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Development of a validated liquid chromatographic method for the quality control of *Prunellae Spica*: Determination of triterpenic acids

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ABSTRACT

A simple and rapid reversed-phase HPLC-UV method was developed for the determination of triterpenic acids in the crude extract of *Prunellae Spica*. Five triterpenic acids were extracted and isolated from *P. Spica* as marker compounds for use in the quality control of herbal medicines. Various solvent extraction techniques were evaluated, and the greatest efficiency was observed with sonication in 100% ethanol. Elemental compositions of the five marker compounds were determined by high-resolution mass spectroscopy. The dynamic range of the HPLC-UV method depended on the specific analyte, and acceptable quantitation was obtained between 10 and 250 $\mu g\,mL^{-1}$ for oleanolic acid, between 10 and 300 $\mu g\,mL^{-1}$ for ursolic acid, between 3 and 75 $\mu g\,mL^{-1}$ for 2α,3α,24-trihydroxyolean-12en-28oic acid, between 5 and 100 $\mu g\,mL^{-1}$ for euscaphic acid, and between 5 and 100 $\mu g\,mL^{-1}$ for 2α,3α-dihydroxyurs-12en-28oic acid. The method was deemed satisfactory by inter- and intra-day validation and exhibited both high accuracy and precision (relative standard deviation <9.4%). Overall limits of quantitation and detection were approximately 0.5–2.5 $\mu g\,mL^{-1}$ at a signal-to-noise ratio (S/N) of 3 and were about 3.0–10.0 $\mu g\,mL^{-1}$ at a S/N of 10. In addition, principal component analysis (PCA) was performed on the analytical data of 15 different *P. Spica* samples in order to classify samples collected from different regions.

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1. 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 *Prunellae Spica* (*Prunellae vulgaris L., Fam. Labiatae*), one of the most important herbal medicines in the treatment of pulmonary tuberculosis, mastitis, infectious hepatitis, and hypertension [3,4]. This herbal medicine contains several active components including triterpenic acids and their glycosidic derivatives, phenolic acids, and saponins [5,6]. Among these components, triterpenic acids such as oleanolic acid, ursolic acid, hydroxyursolic acid, hydroxyoleanolic acid, and euscaphic acid have been selected as marker compounds and have demonstrated anti-inflammatory, diuretic, antitumor, hepatoprotective, and anti-HIV properties [7–9].

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 [10–16]. Recently, identification of bioactive and marker compounds in herbal plants has been performed using tandem mass spectrometry (MS/MS) together with high-resolution mass spectrometry (HRMS) [17,18]. HRMS analysis has been shown to be especially useful in determining the elemental composition of unknown compounds.

Various analytical methods have been reported for the quantification of bioactive constituents and marker compounds in herbal plants [11–16,19–22]. Due to the complex composition of a raw herbal extract and the presence of interfering compounds, several chromatographic methods such as liquid chromatography (LC) coupled with spectrophotometric detectors [11–16], and gas chro-

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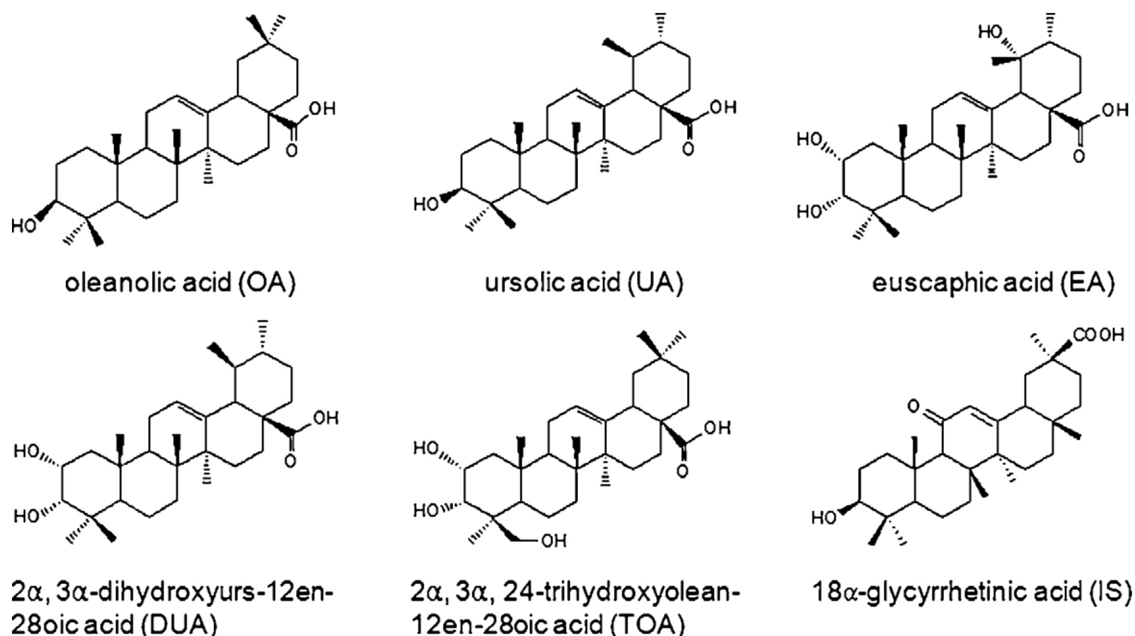

Fig. 1. Chemical structures of the marker compounds isolated from *Prunellae Spica* and the internal standard (IS) used in this study.

matography (GC) using chemical derivatization [19,20], have been widely applied to separate marker and interfering compounds. For the simultaneous determination of triterpenic acid composition in herbal plants, chromatography combined with mass spectrometry (MS) has been introduced [21–23]. In particular, LC–MS with electrospray ionization [21,22] has emerged as a popular technique for determining bioactive compounds in herbal plants. Moreover, GC/MS has served as a suitable and reliable method for the simultaneous determination of volatile compounds and triterpenic acids, especially in the complex natural matrices of plant extracts. Despite the high versatility of GC–MS methods, most require chemical derivatization of analytes prior to analysis [19,20]. HPLC methods generally utilize UV detection at wavelengths specific to the absorbance of target analytes and have a low sensitivity for interfering compounds.

To evaluate the quality control of *P. Spica*, 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 oleanolic and ursolic acids in other herbal medicines using HPLC methods [14–16]. To our knowledge, there has been no analytical method reported which allows the simultaneous determination of ursolic acid (UA), oleanolic acid (OA), euscaphic acid (EA), 2α,3α-dihydroxyurs-12en-28oic acid (DUA), and 2α,3α,24-trihydroxyolean-12en-28oic acid (TOA) levels in *P. Spica*. The quality of *P. Spica* can be predicted by the 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 [24–26]. Due to its usefulness in the differentiation of samples, PCA has recently been applied to the classification of traditional herbal plants from different origins [25,26]. The PCA technique combined with chromatographic data of selected marker compounds can be successfully applied in the quality control of herbal extracts [27].

The purpose of this study was to develop a simple and sensitive HPLC method for the simultaneous determination of five triterpenic acids as marker compounds in *P. Spica*. This method may then be used to ensure the quality of herbal medicines and the chemical standardization of *P. Spica* samples. In addition, the classification of 15 *P. Spica* samples collected from various regions in Korea and China was performed using PCA.

2. Experimental

2.1. Reagents and equipment

All reagents were of analytical grade. Methanol, ethyl acetate, and ethanol were purchased from J.T. Baker (Phillipsburg, NJ, USA). Phosphoric acid was of analytical-reagent grade, and was obtained from Wako (Osaka, Japan). OA and UA were purchased as crystalline powders from Sigma (St. Louis, MO, USA). Other triterpenic acids such as EA, DUA, and TOA were isolated by methods outlined in previous reports [28–30] and their purity was determined by HPLC (purity $\geq$ 90%). An internal standard, 18α-glycyrrhetinic acid was purchased as a crystalline powder from Sigma (St. Louis, MO, USA, purity $\geq$ 98.0%). The chemical structures of five marker compounds and the internal standard are shown in Fig. 1. Stock standard solutions for each of the analytes were prepared at concentrations of 2 mg mL^{-1} in methanol and stored in the dark at −4 °C. Purified water was obtained using the Millipore Alpha-Q Water system (Millipore, Bedford, MA, USA). A rotary evaporator (Büchi, Swiss)

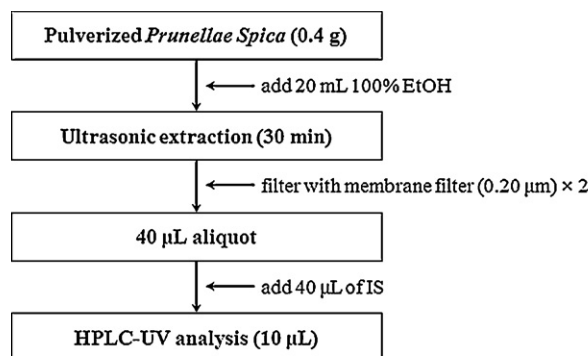

Fig. 2. Overall analytical procedure for the extraction of marker compounds from *Prunellae Spica*.

was used for the concentration of organic solvents. An ultrasonicator (Branson, Danbury, USA) was used for the extraction of marker compounds from *P. Spica*.

2.2. Preparation of crude extract and isolation of marker compounds

Analytical samples of the herbal medicine, *P. Spica* were purchased from various market places in Korea and China. Dried *P. Spica* was pulverized and 0.4 g was placed into 20 mL ethanol in a volumetric flask. The sample mixture was extracted for 30 min in an ultrasonic bath at room temperature. After extraction, the sample mixture was filtered through a 0.2 μm membrane filter, and 10 μL aliquots from the filtrate were then injected into the HPLC system. The overall analytical procedure for the determination of triterpenic acids in *P. Spica* is shown in Fig. 2.

2.3. Preparation of reference standards

From the compounds obtained, 2 mg was dissolved in 1 mL of methanol. For all analyses, the working solutions of the marker compounds were in the range of 50–2000 $\mu g\,mL^{-1}$ in methanol. For target compounds, linearity was established ranging from 3 to 300 $\mu g\,mL^{-1}$ with the addition of 18α-glycyrrhetinic acid as an internal standard. Stock standard and working solutions were stored at −4 °C and used for no longer than 2 months and 1 week, respectively. For the quantitation of marker compounds by HPLC with UV detection, calibration curves with five points were generated by the least squares method.

2.4. HPLC conditions

The apparatus used for HPLC consisted 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 Phenomenex (Torrance, CA, USA) Luna C-18 column (250 × 4.6 mm) 5 μm particle size, end-capped to minimize the effects of unreacted silanol. The isocratic elution system consisted of an aqueous 0.01 M phosphate buffer and methanol (12:88, v/v), maintained at pH 2.8. The flow rate was set at 0.8 $mL\,min^{-1}$ with UV absorbance detection at 210 nm. The operating temperature was maintained at 25 °C. The mobile phase was degassed by filtering through a Millipore HV 0.20 μm pore membrane filter.

2.5. Method validation

Validation of the analytical method for the five triterpenic acids as marker compounds was determined by selectivity, linearity, limit of detection (LOD), limit of quantitation (LOQ), accuracy and precision.

For selectivity validation, a standard solution containing the five triterpenic acids at a concentration of 75 $\mu g\,mL^{-1}$ was dissolved in methanol. A 10 μL volume of the standard mixture was injected into the HPLC column and analyzed using the method described above.

For linearity validation, standard solutions at a concentration range of 10–250 $\mu g\,mL^{-1}$ for OA, 10–300 $\mu g\,mL^{-1}$ for UA, 3–75 $\mu g\,mL^{-1}$ for TOA and 5–100 $\mu g\,mL^{-1}$ for EA and DUA were prepared and injected into the HPLC system. Five calibration solutions were injected in triplicate and five replicate analyses of the calibration solutions were performed. Calibration curves were constructed by linear regression of the peak area-ratios (y) of each triterpenic compound to internal standard, versus the concentration (x) in $\mu g\,mL^{-1}$.

The LOD was calculated by statistical method [31] using a ratio of $3\sigma/s$ (σ: the standard deviation of response, s: slope of the calibration curve). The LOQ was defined as the lowest concentration of a target compound that can be accurately and precisely quantified the noise level. The LOQ was also calculated with a ratio of $10\sigma/s$ according to ICH Q2B method [31].

In order to confirm the reproducibility, the intra- and inter-day precision were estimated by analyzing seven replicates containing the spiked samples at three different concentrations (25, 50, and 75 $\mu g\,mL^{-1}$) in a single day and for seven days, respectively.

2.6. 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.

2.7. Fast atom bombardment (FAB) mass spectrometry

FAB mass spectra were recorded with a JMS-700 Mstation mass spectrometer (JEOL, Tokyo, Japan) using a MS-MP9020D data system. The ion source was operated at 10 kV accelerating voltage with a mass resolution of 1500 (10% valley). Ions were produced by FAB using a cesium ion gun operated at 25 kV. Samples were dissolved in methanol and mixed with 1 μL of triethanolamine (Sigma, St. Louis, MO, USA) on a FAB probe tip. Calibration was performed with an Ultramark 1621 (PCR, Gainseville, FL, USA) in the negative ion mode as standard compound.

FAB exact mass measurement at a resolution of 10000 (10% valley) was used. Samples (1 μL) were mixed with 1 μL of polyethylene glycol (PEG 600, Sigma) as a calibration standard in the NBA matrix.

3. Results and discussion

3.1. Extraction and identification of marker compounds

Isolation of the five triterpenic acids used as marker compounds in *P. Spica* was performed as described in previous reports [28–30]. The purity of the isolated compounds was greater than 90% as evaluated by HPLC with UV detection. These compounds containing one to three carboxylic acids were characterized by the presence of abundant $[M-H]^-$ions detected by FAB-MS in negative ion mode. To elucidate the elemental composition of triterpenic acids isolated from *P. Spica*, exact mass measurements were performed using HRMS at a mass resolution of 10,000. The measured masses of $[M-H]^-$ions were within 2.5 mmu of the calculated masses, showing excellent agreement, as summarized in Table 1.

Both the selected solvent and the extraction method can have a critical affect on the amount of marker compounds extracted from herbal plants. Generally, triterpenic acids are insoluble in water and in non-polar solvents such as hexane and petroleum ether, but are freely soluble in alcoholic solvents. The extracted amounts of marker compounds in herbal medicine were tested

Table 1
Deprotonated molecules and exact mass measurements of $[M-H]^-$ ion (m/z) for marker compounds extracted from *Prunellae Spica*.

Compounds	Formula of $[M-H]^-$	$[M-H]^-$ (m/z)	Exact mass measurement (m/z)		
			Theoretical	Observed	Difference (mmu)
OA	$C_{30}H_{47}O_3$	455	455.3525	455.3511	−1.4
UA	$C_{30}H_{47}O_3$	455	455.3525	455.3519	−0.6
DUA	$C_{30}H_{47}O_4$	471	471.3474	471.3454	−2.0
TOA	$C_{30}H_{47}O_5$	487	487.3423	487.3416	−0.8
EA	$C_{30}H_{47}O_5$	487	487.3423	487.3398	−2.5

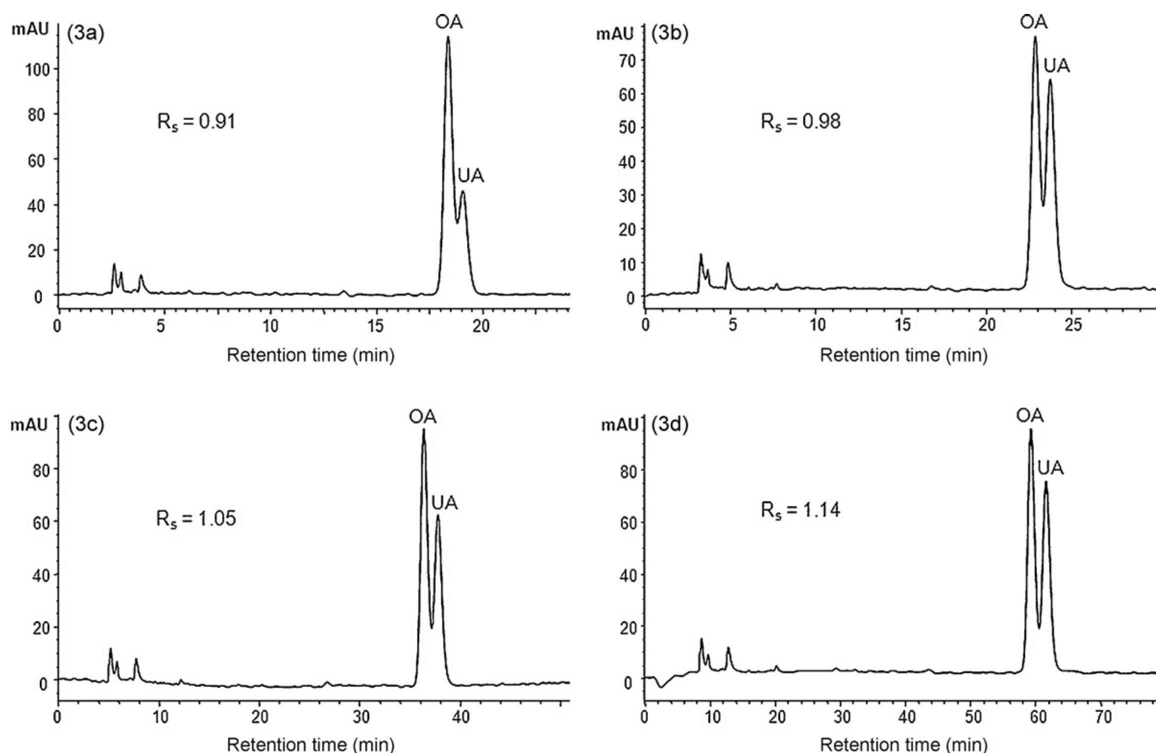

Fig. 3. LC chromatograms of a standard mixture of oleanolic acid and ursolic acid obtained by various flow rates of the mobile phase consisting of MeOH:0.01 M phosphate buffer (88:12, v/v) at 1 mL min^{-1} (3a), 0.8 mL min^{-1} (3b), 0.5 mL min^{-1} (3c), and 0.3 mL min^{-1} (3d).

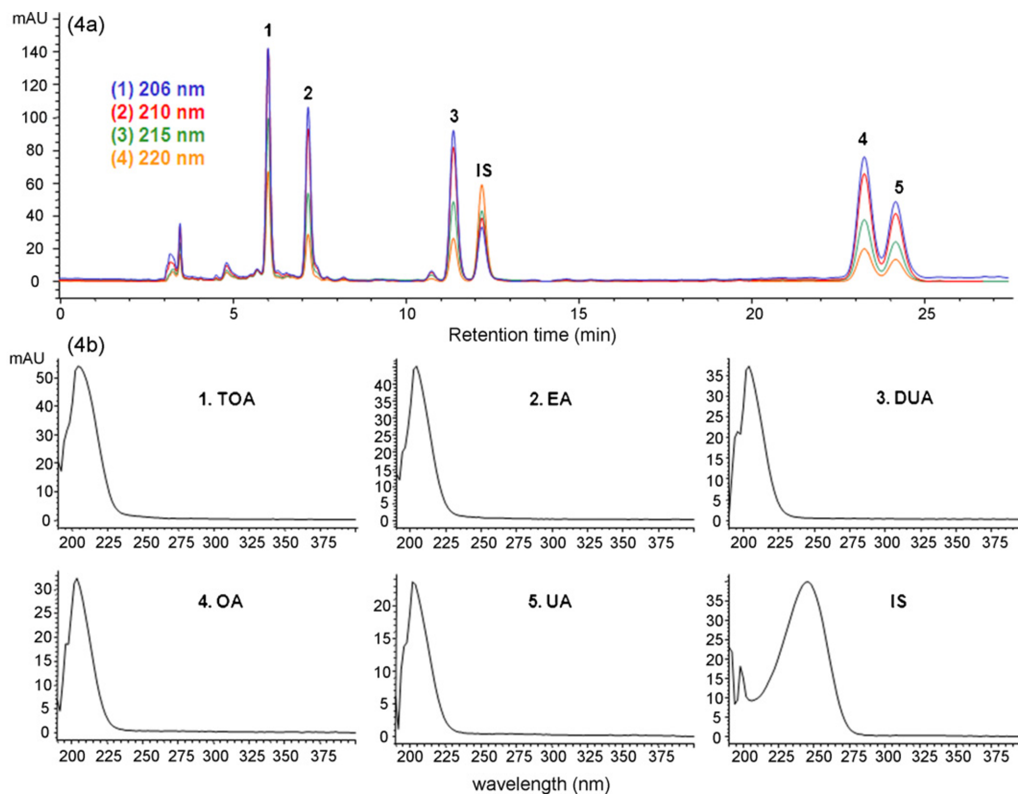

Fig. 4. LC chromatograms (4a) and UV spectra (4b) of triterpenic acids isolated from *Prunellae Spica* and obtained by HPLC-DAD using a 250 mm × 4.6 mm i.d., 5 μm particle, Phenomenex Luna C_{18} column. The mobile phase used was the same as in Fig. 3 at a flow rate 0.8 mL min^{-1}. Peaks were identified as follows: (1) TOA; (2) EA; (3) DUA; (4) OA; and (5) UA. The internal standard (IS) was 18α-glycyrrhetinic acid.

Table 2
Equations of calibration curves, linearity correlation coefficients, LOD, and LOQ for triterpenic acids.

Compounds	Linear range ($\mu g\,g^{-1}$)	Linear equation		Correlation coefficient	LOD ($\mu g\,mL^{-1}$)	LOQ ($\mu g\,mL^{-1}$)
		Slope ± SD[a]	Intercept ± SD			
OA	10.00–125.00	0.0455 ± 0.0011	−0.0940 ± 0.0472	0.997	1.50	3.78
UA	10.00–300.00	0.0333 ± 0.0008	−0.0935 ± 0.0117	0.995	1.16	3.51
EA	5.00–150.00	0.0238 ± 0.0004	−0.0149 ± 0.0051	0.999	0.29	0.88
DUA	5.00–125.00	0.0313 ± 0.0004	0.0026 ± 0.0095	0.999	0.16	0.48
TOA	3.00–100.00	0.0297 ± 0.0008	−0.013 ± 0.0088	0.999	0.42	1.28

[a] Standard deviation.

and compared with 0.4 g of herbal medicine using alcoholic solvents. For the extracted yield of marker compounds, the use of mixture solvents (70% methanol and 70% ethanol) could lead to the extraction of significant amounts of interfering compounds. Although methanol can successfully extract marker compounds, large amounts of interfering compounds are also co-extracted from herbal plants. Consequently, these interfering compounds could adversely effect the qualitative and quantitative analysis of marker compounds in the extract. Ethanol as an extraction solvent is suitable for the extraction of marker compounds and reduces the extraction of polar interfering compounds, compared with other solvents.

Extraction methods such as classical soxhlet, ultrasonication, and shaking were tested and compared. Although not shown here, no significant differences in the extraction yield of marker compounds obtained by these methods were observed. In fact, due to fast, easy and cost-effective extraction, ultrasonication was considered favorable for the extraction of marker compounds.

3.2. Optimization of chromatographic conditions

Approximately 20% of the triterpenic acids present in stock solutions were degraded after 1 week at room temperature. The degradation of some of triterpenic acids stored in clear vial was observed and confirmed by HPLC analysis. Thus, stock solutions were freshly prepared each month and stored at −4 °C. To protect against photolytic degradation, standard solutions were stored in amber bottles.

To simultaneously determine five triterpenic acids, HPLC chromatographic conditions should be optimized to successfully separate these compounds. Initially, a reversed-phase C_{18} column was selected for the LC separation of triterpenic acids based on qualitative assessments of chemical structures, solubility, and acidic properties. 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 various aqueous buffer solutions. The mobile phase was initially acidified since triterpenic acids are partially deprotonated in neutral and basic media. Although most of the acids were well separated, OA and UA could not be separated using a mobile phase with a pH >3.5. This was most likely due to structural similarities between these two isomers, the only difference being the position of a methyl group. To separate OA and UA, the mobile phase pH was reduced to 2.8 using a phosphate buffer. The peak resolution of the two isomers was optimized by adjusting the flow rate of the mobile phase, as shown in Fig. 3. Increasing the flow rate significantly reduced retention times and the overall analysis time, but also reduced peak resolution. Bearing these factors in mind, acceptable results were obtained with a flow rate of 0.8 $mL\,min^{-1}$.

Determining an optimal mobile phase composition was equally important for the establishment of a valid methodology. Using the reversed-phase HPLC procedure described above, the overall retention times of triterpenic acids decreased as the proportion of organic solvent in the mobile phase increased. The mobile phase consisted of organic solvent [either acetonitrile (ACN) or methanol (MeOH)] and aqueous phosphate buffer solution (PB) in an 88:12 (v/v) ratio. The overall analysis time using the ACN/PB blend was approximately 1 h, but was reduced to 25 min using MeOH/PB. The selection of the UV detection wavelength for the simultaneous determination of triterpenic acids represents a compromise between the UV absorption maxima of these compounds and those of interfering components in the extract. The triterpenic acids each exhibit maximum absorbance at 206 nm, as shown in Fig. 4b. How-

Table 3
Intra- and inter-day precision and accuracy for the quantification method of triterpenic acids in spiked sample.

Compounds	Fortified concentration ($\mu g\,mL^{-1}$)[a]	Intra-day (n = 7)			Inter-day (n = 7)		
		Observed concentration ($\mu g\,mL^{-1}$)	Precision (%)	Accuracy (%)	Observed concentration ($\mu g\,mL^{-1}$)	Precision (%)	Accuracy (%)
OA	25.0	24.54	3.84	98.17	24.67	6.21	98.69
	50.0	49.02	4.25	98.04	47.64	6.54	95.27
	75.0	69.92	2.24	93.22	71.35	5.50	95.14
UA	25.0	23.63	2.56	106.52	24.79	5.33	99.15
	50.0	47.03	2.54	94.07	46.57	4.92	93.14
	75.0	75.60	4.86	100.81	76.45	7.11	101.94
EA	25.0	23.51	5.48	94.04	24.45	3.72	94.98
	50.0	51.12	1.80	102.23	50.70	5.61	101.40
	75.0	70.50	3.89	94.01	70.86	5.83	94.48
DUA	25.0	24.73	1.33	98.94	24.37	6.83	97.48
	50.0	48.60	3.08	97.21	48.19	6.90	96.39
	75.0	75.52	3.12	100.69	75.79	4.06	101.05
TOA	25.0	24.31	3.40	97.25	23.93	4.10	104.17
	50.0	47.08	2.91	94.16	52.06	5.77	104.11
	75.0	75.07	3.95	100.09	74.50	5.04	99.33

[a] Conc.: concentration ($\mu g\,mL^{-1}$).

ever, given the stability and reproducibility of our UV source, 210 nm was selected for use in the experiments described herein. Measurements at 210 nm exhibited sufficient sensitivity and a satisfactory chromatographic baseline.

Using a standard mixture of the five triterpenic acids, the above HPLC conditions yielded acceptable retention times and symmetric peaks with reasonable resolution and overall analysis times, as shown in Fig. 4a. The elution order of the triterpenic acids in the chromatogram was closely related to their hydrophobicity. TOA and EA, both with three hydroxyl groups each, were eluted first. DUA, with two hydroxyl groups, was eluted third, and OA and UA, each with one hydroxyl group, were eluted last. As shown in Fig. 4a, all five marker compounds and the internal standard were successfully resolved and eluted within 25 min.

3.3. Method validation

3.3.1. Linearity, detection limit, and quantification limit

Calibration curves were generated using standard solutions containing 10–250 μg mL^{-1} of OA, 10–300 μg mL^{-1} of UA, 3–75 μg mL^{-1} of TOA, and 5–100 μg mL^{-1} of EA and DUA. Five standard solutions 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 0.995 to 0.999, indicating excellent linearity. Line equations representing the calibration curves and their correlation coefficients are summarized in Table 2.

The LOD was calculated by statistical method [31] using the ratio of three standard deviation (σ) of response and slope (s) of calibration curve. The slope and standard deviation can be estimated from the calibration curve of the analyte. LODs of five marker compounds were determined by performing 10 injections of each compound at concentrations incrementally approaching the LOD. LOD values were ranged from 0.16 to 1.16 μg mL^{-1} for five marker compounds, providing a reasonable sensitivity.

The LOQ was defined as the lowest concentration of a target compound that can be accurately and precisely quantified the noise level. The LOQ was also calculated by statistical method [31] using the ratio of 10 standard deviation (σ) of response and slope (s) of calibration curve. LOQ values were determined by performing 10 injections of each compound at concentrations approaching the LOQ. LOQs were ranged from 0.48 to 3.78 μg mL^{-1} for marker compounds used in this study. The LODs and LOQs of five marker compounds are also represented in Table 2. These compounds were shown to have reasonable sensitivities for the quality control of *P. Spica*.

3.3.2. Accuracy and precision

To test the accuracy and precision of the analytical method, five triterpenic acids were fortified at 10, 25, and 50 μg mL^{-1}, respectively, into *Prunealle Spica* samples. The intra- and inter-day variations for five marker compounds in *Prunealle Spica* samples were determined as described in Section 2 and are summarized in Table 3. The precision of the method for simultaneously determining the five marker compounds was acceptable since the relative standard deviation (RSD) did not exceed 8% at concentrations of 25, 50, and 75 μg mL^{-1}. At the same concentrations, the intra-day accuracy ranged from 93.2 to 106.5%, while the inter-day accuracy ranged from 93.1 to 104.2%, indicating an excellent accuracy.

3.3.3. Application of the method

The method developed herein was applied to 15 different samples. The amounts of triterpenic acids in *P. Spica* were determined in samples collected from 10 regions of Korea and from six regions of China (C-1 to C-6). A typical chromatogram of the crude extract is shown in Fig. 5b, indicating the absence of any significant interferents in the quantitation of the five marker compounds. A summary of the regional data in Table 4 shows significant regional variability.

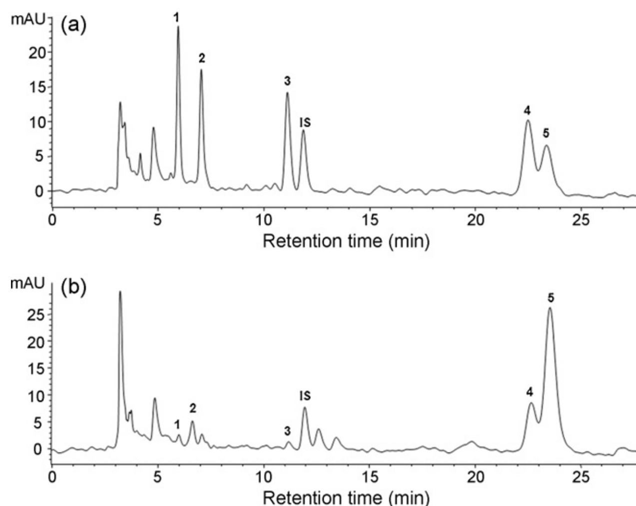

Fig. 5. HPLC chromatograms of the standard mixture (5a) and 100% ethanol extract of *Prunellae Spica* (5b) obtained by HPLC-DAD using a 250 mm × 4.6 mm i.d., 5 μm particle, Phenomenex Luna C_{18} column. Peak identities are the same as those indicated in Fig. 4.

3.4. Quality assessment by PCA

To classify the herbal plants collected from different regions, PCA was performed on the analytical data of all 15 samples. To display the points in two principal components, PC 1 and PC 2 (first and second principal components) were chosen to represent the information. Fig. 6 shows the principal component projection plot of the PC 1 and PC 2 of 15 *P. Spica* samples. It was found that PC 1 and PC 2 reflected 90.1% of the total variance. From the scatter points, the samples could be classified into two groups, indicating a clear differentiation between *P. Spica* collected in Korea and China.

Table 4
The amount of five triterpenic acids in *Prunellae Spica* collected from different regions (n = 5).

Sample number	Mean concentration ± standard deviation (μg g^{-1})				
	OA	UA	EA	DUA	TOA
[a]K-1	37 ± 4	165 ± 4	50 ± 3	19 ± 2	21 ± 2
K-2	21 ± 2	115 ± 4	70 ± 4	31 ± 3	18 ± 1
K-3	24 ± 3	116 ± 4	54 ± 3	18 ± 2	24 ± 2
K-4	12 ± 2	58 ± 4	69 ± 4	57 ± 4	25 ± 2
K-5	31 ± 4	177 ± 5	52 ± 5	23 ± 4	16 ± 2
K-6	29 ± 2	138 ± 4	53 ± 3	18 ± 3	19 ± 2
K-7	29 ± 4	152 ± 5	42 ± 5	17 ± 3	13 ± 2
K-8	29 ± 5	141 ± 4	25 ± 3	18 ± 2	30 ± 1
K-9	26 ± 5	127 ± 4	54 ± 4	20 ± 2	18 ± 2
[b]C-1	44 ± 3	206 ± 4	13 ± 1	7 ± 1	4 ± 2
C-2	33 ± 3	140 ± 4	22 ± 5	8 ± 2	16 ± 3
C-3	38 ± 2	158 ± 3	17 ± 4	11 ± 2	9 ± 2
C-4	42 ± 3	190 ± 4	16 ± 3	10 ± 2	31 ± 3
C-5	46 ± 2	204 ± 5	21 ± 4	8 ± 2	11 ± 4
C-6	44 ± 2	186 ± 3	19 ± 2	9 ± 2	4 ± 2

[a] K-1–9: Korea sample; K-1: Youngchun (flower stalk), K-2: Euisung (aerial stem), K-3: Youngchun (aerial stem), K-4: Youngchun (rhizome), K-5: Youngju (flower stalk), K-6: Jechun (flower stalk), K-7: Inje (flower stalk), K-8: Jeju (flower stalk), K-9: Bonghwa (flower stalk).

[b] C-1–6: China sample (not specified a collected region, flower stalk).

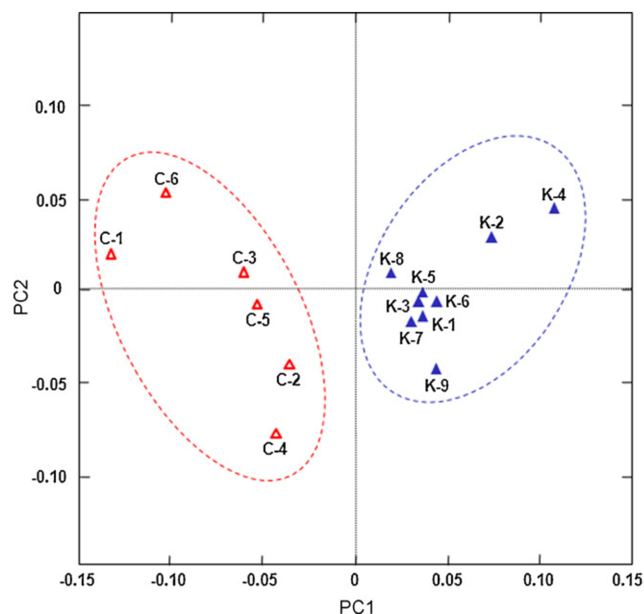

Fig. 6. Representation of 15 chromatographic samples of *Prunellae Spica* on PC1 and PC2 (90.1% variance explained).

4. Conclusion

In this study, we described and validated a HPLC method for separating and quantifying five triterpenic acids in *P. Spica*. The method was found to be suitable for the chemical standardization of herbal medicines obtained from *P. Spica* 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 triterpenic acids in other herbal medicines. In addition, PCA can provide important information on the differentiation of herbal plants from different regions.

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