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A rapid method for simultaneous determination of 15 flavonoids in *Epimedium* using pressurized liquid extraction and ultra-performance liquid chromatography

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Abstract

Herba Epimedii (family Berberidaceae), *Yinyanghuo* in Chinese, is one of commonly used Chinese medicines. Flavonoids are considered as its active components. In this study, a rapid ultra-performance liquid chromatography (UPLC) method was developed for simultaneous determination of 15 flavonoids, including hexandraside E, kaempferol-3-*O*-rhamnoside, hexandraside F, epimedin A, epimedin B, epimedin C, icariin, epimedoside C, baohuoside II, caohuoside C, baohuoside VII, sagittatoside A, sagittatoside B, 2″-*O*-rhamnosyl icariside II and baohuoside I in different species of *Epimedium*. The analysis was performed on Waters Acquity UPLC system with an Acquity UPLC BEH C18 column (50 mm × 2.1 mm I.D., 1.7 μm) and gradient elution of 50 mM acetic acid aqueous solution and acetonitrile within 12 min. All calibration curves showed good linearity ($R^2 > 0.9997$) within test ranges. The LOD and LOQ were lower than 0.13 and 0.52 ng on column, respectively. The R.S.D.s for intra- and inter-day of 15 analytes were less than 5.0% at three levels, and the recoveries were 95.0–103.7%. The validated method was successfully applied to quantitatively analyze 15 flavonoids in different species of *Epimedium*. The results showed there were great variations among the contents of investigated flavonoids. Hierarchical clustering analysis based on characteristics of 15 investigated compounds peaks in UPLC profiles showed that 37 samples were divided into 3 main clusters, which were in accordance with their flavonoids contents. The simulative mean chromatogram of the high content cluster was generated to compare the samples from different species and/or locations of *Epimedium*. Four flavonoids including epimedin A, B, C and icariin were selected as markers for quality control of the species of *Epimedium* used as *Yinyanghuo*.

Keywords: Flavonoids; *Epimedium*; Ultra-performance liquid chromatography (UPLC); Pressurized liquid extraction (PLE); Hierarchical clustering analysis; Similarity evaluation

1. Introduction

The genus *Epimedium* is widespread in Asia, Europe and the Middle and Far East. It comprises about 50 species found throughout the world [1]. According to the Chinese Pharmacopoeia, the dried aerial parts of *Epimedium brevicornu* Maxim., *Epimedium sagittatum* (Sieb. et Zucc.) Maxim., *Epimedium pubescens* Maxim., *Epimedium wushanense* T.S. Ying and *Epimedium koreanum* Nakai were used as *Yinyanghuo*, a well-known Chinese herbal medicine [2]. The flavonoids have been reported possessing multiple biological activities, such as anti-osteoporosis, immunological function modulation and anti-tumor actions [3–7]. In addition, among more than 130 compounds identified in different species of *Epimedium*, most are the flavonoids [8,9]. Thus, determination of flavonoids is necessary for quality control of *Epimedium*. Up to date, a series of methods, including UV–vis spectrophotometry [10–12], thin layer chromatography (TLC) [13–16], high performance liquid chromatography (HPLC) [15,17–20], micellar electrokinetic chromatography (MEKC) [21–24] and capillary zone electrophoresis (CZE) [25–27], have been reported to quantify the level of flavonoids in *Epimedium*. However, these methods suf-

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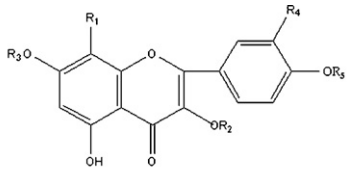
fered from long analysis time, low resolution and sensitivity and/or few analytes.

Ultra-performance liquid chromatography (UPLC) makes it possible to perform very high-resolution separations in short periods of time with little solvent consumption [28–30], which utilizes solid phase particles of 1.7 μm diameter to achieve superior theoretical plates and resolution. And it has attracted wide attention of pharmaceutical and biochemical analysts [31–35]. In addition, it is well known that interaction of multiple chemical compounds contributes to the therapeutic effect of Chinese medicine. Therefore, the analysis of multiple components is necessary and helpful to control the quality of Chinese medicine.

This study developed a rapid and reliable pressurized liquid extraction (PLE) and UPLC method to analyze 15 flavonoids, including hexandraside E, kaempferol-3-*O*-rhamnoside, hexandraside F, epimedin A, epimedin B, epimedin C, icariin, epimedoside C, baohuoside II, caohuoside C, baohuoside VII, sagittatoside A, sagittatoside B, 2″-*O*-rhamnosyl icariside II and baohuoside I, in *Epimedium*. The validated method was applied for assay of 37 samples from 17 species of *Epimedium*. The contents of flavonoids in different species of *Epimedium* were also compared.



Compounds	R_1	R_2	R_3	R_4	R_5	M.W
hexandraside E	$-CH_2CH=C(CH_3)_2$	-glc	-glc	-H	-H	678
kaempferol-3-O-rhamnoside	-H	-rha	-H	-H	-H	432
hexandraside F	$-CH_2CH=C(CH_3)_2$	-rha(3-1)glc	-glc	-H	$-CH_3$	838
epimedin A	$-CH_2CH=C(CH_3)_2$	-rha(2-1)glc	-glc	-H	$-CH_3$	838
epimedin B	$-CH_2CH=C(CH_3)_2$	-rha(2-1)xyl	-glc	-H	$-CH_3$	808
epimedin C	$-CH_2CH=C(CH_3)_2$	-rha(2-1)rha	-glc	-H	$-CH_3$	822
icariin	$-CH_2CH=C(CH_3)_2$	-rha	-glc	-H	$-CH_3$	676
epimedoside C	$-CH_2CH=C(CH_3)_2$	-H	-glc	-H	-H	516
baohuoside II	$-CH_2CH=C(CH_3)_2$	-rha	-H	-H	-H	500
caohuoside C	$-CH_2CH=C(CH_3)_2$	-rha	-H	-OH	$-CH_3$	530
baohuoside VII	$-CH_2CH=C(CH_3)_2$	-rha(4-1)glc	-H	-H	$-CH_3$	676
sagittatoside A	$-CH_2CH=C(CH_3)_2$	-rha(2-1)glc	-H	-H	$-CH_3$	676
sagittatoside B	$-CH_2CH=C(CH_3)_2$	-rha(2-1)xyl	-H	-H	$-CH_3$	646
2''-O-rhamnosyl icariside II	$-CH_2CH=C(CH_3)_2$	-rha(2-1)rha	-H	-H	$-CH_3$	660
baohuoside I	$-CH_2CH=C(CH_3)_2$	-rha	-H	-H	$-CH_3$	514

Fig. 1. Chemical structures of 15 investigated compounds.

2. Experimental

2.1. Chemicals, reagents and materials

Acetonitrile and acetic acid for liquid chromatography were purchased from Merck (Darmstadt, Germany). Absolute ethanol used for extraction was purchased from Riedel-de Haën (Seeize, Germany). Deionized water was prepared using a Millipore Milli Q-Plus system (Millipore, Bedford, MA, USA).

Hexandraside E, kaempferol-3-*O*-rhamnoside, hexandraside F, epimedin A, epimedin B, epimedin C, icariin, epimedoside C, baohuoside II, caohuoside C, baohuoside VII, sagittatoside A, sagittatoside B, 2″-*O*-rhamnosyl icariside II and baohuoside I (Fig. 1) were separated and purified in our lab. The purity of all compounds is more than 95% (determined by HPLC). The structures are confirmed by their UV, MS, ^{1}H NMR and ^{13}C NMR data compared with the literatures [36–46].

The materials of *Epimedium* were collected and identified by Professor Baolin Guo, one of the authors (Table 1). The voucher specimens of these samples were deposited at the Institute of Chinese Medical Sciences, University of Macau, Macao SAR, China.

2.2. Sample preparation

Sample preparation was performed by using pressurized liquid extraction on a Dionex ASE 200 system (Dionex Corp., Sunnyvale, CA, USA) under optimized conditions. In brief, dried powder of *Epimedium* (0.25 g) was mixed with diatomaceous earth in a proportion (1:1) and placed into an 11 ml stainless steel extraction cell, respectively. The extraction cell was extracted under the optimized conditions: solvent, 70% ethanol; particle size, 60–80 mesh; temperature, 120 °C; static extraction time, 10 min; pressure, 1500 psi; static cycle, 1 and the number of extraction times, 1. Then the extract was transferred into a 25 ml volumetric flask which was made up to its volume with extraction solvent and filtered through a 0.2 μm Nylon membrane filter (Whatman, UK) prior to injection into the UPLC system.

2.3. UPLC analysis

All analyses were performed on a Waters Acquity UPLC system (Waters, MA, USA) including binary solvent manager, sampler manager, column compartment and PDA detector, connected to a Waters Empower 2 software. An Acquity UPLC BEH C18 column (50 mm × 2.1 mm I.D., 1.7 μm) also from Waters was used. The column temperature was maintained at 25 °C. The standards and samples were separated using a gradient mobile phase consisting of water with 50 mM acetic acid (A) and acetonitrile (B). The gradient condition is: 0–2 min, 20–24% B; 2–4 min, 24–26% B; 4–5 min, 26–32% B; 5–12 min, 32–35% B; 12–15 min, 35–100% B; and finally, reconditioning the column with 20% B isocratic for 3 min after washing column with 100% B for 2 min. The flow rate was 0.25 ml/min and the injection volume was 1 μl. The peaks were detected at 270 nm.

2.4. Data analysis

Hierarchical clustering analysis was performed by SPSS 14.0 for windows (SPSS Inc., Chicago, IL, USA), which comprise a number of "procedures" – graphical, statistical, reporting, processing and tabulating procedures – that enable simple and rapid data evaluation. Ward's method, a very efficient method for the

Table 1
Summary for the tested samples of *Epimedium*

No.	Code	Samples	Sources	Collection date
1	BR-1	*Epimedium brevicornu* Maxim.	Lingzhou, Shanxi, China	2005.6
2	BR-2	*Epimedium brevicornu* Maxim.	Minxian, Gansu, China	2005.6
3	BR-3	*Epimedium brevicornu* Maxim.	Gansu, China	1986.12
4	SA-1	*Epimedium sagittatum* (Sieb. et Zucc.) Maxim.	Yunshan, Anhui, China	2005.4
5	SA-2	*Epimedium sagittatum* (Sieb. et Zucc.) Maxim.	Anji, Zhejiang, China	2005.4
6	SA-3	*Epimedium sagittatum* (Sieb. et Zucc.) Maxim.	Quanzhou, Guangxi, China	2005.5
7	SA-4	*Epimedium sagittatum* (Sieb. et Zucc.) Maxim.	Sichuan, China	2005.6
8	SA-5	*Epimedium sagittatum* (Sieb. et Zucc.) Maxim.	Chengdu, Sichuan, China	2005.6
9	SA-6	*Epimedium sagittatum* (Sieb. et Zucc.) Maxim.	Commercial	2005.6
10	SA-7	*Epimedium sagittatum* (Sieb. et Zucc.) Maxim.	Xianfeng, Hubei, China	1987.4
11	PU-1	*Epimedium pubescens* Maxim.	Bazhong, Sichuan, China	2005.4
12	PU-2	*Epimedium pubescens* Maxim.	Qionglai, Sichuan, China	2006.5
13	PU-3	*Epimedium pubescens* Maxim.	Commercial	2005.5
14	PU-4	*Epimedium pubescens* Maxim.	Unknown	Unknown
15	PU-5	*Epimedium pubescens* Maxim.	Guanyang, Guangxi, China	1987.4
16	WU-1	*Epimedium wushanense* T.S. Ying	Badong, Hubei, China	2004.4
17	WU-2	*Epimedium wushanense* T.S. Ying	Bazhong, Sichuan, China	2005.4
18	WU-3	*Epimedium wushanense* T.S. Ying	Commercial	2005.6
19	WU-4	*Epimedium wushanense* T.S. Ying	Leye, Guangxi, China	1989.3
20	KO-1	*Epimedium koreanum* Nakai	Fusong, Jilin, China	2005.8
21	KO-2	*Epimedium koreanum* Nakai	Benxi, Liaoning, China	2005.8
22	KO-3	*Epimedium koreanum* Nakai	Hengren, Liaoning, China	2004.6
23	AC-1	*Epimedium acuminatum* Franch.	Xingyi, Guizhou, China	2003.3
24	AC-2	*Epimedium acuminatum* Franch.	Ziyun, Guizhou, China	2003.4
25	AC-3	*Epimedium acuminatum* Franch.	Guiding, Guizhou, China	2003.4
26	AC-4	*Epimedium acuminatum* Franch.	Guizhou, China	1987.4
27	MY-1	*Epimedium myrianthum* Stearn	Yuping, Guizhou, China	2003.4
28	FR-1	*Epimedium franchetii* Stearn	Jianshi, Hubei, China	2004.4
29	ST-1	*Epimedium stellulatum* Stearn	Shiyan, Hubei, China	2004.4
30	ZH-1	*Epimedium zhushanense* K.F. Wu et S.X. Qian	Zhushan, Hubei, China	2004.4
31	LI-1	*Epimedium lishihchenii* Stearn	Lushan, Jiangxi, China	2005.4
32	DA-1	*Epimedium davidii* Franch.	Baoxing, Sichuan, China	2005.5
33	FA-1	*Epimedium fargesii* Franch.	Chengkou, Sichuan, China	1992.3
34	HU-1	*Epimedium hunanense* (Hand.-Mazz.) Hand.-Mazz.	Sanjiang, Guangxi, China	1987.3
35	LE-1	*Epimedium leptorrhizum* Stearn	Baojing, Hunan, China	1987.4
36	PL-1	*Epimedium platypetalum* K. Meyer	Panzhihua, Sichuan, China	1988.5
37	SU-1	*Epimedium sutchuenense* Franch.	Enshi, Hubei, China	1987.3

Table 2
Linear regression data, LOD and LOQ of the investigated compounds

Analytes	Linear regression data			LOD (ng)	LOQ (ng)
	Regressive equation	Test range ($\mu g\,ml^{-1}$)	R^2		
Hexandraside E	$y = 5716x + 3408$	1.80–115.00	0.9998	0.11	0.23
Kaempferol-3-*O*-rhamnoside	$y = 5462x + 4244$	1.84–117.50	0.9997	0.12	0.23
Hexandraside F	$y = 5457x + 2658$	1.81–116.00	0.9998	0.11	0.23
Epimedin A	$y = 5385x + 2700$	2.04–65.25	0.9999	0.05	0.26
Epimedin B	$y = 5923x + 2955$	1.87–119.50	0.9998	0.12	0.23
Epimedin C	$y = 5529x + 2773$	1.98–63.25	0.9999	0.05	0.25
Icariiin	$y = 6927x + 3141$	2.02–129.00	0.9998	0.13	0.25
Epimedoside C	$y = 7008x + 3467$	1.88–120.00	0.9998	0.12	0.23
Baohuoside II	$y = 7114x + 2761$	1.76–112.50	0.9998	0.11	0.22
Caohuoside C	$y = 6358x + 3764$	2.08–133.00	0.9998	0.13	0.26
Baohuoside VII	$y = 6304x + 1445$	1.97–63.00	1.0000	0.05	0.49
Sagittatoside A	$y = 6511x + 2334$	2.09–134.00	0.9998	0.13	0.52
Sagittatoside B	$y = 6836x + 2972$	1.83–58.50	0.9999	0.05	0.46
2”-*O*-rhamnosyl icariside II	$y = 6176x + 2419$	1.91–122.50	0.9998	0.12	0.48
Baohuoside I	$y = 9721x + 2012$	1.90–121.50	0.9998	0.12	0.24

Table 3
Intra- and inter-day precision of the investigated compounds

Analytes	Concentration ($\mu g\,ml^{-1}$)	Intra-day ($n=6$)			Inter-day ($n=6$)		
		Found ($\mu g\,ml^{-1}$)	R.S.D. (%)	Accuracy (%)[a]	Found ($\mu g\,ml^{-1}$)	R.S.D. (%)	Accuracy (%)
Hexandraside E	3.2	3.2	0.7	99.4	3.3	2.4	101.5
	14.4	14.8	1.5	102.9	15.4	4.5	107.0
	57.5	58.9	0.5	102.5	60.1	2.2	104.6
Kaempferol-3-*O*-rhamnoside	3.3	3.2	0.5	97.0	3.3	1.9	99.1
	14.7	15.2	1.6	103.7	15.8	4.4	107.8
	58.8	61.1	0.4	103.9	62.2	2.0	105.9
Hexandraside F	3.3	3.3	0.5	102.0	3.4	2.2	104.0
	14.5	14.9	1.6	102.6	15.5	4.5	106.7
	58.0	59.2	0.4	102.0	60.3	2.1	104.0
Epimedin A	3.7	3.6	0.6	97.8	3.6	2.0	99.4
	16.3	16.1	0.7	98.5	16.4	2.1	100.3
	65.3	65.7	0.6	100.7	67.1	2.4	102.8
Epimedin B	3.4	3.4	0.5	101.1	3.5	2.8	103.5
	14.9	15.3	1.6	102.3	15.9	4.8	106.5
	59.8	60.9	0.4	101.9	62.1	2.1	103.9
Epimedin C	3.6	3.5	0.5	97.6	3.5	1.6	99.6
	15.8	15.6	0.7	98.7	15.9	1.8	100.3
	63.3	63.8	0.5	100.9	65.1	2.3	102.9
Icariiin	3.6	3.8	0.4	103.7	3.8	2.4	105.9
	16.1	16.5	1.6	102.6	17.2	4.9	107.0
	64.5	65.8	0.4	102.0	67.1	2.3	104.1
Epimedoside C	3.4	3.5	0.5	102.9	3.6	3.1	106.1
	15.0	15.7	2.0	104.7	16.4	5.0	109.0
	60.0	62.8	0.4	104.7	64.1	2.1	106.8
Baohuoside II	3.2	3.3	1.0	104.6	3.4	2.8	107.0
	14.1	14.7	1.6	104.5	15.3	4.8	109.0
	56.3	58.5	0.4	104.1	59.6	2.0	106.0
Caohuoside C	3.7	3.8	0.7	102.0	3.9	2.8	104.3
	16.6	17.2	1.5	103.7	17.9	4.6	108.0
	66.5	68.7	0.5	103.4	70.0	2.0	105.3
Baohuoside VII	3.5	3.6	0.8	102.5	3.7	2.0	104.2
	15.8	15.5	0.8	98.2	15.7	1.6	99.9
	63.0	63.9	0.7	101.4	65.3	2.5	103.7
Sagittatoside A	3.8	3.9	3.1	102.3	4.1	4.6	108.2
	16.8	17.3	1.4	103.1	18.0	4.6	107.4
	67.0	68.5	0.4	102.2	69.9	2.2	104.3
Sagittatoside B	3.3	3.2	1.0	98.2	3.3	2.2	100.6
	14.6	14.5	0.8	99.1	14.7	1.6	100.7
	58.5	59.3	0.6	101.4	60.5	2.2	103.4
2″-*O*-rhamnosyl icariside II	3.4	3.6	0.7	104.9	3.7	1.7	106.6
	15.3	15.8	1.7	102.9	16.4	2.1	100.3
	61.3	62.6	0.4	102.3	63.9	2.1	104.2
Baohuoside I	3.4	3.7	1.6	108.8	3.8	2.9	110.3
	15.2	15.7	1.7	103.7	16.3	4.4	107.5
	60.8	62.1	0.5	102.3	63.4	2.1	104.3

[a] Accuracy (%) = 100% × mean of measured concentration/nominal concentration.

analysis of variance between clusters, was applied, and Squared Euclidean distance was selected as measurement for hierarchical clustering analysis.

The correlation coefficients and the similarities of entire chromatographic patterns among tested samples, and the simulative mean chromatogram were calculated and generated using a professional software named "Chromatographic Analysis and Data Management System of Traditional Chinese Medicine" (National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China).

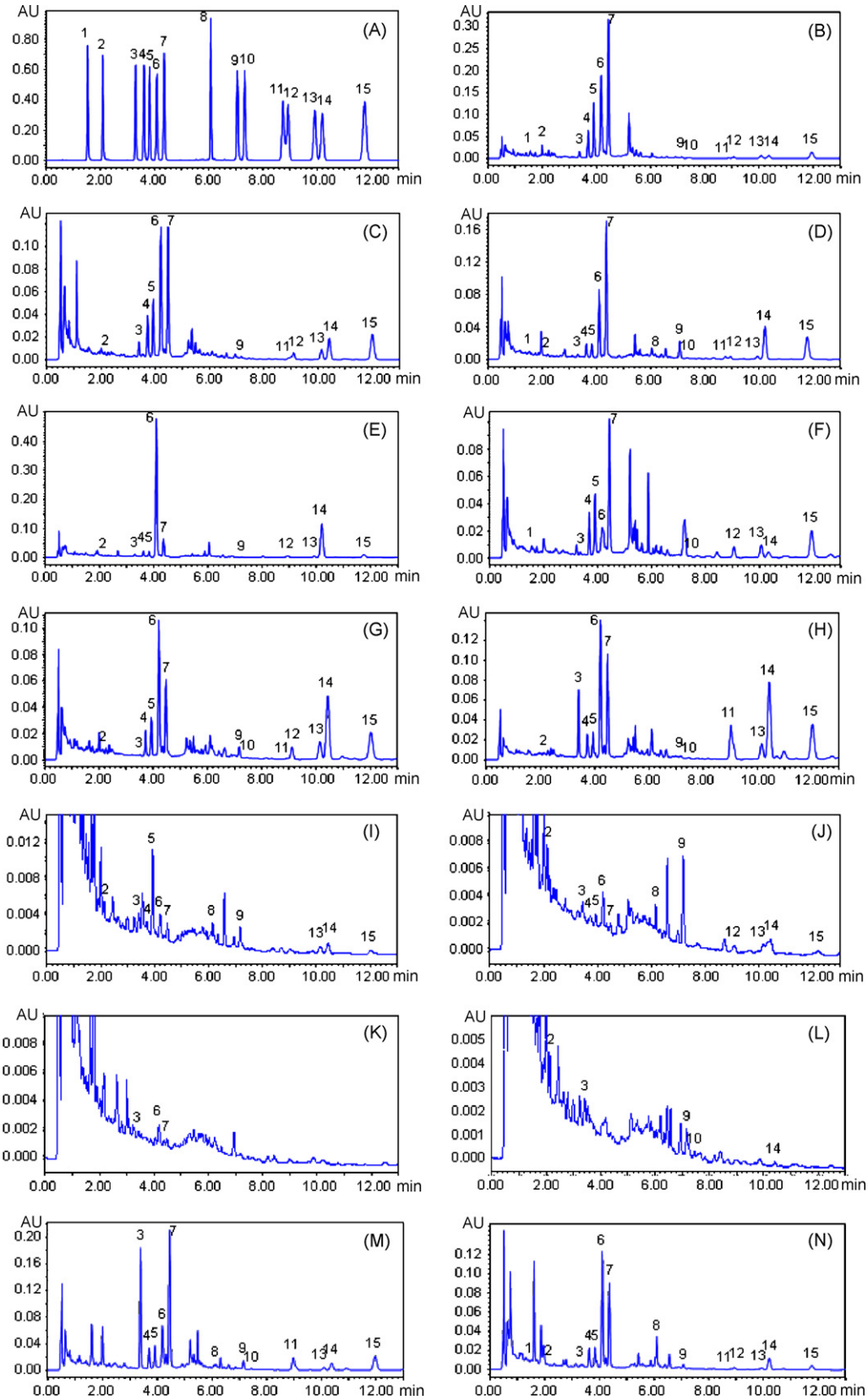


Fig. 2. Typical UPLC chromatograms of (A) mixed standards and PLE extracts of (B) *E. brevicornu*; (C) *E. sagittatum*; (D) *E. pubescens*; (E) *E. wushanense*; (F) *E. koreanum*; (G) *E. acuminatum*; (H) *E. myrianthum*; (I) *E. franchetii*; (J) *E. stellulatum*; (K) *E. zhushanense*; (L) *E. lishihchenii*; (M) *E. davidii*; (N) *E. fargesii*; (O) *E. hunanense*; (P) *E. leptorrhizum*; (Q) *E. platypetalum*; (R) *E. sutchuenense*. (1) hexandraside E; (2) kaempferol-3-*O*-rhamnoside; (3) hexandraside F; (4) pimedin A; (5) epimedin B; (6) epimedin C; (7) icariin; (8) epimedoside C; (9) baohuoside II; (10) caohuoside C; (11) baohuoside VII; (12) sagittatoside A; (13) sagittatoside B; (14) 2″-*O*-rhamnosyl icariside II; (15) baohuoside I.

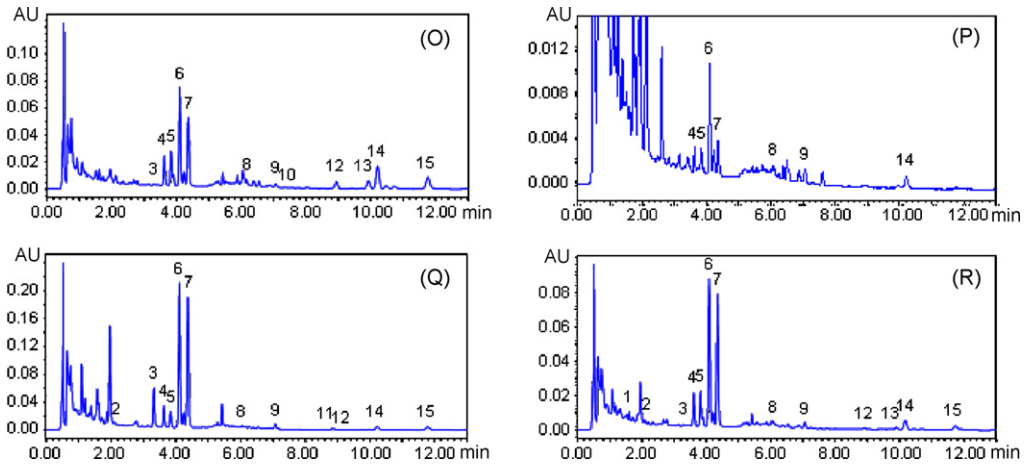


Fig. 2. (*Continued*).

3. Results and discussion

3.1. Calibration curves

Ethanol (70%) stock solutions containing reference compounds (except baohuoside VII and sagittatoside B) were prepared and diluted to appropriate concentrations for the construction of calibration curves. At least six concentrations of the solution were analyzed in duplicates, and then the calibration curves were constructed by plotting the peak areas versus the concentration of each analyte. The calibration curves of baohuoside VII and sagittatoside B were also determined as mentioned above using their mixture solution. The results were shown in Table 2.

3.2. Limits of detection and quantification

The stock solutions mentioned above were diluted to a series of appropriate concentrations with 70% ethanol, and an aliquot 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 a signal-to-noise ratio (S/N) of about 3 and 10, respectively. Table 2 showed the data of LOD and LOQ for each investigated compounds.

3.3. Precision, repeatability and accuracy

Intra- and inter-day variations were chosen to determine the precision of the developed assay. For intra-day variability test, the mixed standards solutions were analyzed for six replicates within 1 day, while for inter-day variability test, the solutions were examined in duplicates for consecutive 3 days. Variations were expressed by the relative standard deviations (R.S.D.) for intra- and inter-day, which were less than 3.1 and 5.0%, respectively. For every calibration curve, the calibration concentrations were back-calculated from the peak area of the analytes. The deviation from the nominal concentration defined as accuracy (Table 3).

Table 4
Recoveries for the assay of 15 compounds in *Epimedium*

Analytes	Original (μg)	Spiked (μg)	Found[a] (μg)	Recovery[b] (%)	R.S.D. (%)
Hexandraside E	–[c]	71.4	69.5	97.4	1.9
Kaempferol-3-*O*-rhamnoside	30.8	26.6	58.4	103.7	4.4
Hexandraside F	+[d]	116.4	111.0	95.4	2.3
Epimedin A	47.5	46.6	93.5	98.7	0.6
Epimedin B	43.8	45.3	87.5	96.5	2.7
Epimedin C	896.4	412.9	1289.6	95.2	3.9
Icariiin	192.4	134.1	320.3	95.3	1.6
Epimedoside C	–	19.2	19.1	99.5	1.0
Baohuoside II	+	26.5	25.2	95.2	1.6
Caohuoside C	+	57.9	58.4	100.9	4.3
Baohuoside VII	+	17.3	17.3	100.1	3.8
Sagittatoside A	+	42.1	42.5	101.1	1.0
Sagittatoside B	+	86.7	85.1	98.1	1.7
2″-*O*-rhamnosyl icariside II	247.9	92.0	335.3	95.0	2.1
Baohuoside I	39.8	42.1	81.3	98.5	0.9

[a] The data was present as average of three determinations.
[b] Recovery (%) = 100% × (amount found − original amount)/amount spiked.
[c] Undetected.
[d] Under the limit of quantitation.

Table 5
Contents (mg/g) of investigated compounds in *Epimedium*

Samples	1[a]	2	3	4	5	6	7	8	9	10	11	12	13	14	15	Sum (4 + 5 + 6 + 7)	Total
BR-1	0.59[b]	–[c]	0.24	1.14	7.45	2.90	8.40	0.22	0.20	0.03	0.09	0.30	1.18	0.76	1.68	19.89	25.19
BR-2	1.11	0.54	0.74	2.44	6.04	9.25	14.24	–	0.12	0.05	0.06	0.23	0.46	0.67	1.12	31.97	37.06
BR-3	0.40	0.43	0.54	0.60	2.75	1.22	2.74	0.04	0.04	–	–	+[d]	0.13	0.11	0.16	7.31	9.15
SA-1	–	0.28	–	–	–	0.07	–	–	0.11	0.02	–	+	–	–	–	0.07	0.48
SA-2	–	0.27	0.66	1.49	2.51	5.35	5.52	–	0.10	0.05	0.06	0.55	0.60	1.91	1.79	14.88	20.88
SA-3	–	0.27	0.52	0.69	1.31	4.06	0.96	–	0.16	0.05	0.69	1.41	1.72	8.32	0.97	7.03	21.13
SA-4	–	0.33	+	0.33	0.39	4.87	1.42	–	+	+	+	+	+	1.77	0.31	7.00	9.41
SA-5	0.39	–	0.09	1.85	2.62	14.64	10.86	–	0.05	0.07	+	0.17	0.13	1.32	0.58	29.96	32.78
SA-6	–	–	0.08	0.91	1.21	20.55	3.41	–	0.02	0.02	–	0.08	0.06	2.17	0.21	26.08	28.72
SA-7	–	–	7.88	0.34	0.13	5.10	0.63	0.82	0.86	–	–	4.65	–	1.97	0.17	6.19	22.54
PU-1	–	0.55	1.54	1.67	3.08	13.31	7.97	–	0.04	0.05	–	0.47	0.43	2.51	0.88	26.04	32.51
PU-2	0.80	–	1.30	1.75	2.79	13.23	10.37	–	0.35	0.10	0.22	0.59	0.42	4.61	2.37	28.13	38.90
PU-3	–	0.41	1.49	1.61	0.80	2.45	1.27	0.05	–	–	0.09	0.06	0.10	0.28	0.07	6.14	8.69
PU-4	–	–	0.20	1.08	1.99	7.20	1.84	0.02	–	–	–	0.07	+	0.29	–	12.11	12.69
PU-5	0.47	0.14	0.29	0.62	0.74	3.17	6.94	0.11	1.01	0.05	0.21	0.25	0.22	3.58	2.03	11.46	19.80
WU-1	–	0.26	1.79	0.55	0.79	5.51	0.91	–	–	0.05	–	0.37	0.18	1.67	0.23	7.75	12.30
WU-2	–	–	1.91	0.60	1.03	17.97	8.79	–	0.14	0.04	0.55	0.19	0.29	7.96	2.39	28.40	41.86
WU-3	–	–	–	2.17	2.14	6.00	2.28	0.26	0.07	0.03	–	0.91	0.51	2.34	0.52	12.59	17.23
WU-4	–	0.51	0.55	0.80	0.93	19.97	2.98	–	0.07	–	–	0.64	0.25	11.41	0.80	24.68	38.92
KO-1	0.65	–	0.15	1.22	2.18	1.80	4.59	–	–	0.06	–	0.57	0.58	0.43	1.53	9.79	13.76
KO-2	0.86	–	0.11	1.73	3.01	2.21	5.81	–	–	0.03	+	0.23	0.25	0.24	0.58	12.76	15.05
KO-3	0.82	–	0.11	1.75	3.04	2.58	6.53	–	–	0.03	–	0.09	0.08	0.14	0.30	13.90	15.47
AC-1	–	0.32	0.04	0.85	1.53	4.96	3.13	–	0.47	0.06	0.06	0.93	1.07	6.04	2.11	10.47	21.57
AC-2	–	–	0.07	1.26	1.83	5.01	2.96	–	0.04	–	–	0.11	0.06	0.31	0.10	11.06	11.73
AC-3	–	0.30	0.23	1.15	1.71	8.23	4.18	0.30	0.10	0.02	–	0.50	0.44	4.10	1.01	15.26	22.25
AC-4	–	0.19	–	0.25	0.05	0.76	0.11	0.54	0.45	–	–	+	–	0.26	–	1.18	2.61
MY-1	–	0.26	3.13	1.10	1.27	5.92	4.59	–	0.19	0.09	3.84	–	1.14	8.32	2.95	12.88	32.80
FR-1	–	0.09	0.19	0.10	0.49	0.17	0.12	0.07	0.12	–	–	–	0.06	0.16	0.05	0.87	1.60
ST-1	–	0.27	0.12	0.04	0.05	0.18	0.05	0.05	0.34	–	–	0.05	+	0.13	–	0.32	1.29
ZH-1	–	–	0.04	–	–	0.09	0.03	–	–	–	–	–	–	–	–	0.12	0.16
LI-1	–	0.09	0.09	–	–	–	–	–	0.06	0.02	–	–	–	+	–	–	0.26
DA-1	–	–	8.32	1.19	1.76	3.20	9.16	0.25	0.55	0.08	1.76	–	0.22	0.97	1.56	15.31	29.01
FA-1	0.21	0.13	0.11	0.76	0.96	4.84	3.70	1.06	0.21	–	+	0.18	0.09	1.06	0.28	10.27	13.59
HU-1	–	–	0.06	0.84	1.15	2.91	2.20	0.17	0.16	0.02	–	0.36	0.34	1.50	0.57	7.10	10.27
LE-1	–	–	–	0.09	0.14	0.42	0.16	0.03	0.06	–	–	–	–	0.06	–	0.81	0.96
PL-1	–	0.43	2.64	1.24	1.05	8.48	8.10	0.09	0.34	–	0.09	+	–	0.43	0.28	18.87	23.18
SU-1	0.39	0.18	0.05	0.85	1.10	3.95	3.84	0.09	0.15	–	–	0.08	0.08	0.56	0.18	9.75	11.52

[a] 1–15 are hexandraside E, kaempferol-3-*O*-rhamnoside, hexandraside F, epimedin A, epimedin B, epimedin C, icariin, epimedoside C, baohuoside II, caohuoside C, baohuoside VII, sagittatoside A, sagittatoside B, 2″-*O*-rhamnosyl icariside II and baohuoside I, respectively.
[b] The data was present as average of duplicates.
[c] Undetected.
[d] Under the limit of quantification.

The repeatability of the developed method was evaluated at three levels (0.10, 0.25 and 0.40 g) of the sample SA-4. The samples of each level were extracted and analyzed triplicates as mentioned above. The repeatability present as R.S.D. ($n = 3$) was less than 4.7, 3.8 and 3.4%, respectively.

The recovery was preformed by adding a known amount of individual standards into a certain amount (0.13 g) of SA-4. The mixture was extracted and analyzed using the method mentioned above. Three replicates were performed for the test. Table 4 shows the recoveries of the 15 investigated compounds.

3.4. Quantitation of the investigated flavonoids in Epimedium

The investigated flavonoids in *Epimedium* were well separated using the developed UPLC method. Typical chromatograms of the PLE extracts from different species of *Epimedium* were shown in Fig. 2. The identification of investigated compounds was carried out by comparison of their retention time and UV spectra with those obtained injecting standards in the same conditions, or by spiking the samples with stock standard solutions.

The developed UPLC method was applied to analyze 15 flavonoids in 37 samples of *Epimedium*. The data were summarized in Table 5. The results showed that there were great variations among the contents of the 15 investigated flavonoids in *Epimedium* from different species, collection and/or storage times and/or locations.

3.5. Comparison of different species of Epimedium

3.5.1. Hierarchical clustering analysis

In China, five species of *Epimedium*, including *E. brevicornu*, *E. sagittatum*, *E. pubescens*, *E. wushanense* and *E. koreanum*, are listed as *Yinyanghuo* in China pharmacopeia [1]. However, this work showed that the chemical variation is obvious among the different species and/or locations of *Epimedium*. Therefore, the exact identity is assurance of safety and efficacy of medication. In order to evaluate the variation of *Epimedium*, hierarchical cluster analysis was performed based on 15 investigated components characteristics from UPLC profiles of 37 tested samples. Fig. 3A shows the result on the 37 tested samples of *Epimedium*, which are divided into three main clusters where the contents of flavonoids were different (low to high). Especially, most sam-

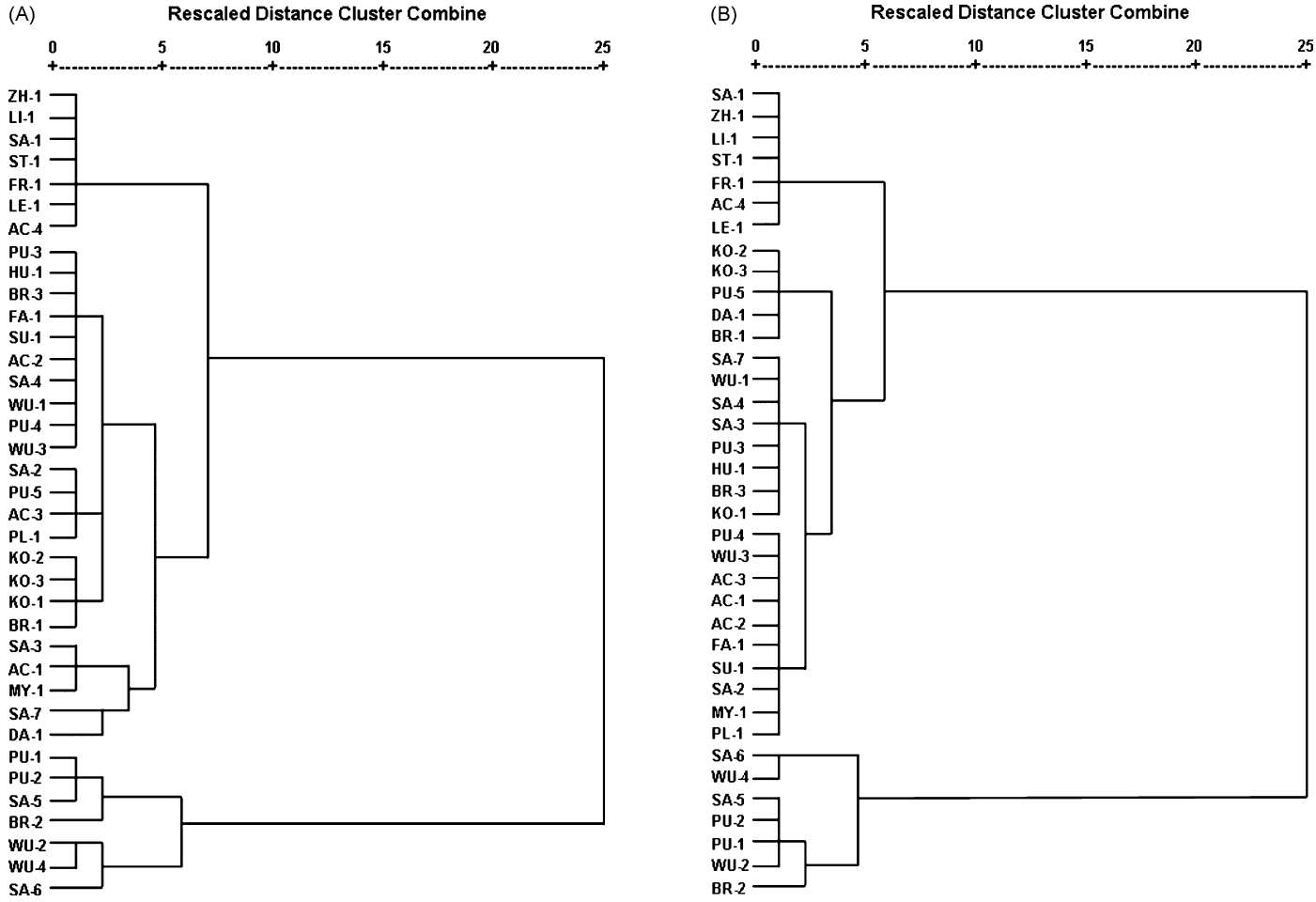


Fig. 3. Dendrograms of hierarchical cluster analysis for the 37 tested samples of *Epimedium*. The hierarchical clustering was done by SPSS software. Ward's method was applied, and Squared Euclidean distance was selected as measurement. (A) Dendrogram resulting from the 15 investigated compounds peaks' area derived from UPLC profiles of the tested samples. (B) Dendrogram resulting from the characteristics of four peaks, epimedin A, epimedin B, epimedin C and icariin, derived from UPLC profiles of the tested samples. The 37 samples are the same as Table 1.

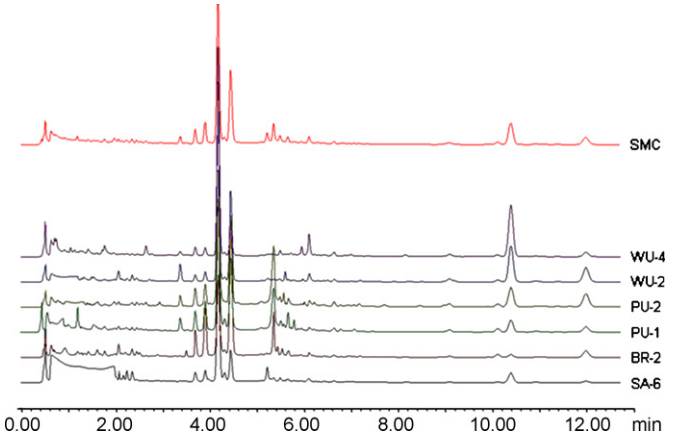


Fig. 4. UPLC chromatograms of six samples in high content cluster and their simulative mean chromatogram (SMC).

ples in the cluster with low content of flavonoids are the species of *Epimedium* not recorded in China pharmacopeia. Using the peak's characteristics of epimedin A, B, C and icariin, hierarchical cluster analysis was also performed as mentioned above. The result was very similar to the one derived from 15 compounds characteristics (Fig. 3B). Therefore, epimedin A, B, C and icariin could be used as markers for quality control of *Epimedium* used as *Yinyanghuo*.

3.5.2. Similarity evaluation

The similarity of the 37 tested *Epimedium* samples was pretty low. Based on the hierarchical clustering analysis, simulative mean chromatogram of the cluster with high content of flavonoids (high content cluster) was generated using six samples' chromatograms, where one sample left was used for test (Fig. 4). Then the similarity calculation was carried out after the UPLC profiles were standardized. The correlation coefficient (entire chromatogram) of test sample to mutual mode of high content cluster was 0.96, while the value for samples of middle and low content clusters were 0.70 ± 0.15 and 0.18 ± 0.03, respectively. Generally, the results of similarity evaluation were in accordance with those of hierarchical clustering analysis except one sample (AC-3). The correlation coefficient of sample AC-3 to mutual mode was 0.98, higher than that of test sample in high content cluster. It may due to that hierarchical clustering analysis was just based on the characteristics of 15 peaks of investigated samples, while the similarity evaluation was based on their entire chromatogram.

4. Conclusion

A rapid and reliable UPLC method is first developed for simultaneously quantitative determination of 15 flavonoids in 17 species of *Epimedium* which were divided into 3 clusters based on their flavonoids contents. The result has a good correlation with that of similarity evaluation based on the entire chromatogram. Epimedin A, B, C and icariin are selected as the markers for quality control of *Epimedium* used as *Yinyanghuo*, which is helpful to control their quality.

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