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HPLC-tandem Mass Spectrometric Analysis of the Marker Compounds in Forsythiae Fructus and Multivariate Analysis

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Abstract – A high-performance liquid chromatography-electrospray ionization-tandem mass spectrometric method was developed to determine simultaneously eight marker constituents of Forsythiae fructus, and subsequently applied it to classify its two botanical origins. The marker compounds of *Forsythia suspensa* were phillyrin, pinoresinol, phillygenin, lariciresinol and forsythiaside; those of *F.viridissima* were arctiin, arctigenin and matairesinol. Separation of the eight analytes was achieved on a phenyl-hexyl column (150 × 2.0 mm i.d., 3 μm) using gradient elution with the mobile phase: (A) 10% acetonitrile in 0.5% acetic acid, (B) 40% aqueous acetonitrile. A few fragment ions specific to the types of lignans, among the product ions generated by collisonally induced dissociation (CID) of molecular ion clusters, such as $[M-H]^-$ or $[M+OAc]^-$ were used not only for fingerprinting analysis but for the quantification of each epimer by using multiple-reaction monitoring mode. It was shown good linearity ($r^2 \geq 0.9998$) over the wide range of all analytes; intra- and inter-day precisions (RSD, %) were within 9.14% and the accuracy ranged from 84.3 to 115.1%. The analytical results of 40 drug samples, combined with multivariate statistical analyses - principal component analysis (PCA) and hierarchical cluster analysis (HCA) - clearly demonstrated the classification of the test samples according to their botanical origins. This method would provide a practical strategy for assessing the authenticity or quality of the herbal drug.
Key words – Tandem mass spectrometry, Forsythiae fructus, *F.viridissima*, *F.suspensa*, lignans; forsythiaside, multivariate analysis

Introduction

Forsythiae fructus (F. fructus), the fruits of *Forsythia viridissima (F. viridissima)* Lindley or *Forsythia suspensa (F. suspensa)* Vahl, is a well-known herbal drug widely used in Korea (Korean name, "Yeon-Gyo"), Japan and China. *F. viridissima and F. suspensa are* officially listed Forsythiae fructus in Korea and Japan. Only *F. suspensa* is officially listed Forsythiae fructus in China (Chinese name, "Lianqio"). *F. suspensa* is widely distributed in Korea, China and Japan (Namba, 1993); *F. viridissima* is mostly being cultivated in Korea but grows wild in certain regions in China (Lee *et al.*, 1988).

The oriental medicine has long been used for the treatments of carbuncle, lymphadenitis, mastitis and suppuration because of its anti-inflammatory, diuretic, antidotal, and antipyretic, and anti-bacterial properties (WHO *et al.*, 1998; Lee *et al.*, 1996). Earlier studies on the crude drug have revealed its additional properties, such as choleretic (Miura *et al.*, 1987), antispasmodic (Woo *et al.*, 1979), antifungal properties (Rho, 1975). The dried fruit of *F. suspensa*, has also been used in Chinese folk medicine to treat gonorrhea, nephritis, erysipelas, inflammation, pharyngitis, pyrexia, tonsilitis, and ulcer (Takagi *et al.*, 1982; Xinyixueyuan, 1977). Furthermore, the extract showed potential antibacterial, antiviral, cholerectic and antipyretic, and antipsoriatic effects (Miura *et al.*, 1987; Ishizuka *et al.*, 1992; Preto *et al.*, 2003; Nishibe *et al.*, 1982a; Ozaki *et al.*, 2000; Schinella *et al.*, 2003; Hikino *et al.*, 1982).

More recently, various biological activities have been reported for the major constituents of F. fructus (respectively shown in the parentheses): Anti-oxidant, anti-inflammatory and cAMP-phosphodiesterase inhibitory activities (phillyrin, pinoresinol, phillygenin) (Nikaido *et al.*, 1981; Kim *et al.*, 2008; Chen *et al.*, 1999); 5-

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lipoxygenase-inhibitory, antibacterial, anti-inflammatory, and antioxidant activities (caffeoyl glycosides, forsythiaside and suspensaside) (Kimura *et al.*, 1987; Chiba *et al.*, 1978; Chiba *et al.*, 1979; Nishibe *et al.*, 1982b; Nishibe *et al.*, 1982a; Kitagawa *et al.*, 1987); anti-carcinogenic effects (arctiin) (Hirose, *et al.*, 2000; Takasaki *et al.*, 2000; Huang *et al.*, 2004); anti-inflammatory, cytostatic, immunoregulatory, and hepatoprotective activities (arctigenin) (Kang *et al.*, 2008; Vlietinck *et al.*, 1998; Eich *et al.*, 1996; Cho *et al.*, 2002; Kim *et al.*, 2003; Cho *et al.*, 1999); neuroprotective activities (dibenzylbutyrolactone lignans) (Jang *et al.*, 2001); potent inhibitory activities on casein kinase and HIV replication (matairesinol) (Ishida *et al.*, 2001).

Previous chemical studies on the two species of the herbal drug have discovered the major chemical classes different from each other (Chiba *et al.*, 1978; Chiba *et al.*, 1979; Nishibe *et al.*, 1982b; Nishibe *et al.*, 1982a; Nishibe *et al.*, 1977a; Nishibe *et al.*, 1977b; Kitagawa *et al.*, 1984) as well as some constituents common to both species, such as triterpenes (betulinic acid, ursolic acid, oleanolic acids) and a flavonoid (rutin). The main constituents of *F. suspensa* are the furo-furano lignans with their glycosides (such as phillygenin, (+)-pinoresinol, phillyrin, (+)-pinoresinol-β-D-glucoside), and the phenylethanoide glucosides (such as forsythiaside and suspensaside). Meanwhile, those characteristics to *F. virridisimia* were found to be the diarybutyro-lactone lignans with their glycosides (i.e., arctiin, arctignein, matairesinol, matairesinoside) and the phenylethanoid glycosides (aceteoside, b-hydroxyaceteoside). On the other hand, *F. koreana*, an endemic species of Korea, is known to contain both classes of the compounds (Chiba *et al.*, 1979). Once it has been used, but currently not being found in the herbal markets of Asia (Nishibe, 2002).

Although the two different species of this herbal drug distinctly have differences in their biological effects as shown above, they are currently being traded indiscriminately between countries to meet the demand for clinical uses or pharmaceutical products. Therefore, a new method for confirmation of the species origin and subsequent evaluation of the individual herbal drugs by chemical analysis of the respective bioactive marker constituents is essential to assure quality of the herbal drug.

Up to the present, chemical analyses of the herbal drug were largely confined to quantitative determination of a single or a few constituents of *F. suspensa* by using TLC (Nishibe *et al.*, 2001), HPLC (Noro *et al.*, 1991; Tokar *et al.*, 2004; Pharmacopoeia Commission of PRC, 2005) and HPLC-ESI-MS (Huanhuan *et al.*, 2008; Guo *et al.*, 2007). Therefore, there was an urgent need for a sensitive analytical method that enables to determine all of the respective marker compounds simultaneously in a drug sample. To meet these requirements, HPLC-tandem MS analysis is a powerful approach to selectively quantify individual ingredients of this herbal medicine. From our previous works (unpublished results), we already have isolated and purified eight marker compounds from the two individual fruits of forsythia species (Fig. 1). To our knowledge, no reports are yet available for a feasible method of quantifying the marker compounds of the herbal fruit that will enable subsequent discrimination of the species origin of the plant. In this paper, we describe a LC-tandem MS spectrometric method to quantify the eight marker compounds and perform a fingerprinting analysis of the herbal drug. A multivariate statistical analysis on the base of the analytical results, unsupervised clustering analysis such as hierarchical cluster analysis (HCA) and principal component analysis (PCA) were successfully employed to evaluate the drug samples to determine their origin of the species and habitats.

Experimental

Chemicals and Materials – The five reference standards of the F. fruit isolated from *F. suspena* (forsythiaside, lariciresinol, phillyrin, pinoresinol and phillygenin) were provided by Prof. Lee, S.H., Yeoungnam University, Daegu, Korea. The three marker lignans of *F. viridissima* (arctiin, matairesinol and arctigenin) were provided by Prof. Woo, M.H., Daegu Catholic University, Daegu, Korea. The purity of all reference standards was over than 95% as determined by HPLC. The internal standard (IS), enterolactone (HPLC purity ~95%) was purchased from Biochemika (Sigma-Aldrich, Switzerland). The structures of the nine standards including the internal standard are shown in Fig. 1.

Acetonitrile and methanol were HPLC-grade solvents purchased from Fischer Scientific Co. (FairLawn, NJ, USA). Water was prepared using the Milli-Q water purification system (Millipore, Bedford, MA, USA). Acetic acid was purchased from Merck Company (Darmstadt, Germany). All other reagents were of analytical grade.

The forty drug samples, whether they are from *F. suspensa* (Chinese habitat) or from *F. viridissima* (Korean habitat), were collected from local herbal drug stores located in several regions of Korea and China, then identified using the morphological characteristics by Prof.

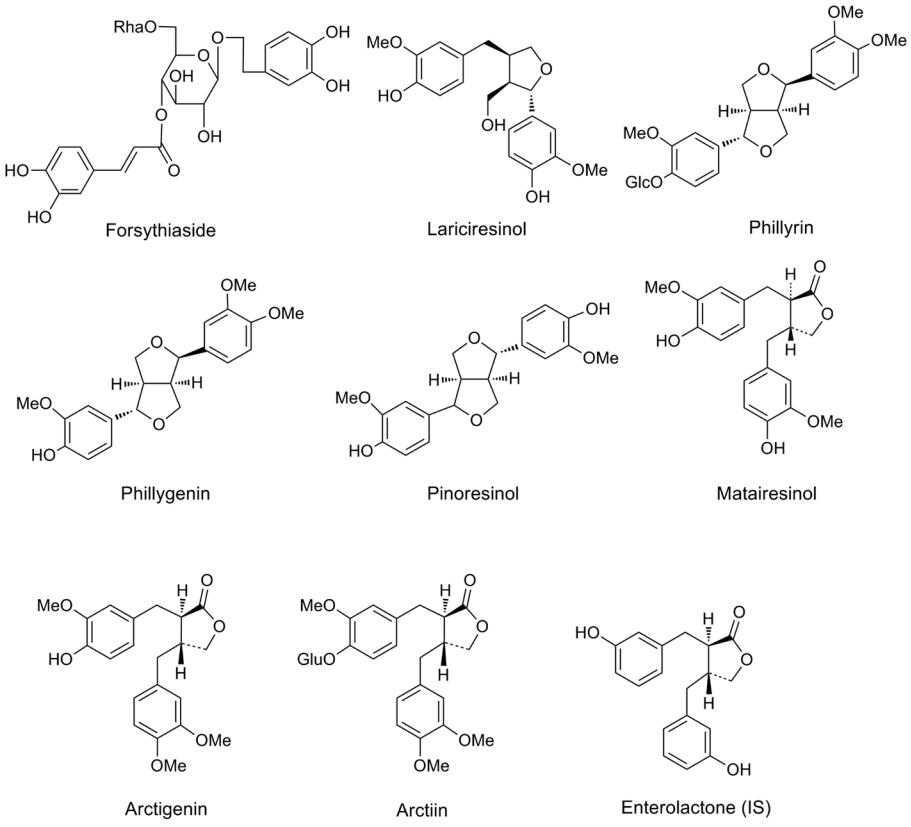

Fig. 1. The eight marker compounds of Forsythia fructus (from *F. suspense* and *F. viridissimia*) and enterolactone (internal standard).

Lee, J.H, Dongguk University, Seoul. The 20 Korean habitat samples (labeled K1-K20) were collected from the local region of Euisung (K1-K5, K8, K10-K12, and K14-K19), Euiryeung (K6), Daegu (K7), Wonju (K9), Youngcheon (K13) and Andong (K20) in Korea, respectively. Chinese habitat samples (labeled C1-C20) were collected from such region as Shenyang (C9), Henan (C10), Simyang (C11), Hebei (C12), and Yanji (C13-C14) in China; the other samples of the Chinese habitat were gathered from various stores in Daegu (C1-C6, C8, and C15-C20) and Yeungcheon (C7) in Korea. All of the test drugs were air-dried in the shade before analysis.

Standard solutions – The stock solutions of the standards and the internal standard were prepared with methanol to the concentration as indicated in parenthesis: forsythiaside (106000 ng/mL), lariciresinol (97000 ng/mL), phillyrin (95000 ng/mL), pinoresinol (98000 ng/mL), phillygenin (108000 ng/mL), arctiin (97000 ng/mL), matairesinol (77000 ng/mL), arctigenin (88000 ng/mL) and enterolactone (IS, 106000 ng/mL), then kept under 4 °C prior to analysis. Working solutions were prepared by serial dilution of each stock solution with the mobile phase to be within the concentration range of the real samples: forsythiaside, 164.8-41200 ng/mL; lariciresinol, 3.9-388 ng/mL; phillyrin, 19-1900 ng/mL; pinoresinol, 3.9-980 ng/mL; phillygenin, 4.3-432 ng/mL; arctiin, 19.4-1940 ng/mL; matairesinol, 6.2-616 ng/mL; arctigenin, 17.6-1760 ng/mL. The working solutions were filtered through a 0.45 μm membrane filter before HPLC analysis.

Sample preparation – The dried samples were ground to fine powder, screened through standard No.25 sieve (0.71 mm metric) to obtain homogenous size. Approximately 0.1 g of the pulverized powder was accurately weighted into a 100-mL centrifuge tube, then 50 mL of 50% methanol was added and then weighed. After sonicated at 42 kHz for 1 hr, the sample mixture was cooled and the loss of weight due to evaporation of solvent was replenished with 50% methanol. The extract was centrifuged at 3600 rpm for 10 min, and then the supernatant was filtered though 0.20 μm membrane filter.

We prepared final test solutions by diluting the filtered extracts with the mobile phase, but dilution ratios were different depending on the sample origins as been expected because the contents of lignans in samples are very low in comparison to those of forsythiaside. For the Chinese habitat samples, 0.5 mL of the filtrate was

transferred into 5.0-mL volumetric flask and diluted to 5.0 mL using the mobile phase; for those of Korean habitat, 0.25 mL of the filtrate was diluted to 10.0 mL using the same solvent.

HPLC separation – HPLC separation was accomplished on a LUNA phenyl-hexyl column (150 × 2.0 mm, 3 μm, 100Å, Phenomenex, Torrance, CA, USA) with guard column of phenyl-propyl cartridge (4.0 × 2.0 mm, I.D.). The column temperature was held at 25ºC. The mobile phases were consisted of (A) 10% acetonitrile with 0.5% acetic acid and (B) 40% acetonitrile. The gradient elution was made at the flow-rate of 0.2 mL/min: 0-15 min, 20% (B), 15-25 min, 20-100% (B), 25-40 min, 100% (B), 40-45 min, 100-20% (B), 45-60 min, 20% (B). 10 μL aliquots of the final test solutions were injected into the HPLC system through the autosampler maintained at 10 ºC for each analysis.

ESI-tandem MS analysis – LC-ESI-tandem-MS analysis was performed on Applied Bio systems Sciex API 3000 triple quadrupole mass spectrometer (Applied Biosystems, MDS Sciex, Concord, Canada) coupled with an Agilent 1100 series HPLC system (Agilent Technologies, Palo Alto, CA, USA) that comprises of a degasser, a binary pump, an autosampler and a column oven controller. Infusion of analytes to obtain the MS spectra of all compounds was performed using a syringe pump (Harvard, Holliston, MA, USA), while the effluent from the HPLC column was directed into the ESI probe in quantitation works. Data treatment was carried out using the Analyst software (ver. 1.4.1).

Mass spectrometer conditions were optimized to obtain maximal sensitivity. The ion spray interface was operated in the negative ion mode at –4.5 kV with a turbo gas temperature at 350 ºC. The MS operating condition was optimized by flow injection of a mixture of all analytes as follows: nebulizing gas flow, 1.16 L/min (setting 9); auxiliary gas flow, 6.1 L/min; curtain gas flow, 1.08 L/min (setting 9); collision gas (nitrogen) pressure, 3.58×10^{-5} Torr (setting 7); dwell time, 200 ms. Unit mass resolution was used in Q1 and Q3. Quantification was performed by the negative ion ESI-MS analysis using the multiple reaction monitoring (MRM) mode (Table 1).

Statistical analysis – The SPSS (version 12.0, SPSS Inc., Chicago, USA) software was used for statistical analyses of the data. The contents of eight components in *F. Fructus* were used as variables for data analysis. Multivariate statistical analysis, such as hierarchical cluster analysis (HCA) and principal component analysis (PCA) were applied to assess the species origins. The HCA result was obtained by the Ward's method using squared euclidian distance as a measure of similarity. For PCA analysis, the cumulative proportions of Eigen values ≥ 80% were considered sufficiently conspicuous for interpretation. The PC1 and PC2 explained an estimated 83.8% of the total variance. The Kaiser-Meyer-Olkin measure of sampling adequacy was 0.839 and Bartlett's test of sphericity ($\chi^2 = 350.567$, $p < 0.001$) was less than the level of significance ($\alpha = 0.05$).

Results and discussion

ESI -MS/MS spectra – The lignans of our concerns in this study were free phenolic groups, which were showed better sensitivity and specificity in the ESI-negative ion mode rather than by the positive ion detection. However, the infusion matrices affected the sensitivity of the

Table 1. Multiple reaction monitoring (MRM) parameters

Compound	RT (min)	Nominal mass (amu)	Q1 mass (amu)	Q3 mass (amu)	MRM parameters				Other Q3 ions, m/z
					DP (V)	FP (V)	CE (eV)	CXP (V)	
Forsythiaside	16.0	624	623	161	-71	-280	-46	-13	**135, 179,** 461
Lariciresinol	28.2	360	359	329	-33	-195	-13	-14	**160, 175,** 178, 192, 313
Phillyrin	29.0	534	593	371	-36	-190	-44	-5	**356**, **161,** 121
Arctin	29.5	534	593	371	-36	-220	-24	-7	**59, 83**, 136, 151, 533
Pinoresinol	31.0	358	357	151	-51	-210	-24	-11	**121, 136**, 342
Matairesinol	33.2	358	357	83	-41	-250	-38	-5	**122, 137,** 342, 121, 136, 151
Enterolacton (IS)	34.2	298	297	253	-41	-190	-28	-7	**107, 121,** 133, 145, 188
Phillygenin	35.5	372	371	356	-51	-260	-28	-19	**93, 121**, 136, 151, 326
Arctigenin	36.3	372	371	136	-38	-220	-36	-11	**83**, **121,** 136, 151, 223

DP = Declustering potential; FP = focusing potential; CE = collision energy; CXP=Collision cell exit potential
The negative ion mode fragmentation was measured by the direct infusion of analytes in 70% acetonitrile with 20 mM ammonium acetate; Forsythiaside was measured with 70% acetonitrile in 0.5% acetic acid.
The bold letter in other Q3 ions are the characteristic ions used for differentiation of the respective analytes

Table 2. Calibration graphs, linear range, LOD and LOQ

Compound	Regression equation	r^2	Linear range (ng/mL)	LOD (ng/mL)	LOQ (ng/mL)
Forsythiaside	y = 0.0001x - 0.0152	0.9998	164.8 - 41200	115.0	318.0
Lariciresinol	y = 0.0002x - 0.0004	1.0000	3.9 - 388	2.1	7.1
Phillyrin	y = 0.0002x + 0.0015	0.9999	19 - 1900	4.2	14.1
Pinoresinol	y = 0.0001x + 0.0002	0.9999	3.9 - 980	2.1	7.0
Phillygenin	y = 0.0004x - 0.0005	1.0000	4.3 - 432	2.7	10.8
Arctiin	y = 0.0012x + 0.0171	0.9999	19.4 - 1940	0.5	1.2
Matairesinol	y = 0.0014x + 0.0015	1.0000	6.2 - 616	2.1	7.7
Arctigenin	y = 0.0021x + 0.0507	0.9999	17.6 - 1760	1.1	3.5

LOD=3 S/N; LOQ=10 S/N

molecular and fragment ions for each analyte. We obtained the Q1 and Q3 mass spectra by introducing the analytes (1000-2000 ng/mL) in the infusion solvent consisting of 70% acetonitrile in 20 mM ammonium acetate. The eight compounds were obviously identified by matching the mass spectra with the chemical structures, and by comparing the retention time. The non-phenolic lignan glycosides (phillyrin and arctiin) showed their acetate ion adducts $[M+OAc]^-$ with sufficient abundances, and the adduct ions initially lost the sugar unit before further fragmentations. The Q3 ions generated from the collisionally induced dissociation (CID) of the selected Q1 ions are shown in Table 2.

The negative ion CID mass spectra of the furofuran lignans mainly showed the $[M-H-CH_3]^-$ as the primary product ions. Other ions at m/z 151 and m/z 136 from the guaiacyl (3-hydroxy-4-methoxyphenyl) moiety together with the ions at m/z 121 and m/z 163 from the dimethoxyphenyl moiety were observed as the previous explanation of the fragment pathway. The dimethoxy-phenyl unit is more readily cleaved from the lignan skeleton, and rearranged to give the fragment ions (Eklund *et al.*, 2008).

For the fragmentation of the DBL-type lignans (arctiin, arctigenin and matairesinol), both the molecular ion clusters ($[M-H]^-$ and $[M+OAc]^-$) were observed on the Q1 spectra. A few structurally diagnostic ions (Q3 ions) were generated by the CID of the $[M-H]^-$ ions: the ions at the $[M-H-CH_3]^-$, m/z 83 [butyrolactone moiety-H]$^-$, m/z 136 [dimethoxybenzene-H]$^-$ and m/z 121 [dimethoxy-benzne-H-CH_3]$^-$. Therefore, the DBL-lignans could be distinguished from the isomeric furofuran lignans by the presence of these characteristic fragment ions between the isomeric pairs, i.e. artigenin/ phillygenin, arctiin/ phillyrin, and matairesinol/ pinoresinol. The intense characteristic fragment of m/z 83 was presumed to be formed from the cleavage of the butyrolactone moiety. For the internal standard (enterolactone), the $[M-H-CO_2]^-$, m/z 189 $[C_{11}H_9O_3]^-$, m/z 121 $[C_8H_9O]^-$ and m/z 107 $[C_7H_7O]^-$ were observed as the product ions (Q3 ions) by the ion transition of the $[M-H]^-$ ion.

The CID of the $[M-H]^-$ ion (m/z 359) of lariciresinol (a tetrahydro-furano lignan) afforded the primary production ion, $[M-H-CH_3OH]^-$ at m/z 329; The minor ions at m/z 193 ([3-hydroxy-2-methoxybutanol-H]$^-$; i.e. 359-166), m/z 178 (i.e. 359-166-CH_3) and m/z 160 (i.e. 359-166-CH_3-H_2O) were attributed to the cleavage of the furan ring.

Forsythiaside (a phenylethanoid glycoside) showed the $[M-H]^-$ ion at m/z 623, and the Q3 product ions at m/z 451, 179 and 161 were assigned to [M-H-caffeoyl]$^-$, [glc-H]$^-$ and [glc-H-H_2O]$^-$, respectively.

Recent reports, by using ESI ion-trap mass spectrometry, have presented on the fragmentation pathways for various types of lignans, but they showed large variation in the fragmentation behavior among the compounds even in the same classes (Eklund *et al.*, 2008; Ricci *et al.*, 2008; Guo *et al.*, 2007; Ye *et al.*, 2005; Schmidt *et al.*, 2006). Small changes in the structure could lead to different fragmentation behavior. Therefore, identification of the compounds was made by the observed fragmentation pattern in combination of retention times, molecular ion species (protonated or deprotonated) and compound-specific product ions produced in reference to the compounds used here. For the quantification works, the MRM parameters were optimized to determine the eight analytes simultaneously (Table 1). When the LC-separation was feasible between isomeric pairs, the Q3 product ions showing maximal intensity were selected; otherwise, the compound-specific ions were selected to discriminate the plant species.

HPLC separation – Optimization of LC separations was guided by the requirements to fulfill the complete separation with adequate sensitivity for the eight analytes. As far as we know, there are no reports available to

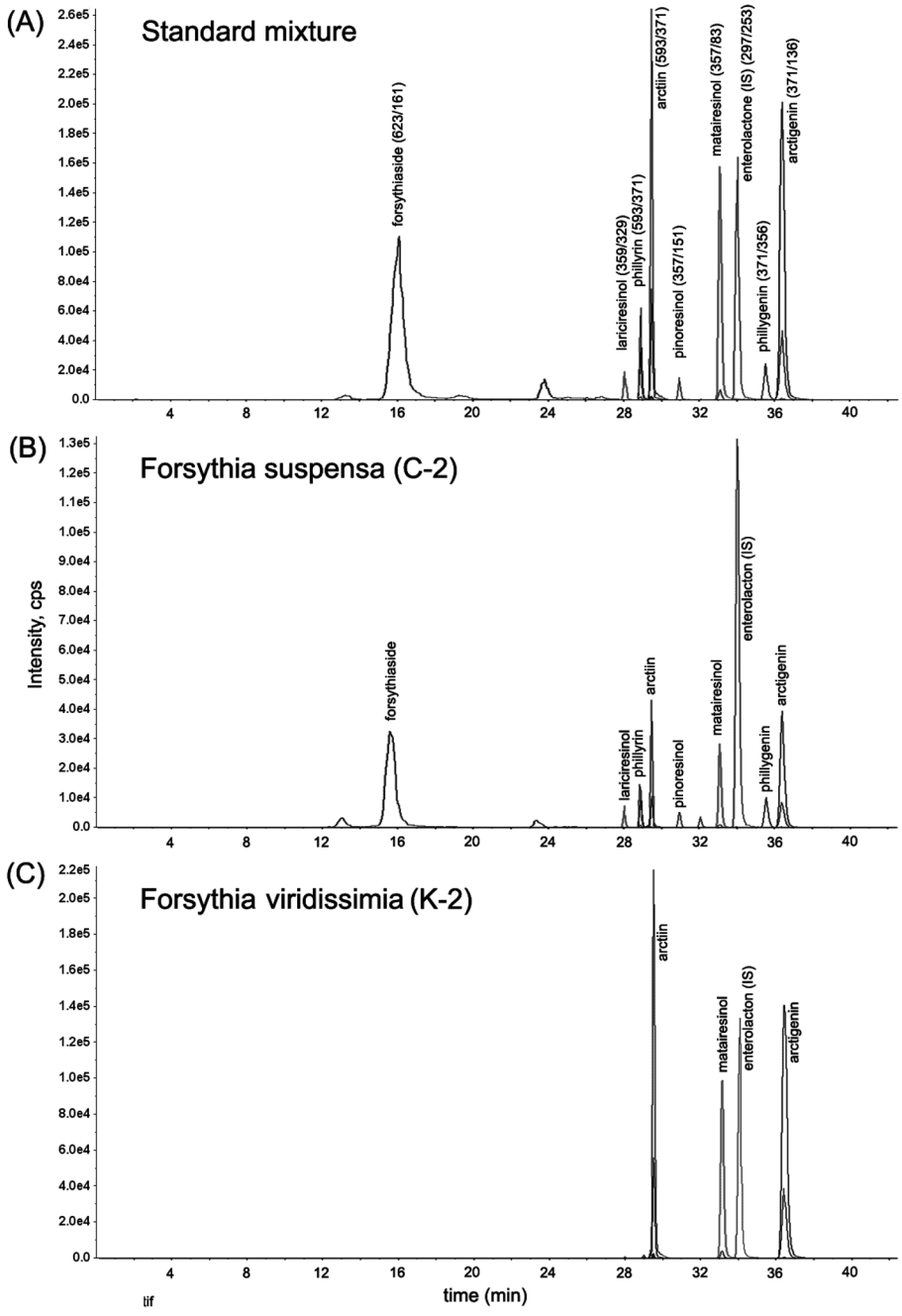

Fig. 2. The combined LC-tandem MS chromatograms of standard mixture (A), a sample of Chinese habitat (B), and a sample of Korean habitat (C).

analyze the various types of lignans and forsythiaside simultaneously in plant samples. Complete separation of the eight analytes in the complex sample was achieved by gradient elution. We established the optimal chromatographic condition after several trials of the eluting systems of acetonitrile-water, methanol-water, and the aqueous acetonitrile that contain variable proportions of the acidic modifiers, such as formic and acetic acids. Although the seven lignans were completely separated all showing with adequate sensitivity in the neutral mobile phases, but peak deformation or broadening was observed for forsythiaside whether the mobile phase contains volatile salts or not. It means the ion formation of forsythiaside can be obtainable in acidic mobile phases.

Control of the mobile phases with gradient flow was employed to allow the simultaneous separation and

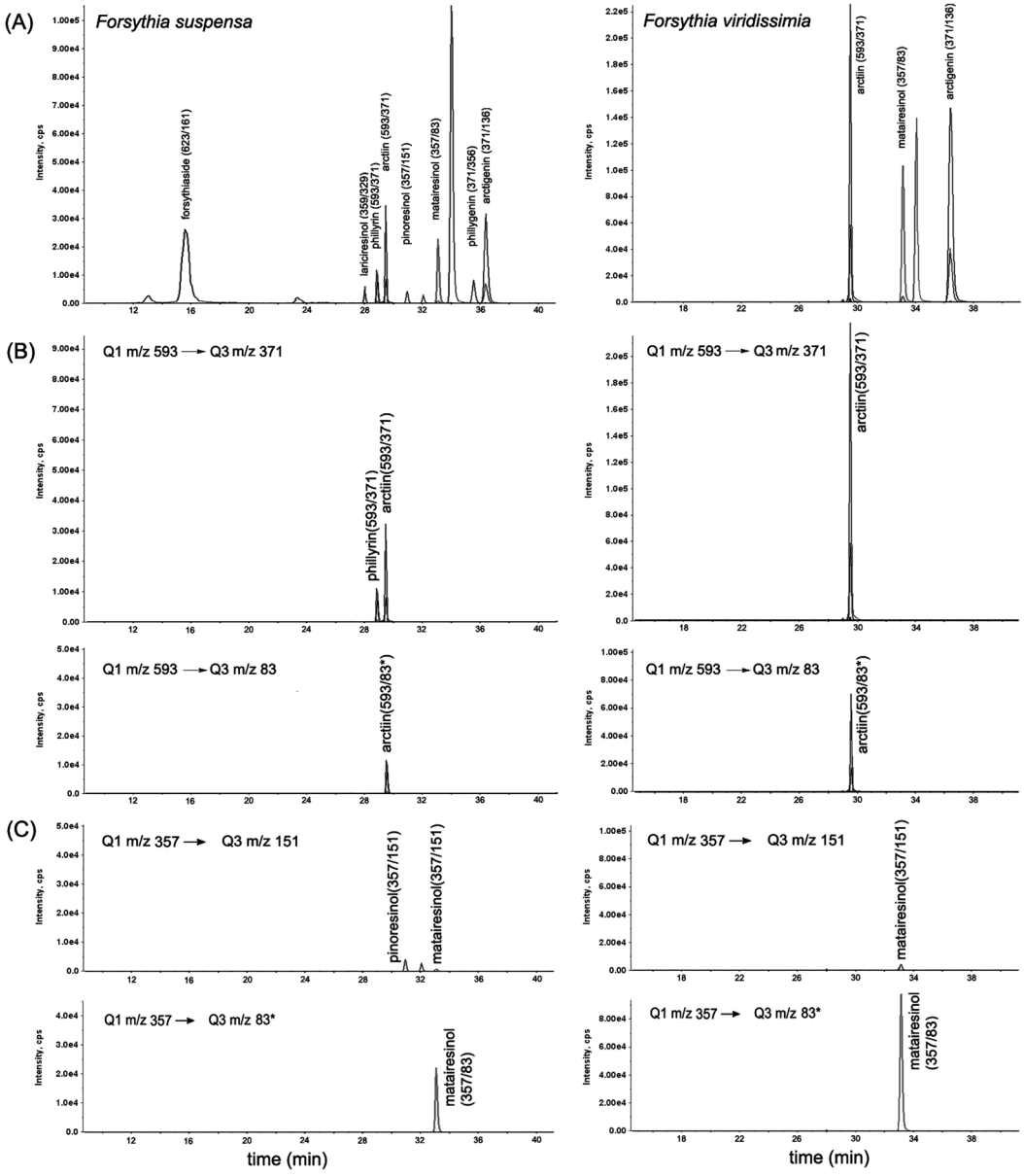

Fig. 3. The extracted ion mass chromatograms to differentiate the isomeric compounds in Forsythia fructus. (A) Combined LC-MS/MS chromatograms; (B) Q3 MS spectra to differentiate phillyrin and arctiin at m/z 83 (59383); (C) Q3 MS spectra to differentiate pinoresinol and matairesinol at m/z 83 (35783).

detection for both classes of the compounds. The mobile phase constituent was kept in 10% acetonitrle with 0.5% acetic acid (A) until complete elution of forsythiaside, subsequently altered into 40% acetonitrile (B) to achieve good separation condition. The gradient flows led to good detection of forsythiaside without sacrificing the separational efficiency and sensitivity of the lignans. Peak tailing or lags during the elution of the lignans was not observed with the change rate of the organic phase (acetonitrile %, 16 → 40% between 15 and 25 min).

We compared two reversed-phase minibore columns (octadecyl and phenyl-hexyl) of the same dimensions for the separation of all anlaytes: (1) C18 column (150 × 2.0 mm, 3 μm, 100 Å, Phenomenex, Torrance, CA, USA); (2) phenyl-hexyl column (150 × 2.0 mm, 3 μm, 100 Å, Phenomenex, Torrance, CA, USA). All analytes were eluted within 40 min with both columns, but the separation between phylligenin and arctigenin was not attainable with the C18 column, while complete separation of all analytes was achieved with the phenyl-

hexyl phase. The separation was enough when the column temperature was kept at the room temperature around 25 °C. The representative chromatograms for the separation of standard mixture and the two samples of Korean and Chinese origins are shown in Fig. 2.

From the chromatogram of Figure 2(B), forsythiaside (RT, ~16 min), lariciresinol (28 min), phillyrin (29 min), pinoresinol (31 min), and phillygenin (35.6 min) could be identified as the major constituent of the drug sample of Chinese origin. The small amounts of arctiin (29.5 min) and arctigenin (36.5 min) were also contained in Chinese samples, which the minor compounds might be adulterants or contaminated from the other species. It was clear that the major components of the drugs from Korean habitat were arctiin, arctigenin and matairesinol. Besides, the two peaks at the respective retention times ~13 and 23.5 min were presumed as the isomers of forsythiaside (acetoside) to confirm the same mass spectral patterns with the same molecular weights from TIC-SIM mass chromatograms. The other small peaks in the sample chromatograms were assumed epipinoresinol, forsythin, forsythigenin, the isomeric analogues of the corresponding phillyrin, pinoresinol, phillygenin.

Identification of isomeric compounds – The fragmentation patterns of the reference compounds made feasible for the identification of different type of lignans. Discrimination between the respective isomeric pairs (pinoresinol/ matairesinol, phillyrin/ arctiin and phillygenin/ artcigenin) was easily made through extracting specific ions by multiple reaction monitoring (MRM) mode (Fig. 3). Both phillyrin and arctiin were detected simultaneously by the ion transition (Q1 m/z 593 → Q3 m/z 371), but each one was separated in the LC-MS chromatograms. Further identification of arctiin from the two peaks appeared in real samples was made by the specific ion transition (Q1 m/z 593 → Q3 m/z 83). Likewise, the isomeric pinoresinol and matairesinol were detected together by the ion transitions (Q1 m/z 357 → Q3 m/z 151); but the latter was only identified by the selective ion transition (Q1 m/z 357 → Q3 m/z 83). Both phillygenin and arctigenin were detected simultaneously by the ion transition (Q1 m/z 371 → Q3 m/z 356); arctigenin was detected by using the two specific transitions, Q1 m/z 371 → Q3 m/z 136 and Q1 m/z 371 → Q3 m/z 83. It was apparent that use of the ion transition (m/z 371 → m/z 136) gave better sensitivity of detection than by the ion transition (m/z 371 → m/z 83).

Method validation – Calibration graphs were prepared from the lower limits of quantitation (LLOQ) to the upper range, the two or three folds of the expected level of each compound in real samples. The linear calibration curves were constructed by at least five concentration levels of each analyte in quintuplicate. They were constructed by plotting the integrated chromatographic peak area ratios (y) versus the corresponding concentration of the injected standards (x). The high correlation coefficient values ($r^2 \geq 0.9998$) indicated good linearity over the wide concentration ranges for each analyte. The LOD and LOQ were calculated on the basis of the signal-to-noise ratio (S/N) of 3 and 10, respectively (Table 2).

The precision and accuracy of the method were determined by standards addition method. Three reference samples were prepared by spiking each analyte at three different concentration levels (high, medium and low) into both the samples (C2 and K2), then were analyzed in triplicates according to the proposed procedure. For comparison, unspiked samples were concurrently prepared and analyzed by the same manner. The intra-day precision was examined over the results of three individual samples within a day, and the inter-day precision was determined by the results on three independent days. The precision (expressed as RSD %) was less than 9.1% for all test samples, and accuracy over the concentration range of the analytes in both samples was in the range of 91-115% (Table 3).

Sample analysis – The developed method was applied for the simultaneous determination of the eight marker compounds for the 40 herbal drugs, which enabled us to make distinction of the drug origins at glance whether they are from Korean (*F.viridissima*) or from Chinese (*F.suspensa*) (Table 4).

We can ascertain that the content variation of forsythiaside, the most abundant component of *F.suspensa*, ranged between 2-8% in the samples of the *Chinese* origins (C1-C20), whereas showing with the much lower content in the samples of the *Korean* origins (K1-K20). The content of the furano, furo-furano lignans and its glycoside (lariciresinol, phillygenin, pinoresinol, and phillyrin) was distinctively higher in the samples of the *Chinese* origin than in those of the *Korean* origin. These remarkable constituents were not at all detectable in some of the latter origin. The DBL-type lignans and the glycoside (matairesinol, arctigenin, and arctiin) in the samples of the *Korean* origin were found to be in the range of 0.5-3.6% (w/w), comparable with the approximately 0.2% in the *Chinese* samples (*F.suspensa*), which clearly represent they are the most remarkable constituents of the *Korean* samples (*F.viridissima*). It is certain that the phenylethanoid glycoside (forsythiaside) should be the representative marker compounds to

Table 3. Precision and accuracy

(A) F. suspensa

Analytes	Fortified conc. (ng/mL)	Intra - day (n=3)			Inter - day (n=3)		
		Found (ng/mL)	RSD (%)	Accuracy (%)	Found (ng/mL)	RSD (%)	Accuracy (%)
Forsythiaside	949.0	1008.8 ± 4.8	0.5	106.3	1053.0 ± 36.1	3.4	111.0
	1889.0	1851.8 ± 74.3	4.0	98.0	1757.8 ± 95.6	5.4	93.1
	2829.0	2617.3 ± 91.3	3.5	92.5	2636.7 ± 73.7	2.8	93.2
Lariciresinol	15.5	14.8 ± 0.1	0.7	95.5	14.3 ± 0.4	2.5	92.4
	38.0	38.8 ± 2.9	7.5	102.2	40.0 ± 2.5	6.3	105.4
	60.5	59.4 ± 1.3	2.1	98.2	61.4 ± 1.5	2.5	101.5
Phillyrin	105.0	107.6 ± 5.1	4.7	102.4	118.3 ± 5.7	4.8	112.7
	146.5	129.2 ± 4.5	3.5	88.2	134.3 ± 8.8	6.6	91.7
	188.0	186.5 ± 6.8	3.7	99.2	195.1 ± 4.9	2.5	103.8
Pinoresinol	66.6	69.1 ± 2.5	3.7	103.7	72.2 ± 1.1	1.5	108.3
	100.0	102.7 ± 5.7	5.5	102.8	98.7 ± 3.7	3.8	98.8
	133.3	129.3 ± 0.8	0.6	97.0	124.6 ± 6.2	4.9	93.5
Phillygenin	13.5	14.2 ± 0.3	2.0	105.3	14.5 ± 0.0	0.0	107.4
	25.8	27.2 ± 1.3	4.6	105.8	29.0 ± 0.7	2.5	112.6
	38.0	36.5 ± 1.8	5.0	96.1	36.9 ± 1.9	5.0	97.1
Arctiin	76.0	76.4 ± 2.5	3.3	100.5	80.6 ± 3.8	4.7	106.0
	121.0	117.7 ± 5.5	4.7	97.3	111.9 ± 5.8	5.2	92.5
	166.0	159.0 ± 8.9	5.6	95.8	171.2 ± 4.3	2.5	103.2
Matairesinol	25.5	25.4 ± 0.5	2.1	99.5	27.3 ± 1.2	4.4	107.0
	38.5	40.7 ± 2.4	5.9	105.7	39.7 ± 1.7	4.2	103.0
	51.5	50.7 ± 1.3	2.5	98.4	51.4 ± 2.2	4.3	99.7
Arctigenin	26.0	29.0 ± 0.8	2.8	111.6	27.8 ± 0.5	1.9	106.8
	61.0	54.1 ± 1.4	2.6	88.8	55.4 ± 2.1	3.9	90.8
	96.0	91.1 ± 2.2	2.4	94.9	90.8 ± 3.2	3.5	94.5

(B) F.viridissima

Analytes	Fortified conc. (ng/mL)	Intra - day (n=3)			Inter - day (n=3)		
		Found (ng/mL)	RSD (%)	Accuracy (%)	Found (ng/mL)	RSD (%)	Accuracy (%)
Forsythiaside	70.0	77.9 ± 4.6	5.9	111.2	80.6 ± 2.3	2.8	115.1
	105.1	88.6 ± 6.4	7.2	84.3	95.3 ± 1.5	1.5	90.7
	140.1	122.6 ± 8.8	7.1	87.5	127.3 ± 8.0	6.3	90.9
Lariciresinol	2.1	2.1 ± 0.1	5.7	100.4	2.3 ± 0.1	5.1	107.0
	3.2	3.2 ± 0.2	6.7	100.5	3.2 ± 0.2	6.7	98.7
	4.3	4.1 ± 0.3	6.8	96.9	4.3 ± 0.1	2.6	101.6
Phillyrin	8.0	7.8 ± 0.4	4.5	97.9	7.2 ± 0.3	4.5	89.5
	14.0	13.7 ± 1.3	9.1	98.0	13.1 ± 0.1	1.1	93.7
	20.0	21.1 ± 0.8	3.7	105.5	19.8 ± 1.7	8.7	99.2
Pinoresinol	2.4	2.4 ± 0.1	4.1	101.3	2.6 ± 0.1	3.8	110.3
	3.1	3.1 ± 0.2	4.9	100.9	3.1 ± 0.2	6.4	102.7
	3.7	3.7 ± 0.2	4.5	99.4	3.7 ± 0.2	6.7	98.7
Phillygenin	1.2	1.1 ± 0.1	6.8	93.3	1.2 ± 0.0	2.6	98.3
	2.1	1.9 ± 0.1	5.0	89.6	2.0 ± 0.0	0.9	94.2
	3.0	3.1 ± 0.2	5.1	102.0	3.2 ± 0.2	6.9	106.9
Arctiin	155.2	156.1 ± 11.7	7.5	100.6	165.8 ± 1.7	1.0	106.8
	232.8	217.4 ± 8.8	4.1	93.4	237.9 ± 11.2	4.7	102.2
	310.4	331.1 ± 20.2	6.1	106.7	315.5 ± 23.0	7.3	101.7
Matairesinol	37.0	36.4 ± 2.4	6.7	98.5	38.6 ± 1.9	4.8	104.2
	44.0	45.1 ± 3.9	8.6	102.5	43.9 ± 3.5	8.0	99.8
	51.0	47.2 ± 1.1	2.3	92.5	51.9 ± 3.5	6.8	101.7
Arctigenin	93.0	90.0 ± 2.7	3.0	96.8	94.6 ± 5.4	5.7	101.8
	115.0	114.8 ± 2.7	2.3	99.8	107.0 ± 4.7	4.3	93.1
	137.0	141.2 ± 2.7	1.9	103.1	148.9 ± 4.7	3.1	108.7

Table 4. Assay results (mean ± SD, wt %, *n*=3)

Sample	Forsythiaside	Lariciresinol	Phillyrin	Arctiin	Pinoresinol	Matairesinol	Phillygenin	Arctigenin
C1	2.254 ± 0.154	0.070 ± 0.011	0.098 ± 0.010	0.044 ± 0.003	0.091 ± 0.014	0.041 ± 0.003	0.082 ± 0.005	0.046 ± 0.003
C2	1.736 ± 0.315	0.054 ± 0.007	0.103 ± 0.007	0.034 ± 0.002	0.212 ± 0.036	0.027 ± 0.001	0.049 ± 0.001	0.030 ± 0.004
C3	6.430 ± 0.464	0.041 ± 0.005	0.733 ± 0.061	0.000 ± 0.000	0.074 ± 0.009	0.005 ± 0.001	0.063 ± 0.005	0.000 ± 0.000
C4	8.251 ± 0.394	0.036 ± 0.002	0.883 ± 0.044	0.000 ± 0.000	0.097 ± 0.009	0.005 ± 0.000	0.077 ± 0.006	0.000 ± 0.000
C5	4.327 ± 0.322	0.053 ± 0.006	0.512 ± 0.007	0.000 ± 0.000	0.143 ± 0.009	0.010 ± 0.001	0.135 ± 0.011	0.000 ± 0.000
C6	3.420 ± 0.204	0.050 ± 0.004	0.171 ± 0.004	0.000 ± 0.000	0.171 ± 0.007	0.016 ± 0.001	0.108 ± 0.006	0.000 ± 0.000
C7	1.668 ± 0.133	0.031 ± 0.002	0.073 ± 0.001	0.000 ± 0.000	0.043 ± 0.005	0.004 ± 0.000	0.053 ± 0.001	0.000 ± 0.000
C8	2.258 ± 0.300	0.030 ± 0.001	0.108 ± 0.004	0.000 ± 0.000	0.058 ± 0.001	0.007 ± 0.000	0.127 ± 0.004	0.000 ± 0.000
C9	0.959 ± 0.090	0.020 ± 0.002	0.071 ± 0.003	0.000 ± 0.000	0.035 ± 0.003	0.050 ± 0.002	0.048 ± 0.001	0.000 ± 0.000
C10	2.324 ± 0.071	0.042 ± 0.002	0.157 ± 0.007	0.000 ± 0.000	0.054 ± 0.005	0.008 ± 0.001	0.075 ± 0.002	0.000 ± 0.000
C11	4.116 ± 0.122	0.034 ± 0.005	0.319 ± 0.027	0.043 ± 0.004	0.036 ± 0.005	0.016 ± 0.001	0.042 ± 0.003	0.039 ± 0.002
C12	4.662 ± 0.178	0.072 ± 0.004	0.286 ± 0.017	0.000 ± 0.000	0.074 ± 0.001	0.009 ± 0.001	0.081 ± 0.003	0.000 ± 0.000
C13	2.048 ± 0.037	0.034 ± 0.003	0.073 ± 0.008	0.000 ± 0.000	0.021 ± 0.002	0.005 ± 0.001	0.033 ± 0.002	0.000 ± 0.000
C14	8.436 ± 0.445	0.034 ± 0.003	0.699 ± 0.021	0.000 ± 0.000	0.057 ± 0.004	0.009 ± 0.000	0.074 ± 0.003	0.000 ± 0.000
C15	4.824 ± 0.071	0.039 ± 0.004	0.619 ± 0.004	0.000 ± 0.000	0.063 ± 0.003	0.010 ± 0.000	0.062 ± 0.005	0.000 ± 0.000
C16	4.591 ± 0.461	0.042 ± 0.001	0.597 ± 0.015	0.000 ± 0.000	0.090 ± 0.003	0.011 ± 0.001	0.087 ± 0.003	0.000 ± 0.000
C17	4.883 ± 0.505	0.043 ± 0.002	0.456 ± 0.014	0.000 ± 0.000	0.047 ± 0.004	0.006 ± 0.001	0.040 ± 0.001	0.000 ± 0.000
C18	4.886 ± 0.046	0.037 ± 0.005	0.644 ± 0.037	0.000 ± 0.000	0.055 ± 0.003	0.006 ± 0.000	0.056 ± 0.003	0.000 ± 0.000
C19	2.696 ± 0.114	0.016 ± 0.001	0.407 ± 0.041	0.000 ± 0.000	0.031 ± 0.001	0.006 ± 0.000	0.037 ± 0.003	0.000 ± 0.000
C20	2.116 ± 0.037	0.010 ± 0.001	0.277 ± 0.028	0.000 ± 0.000	0.024 ± 0.001	0.003 ± 0.000	0.044 ± 0.001	0.000 ± 0.000
K1	0.385 ± 0.030	0.015 ± 0.001	0.000 ± 0.001	0.624 ± 0.019	0.011 ± 0.004	0.515 ± 0.024	0.013 ± 0.001	1.042 ± 0.037
K2	0.347 ± 0.011	0.008 ± 0.001	0.026 ± 0.002	1.824 ± 0.053	0.012 ± 0.003	0.962 ± 0.010	0.009 ± 0.000	1.538 ± 0.067
K3	0.000 ± 0.000	0.002 ± 0.003	0.000 ± 0.000	0.613 ± 0.027	0.000 ± 0.000	0.432 ± 0.018	0.000 ± 0.000	1.178 ± 0.047
K4	0.000 ± 0.000	0.004 ± 0.003	0.000 ± 0.000	1.368 ± 0.034	0.000 ± 0.000	0.686 ± 0.032	0.000 ± 0.000	1.483 ± 0.062
K5	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	1.394 ± 0.059	0.000 ± 0.000	0.575 ± 0.017	0.000 ± 0.000	1.284 ± 0.009
K6	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.899 ± 0.040	0.000 ± 0.000	1.026 ± 0.076	0.000 ± 0.000	2.674 ± 0.120
K7	0.396 ± 0.017	0.000 ± 0.000	0.000 ± 0.000	2.417 ± 0.183	0.000 ± 0.000	0.585 ± 0.024	0.000 ± 0.000	1.510 ± 0.014
K8	0.456 ± 0.013	0.000 ± 0.000	0.000 ± 0.000	2.522 ± 0.092	0.000 ± 0.000	0.735 ± 0.024	0.000 ± 0.000	2.295 ± 0.104
K9	0.301 ± 0.006	0.000 ± 0.000	0.000 ± 0.000	1.084 ± 0.101	0.000 ± 0.000	0.614 ± 0.024	0.000 ± 0.000	1.504 ± 0.056
K10	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.809 ± 0.095	0.000 ± 0.000	0.456 ± 0.015	0.000 ± 0.000	1.222 ± 0.102
K11	0.306 ± 0.003	0.000 ± 0.000	0.000 ± 0.000	1.042 ± 0.039	0.000 ± 0.000	0.659 ± 0.039	0.000 ± 0.000	2.382 ± 0.152
K12	0.332 ± 0.009	0.000 ± 0.000	0.000 ± 0.000	2.228 ± 0.157	0.000 ± 0.000	0.558 ± 0.033	0.000 ± 0.000	1.864 ± 0.071
K13	0.292 ± 0.004	0.000 ± 0.000	0.000 ± 0.000	0.821 ± 0.034	0.000 ± 0.000	1.184 ± 0.048	0.000 ± 0.000	2.690 ± 0.034
K14	0.300 ± 0.002	0.000 ± 0.000	0.000 ± 0.000	1.019 ± 0.103	0.000 ± 0.000	0.768 ± 0.018	0.000 ± 0.000	2.500 ± 0.097
K15	0.289 ± 0.002	0.000 ± 0.000	0.000 ± 0.000	2.040 ± 0.171	0.000 ± 0.000	0.599 ± 0.013	0.000 ± 0.000	1.796 ± 0.085
K16	0.322 ± 0.003	0.000 ± 0.000	0.000 ± 0.000	1.430 ± 0.101	0.000 ± 0.000	0.673 ± 0.050	0.000 ± 0.000	2.615 ± 0.101
K17	0.368 ± 0.003	0.000 ± 0.000	0.000 ± 0.000	3.770 ± 0.134	0.000 ± 0.000	0.409 ± 0.021	0.000 ± 0.000	2.075 ± 0.119
K18	0.295 ± 0.001	0.000 ± 0.000	0.000 ± 0.000	2.495 ± 0.179	0.000 ± 0.000	0.691 ± 0.041	0.000 ± 0.000	2.811 ± 0.092
K19	0.282 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	2.306 ± 0.155	0.000 ± 0.000	0.689 ± 0.073	0.000 ± 0.000	2.826 ± 0.214
K20	0.282 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.720 ± 0.014	0.000 ± 0.000	0.221 ± 0.010	0.000 ± 0.000	0.514 ± 0.016

evaluate the quality of *F.suspensa* because of its diverse pharmacological properties previously described. Besides, the other lignans, such as phillyrin, pinoresinol, lariciresinol, and phillygenin are also significant bioactive compounds of the herbal drug, thus they are also should be treated as the marker compounds to evaluate the quality of the *Chinese* drug. While the DBL-lignans together with the glycoside (arctiin, artctigenin, and matairesinol) showing diverse pharmacological effects, clearly should be the marker constituents of *F. viridissima*. The results clearly

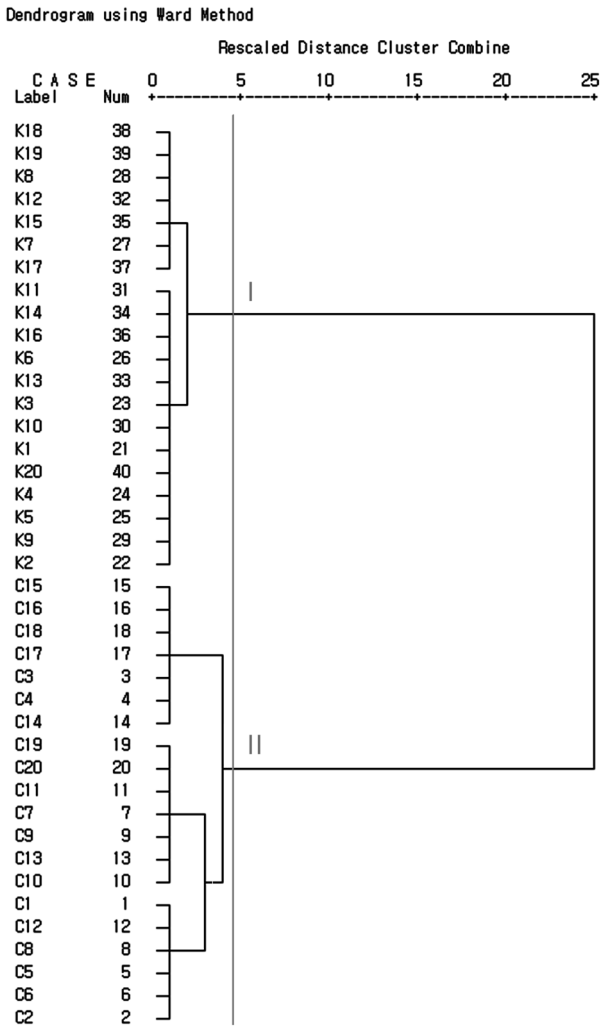

Fig. 4. Dendrogram of hierarchical cluster analysis for 40 samples of F. fructus.

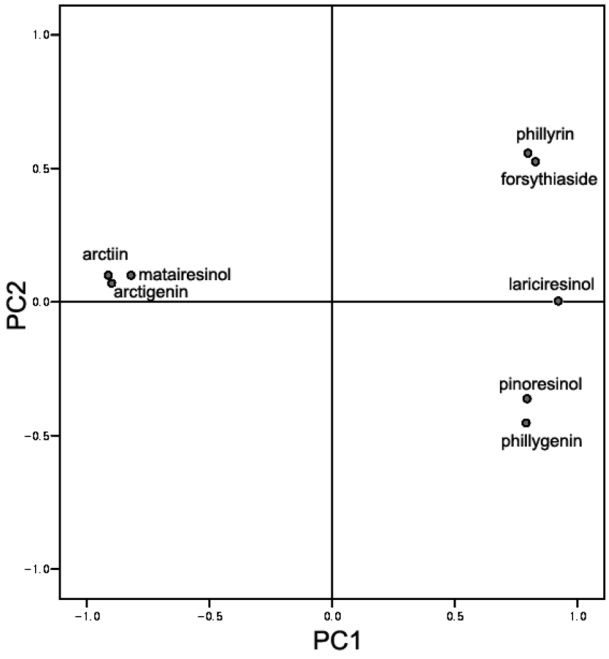

Fig. 5. The factor score plot.

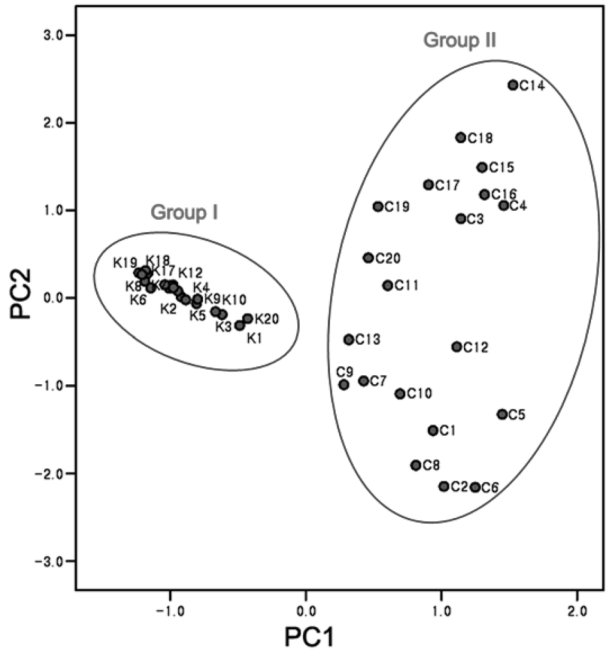

Fig. 6. The principal component analysis (PCA).

suggest that the two species of F. Fructus should be treated separately because they might have quite different medicinal properties. It should be accompanied by further researches to reveal the relationship between the contents of the marker compounds and the pharmacological effects of the herbal drug.

Statistical analysis – The dendrogram from the hierarchical cluster analysis is shown in Fig. 4. All samples were clustered into two groups in which the Korean habitat samples (K1-K20) were classified into the group I; the Chinese samples (C1-C20) into the group II. The principal component analysis (PCA) was carried out using the new variables (PC1 and PC2) produced by the factor analysis (FA) for the eight valuables. The Kaiser-Meyer-Olkin (KMO) measure of sampling adequacy was 0.839 meritoriously with Bartlett's test of sphericity ($\chi^2 = 350.567$, $p < 0.001$) less than the significance level ($\alpha = 0.05$), which means the PCA is appropriate for the data analysis. The two principal components (PC1 and PC2) explain an estimate of 83.8% of the total variances. The factor scores plot (Fig. 5), which explains the correlations between the PC components and the variables,

well designate the featured variables to characterize the sample clusters. The PC1 is influenced negatively by arctigenin, arctiin and matairesinol but positively by the other 5 compounds; The PC2 is mainly influenced positively by phillyrin and forsythiaside but negatively by pinoresinol and phyllygenin. The two-dimensional PCA plot of the 40 samples is shown in Fig. 6. All samples of the Korean and Chinese habitat were clearly grouped by the PC1 into the respective ones, where the samples presumed as *F. viridissima* have negative scores; while those of *F. suspensa* showed positive values (Fig. 6). Moreover, the Chinese habitat samples (C1-C20) were further classified into the subgroups by the PC2 that is influenced by the two pairs of the compounds: (1) phillyrin and forsythiaside, (2) pinoresinol and phillygenin. The clustering from PCA showed the same result with that of HCA in Fig. 4.

Conclusion

A HPLC-tandem mass spectrometric method was developed to quantify simultaneously the eight bioactive maker compounds of F. fructus. This method could evaluated its quality and assess the origin of the herbal drug using the multivariate statistical analysis based on the analytical results. The minor isomeric components that appear in the *Chinese* habitat samples could be distinguished from the major marker compounds of *F.suspensa* by the highly sensitive and selective analytical method. The analytical results of the forty commercial samples combined with statistical interpretation clearly demonstrated the evaluation of the F. fructus according to both the origin and the contents of the marker constituents. The analytical method with pattern recognition method would provide a practical strategy for assessing the authenticity or quality of the herbal drug.

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