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Quantitative Determination of Compounds from *Akebia quinata* by High-Performance Liquid Chromatography

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To provide the scientific corroboration of the traditional uses of *Akebia quinata* (Thunb.) Decne., a detailed analytical examination of *A. quinata* stems was carried out using a reversed-phase high performance liquid chromatography (RP-HPLC) method coupled to photodiode array detector (PDA) for the simultaneous determination of four phenolic substances; cuneataside D (**1**), 2-(3,4-dihydroxyphenyl)ethyl-*O*-*β*-D-glucopyranoside (**2**), 3-caffeoylquinic acid (**3**) and calceolarioside B (**4**). Particular attention was focused on the main compound, 3-caffeoylquinic acid (**3**), which has a range of biological functions. In addition, 2-(3,4-dihydroxyphenyl)ethyl-*O*-*β*-D-glucopyranoside (**2**) was considered as a discernible marker of *A. quinata* from its easy confuse plants. The contents of compounds **2** and **3** ranged from 0.72 to 2.68 mg/g and from 1.66 to 5.64 mg/g, respectively. The validation data indicated that this HPLC/PDA assay was used successfully to quantify the four phenolic compounds in *A. quinata* from different locations using relatively simple conditions and procedures. The pattern-recognition analysis data from 53 samples classified them into two groups, allowing discrimination between *A. quinata* and comparable herbs. The results suggest that the established HPLC/PDA method is suitable for quantitation and pattern-recognition analyses for a quality evaluation of this medicinal herb.

Key Words : *Akebia quinata*, Lardizabalaceae, HPLC, Pattern-recognition analyses, 3-Caffeoylquinic acid

Introduction

Akebia quinata (Thunb.) Decne. is the most studied species of the Lardizabalaceae family. The herb was used as an important crude drug for promoting urination and counteracting inflammation in traditional Chinese medicine.[1] *Akebia quinata*, which is also known as the chocolate vine, is a woody perennial plant that grows either as a twining vine or as groundcover. The plant is distributed widely in East Asia, including: China, Japan and Korea.[2] Although extensive phytochemical analyses of the stems, pericarps and seeds of *A. quinata* were carried out in the 1970's with particular attention paid to the triterpenes and triterpene saponins,[3] there is no report of an analytical method for the quantitation of this plant. In 2009, Fumiyo Kikaota *et al*.[1] performed a molecular biological identification of *Akebia* plant species and discriminating Akebiae Caulis from non-*Akebia* plants. In a report on the characterization of the stems of *Akebia trifoliata*, Yoshihiro Mimaki *et al*.[3] identified four saponins that were obtained in good yield but were not isolated from *A. quinata* stems. They concluded that these compounds are suitable marker compounds for chemically distinguishing between *A. trifoliata* and *A. quinata* by a conventional TLC examination. Despite this, it is still necessary to develop a quality evaluation method for this herb. Furthermore, discriminating *A. quinata* from other related crude drugs originating from non-*Akebia* plants is needed. The Chinese name of *A. quinata* is "Mutong", which was originally called "Tongcao" in history. In modern times, however, the name "Tongcao" has been given to the herb, *Tetrapanax papyriferus*. Other plants that have also been known by the name, Mu Tong, inlcude *Aristolichiae manshuriensis* caulis (Guan-Mutong) and *Clematidis armandii* caulis (Chuan-Mutong). One study reported that *A. manshuriensis* (Aristolochiaceae) is the source of *Akebia* plants, Akebiae Caulis sometimes causes renal failure due to the constituent, aristolochic acid,[4] and has been banned from clinical use in China.[5] Therefore, in this study, the stems of *A. manshuriensis, C. armandii* and *T. papyriferus* were used as comparative herbs of *A. quinata.*

Almost a hundred triterpenes or triterpene saponins have been isolated and elucidated from the stems of *A. quinata*.[3,6-8] On the other hand, compounds, such as saponins in *A. quinata* with very few chromophore groups, will poorly absorb UV radiation and are difficult to detected using this type of detector. Therefore, a simple, low-cost, and reasonable analytical method is needed for a quality evaluation of *A. quinata* through the simultaneous determination of four phenolic

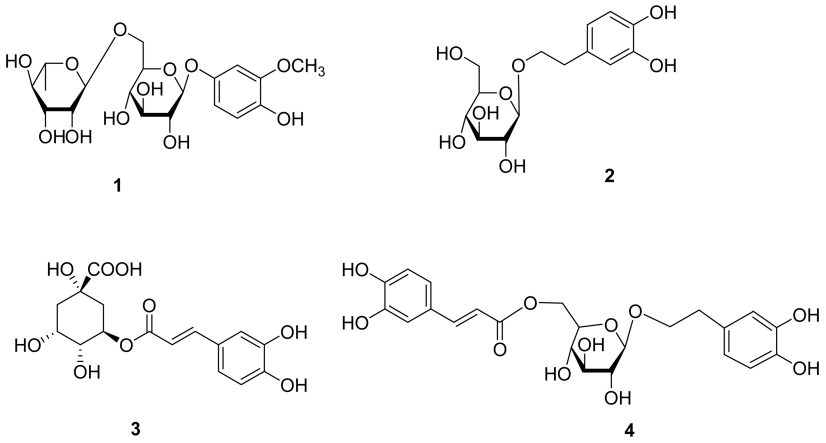

Figure 1. Chemical structures of marker compounds **1-4**.

compounds that have strong UV absorbance. Among them, 3-caffeoylquinic acid has been reported to have a variety of bioactivities: anti-amnesic action by inhibiting acetylcholinesterase,[9] anti-nociceptive and anti-pyretic actions by inhibiting inflammation,[10] anti-ischemic action by inhibiting oxidative stress and apoptosis,[11] and anti-anxyolytic action by inhibiting oxidative stress.[12] Caffeoylquinic acids are also believed to be responsible for the digestive and hepatoprotective activities in some medicinal plants.[13,14] In a worthy addition, calceolarioside B was reported to exhibit moderate binding affinity on HIV gp41 with IC_{50} values of 0.1 mg/mL.[15] By focusing on some index compounds, cuneataside D (**1**), 2-(3,4-dihydroxyphenyl)ethyl-*O*-*β*-D-glucopyranoside (**2**), 3-caffeoylquinic acid (**3**) and calceolarioside B (**4**) (Figure 1) were selected as marker compounds to establish a comprehensive quality evaluation method for 46 samples of *A*. *quinata* and 7 comparative herbal samples including two same species of *A*. *trifloliata* with 5 different species of *T. papyriferus*, *A. manshuriensis* and *C. armandii*.

Experimental

Materials. HPLC grade methanol was purchased from Fisher Scientific Korea Ltd. Distilled and deionized water were obtained from the instrument center (Catholic University of Daegu, Daegu, Korea) and used throughout the study. Trifluoroacetic acid (TFA) was obtained from Sigma-Aldrich (USA). Others solvents and reagents were of analytical grade. The reference standards of compounds (**1-4**) were supplied from Prof. Eun Rhan Woo, Chosun University, Gwangju, Korea. The purities of these marker compounds were determined to be greater than 95% by normalization of the peak areas detected by HPLC analyses. The internal standard of methyl *p*-hydroxyl benzoate (methyl paraben) was purchased from Sigma-Aldrich (German). Fifty three samples cultured in different regions in Korea and China were collected in 2013 corresponding to forty six *A*. *quinata* samples (AQ1-AQ41; AQ44-AQ48), two *C. armandii* samples (CA42, CA49), two *A*. *trifloliata* samples (AT50, AT51), two *A. manshuriensit* samples (AM52, AM53) and a sample of *T.papyriferus* (TP43). The origin of sample was identified by Prof. Je Hyun Lee, Dongguk University, Korea and voucher specimens were deposited in Catholic University of Daegu.

HPLC Apparatus and Chromatographic Conditions. HPLC analysis was performed on a Waters Alliance 2695 chromatograph equipped with a photodiode array detector (PDA; UV/UV Waters 2996). It was used to check the purity and verify the specificity of the evaluated compounds. UV spectra were recorded in range 190-400 nm. The chromatographic separation of analyses was carried out on an Agilent Eclipse XDB-C18 column (5.0 μm, 150 × 4.6 mm i.d.) performed at ambient temperature using a Thermo (Waters Corp.). Equipment control, data acquisition and integration were performed with Waters Empower software. The mobile phase consisting of gradient elution of 0.05% (v/v) TFA in water (solvent A, pH 2.3) and methanol (solvent B) was run with gradient elution at a flow rate of 1.0 mL/min. The linear gradient elution was set as follows: 0-15 min, form 5 to 20% of B; 15-25 min, 20-27% of B; 25-50 min, 27-60% of B. The injection volume was 10 μL. The column temperature was maintained at 30 °C. UV absorption was monitored at 280 nm. The existence of four marker compounds in the extract of *A. quinata* was identified by comparing their retention times with the standards (Figure 2). The presence of standard compounds was confirmed again by HPLC recovery test and co-TLC. Four compounds were used for HPLC analysis to determine their quantities in *A*. *quinata.* Quantification was conducted using an internal standard method based on the peak area ratio of the analyte/I.S. *vs* the amount of each analyte.

Sample Preparation. To determine the content of four marker compounds and pattern recognition analysis of *A. quinata* samples, the dried stems powder were used for each extraction. *A. quinata* stems samples were powdered and sieved through 50 mesh, and about 0.20 g of the powder were accurately weighed and added 10 mL of 70% methanol, accurately measured weight and ultrasonically extracted for 60 minutes at 40 °C. The solution was cooled, weighed again, and made up the loss in weight with 70% meth-

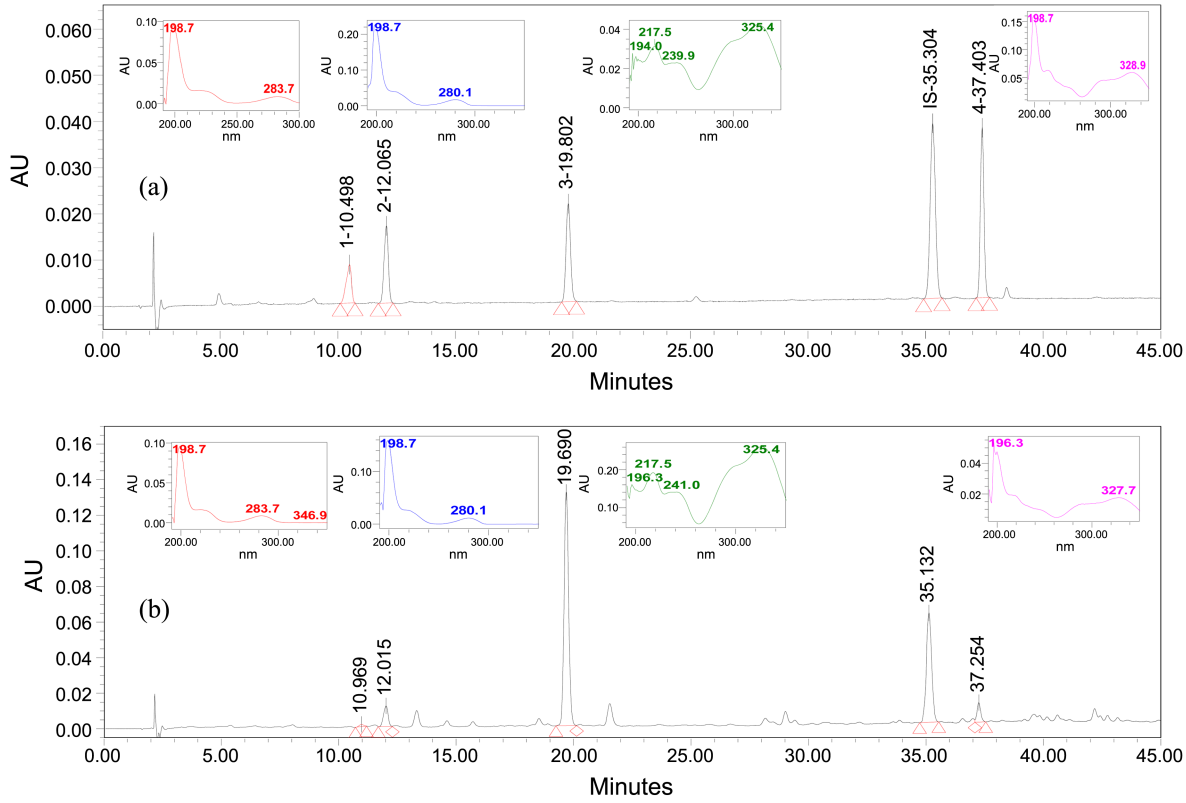

Figure 2. Representative HPLC chromatograms of mixed standards (a) and the extract of *A. quinata* stems (b) at 280 nm. The UV spectra corresponding to the phenolic compounds are shown above the chromatogram. Key to peak identification: 1, cuneataside D; 2, 2-(3,4-dihydroxyphenyl)ethyl-*O*-*β*-D-glucopyranoside; 3, 3-caffeoylquinic acid; 4, calceolarioside B and peak 'IS' was internal standard.

anol. The sample mixture was filtered through 0.45 μM membrane filter (Whatman), then 10 μL of aliquot from the filtrate was subjected to HPLC analysis.

Preparation of Standard Solutions. Based on the solubility of each component in MeOH, standard stock solutions (1000 μg/mL) were prepared by dissolving each reference compound in MeOH and then preserved in a refrigerator at less than 4 °C. Working standard solutions were prepared by serial dilution of stock solutions with MeOH. Linear regression equations were determined by calculation of integrated peak areas (y) of six serial minimum concentrations versus each concentration (x, μg/mL).

Linearity, Calibration Range, Limits of Detection, and Quantification. MeOH stock solution, which contained four compounds, was prepared and diluted to an appropriate concentration for the construction of calibration curves. Six concentration levels (3.125, 6.25, 12.5, 25.0, 50.0 and 100.0 μg/mL) of the mixed standard solution were injected in triplicate. The calibration curves were constructed by plotting the peak area ratio (phenolic/IS) versus the amount of each compound. The good linearity (correlation coefficient values $R^2 > 0.9983$) was achieved in relatively wide concentration ranging from 3.125 to 100.0 μg/mL for all the compounds. The lowest concentration of working solution was diluted with methanol to yield a series of appropriate concentrations, and the limit of detection (LOD) and quantification (LOQ) under the chromatographic conditions were separately determined at signal-to-noise ratio (S/N) of about 3 and 10, respectively. The data are summarized in Table 1.

Precision and Accuracy. The precision and accuracy of the method were assessed by analyzing three control samples at concentrations of 6.25; 50.0; 100.0 μg/mL. Intra-day precision and accuracy were determined by analyzing five replicates of each of control samples described above on a single day. Accuracy was calculated by dividing the measured mean phenolic concentration by the theoretical mean phenolic concentration. Precision was expressed as the coefficient of variation (CV, %), calculated as the ratio of the standard deviation to the measure mean phenolic concentration. The remaining quality control samples had intra-assay precision below 3.91% and accuracy between 98.61% and 105.79%. The inter-day precision and accuracy were evaluated from the variability of multiple analyses (n = 5) of quality control samples analyzed on a single analytical run and extended for three days. The remaining quality control samples had inter-assay precision below 3.91% and accuracy between 93.33% and 105.34%. Precision and accuracy data are presented in Table 2.

Stability. The mixture of four marker compounds was divided into two parts: one was stored at 5 °C and another was kept at room temperature. Two parts were analyzed in triplicate at 0, 1, 3, 5, 10, 15, 30 days. The resulting data indicated that marker compounds **1** and **2** remained stable more than 96.36% while compounds **3** and **4** signified

Table 1. Calibration data for 4 marker compounds **1-4** (n = 3)

Compound	Regression equation ($y = ax + b$)[a]	R^2	Linear range (μg/mL)	LOD (ng/mL)	LOQ (ng/mL)
1	$y = 0.0054x + 0.0099$	0.9988	3.125-100	89.58	298.60
2	$y = 0.0062x + 0.0116$	0.9983	3.125-100	70.35	235.51
3	$y = 0.0277x + 0.0258$	0.9993	3.125-100	16.34	54.46
4	$y = 0.0321x + 0.0393$	0.9995	3.125-100	13.31	44.35

[a]y is the peak area ratio (peak area/IS area), x is the corresponding injection concentration (μg/mL), a is the slope and b is the intercept of the regression line, R^2 is correlation coefficient.

Table 2. Precision and accuracy of analytical results

Compounds	Norminal Conc. (μg/mL)	Intra- day			Inter- day		
		Observed (μg/mL)	Accuracy (%)	Precision (C.V., %)	Observed (μg/mL)	Accuracy (%)	Precision (C.V., %)
1	100.00	104.04	104.04	1.83	104.30	104.30	3.63
	50.00	52.89	105.79	3.91	51.79	103.57	5.16
	6.25	6.44	103.13	2.50	6.52	104.32	2.77
2	100.00	102.09	102.11	1.82	103.52	103.52	2.31
	50.00	49.02	98.61	1.51	46.66	93.33	1.87
	6.25	6.03	103.59	3.23	6.27	100.31	1.81
3	100.00	101.81	101.81	3.35	105.34	105.34	5.38
	50.00	52.35	104.03	1.22	49.26	98.53	1.48
	6.25	6.55	104.89	3.54	6.07	97.19	0.68
4	100.00	103.54	103.54	2.51	104.40	104.40	2.53
	50.00	50.91	101.83	0.90	49.20	98.40	1.08
	6.25	6.39	102.34	3.59	5.99	95.97	0.60

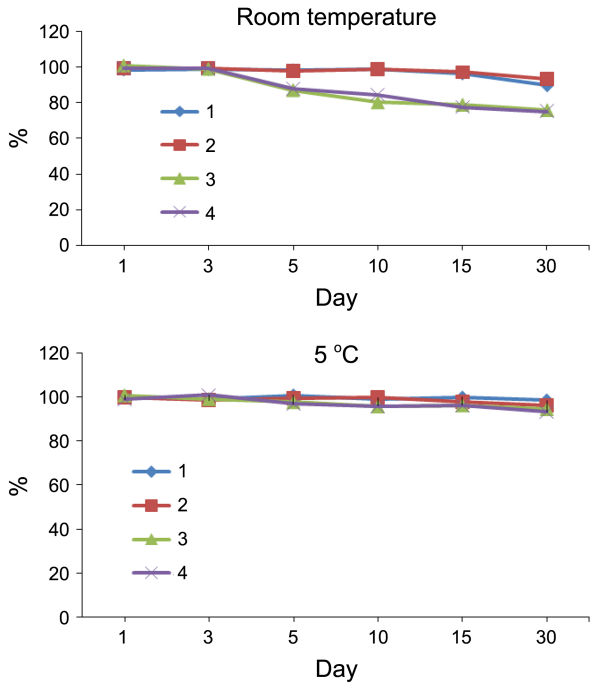

Figure 3. Stability of marker compounds **1-4**.

lability in room temperature during the experiment period (Figure 3).

Recovery. To determine the effectiveness of this method,

Table 3. Recovery of marker compounds through addition standard (n = 5)

Compounds	Original (μg/mL)	Added (μg/mL)	Calconc. (μg/mL)	Recovery (%)	SD
1	0.00	12.5	11.56	92.47	1.27
		25.0	23.37	93.48	1.60
		50.0	48.76	97.52	2.86
2	25.10	12.5	37.53	99.43	4.58
		25.0	48.09	91.96	1.50
		50.0	72.30	94.40	1.09
3	268.12	12.5	279.65	92.25	2.18
		25.0	294.31	104.79	1.94
		50.0	317.59	98.95	4.87
4	9.46	12.5	22.80	106.76	2.22
		25.0	35.23	103.12	0.30
		50.0	61.42	103.93	3.49

recovery experiment was carried out. Accurate amounts of the four standards were added into a sample of *A. quinata*, which was quantified previously. The mixture was extracted and compounds using the above-established method. Each sample was analyzed in triplicate. For comparison, a blank sample (not spiked with standard compounds) was prepared and analyzed. The average percentage recoveries were evaluated by calculating the ratio of detected amount *versus* added amount. As shown in Table 3, the recovery rates were

Table 4. Robustness

		Analytes							
		1		**2**		**3**		**4**	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
Column	**Theoretical plate (*N*)**								
	Aligent	7465	1018	9716	1674	11319	626	34345	1868
	Xselect	9353	992	11371	695	13634	1684	51189	2574
	Shishedo	4510	635	4742	131	14958	119	28001	3039
	Capacity factor (*k'*)								
	Aligent	3.45	0.07	4.12	0.09	7.28	0.18	13.23	0.42
	Xselect	3.34	0.02	4.15	0.01	7.07	0.01	12.92	0.03
	Shishedo	3.12	0.08	3.72	0.10	7.04	0.21	11.80	0.48
	Separation factor (α)								
	Aligent	1.15	0.00	1.62	0.01	1.72	0.01	0.96	0.01
	Xselect	1.19	0.00	1.57	0.00	1.72	0.00	1.02	0.00
	Shishedo	1.15	0.00	1.70	0.01	1.59	0.02	0.98	0.00
	Resolution (*Rs*)								
	Aligent	3.21	0.24	12.15	0.45	19.21	0.23	2.06	0.34
	Xselect	4.38	0.08	12.40	0.24	23.49	0.82	1.40	0.03
	Shishedo	2.31	0.07	9.07	0.03	12.54	0.07	0.91	0.17
Flow rate (mL/min)	**Theoretical plate (*N*)**								
	0.8	6013	381	9741	887	11443	1931	40305	8570
	1.00	7465	1018	9716	1674	11319	626	34345	1868
	1.2	3997	451	6945	177	9294	138	21608	2204
	Capacity factor (*k'*)								
	0.8	3.62	0.11	4.32	0.11	7.59	0.20	13.33	0.45
	1.00	3.45	0.07	4.12	0.09	7.28	0.18	13.23	0.42
	1.2	4.64	0.13	5.57	0.14	10.37	0.29	19.26	0.61
	Separation factor (α)								
	0.8	1.15	0.00	1.61	0.00	1.67	0.02	0.97	0.01
	1.00	1.15	0.00	1.62	0.01	1.72	0.01	0.96	0.01
	1.2	1.17	0.00	1.73	0.01	1.78	0.01	0.89	0.01
	Resolution (*Rs*)								
	0.8	3.10	0.07	12.14	0.79	18.82	1.46	1.34	0.38
	1.00	3.21	0.24	12.15	0.45	19.21	0.23	2.06	0.34
	1.2	2.78	0.07	12.18	0.07	17.38	0.59	5.59	0.43

in the range 91.96-104.79%, and their RSD values were less than 5%.

Robustness. The robustness of the method was studied by introducing changes in column (*i.e.* Agilent Eclipse XDB-C18 (5.0 μm, 150 × 4.6 mm i.d.), Shisheido Capcell pak C18 (5.0 μm, 250 × 4.6 mm i.d.), Waters Xselect HSS C18 (5.0 μm, 250 × 4.6 mm i.d.), separation flow (*i.e.* 0.8 mL/min, 1.0 mL/min, 1.2 mL/min) (Table 4) and separation temperature (*i.e.* 25, 30, 35, and 40 °C).

Pattern Recognition Analysis. Hierarchical clustering analysis (HCA) is a multivariate analysis technique that is used to sort samples into groups. Owing to its unsupervised character, HCA is a pattern recognition technique that performed on autoscaled data, sample similarities were calculated on the basis of the squared Euclidean distance and the Ward hierarchical agglomerative method was used to establish clusters. The similarity or dissimilarity between samples is usually represented in a dendrogram for ease of interpretation. Each sample is similar to the others within a group but different from those in other groups with respect to a predetermined selection criterion.[16] In this study we used four marker compound contents [cuneataside D (**1**), 2-(3,4-dihydroxyphenyl)ethyl-*O*-*β*-D-glucopyranoside (**2**), 3-caffeoylquinic acid (**3**), and calceolarioside B (**4**)] for conducting pattern recognition analysis using software package IBM SPSS Statistics 19.0 to evaluate the phyto-chemical equivalency among fifty three samples [forty six *A. quinata* samples (AQ1-AQ41; AQ44-AQ48), two *C. armandii* samples (CA42, CA49), two *A. trifloliata* samples (AT50, AT51), two *A. manshuriensit* samples (AM52, AM53) and a sample of *T. papyriferus* (TP43)] (Figure 6).

Results and Discussion

Optimization of Extraction Method. To achieve a complete extraction of the components studied from *Akebia*

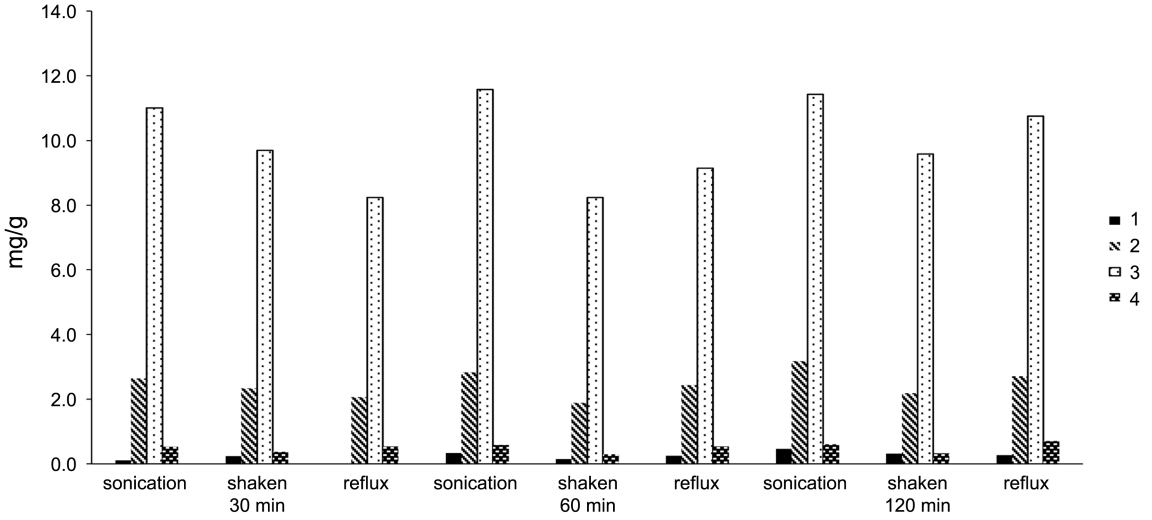

Figure 4. Effect of extraction time and method on the yields (mg/g) of marker compounds **1-4**.

quinata, four solvent systems including methanol, 70% methanol, ethanol, and 70% ethanol were used for the test. The extraction efficiencies of all the components from each solvent extraction systems were obtained and compared. The results indicated that for compound **1**, the methanol and 70% methanol solvent systems were more efficient than the ethanol and 70% ethanol solvent systems. For compounds **2** and **3**, 70% methanol was most efficient. In addition, the effects of the extraction time and methods on the extraction efficiency were investigated using three different methods, i.e. shaking, refluxing and sonication for 30, 60 and 120 min. The results showed that sonication for 60 min using 70% methanol was the preferred procedure (Figure 4). Plant materials contain a wide variety of different ballast compounds that can interfere with the analytical compounds and can damage the analytical column. Therefore, a series of concentration extracts of *A. quinata* were tested to determine a more appropriate level. A 20 μg/mL extract of *A. quinata* was obtained by sample preparation that showed good separation and prevented overlap of the peaks due to overload.

Optimization of HPLC Conditions. A HPLC method was developed to separate and quantify all the analyzed ingredients of *A. quinata.* The effects of the composition of the mobile phase on separation were tested to optimize the chromatographic condition. At the beginning, acetonitrile was used because of its lower viscosity. This helps reduce the back pressure and obtain results with a slightly better peak shape. Unfortunately, cuneataside D (**1**) and 2-(3,4-dihydroxyphenyl)ethyl-*O*-*β*-D-glucopyranoside (**2**) are polar. Therefore, it is important to reduce the percentage of organic components in the mobile phase to increase the compound's retention. With 2% acetonitrile, compounds **1** and **2** were eluted at 6.25 and 7.25 min, respectively, with just a 20 minute total runtime. Because a low percentage of organic solvent was used continuously, the de-wetting problem occurred. The chromatogram showed complete loss of retention because the low organic or pure aqueous mobile phase is expelled from the pores (dewetted) whereas the retentivity is a function of the surface area and ligand density. On the other hand, if the surface is non-wetted, then the effective chromatographic surface area is reduced > 95%. Therefore, the retentivity of the analyte was reduced. This means that the poor capture or otherwise is "hydrophobic collapse". For this reason, methanol was used instead. Equilibration of the column with 5% methanol allowed superior separation and the more economical use of methanol for routine analysis than acetonitrile. The retention time of compounds **1** and **2** was 10.49 and 12.06 min, respectively (Figure 2). Furthermore, initial screening experiments showed that the mobile phase needed to be acidic. As a result, methanol and 0.05% TFA aqueous were chosen as the eluting solvent system to achieve the desired separation and acceptable tailing factor within a running time of 50 min.

The robustness was determined to evaluate the reliability of established HPLC methods. The theoretical plate number (N), capacity factor (k'), separation factor (α), and resolution (Rs) were evaluated. To examine the suitability of three different columns, Eclipse XDB C18, Capcell pak C18 and Xselect HSS C18 columns with a particle size of 5 μm were used. The column temperature was controlled to 30 °C and the flow rate was kept at 1.0 mL/min. All these compounds showed a capacity for separation of the band center. Although the Water Xselect HSS C18 achieves a high resolution and good peak sharp as Agilent Eclipse XDB-C18, it takes longer time for elution. The Xselect HSS C18 column and Capcell pak C18 did not separate compound **4** and the internal standard at a 1.2 and 0.8 mL/min flow rate, respectively. In contrast, the Agilent Eclipse XDB-C18 column showed good resolution at all flow rates. With a flow rate of 1.0 mL/min, the Agilent column had the highest theoretical plate with good resolution of most analytes. The separation temperature was evaluated at 25, 30, 35, and 40 °C with regard to four analytical factors using the Agilent column. The retention time of the marker compounds, **1-4**, decreased with increasing separation temperature (Figure 5(a)). Figure

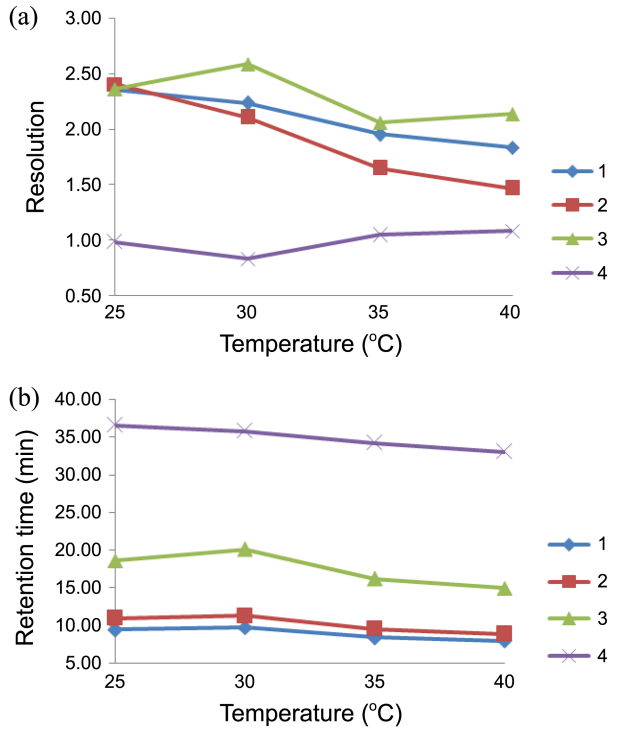

Figure 5. Effect of separation temperature on the retention time of four marker compounds (a) and on the resolution of the adjacent peaks (b).

5(b) showed the effects of the separation temperature on the resolution of the four marker compounds with their corresponding adjacent peaks. The compounds displayed good separation and resolution with adjacent peaks at all separation temperatures. The chromatographic parameters were optimized, but the four analytical factors did not differ significantly. Therefore, these experiment conditions were considered sufficiently robust.

According to the UV spectra of the marker compounds, **1-4**, in the range from 200 to 600 nm, 280 nm exhibited the highest sensitivity and was used to monitor the four phenolic compounds. The HPLC-UV chromatogram revealed the presence of four identical peaks (**1-4**) and internal standard (Figure 2(a)) in addition to four major peaks in the extract of *A. quinata* stems (Figure 2(b)). The separated components showed phenolic type UV spectra with two main absorption bands. For the first two peaks (corresponding to compounds **1** and **2**), two maxima were recorded at *ca.* 198 and 280 nm. For the other peaks, **3** and **4**, two bands were present at ca. 217 and 325 nm and 198 and 328 nm, respectively. The peaks of the four compounds were assigned by spiking the samples with the reference standards and a comparison of both UV spectra and retention times. The UV spectra paten is also good complement factor to identify compounds, particularly in compounds **1** and **4** in our experiments. These

Table 5. Contents (mg/g) of four phenolic compounds in forty six *A. quinata* samples (AQ1-AQ41; AQ44-AQ48), two *C. armandii* samples (CA42, CA49), two *A. trifloliata* samples (AT50, AT51), two *A. manshuriensit* samples (AM52, AM53) and a sample of *T. papyriferus* (TP43) (n = 3)

Sample	Contents (mg/g)											
	1			**2**			**3**			**4**		
	Mean	SD	RSD	Mean	SD	RSD	Mean	SD	RSD	Mean	SD	RSD
AQ01	0.190	0.008	4.25	1.433	0.044	3.04	3.151	0.014	0.45	0.000	0.000	0.00
AQ02	0.000	0.000	0.00	0.000	0.000	0.00	1.508	0.038	2.55	0.000	0.038	2.55
AQ03	0.140	0.007	5.01	2.485	0.066	2.67	6.377	0.094	1.47	0.126	0.002	1.66
AQ04	0.126	0.003	2.47	1.290	0.090	6.98	2.373	0.113	4.77	0.028	0.002	7.28
AQ05	0.000	0.000	0.00	3.530	0.046	1.30	5.048	0.048	0.95	0.072	0.001	1.05
AQ06	0.000	0.000	0.00	1.173	0.011	0.91	2.774	0.012	0.44	0.000	0.000	0.00
AQ07	0.505	0.007	1.39	1.645	0.060	3.64	3.614	0.021	0.58	0.082	0.005	5.96
AQ08	0.000	0.000	0.00	2.531	0.059	2.35	4.817	0.074	1.54	0.070	0.075	1.10
AQ09	0.162	0.006	3.16	1.709	0.024	1.42	3.987	0.015	0.38	0.220	0.016	7.27
AQ10	0.000	0.000	0.00	2.040	0.132	6.45	3.337	0.063	1.90	0.185	0.003	1.60
AQ11	0.021	0.001	3.60	0.417	0.020	4.87	2.138	0.041	1.95	0.000	0.000	0.00
AQ12	0.183	0.007	5.31	2.609	0.012	0.48	4.628	0.073	1.58	0.170	0.002	1.24
AQ13	0.113	0.005	4.23	3.082	0.030	0.97	7.346	0.035	0.48	0.000	0.000	0.00
AQ14	0.204	0.011	4.55	2.324	0.058	2.50	5.139	0.094	1.82	0.069	0.003	4.97
AQ15	0.156	0.040	8.40	0.969	0.081	8.39	2.761	0.016	0.57	0.010	0.001	5.95
AQ16	0.172	0.005	2.64	0.624	0.005	0.62	2.794	0.043	1.26	0.000	0.000	0.00
AQ17	0.240	0.026	4.82	0.622	0.039	6.30	2.875	0.261	9.09	0.077	0.005	6.32
AQ18	0.128	0.004	3.34	1.133	0.035	3.10	2.397	0.034	1.41	0.034	0.002	5.68
AQ19	0.126	0.010	8.18	1.780	0.064	3.60	3.080	0.029	0.93	0.031	0.001	2.08
AQ20	0.000	0.000	0.00	2.496	0.119	7.95	4.162	0.307	7.37	0.827	0.083	10.05
AQ21	0.245	0.001	6.59	0.114	0.006	5.97	2.989	0.049	1.63	0.000	0.000	0.00
AQ22	0.104	0.011	9.99	1.380	0.107	7.75	3.202	0.057	1.77	0.086	0.006	6.40
AQ23	0.192	0.017	8.87	1.857	0.110	5.93	4.481	0.143	3.20	0.000	0.000	0.00

Table 5. Continued

Sample	Contents (mg/g)											
	1			**2**			**3**			**4**		
	Mean	SD	RSD	Mean	SD	RSD	Mean	SD	RSD	Mean	SD	RSD
AQ24	0.260	0.009	3.11	0.500	0.025	5.09	4.153	0.015	0.35	0.000	0.000	0.00
AQ25	0.000	0.000	0.00	2.018	0.006	3.29	3.463	0.043	1.23	0.015	0.000	2.25
AQ26	0.135	0.020	3.84	1.549	0.031	1.97	2.966	0.054	1.83	0.000	0.000	0.00
AQ27	0.000	0.016	2.97	0.523	0.017	3.49	3.247	0.090	2.78	0.000	0.000	0.00
AQ28	0.118	0.004	3.38	2.305	0.069	3.00	4.447	0.169	3.79	0.050	0.005	10.11
AQ29	0.000	0.000	0.00	1.328	0.029	2.17	2.455	0.042	1.69	0.014	0.001	9.57
AQ30	0.000	0.000	0.00	2.141	0.045	2.09	3.314	0.016	0.49	0.010	0.001	8.88
AQ31	0.000	0.000	0.00	2.153	0.021	0.99	3.240	0.030	0.92	0.023	0.001	4.53
AQ32	0.000	0.000	0.00	1.501	0.008	0.55	4.603	0.011	0.24	0.000	0.000	0.00
AQ33	0.134	0.003	2.49	1.217	0.041	3.34	3.469	0.069	1.98	0.000	0.000	0.00
AQ34	0.155	0.006	9.74	1.479	0.047	3.36	2.691	0.237	8.79	0.061	0.007	10.24
AQ35	0.000	0.000	0.00	3.179	0.038	1.18	4.259	0.012	0.28	0.000	0.000	0.00
AQ36	0.000	0.000	0.00	0.634	0.018	2.85	4.044	0.053	1.32	0.357	0.014	4.05
AQ37	0.000	0.000	0.00	0.947	0.050	5.29	2.108	0.028	1.31	0.023	0.002	8.92
AQ38	0.000	0.000	0.00	2.700	0.048	1.79	3.718	0.003	0.08	0.088	0.004	4.24
AQ39	0.000	0.000	0.00	2.425	0.067	2.78	3.910	0.073	1.86	0.073	0.006	8.69
AQ40	0.100	0.002	2.29	0.226	0.020	8.67	1.587	0.024	1.53	0.087	0.002	2.25
AQ41	0.120	0.002	8.79	1.021	0.065	6.35	1.900	0.044	2.32	0.066	0.005	8.16
CA42	0.000	0.000	0.00	0.000	0.000	0.00	0.000	0.000	0.00	0.000	0.000	0.00
TP43	0.000	0.000	0.00	0.000	0.000	0.00	0.920	0.028	2.80	2.448	0.226	9.23
AQ44	0.000	0.000	0.00	0.508	0.017	3.37	4.055	0.343	8.25	0.066	0.006	9.82
AQ45	0.000	0.000	0.00	4.223	0.088	2.08	5.081	0.107	2.11	0.077	0.005	6.46
AQ46	0.000	0.000	0.00	2.698	0.104	3.87	5.611	0.170	3.02	0.758	0.008	1.11
AQ47	0.000	0.000	0.00	3.128	0.180	5.74	4.801	0.380	7.91	0.000	0.000	0.00
AQ48	0.000	0.012	8.96	2.629	0.135	5.15	3.859	0.201	5.20	0.000	0.000	0.00
CA49	0.000	0.000	0.00	0.000	0.000	0.00	0.000	0.000	0.00	0.000	0.000	0.00
AT50	0.000	0.000	0.00	0.000	0.000	0.00	0.854	0.085	9.29	0.654	0.065	9.97
AT51	0.000	0.000	0.00	0.000	0.000	0.00	0.180	0.013	7.34	0.320	0.021	6.68
AM52	0.000	0.000	0.00	0.000	0.000	0.00	0.000	0.000	0.00	0.573	0.015	2.64
AM53	0.000	0.000	0.00	0.000	0.000	0.00	0.000	0.000	0.00	0.855	0.026	3.01

compounds are found in small amounts and are difficult to recognize them among closely eluting compounds.

Sample Analysis. The established analytical method was then used to quantitatively analyze four marker compounds **1-4** in 46 samples of *A. quinata* (AQ1-AQ41; AQ44-AQ48) and 7 comparative samples including 2 samples of the same genus [*A. trifloliata* samples (AT50, AT51)] and 5 different genus samples [one *T. papyriferus* (TP43), two *A. manshuriensis* (AM52, AM53) and two *C. armandii* (CA42, CA49)] using the regression equation described above. Each sample was analyzed in triplicate to ensure reproducibility of the quantitative result. Table 5 lists their contents. The data revealed the highly variable contents of the four marker compounds in samples. In 46 samples of *A. quinata,* the contents of compound **3** ranged from 1.66 to 5.64 mg/g. This was found to be the most abundant component in most samples, whereas compounds **1** and **4** were found in small amounts. In addition, the content of compound **2**, ranging from 0.72 to 2.68 mg/g was also high in large samples.

In the comparative group, none of the four marker compounds were detected in *C. armandii*, whereas compound **4** was detected in the *A. manshuriensis* samples. Two samples of *A. trifloliata* and one sample of *T. papyriferus* contained only compounds **3** and **4**. Compound **2** was observed in all *A. quinata* samples but was not detected in the comparative samples. This discrepancy induced discrimination between *A. quinata* and comparative herbs.

Pattern Recognition Analysis. A hierarchical clustering analysis of 53 samples by using IBM SPSS Statistics 19.0 software was performed (Figure 6). The original data for HCA were exported from contents of the four marker compounds in samples (Table 5). As is shown in Fig. 6, the samples could be clustered into two groups, A (*A. quinata*) and B (comparative samples). There were two main clusters corresponding to different origins of samples in dendrogram, while the subsets gave more information on the samples of different batches. Therefore, pattern-recognition analysis can provide more comprehensive information for the chemical equivalency that can identify *Akebia* species and discriminate *A. quinata* from the other plants.

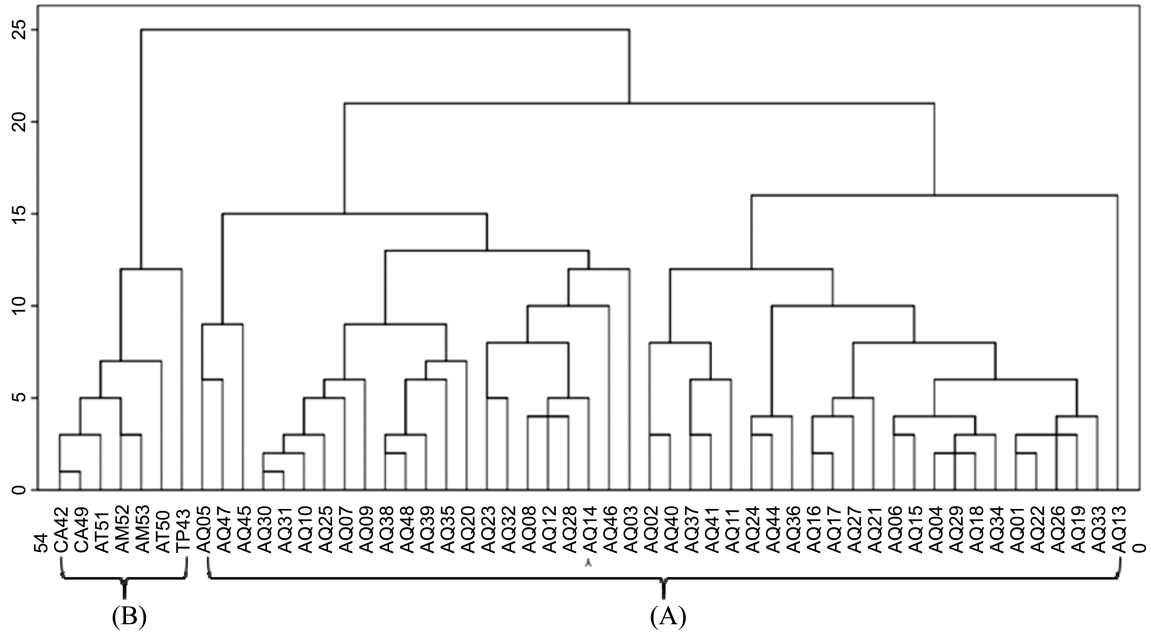

Figure 6. Pattern analysis of 53 samples. (A) *A. quinata* (AQ1-AQ41; AQ44-AQ48), (B) [*C. armandii* (CA42, CA49), *A. trifloliata* (AT50, AT51), *A. manshuriensit* (AM52, AM53) and *T. papyriferus* (TP43)].

Conclusion

In this study, qualitative and quantitative analyses of the phenolic substances of *A. quinata* were performed including optimization of the extraction method and the experiment for validation along with the pattern-recognition method. Based on these results, this HPLC method was proven to be a simple, accurate and reasonable analytical method for the simultaneous quantification of the four phenolic compounds in the *A. quinata*. The developed assay was applied successfully to quantify the phenolic compounds in 46 batches of the *A. quinata* collected from different locations in Korea and China. The variation in the contents of the main compounds affects the quality, stability and therapeutic effects of this medicinal herb. Therefore, the simultaneous determination of the major-components can play an important role in a quality evaluation, used part, and on guidance for the good crude drug source of *A. quinata*. These results confirm that compound **2** can serve as a marker compound to distinguish *A. quinata* and some plants prescribed as the source plants of *Akebia* plants *e.g. A. manshuriensis*, Aristolochiaceae, and *Clematis* spp., Ranunculaceae. Furthermore, the combination of quantitative analysis and pattern-recognition analysis was found to be a more comprehensive technique than quantitative analysis alone for quality control and species identification of the *Akebia quinata* herbal drug.

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