

## Quantitative and Pattern Recognition Analyses for the Quality Evaluation of Herba Epimedii by HPLC

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Received August 7, 2008, Accepted November 19, 2008

In this study, quantitative and pattern recognition analyses for the quality evaluation of Herba Epimedii using HPLC was developed. For quantitative analysis, five major bioactive constituents, hyperin, epimedin A, epimedin B, epimedin C, and icariin were determined. Analysis was carried out on Capcell pak C<sub>18</sub> column (250×4.6 mm, 5 μm) with a mobile phase of mixture of acetonitrile and 0.1% formic acid, using UV detection at 270 nm. The linear behavior was observed over the investigated concentration range (2–50 μg/mL;  $r^2 > 0.99$ ) for all analytes. The intra- and inter-day precisions were lower than 4.3% (as a relative standard deviation, RSD) and accuracies between 95.1% and 104.4%. The HPLC analytical method for pattern recognition analysis was validated by repeated analysis of one reference sample. The RSD of intra- and inter-day variation of relative retention time (RRT) and relative peak area (RPA) of the 12 selected common peaks were below 0.8% and 4.7%, respectively. The developed methods were applied to analysis of twenty Herba Epimedii extract samples. Contents of hyperin, epimedin A, epimedin B, epimedin C, and icariin were calculated to be 0–0.79, 0.69–1.91, 0.93–9.58, 0.65–3.05, and 2.43–11.8 mg/g dried plant. Principal component analysis (PCA) showed that most samples were clustered together with the reference samples but several apart from the main cluster in the PC score plot, indicating differences in overall chemical composition between two clusters. The present study suggests that quantitative determination of marker compounds combined with pattern-recognition method can provide a comprehensive approach for the quality assessment of herbal medicines.

**Key Words:** Herba Epimedii, HPLC, Pattern recognition, Quality control

### Introduction

Herbal medicines have a long history in therapeutic field and they are attracting considerable attention because of low toxicity and excellent therapeutic benefit. "Quality control" is one of the important issues to assure the efficacy of herbal medicines. Quality control in synthetic drugs is conducted by measuring their medicinal components whereas quality control in herbal medicines traditionally measuring a representative compound (a marker compound) contained in the herbal medicines. In other words, one or a few major constituents have been determined for identification and quality assessment of herbal medicine.<sup>1</sup> However, herbal drugs, individually and in combination, contain a myriad of compounds in complex matrices in which no single active constituent is responsible for the overall pharmacological efficacy.<sup>2</sup> Furthermore, in some cases, the marker compounds do not even relate to the pharmacological effects of the herbal medicines. Therefore, quantitation of one or few components will not an adequate approach for quality control of herbal medicines. In this regard, pattern recognition approach with chemometric analysis of the herbal medicine can be considered as an alternate and effective strategy. Fingerprint analysis/pattern recognition with multivariate statistical analysis can provide the information of overall chemical composition of herbal medicines including the marker compounds traditionally used for quality control. This approach has been extensively used in the quality studies of diverse herbal drugs<sup>3–6</sup> and recently has been

suggested to check the authenticity or quality evaluation of the herbal medicines by FDA, WHO and the State Food and Drug Administration of China.<sup>7–9</sup>

Herba Epimedii (Berberidaceae) is a well known Korean traditional medicine, prepared from the dried aerial parts of *Epimedium koreanum* Nakai or closely related species, such as *E. brevicornum* Maxim., *E. sagittatum* Sieb. et Zucc., *E. pubescens* Maxim., and *E. wushanense* T.S. Ying.<sup>10</sup> Herba Epimedii has attractive potential for the treatment of heart, cerebrovascular and other related diseases due to influence on Ca<sup>2+</sup> influx and efflux system.<sup>11</sup> Flavonoids, alkaloids and lignans have been identified as the constituents of this medicinal plant.<sup>12</sup> Especially, icariin, epimedin A, epimedin B, epimedin C and hyperin are known to be the biologically active flavonoid glycosides from this plant and these flavonoid compounds have mainly been considered as marker compounds of Herba Epimedii.<sup>13–30</sup>

Analytical tools such as thin-layer chromatography, high performance liquid chromatography, capillary zone electrophoresis, fourier transform infrared spectroscopy, micellar electrokinetic capillary chromatography and mass spectrometry have been developed for the analysis of Herba Epimedii and its related products.<sup>31–37</sup> However, these studies focused only quantitative analysis of selected marker compounds which are not promising approaches for the quality control of multi-component herbal drugs. In the present study, a simple, sensitive and precise reverse-phase high performance liquid chromatographic method with ultra-violet detection has been

developed for the quantitative determination of five marker flavonoid glycosides, namely hyperin, epimedin A, epimedin B, epimedin C and icariin along with pattern-recognition method for the quality control of Herba Epimedii extract. Total fifteen Herba Epimedii samples collected from the market were analyzed by HPLC after extraction with 70% ethanol. Subsequent principal component analysis (PCA) was applied to assess the comprehensive quality of Herba Epimedii.

### Experimental

**Plant materials.** The Herba Epimedii samples were purchased from various oriental herb stores in Korea and voucher specimens including the authenticated five reference samples (EP-ST-001-005 and EP-SP-001-015) were deposited at the Herbarium of Korea Institute of Science and Technology (Seoul, Korea).

**Reagents.** The standard compounds of hyperin, epimedin A, epimedin B, epimedin C and icariin were provided by Korea University, Seoul, Korea. Purity of standard compounds was estimated to be higher than 95% based on HPLC and liquid chromatography-mass spectrometry (LC-MS) analyses. Internal standard  $\alpha$ -naphthoflavone was purchased from Sigma Chemicals (St. Louis, MO, USA). Methanol and acetonitrile of HPLC grade were purchased from JT Baker (NJ, USA). All other chemicals used were of analytical grade unless otherwise noted. Distilled water was prepared using Milli-Q purification system (Millipore, Bedford, MA, USA).

**Sample preparation.** To determine the content of five marker compounds and pattern-recognition analysis of *E. koreanum* preparations, dried leaves powder were used for each extraction. One hundred grams of each Herba Epimedii sample was extracted with 70% ethanol (700 mL) by refluxing for 3 hr at 80°C. After cooling, the samples were filtered and the supernatant was collected. The supernatant was then concentrated and freeze-dried to produce dried powder. The sample solution for analysis was prepared to dissolve the extract in methanol at concentration of 2 mg/mL and suitably diluted to fit the calibration curve. An aliquot of the sample (100  $\mu$ L) was spiked with internal standard  $\alpha$ -naphthoflavone (100  $\mu$ g/mL) and filtered through a 0.22  $\mu$ m membrane filter to remove the undissolved particles before analysis. For Pattern-recognition analysis, samples are analyzed without addition of internal standard. Ten microliter of sample was subjected to injection into the HPLC system.

**HPLC condition.** Chromatography procedure was performed with Nanospace SI-1 HPLC system (Shiseido, Tokyo, Japan). The chromatographic separation of compounds was achieved using a Capcell Pak C<sub>18</sub> column (4.6 mm I.D.  $\times$  250 mm, 5  $\mu$ m, Shiseido, Tokyo, Japan) with column oven temperature maintained at 40°C. The mobile phase was consisted of 0.1% formic acid (A) and 90% acetonitrile containing 0.1% formic acid (B). Elution was performed at a flow rate of 1 mL/min in a binary gradient mode. The solvent gradient changed according to the following schedule: from 75%(A): 25%(B) to 50%(A):50%(B) in 12 min; to 85%(B): 15%(A) in 3 min; maintained for 5 min, followed by 10 min of column re-equilibrium with total run time of 30 min. Chromatograms

were acquired at 270 nm in UV detector. The signals from the detector were collected and analyzed with a computer equipped with software of SMC21 system (Shiseido, Tokyo, Japan).

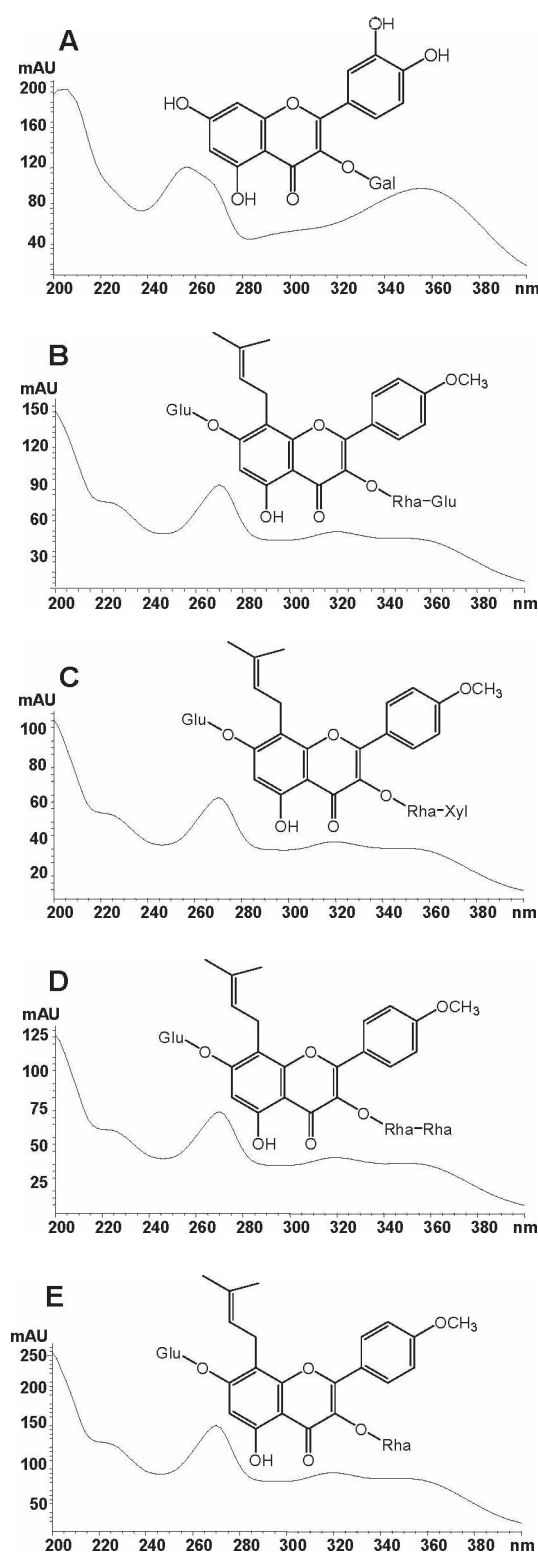
**Data analysis.** For pattern recognition analysis, 12 common peaks were selected based on the relative retention time (RRT) for icariin peak and used for a dataset. The peak area for each peak was calculated and then the resulting peak area was log-transformed. With the log-transformed peak area values for the selected 12 peaks, PCA was conducted using software package SAS 8.02 (SAS Institute Inc., Cary, NC).

### Results and Discussion

**Chromatography.** For the optimization of chromatographic condition, initially, the effect of the composition of mobile phase on the separation was examined. Mobile phase of water and methanol did not result in the satisfactory separation of structurally similar compounds such as epimedin A, epimedin B, and epimedin C. Acetonitrile as an organic modifier demonstrated a significant improvement in separation, but bad peak shape and tailing of the major analyte were still observed. The addition of 0.1% formic acid to the mobile phase to minimize the ionization of phenol group of flavonoid compounds resulted in a good resolution ( $R_s > 1.8$ ), as well as satisfactory peak symmetry and shape. For the choice of detection wavelength, extract sample was scanned between 200-400 nm using DAD detector. All components could be resolved with baseline separation at 270 nm with the maximum absorption shown for five major bioactive constituents: hyperin, epimedin A, epimedin B, epimedin C, and icariin (Fig. 1). Hence, characteristic chromatographic patterns were obtained at 270 nm. The typical chromatograms of samples and standard mixture are shown in Fig. 2, from which one can observe that all target compounds and internal standard are completely separated within 20 minutes. Hydrophobic and synthetic  $\alpha$ -naphthoflavone was selected as internal standard so that it could elute after complete elution of analytes to avoid the possible interference of compounds present in extract samples. The chromatographic peaks of the analytes in sample solution were identified by comparing their retention time and UV spectra with those of the reference standards and further confirmed by spiking samples with the reference compounds.

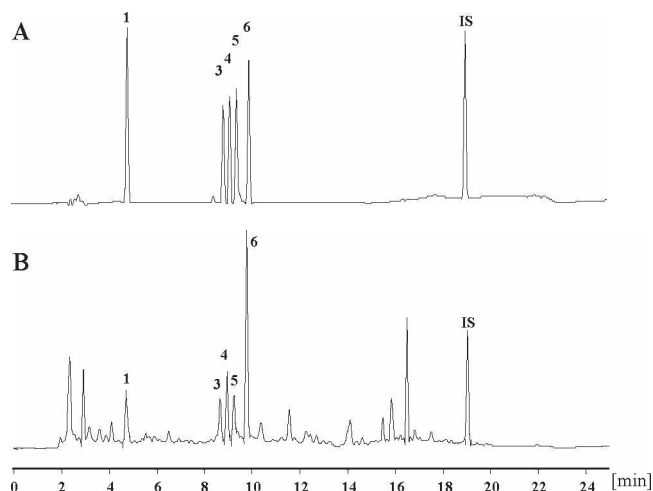
**Validation.** The calibration curves for hyperin, epimedin A, epimedin B, epimedin C, and icariin were generated by plotting the peak area ratio for analyte to internal standard versus the concentration by least-square regression analysis. Each calibration curve was obtained using five calibration standards at concentration of 2, 5, 10, 20 and 50  $\mu$ g/mL in triplicate. The range of calibration curve (2-50  $\mu$ g/mL) was found to be adequate for the analysis of Herba Epimedii extract used in this study. The linear correlation co-efficient ( $r^2$ ) for all calibration curves were greater than 0.998, indicating a good linearity in the proposed investigation range, and intercept was closer to zero (Table 1).

Precision and accuracy were determined by multiple analysis ( $n=5$ ) of quality control samples prepared at lower, medium and higher concentration spanning the calibration range (2, 10 and 50  $\mu$ g/mL). Intra-assay precision and accuracy



**Figure 1.** Chemical structure of marker compounds hyperin (A), epimedinin A (B), epimedinin B (C), epimedinin C (D), and icariin (E) along with UV spectra.

were determined from the variability of replicate analyses of quality control samples analyzed within the same analytical run. The quality control samples prepared at the lower limit of calibration curve had an intra-assay precision (RSD) lower than 3.33% and accuracy remained between 102.8% and



**Figure 2.** Representative HPLC chromatograms of standard mixture (A) and *Herba Epimedii* sample (B). Hyperin (1), epimedinin A (3), epimedinin B (4), epimedinin C (5), and icariin (6).

103.5% for all compounds. The remaining quality control samples had the intra-assay RSD below 1.17% and accuracy between 95.8% and 102.1%. Inter-assay precision and accuracy were evaluated from the variability of replicate analyses of quality control samples analyzed on single analytical run and extended for consecutive four days. The quality control samples prepared at the lower limit of calibration curve had the inter-assay RSD lower than 2.86% and accuracy between 102.1% and 104.4% for all analyzed compounds. The other quality control samples had the inter-assay RSD below 4.38% and accuracy between 95.1% and 104.9%. The above data reflects that the developed method is highly reproducible and precise for all flavonoid compounds tested. All intra and inter-assay precision and accuracy data are presented in Table 2.

A recovery test was performed to examine any effect of the matrix of plant extract on the analysis of analytes. In this validation test, a known amount at three different level of each standard compound stock solutions were spiked into *Herba Epimedii* sample and extracted described in preparation of sample solutions. For comparison, the blank sample spiking with 70% methanol was prepared and analyzed. Then, the quantitation of each component was subsequently achieved from the corresponding calibration curves. The recoveries of the all components were between 94.0% and 104.0% ( $n = 4$ ) with RSD < 6.79%. Detail results are shown in Table 3.

The stock solutions of standard compounds were diluted to a series of appropriate concentrations with 70% methanol, and an aliquot of diluted solutions were injected into HPLC system for analysis. The limits of detection (LOD) were evaluated based on the lowest detectable peak in the chromatogram having a signal-to-noise (S/N) ratio of 3. Under our experimental conditions, listed in Table 3, we found that LODs were of 0.11, 0.15, 0.21, 0.18 and 0.10  $\mu\text{g}/\text{mL}$  for hyperin, epimedinin A, epimedinin B, epimedinin C, and icariin respectively. The limits of quantitation (LOQ) were assessed based on the lowest quantitative level having a S/N ratio 10. LOQs of hyperin, epimedinin A, epimedinin B, epimedinin C, and icariin were 0.38, 0.50, 0.70, 0.60 and 0.34  $\mu\text{g}/\text{mL}$  respectively. The obtained values for both LOD and LOQ for these three stand-

**Table 1.** Calibration curve data for hyperin, epimedini A, epimedini B, epimedini C and icariin

Compounds	Linear range ( $\mu\text{g/mL}$ )	Slope $A^a$	Intercept $B^a$	Correlation Coefficient, $r^2$	LOD ( $\mu\text{g/mL}$ )	LOQ ( $\mu\text{g/mL}$ )
Hyperin	2-50	0.0453	-0.0091	0.9996	0.11	0.38
Epimedini A	2-50	0.0268	0.0092	0.9999	0.15	0.50
Epimedini B	2-50	0.0302	0.0049	0.9992	0.21	0.70
Epimedini C	2-50	0.0294	0.0250	0.9999	0.18	0.60
Icariin	2-50	0.0367	0.0113	0.9997	0.10	0.34

<sup>a</sup>Values are mean of three calibration curves: Slope and intercept refer to the regression equation:  $Y = Ax + B$ .

**Table 2.** Intra- and inter-day precision and accuracy for determination of hyperin, epimedini A, epimedini B, epimedini C and icariin

Compounds	Nominal conc. ( $\mu\text{g/mL}$ )	Intra-day			Inter-day		
		Conc. found ( $\mu\text{g/mL}$ ) Mean $\pm$ SD, n = 5	Accuracy (%)	Precision (%)	Conc. found ( $\mu\text{g/mL}$ ) Mean $\pm$ SD, n = 5	Accuracy (%)	Precision (%)
Hyperin	2	2.07 $\pm$ 0.01	103.5	0.34	2.04 $\pm$ 0.02	102.1	0.80
	10	9.59 $\pm$ 0.07	95.8	0.69	9.51 $\pm$ 0.22	95.1	2.27
	50	48.82 $\pm$ 0.09	97.6	0.19	48.54 $\pm$ 0.23	97.7	0.47
Epimedini A	2	2.07 $\pm$ 0.07	103.5	3.33	2.09 $\pm$ 0.05	104.4	2.43
	10	10.08 $\pm$ 0.12	100.8	1.17	9.97 $\pm$ 0.24	99.7	2.36
	50	50.86 $\pm$ 0.37	101.7	0.73	50.49 $\pm$ 0.23	100.8	0.46
Epimedini B	2	2.07 $\pm$ 0.03	103.3	1.39	2.07 $\pm$ 0.06	103.0	2.86
	10	10.06 $\pm$ 0.09	100.6	0.93	9.99 $\pm$ 0.29	99.9	2.94
	50	50.91 $\pm$ 0.20	101.8	0.40	52.45 $\pm$ 2.30	104.9	4.38
Epimedini C	2	2.06 $\pm$ 0.01	102.8	0.27	2.05 $\pm$ 0.03	102.3	1.64
	10	10.01 $\pm$ 0.08	100.1	0.77	9.94 $\pm$ 0.31	99.3	3.13
	50	50.91 $\pm$ 0.20	101.8	0.39	51.37 $\pm$ 2.23	102.7	4.34
Icariin	2	2.03 $\pm$ 0.04	103.0	2.12	2.04 $\pm$ 0.02	102.5	0.89
	10	10.12 $\pm$ 0.04	101.2	0.38	10.00 $\pm$ 0.28	98.6	2.78
	50	51.06 $\pm$ 0.35	102.1	0.69	51.19 $\pm$ 1.19	102.8	3.71

ards were shown to be low enough to detect traces of these flavonoid compounds in either crude extract or its preparation.

For validation of pattern-recognition analysis, the RRT and relative peak area (RPA) of the 12 selected peaks were used as parameters. The developed analytical method was validated by repeated analysis for one reference sample, ST3. The intra-day variation was determined by replicate analyses of the same sample six times in one day. The RSD of RRT and RPA for the 12 common peaks were lower than 0.78% and 3.98% respectively. The inter-day variation was determined by replicate analyses during five consecutive days. The RSDs of RRT and RPA were observed lower than 0.63% and 4.65% respectively. The results from intra- and inter-day variations reflected that this analytical method was suitable for obtaining reproducible chromatograms of the extracts (Table 4).

The sample stability test was determined with one sample, ST3 at 0, 12, 24, 36, 48, and 120 hr. During this period, the solution was stored at room temperature and 4°C. The resulting data indicated that all marker analytes remained stable more than 97% during the experimental period.

**Sample analysis.** The developed analytical method was subsequently applied to the simultaneous determination of the five components in *Herba Epimedii* extract. The quantity

**Table 3.** Recovery of hyperin, epimedini A, epimedini B, epimedini C and icariin from sample matrix

Compounds	Original mean ( $\mu\text{g/mL}$ )	Spiked ( $\mu\text{g/mL}$ ) (n=3)	Detected mean ( $\mu\text{g/mL}$ )	Recovery mean (%)	RSD(%)
Hyperin	4.65	2	6.70	102.5	6.79
		10	14.60	99.5	4.33
		25	29.43	99.1	1.95
Epimedini A	5.51	2	7.59	104.0	4.29
		10	15.35	98.4	5.73
		25	30.81	101.2	2.72
Epimedini B	7.43	2	9.38	97.5	5.10
		10	17.73	103.0	5.34
		25	32.06	98.5	3.91
Epimedini C	5.36	2	7.24	94.0	5.73
		10	15.65	102.9	4.11
		25	30.70	101.6	2.29
Icariin	19.45	2	21.54	104.0	5.71
		10	29.67	102.0	6.39
		25	44.03	98.3	3.76

$$\text{Recovery}(\%) = (\text{Amount}_{\text{determined}} - \text{Amount}_{\text{original}}) / \text{Amount}_{\text{spiked}} \cdot 100$$

**Table 4.** Intra- and inter-day variability of relative retention time (RRT) and relative peak area (RPA) of common peaks

Peak No.	Intra-day (n = 6)				Inter-day (n = 6)				Compounds identification
	RRT	RSD(%)	RPA	RSD(%)	RRT	RSD(%)	RPA	RSD(%)	
1	0.499	0.37	0.143	1.48	0.498	0.61	0.140	2.06	Hyperin
2	0.668	0.40	0.048	3.98	0.667	0.47	0.047	4.65	Unknown
3	0.881	0.39	0.178	2.96	0.880	0.37	0.188	4.61	Epimedin A
4	0.912	0.22	0.257	2.27	0.911	0.24	0.267	3.08	Epimedin B
5	0.942	0.19	0.180	1.59	0.942	0.29	0.178	3.25	Epimedin C
6	1.000	-	1.000	-	1.000	-	1.000	-	Icariin
7	1.057	0.21	0.076	2.21	1.057	0.29	0.078	4.28	Unknown
8	1.183	0.18	0.228	3.83	1.183	0.13	0.238	3.31	Unknown
9	1.428	0.12	0.177	1.79	1.437	0.20	0.174	2.94	Unknown
10	1.647	0.78	0.132	2.28	1.647	0.13	0.130	3.90	Unknown
11	1.728	0.18	0.337	1.89	1.726	0.47	0.341	3.24	Unknown
12	1.821	0.21	0.642	1.36	1.817	0.63	0.643	2.10	Unknown

Retention time of peak 6 (icariin) considered as reference peak

of each compound present in samples was determined and the results are summarized in Table 5. Each sample was analyzed in triplicate to ensure the reproducibility of the quantitative result. The results indicated that, four compounds except hyperin, were detected in all analyzed samples. Icariin was found to be the most abundant component (2.43–11.8 mg/g dried plant). The highest amount of marker compounds (epimedin A, epimedin B, epimedin C, and icariin) were found in samples EP9, EP10 and EP11. The overall results were consistent with our previous results using LC-MS/MS.<sup>36</sup> Generally, HPLC method has several limitations such as lower sensitivity and selectivity and longer analytical time than LC-MS/MS methods. Compared to the our previous study using LC-MS/MS<sup>36</sup> in technical aspects, the most significant differ-

ence between the two methods was shown in total analytical time: 6 min vs 30 min for LC-MS/MS and HPLC methods, respectively. However, in other points such as sensitivity and selectivity the HPLC method showed the results comparable to those obtained from the LC-MS/MS analysis. Therefore it is considered that the present HPLC method is effective enough to be employed at least for the quality control of *Herba Epimedii*.

**Pattern recognition analysis.** Mostly, *E. koreanum* is used as *Herba Epimedii* in Korea and thus the five authentic samples of *E. koreanum* were chosen as references for the quality control of *Herba Epimedii*. To evaluate the phytochemical-equivalency between the references and the fifteen samples from the market, a pattern recognition analysis was conducted.

**Table 5.** Determination of hyperin, epimedin A, epimedin B, epimedin C and icariin contents in crude extract samples of *Herba epimedii* (mg/g of dried plant)

Code No.	Flavonoids content				
	Hyperin	Epimedin A	Epimedin B	Epimedin C	Icariin
ST1	0.34 ± 0.02	0.77 ± 0.05	1.00 ± 0.04	0.90 ± 0.08	2.75 ± 0.13
ST2	0.58 ± 0.03	0.69 ± 0.04	0.93 ± 0.07	0.66 ± 0.02	2.43 ± 0.21
ST3	0.58 ± 0.05	0.93 ± 0.06	0.98 ± 0.05	0.66 ± 0.01	2.56 ± 0.17
ST4	ND	1.02 ± 0.09	1.44 ± 0.11	2.26 ± 0.21	4.05 ± 0.27
ST5	ND	0.80 ± 0.03	1.14 ± 0.07	1.75 ± 0.10	4.46 ± 0.02
EP1	ND	0.88 ± 0.06	1.27 ± 0.03	0.96 ± 0.12	5.87 ± 0.22
EP2	0.42 ± 0.02	0.99 ± 0.05	1.26 ± 0.09	0.44 ± 0.01	5.28 ± 0.17
EP3	0.46 ± 0.04	0.77 ± 0.03	1.08 ± 0.06	0.34 ± 0.02	3.80 ± 0.12
EP4	0.46 ± 0.03	0.76 ± 0.05	1.04 ± 0.04	0.30 ± 0.02	3.14 ± 0.14
EP5	0.21 ± 0.01	1.47 ± 0.10	2.07 ± 0.14	1.81 ± 0.11	3.18 ± 0.19
EP6	0.52 ± 0.03	1.17 ± 0.07	1.57 ± 0.05	0.73 ± 0.02	5.40 ± 0.25
EP7	0.55 ± 0.01	1.04 ± 0.03	1.32 ± 0.02	0.82 ± 0.06	3.99 ± 0.21
EP8	0.44 ± 0.02	0.96 ± 0.05	1.37 ± 0.09	1.18 ± 0.05	3.93 ± 0.12
EP9	ND	1.91 ± 0.11	7.83 ± 0.31	3.05 ± 0.22	9.45 ± 0.40
EP10	ND	1.75 ± 0.09	7.18 ± 0.29	2.68 ± 0.17	9.72 ± 0.34
EP11	ND	1.77 ± 0.13	9.58 ± 0.37	3.66 ± 0.22	11.8 ± 0.45
EP12	0.53 ± 0.04	0.99 ± 0.02	1.33 ± 0.07	0.72 ± 0.04	5.71 ± 0.23
EP13	0.79 ± 0.03	1.20 ± 0.05	1.66 ± 0.05	0.93 ± 0.04	4.96 ± 0.28
EP14	0.55 ± 0.01	0.95 ± 0.04	1.25 ± 0.07	0.73 ± 0.02	3.77 ± 0.21
EP15	0.69 ± 0.05	0.82 ± 0.07	1.32 ± 0.10	0.65 ± 0.03	2.91 ± 0.17

Data presented as the mean ± SD. (n = 3); ND indicated below lower limit of quantitation.

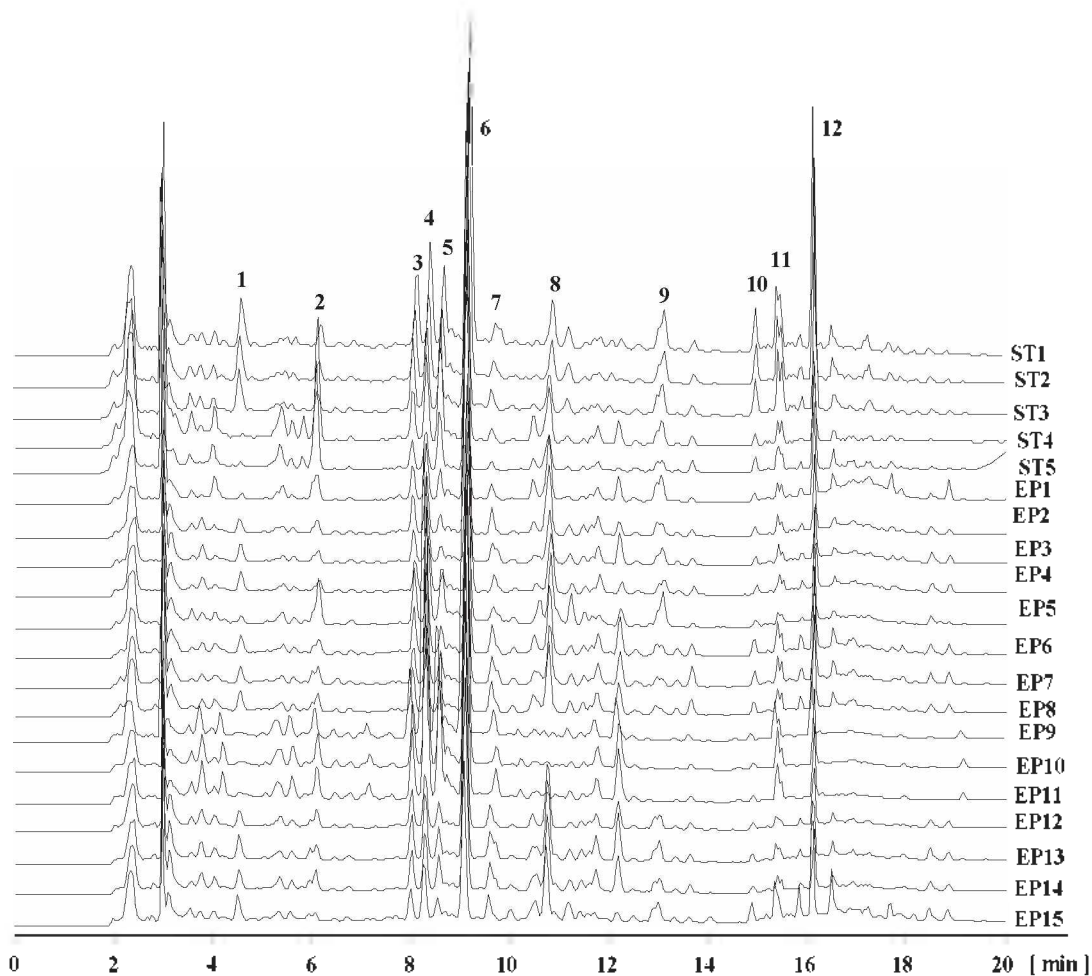


Figure 3. Overlaid chromatograms of twenty samples of Herba Epimedii including five reference samples collected from the market.

The fifteen Herba Epimedii samples collected from the market showed roughly similar chromatographic pattern but some differences were observed among the samples (Fig. 3). This may be a result of various factors such as origins, cultivation areas, harvesting season, climatic conditions, collection, washing, drying, preservation procedures, handling, transportation and storage conditions. First, PCA was conducted for twenty Herba Epimedii samples including five reference samples. As a result, the first three PCs (PC1, PC2, and PC3) could explain more than 85% of the total variability (data not shown). Therefore, the resulting score plots were presented for the first three PCs (Fig. 4). Most samples were clustered together with the five reference samples in the PC score plots, which indicated that these samples have similar chemical profiles to the references of *E. koreanum*. Meanwhile, the three samples, EP9, EP10, and EP11 were apart from the main cluster, revealing that those have somewhat different chemical profiles compared to other investigated samples including the reference samples. The seventeen samples except EP9, EP10, and EP11 were characterized by negative scores on PC1, while EP9, EP10, and EP11 showed positive scores on PC1. For PC2 or PC3, obvious separation between samples was not shown. EP9, EP10, and EP11 can be distinguished from other samples due to higher content of the marker compounds and lack of hyperin as shown in the quantitative result of Table 5

but may be considered to be high quality-herbal extract by the conventional guidance only requiring the minimum content of marker compound(s). However, PC1 loading plot (Fig. 5)

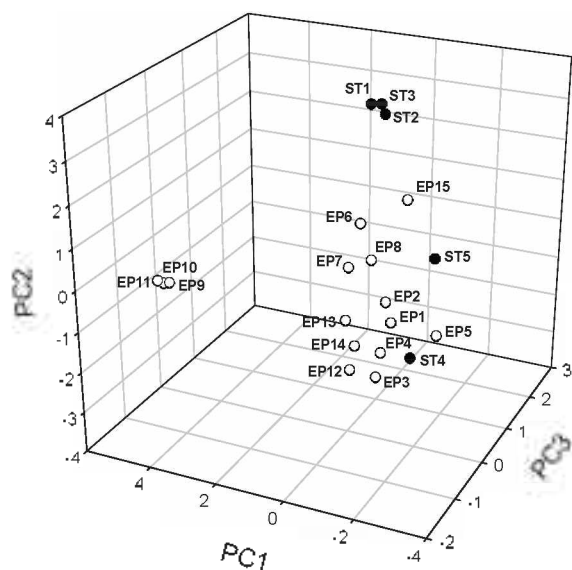


Figure 4. PC score plot of the first three PCs from PCA of 20 Herba Epimedii extract samples.

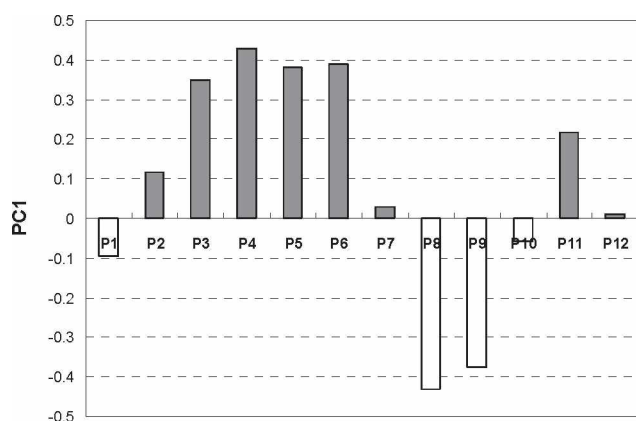


Figure 5. PC1-loading plot for the 12 common peaks of Herba Epimedii samples.

revealed that peak 8, peak 9, and peak 10 as well as peak 1 (hyperin) were important components characterizing the general Herba Epimedii extracts exhibiting the negative scores on the PC1. It simultaneously indicates that EP9, EP10, and EP11 have relatively low contents of these components, which is corresponding to the chromatographic data (Fig 3). Especially peak 8 and peak 9 rather than hyperin were found to be more contributive to clustering Herba Epimedii samples in the PC1 loading plot. Therefore, this result demonstrated that pattern recognition analysis can provide more comprehensive information for the chemical equivalency which can be omitted in the general simultaneous quantitative analyses as well as complement the conventional quality control approach of herbal medicine.

### Conclusion

A rapid and optimized chromatographic method with UV detection was designed for the quality control of Herba Epimedii, a well-known Korean traditional medicine. Validation data indicates that the developed analytical methods are suitable to measure the concentration of hyperin, epimedin A, epimedin B, epimedin C, and icariin and to apply to pattern recognition analysis of Herba Epimedii. Quantitative analysis of Herba Epimedii samples exhibits that Samples EP9, EP10 and EP11 contained the highest amount of marker compounds such as epimedin A, epimedin B, epimedin C and icariin among all analyzed samples. However, these three samples showed different chemical equivalent from other samples including the reference extracts in pattern recognition analysis. This implies that only quantitative determination of selected compounds is not sufficient to perform proper quality control tests for herbal medicine. The developed HPLC method for quantitative analysis of major bioactive compounds, along with a pattern-recognition method, can provide the promising prospect to comprehensive quality control of Herba Epimedii and its related herbal medicine.

**Acknowledgments.** This work was supported partly by a grant from Seoul Research and Business Program and partly by an intramural grant from the Korea Institute of Science and

Technology. The authors would like to thank Emeritus Professor D.S. Han (Seoul National University, Seoul, Korea) for identification of *Epimedium koreanum* Nakai plant.

### References

- Kim, J. H.; Park, S. Y.; Lim, H. K.; Park, A. Y.; Kim, J. S.; Kang, S. S.; Youm, J. R.; Han, S. B. *Bull. Korean Chem. Soc.* **2007**, *28*, 1187.
- Giordano, J.; Garcia, M. K.; Strickland, G. J. *Altern. Complement. Med.* **2004**, *10*, 706.
- Yan, S. K.; Zhang, W. D.; Liu, R. H.; Zhan, Y. C. *Chem. Pharm. Bull.* **2006**, *54*, 1058.
- Qian, G.; Leung, S. Y.; Lu, G.; Leung, K. S. Y. *Chem. Pharm. Bull.* **2006**, *54*, 1179.
- Piao, X. L.; Park, J. H.; Cui, J.; Kim, D. H.; Yoo, H. H. *J. Pharm. Biomed. Anal.* **2007**, *44*, 1163.
- Whang, W. H.; Lee, M. W.; Choi, H. K. *Bull. Korean Chem. Soc.* **2007**, *28*, 557.
- World Health Organization *Guidelines for the Assessment of Herbal Medicines*; WHO: Munich, Geneva, 1991.
- Guidance for Industry *Botanical Drug Products*; Food and Drug Administration: USA, June, 2004.
- Requirement for Studying Fingerprint of Traditional Chinese Medicine Injections (Draft)*; Drug Administration Bureau of China: 2000.
- Guo, B. L.; Xia, P. G. *China J. Chin. Mater. Med.* **2003**, *28*, 303.
- Yang, Y.; Liu, N.; Mo, Z.; Xie, J.; Liao, J.; Mo, S. *Appl. Radiat. Isot.* **2006**, *64*, 241.
- Sun, P.; Ye, W.; Zhao, J.; Pei, Y.; Wang, Z.; Chen, Y.; Ogihara, Y.; Takeda, T. *Chem. Pharm. Bull.* **1995**, *43*, 703.
- Liu, J.; Lou, Y. J. *J. Pharm. Biomed. Anal.* **2004**, *36*, 365.
- Liu, J. J.; Li, S. P.; Wang, Y. T. *J. Chromatogr. A* **2006**, *1103*, 344.
- Sha, A.; Cao, A.; Yang, S. *China J. Chin. Mater. Med.* **1995**, *20*, 357.
- Liu, W. J.; Xin, Z. C.; Xin, H.; Yuan, Y. M.; Tian, L.; Guo, Y. L. *Asian J. Androl.* **2005**, *7*, 381.
- Xin, Z. C.; Kim, E. K.; Lin, C. S.; Liu, W. J.; Tian, L.; Yuan, Y. M.; Fu, J. *Asian J. Androl.* **2003**, *5*, 15.
- Pan, Y.; Kong, L.; Xia, X.; Zhang, W.; Xia, Z.; Jiang, F. *Pharmacol. Biochem. Behav.* **2005**, *82*, 686.
- Lee, M. K.; Choi, Y. J.; Sung, S. H.; Shim, D. I.; Kim, J. W.; Kim, Y. C. *Planta Med.* **1995**, *61*, 523.
- Liu, Z. Q.; Luo, X. Y.; Sun, Y. X.; Wu, W.; Liu, C. M.; Liu, Z. Q.; Liu, S. Y. *J. Pharm. Pharmacol.* **2004**, *56*, 1557.
- Zheng, X.; Hu, Y.; Liu, J.; Ouyang, K. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 4531.
- Zhang, Z.; Li, Y.; Yang, X.; Wang, K.; Ni, J.; Qu, X. *Life Sci.* **2005**, *78*, 180.
- Zhu, D.; Qu, L.; Zhang, X.; Lou, Y. *Eur. J. Pharmacol.* **2005**, *514*, 99.
- Liu, T. Z.; Chen, C. Y.; Yiin, S. J.; Chen, C. H.; Cheng, J. T.; Shih, M. K.; Wang, Y. S.; Chen, C. L. *Food Chem. Toxicol.* **2006**, *44*, 227.
- Meng, F. H.; Li, Y. B.; Xiong, Z. L.; Jinag, Z. M.; Li, F. M. *Pharm. Biol.* **2005**, *43*, 92.
- Huang, J.; Yuan, L.; Wang, X.; Zhang, T. L.; Wang, K. *Life Sci.* **2007**, *81*, 832.
- Butterweck, V.; Jurgenliemk, G.; Nahrstedt, A.; Winterhoff, H. *Planta Med.* **2000**, *66*, 3.
- Wu, L. L.; Yang, X. B.; Huang, Z. M.; Liu, H. Z.; Wu, G. X. *Acta Pharmacol. Sin.* **2007**, *28*, 404.
- Liu, Z.; Tao, X.; Zhang, C.; Lu, Y.; Wei, D. *Biomed. Pharmacother.* **2005**, *59*, 481.
- Lee, S.; Jung, S. H.; Lee, Y. S.; Yamada, K.; Kim, B. K.; Ohuchi, K.; Shin, K. H. *Arch. Pharm. Res.* **2004**, *27*, 628.

31. Ding, C. *China J. Chin. Mater. Med.* **1990**, *15*, 604.
  32. Ito, Y.; Hirayama, F.; Suto, K.; Shima, T.; Sagara, K.; Yoshida, T. *J. Chromatogr.* **1988**, *456*, 392.
  33. Pozharitskaya, O. N.; Kosman, V. M.; Shikov, A. N.; Demchenko, D. V.; Eschenko, A. Y.; Makarov, V. G. *J. Sep. Sci.* **2007**, *30*, 708.
  34. Pei, L. K.; Sun, S. Q.; Guo, B. L.; Huang, W. H.; Xiao, P. G. *Spectrochim. Acta Part A: Mol. Biomol. Spectrosc.* **2007**, Doi: 10.1016/j.saa.2007.07.053.
  35. Chen, X. J.; Ji, H.; Wang, Y. T.; Li, S. P. *J. Sep. Sci.* **2008**, *31*, 881.
  36. Islam, N. M.; Yoo, H. H.; Lee, M. W.; Dong, M. S.; Park, Y. I.; Jeong, H. S.; Kim, D. H. *Phytochem. Anal.* **2008**, *19*, 71.
  37. Liu, Z.; Ding, L.; Zhang, H.; Hu, X.; Bu, F. *J. Liq. Chromatogr. Relat. Technol.* **2006**, *29*, 719.
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