Simultaneous Determination of Biological Marker Compounds in *Ostericum koreanum* by HPLC Method and Discrimination by Principal Component Analysis

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An analytical method for the simultaneous determination of four biological marker compounds including bisabolangelone, oxypeucedanin, imperatorin, and isoimperatorin extracted from *Ostericum koreanum* has been developed by HPLC-UV detection at a wavelength 254 nm. Moreover, biological active compounds have been identified by electrospray ionization tandem mass spectrometry (ESI-MS/MS). In ESI-MS/MS spectra, these compounds produced several characteristic ions through collisional-induced dissociation, enabling to clarify these compounds. HPLC chromatographic separation was successfully achieved with a Develosil RPAQUEOUS C_{30} (4.6 × 250 mm, 5 μm) column and a mobile phase of acetonitrile-water (60:40, v/v). This developed analytical method was validated with specificity, selectivity, accuracy and precision. The method was deemed satisfactory by inter- and intra-day validation and exhibited both high accuracy and precision (relative standard deviation < 9.2%). Overall limits of quantitation and detection were approximately 0.03-0.05 μg/mL at a signal-to-noise ratio (S/N) of 3 and were about 0.10-0.25 μg/mL at a S/N of 10. In addition, principal component analysis (PCA) was performed on the analytical data of 14 different *Ostericum koreanum* samples in order to classify samples collected from different regions. This method has been successfully applied to quality control of *Ostericum koreanum* extracts.

Key Words : *Ostericum koreanum*, HPLC, ESI-MS/MS, Method validation, Principal component analysis

Introduction

Herbal medicines have a long history in South Korea and have been widely used worldwide to prevent and treat human disease or maintain health. In general, the composition of herbal medicines is complex and active components are rarely identified. In addition, the quantities of active compounds and/or marker compounds in herbal medicines are dependent on intraspecies variability, environmental conditions, harvest period, storage time, and processing method.[1,2] Besides these factors, the extraction methods used to process the herbal plants can also affect the quantities of biologically active compounds in the extract. Thus, the quality control of active constituents or marker compounds in the herbal extract is of great importance in medicinal and dietary applications.

This study focuses on *Ostericum koreanum* (*Angelicae koreanae* Radix, Fam. Umbelliferae), one of the most important herbal medicines in the treatment of common cold, headache, neuralgia and arthritis.[3,4] This herbal medicine contains several active components including essential oil, coumarins (oxypeucedanin, imperatorin, isoimperatorin) and marmesinin.[5,6] Among these components, coumarins and structurally related compounds have been recently shown to inhibit human immunodeficiency virus, type 1 (HIV-1) activity.[7]

The isolation and identification of marker compounds in herbal medicines is a prerequisite in quality control since most of these compounds are not commercially available. Extraction and isolation methods including various liquid-liquid partition and chromatographic methods to obtain marker compounds from herbal medicines have been extensively reported.[6,8]

Various analytical methods have been reported for the quantification of bioactive constituents and marker compounds in herbal plants.[9-15] Several methods have been developed for the determination of coumarins in herbal medicines. The majority of these have been performed by using thin-layer chromatography (TLC),[11,12] reversed-phase high performance liquid chromatography (RP-HPLC),[11-15] high performance liquid chromatography-mass spectrometry (HPLC-MS),[15,16] gas chromatography-mass spectrometry (GC-MS),[17] pressurized capillary electrochromatography (pCEC),[18] and high-speed counter-current chromatography.[19]

To evaluate the quality control of *Ostericum koreanum*, the development of an HPLC analytical method to simultaneously determine marker compounds in the herbal extract is preferred. Several reports in the primary literature have discussed the analysis of oxypeucedanin and imperatorin in other herbal medicines using HPLC methods.[20,21] To our knowledge, there has been no analytical method reported which allows the simultaneous determination of bisabolangelone, oxypeucedanin, imperatorin, and isoimperatorin levels in *Ostericum koreanum*. Thus, it is necessary to

develop the analytical method that can simultaneously be separated and quantified these marker compounds by convenient HPLC-UV method for the evaluation of this herbal drug. The quality of *Ostericum koreanum* can be predicted by the quantitative analysis of these compounds. Based on chromatographic data of selected marker compounds obtained by the developed HPLC method, the quality control of herbal extracts can be evaluated. In addition, principal component analysis (PCA) can be used to provide a convenient visual aid for identification of inhomogeneity in the data sets.[22-25] Due to its usefulness in the differentiation of samples, PCA has recently been applied to the classification of traditional herbal plants from different origins.[24,25] The PCA technique combined with chromatographic data of selected marker compounds can be successfully applied in the quality control of herbal extracts.

The purpose of this study was to develop a simple and sensitive HPLC method for the simultaneous determination of four marker compounds in *Ostericum koreanum*. This method can be used to ensure the quality of herbal medicines and the chemical standardization of *Ostericum koreanum* samples. In addition, the classification of 14 *Ostericum koreanum* samples collected from various regions in Korea and China was performed using PCA. Moreover, biological active compounds have been identified by electrospray ionization tandem mass spectrometry (ESI-MS/MS). In ESI-MS/MS spectra, these compounds produced several characteristic ions through collisional-induced dissociation, enabling to clarify these compounds.

Experimental

Reagents and equipment. All reagents were of analytical grade. Acetonitrile, methanol and ethanol were purchased from J.T. Baker (Phillipsburg, NJ, USA). Biological marker compounds such as bisabolangelone, oxypeucedanin, imperatorin and isoimperatorin were isolated by methods outlined in previous reports[26-28] and their purity was determined by HPLC (purity ≥ 98%). An internal standard, propylparaben was purchased as crystalline powders from Sigma (St. Louis, MO, USA). The chemical structures of four marker compounds and internal standard are shown in Figure 1. Stock standard solutions for each of the analyte were prepared at concentrations of 1 mg/mL in acetonitrile and stored in the dark at −4 °C. Purified water was obtained from Millipore Alpha-Q Water system (Millipore, Bedford, MA, USA). A rotary evaporator (Büchi, Swiss) was used for the concentration of organic solvents. A soxhlet extractor (Buil Chemicals, Korea) was used for extraction of marker compounds from *Ostericum koreanum*.

Preparation of crude extract and isolation of marker compounds. Analytical samples of the herbal medicine, *Ostericum koreanum* was purchased from various market places in Korea and China. Dried *Ostericum koreanum* was pulverized to get homogenize the sample. Thirty grams of ground sample was taken and extracted with 300 mL 70% ethanol for 7 hr by soxhlet extraction method. After extraction, the extract was dried by rotary evaporator and then redissolved with HPLC mobile phase. The sample extract was filtered through 0.2 μm membrane filter, and then 10 μL aliquots from the filtrate were injected into the HPLC system. The overall analytical procedure for the determination of marker compounds extracted from *Ostericum koreanum* is indicated in Figure 2.

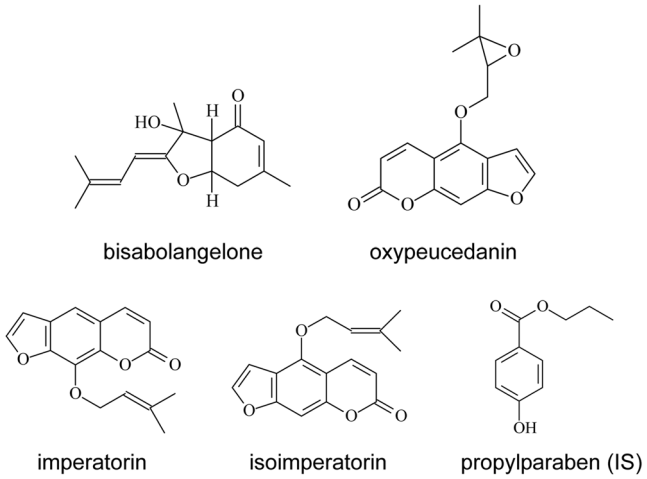

Figure 1. Chemical structures of the marker compounds isolated from *Ostericum koreanum* and internal standard (IS) used in this study.

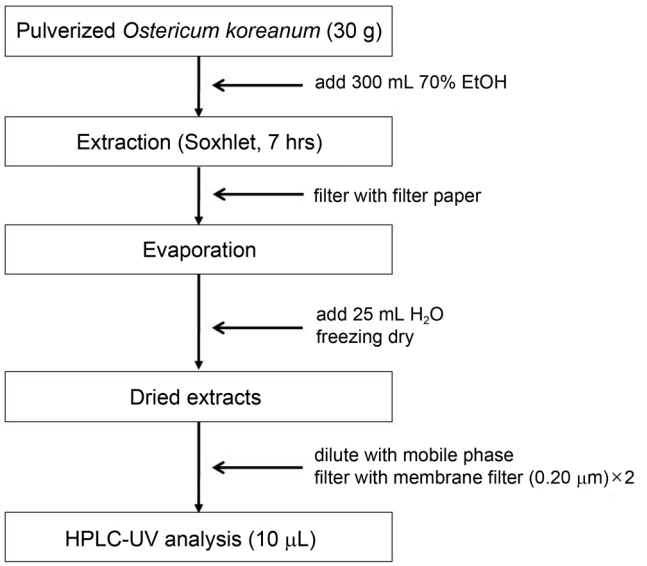

Figure 2. Overall analytical procedure for the extraction of marker compounds from *Ostericum koreanum*.

Preparation of reference standards. From the compounds obtained, 1 mg was dissolved in 1 mL of acetonitrile. These stock and working solutions were stored at −4 °C and used for no longer 2 months and 1 week, respectively. Each stock solution was diluted to the appropriate concentration range for the preparation of the calibration curves. For the quantitation of marker compounds by HPLC with UV detection, calibration curves with a five point were generated by the least squares method.

HPLC conditions. The apparatus used for HPLC con-

sisted of an Agilent 1100 series (Palo Alto, CA, USA) a quaternary pump with vacuum degasser, a thermostated column compartment, an autosampler and a diode array detector (DAD). Separation was achieved on a Develosil (Seto, Aichi, Japan) RPAQUEOUS C_{30} column (150 × 4.6 mm) 5 μm particle size, end capped to minimize unreacted silanol effects. The isocratic elution system was consisting of an aqueous acetonitrile and water (60:40, v/v). The flow rate was set at 1.0 mL/min with UV absorbance detection at 254 nm. The operation temperature was maintained at 25 °C. The mobile phase was degassed by filtering through a Millipore HV 0.20 μm pore membrane filter.

Method validation. Validation of the analytical method for the four coumarins and sesquiterpene as marker compounds examined for selectivity, linearity, limit of detection (LOD), limit of quantitation (LOQ), accuracy and precision.

For selectivity validation, a standard solution was composed of four marker compounds at the concentration of 25 μL/mL to be dissolved with acetonitrile. A volume of 10 μL of standard mixture was injected into the HPLC column and analyzed using an HPLC method as described above.

For linearity validation, standard solutions in the concentration range of 0.25-50 μg/mL for marker were prepared and injected into the HPLC system. The peak area-ratios of analytes to internal standard were plotted against the corresponding concentrations of the analytes and calibration curves constructed by means of the least-squares method.

The limit of detection (LOD) is defined as the concentration of the standard solution with a single-to-noise ratio > 3 (S/N > 3). The limit of quantification (LOQ) is defined as the concentration of standard solution with a signal-to-noise ratio > 10 (S/N > 10).

In order to confirm the reproducibility, the intra- and inter-day precision were estimated by analyzing five replicates containing the standard compounds at three different concentrations (10, 25, and 50 μg/mL) in a single day and repeating this analysis for five days, respectively.

Principal component analysis (PCA). PCA using the singular value decomposition method was performed by the Multivariate Statistical Package program (MVSP, Kovach Computing Service, Anglesey, Wales). For the classification of herbal medicines, PCA was performed by applying the peak area of selected marker compounds obtained from the HPLC analysis.

Electrospray ionization tandem mass spectrometry (ESI-MS/MS). ESI mass Spectra were acquired using an LCQ DECA XP (Thermo Finnigan, San Jose, CA, USA) mass spectrometer, equipped with an ESI source. Mass spectrometer was operated in full scan mode (positive and negative ions) over range of m/z 50-600 amu. The discharge and capillary temperature were set at 3 kV and 100 °C, respectively. The CID spectra were obtained in the MS/MS mode, which was triggered by when the intensity of an ion exceeded a preset threshold. The collision gas was high purity helium and adjusted the pressure of collision chamber by 50% reducing the intensity of precursor ion.

Results and Discussion

Isolation and identification of marker compounds. Isolation of the three coumarins and sesquiterpene (bisabolangelone) used as marker compounds of *Ostericum*

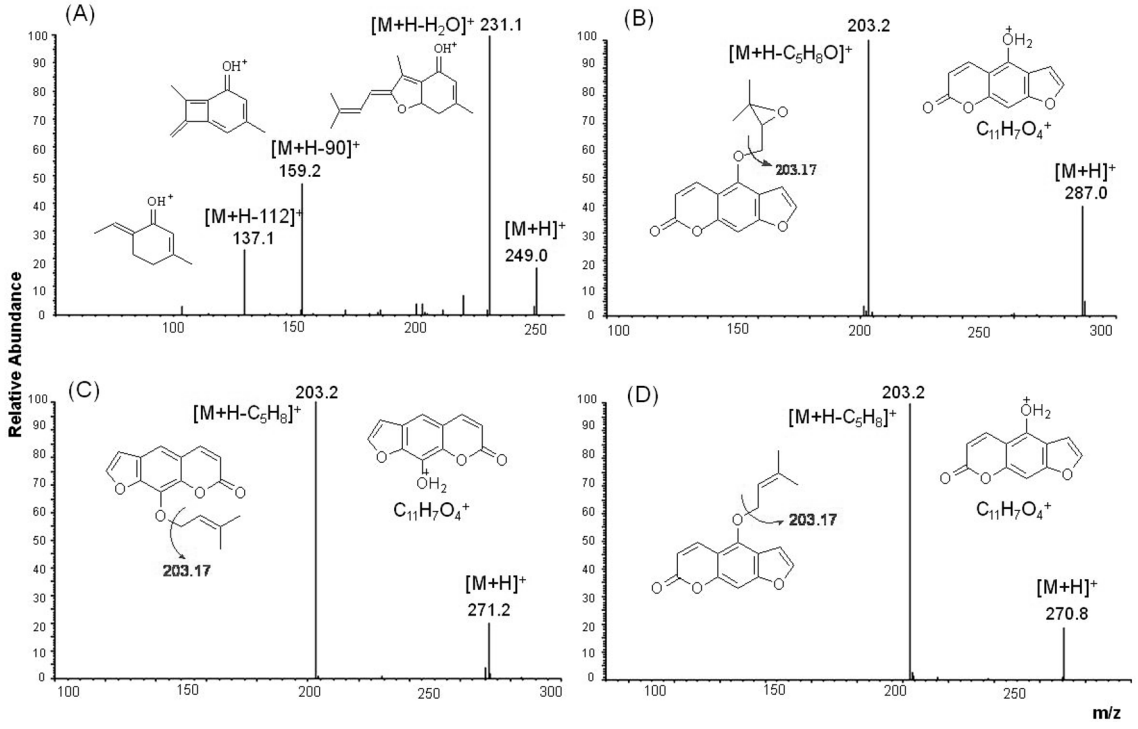

Figure 3. LC-electrospray ionization tandem mass spectra of $[M+H]^+$ at m/z 249 for bisabolangelone (A), m/z 287 for oxypeucedanin (B), m/z 271 for imperatorin (C) and m/z 271 for isoimperatorin (D).

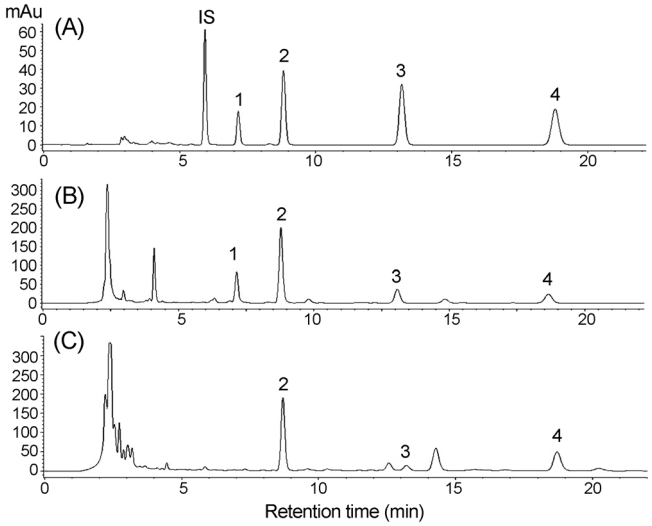

Figure 4. HPLC chromatograms of the standard mixture (A), 70% ethanol extract of Korea sample (B) and China sample (C) obtained by HPLC-DAD using a 250 mm × 4.6 mm i.d., 5 μm particle, Develosil RPAQUEOUS C_{30} column. The mobile phase was 60:40 (v/v) acetonitrile-water. Peaks are identified as follows: 1. bisabolangelone; 2. oxypeucedanin; 3. imperatorin; and 4. isoimperatorin. The internal standard (IS) is propylparaben.

koreanum was performed as described in previous reports.[26-28] The purity of the isolated compounds was greater than 98% as evaluated by HPLC with UV detection. These compounds were characterized by the presence of abundant $[M+H]^+$ ions in positive ion mode ESI-MS analysis, due mainly to protonation at oxygen atom bonded furocoumarin ring. On the other hand, the marker compounds exhibited poor sensitive detection in negative ion mode ESI-MS analysis because these compounds have no-active hydrogen atom to occur deprotonation.

To elucidate the chemical structures of furocoumarins, ESI CID-MS/MS technique was applied. The MS/MS spectra of $[M+H]^+$ ions for furocoumarins produced common product ion at m/z 203 ($C_{11}H_7O_4$) that is formed through the loss of side-chain from furocoumarins, as indicated in Figure 3. This characteristic ion can be used as diagnostic ion for the presence of a furocoumarins ring moiety in complicate herbal extract. In the case of MS/MS spectrum for bisabolangelone, the characteristic ions were formed at the ions m/z 231, 159 and 137 through dehydration, the losses of 90u and 112u from $[M+H]^+$ ion. The chemical structures of these product ions are indicated in Figure 3-A.

Optimization of chromatographic conditions. In order to simultaneously determine four marker compounds, HPLC chromatographic conditions should be optimized to successfully separate these compounds. At first, reversed phase C_{30} column was selected for the LC separation of furocoumarins and sesquiterpenes based on qualitative assessments of chemical structures and solubility. Optimization of HPLC conditions was based on the peak resolution and retention time and performed using a mixed mobile phase consisting of acetonitrile and methanol with water. The mobile phase consisted of organic solvent acetonitrile-water (60:40, v/v).

Bearing these factors in mind, acceptable results were obtained with a flow rate of 1.0 mL/min. The coumarin compounds each exhibit maximum absorbance at 254 nm. Using a standard mixture of the four marker compounds, the above HPLC conditions yielded acceptable retention times and symmetric peaks with reasonable resolution and overall analysis times, as shown in Figure 4-A. As shown in Figure 4, all four marker compounds and the internal standard were successfully resolved and eluted within 20 min.

Linearity, detection limit, and quantification limit. Calibration curves were generated using standard solutions containing 0.25-50 μg/mL of oxypeucedanin and imperatorin, and 0.50-50 μg/mL of bisabolangelone and isoimperatorin of marker compounds. Standard mixture solutions at five concentrations were analyzed in triplicate. Multi-point calibration curves were constructed by linear regression analysis of the peak area ratios of each analyte to the internal standard, versus concentration. The calibration curves of the analytes showed good linearity within given concentration ranges. Correlation coefficients ranged from 0.996 to 0.999, indicating excellent linearity of this method. Line equations representing the calibration curves and their correlation coefficients are summarized in Table 1.

The limit of detection (LOD) was defined as the concentration at which a signal three times higher than the noise level was generated. LODs were determined by performing five injections of each compound at concentrations incrementally approaching the LOD. LOD values were 0.03 μg/mL for oxypeucedanin and imperatorin, and 0.05 μg/mL for bisabolangelone and isoimperatorin.

The limit of quantitation (LOQ) was defined as the lowest concentration of a target compound that can be accurately and precisely quantified, in this case ten times the noise level. LOQ values were determined by performing five injections of each compound at concentrations approaching the LOQ. LOQs were 0.10 μg/mL for oxypeucedanin and imperatorin, and 0.25 μg/mL for bisabolangelone and isoimperatorin. The LODs and LOQs of four marker compounds are also represented in Table 1. These compounds

Table 1. Equations of calibration curves, linearity correlation coefficients, LOD and LOQ for marker compounds

Compounds	Linear range (μg/g)	Slope	Intercept	Correlation coefficient	LOD (μg/g)	LOQ (μg/g)
bisabolangelone	0.50 - 50.00	0.092	−0.187	0.996	0.05	0.25
oxypeucedanin	0.25 - 50.00	0.072	−0.006	0.999	0.03	0.10
imperatorin	0.25 - 50.00	0.086	−0.008	0.999	0.03	0.10
isoimperatorin	0.50 - 50.00	0.076	−0.009	0.999	0.05	0.25

Table 2. Intra- and inter-day precision and accuracy for the quantification method of marker compounds in standard solution

Compounds	Fortified conc. (μg/mL)	Intra-day (n = 5)			Inter-day (n = 5)		
		Observed conc. (μg/mL)	Precision (%)	Accuracy (%)	Observed conc. (μg/mL)	Precision (%)	Accuracy (%)
bisabolangelone	10	9.46	2.72	94.67	10.78	9.18	107.79
	25	23.89	1.38	95.56	26.75	7.28	107.00
	50	51.12	0.84	102.25	52.85	3.36	105.72
oxypeucedanin	10	9.97	0.05	99.71	9.95	0.20	99.49
	25	25.43	0.15	101.71	25.42	0.26	101.69
	50	50.57	0.11	101.14	50.43	0.27	100.87
imperatorin	10	9.96	0.05	99.57	9.94	0.15	99.42
	25	25.34	0.12	101.36	25.33	0.14	101.40
	50	50.41	0.16	100.81	50.21	0.33	100.42
isoimperatorin	10	9.96	0.05	99.62	9.93	0.24	99.27
	25	25.37	0.08	101.49	25.32	0.23	101.25
	50	50.50	0.23	101.01	50.31	0.42	100.62

were shown reasonable sensitivities for quality control of *Ostericum koreanum*.

Accuracy and precision. To test the accuracy and precision of the analytical method, the intra- and inter-day variations for four marker compounds were determined and are summarized in Table 2. The precision of the method for simultaneously determining the five marker compounds was acceptable since the relative standard deviation (RSD) did not exceed 10% at concentrations of 10, 25, and 50 μg/mL. At the same concentrations, the intra-day accuracy ranged from 94.6 to 102.2%, while the inter-day accuracy ranged from 99.2 to 107.8%.

Application of the method. The method developed herein was applied to 14 different samples. The amounts of marker compounds in *Ostericum koreanum* were determined in samples collected from nine samples of Korea and from five samples of China (C-1 to C-5). A typical chromatogram of the crude extract is shown in Figure 4-B and C, indicating the absence of any significant interferents for the quantitation of the four marker compounds. As shown in Figure 4-B and C, the amount of some marker compounds in HPLC chromatograms exhibited a significant difference between Korea sample (B chromatogram) and China sample (C chromatogram).

A summary of the regional data in Table 3 shows significant regional variability. In difference between Korea and Chinese samples, the overall amount of bisabolangelone in Korean samples was greatly higher than those in Chinese samples. On the other hand, the average amount of isoimperatorin in Chinese samples (7.80%, w/w) was about 4.7 times higher than those in Korean samples (1.66%, w/w). The amounts of oxypeucedanin and imperatorin are shown a similar distribution for both samples.

Quality assessment by PCA. Although great difference in the amount of bisabolangelone between Korean samples and Chinese samples was observed, the regional differentiation of *Ostericum koreanum* could not be performed by just comparing the amount of this compound. Reasonable criterion on the amount of bisabolangelone was not provided for the differentiation of herbal plants at this present time. To clearly classify the herbal plants collected from different regions, PCA was performed on the analytical data of all 14 samples. The amount of four marker compounds in Table 3 measured in n samples (n = 14) having two different origins represented the values of p variables (p = 4) and it made $n \times p$ matrix. From this matrix, principal component scores were computed. To display the points in two principal components, PC 1 and PC 2 (first and second principal components) were chosen to represent the information. In this study, the marker compounds and their corresponding w/w % obtained from HPLC chromatographic analysis were selected as PCA parameters. When chromatographic data for four marker compounds were selected, the scatter points were shown as random distribution, not enabling classifi-

Table 3. The amount of four marker compounds in *Ostericum koreanum* collected from different regions (n = 3)

Sample number	Analytes, contents (w/w, %)			
	bisabol-angelone	oxypeuce-danin	impera-torin	isoimpera-torin
[a]K-1	17.22 ± 0.10	0.99 ± 0.01	3.00 ± 0.05	0.45 ± 0.03
K-2	24.66 ± 0.07	0.76 ± 0.01	3.41 ± 0.01	0.42 ± 0.01
K-3	13.30 ± 0.01	1.02 ± 0.01	1.57 ± 0.10	0.59 ± 0.01
K-4	19.84 ± 0.05	1.76 ± 0.01	3.34 ± 0.02	1.18 ± 0.01
K-5	16.24 ± 0.04	1.42 ± 0.01	2.95 ± 0.03	0.63 ± 0.03
K-6	4.29 ± 0.02	13.29 ± 0.07	4.88 ± 0.02	5.23 ± 0.04
K-7	8.07 ± 0.14	21.11 ± 0.07	5.60 ± 0.01	4.95 ± 0.03
K-8	8.98 ± 0.02	0.70 ± 0.02	0.79 ± 0.07	0.68 ± 0.06
K-9	11.01 ± 0.02	1.00 ± 0.03	1.62 ± 0.04	0.48 ± 0.02
[b]C-1	ND	ND	6.98 ± 0.19	3.24 ± 0.12
C-2	0.57 ± 0.01	2.81 ± 0.01	4.78 ± 0.05	10.74 ± 0.15
C-3	0.73 ± 0.02	2.85 ± 0.01	4.69 ± 0.04	10.70 ± 0.11
C-4	ND	12.60 ± 0.10	1.65 ± 0.02	6.49 ± 0.03
C-5	0.52 ± 0.03	1.02 ± 0.01	5.88 ± 0.10	14.42 ± 0.21

[a]K-1~9: Korea sample; K-1: Jeongseon (1), K-2: Jechun, K-3: Youngju, K-4: Bonghwa, K-5: Inje, K-6: Jeongseon (2), K-7: Youngchun (1), K-8: Youngchun (2), K-9: Geumsan. [b]C-1~5: China sample (not specified a collected region)

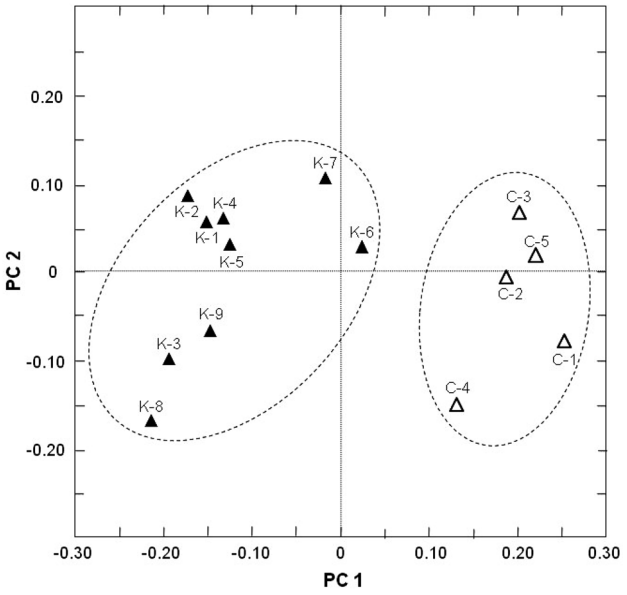

Figure 5. Representation of 14 chromatographic samples of *Ostericum koreanum* on PC 1 and PC 2 (99% variance explained).

cation according to regional samples (not shown data here). Hence, three marker compounds except for isoimperatorin were selected since its amount was wide range for all samples. Following PCA approach, a decision must be made on how many PC scores should be retained. Generally, sufficient components should be retained to account for a specified percentage of the total variance, say 80%.[29,30] According to this criterion, the first two PC scores were chosen for the plotting of PCA plot. Figure 5 shows the principal component projection plot of the PC 1 and PC 2 of 14 *Ostericum koreanum* samples. It was found that PC 1 and PC 2 reflected 99% of the total variance. From the scatter points, the samples could be classified into two groups, indicating a clear differentiation between *Ostericum koreanum* collected in Korea and China regions.

Conclusion

In this study, we described and validated a HPLC method for separating and quantifying four marker compounds in *Ostericum koreanum*. The method was found to be suitable for the chemical standardization of herbal medicines obtained from *Ostericum koreanum* regardless of geographic origin. The simplicity of the procedure, combined with excellent sensitivity, resolution, and short analysis time, makes this method a useful tool for characterizing furanocoumarins and sesquiterpen compounds in other herbal medicines. The structural information of marker compounds was obtained by ESI-MS/MS technique, providing diagnostic ions for furanocoumarins. In addition, PCA can provide important information on the differentiation of herbal plants from different regions.

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