JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2009, 57, 6879–6887 6879
DOI:10.1021/jf901462s

Chemical Characteristics of *Salvia miltiorrhiza* (Danshen) Collected from Different Locations in China

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The chemical characteristics of *Salvia miltiorrhiza*, also called "danshen" in China, were investigated on the basis of the simultaneous quantitative determination of 13 hydrophilic and lipophilic compounds, namely, protocatechuic acid, protocatechuic aldehyde, caffeic acid, ferulic acid, isoferulic acid, rosmarinic acid, salvianolic acid B, salvianolic acid A, dihydrotanshinone I, przewalskin, cryptotanshinone, tanshinone I, and tanshinone IIA, in 74 samples collected from different locations using ultraperformance liquid chromatography (UPLC). Hierarchical clustering analysis based on 13 investigated compounds and the similarity of the entire chromatographic pattern showed that *S. miltiorrhiza* was significantly different from *Salvia przewalskii*, an adulterant of danshen. The chemical characteristics of *S. miltiorrhiza* collected from different locations in China were revealed, and salvianolic acid B, rosmarinic acid, cryptotanshinone, and tanshinones I and IIA were optimized as markers for the evaluation, which is helpful in the quality control of *S. miltiorrhiza*.

KEYWORDS: *Salvia*; chemical characteristics; ultraperformance liquid chromatography (UPLC); quality control; hierarchical clustering analysis

INTRODUCTION

The root and rhizome of *Salvia miltiorrhiza* Bge. (red sage, family Labiatae), known as "danshen" in Chinese, have been used for hundreds of years for the treatment of numerous ailments, especially cardiovascular diseases (*1*−*4*). It has also been used as a new dietary ingredient (www.fda.gov/Food/DietarySupplements/ucm109764.htm#whatnew) and employed as a natural food preservative and dietary herbal supplement (*5*, *6*). In recent decades, danshen has been cultivated widely in China, such as in Shandong, Sichuan, Henan, and Shaanxi, with the decrease of wild resource and great industrial consumption (*7*, *8*). It is well-known that two main types of compounds, hydrophilic (e.g., phenolic acids) and lipophilic (e.g., tanshinones) components, contribute to the curative effects of danshen (*9*, *10*). However, the contents of these components in danshen are obviously variant, which inevitably affects its pharmacological activities, due to the different locations and collection time (*11*). Several methods, including HPTLC (*12*, *13*), HPLC (*14*, *15*), LC-MS (*16*, *17*), UPLC (*18*, *19*), CE (*20*−*22*), and CEC (*23*), have been used for qualitative (*12*−*14*, *16*, *17*, *19*) and/or quantitative (*12*−*16*, *18*−*23*) analysis of chemical components in danshen. However, because of small sample size, few analytes, and deficiency of statistical analysis, there are few comprehensive reports on the chemical characteristics of danshen produced and used in China.

In the present study, 74 samples of danshen and *Salvia przewalskii* Maxim from different cultivation locations and provincial hospitals in China were collected, and the contents of 13 compounds including hydrophilic and lipophilic components such as protocatechuic acid (**1**), protocatechuic aldehyde (**2**), caffeic acid (**3**), ferulic acid (**4**), isoferulic acid (**5**), rosmarinic acid (**6**), salvianolic acid B (**7**), salvianolic acid A (**8**), dihydrotanshinone I (**9**), przewalskin (**10**), cryptotanshinone (**11**), tanshinone I (**12**), and tanshinone IIA (**13**) were quantitatively analyzed using UPLC. The chemical characteristics of danshen were also compared using hierarchical cluster analysis.

EXPERIMENTAL PROCEDURES

Plant Materials and Chemicals. The materials were collected from different cultivation locations and provincial hospitals in China, which are list in **Table 1**. Species identification was performed by the corresponding author. Voucher specimens of these samples were deposited at the Institute of Chinese Medical Sciences, University of Macau, Macao SAR, China. The materials were dried to constant weight at 36 °C in an oven before use.

Protocatechuic acid was purchased from ChromaDex (Irvine, CA). Protocatechuic aldehyde, caffeic acid, isoferulic acid, rosmarinic acid, and dihydrotanshinone I were purchased from International Laboratory (Lexington, KY). Ferulic acid and tanshinone I were from the National Institute for the Control of Pharmaceutical and Biological Prouducts (Beijing, China). Salvianolic acid B and salvianolic acid A were purchased from Shanghai Ronghe Pharmaceutical Corp. (Shanghai, China). Przewalskin, cryptotanshinone, and tanshinone IIA were separated and purified in our laboratory. Their structures were confirmed by comparison of their spectroscopic data including UV, IR, MS, and ^{1}H and ^{13}C NMR with the literature (*24*−*26*). The purity of all compounds was > 95% (determined by HPLC). Acetonitrile and phosphoric acid for liquid chromatography were purchased from Merck (Darmstadt, Germany). Deionized water was prepared using a Millipore Milli-Q Plus system (Millipore, Bedford, MA).

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Published on Web 07/14/2009

Table 1. Tested Samples of *Salvia*

code	source	code	source	code	source
Salvia miltiorrhiza		NC-H2	Nanchang, Jiangxi	NJ-H1	Nanjing, Jiangsu
HF-1	Lu′an, Anhui (cultivated)	XA-H1	Xi′an, Shaanxi	NJ-H2	Nanjing, Jiangsu
ZZ-1	Luoyang, Henan (wild)	XA-H2	Xi′an, Shaanxi	HHHT-H1	Huhehaote, Neimenggu
ZZ-2	Luoyang, Henan (cultivated)	WH-H1	Wuhan, Hubei	HHHT-H2	Huhehaote, Neimenggu
ZZ-3	Luoyang, Henan (cultivated)	WH-H2	Wuhan, Hubei	GY-H1	Guiyang, Guizhou
ZZ-4	Henan (cultivated)	WH-H3	Wuhan, Hubei	GY-H2	Guiyang, Guizhou
ZZ-5	Henan (cultivated)	CS-H1	Changsha, Hunan	CD-H1	Chengdu, Sichuan
JN-1	Linju, Shandong (cultivated)	CS-H2	Changsha, Hunan	CD-H2	Chengdu, Sichuan
JN-2	Linju, Shandong (cultivated)	LZ-H1	Lanzhou, Gansu	CD-H3	Chengdu, Sichuan
JN-3	Shandong (cultivated)	LZ-H2	Lanzhou, Gansu	HK-H1	Haikou, Hainan
JN-4	Shandong (cultivated)	TJ-H1	Tianjin	HK-H2	Haikou, Hainan
JN-5	Shandong (cultivated)	SH-H1	Shanghai	SY-H1	Shenyang, Liaoning
XA-1	Shaanxi (cultivated)	SH-H2	Shanghai	SY-H2	Shenyang, Liaoning
XA-2	Shaanxi (cultivated)	SH-H3	Shanghai	NN-H1	Nanning, Guangxi
XA-3	Tianshili base, Shaanxi (cultivated)	JN-H1	Jinan, Shandong	NN-H2	Nanning, Guangxi
CD-1	Zhongjiang, Sichuan (cultivated)	JN-H2	Jinan, Shandong	WLMQ-H1	Wulumuqi, Xinjiang
Salvia miltiorrhiza (**purchased from hospitals**)		HZ-H1	Hangzhou, Zhejiang	WLMQ-H2	Wulumuqi, Xinjiang
KM-H1	Kunming, Yunan	HZ-H2	Hangzhou, Zhejiang	TY-H1	Taiyuan, Shanxi
KM-H2	Kunming, Yunan	HZ-H3	Hangzhou, Zhejiang	TY-H2	Taiyuan, Shanxi
KM-H3	Kunming, Yunan	HF-H1	Hefei, Anhui	BJ-H1	Beijing
CQ-H1	Chongqing	HF-H2	Hefei, Anhui	HEB-H1	Haerbing, Heilongjiang
CQ-H2	Chongqing	HF-H3	Hefei, Anhui	***Salvia przewalskii***	
GZ-H1	Guangzhou, Guangdong	ZZ-H1	Zhengzhou, Henan	SP	Xining, Qinghai (cultivated)
GZ-H2	Guangzhou, Guangdong	ZZ-H2	Zhengzhou, Henan	***Salvia przewalskii*** (**purchased from hospitals**)	
GZ-H3	Guangzhou, Guangdong	ZZ-H3	Zhengzhou, Henan	XN-H1	Xining, Qinghai
NC-H1	Nanchang, Jiangxi	ZZ-H4	Zhengzhou, Henan	XN-H2	Xining, Qinghai

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

	linear regression data				
analyte	regression eq	test range (μg/mL)	R^2	LOD (μg/mL)	LOQ (μg/mL)
protocatechuic acid	$y = 3944.3x + 1331.4$	0.34−86.92	0.9998	0.05	0.09
protocatechuic aldehyde	$y = 12203.0x + 8306.3$	0.65−164.62	0.9997	0.04	0.08
caffeic acid	$y = 8424.5x + 4702.4$	0.73−186.92	0.9997	0.05	0.09
ferulic acid	$y = 7953.3x + 1177.8$	0.38−97.69	0.9999	0.03	0.06
isoferulic acid	$y = 8842.6x + 1103.3$	0.30−76.92	0.9998	0.02	0.05
rosmarinic acid	$y = 2705.3x - 642.84$	0.41−104.62	0.9995	0.01	0.07
salvianolic acid B	$y = 2468.8x - 9088.5$	2.99−763.85	0.9999	0.35	0.59
salvianolic acid A	$y = 7687.9x - 1786.6$	0.44−111.54	0.9998	0.10	0.22
dihydrotanshinone I	$y = 6780.6x - 461.39$	0.22−6.88	0.9999	0.05	0.18
przewalskin	$y = 1914.1x + 1352.6$	0.43−110.77	0.9998	0.04	0.07
cryptotanshinone	$y = 6585.1x + 1727.8$	0.38−97.69	0.9999	0.03	0.09
tanshinone I	$y = 20511.0x + 624.82$	0.15−9.65	0.9998	0.01	0.06
tanshinone IIA	$y = 7385.3x - 1191.1$	0.15−36.92	0.9997	0.01	0.04

Table 3. Precision and Recovery of 13 Investigated Compounds

	precision					
	intraday		interday		recovery[a] (%, $n = 3$)	
analyte	accuracy[b] (%)	RSD (%, $n = 6$)	accuracy (%)	RSD (%, $n = 6$)	mean	RSD (%)
protocatechuic acid	103.5	1.02	102.8	1.19	104.2	0.62
protocatechuic aldehyde	99.9	0.26	104.9	3.97	103.3	1.82
caffeic acid	98.6	0.30	104.0	4.14	91.1	1.51
ferulic acid	97.5	0.35	103.3	4.85	96.3	2.26
isoferulic acid	100.2	1.16	106.0	4.65	99.7	1.31
rosmarinic acid	99.6	0.43	104.9	4.38	101.8	1.79
salvianolic acid B	102.1	0.40	109.5	2.77	100.0	0.66
salvianolic acid A	95.3	0.57	96.7	2.24	99.8	1.37
dihydrotanshinone I	98.9	3.19	99.4	2.19	100.0	1.38
przewalskin	98.7	0.95	99.2	2.53	98.7	1.46
cryptotanshinone	104.0	0.38	109.6	4.19	99.8	1.13
tanshinone I	103.6	0.73	108.4	3.84	100.0	1.76
tanshinone IIA	100.9	2.01	104.1	4.14	99.8	1.25

[a] Recovery (%) = 100 × (amount found − original amount)/amount spiked. [b] Accuracy (%) = 100 × (mean of measured concentration/nominal concentration).

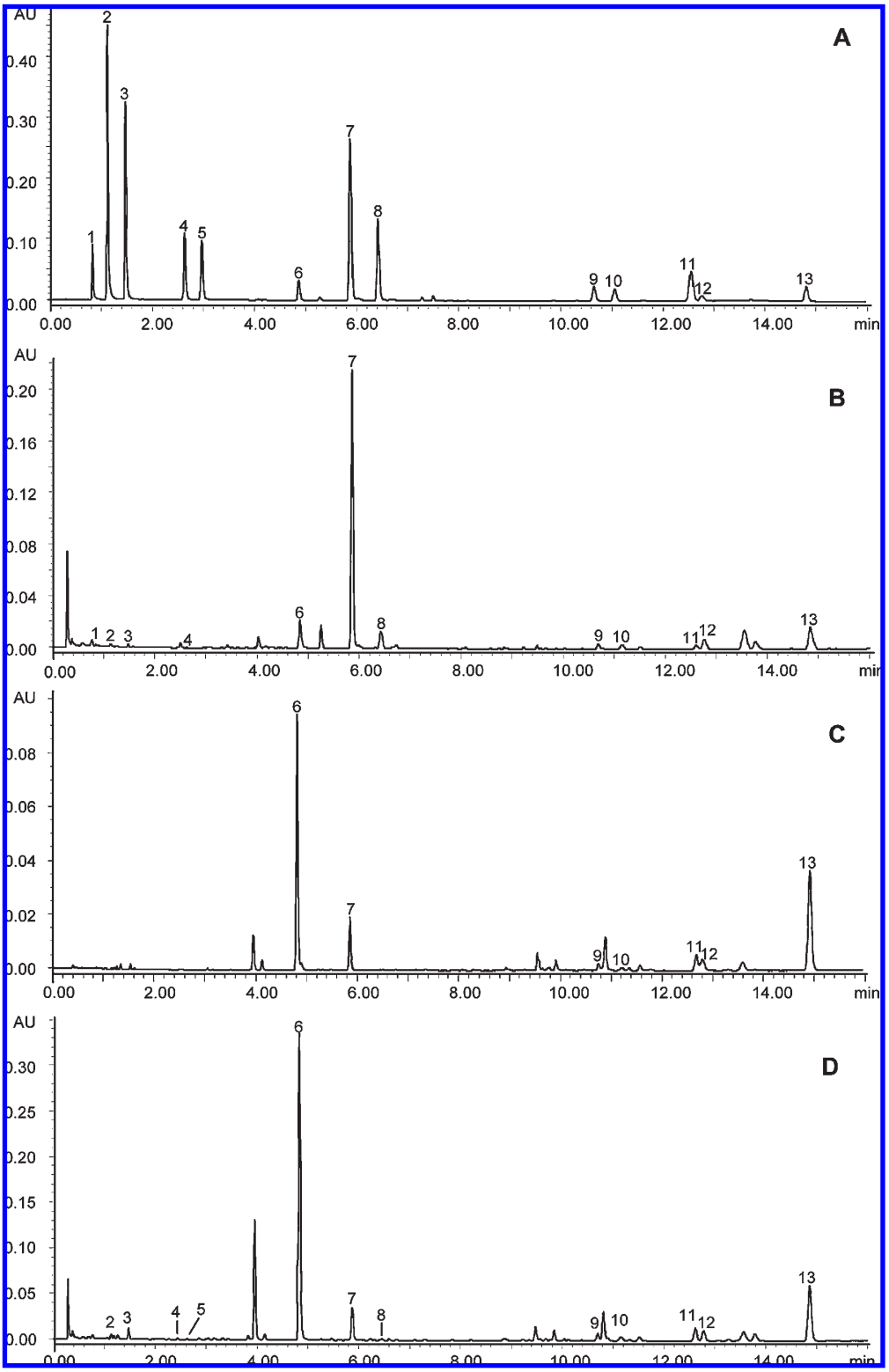

Figure 1. Typical UPLC chromatograms of (**A**) mixed standards, (**B**) *S. miltiorrhiza* (GZ-H2, collected from Guangzhou, Guangdong Province), (**C**) *S. przewalskii* (SP, collected from Xining, Qinghai Province), and (**D**) the sample collected from the hospital (XN-H1). Peaks: 1, protocatechuic acid; 2, protocatechuic aldehyde; 3, caffeic acid; 4, ferulic acid; 5, isoferulic acid; 6, rosmarinic acid; 7, salvianolic acid B; 8, salvianolic acid A; 9, dihydrotanshinone I; 10, przewalskin; 11, cryptotanshinone; 12, tanshinone I; 13, tanshinone IIA.

Sample Preparation. Dried sample powder (0.50 g, 0.2−0.3 mm particle size) was mixed with 25 mL of methanol in a sealed tube. The solution was treated in an ultrasonic apparatus (881 w, 43 kHz, Branson, 8510E-DTH) for 45 min at room temperature. Then appropriate amount of methanol was added to compensate for the weight lost during extraction. Finally, the extract was filtered through a 0.2 μm nylon membrane filter (Whatman, Maidstone, U.K.) prior to injection into the UPLC system.

UPLC Analysis. All analyses were performed on a Waters Acquity UPLC system (Waters, Milford, MA), including a binary solvent manager, sampler manager, column compartment, and photodiode array (PDA) detector, connected to Waters Empower 2 software. An Acquity UPLC

Table 4. Contents (Milligrams per Gram) of 13 Investigated Compounds in Tested *Salvia*

	investigated compounds													total		sum (**6** + **7** + **11** + **12** + **13**)	
code	**1**[a]	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	content	av (RSD, %)	content	av (RSD, %)
S. miltiorrhiza																	
CS-H2	−[b]	+[c]	+	+	0.02[d]	1.09	17.08	0.11	0.15	0.43	0.23	0.14	0.88	20.14		19.43	
ZZ-H4	−	+	+	+	+	1.11	16.91	0.09	0.15	0.47	0.22	0.13	0.88	19.96		19.25	
CQ-H1	−	+	+	+	0.02	0.85	14.53	0.10	0.14	0.63	0.17	0.08	0.74	17.26		16.37	
GZ-H1	+	+	+	+	0.02	1.02	14.59	0.08	0.13	0.43	0.10	0.05	0.41	16.83		16.16	
ZZ-H1	−	+	+	+	+	0.88	13.87	0.10	0.12	0.40	0.15	0.08	0.66	16.26		15.63	
NC-H1	0.02	+	+	+	0.02	1.22	15.05	0.13	0.43	1.40	0.36	0.15	0.87	19.64		17.64	
SY-H2	+	+	+	+	0.02	1.43	15.53	0.12	0.31	1.21	0.27	0.13	0.75	19.77		18.11	
SH-H2	−	+	+	+	0.02	1.01	15.43	0.12	0.24	0.62	0.63	0.22	1.47	19.88		18.76	
CD-H2	−	+	+	+	−	0.93	15.39	0.08	0.15	0.49	0.39	0.12	1.99[e]	19.54		18.82	
ZZ-4	−	+	+	−	+	1.37	15.35	0.24	0.19	0.54	0.40	0.10	0.75	18.94		17.97	
TY-H1	+	+	+	+	+	1.48	14.78	0.27	0.05	0.09	0.22	0.06	1.19	18.14		17.73	
NJ-H1	−	+	+	+	+	0.09	12.89	0.19	0.24	0.45	0.48	0.15	1.15	15.64		14.77	
HHHT-H1	+	+	+	+	+	1.30	10.63	0.09	0.06	0.32	0.12	0.08	0.60	13.20		12.73	
XA-3	−	+	+	−	−	0.71	10.31	0.08	0.13	0.28	0.26	0.07	0.62	12.46		11.98	
CS-H1	−	+	+	-	0.02	0.79	11.87	0.04	0.07	0.21	0.14	0.06	0.68	13.87		13.54	
ZZ-H2	−	+	+	+	+	0.75	11.78	0.09	0.13	0.43	0.12	0.08	0.75	14.12	14.32 (29.01)	13.48	13.45 (29.76)
CD-H3	+	+	+	+	−	0.88	12.04	0.11	0.06	0.20	0.20	0.04	1.02	14.54		14.18	
HZ-H3	+	+	+	+	−	0.83	11.50	0.10	0.07	0.18	0.14	0.07	0.60	13.48		13.14	
HF-H2	+	+	+	+	+	1.11	11.40	0.15	0.13	0.36	0.19	0.08	0.58	14.00		13.36	
XA-H1	−	+	+	+	+	0.92	11.47	0.13	0.26	0.71	0.39	0.14	0.98	15.00		13.90	
ZZ-5	−	−	+	−	−	0.49	11.35	0.09	0.25	2.01	1.07	0.16	2.02[e]	17.44		15.09	
SH-H1	−	+	+	+	+	0.95	12.06	0.14	0.62	1.49	0.49	0.20	1.32[e]	17.28		15.02	
BJ-H1	−	+	+	+	+	0.95	13.20	0.09	0.08	0.32	0.10	0.07	0.51	15.30		14.82	
HEB-H1	−	+	+	+	+	0.86	13.17	0.06	0.12	0.52	0.24	0.08	0.80	15.85		15.16	
XA-2	−	−	+	−	+	0.82	12.77	0.13	0.14	0.67	0.38	0.08	0.86	15.85		14.91	
NJ-H1	−	+	+	+	+	0.09	12.89	0.19	0.24	0.45	0.48	0.15	1.15	15.64		14.77	
HHHT-H2	+	+	+	+	+	0.09	12.12	0.07	0.13	0.36	0.22	0.09	0.78	13.88		13.31	
KM-H3	−	+	+	+	0.10	0.36	3.26	0.11	0.23	0.39	0.13	0.16	1.70	6.33		5.60	
LZ-H1	+	+	+	+	−	0.79	4.48	0.02	0.17	0.41	0.22	0.09	0.76	6.94		6.33	
JN-H1	−	+	+	+	−	0.10	1.95	0.06	0.49	1.13	0.86	0.24	1.69	6.52		4.84	
SH-H3	−	+	+	+	+	0.54	6.23	0.05	0.12	0.41	0.23	0.08	0.74	8.39		7.81	
NN-H1	+	+	+	+	+	0.65	5.86	0.09	0.11	0.34	0.17	0.10	0.89	8.21		7.67	
GZ-H2	0.03	+	+	+	−	0.65	6.08	0.17	0.05	0.19	0.04	0.04	0.33	7.56		7.13	
CD-1	−	−	−	−	−	0.49	8.34	0.05	0.02	0.10	0.06	0.01	0.32	9.39		9.22	
HF-H3	+	+	+	0.02	+	0.41	8.25	0.07	0.07	0.21	0.08	0.05	0.47	9.63		9.26	
HZ-H2	−	+	+	0.03	−	0.66	8.19	0.07	0.22	0.62	0.19	0.07	0.59	10.63		9.70	
HZ-H1	+	+	+	+	−	0.34	8.94	0.07	0.13	0.22	0.37	0.14	1.05	11.27		10.84	
JN-H2	−	+	+	+	+	0.74	7.56	0.11	1.02[e]	1.44	1.24	0.36	2.71[e]	15.19		12.62	
CD-H1	−	+	+	+	+	1.47	21.58	0.14	0.11	0.22	0.34	0.07	2.20	26.12		25.65	
HK-H1	−	+	+	+	+	1.45	21.89	0.14	0.29	0.71	0.45	0.20	1.60	26.72		25.58	
XA-H2	+	+	+	+	+	2.34	21.75	0.08	0.14	0.36	0.12	0.07	0.51	25.36		24.78	
TJ-H1	−	+	+	+	0.05	1.57	22.49	0.13	0.17	0.53	0.16	0.08	0.68	25.85		24.98	
WH-H2	−	+	+	+	+	1.14	19.71	0.15	0.34	0.78	0.70	0.27	1.86[e]	24.94		23.67	
GY-H1	−	+	+	+	+	1.44	20.48	0.16	0.20	0.52	0.51	0.11	2.33[e]	25.65		24.77	
LZ-H2	−	+	+	+	+	1.13	19.67	0.12	0.25	0.94	0.26	0.13	1.13	23.62		22.31	
TY-H2	−	+	+	−	+	0.93	18.90	0.07	0.27	0.80	0.42	0.19	1.31	22.89		21.75	
GY-H2	−	+	+	+	0.16	1.49	19.26	0.37	0.46[e]	1.45	0.45	0.24	1.52	25.38		22.94	
WH-H3	−	+	+	+	+	1.06	18.84	0.10	0.08	0.28	0.09	0.05	0.47	20.95		20.50	
KM-H1	−	+	+	+	+	1.13	24.11	0.11	0.15	0.50	0.29	0.17	1.54	28.00		27.25	
HK-H2	−	+	+	+	+	1.62	23.64	0.16	0.30	0.89	0.32	0.18	1.31	28.44		27.08	
GZ-H3	−	+	+	+	0.03	1.42	24.32	0.13	0.11	0.30	0.39	0.07	2.31[e]	29.07	31.42 (23.38)	28.51	29.79 (21.86)
NC-H2	−	+	+	+	+	1.82	24.68	0.26	0.49[e]	0.82	1.12	0.43	3.00[e]	32.62		31.05	
WLMQ-H2	+	+	+	+	0.03	1.74	24.92	0.21	0.58	1.40	0.97	0.37	2.47[e]	32.68		30.47	
WLMQ-H1	−	+	+	+	0.06	1.73	23.92	0.22	0.62[e]	1.54	1.62	0.59[e]	3.93[e]	34.22		31.79	
JN-5	−	−	−	−	+	3.05	28.43	0.85	0.26	0.49	0.34	0.27	1.60	35.29		33.69	
WH-H1	−	+	+	+	0.06	2.11	30.06	0.22	0.28	0.89	0.60	0.31	2.01[e]	36.55		35.09	
KM-H2	−	+	−	−	0.04	1.79	27.40	0.20	0.76[e]	2.18	0.75	0.36	2.30[e]	35.76		32.59	
SY-H1	−	+	+	+	+	1.66	26.17	0.14	0.16	0.40	0.30	0.14	1.22	30.20		29.49	
HF-1	−	−	+	−	−	5.45[e]	32.55	0.47	0.27	0.68	0.18	0.33	1.14	41.07		39.64	
CQ-H2	−	+	+	+	+	2.09	33.42	0.16	0.27	0.49	0.25	0.19	2.04[e]	38.90		37.98	
ZZ-3	−	−	+	−	+	2.10	31.29	0.55	0.66[e]	1.38	1.64	0.53[e]	5.52[e]	43.67		41.08	
JN-2	+	−	−	−	+	2.62	30.92	0.49	1.10[e]	1.38	2.40	0.50[e]	3.74[e]	43.15		40.18	
JN-4	−	−	−	−	0.02	2.78	28.27	0.83	2.61[e]	3.04	5.05[e]	0.83[e]	4.92[e]	48.36		41.86	

Table 4. Continued

	investigated compounds													total		sum (6 + 7 + 11 + 12 + 13)	
code	1[a]	2	3	4	5	6	7	8	9	10	11	12	13	content	av (RSD, %)	content	av (RSD, %)
ZZ-2	−	−	−	−	+	2.31	40.44[e]	0.57	0.42[e]	0.78	0.83	0.32	2.63[e]	48.31	59.03 (32.83)	46.54	57.28 (32.23)
HF-H1	−	+	+	+	0.03	1.93	40.11[e]	0.22	0.16	0.54	0.19	0.14	1.32	44.65		43.69	
NN-H2	−	+	+	+	0.03	2.70	37.70	0.24	0.08	0.18	0.11	0.07	0.78	41.89		41.36	
JN-3	−	−	−	−	0.02	2.63	43.39[e]	0.64	0.40[e]	0.58	0.73	0.27	2.55[e]	51.20		49.57	
ZZ-1	+	−	−	−	+	3.17	58.69[e]	0.60	0.11	0.18	0.19	0.15	1.15	64.25		63.36	
XA-1	−	−	−	−	+	2.56	53.36[e]	0.83	0.85[e]	1.21	1.64	0.54[e]	4.01[e]	65.00		62.11	
JN-1	−	−	−	−	0.04	7.38	82.29[e]	1.50	0.72[e]	1.36	1.06	0.39	3.20[e]	97.94		94.32	
ZZ-H3	−	+	+	+	0.04	2.63	181.41[e]	0.26	0.45[e]	1.19	0.42	0.12	1.43	187.95		186.00	
S. przewalskii																	
XN-H1	−	+	0.06	+	+	9.27[e]	1.10	0.01	0.10	0.25	0.22	0.06	0.96	12.03		11.60	
XN-H2	−	+	0.01	+	+	6.87[e]	2.24	−	0.04	0.24	0.30	0.05	1.56	11.30	9.98 (29.50)	11.02	9.69 (29.25)
SP	−	−	−	−	−	3.97	1.11	−	0.07	0.11	0.18	0.05	1.12	6.61		6.43	

[a] **1**, protocatechuic acid; **2**, protocatechuic aldehyde; **3**, caffeic acid; **4**, ferulic acid; **5**, isoferulic acid; **6**, rosmarinic acid; **7**, salvianolic acid B; **8**, salvianolic acid A; **9**, dihydrotanshinone I; **10**, przewalskin; **11**, cryptotanshinone; **12**, tanshinone I; **13**, tanshinone IIA. [b] Undetected. [c] Under the limit of quantification. [d] Data are presented as the average of duplicates. [e] Quantification was performed after appropriate dilution.

BEH C18 column (50 mm × 2.1 mm i.d., 1.7 μm), also from Waters, was used. The column temperature was maintained at 35 °C. Separation of analytes was achieved using a gradient mobile phase consisting of 0.5% (v/v) phosphoric acid in water (A) and acetonitrile (B), which was modified on the basis of a previous paper (*18*). In brief, the gradient condition is as follows: 0−0.5 min, 5−10% B; 0.5−6.0 min, 10−23% B; 6.0−7.8 min, 23−30% B; 7.8−9.0 min, 30−42% B; 9.0−11.5 min, 42−45% B; 11.5 −14.5 min, 45−46% B; 14.5−17.5 min, 46−85% B and finally, reconditioning of the column with 5% B isocratic for 2 min after the column had been washed with 100% B for 3 min. The flow rate was set at 0.5 mL/min, and the injection volume was 2 μL. The sample manager temperature was set at 10 °C. The peaks were detected at 280 nm.

Calibration Curves. Methanol stock solutions containing the 13 reference compounds were prepared and diluted to appropriate concentrations for the construction of calibration curves. At least six concentrations of the solution were analyzed in triplicates, and then the calibration curves were constructed by plotting the peak area versus the concentration of each analyte.

Limits of Detection and Quantification. The stock solution containing the 13 reference compounds was diluted to a series of appropriate concentrations with methanol, and an aliquot of the diluted solutions was injected into the 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, respectively.

Precision, Accuracy, Repeatability, and Recovery. Intraday and interday variations were used for evaluation of precision. For intraday variation, the mixed standard solutions were analyzed for six replicates within one day, whereas for the interday variability test, the solutions were examined in duplicates for three consecutive days. Variations were expressed as the relative standard deviations (RSD). For every calibration curve, the calibration concentrations were back-calculated from the peak area of the analytes. The deviation from the nominal concentration was defined as accuracy.

The repeatability of the developed method was evaluated at three levels (0.40, 0.50, and 0.60 g) using *S. miltiorrhiza* (HF-1), and each level of sample was extracted and analyzed in triplicates as mentioned above. The repeatability was also presented as RSD ($n = 3$).

To determine the recovery, a known amount of individual standards was added into a certain amount (0.25 g) of *S. miltiorrhiza* raw material (XA-2). The mixture was extracted and analyzed using the method mentioned above. Three replicates were performed for the test.

Statistical Analysis. Statistical analysis was carried out by the SPSS 13.0 for Windows program (SPSS Inc., Chicago, IL). SPSS comprises a number of "procedures"—graphical, statistical, reporting, processing, and tabulating procedures—that enable simple and rapid data evaluation.

The correlation coefficients, the similarities of entire chromatographic patterns among tested samples, and the simulative mean chromatogram were calculated and generated using professional software named "Chromatogram Analysis and Data Management System for Traditional Chinese Medicine" (version 2004), which was recommended by the National Institute for the Control of Pharmaceutical and Biological Prouducts (Beijing, China).

RESULTS AND DISCUSSION

Optimization of Ultrasonic Extraction. Ultrasonic extraction, rapid and accurate, has been widely used for sample preparation (*13*, *16*, *17*, *19*, *31*). Ultrasonic extraction was also employed in our study. The optimization was performed using the sample HF-1 (*S. miltiorrhiza* from Anhui province). The parameters, including the type of solvent (methanol, 70% methanol−water solution and water), extraction time (30, 45, and 60 min), and solid−liquid ratio (1:25, 1:50, and 1:100), were investigated using a univariate approach. The amount of the 13 investigated compounds was used as the marker for evaluation of extraction efficiency. The results showed that the highest extraction efficiency was obtained when the sample was ultrasonicated with methanol for 45 min at the solid−liquid ratio of 1:50.

Validation of the Method. The linearity, regression, and linear ranges of 13 analytes were determined using the developed UPLC method. The results indicate a good relationship between the investigated compounds' concentrations and their peak areas within the test ranges, and the LOD and LOQ were less than 0.35 and 0.59 μg/mL (**Table 2**), and the overall intra- and interday variations (RSD) of the 13 analytes were less than 3.19 and 4.85%, respectively (**Table 3**). The repeatability (RSD, $n = 3$) at high, medium, and low levels of sample amount was less than 3.07, 3.36, and 4.96%, respectively. The stability assay showed that all analytes were stable, overall peak area RSD < 4.72%, in the mixed standard solution at high level of concentration in 20 h. The analyte recoveries were between 91.1 and 104.2% with RSD < 2.26% (**Table 3**).

Quantification of the Investigated Compounds in *Salvia*. The developed UPLC method was applied to the simultaneous determination of 13 hydrophilic and lipophilic components in 74 samples of *Salvia*. Their typical chromatograms are shown in **Figure 1**. The identification of the investigated compounds was carried out by comparison of their retention time and UV spectra with those obtained by injecting standards under the same conditions, as well as by spiking the samples with stock standard solutions. By using the calibration curves of the analytes, their contents in different samples were analyzed, which are summarized in **Table 4**. Briefly, the contents of the 13 investigated

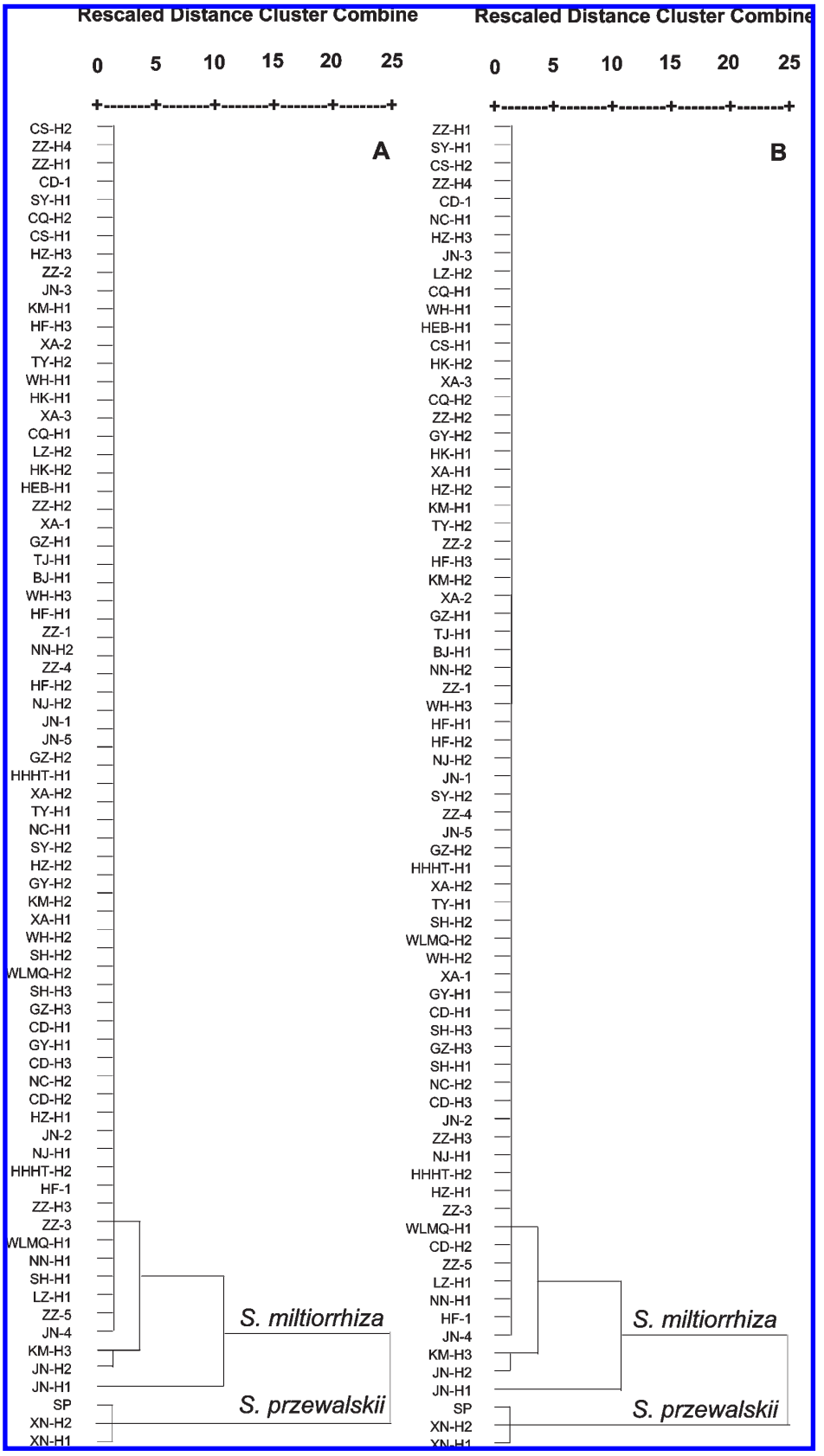

Figure 2. Dendrograms resulting from single linkage between groups by hierarchical cluster analysis. The hierarchical clustering was done by SPSS 13.0 for Windows. A method called Between Groups Linkage was applied, and Pearson correlation was selected as measurement. The dendrograms resulted from the characteristics of (**A**) 13 investigated peaks and (**B**) 5 peaks of rosmarinic acid, salvianolic acid B, cryptotanshinone, and tanshinones I and IIA derived from UPLC profiles of all tested *Salvia* samples. The samples are the same as in **Table 1**.

compounds in tested samples of *Salvia* were variant. Generally, salvianolic acid B was the richest component in all tested samples of *S. miltiorrhiza*, followed by rosmarinic acid and salvianolic acid A. In addition, tanshinones such as dihydrotanshinone I,

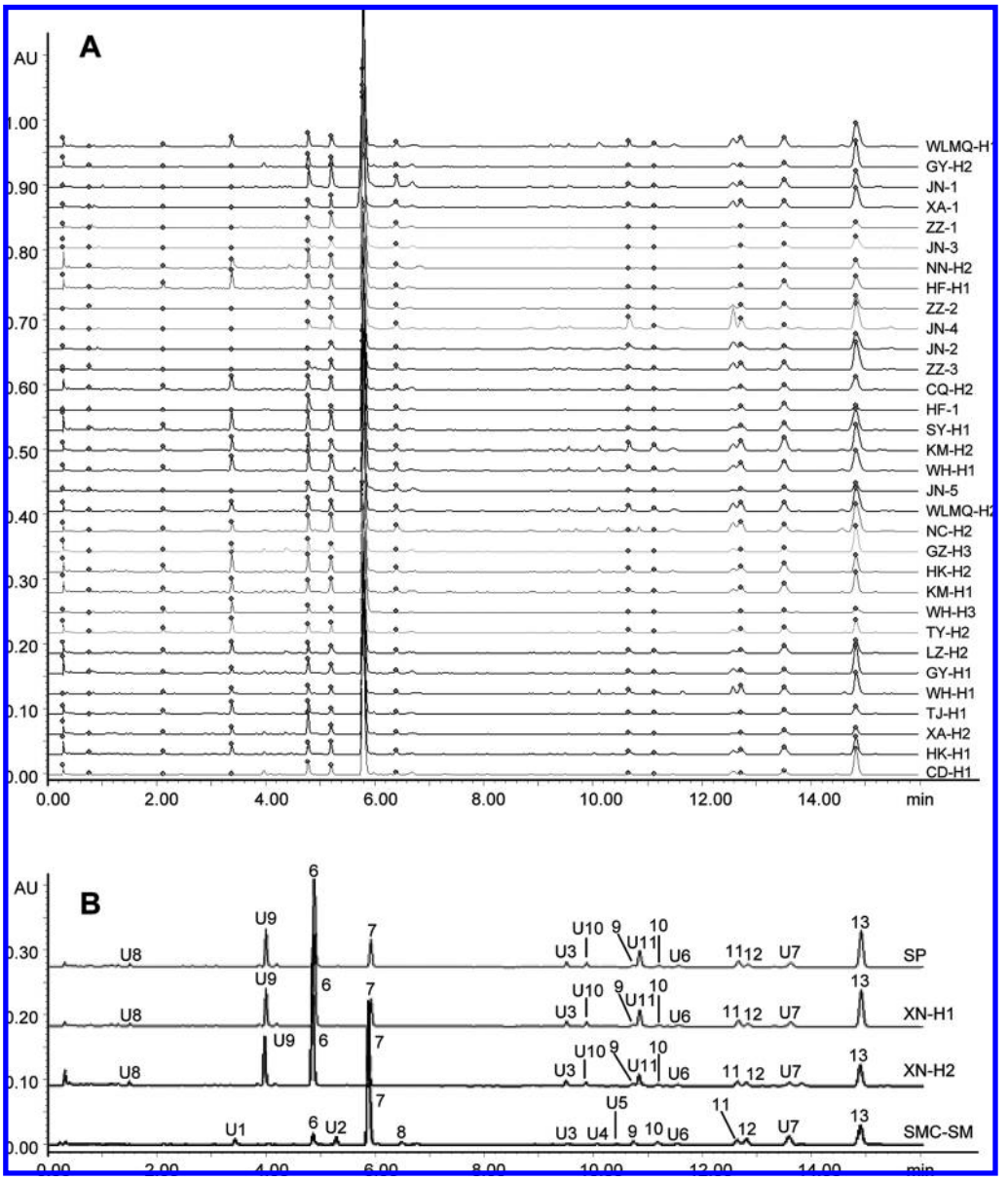

Figure 3. Profiles of (**A**) UPLC chromatograms for partial samples of *S. miltiorrhiza* and (**B**) the simulative mean chromatogram of *S. miltiorrhiza* and chromatograms for three samples of *S. przewalskii*. Peaks: 6−13, same as in **Figure 1**; U1−U11, unknown peaks detected in samples of *S. miltiorrhiza* and/or *S. przewalskii*. SMC-SM, simulative mean chromatogram of *S. miltiorrhiza*. The samples are the same as in **Table 1**.

cryptotanshinone, and tanshinones I and IIA were also major components in *S. miltiorrhiza*.

Chemical Characteristics of Danshen Used in China. Besides *S. miltiorrhiza* (red sage), some other species of *Salvia* including *S. officinalis* L. (common sage), *S. triloba* L. (sage), and *S. przewalskii* are also used for antioxidation (*27*−*29*) and prevention of type 2 diabetes (*30*). However, the chemical components and their amounts in these species of *Salvia* are obviously different (*11*). In the present study, the samples of danshen collected from the provincial hospitals in Xining were actually identified as *S. przewalskii*; its chemical characteristics were also similar to that of *S. przewalskii* Maxim collected from Qinghai (**Figure 1D**), which were different from those of *S. miltiorrhiza*. Indeed, the content of rosmarinic acid in *S. przewalskii* was much higher than that of salvianolic acid B, which was different from that in *S. miltiorrhiza* (**Figure 1**; **Table 4**).

Hierarchical cluster analysis based on 13 peak characteristics from UPLC profiles were used for investigating the chemical characteristics of the tested samples. Between Groups Linkage was applied, and Pearson correlation was selected as measurement. **Figure 2A** shows the result of the 74 tested samples of *Salvia*, which were divided into two main clusters, samples of *S. miltiorrhiza* and *S. przewalskii*. Actually, five typical peaks of rosmarinic acid (**6**), salvianolic acid B (**7**), cryptotanshinone (**11**), tanshinone I (**12**), and tanshinone IIA (**13**) could be optimized on the basis of the cluster analysis of 13 peaks and then principal component analysis. Using the peak characteristics of rosmarinic acid, salvianolic acid B, cryptotanshinone, and tanshinones I and IIA, hierarchical cluster analysis of the tested 74 samples was performed as before. The result was very similar to the one derived from 13 peak characteristics (**Figure 2B**). Therefore, the characteristics of peaks for the investigated compounds, especially rosmarinic acid, salvianolic acid B, cryptotanshinone, and tanshinones I and IIA, from UPLC chromatogram profiles could be used as markers for discrimination and quality control of danshen, *Salvia miltiorrhiza* (*31*).

The chemical characteristics of 71 samples of *S. miltiorrhiza* were very similar. The similarities of their entire chromatographic

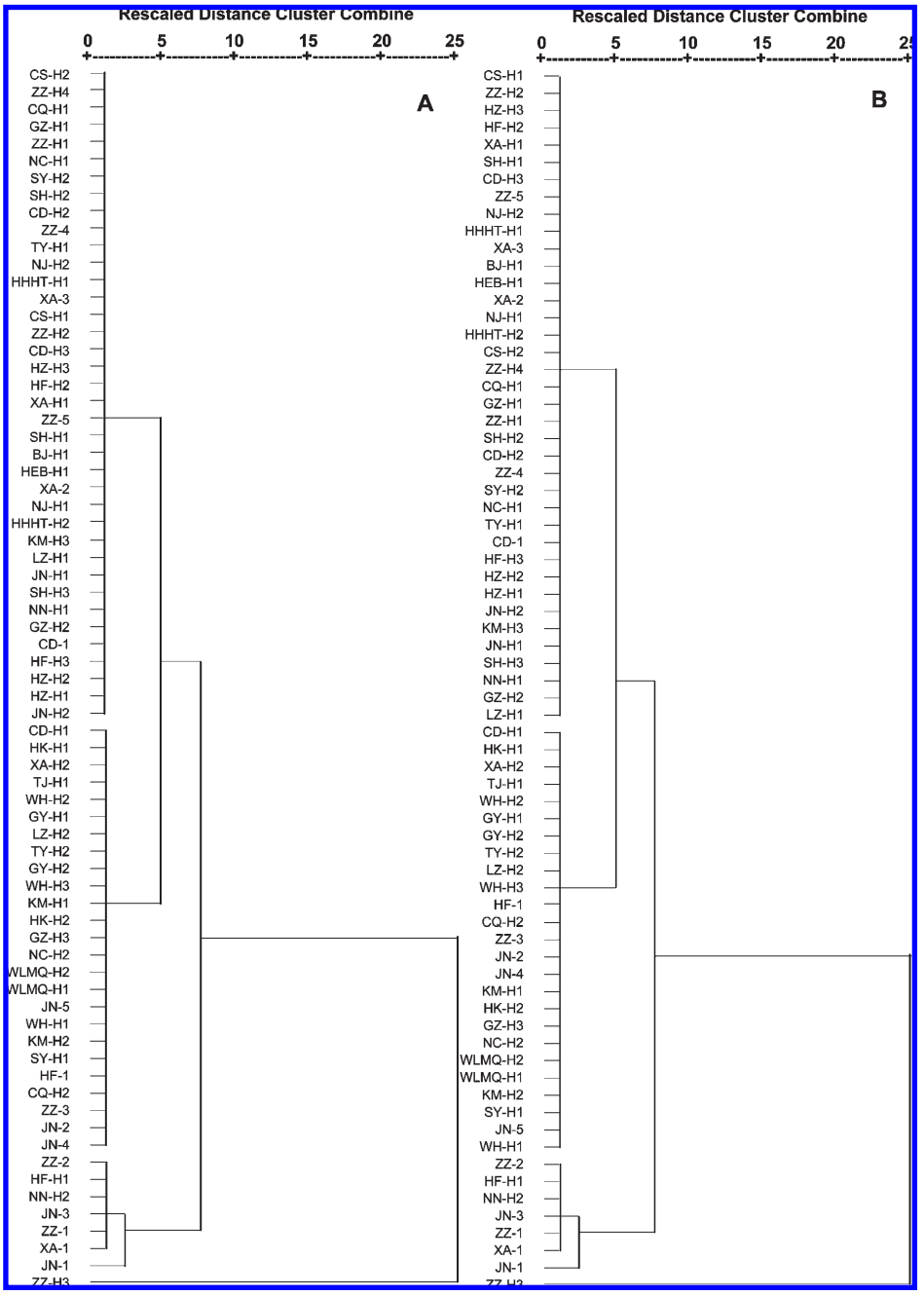

Figure 4. Dendrograms resulting from single linkage between groups by hierarchical cluster analysis. The hierarchical clustering was done by SPSS 13.0 for Windows. A method called Ward's method was applied, and Squared Euclidean distance was selected as measurement. The dendrograms resulted from the characteristics of (**A**) 13 investigated peaks and (**B**) 5 peaks of rosmarinic acid, salvianolic acid B, cryptotanshinone, and tanshinones I and IIA derived from UPLC profiles of tested *S. miltiorrhiza* samples. The samples are the same as in **Table 1**.

patterns were evaluated by using "Chromatogram Analysis and Data Management System for Traditional Chinese Medicine" (version 2004). The correlation coefficient of each chromatogram to their simulative mean chromatogram was 0.952 ± 0.096 (mean $\pm$ SD, $n = 71$). **Figure 3A** shows a part of the UPLC profile of samples of *S. miltiorrhiza*. However, chemical characteristics of *S. miltiorrhiza* and *S. przewalskii* were significantly different. **Figure 3B** shows the simulative mean chromatogram for *S. miltiorrhiza* and the chromatograms of the tested three samples of *S. przewalskii*. The correlation coefficients of the three chromatograms of *S. przewalskii* to the simulative mean chromatogram of *S. miltiorrhiza* were 0.199, 0.366, and 0.200, respectively.

Furthermore, to investigate the difference of chemical amount in the tested samples of *S. miltiorrhiza*, hierarchical cluster analysis based on 13 peaks from UPLC profiles was also performed with Ward's method, a very efficient method for the analysis of variance between clusters, and squared Euclidean distance was selected as measurement (*32*). **Figure 4A** shows the result of the 71 tested samples of *S. miltiorrhiza*, which are divided into three main clusters in which the contents of the investigated compounds were significantly different (low to high). Of course, a similar result was also obtained when the contents of rosmarinic acid, salvianolic acid B, cryptotanshinone, and tanshinones I and IIA instead of those of 13 investigated compounds were used for the analysis (**Figure 4B**). The total amounts of the 13 investigated compounds in danshen, *S. miltiorrhiza*, among the three clusters were significantly different (**Table 4**).

Concluding Remarks. The chemical characteristics of *S. miltiorrhiza* used in China were determined by UPLC and are strongly correlated with the contents of salvianolic acid B, rosmarinic acid, cryptotanshinone, and tanshinones I and IIA. The results suggest that these five components may be appropriate markers for quality control of *S. miltiorrhiza*.

ACKNOWLEDGMENT

We are grateful to Leon Lai from our institute for expert technical assistance.

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Received May 2, 2009. Revised manuscript received June 30, 2009. Accepted June 30, 2009. The research was supported by grants from the University of Macau (UL015/09-Y1/CMS/LSP01) to S.P. Li.