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Quality Evaluation and Pattern Recognition Analyses of Bioactive Marker Compounds from Farfarae Flos Using HPLC/PDA

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The flower bud of *Tussilago farfara* L., called Farfarae Flos, has traditionally been used in Oriental medicine for the treatment of bronchitis and asthma. To establish a standard for quality control as well as the reliable identification of Farfarae Flos, the contents of five standards, rutin (1), isoquercetin (2), 3,5-dicaffeoylquinic acid (3), tussilagone (4), and tussilagonone (5), were determined by quantitative high-performance liquid chromatography (HPLC)/photodiode array (PDA) analysis. The five standards were separated on a YoungJinBioChrom Aegispak C18-L (250-mm×4.6-mm, 5-μm) column by gradient elution using 0.03% tri-fluoroacetic acid in water (A), with acetonitrile (B) as the mobile phase. The flow rate was 1.0 mL/min, and the UV detector wavelength was set at 220 nm. The method was successfully used in the analysis of Farfarae Flos from different geographic origins with relatively simple conditions and procedures, and the results demonstrated satisfactory linearity, recovery, precision, accuracy, stability, and robustness. The HPLC analytical method for pattern recognition analysis was validated by repeated analysis of 62 Farfarae Flos samples. This result indicated that the established HPLC/PDA method is suitable for quantitation and pattern recognition analyses for the quality evaluation of Farfarae Flos.

Key words Farfarae Flos; HPLC; pattern recognition; quality control

Herbal medicines have a long history of use in therapeutic fields, and are attracting considerable attention because of their low toxicity and excellent therapeutic benefits. Quality control of herbal medicines is more difficult than synthetic drugs due to the chemical complexity of the material, wherein a single active substance is not responsible for the overall pharmacological potency. Pattern recognition analysis of quality control for traditionally used herbs can provide information on the overall chemical composition.¹⁾ *Tussilago farfara* L., one of the Chinese traditional medicines, is widely spread in China, North Africa, Siberia, and Europe. The flower buds of *T. farfara* L. (Compositae) are called Farfarae Flos, and have been used for the treatment of cough, bronchitis, and asthmatic disorders in Asia.^{2,3)} This bud, also known as Kuan-Dong-Hua in China, is a popular folk medicine, and has been studied for its pharmacological activities, including anti-oxidative, anti-microbial, anti-inflammatory, anti-diabetic, anti-hypertension, platelet anti-aggregation, and anti-cancer functions.^{4–8)}

Farfarae Flos is the flower bud of *T. farfara* L. in the Chinese Pharmacopoeia (C.P. 2010) and the Korean Pharmacopoeia (K.P. 2014).^{9,10)} The regulation of Farfarae Flos content in the C.P. was already stipulated in 2010, which prescribed that more than 0.07% tussilagone from Farfarae Flos must be included. However, the K.P. has no stipulations on the main compounds contained in Farfarae Flos. Therefore, the purpose of this study was to establish a reliable HPLC method for the quantitative analysis of the major compounds in Farfarae Flos, and to provide analytical methods which can be used as the official analytical method of the K.P.

Previously, a few researchers quantified only the amount of

rutin, one of the bioactive components in Farfarae Flos.^{11–13)} However, they ignored other reasonable component, such as tussilagone. Wu *et al.* established a method for the quantitative analysis of rutin, isoquercitrin, and chlorogenic acid in 50 min at 255 nm using three solvent system such as methanol, acetonitrile, and 0.2% formic acid in water.¹⁴⁾ Zhang *et al.* developed a reverse phase (RP)-HPLC method for the simultaneous determination of chlorogenic acid and rutin in Farfarae Flos by HPLC with an RP-C18 column and a UV detector at 254 nm, but this method was unreliable, because the first compound (chlorogenic acid) came out too early, near the solvent peak at about 5 min.¹⁵⁾ Ma *et al.* later established a method for the quantitative analysis of the quercetin and kaempferide in Farfarae Flos, in which two marker compounds were not considered as characteristic components.¹⁶⁾ As was stated above, their studies were focused on the quantification of only flavonoid and phenolic compounds without unique alkaloid components such as tussilagone and tussilagonone in Farfarae Flos.

In this study, the developed method not only had short analytical time, but also showed good resolution. An HPLC/photodiode array (PDA) method was established for analysis of rutin, isoquercetin, 3,5-dicaffeoylquinic acid, tussilagone, and tussilagonone as marker compounds. The methods for plant extraction and HPLC analysis were optimized and validated. A calibration curve of the marker compounds was also made, and variations of the retention times according to the pH of the mobile phase as well as the stability of marker compounds in solution were investigated. We therefore suggest a suitable analytical method for quantitative and pattern recognition analyses of Farfarae Flos, together with the establishment of adequate marker compounds [rutin (1), isoquercetin (2),

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3,5-dicaffeoylquinic acid (**3**), tussilagone (**4**), and tussilagonone (**5**)] to distinguish between Chinese and Korean Farfarae Flos.

Experimental

Materials All of the standard compounds were provided by Prof. Eun Kyoung Seo, Ewha Womans University, Seoul, Korea. Their structures were unambiguously identified as rutin (**1**), isoquercetin (**2**), 3,5-dicaffeoylquinic acid (**3**), tussilagone (**4**), and tussilagonone (**5**). The standard compound structures are shown in Fig. 1. Purity of standard compounds was estimated to be higher than 95% based on HPLC analysis. Internal standard, amiodarone hydrochloride, was purchased from Sigma Chemicals (St. Louis, MO, U.S.A.). Acetonitrile was purchased from Merck K GaA (Darmstadt, Germany). All other chemicals used were of analytical grade unless otherwise noted. Distilled water was prepared using the Milli-Q purification system (Millipore, Bedford, MA, U.S.A.). Sixty-two samples cultured in different regions were provided from Prof. Jae-Hyun Lee, College of Oriental Medicine, Dongguk University at Gyeongju, Korea. This study adopted the following sixty-two samples corresponding to Farfarae Flos [six ones (K01–K06) from Korea and fifty-six ones (C07–C62) from China].

Sample Preparation The dried Farfarae Flos was pulverized and passed through a 50 mesh sieve. About 2.0 g of the powder, accurately weighed, were added to 40 mL of 95% methanol containing an internal standard (I.S.: 50 ppm amio-

darone hydrochloride) by sonication for 60 min at room temperature. After extraction, each sample solution was adjusted to the original volume and filtered through 0.45 μ m membrane, and an aliquot (10 μ L) of the filtrate was injected into HPLC.

HPLC/PDA Conditions The HPLC equipment was a Waters 2695 Separations Module (Waters, Milford, MA, U.S.A.) with Waters 996 Photodiode Array Detector. YoungJinBioChrom Aegispak C18-L (250 mm $\times$ 4.6 mm, 5 μ m), Phenomenex Gemini C18 110A (250 mm $\times$ 4.6 mm, 5 μ m) and Shiseido C18 (250 mm $\times$ 4.6 mm, 5 μ m) columns were tested with the guard column (3.9 mm $\times$ 20 mm XTerra RP18, 5 μ m) filled with the same stationary phase. The mobile phase consisted of water containing 0.03% trifluoroacetic acid (A) and acetonitrile (B). Elution was performed at a flow rate of 1.0 mL/min in gradient and isocratic modes. The solvent gradient was changed according to the following program: [0–15 min, 15–30% (B); 15–20 min, 30–73% (B) and 20–55 min, 73% (B)]. The column was washed by 100% B for 20 min. And each run was followed by a 20 min re-equilibration. Equilibration time for the next run is 20 min. The mobile phase was filtered under vacuum through a 0.21- μ m membrane filter and degassed prior to use. The analysis was carried out at a flow rate of 1.0 mL/min with PDA detection absorbance at 220 nm. The chromatograms were processed using Empower pro software, Build 1154 (Waters).

Analytical Method Validation The standards of rutin (**1**), isoquercetin (**2**), 3,5-dicaffeoylquinic acid (**3**), tussilagone (**4**), and tussilagonone (**5**) were each accurately weighed and then

Fig. 1. Structures of Standards and an Internal Standard

dissolved with 95% methanol containing I.S. (50ppm amiodarone hydrochloride) to produce stock standard solutions of 500ppm. The calibration curves were generated after diluting the stock solution with 95% methanol containing I.S. (50ppm amiodarone hydrochloride). A reference solution at concentrations of 0.625–500.0 $\mu\text{g/mL}$ was analyzed by HPLC/PDA. The regression equations were calculated in the form of $y=ax+b$, where y and x correspond to peak ratio (compound area/I.S. area) and compound concentration, respectively. Limits of detection (LOD) and limits of quantification (LOQ) were obtained based on the concentrations at three times signal-to-noise value ($3=S/N$) and ten times ($10=S/N$), respectively.

Recovery tests were executed by mixing a powdered sample (2.0 g) of the reference compounds at three control levels (near the LOQ, medium and higher concentrations from the calibration curves). The mixture was then extracted by sonication in 40 mL of 95% methanol containing I.S. (50ppm amiodarone hydrochloride) at room temperature (25°C) for 60 min. The extract solution was filtered through a 0.45- μm membrane. The HPLC/PDA analysis experiments were performed in triplicate for each control level. The data from the standard solution and the extracted sample were compared.

Precision and accuracy were determined by multiple analyses ($n=5$) of quality control samples prepared at low, medium and high concentrations spanning the calibration range.

The robustness of the method was studied by introducing changes in columns (*i.e.*, Aegispak, Gemini and Shiseido), separation temperatures (*i.e.*, 25, 30, 35°C) and three different flow rates (*i.e.*, 0.8, 1.0, 1.2 mL/min).

Stability was tested with mixed standard solution stored in the dark at 4°C and room temperature (25°C). The two samples were analyzed in triplicate at 0, 1, 3, 7, 15, and 30 d.

Pattern Recognition Analysis A pattern recognition analysis was conducted to evaluate the phytochemical equivalency among the 62 Farfarae Flos samples. We used five major marker compounds [rutin (1), isoquercetin (2), 3,5-dicaffeoylquinic acid (3), tussilagone (4), and tussilagonone (5)] for the pattern recognition analysis using IBM SPSS Statistics Version 19 software (SPSS, Inc., Chicago, IL, U.S.A.).

Results and Discussion

Optimization of Chromatographic Conditions HPLC conditions were selected to obtain good resolution on the chromatograms within a short retention time. For optimization of the chromatographic conditions, the effects of the composition of the mobile phase on separation were examined. A mobile phase of water–methanol proved unsatisfactory for separating the structurally similar compounds. Acetonitrile, as an organic modifier, demonstrated significantly improved separation. We also tested the addition of 0.03, 0.1, and 1% acid (acetic acid, formic acid, and trifluoroacetic acid) to the mobile phase. The addition of 0.03% trifluoroacetic acid resulted in good resolution of all compounds, as well as satisfactory peak symmetry and shape. The extracted sample was scanned between 200–400 nm using a PDA detector to determine the appropriate detection wavelength. The reasonable components, such as tussilagone and tussilagonone, showed maximum absorption at 220 nm. Besides these, rutin, isoquercetin, and 3,5-dicaffeoylquinic acid could also be detected well at about 220 nm. Wu *et al.* had reported LOD value of rutin as 200 ng/mL at 255 nm, exhibiting about 20 times higher than our LOD value (8.58 ng/mL) of rutin at 220 nm.¹⁴ Therefore, our method using the detection wavelength at 220 nm is more sensitive than the analytical method at 255 nm. Therefore, all components could

Fig. 2. The HPLC Chromatograms of Standards Mixture (A) and Farfarae Flos Samples [Chinese (B), and Korean (C)]

Rutin (1), isoquercetin (2), 3,5-dicaffeoylquinic acid (3), tussilagone (4), and tussilagonone (5).

be shown with baseline separation at 220 nm (data not shown). The typical chromatograms of the sample and standard mixtures are shown in Fig. 2, from which it can be seen that all target compound peaks and internal standard peak were clearly separated within 45 min. Amiodarone hydrochloride was selected as an internal standard. The chromatographic peaks of the analytes in the sample solution were identified by comparing their retention times with those of the reference standards, and were further confirmed by spiking the samples with the reference compounds.

Optimization of Sample Preparation Conditions Six solvents for extraction, including 95% ethanol, 75% ethanol, 50% ethanol, 95% methanol, 75% methanol, and 50% methanol, were compared in sample assays after extraction by sonication for 1 h at room temperature. When the extraction was carried out with 95% methanol, the sample assay (compound area/I.S. area) showed higher results than obtained by the other solvent samples. Thus, 95% methanol was used as the extraction solvent throughout this study. Two extraction methods, ultra-sonication and reflux using 95% methanol extraction solvent, were also compared. The sample assay results were superior after extraction with sonication than with reflux. To determine the time needed for complete extraction, extraction was carried out for five different lengths of time (30, 45, 60, 75, 90 min) in 95% methanol *via* sonication at room temperature. The extraction time of 60 min yielded similar sample

assay results as 75 min. Therefore, the compounds were thoroughly extracted when the extraction time was 60 min (data not shown).

Validation of the Method Each coefficient of correlation (r^2) was >0.999 , as determined by least square analysis, suggesting good linearity between the peak area ratio and the compound concentrations. The LOD and LOQ were evaluated at signal-to-noise (S/N) ratios of 3 and 10, respectively. The LOD and LOQ obtained under our experimental conditions are listed in Table 1. All LOD and LOQ values obtained for these five standards were low enough to allow detection of traces of these compounds in either a crude extract or its preparation.

Precision and accuracy were determined by multiple analysis ($n=5$) of quality control samples prepared at lower, medium and higher concentrations (1, 50, 100 $\mu\text{g/mL}$) spanning the calibration range. Intra-assay precision and accuracy were determined from multiple analyses ($n=5$) of the quality control samples, carried out within the same analytical run. The quality control samples had intra-assay precision of $<4.18\%$ and accuracy of 90.9–106.7%. Inter-assay precision and accuracy were evaluated from multiple analyses ($n=5$) of the quality control samples, analyzed in a single analytical run for five consecutive days. The quality control samples had an inter-assay precision of $<4.42\%$ and accuracy of 94.9–109.7%. Thus, the method developed was highly reproducible. The pre-

Table 1. Linearity, Linear Ranges, LOD, and LOQ

Analytes	Linear range ($\mu\text{g/mL}$)	Slope	Intercept	Correlation coefficient (r^2)	LOD (ng/mL)	LOQ (ng/mL)
1	0.625–500	0.0220	0.0157	0.9998	8.58	28.61
2	0.625–500	0.0197	0.0111	0.9998	10.22	34.08
3	0.625–500	0.0249	−0.0182	0.9996	11.04	36.79
4	0.625–500	0.0237	0.0191	0.9998	7.04	23.47
5	0.625–500	0.0223	0.0002	0.9997	12.65	42.17

Rutin (1), isoquercetin (2), 3,5-dicafeoylquinic acid (3), tussilagone (4), and tussilagonone (5).

Table 2. The Results of Precision and Accuracy

Analyte	Fortified conc. ($\mu\text{g/mL}$)	Sample conc. ($\mu\text{g/mL}$)	Intra-day ($n=5$)				Sample conc. ($\mu\text{g/mL}$)	Inter-day ($n=5$)			
			Observed ($\mu\text{g/mL}$)	S.D.	Accuracy (%)	Precision (%)		Observed ($\mu\text{g/mL}$)	S.D.	Accuracy (%)	Precision (%)
1	1	36.48	37.39	1.65	91.30	1.81	36.72	37.72	1.65	99.72	1.66
	50	36.48	87.28	1.73	101.61	1.70	36.72	88.11	0.89	102.79	0.86
	100	36.48	130.81	1.25	94.34	1.32	36.72	130.85	1.27	94.13	1.35
2	1	33.07	34.05	1.56	98.12	1.59	32.91	34.01	1.95	109.70	1.78
	50	33.07	84.04	1.17	101.95	1.15	32.91	84.39	0.81	102.97	0.79
	100	33.07	126.77	3.11	93.70	3.32	32.91	127.18	3.46	94.27	3.67
3	1	249.42	250.45	1.70	102.77	1.65	249.87	250.82	4.22	95.35	4.42
	50	249.42	299.37	2.52	99.90	2.52	249.87	299.53	4.22	99.32	4.24
	100	249.42	356.13	4.46	106.71	4.18	249.87	356.87	4.65	107.00	4.35
4	1	22.85	23.81	1.65	96.39	1.72	22.95	23.93	2.02	98.51	2.05
	50	22.85	69.51	1.24	93.33	1.33	22.95	70.18	1.17	94.46	1.24
	100	22.85	122.65	3.39	99.80	3.40	22.95	122.65	3.52	99.71	3.53
5	1	8.21	9.12	1.57	90.87	1.73	8.00	8.95	1.66	94.91	1.74
	50	8.21	59.32	1.97	102.23	1.92	8.00	58.65	1.73	101.31	1.71
	100	8.21	110.65	2.37	102.45	2.32	8.00	109.65	2.38	101.65	2.34

Rutin (1), isoquercetin (2), 3,5-dicafeoylquinic acid (3), tussilagone (4), and tussilagonone (5).

cision and accuracy data are presented in Table 2.

The extraction recovery test was performed by extracting a known amount of the five marker compounds from sample C01. Known amounts of each standard compound, included at

three levels, were mixed with the sample powder and extracted with 95% methanol containing the internal standard (50 ppm amiodarone hydrochloride). For comparison, the blank sample was prepared by spiking with 95% methanol containing the

Table 3. Robustness of Marker Compounds According to Columns, Temperatures and Flow Rates ($n=3$)

Marker compounds	Analytical condition		Theoretical plate (N)	Capacity factor (k')	Separation factor (α)	Resolution (R_s)
			Mean $\pm$ S.D.	Mean $\pm$ S.D.	Mean $\pm$ S.D.	Mean $\pm$ S.D.
1	Column ^{a)}	Aegispak	9397 $\pm$ 67	3.63 $\pm$ 0.01	1.11 $\pm$ 0.00	1.94 $\pm$ 0.02
		Gemini	4724 $\pm$ 98	3.42 $\pm$ 0.05	1.09 $\pm$ 0.01	1.69 $\pm$ 0.19
		Shiseido	5218 $\pm$ 37	2.60 $\pm$ 0.03	1.11 $\pm$ 0.00	1.74 $\pm$ 0.04
	Temperature (°C)	25	9397 $\pm$ 67	3.63 $\pm$ 0.01	1.11 $\pm$ 0.00	1.94 $\pm$ 0.02
		30	9329 $\pm$ 37	3.49 $\pm$ 0.01	1.10 $\pm$ 0.00	2.59 $\pm$ 0.03
		35	9899 $\pm$ 50	3.32 $\pm$ 0.01	1.10 $\pm$ 0.00	2.13 $\pm$ 0.07
	Flow rate (mL/min)	0.8	10443 $\pm$ 31	3.21 $\pm$ 0.03	1.10 $\pm$ 0.00	2.42 $\pm$ 0.49
		1.0	9397 $\pm$ 67	3.63 $\pm$ 0.01	1.11 $\pm$ 0.00	1.94 $\pm$ 0.02
		1.2	7164 $\pm$ 31	3.94 $\pm$ 0.02	1.11 $\pm$ 0.00	2.29 $\pm$ 0.02
2	Column	Aegispak	4261 $\pm$ 98	4.12 $\pm$ 0.01	1.17 $\pm$ 0.00	2.87 $\pm$ 0.29
		Gemini	4583 $\pm$ 25	3.83 $\pm$ 0.01	1.16 $\pm$ 0.00	2.46 $\pm$ 0.03
		Shiseido	3842 $\pm$ 163	3.00 $\pm$ 0.03	1.19 $\pm$ 0.00	3.24 $\pm$ 0.01
	Temperature (°C)	25	4261 $\pm$ 98	4.12 $\pm$ 0.01	1.17 $\pm$ 0.00	2.87 $\pm$ 0.29
		30	4773 $\pm$ 22	3.95 $\pm$ 0.01	1.16 $\pm$ 0.00	4.11 $\pm$ 0.03
		35	6601 $\pm$ 64	3.75 $\pm$ 0.02	1.15 $\pm$ 0.00	3.43 $\pm$ 0.13
	Flow rate (mL/min)	0.8	4506 $\pm$ 102	3.63 $\pm$ 0.03	1.15 $\pm$ 0.00	3.11 $\pm$ 0.14
		1.0	4261 $\pm$ 98	4.12 $\pm$ 0.01	1.17 $\pm$ 0.00	2.87 $\pm$ 0.29
		1.2	6994 $\pm$ 54	4.47 $\pm$ 0.02	1.17 $\pm$ 0.00	3.01 $\pm$ 0.01
3	Column	Aegispak	7860 $\pm$ 35	4.97 $\pm$ 0.02	1.58 $\pm$ 0.00	16.84 $\pm$ 2.27
		Gemini	4803 $\pm$ 47	4.62 $\pm$ 0.01	1.49 $\pm$ 0.00	9.55 $\pm$ 0.07
		Shiseido	7549 $\pm$ 15	3.77 $\pm$ 0.04	1.76 $\pm$ 0.01	18.97 $\pm$ 0.62
	Temperature (°C)	25	12860 $\pm$ 35	4.97 $\pm$ 0.02	1.58 $\pm$ 0.00	16.84 $\pm$ 2.27
		30	11928 $\pm$ 39	4.72 $\pm$ 0.01	1.65 $\pm$ 0.00	20.68 $\pm$ 0.24
		35	14107 $\pm$ 127	4.47 $\pm$ 0.01	1.72 $\pm$ 0.00	24.64 $\pm$ 0.51
	Flow rate (mL/min)	0.8	7158 $\pm$ 26	4.34 $\pm$ 0.04	1.50 $\pm$ 0.01	12.99 $\pm$ 1.93
		1.0	7860 $\pm$ 35	4.97 $\pm$ 0.02	1.58 $\pm$ 0.00	16.84 $\pm$ 2.27
		1.2	4315 $\pm$ 12	5.41 $\pm$ 0.03	1.69 $\pm$ 0.01	15.06 $\pm$ 0.20
4	Column	Aegispak	37049 $\pm$ 22	10.78 $\pm$ 0.02	1.29 $\pm$ 0.00	10.92 $\pm$ 0.09
		Gemini	15809 $\pm$ 45	9.78 $\pm$ 0.02	1.21 $\pm$ 0.00	5.98 $\pm$ 0.03
		Shiseido	43661 $\pm$ 21	9.76 $\pm$ 0.03	1.29 $\pm$ 0.00	9.67 $\pm$ 0.15
	Temperature (°C)	25	67049 $\pm$ 22	10.78 $\pm$ 0.02	1.29 $\pm$ 0.00	10.92 $\pm$ 0.09
		30	79908 $\pm$ 27	10.67 $\pm$ 0.01	1.27 $\pm$ 0.00	12.48 $\pm$ 0.24
		35	75155 $\pm$ 26	10.54 $\pm$ 0.01	1.25 $\pm$ 0.00	11.73 $\pm$ 0.26
	Flow rate (mL/min)	0.8	49874 $\pm$ 17	9.35 $\pm$ 0.01	1.32 $\pm$ 0.00	13.05 $\pm$ 0.52
		1.0	37049 $\pm$ 22	10.78 $\pm$ 0.02	1.29 $\pm$ 0.00	10.92 $\pm$ 0.09
		1.2	43237 $\pm$ 101	12.15 $\pm$ 0.02	1.26 $\pm$ 0.00	9.20 $\pm$ 0.13
5	Column	Aegispak	34151 $\pm$ 10	14.20 $\pm$ 0.04	1.07 $\pm$ 0.00	3.18 $\pm$ 0.14
		Gemini	27621 $\pm$ 63	12.01 $\pm$ 0.03	1.06 $\pm$ 0.00	2.26 $\pm$ 0.02
		Shiseido	21748 $\pm$ 94	12.89 $\pm$ 0.06	1.09 $\pm$ 0.02	3.84 $\pm$ 0.71
	Temperature (°C)	25	44151 $\pm$ 10	14.20 $\pm$ 0.04	1.07 $\pm$ 0.00	3.18 $\pm$ 0.14
		30	46216 $\pm$ 24	13.81 $\pm$ 0.01	1.07 $\pm$ 0.00	3.06 $\pm$ 0.05
		35	47405 $\pm$ 25	13.43 $\pm$ 0.01	1.06 $\pm$ 0.00	3.00 $\pm$ 0.06
	Flow rate (mL/min)	0.8	38844 $\pm$ 51	12.64 $\pm$ 0.03	1.07 $\pm$ 0.00	3.22 $\pm$ 0.08
		1.0	44151 $\pm$ 10	14.20 $\pm$ 0.04	1.07 $\pm$ 0.00	3.18 $\pm$ 0.14
		1.2	38514 $\pm$ 12	15.51 $\pm$ 0.05	1.07 $\pm$ 0.00	2.79 $\pm$ 0.09

Rutin (1), isoquercetin (2), 3,5-dicaffeoylquinic acid (3), tussilagone (4), and tussilagone (5). ^{a)} YoungJinBioChrom Aegispak C18-L (250 mm $\times$ 4.6 mm, 5 μ m), Phenomenex Gemini C18 110A (250 mm $\times$ 4.6 mm, 5 μ m), and Shiseido C18 (250 mm $\times$ 4.6 mm, 5 μ m).

Table 4. Stability of Marker Compounds

Compounds	Temperature (°C)	Day (%)						Mean (n=6)	R.S.D. ^{a)} (%)
		0	1	3	7	15	30		
1	4	100 ^{b)}	98.51	101.99	99.33	101.11	97.12	99.68	1.77
	25	100	96.86	95.61	98.56	97.96	101.43	98.40	2.14
2	4	100	98.29	100.71	97.44	99.77	95.57	98.63	1.95
	25	100	95.25	95.97	97.29	98.22	100.17	97.82	2.08
3	4	100	99.16	91.26	86.15	84.98	83.70	90.87	7.95
	25	100	87.19	84.86	79.49	66.18	66.23	80.66	16.20
4	4	100	98.45	101.73	99.48	98.72	95.76	99.02	2.00
	25	100	98.04	95.72	98.12	100.99	99.81	98.78	1.91
5	4	100	98.28	97.95	99.19	95.74	95.02	97.70	1.99
	25	100	95.15	95.72	98.11	98.11	95.81	97.15	1.94

Rutin (1), isoquercetin (2), 3,5-dicaffeoylquinic acid (3), tussilagone (4), and tussilagonone (5). a) R.S.D. (%)=S.D./mean×100. b) Percentage of peak ratio of marker compound to internal standard according to days.

internal standard (50 ppm amiodarone hydrochloride). The quantitation of each component was subsequently achieved from the corresponding calibration curves. The recoveries of the all components were between 93.9% and 106.7% ($n=6$), with a relative standard deviation (R.S.D.) <4.4%.

The robustness was determined in order to evaluate the reliability of the established HPLC method. All parameters were maintained so there would be no interference with the other peaks for the Farfarae Flos. The experimental conditions, such as column temperature, column species and mobile phases, were purposefully altered. Evaluation was carried out on the theoretical plate (N), capacity factor (k'), separation factor (α) and resolution (R_s). To evaluate the suitability of three different columns, YoungJinBioChrom Aegispak, Phenomenex Gemini, and Shiseido, the performances were compared with regard to four analytical factors (N , k' , α , and R_s) at the column temperature of 25°C. The result showed no large differences in the factors depending on the column. Comparison of the factors was then done for the Aegispak column at three different column temperatures, 25, 30, and 35°C. Similarly, the results indicated that great variation in the factors did not occur depending on the column temperature (25, 30, 35°C). Further comparison of three different flow rates, 0.8, 1.0 and 1.2 mL/min, for the Aegispak column at 25°C also revealed that the four analytical factors did not differ greatly depending on the flow rates (0.8, 1.0, 1.2 mL/min). Optimization was also carried out by changing the chromatographic parameters, but no great variation was observed. Therefore, this experimental condition was sufficiently robust (Table 3).

The sample stability test was carried out with a standard mixture solution at 0, 1, 3, 7, 15, and 30 d. During this period, the solution was stored in lightless conditions at room temperature (25°C) and 4°C. The recovery rate of 3,5-dicaffeoylquinic acid (3) was found to drop below 90% after 1 d at room temperature; Even a recovery of this compound was confirmed as 91.3% after 3 d at 4°C, respectively. After 30 d, the recovery rates were down to 66.23 and 83.70% at room temperature and 4°C, respectively. At 4°C, this compound demonstrated 99.2% recovery for 24 h. Thus, the content of this compound in the extract should be measured within 24 h of storage at 4°C. The other marker compounds demonstrated stability for 30 d, as shown in Table 4.

Sample Analysis The established HPLC/PDA analytical method was applied for the simultaneous determination of five standard compounds, rutin (1), isoquercetin (2), 3,5-dicaffeoylquinic acid (3), tussilagone (4), and tussilagonone (5), in Farfarae Flos [Korean products (K01–K06), and Chinese products (C7–C62)]. The quantity of each compound present in the samples was determined, and the results are summarized in Table 5. Each sample was analyzed in triplicate to ensure reproducibility of the quantitative result. The average contents of rutin ($0.090 \pm 0.055\%$) and tussilagonone ($0.021 \pm 0.009\%$) in the Chinese Farfarae Flos samples were not significantly different from those of rutin ($0.078 \pm 0.015\%$) and tussilagonone ($0.019 \pm 0.015\%$) in the Korean Farfarae Flos samples. However, the average isoquercetin ($0.046 \pm 0.034\%$) and 3,5-dicaffeoylquinic acid ($0.537 \pm 0.146\%$) content in the Chinese Farfarae Flos were lower than those of isoquercetin ($0.086 \pm 0.037\%$) and 3,5-dicaffeoylquinic acid ($0.696 \pm 0.117\%$) in Korean Farfarae Flos. Interestingly, the average tussilagone content in Chinese Farfarae Flos was found to be $0.101 \pm 0.029\%$, whereas little or no detection was observed for the Korean samples (Table 6). This result should be confirmed with more data from a greater number of Korean samples.

Pattern Recognition Analysis A pattern recognition analysis method using the contents of five markers [rutin (1), isoquercetin (2), 3,5-dicaffeoylquinic acid (3), tussilagone (4), and tussilagonone (5)] was carried out for quality evaluation of the Farfarae Flos. All samples were clustered into two groups by hierarchical cluster analysis in SPDD, according to geographic origin: Chinese Farfarae Flos (group A; C07–C62), and Korean Farfarae Flos (group B; K01–K06) (Fig. 3). Thus, these pattern analysis results could be used for the quality control of Farfarae Flos samples.

Conclusion

An analytical method for Farfarae Flos by HPLC was developed for the separation of five marker compounds [rutin (1), isoquercetin (2), 3,5-dicaffeoylquinic acid (3), tussilagone (4), and tussilagonone (5)] using amiodarone hydrochloride as an internal standard. HPLC separation was carried out using the Aegispak C18-L (250×4.6 mm, 5 μ m) column with a three-step solvent system by PDA. The results suggested that the established HPLC/PDA method was suitable for the quantitative

Table 5. The Contents (wt%) of 1–5 in Oriental Medicinal Drugs of Farfarae Flos Samples

Samples	Compounds					Origin	Samples	Compounds					Origin
	1	2	3	4	5			1	2	3	4	5	
K01	0.065±0.001	0.057±0.001	0.819±0.006	0.002±0.000	0.031±0.001	Korea	C32	0.061±0.001	0.028±0.001	0.529±0.006	0.115±0.001	0.024±0.001	China
K02	0.086±0.002	0.065±0.002	0.740±0.011	0.004±0.000	0.040±0.002	Korea	C33	0.141±0.002	0.054±0.002	0.626±0.011	0.105±0.003	0.039±0.001	China
K03	0.085±0.002	0.149±0.005	0.764±0.020	0.002±0.000	0.021±0.001	Korea	C34	0.043±0.000	0.020±0.001	0.412±0.004	0.080±0.001	0.018±0.001	China
K04	0.070±0.005	0.097±0.002	0.711±0.017	—	0.004±0.000	Korea	C35	0.029±0.001	0.013±0.000	0.348±0.002	0.063±0.001	0.015±0.001	China
K05	0.101±0.000	0.099±0.001	0.658±0.005	—	0.007±0.000	Korea	C36	0.026±0.000	0.012±0.001	0.258±0.002	0.118±0.000	0.025±0.000	China
K06	0.060±0.002	0.049±0.002	0.483±0.005	0.003±0.000	0.009±0.000	Korea	C37	0.035±0.001	0.015±0.000	0.415±0.004	0.085±0.001	0.020±0.000	China
C07	0.116±0.003	0.045±0.002	0.623±0.011	0.080±0.001	0.025±0.001	China	C38	0.054±0.002	0.031±0.001	0.327±0.010	0.054±0.002	0.015±0.001	China
C08	0.074±0.002	0.040±0.002	0.511±0.009	0.061±0.001	0.020±0.000	China	C39	0.067±0.002	0.030±0.001	0.542±0.005	0.103±0.001	0.022±0.001	China
C09	0.037±0.001	0.017±0.000	0.470±0.005	0.096±0.002	0.022±0.001	China	C40	0.121±0.003	0.058±0.003	0.717±0.015	0.130±0.004	0.026±0.000	China
C10	0.030±0.001	0.013±0.000	0.370±0.008	0.076±0.004	0.017±0.001	China	C41	0.075±0.002	0.035±0.002	0.636±0.020	0.109±0.001	0.023±0.002	China
C11	0.092±0.003	0.036±0.000	0.716±0.012	0.080±0.002	0.015±0.001	China	C42	0.061±0.001	0.031±0.001	0.448±0.001	0.060±0.001	0.021±0.000	China
C12	0.231±0.010	0.172±0.004	1.032±0.015	0.146±0.006	0.033±0.002	China	C43	0.116±0.001	0.052±0.001	0.588±0.008	0.098±0.001	0.035±0.002	China
C13	0.032±0.001	0.009±0.000	0.295±0.010	0.123±0.002	0.023±0.001	China	C44	0.047±0.001	0.021±0.000	0.454±0.005	0.107±0.002	0.007±0.000	China
C14	0.182±0.002	0.089±0.003	0.679±0.011	0.153±0.002	0.039±0.002	China	C45	0.162±0.002	0.103±0.004	0.730±0.020	0.109±0.001	0.006±0.000	China
C15	0.217±0.006	0.108±0.004	0.745±0.027	0.173±0.008	0.056±0.001	China	C46	0.040±0.001	0.020±0.000	0.417±0.002	0.104±0.000	0.010±0.000	China
C16	0.098±0.000	0.040±0.002	0.609±0.016	0.074±0.003	0.028±0.001	China	C47	0.101±0.003	0.043±0.002	0.711±0.019	0.052±0.003	0.013±0.000	China
C17	0.204±0.002	0.132±0.002	0.778±0.002	0.133±0.004	0.047±0.002	China	C48	0.043±0.001	0.020±0.001	0.406±0.007	0.097±0.000	0.011±0.000	China
C18	0.095±0.001	0.041±0.002	0.644±0.005	0.075±0.001	0.025±0.000	China	C49	0.137±0.004	0.063±0.002	0.535±0.008	0.095±0.002	0.015±0.001	China
C19	0.039±0.001	0.013±0.000	0.484±0.010	0.107±0.005	0.021±0.001	China	C50	0.126±0.002	0.061±0.001	0.585±0.004	0.181±0.002	0.018±0.000	China
C20	0.032±0.001	0.013±0.000	0.418±0.007	0.079±0.002	0.019±0.000	China	C51	0.116±0.004	0.060±0.002	0.496±0.011	0.090±0.001	0.015±0.000	China
C21	0.090±0.003	0.052±0.002	0.637±0.013	0.128±0.002	0.029±0.001	China	C52	0.043±0.001	0.022±0.001	0.482±0.008	0.141±0.005	0.014±0.001	China
C22	0.121±0.002	0.050±0.001	0.718±0.006	0.074±0.001	0.019±0.000	China	C53	0.218±0.001	0.117±0.003	0.665±0.017	0.112±0.004	0.030±0.001	China
C23	0.039±0.001	0.019±0.000	0.383±0.003	0.109±0.003	0.018±0.000	China	C54	0.125±0.001	0.061±0.001	0.638±0.001	0.140±0.001	0.016±0.001	China
C24	0.026±0.001	0.010±0.000	0.304±0.001	0.082±0.002	0.017±0.001	China	C55	0.041±0.001	0.018±0.000	0.513±0.002	0.099±0.002	0.011±0.000	China
C25	0.030±0.001	0.012±0.001	0.372±0.004	0.074±0.001	0.017±0.000	China	C56	0.046±0.000	0.024±0.001	0.422±0.005	0.107±0.004	0.009±0.000	China
C26	0.071±0.003	0.036±0.000	0.420±0.009	0.082±0.003	0.021±0.001	China	C57	0.095±0.002	0.046±0.002	0.542±0.008	0.082±0.001	0.016±0.001	China
C27	0.063±0.000	0.034±0.002	0.538±0.004	0.130±0.001	0.022±0.000	China	C58	0.163±0.002	0.108±0.003	0.710±0.006	0.115±0.002	0.010±0.000	China
C28	0.062±0.002	0.034±0.001	0.509±0.007	0.082±0.001	0.018±0.001	China	C59	0.038±0.000	0.018±0.000	0.410±0.004	0.085±0.001	0.011±0.000	China
C29	0.141±0.001	0.074±0.003	0.552±0.005	0.106±0.001	0.026±0.000	China	C60	0.142±0.001	0.074±0.001	0.549±0.006	0.111±0.002	0.018±0.001	China
C30	0.102±0.001	0.063±0.002	0.447±0.007	0.066±0.001	0.020±0.000	China	C61	0.124±0.005	0.067±0.002	0.646±0.004	0.160±0.001	0.019±0.001	China
C31	0.064±0.001	0.033±0.001	0.483±0.002	0.103±0.003	0.023±0.001	China	C62	0.119±0.001	0.060±0.002	0.565±0.008	0.084±0.000	0.013±0.001	China

Data are presented as the mean±S.D. (*n*=3). Rutin (1), isoquercetin (2), 3,5-dicafeoylquinic acid (3), tussilagone (4), and tussilagone (5).

Fig. 3. Hierarchical Cluster Analysis by SPSS of 62 Samples Based on the Contents of Marker Compounds

A: Chinese Farfarae Flos (C07–C62), B: Korean Farfarae Flos (K01–K06).

Table 6. The Average Content (wt%) of Marker Compounds ($n=3$)

Compounds	Chinese Farfarae Flos samples ($n=56$)	Korean Farfarae Flos samples ($n=6$)	Total ($n=62$)
1	0.090 ± 0.055 (0.026–0.231)	0.078 ± 0.015 (0.060–0.101)	0.089 ± 0.052 (0.026–0.231)
2	0.046 ± 0.034 (0.009–0.172)	0.086 ± 0.037 (0.049–0.149)	0.050 ± 0.036 (0.009–0.172)
3	0.537 ± 0.146 (0.258–1.032)	0.696 ± 0.117 (0.483–0.819)	0.552 ± 0.150 (0.258–1.032)
4	0.101 ± 0.029 (0.052–0.181)	0.002 ± 0.002 (0.000–0.004)	0.092 ± 0.041 (0.000–0.181)
5	0.021 ± 0.009 (0.006–0.056)	0.019 ± 0.015 (0.004–0.040)	0.021 ± 0.010 (0.004–0.056)

Rutin (1), isoquercetin (2), 3,5-dicaffeoylquinic acid (3), tussilagonone (4), and tussilagonone (5).

analysis and quality control of Farfarae Flos. Based on the detected quantities of the five standard compounds, the Korean and the Chinese Farfarae Flos samples were distinguished by pattern analysis. The 3,5-dicaffeoylquinic acid content was the highest, but this compound was confirmed to undergo rapid decomposition. Large variation was observed in the tussilagonone content, ranging from 0.004 to 0.056%. Therefore, 3,5-dicaffeoylquinic acid and tussilagonone were not suitable as marker compounds. Consequently, we suggest that the regulation of tussilagonone, rutin and isoquercetin as marker compounds is more reasonable compared with the use of only one marker compound (tussilagonone), as currently employed in the C.P. In addition, the tussilagonone content in the Korean Farfarae Flos was low or could not be detected. It is considered that *T. farfara* is not suitable for cultivation in Korea because of climatic conditions. Actually, all Farfarae Flos circulating in the Korean market is currently imported from China.

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Conflict of Interest The authors declare no conflict of interest.

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