Optimizing Ultraperformance Liquid Chromatographic Analysis of 10 Diterpenoid Compounds in Salvia miltiorrhiza Using Central Composite Design

ARTICLE *in* JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY · MARCH 2008

Impact Factor: 2.91 · DOI: 10.1021/jf073020u · Source: PubMed

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Optimizing Ultraperformance Liquid Chromatographic Analysis of 10 Diterpenoid Compounds in *Salvia miltiorrhiza* Using Central Composite Design

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A rapid, sensitive, reproducible, and accurate ultraperformance liquid chromatographic (UPLC) method was developed for the simultaneous determination of 10 diterpenoid compounds (tanshinone I, tanshinone IIA, cryptotanshinone, dihydrotanshinone I, 1,2-dihydrotanshinquinone, methylenetanshinquinone, miltirone, 5,6-dehydrosugiol, sugiol, and przewalskin) in *Salvia miltiorrhiza* for the first time. Central composite design was applied as a powerful tool to optimize the most dominant parameters that influence the resolution of UPLC, that is, gradient, flow rate, and column temperature. Under optimum conditions, all peaks except 1,2-dihydrotanshinquinone and methylenetanshinquinone could be baseline separated within 8 min. Furthermore, the contents of these compounds in *S. miltiorrhiza* samples collected from different provinces of China have also been compared. The results showed that UPLC is one of the most efficient methods for the analysis of diterpenoid compounds in *S. miltiorrhiza* and that it is a potential method for quality control of this valuable traditional Chinese medicine.

KEYWORDS: Ultraperformance liquid chromatography (UPLC); diterpenoid; *Salvia miltiorrhiza*; central composite design

INTRODUCTION

The dried root and rhizome of *Salvia miltiorrhiza*, which are called danshen, have been used clinically in traditional Chinese medicine for thousands of years and are now widely used for promoting circulation and removing blood stasis (*1*). In addition, to a lesser extent, *S. miltiorrhiza* has been used as an effective herbal medicine for the treatment of cardiovascular and cerebrovascular diseases in Japan, the United States, and some European countries (*2*).

The chemical components of *S. miltiorrhiza* could be classified as hydrophobic and hydrophilic parts. To date, more than 70 hydrophobic compounds have been isolated and identified from *S. miltiorrhiza*, most of which are diterpenoids involving tanshinones of *o*-quinonoids, royleanones of *p*-quinonoids, and other types. Some of these diterpenoid compounds are believed to be the major bioactive ingredients in *S. miltiorrhiza* with antioxidative, anti-inflammatory, antitumor, and angiogenesis-modulating activities (*3–10*). Due to the importance of these compounds in the quality control of *S. miltiorrhiza* and related preparations, several analytical techniques including HPLC (*11, 12*), LC-MS (*13*), high-speed countercurrent chromatography (HSCCC) (*14, 15*), and capillary electrochromatography (CEC) (*16*) have been developed for the qualitative and quantitative analysis of these diterpenoids in recent years.

Ultraperformance liquid chromatography (UPLC) is a newly developed analytical technology that takes advantage of small 1.7 μm particles operated at elevated pressures to achieve uncompromised separation speed, resolution, and sensitivity. In this paper, a rapid, sensitive, accurate, and reproducible UPLC method was developed for the simultaneous determination of 10 diterpenoid compounds in *S. miltiorrhiza* for the first time. Using a univariate approach, we found that the peaks of cryptotanshinone and tanshinone I could not be baseline separated within 10 min. Therefore, a more specific central composite design (CCD) was applied to optimize the most dominant parameters that influence the resolution of UPLC, that is, gradient condition, flow rate, and column temperature. The

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10.1021/jf073020u CCC: $40.75
Published on Web 01/17/2008

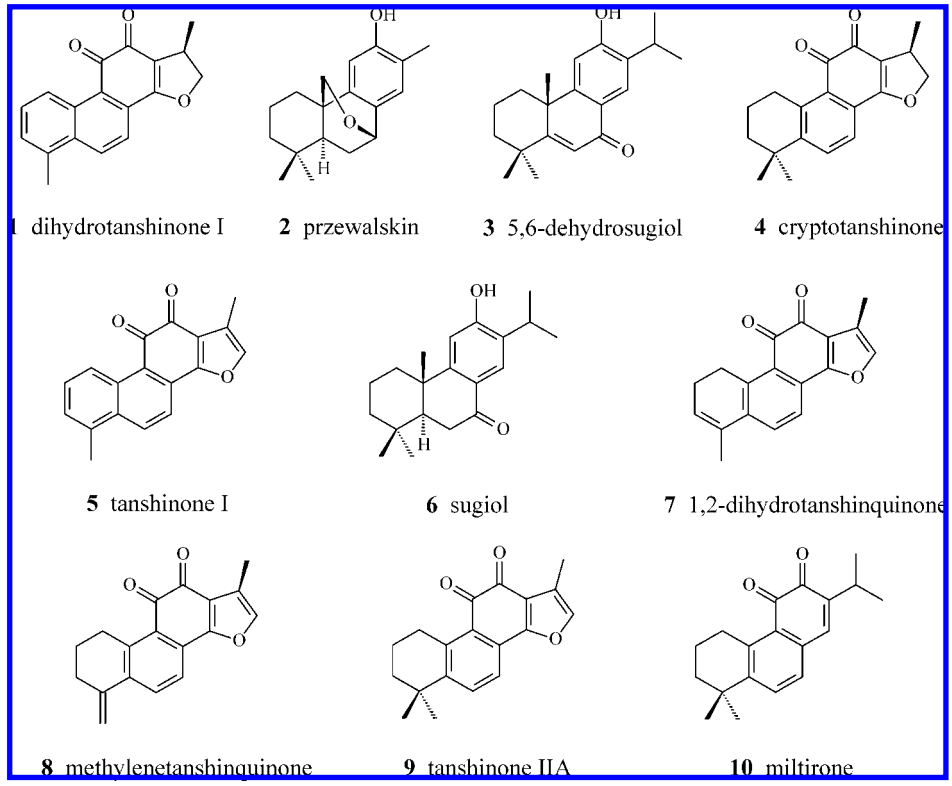

Figure 1. Chemical structures of 10 diterpenoids in *S. miltiorrhiza*.

Table 1. Central Composite Design Matrix of Three Test Variables in Coded and Natural Units along with the Observed Responses

no.	time$_{(B: 45–56\%)}$ (min)[a]	flow rate (mL/min)	temperature (°C)	Rs$_{(4\&5)}$[b]	Rt$_{(10)}$ (min)[c]
1	3.4[d] (−1[e])	0.24 (−1)	29 (−1)	1.46	7.97
2	4.6 (1)	0.24 (−1)	29 (−1)	1.49	9.05
3	3.4 (−1)	0.36 (1)	29 (−1)	1.38	7.03
4	4.6 (1)	0.36 (1)	29 (−1)	1.36	7.83
5	3.4 (−1)	0.24 (−1)	41 (1)	0.64	7.85
6	4.6 (1)	0.24 (−1)	41 (1)	0.68	8.85
7	3.4 (−1)	0.36 (1)	41 (1)	0.63	6.57
8	4.6 (1)	0.36 (1)	41 (1)	0.70	8.06
9	3 (−1.668)	0.3 (0)	35 (0)	1.12	7.22
10	5 (1.668)	0.3 (0)	35 (0)	1.14	8.89
11	4 (0)	0.2 (−1.668)	35 (0)	1.05	8.90
12	4 (0)	0.4 (1.668)	35 (0)	1.09	7.49
13	4 (0)	0.3 (0)	25 (−1.668)	1.92	8.61
14	4 (0)	0.3 (0)	45 (1.668)	0.69	7.99
15–20	4 (0)	0.3 (0)	35 (0)	1.06	8.08

[a] Persistent time during the gradient of B from 45 to 56%. [b] Resolution of the peaks 4 and 5. [c] Retention time of peak 10. [d] Test variable in natural value. [e] Test variable in coded value.

resolution of cryptotanshinone with tanshinone I and the retention time of miltirone were employed to evaluate the response function.

EXPERIMENTAL PROCEDURES

Materials. Samples of *S. miltiorrhiza* were purchased from Ji'nan (Shandong), Yong'an (Fujian), Beihai (Guangxi), Chengdu (Sichuan), Zhengzhou (Hebei), Hangzhou (Zhejiang), Nanjing (Jiangsu), Heifei (Anhui), and Chongqing districts of China. The botanical origins of materials were identified by Prof. Shengwu Tang, Chengdu University of Traditional Chinese Medicine, Chengdu, China. The voucher specimens (no. SM 05001-009) were deposited at the Institute of Chinese Medical Sciences, University of Macau, Macau, China. Ten diterpenoids involving dihydrotanshinone I (**1**), przewalskin (**2**), 5,6-dehydrosugiol (**3**), cryptotanshinone (**4**), tanshinone I (**5**), sugiol (**6**), 1,2-dihydrotanshinquinone (**7**), methylenetanshinquinone (**8**), tanshinone IIA (**9**) and miltirone (**10**) (structures shown in **Figure 1**) were isolated from *S. miltiorrhiza*. Their structures have been elucidated by comparison of their spectroscopic data including UV, IR, MS, ^{1}H and ^{13}C NMR with literature data (*17–23*). HPLC-grade acetonitrile was a product of Merck (Darmstadt, Germany), and deionized water was purified by a Milli-Q purification system (Millipore, Bedford, MA).

Sample Preparation. Sample preparation was performed with a pressurized liquid extraction (PLE) technique using a Dionex ASE 200 (Sunnyvale, CA) system equipped with a 24 sample carousel. Dried powder of *S. miltiorrhiza* (0.5 g) was mixed with diatomaceous earth in a proportion of 1:2 and placed into an 11 mL stainless steel extraction cell. The extraction cells were placed into the carousel, and the samples were extracted under the optimized conditions: solvent, ethanol; temperature, 100 °C; static extraction time, 10 min; one cycle and one time extraction. Pressure and flush volume were set at their default values, that is, 1500 psi and 60%, respectively. The extract was transferred to a 25 mL volumetric flask, which was brought up to volume with extraction solvent and filtered through a 0.2 μm nylon membrane filter (Whatman, Maidstone, U.K.) prior to injection into the UPLC system.

UPLC Analysis. The quantitative analyses were performed on a Waters Acquity UPLC system (Waters, Milford, MA), equipped with binary solvent manager, sampler manager, column compartment, and PDA detector, connected to Waters Empower 2 software. An Acquity UPLC BEH C18 column (50 mm × 2.1 mm i.d., 1.7 μm) was used. The standards and samples were separated using a gradient mobile phase consisting of water (A) and acetonitrile (B). The sample manager temperature was set at 15 °C, and the injection volume was 1 μL. Peaks were detected at 270 nm. The gradient condition, the flow rate, and the column temperature were optimized by central composite design.

Statistical Analysis. Statistical analysis was carried out by Statistica data analysis software system for Windows, version 6.0 (StatSoft, Inc., 2001), which comprises a number of "procedures"—graphical, statistical, reporting, processing, and tabulating procedures—that enable simple and rapid data evaluation.

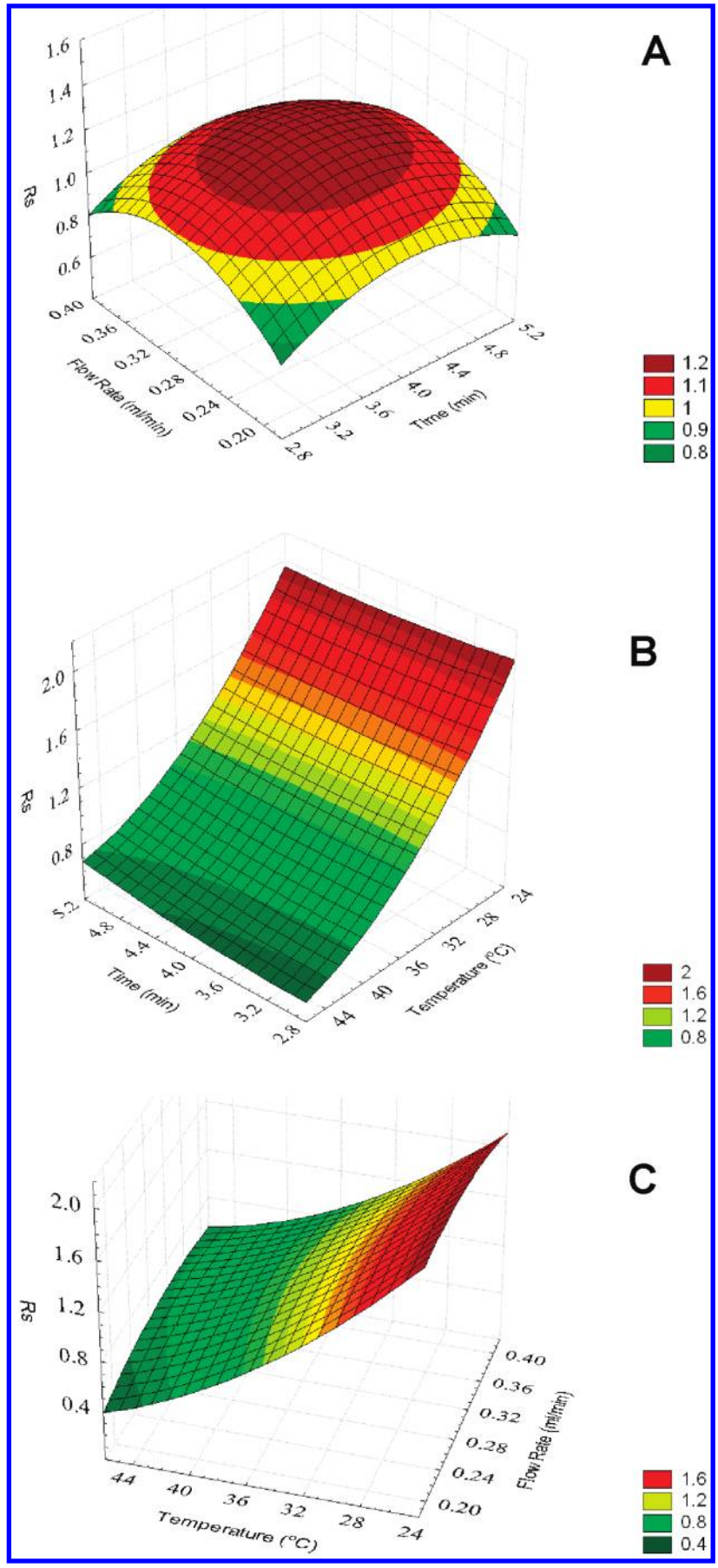

Figure 2. Response surface for resolution response function of cryptotanshinone (**4**) and tanshinone I (**5**): (**A**) $time_{(B:\ 45–56\%)}$ (min) versus flow rate (mL/min); (**B**) $time_{(B:\ 45–56\%)}$ (min) versus column temperature (°C); (**C**) flow rate (mL/min) versus column temperature (°C).

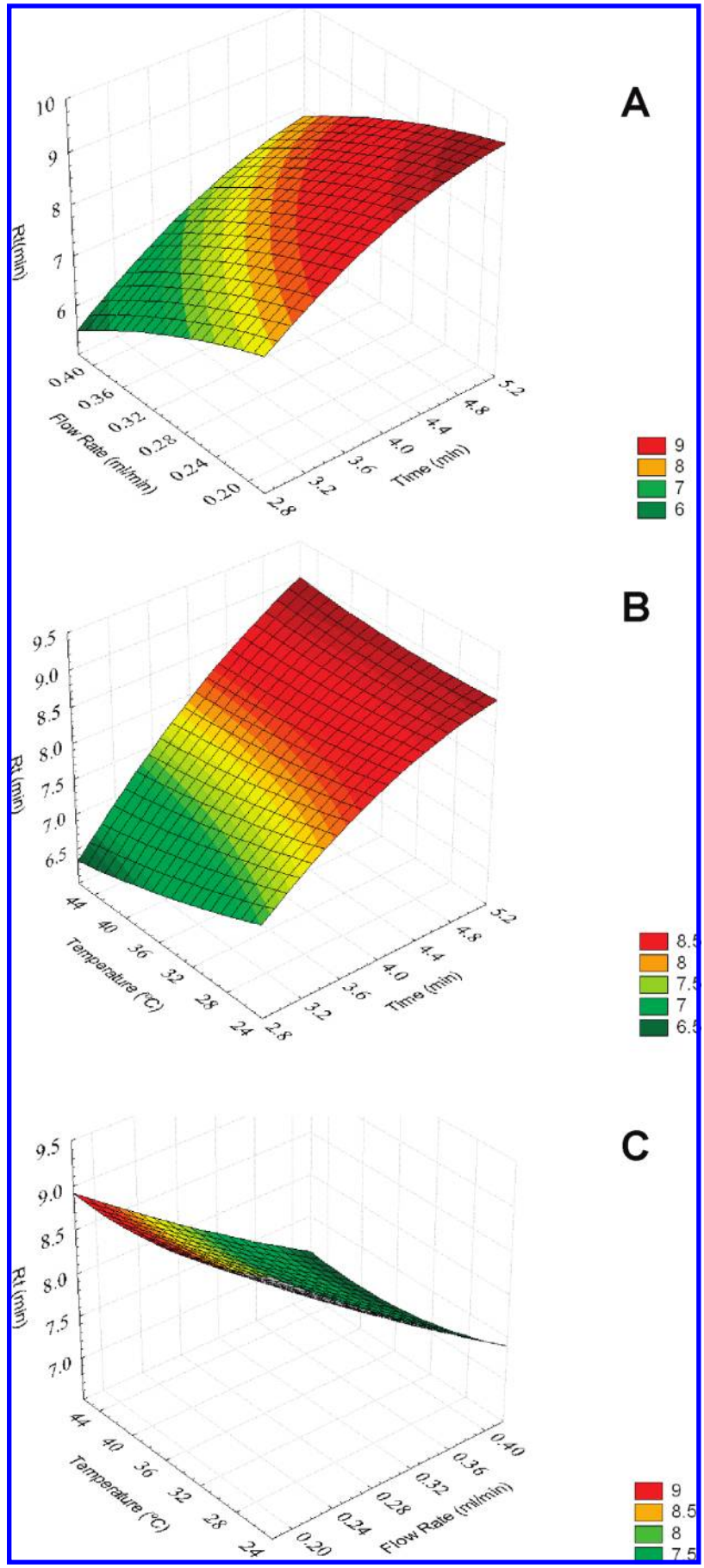

Figure 3. Response surface for the retention time of miltirone ($Rt_{(10)}$): (**A**) $time_{(B:\ 45–56\%)}$ (min) versus flow rate (mL/min); (**B**) $time_{(B:\ 45–56\%)}$ (min) versus column temperature (°C); (**C**) flow rate (mL/min) versus column temperature (°C).

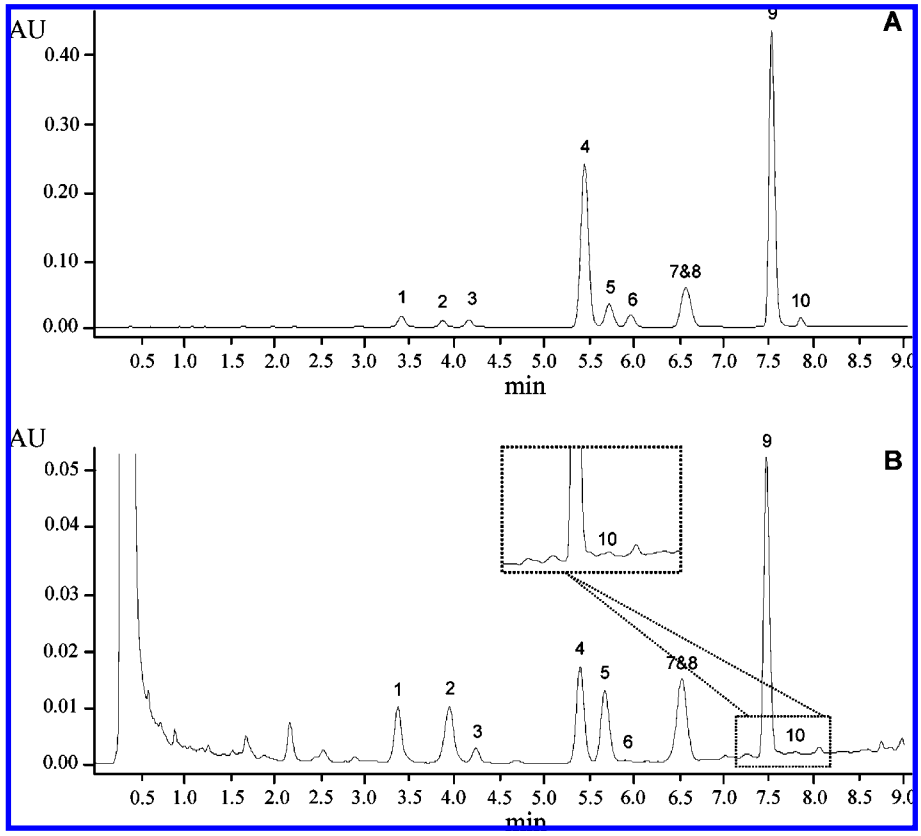

Figure 4. Typical UPLC chromatograms of (**A**) mixed standards and (**B**) PLE extract of *S. miltiorrhiza* from Guangxi. Peaks: 1, dihydrotanshinone I; 2, przewalskin; 3, 5,6-dehydrosugiol; 4, cryptotanshinone; 5, tanshinone I; 6, sugiol; 7, 1,2-dihydrotanshinquinone; 8, methylenetanshinquinone; 9, tanshinone IIA; 10, miltirone.

Table 2. Linear Regression Data, LOD, and LOQ of Investigated Compounds

	linear regression data					
analyte	regression eq	test range (μg/mL)	R^2	LOD (μg/mL)	LOQ (μg/mL)	recovery (%) (RSD%, $n = 3$)
dihydrotanshinone I	$y = 5284.6x + 1289.7$	0.46−7.35	0.9998	0.05	0.18	105.50 (3.65)
przewalskin	$y = 3093.9x - 212.18$	1.07−34.20	0.9998	0.21	0.72	104.33 (2.66)
5,6-dehydrosugiol	$y = 3396.2x - 144.57$	0.93−29.60	0.9998	0.19	0.84	95.96 (3.32)
cryptotanshinone	$y = 22556x - 8764.4$	3.66−117.00	0.9998	0.03	0.09	100.98 (1.17)
tanshinone I	$y = 20095x + 936.24$	0.48−15.20	0.9996	0.02	0.06	98.35 (1.92)
sugiol	$y = 10159x - 456.78$	0.76−24.40	0.9996	0.06	0.31	96.47 (3.64)
1,2-dihydrotanshinquinone/ methylenetanshinquinone	$y = 87859x - 4315.5$	0.41−13.20	0.9996	0.01	0.03	103.39 (2.84)
tanshinone IIA	$y = 28092x - 19064$	4.50−144.00	0.9995	0.02	0.04	101.27 (0.87)
miltirone	$y = 2283.8x + 356.36$	0.63–20.20	1	0.13	0.38	96.64 (2.95)

RESULTS AND DISCUSSION

Chromatographic Optimization by Central Composite Design. For obtaining the best separation results, the chromatographic conditions of UPLC should be optimized. Solvents that constituted the mobile phase were A (water) and B (acetonitrile). In the preliminary experiments, the elution conditions applied were as follows: 0–2.5 min, linear gradient 42–45% B; 2.5–5.5 min, linear gradient 45–56% B; 5.5–8.0 min, linear gradient 56–85% B; 8.0–10.0 min, linear gradient 85–100% B; and, finally, reconditioning steps of the column was 42% B isocratic for 3 min after washing column with 100% B for 2 min. The flow rate was 0.3 mL/min, and the system operated at 25 °C. Using the univariate approach, all peaks had good resolution in a shorter analytical run time under the optimized chromatographic conditions except cryptotanshinone (**4**) and tanshinone I (**5**), which could not be baseline separated within 10 min. Although these two compounds could be separated from each other while the analytical run time was increased to 15 min, it negated the superiority of UPLC compared to HPLC. The persistent time of the duration of gradient of B from 45 to 56% ($time_{(B:\ 45–56\%)}$), the flow rate, and the column temperature were found to be the most dominant parameters that influenced the resolution of the peaks 4 and 5 ($Rs_{(4\&5)}$). In addition, the retention time of peak 10 ($Rt_{(10)}$) was another remarkably significant factor, which corresponded directly to the entire analytical run time. Therefore, a more specific central composite design (CCD) was applied to optimize these three parameters, that is, the $time_{(B:\ 45–56\%)}$), the flow rate, and the column temperature. The $Rs_{(4\&5)}$ and $Rt_{(10)}$ were employed to evaluate the response function.

The levels of the variables ($time_{(B:\ 45–56\%)}$, flow rate, and column temperature) investigated in this study are given in **Table 1**. Each factor in the design was studied at five different levels (−1.668, −1, 0, 1, 1.668). All variables were taken at a central coded value considered as zero. In general, CCD is constructed in such a way that $2^f + 2f + 1$ experiments are

Table 3. Intra- and Interday Precision of the Investigated Compounds

		intraday ($n = 6$)			interday ($n = 6$)		
analyte	concn (μg/mL)	found (μg/mL)	RSD (%)	accuracy (%)	found (μg/mL)	RSD (%)	accuracy (%)
dihydrotanshinone I	7.35	7.50	3.51	102.0	7.31	2.28	99.4
	3.68	3.79	2.63	103.0	3.82	2.71	103.8
	0.92	0.92	3.29	100.5	0.93	3.05	101.4
przewalskin	34.20	34.89	3.34	102.0	35.08	3.68	100.5
	8.55	8.94	1.67	104.6	9.02	2.35	105.6
	2.14	2.03	3.62	95.0	2.02	2.53	94.4
5,6-dehydrosugiol	29.60	30.09	3.15	101.7	30.75	3.79	103.9
	7.40	7.84	3.52	106.0	7.68	4.66	103.8
	1.85	1.77	1.58	95.4	1.79	3.95	97.0
cryptotanshinone	117.00	118.20	1.06	101.0	119.37	1.70	102.0
	29.25	30.25	0.16	103.4	30.27	0.11	103.5
	7.31	7.04	2.63	96.3	6.91	2.41	94.6
tanshinone I	15.20	14.93	3.80	98.2	14.95	3.05	98.4
	3.80	3.73	2.29	98.2	3.73	2.37	98.3
	0.95	1.01	1.92	106.3	0.99	1.83	104.2
sugiol	24.40	24.57	0.37	100.7	24.59	0.35	100.8
	6.10	6.34	0.46	103.9	6.33	0.48	103.8
	1.53	1.47	2.32	96.1	1.47	2.27	95.8
1,2-dihydrotanshinquinone/ methylenetanshinquinone	13.20	13.31	0.63	100.8	13.38	0.91	101.3
	3.30	3.43	0.34	103.8	3.44	0.44	104.1
	0.83	0.79	3.03	95.6	0.81	2.77	97.6
tanshinone IIA	144.00	145.00	0.19	100.7	145.17	0.23	100.8
	36.00	37.07	0.31	102.9	37.03	0.39	102.9
	9.00	8.86	3.28	98.5	8.80	2.15	97.7
miltirone	10.10	9.84	4.04	97.5	9.92	3.52	98.3
	5.05	5.18	2.62	102.6	5.38	2.86	106.5
	1.26	1.30	2.89	103.1	1.29	2.34	102.2

Table 4. Contents (Micrograms per Gram) of the 10 Diterpenoid Compounds in *S. miltiorrhiza*[a]

analyte	Shandong	Fujian	Guangxi	Sichuan	Hebei	Zhejiang	Jiangsu	Anhui	Chongqing
dihydrotanshinone I	94.9	163.1	56.9	25.9	190.7	76.4	156.6	112.9	34.8
przewalskin	582.8	827.3	625.7	103.1	1223.6	812.4	1142.5	663.6	187.6
5,6-dehydrosugiol	647.6	816.3	234.8	267.1	1299.9	667.8	777.3	746.7	258.2
cryptotanshinone	750.1	739.9	259.7	359.8	1794.4	503.7	868.7	910.0	456.5
tanshinone I	568.3	727.1	207.2	251.8	1705.1	594.2	671.6	811.0	222.3
sugiol	46.0	61.3	+[b]	+	82.1	+	+	61.4	+
1,2-dihydrotanshinquinone/ methylenetanshinquinone	118.4	134.9	71.9	33.9	192.5	192.1	143.3	158.8	51.8
tanshinone IIA	1390.2	1479.0	492.5	1807.1	3128.9	1304.9	1340.6	1783.1	1805.0
miltirone	100.6	37.9	+	+	580.0	142.2	154.9	156.4	51.2

[a] Data are presented as the average of triplicates. [b] +, under the limits of quantification.

required, where f represents the number of factors to be studied (*24*). Therefore, a three-factor CCD requires 15 experimental points, each being the result of different experimental conditions. Five additional experiments were carried out at the center point to estimate the overall error; the total number of experiments thus amounted to 20. The results of $Rs_{(4\&5)}$ and $Rt_{(10)}$ are presented in **Table 1**. The experiments were performed in random order to avoid systematic error.

Because the quadratic response surface is calculated in (f + 1) dimensions, where f is the number of factors in the CCD, the quadratic response surface for the three factors involved generates a four-dimensional response surface, which can be readily visualized in a three-dimensional (3-D) response surface. In the present work, the software of Statistica was used to depict the 3-D response function of $Rs_{(4\&5)}$ and $Rt_{(10)}$ in **Figures 2** and **3**, respectively.

As shown in **Figure 2**, when $time_{(B:\ 45–56\%)}$ was between 3.2 and 5.0 min, the flow rate was from 0.23 to 0.34 mL/min, and the temperature was in the range of 24−25 °C, the resolution of the peaks 4 and 5 ($Rs_{(4\&5)}$) was greater than 1.2. From **Figure 3**, to obtain as short as possible a retention time of peak 10 ($Rt_{(10)}$), the conditions would be 2.8 min < $time_{(B:\ 45–56\%)}$ < 3.2 min, 0.34 mL/min < flow rate < 0.42 mL/min, and 24 °C < temperature < 46 °C. By comprehensive consideration of the resolution and the whole analytical time, the optimum conditions were confirmed as follows: $time_{(B:\ 45–56\%)}$ = 3.2 min, flow rate = 0.34 mL/min, and temperature = 24 °C. Therefore, the final elution conditions were adjusted to 0–2.5 min, linear

gradient 42–45% B; 2.5–5.7 min, linear gradient 45–56% B; 5.7–8.2 min, linear gradient 56–85% B; 8.2–10.0 min, linear gradient 85–100% B; and, finally, reconditioning steps of the column was 42% B isocratic for 3 min after the column had been washed with 100% B for 2 min. Under these optimum conditions, the peaks of cryptotanshinone (**4**) and tanshinone I (**5**) could be baseline separated ($Rs_{(4\&5)} = 1.88$) and the separation of the investigated compounds could be achieved within 8 min (**Figure 4A**).

Furthermore, the two diterpenoids of 1,2-dihydrotanshinquinone (**7**) and methylenetanshinquinone (**8**) are a pair of isomers first isolated from an ether extract of *S. miltiorrhiza* in 1980, which are hardly separated in commonly normal or reverse chromatography, whereas both of them could be obtained by stepwise recrystallization with benzene and ethanol (*22, 23*). These two compounds also failed to be separated from each other in the current study.

Identification of Investigated Compounds in *S. miltiorrhiza.* The identification of investigated compounds was carried out by comparison of their retention time and UV spectra with those obtained by injecting standards in the same conditions or by spiking *S. miltiorrhiza* samples with stock standard solutions.

Calibration Curves. As mentioned above, because 1,2-dihydrotanshinquinone (**7**) and methylenetanshinquinone (**8**) could not be separated from each other in the current study, the calibration curves of these two compounds were determined using 1,2-dihydrotanshinquinone (**7**) as the reference. Therefore, the stock solution of mixed standards was prepared by dissolving appropriate amounts of the nine diterpenoids, that is, dihydrotanshinone I (**1**), przewalskin (**2**), 5,6-dehydrosugiol (**3**), cryptotanshinone (**4**), tanshinone I (**5**), sugiol (**6**), 1,2-dihydrotanshinquinone (**7**), tanshinone IIA (**9**), and miltirone (**10**) with methanol. For the construction of calibration curves, the stock solution of mixed standards was diluted with methanol to six different concentrations; then triplicate injections were made for each concentration of mixed standard solution. The calibration curves were constructed by plotting the peak areas versus the concentration of each analyte. The results are shown in **Table 2**.

Limits of Detection and Quantification. The stock solution containing nine reference compounds was diluted to a series of appropriate concentrations with methanol, and aliquots of the diluted solutions were injected into UPLC for analysis. The limits of detection (LOD) and quantification (LOQ) under the present chromatographic conditions were determined at signal-to-noise ratios (S/N) of about 3 and 10. As indicated in **Table 2**, the method was very sensitive with overall LOD and LOQ of no more than 0.21 and 0.84 μg/mL, respectively.

Precision, Accuracy, and Recovery. Intra- and interday variations were chosen to determine the precision of the developed assay. For intraday variability test, three concentrations of the mixed standards solution were analyzed for six replicates, respectively, within one day, whereas for the interday variability test, the solutions were examined in duplicate for a consecutive three days. Variations were expressed by the relative standard deviations (RSD) for intra- and interday, which were <4.04 and $<4.66\%$, respectively. For every calibration curve, the calibration concentrations were back-calculated from the relative peak area of the analytes. The deviation from the nominal concentration was defined as accuracy (as indicated in **Table 3**).

To determine the recovery, a known amount of investigated components was added into a certain amount (0.5 g) of *S. miltiorrhiza* material. The mixture was extracted and analyzed with the method mentioned above. Three replicates were performed for the test. **Table 2** shows the recoveries of the investigated compounds.

These results indicated that the current UPLC method was sensitive, repeatable, and accurate for the quantitative determination of diterpenoids in *S. miltiorrhiza*.

Quantification of Diterpenoid Compounds in *S. miltiorrhiza.* The UPLC method developed at our Institute was applied to the quantitative analysis of 10 diterpenoids in *S. miltiorrhiza* samples collected from different districts of China, namely, Shandong, Fujian, Guangxi, Sichuan, Hebei, Zhejiang, Jiangsu, Anhui, and Chongqing. As mentioned above, the investigated diterpenoid compounds in *S. miltiorrhiza* were well separated using the developed UPLC method except 1,2-dihydrotanshinquinone (**7**) and methylenetanshinquinone (**8**). Therefore, the total amount of these two compounds in *S. miltiorrhiza* was estimated utilizing 1,2-dihydrotanshinquinone as the reference. The representatinve chromatogram of the PLE extract of *S. miltiorrhiza* from Guangxi is shown in **Figure 4**, and the contents of the 10 diterpenoid compounds of different *S. miltiorrhiza* samples are summarized in **Table 4**.

Concluding Remark. Simultaneous determination of 10 diterpenoid compounds in *S. miltiorrhiza* by ultraperformance liquid chromatography was optimized and performed for the first time. This new approach has been validated as a rapid, sensitive, accurate, and reproducible method, which may serve as a potential technique for quality control of this valued traditional Chinese medicine.

ACKNOWLEDGMENT

We thank Sandy Lao and Leon Lai from our Institute for their technical assistance.

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Received for review October 13, 2007. Revised manuscript received December 12, 2007. Accepted December 17, 2007. The research was supported by a grant from the Macao Science and Technology Development Fund (049/2005/A-R1 to Y.T.W.).

JF073020U