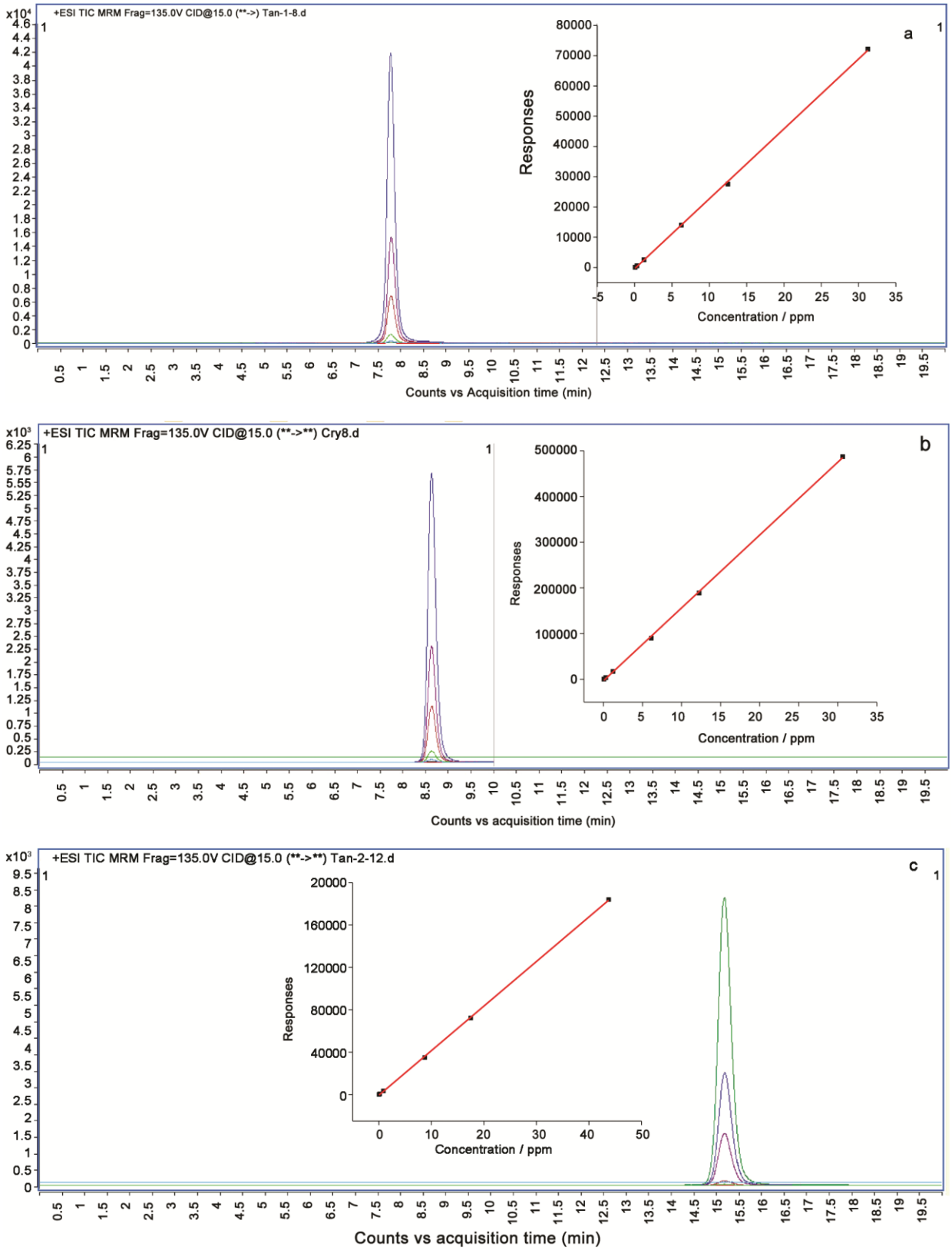

Fig. 2 — Graph showing the standard curves of (a) tanshinone I, (b) cryptotanshinone and (c) tanshinone IIA.

analyzed until the signal in the chromatogram obtained has S/N (signal height/noise) ratio of about 2-3. The determined concentration is LOD of the method for each substance. LOQ of the method is determined based on LOD following the equation LOQ = 3.3 × LOD. Results of the analysis show that the LODs for tanshinone I, cryptotanshinone, tanshinone IIA are 0.39, 0.4, and 0.35 ng/mL with LOQs of 1.3, 1.4, and 1.2 ng/mL, respectively.

Correctness

The correctness of the tanshinone I, cryptotanshinone, tanshinone IIA analyzes is determined by the recovery formula:

$$\mathrm{Re}\,v(\%) = \frac{C_2 - C_1}{C_o} \times 100$$

Here C_0 is the concentration of the analyte added in the sample; C_1 is the concentration of the analyte in the sample; C_2 is the concentration of the analyte in the sample that has been added. The recovery of the method is also determined by carrying out the analysis in triplicate according to the procedures shown above. Then a quantity of standard reagent is added followed by analysis in triplicate, and the mean results are calculated. The recovery results of the method are given in Tables 1-3. From the calculated results in Tables 1-3, the developed method for determining the contents of tanshinone I, cryptotanshinone, and tanshinone IIA displays high recovery percentages ranging from 96.0 to 104.0 %, in accordance with the requirements of AOAC[24,25]. Therefore, the LC-MS/MS method achieves good accuracy, so it can be applied to analyze tanshinone I, cryptotanshinone, tanshinone IIA in *S. miltiorrhiza* samples.

Repeatability

The samples containing tanshinone I, cryptotanshinone, and tanshinone IIA are analyzed for six replications. Concentrations of tanshinone I, cryptotanshinone, and tanshinone IIA are 12.25, 12.5, and 8.75 µg/mL (Fig. 3), and RSD values are 0.03 for tanshinone I, 0.02 for cryptotanshinone, 0.04 for tanshinone IIA, respectively. RSD values are less than 1/2 RSD_H and it indicates good repeatability and stability for the present method[24,25].

Analysis of tanshinone derivatives in real samples

The contents of tanshinone I, cryptotanshinone, and tanshinone IIA in the real danshen samples are determined under optimal conditions (Fig. 4). The linear regression line is constructed and the interpolation method is used to analyze the content of tanshinone I, cryptotanshinone, tanshinone IIA in the samples. The results are presented in Tables 4-6 and these data show that the LC-MS/MS method for the analysis of the medical plant samples of *S.*

Table 1 — Evaluation of accuracy for tanshinone I*

Sample	C_x	0.3			1.0			2.0			Rev_{TB}
	Experiment	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	(%)
	C_0	0.7997	0.8000	0.8001	0.7997	0.8000	0.8001	0.7997	0.8000	0.8001	
Sapa	C	1.1012	1.0941	1.1109	1.8211	1.7809	1.8321	2.8751	2.8112	2.7501	
	Rev	100.50	98.03	103.60	102.14	98.09	103.20	103.77	100.56	97.50	100.82
	C_0	1.4218	1.4212	1.4217	1.4218	1.4212	1.4217	1.4218	1.4212	1.4217	
Lam Dong	C	1.7092	1.7094	1.7139	2.4511	2.4059	2.4121	3.4751	3.4512	3.3501	
	Rev	95.80	96.06	97.40	102.93	98.47	99.04	102.66	101.50	96.42	98.92
	C_0	1.0996	1.0989	1.0991	1.0996	1.0989	1.0991	1.0996	1.0989	1.0991	
Ha Giang	C	1.3892	1.3941	1.3909	2.1211	2.1059	2.0821	3.0751	3.1512	3.0501	
	Rev	96.53	98.40	97.26	102.15	100.70	98.30	98.77	102.62	97.55	99.14
	C_0	0.8887	0.8884	0.8889	0.8887	0.8884	0.8889	0.8887	0.8884	0.8889	
Moc Chau	C	1.1792	1.1841	1.1809	1.9211	1.9059	1.8721	2.8751	2.9512	2.9301	
	Rev	96.83	98.56	97.33	103.24	101.75	98.32	99.32	103.14	102.06	100.06
	C_0	0.4978	0.4984	0.4976	0.4978	0.4984	0.4976	0.4978	0.4984	0.4976	
Guangxi	C	0.7921	0.7892	0.7879	1.5211	1.5059	1.4872	2.4751	2.5121	2.5301	
	Rev	98.10	96.93	96.76	102.33	100.75	98.96	98.86	100.68	101.62	99.45
	C_0	0.4080	0.4080	0.4087	0.4080	0.4080	0.4087	0.4080	0.4080	0.4087	
Muong Long	C	0.7201	0.6992	0.7009	1.4211	1.4059	1.4172	2.4751	2.3901	2.4331	
	Rev	98.03	97.06	97.40	101.31	99.79	100.85	103.35	99.10	101.22	99.79

(*) C_x: *Concentration of the standard added to the sample*; C_o: *Concentration in the sample (µg / mL)*; C: *Concentration in the sample after added standard*; Rev: *Recovery*; Rev_{TB}: *Medium recovery* (n = 9). C_x, $C_{0,}$ and C is in ug/mL and Rev is in %

Table 2 — Evaluation of accuracy for cryptotanshinone

Sample	C_x	0.5			1.5			3.0			Rev_{TB} (%)
	Experiment	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	
Sapa	C_0	1.5126	1.5128	1.5130	1.5126	1.5128	1.5130	1.5126	1.5128	1.5130	
	C	2.0021	2.0173	1.9992	3.0511	2.9759	3.0172	4.4751	4.5901	4.4331	
	Rev	97.90	100.90	97.24	102.56	97.54	100.28	98.75	102.57	97.33	99.45
Lam Dong	C_0	2.6190	2.6188	2.6192	2.6190	2.6188	2.6192	2.6190	2.6188	2.6192	
	C	3.1071	3.1133	3.1072	4.0913	4.1705	4.1004	5.5955	5.6514	5.5331	
	Rev	97.62	98.90	97.60	98.15	103.44	98.74	99.21	101.08	97.13	99.10
Ha Giang	C_0	1.6173	1.6178	1.6182	1.6173	1.6178	1.6182	1.6173	1.6178	1.6182	
	C	2.1017	2.1343	2.1127	3.1023	3.1695	3.1064	4.5458	4.6574	4.5371	
	Rev	96.88	103.30	98.90	99.00	103.45	99.21	97.62	101.32	97.30	99.66
Moc Chau	C_0	1.2853	1.2849	1.2850	1.2853	1.2849	1.2850	1.2853	1.2849	1.2850	
	C	1.7722	1.8034	1.7912	2.7628	2.7865	2.8336	4.2544	4.1697	4.3712	
	Rev	97.38	103.70	101.24	98.50	101.11	103.24	98.97	96.16	102.87	100.24
Guangxi	C_0	0.9164	0.9159	0.9163	0.9164	0.9159	0.9163	0.9164	0.9159	0.9163	
	C	1.4042	1.4139	1.3996	2.4682	2.4405	2.3963	4.0374	3.9677	3.8379	
	Rev	97.56	99.60	96.66	103.45	101.64	98.66	104.03	101.72	97.40	100.10
Muong Long	C_0	1.2509	1.2501	1.2505	1.2509	1.2501	1.2505	1.2509	1.2501	1.2505	
	C	1.7394	1.7391	1.7350	2.7692	2.7185	2.7936	4.2037	4.2647	4.3097	
	Rev	97.70	97.80	96.90	101.22	97.89	102.87	98.43	100.50	101.98	99.50

Where C_x, C_0 and C is in ug/mL and Rev is in %

Table 3 — Evaluation of accuracy for tanshinone IIA

Sample	C_x	1.0			3.0			6.0			Rev_{TB} (%)
	Experiment	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	
Sapa	C_0	2.9863	2.9865	2.9865	2.9863	2.9865	2.9865	2.9863	2.9865	2.9865	
	C	3.9754	3.9639	3.9753	6.0692	5.9155	5.9376	9.0203	9.1264	9.0309	
	Rev	98.91	97.74	98.88	102.76	97.63	98.37	100.56	102.33	100.34	99.77
Lam Dong	C_0	3.1757	3.1775	3.1775	3.1757	3.1775	3.1775	3.1757	3.1775	3.1775	
	C	4.1504	4.1603	4.1735	6.1959	6.1135	6.0937	9.1020	9.2264	9.2430	
	Rev	97.47	98.28	99.60	100.67	97.87	97.20	98.77	100.82	101.10	99.10
Ha Giang	C_0	4.0510	4.0507	4.0507	4.0510	4.0507	4.0507	4.0510	4.0507	4.0507	
	C	5.0314	5.0196	5.0435	7.0915	6.9853	7.1032	10.1202	10.0296	10.2243	
	Rev	98.04	96.89	99.28	101.35	97.82	101.75	101.15	99.65	102.89	99.87
Moc Chau	C_0	2.5823	2.5826	2.5826	2.5823	2.5826	2.5826	2.5823	2.5826	2.5826	
	C	3.5641	3.5609	3.5705	5.6191	5.4985	5.6503	8.5247	8.6297	8.7224	
	Rev	98.18	97.83	98.79	101.23	97.20	102.26	99.04	100.78	102.33	99.74
Guangxi	C_0	1.5347	1.5348	1.5348	1.5347	1.5348	1.5348	1.5347	1.5348	1.5348	
	C	2.4991	2.4965	2.5071	4.6019	4.4968	4.4543	7.5284	7.6029	7.7022	
	Rev	96.44	96.17	97.23	102.24	98.73	97.32	99.89	101.14	102.79	99.11
Muong Long	C_0	1.2288	1.2286	1.2286	1.2288	1.2286	1.2286	1.2288	1.2286	1.2286	
	C	2.1909	2.2096	2.2237	4.2192	4.2968	4.1904	7.2148	7.1672	7.3012	
	Rev	96.21	98.10	99.51	99.68	102.27	98.72	99.76	98.98	101.21	99.40

Where C_x, C_0 and C is in ug/mL and Rev is in %

miltiorrhiza achieved a high precision with RSD of less than 1%. One-way ANOVA was used to evaluate the differences between the analytical results within the significance level. Results showed that the contents of tanshinone I, cryptotanshinone, and tanshinone IIA in the samples collected in different locations (Tables 4-6) were statistically different (tanshinone I: $F(5) = 3.81$, $p < 0.001$; cryptotanshinone: $F(5) = 1.13$, $p < 0.001$; tanshinone IIA: $F(5) = 1.58$, $p < 0.001$). The present experimental results show that the highest content of tanshinone I in the sample collected in Lam Dong (4.4286±0.0009 µg/mg), and the lowest was found in the sample from Muong Chong (1.2717±0.0013 µg/mg). Cryptotanshinone content is highest in the sample from Lam Dong (8.1589±0.0006 µg/mg), and the lowest was found in the sample from Guangxi-China (2.88630±0.0008 µg/mg). For tanshinone IIA,

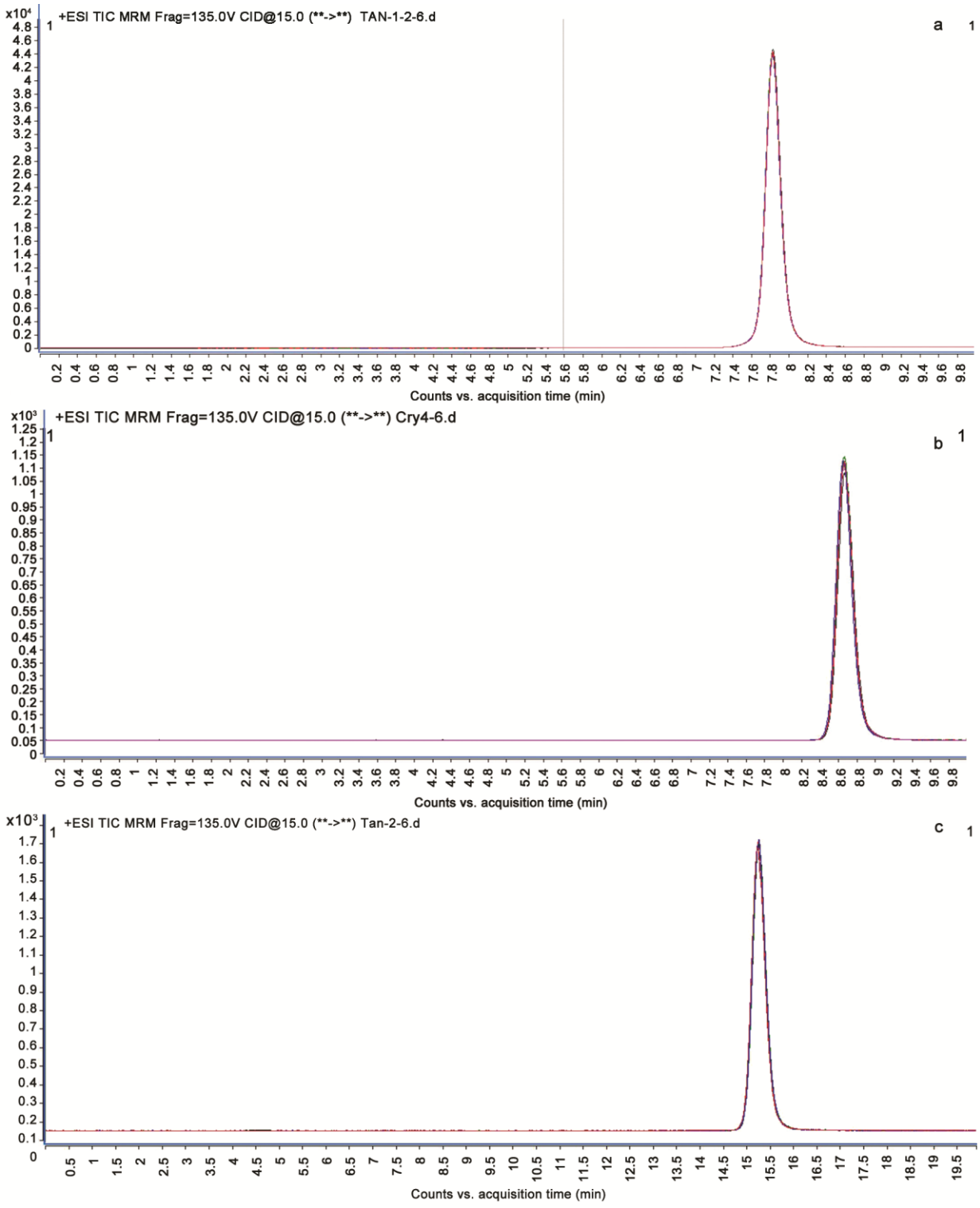

Fig. 3 — Repeatability of the signal (a) tanshinone I, (b) cryptotanshinone and (c) tanshinone IIA.

the highest content is shown in the sample from Ha Giang (13.0252±0.0004 µg/mg), and the lowest sampling from Muong Long (3.8278±0.0003 µg/mg). Danshen samples collected in Lam Dong, Ha Giang have high contents of three diterpenoids tanshinone I, cryptotanshinone, and tanshinone IIA, which indicates the quality of medicinal herbs collected in these provinces are excellent.

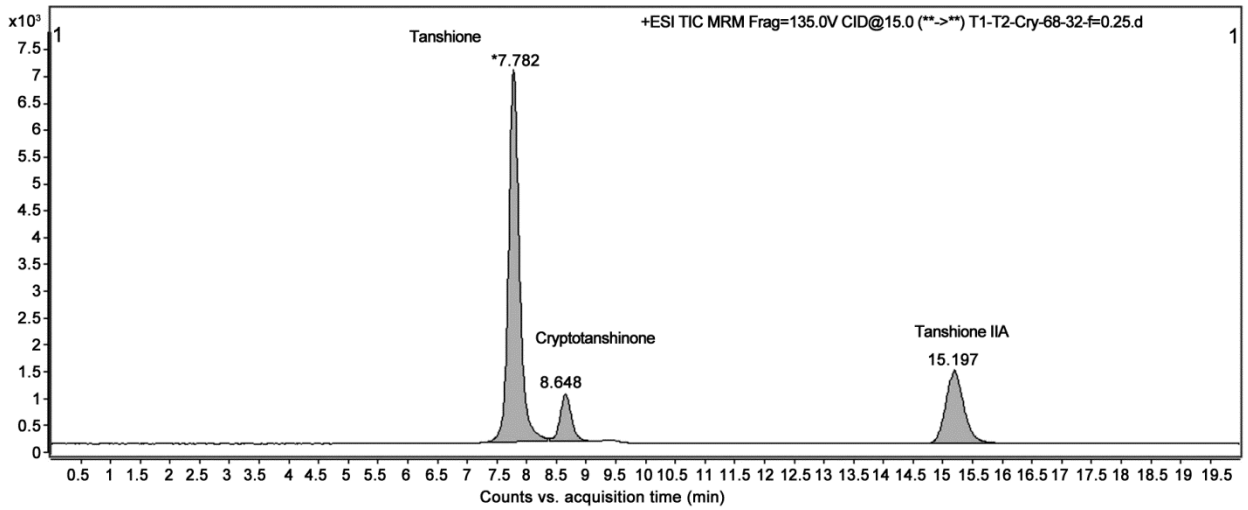

Fig. 4 — Chromatography of the standard substance.

Table 4 — Determined contents for tanshinone I in the samples

Sample	Calculated tanshinone I content, µg/mg					
	1st	2nd	3rd	Average ± SD*	RSD** (%)	1/2.RSD$_H$ (%)
Sa Pa	2.4606	2.4614	2.4619	2.4613±0.0007	0.03	2.47
Lam Dong	4.4293	4.4275	4.4289	4.4286±0.0009	0.02	2.26
Ha Giang	3.5357	3.5335	3.5342	3.5345±0.0011	0.03	2.34
Moc Chau	2.8853	2.8843	2.8861	2.8852±0.0009	0.03	2.41
Guangxi	1.5555	1.5574	1.5549	1.5559±0.0013	0.08	2.65
Muong Long	1.2711	1.2709	1.2732	1.2717±0.0013	0.10	2.73

*SD: Standard deviation, **RSD: Relative standard deviation

Table 5 — Determined contents for cryptotanshinone in the samples

Sample	Calculated cryptotanshinone content, µg/mg					
	1st	2nd	3rd	Average ± SD	RSD (%)	1/2.RSD$_H$ (%)
Sa Pa	4.6543	4.6549	4.6555	4.6549±0.0006	0.01	2.24
Lam Dong	8.1590	8.1583	8.1595	8.1589±0.0006	0.01	2.06
Ha Giang	5.2002	5.2019	5.2031	5.2017±0.0015	0.03	2.21
Moc Chau	4.1729	4.1719	4.1722	4.1723±0.0005	0.01	2.28
Guangxi	2.8636	2.8621	2.8634	2.8630±0.0008	0.03	2.41
Muong Long	3.8969	3.8945	3.8955	3.8956±0.0012	0.03	2.30

Table 6 — Determined contents for tanshinone IIA in the samples

Sample	Calculated tanshinone IIA content, µg/mg					
	1st	2nd	3rd	Average ± SD	RSD (%)	1/2.RSD$_H$ (%)
Sa Pa	9.1887	9.1889	9.1891	9.1889±0.0002	0.002	2.03
Lam Dong	9.893	9.8991	9.8989	9.8970±0.0035	0.035	2.00
Ha Giang	13.0257	13.0251	13.0249	13.0252±0.0004	0.003	1.92
Moc Chau	8.3841	8.3839	8.3851	8.3844±0.0006	0.008	2.05
Guangxi	4.7958	4.7951	4.7964	4.7958±0.0007	0.014	2.23
Muong Luong	3.8279	3.8281	3.8275	3.8278±0.0003	0.008	2.31

Conclusion

By using chromatography methods, we isolated the principal compounds tanshinon I, cryptotanshinone, and tanshinone IIA from *Salvia miltiorrhiza* Bunge. Based on the spectroscopic data (^{1}H-NMR, ^{13}C-NMR and ESI-MS mass spectra), the structure was determined. The compound was purified (purity >99.8%) by Agilent 218 purification system, which was used as a standard for analyzing tanshinon I, cryptotanshinone, tanshinone IIA in six samples. The tanshinone diterpenoids are the main active ingredients that are considered as the specific

medicinal substances of *S. miltiorrhiza*. The present analytical method for tanshinone I, cryptotanshinone, and tanshinone IIA has high correlation coefficients, tight linearity, wide linear range, and good qualitative & quantitative limits. In conclusion, this developed LC-MS/MS method has the potential to analyze tanshinone I, cryptotanshinone, and tanshinone IIA in real danshen samples.

Conflicts of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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