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Simultaneous determination of chromones and coumarins in Radix Saposhnikoviae by high performance liquid chromatography with diode array and tandem mass detectors

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ABSTRACT

Methods using high performance liquid chromatography with diode array detection (HPLC-DAD) and tandem mass spectrometry (HPLC–MS/MS) were developed and validated for the simultaneous determination of 5 chromones and 6 coumarins: prim-*O*-glucosylcimifugin (1), cimifugin (2), nodakenin (3), 4′-*O*-β-D-glucosyl-5-*O*-methylvisamminol (4), sec-*O*-glucosylhamaudol (5), psoralen (6), bergapten (7), imperatorin (8), phellopterin (9), 3′-*O*-angeloylhamaudol (10) and anomalin (11), in Radix Saposhnikoviae. The separation conditions for HPLC-DAD were optimized using an Ascentis Express C18 (4.6 mm × 100 mm, 2.7 μm particle size) fused-core column. The mobile phase was composed of 10% aqueous acetonitrile (A) and 90% acetonitrile (B) and the elution was performed under a gradient mode at a flow rate of 1.0 mL/min. The detection wavelength was set at 300 nm. The HPLC-DAD method yielded a base line separation of the 11 components in 50% methanol extract of Radix Saposhnikoviae with no interfering peaks detected. The HPLC-DAD method was validated in terms of linearity, accuracy and precision (intra- and inter-day), limit of quantification (LOQ), recovery, and robustness. Specific determination of the 11 components was also accomplished by a triple quadrupole tandem mass spectrometer equipped with an electrospray ionization (ESI) source. This HPLC–MS/MS method was also validated by determining the linearity, limit of quantification, accuracy, and precision. Quantification of the 11 components in 51 commercial Radix Saposhnikoviae samples was successfully performed using the developed HPLC-DAD method. The identity, batch-to-batch consistency, and authenticity of Radix Saposhnikoviae were successfully monitored by the proposed HPLC-DAD and HPLC–MS/MS methods.

1. Introduction

Radix Saposhnikoviae, the dried root of *Saposhnikovia divaricata* (Turcz.) Schischk. (Umbelliferae), is a popular traditional herbal medicine that has been used for thousands of years in China, Japan, and Korea [1,2]. It has been widely applied for the treatment of pyrexia, rheumatism, headache, vertigo, generalized aching, and arthralgia in traditional medicine [3]. It is also used in prescriptions for the treatment of generalized aches, inflammatory symptoms, and cardiovascular diseases [4].

Despite its long historical usage in traditional medicine, relatively little scientific research has been conducted on this herbal medicine. Several pharmacological studies have provided evidence of various activities, including analgesic, anti-oxidant, anti-inflammatory, anti-proliferative, and anti-cancer effects [5–9]. Phytochemical studies showed that Radix Saposhnikoviae contains many types of compounds, including chromones, coumarins, and polyacetylenes. Of these molecules, chromones are considered to be the bioactive constituents most relevant to pharmacological efficacy, mediating such activities as anti-clotting and immune regulation [2,4,10–15]. However, other minor constituents, such as coumarins, could also contribute to the overall therapeutic effects [1,9].

Two chromones, prim-*O*-glucosylcimifugin and 4′-*O*-β-D-glucosyl-5-*O*-methylvisamminol, were selected as marker compounds for quality control of Radix Saposhnikoviae in the Chinese Pharmacopoeia [16], while specific marker compounds were not described in the Japanese and Korean Pharmacopoeia [17,18]. Since

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Radix Saposhnikoviae has been rarely available in Korea, Radix Glehniae (*Glehnia littoralis* F. Schmidt ex Miq.; Umbelliferae) and Radix Peucedani (*Peucedanum japonicum* Thunb.; Umbelliferae) have been wrongly used for the same purposes, although these substitutes have different phytochemical profiles and probably different therapeutic potential [2]. In addition, some of the bioactive components in Radix Saposhnikoviae, e.g. coumarins, are also found in Radix Glehniae and Radix Peucedani [13,19–21]. Therefore, the simultaneous quantification of chromones and coumarins would be helpful for the discrimination of Radix Saposhnikoviae from its substitutes.

Only a few analytical methods have been developed for the quantitative determination of bioactive constituents in Radix Saposhnikoviae. These analytical procedures include the determination of panaxynol by gas chromatography–mass spectrometry (GC–MS) [22], the simultaneous determination of two chromones by high performance liquid chromatography–ultraviolet detection (HPLC-UV) [23,24] and four chromones by high performance liquid chromatography-electrospray-mass spectrometry (HPLC-ESI-MS) [25]. It is generally accepted that the therapeutic efficacy of herbal medicines is based on the synergistic effects of their multiple constituents [26,27]. Accordingly, these methods may be insufficient to fulfill the requirements for the quality control of Radix Saposhnikoviae, as other constituents in Radix Saposhnikoviae may contribute to its overall effectiveness. Therefore, a comprehensive qualification method based on the simultaneous determination of both major and minor constituents is required in order to evaluate variation among samples of Radix Saposhnikoviae, for identification, for control of batch-to-batch consistency, and for authenticity. However, to the best of our knowledge, no such analytical methods have been developed for the simultaneous quantification of multi-constituents in Radix Saposhnikoviae.

In the present study, sensitive and reliable quantification methods were developed and validated for the simultaneous determination of five chromones (prim-*O*-glucosylcimifugin, 4′-*O*-β-D-glucosyl-5-*O*-methylvisamminol, 3′-*O*-angeloylhamaudol, sec-*O*-glucosylhamaudol and cimifugin) and six coumarins (nodakenin, psoralen, bergapten, imperatorin, phellopterin and anomalin) in Radix Saposhnikoviae using HPLC-DAD and HPLC–MS/MS. In addition, our HPLC-DAD method was successfully applied to quantify multi-constituent levels in 51 batches of Radix Saposhnikoviae obtained from Chinese and Korean herbal markets.

2. Experimental

2.1. Chemicals and materials

Psoralen and bergapten were obtained from Sigma–Aldrich (St. Louis, MO, USA). Nodakenin and cimifugin were supplied by Wako (Osaka, Japan) and Shanghai Tauto Biotech (Pudong, Shanghai, China), respectively. Anomalin, sec-*O*-glucosylhamaudol, prim-*O*-glucosylcimifugin, 4′-*O*-β-D-glucosyl-5-*O*-methylvisamminol, imperatorin, phellopterin, 3′-*O*-angeloylhamaudol, and an internal standard, byakangelicol, were kindly provided by the College of Pharmacy, Seoul National University.

Fifty-one batches of Radix Saposhnikoviae were collected from different geographic regions, four from Korea and 47 from China (eight provinces: Inner Mongolia, Hebei, Northeast, Hunan, Guangxi, Anhui, Gansu, and Yunnan). Radix Glehniae and Radix Peucedani were also collected from local herbal medicine markets in Korea. Organoleptic authentication of Radix Saposhnikoviae, Radix Glehniae, and Radix Peucedani was performed by Professor Jae Hyun Lee (College of Oriental Medicine, Dongguk University, Kyungju, South Korea).

HPLC-grade acetonitrile, methanol, and water were obtained from Fisher Scientific (Fair Lawn, NJ, USA). HPLC-grade acetic acid and formic acid were purchased from Sigma (St. Louis, MO, USA). Nylon membrane filters (0.2 μm) were obtained from Whatman (Maidstone, UK).

2.2. Instrumentation

The HPLC-DAD method was performed using a Waters 1525 pump (Waters, Milford, MA, USA), a Waters 996 photodiode array (PDA) detector, a Waters temperature control module column oven, and a Waters 717 plus auto-sampler. Analytes were chromatographed on a number of column types including Ascentis Express C18 (4.6 mm × 100 mm, 2.7 μm, Supelco, Bellefonte, PA, USA), Hypersil GOLD C18 (4.6 mm × 150 mm, 5 μm, Thermo Scientific, Waltham, MA, USA), Luna 5 μ C18 (4.6 mm × 150 mm, 5 μm, Phenomenex, Torrance, CA, USA), Luna phenyl-hexyl (4.6 mm × 150 mm, 5 μm, Phenomenex, Torrance, CA, USA), and Allure Biphenyl (4.6 mm × 250 mm, 5 μm, Restek, Bellefonte, PA, USA) columns. The data analysis was performed using Empower software (Waters, version 5.00.00.00).

HPLC–MS/MS analysis was conducted on a Waters Quattro Micro API tandem mass spectrometer (Milford, MA, USA) equipped with an ESI source and a Waters Alliance 2795 HPLC system that consisted of a quaternary pump, an auto-sampler, a degasser, and an automatic thermostatic column oven. The samples were separated on Hypersil GOLD C18 (2.1 mm × 150 mm, 5 μm, Thermo Scientific, Waltham, MA, USA), Atlantis dC18 (2.1 mm × 150 mm, 3 μm, Waters, Milford, MA, USA) and Kinetex C18 (2.1 mm × 100 mm, 2.6 μm, Phenomenex, Torrance, CA, USA) columns. MassLynx software (Waters, version 4.1) was used for the instrument control and data analysis.

2.3. Preparation of standard solutions

Stock solutions of the standards and internal standard (byakangelicol) were prepared at a concentration of 5 mg/mL in methanol, filtered through 0.2 μm nylon membrane filters, and stored at −70 °C until use.

Standard working solutions for HPLC-DAD were prepared by mixing stock solutions and then diluting in 50% methanol to obtain concentration ranges of 1–500 μg/mL (1, 2, 10, 100, 250, and 500 μg/mL) for prim-*O*-glucosylcimifugin and 4′-*O*-β-D-glucosyl-5-*O*-methylvisamminol, 0.5–250 μg/mL (0.5, 1, 5, 50, 125, and 250 μg/mL) for cimifugin, psoralen, bergapten, imperatorin, and phellopterin, 1–100 μg/mL (1, 2, 10, 20, 50, and 100 μg/mL) for nodakenin and sec-*O*-glucosylhamaudol, 1–50 μg/mL (1, 2, 5, 10, 25, and 50 μg/mL) for 3′-*O*-angeloylhamaudol, and 10–500 μg/mL (10, 20, 50, 100, 250, and 500 μg/mL) for anomalin. A stock solution of the internal standard was diluted with 50% methanol to a final concentration of 500 μg/mL. All working solutions were stored at 4 °C until use.

Standard working solutions for HPLC–MS/MS were prepared by mixing stock solutions and then diluted in 50% methanol to obtain concentration ranges of 10–1000 ng/mL (10, 20, 100, 200, 500, and 1000 ng/mL) for prim-*O*-glucosylcimifugin, 10–500 ng/mL (10, 20, 50, 100, 250, and 500 ng/mL) for 4′-*O*-β-D-glucosyl-5-*O*-methylvisamminol and cimifugin, 5–500 ng/mL (5, 10, 50, 100, 250, and 500 ng/mL) for sec-*O*-glucosylhamaudol, 2–100 ng/mL (2, 4, 10, 20, 50, and 100 ng/mL) for psoralen and phellopterin, 1–100 ng/mL (1, 2, 10, 20, 50, and 100 ng/mL) for imperatorin and 3′-*O*-angeloylhamaudol, 40–1000 ng/mL (40, 80, 100, 200, 500, and 1000 ng/mL) for nodakenin, 2.5–250 ng/mL (2.5, 5, 25, 50, 125, and 250 ng/mL) for bergapten, and 15–750 ng/mL (15, 30, 75, 150, 375, and 750 ng/mL) for anomalin. All working solutions were stored at 4 °C until use.

2.4. Preparation of sample solutions

The dried roots were pulverized by a grinder, and sieved through a No. 70 mesh (<380 μm). Two hundred fifty milligrams of the powder were extracted with 0.5 mL of internal standard solution and 4.5 mL of 50% methanol in an ultrasonic bath at ambient temperature for 60 min. The resultant solution was filtered through a 0.2 μm membrane filter before injection into the HPLC-DAD system for analysis. An aliquot of these sample solutions were 1000-fold diluted with 50% methanol, and then used for the HPLC–MS/MS analysis. The final concentrations of byakangelicol (IS) in sample solutions for the HPLC-DAD and HPLC–MS/MS analysis were 50 μg/mL and 50 ng/mL, respectively.

2.5. Operation conditions for HPLC-DAD

The chromatographic analysis was performed on a Supelco Ascentis Express C18 (4.6 mm × 100 mm, 2.7 μm) column. The binary mobile phase, which was filtered through a 0.2 μm membrane filter, consisted of 10% (v/v) aqueous acetonitrile as solvent A and 90% (v/v) acetonitrile as solvent B. A flow rate of 1 mL/min was used for the sample analysis with the gradient program as follows: 0–20% solvent B at 0–25 min; 20–40% solvent B at 25–35 min; 40–55% solvent B at 35–40 min; 55% solvent B at 40–45 min; 55–80% solvent B at 45–65 min; 0% solvent B at 65–80 min (equilibration time). The column temperature was maintained at 25 °C and the injection volume was 10 μL. The wavelength of the PDA detector was set at 300 nm and the on-line UV spectra were acquired in the range of 200–400 nm.

2.6. Operation conditions for HPLC–MS/MS

The chromatographic separation was achieved on a Hypersil GOLD C18 (2.1 mm × 150 mm, 5 μm) at a column temperature of 30 °C. The mobile phase, which was filtered through a 0.2 μm membrane filter, consisted of 0.1% (v/v) aqueous acetic acid (solvent A) and acetonitrile (solvent B). The flow rate was 200 μL/min. The gradient program was applied as follows: 15–30% B at 0–5 min, 30–50% B at 5–7 min, 50–90% B at 7–13 min, 90% B at 13–15 min and 0% B at 15–20 min (equilibration time). The injection volume was 10 μL and the temperature of the auto-sampler was kept at 4 °C.

The detection was performed with triple quadrupole tandem mass spectrometry using multiple-reaction monitoring (MRM). The instrument was operated with electrospray in positive ion mode to produce $[M+H]^+$ and $[M+Na]^+$ ions. Nitrogen gas was used as a nebulization and desolvation gas and argon gas was used as a collision gas. The conditions of the electrospray ionization source were optimized as follows: desolvation temperature, 400 °C; source temperature, 100 °C, desolvation gas flow, 600 L/h; cone gas flow, 50 L/h. MS/MS parameters were set as follows: capillary voltage at 3.5 kV; extractor at 3.0 V; RF lens at 0.0 V; collision cell entrance potential at −2.0 V; collision cell exit potential at 1.0 V; multiplier at 650 V.

2.7. Validation of the HPLC-DAD and HPLC–MS/MS methods

The HPLC-DAD method and HPLC–MS/MS method were validated for linearity, intra-day and inter-day precision, accuracy, and limit of quantification (LOQ). Calibration curves for HPLC-DAD were constructed with six different concentration levels from the following concentration ranges; 1–500 μg/mL for prim-*O*-glucosylcimifugin and 4′-*O*-β-D-glucosyl-5-*O*-methylvisamminol, 0.5–250 μg/mL for cimifugin, psoralen, bergapten, imperatorin, and phellopterin, 1–100 μg/mL for nodakenin and sec-*O*-glucosylhamaudol, 1–50 μg/mL for 3′-*O*-angeloylhamaudol, and 10–500 μg/mL for anomalin.

Calibration curves for HPLC–MS/MS were constructed with six different concentration levels from the following concentration ranges; 10–1000 ng/mL for prim-*O*-glucosylcimifugin, 10–500 ng/mL for 4′-*O*-β-D-glucosyl-5-*O*-methylvisamminol and cimifugin, 5–500 ng/mL for sec-*O*-glucosylhamaudol, 2–100 ng/mL for psoralen and phellopterin, 1–100 ng/mL for imperatorin and 3′-*O*-angeloylhamaudol, 40–1000 ng/mL for nodakenin, 2.5–250 ng/mL for bergapten, and 15–750 ng/mL for anomalin. Calibration curves were composed by plotting the peak area ratios of analyte to internal standard against the analyte concentrations.

The intra- and inter-day precision (CV) and accuracy (%) were estimated by analyzing five replicates at four different concentrations all within one day or over five days. The limit of quantification (LOQ) was accepted in which the precision was less than 20% and the accuracy was between 80 and 120% for both intra- and inter-day assays with a signal-to-noise (S/N) ratio greater than 10.

Recovery was calculated by analyzing the peak areas of plant extract with standard added and peak areas of plant extract alone, with peak areas of standard used as true concentrations. Three replicates were measured at three different concentration levels to determine the recovery.

Robustness was determined by varying the experimental conditions including column temperature (25 ± 2.5 °C), flow rate (1 ± 0.1 mL/min), and column vendor. The analysis was performed in triplicate and the parameters, including peak area precision (CV), relative retention time (RRT), theoretical plate number (N), and peak-tailing factor (T), were compared to determine the robustness of the assay.

3. Results and discussion

3.1. Optimization of sample extraction conditions

Four variables were investigated in order to identify the optimal extraction conditions: extraction method, extraction solvent, solvent composition, and extraction time. For comparisons of extraction efficiency, the six major bioactive components in Radix Saposhnikoviae, prim-*O*-glucosylcimifugin, 4′-*O*-β-D-glucosyl-5-*O*-methylvisamminol, sec-*O*-glucosylhamaudol, cimifugin, 3′-*O*-angeloylhamaudol, and anomalin, were quantified using the HPLC-DAD method described above. Deionized water, methanol, and ethanol were tested as the extraction solvents, and sonication, reflux, and maceration at room temperature were the extraction methods investigated. The results indicate that sonication with 50% methanol allowed for better extraction than was achieved with other solvents and methods. To determine the optimal extraction time, 250 mg aliquots of pulverized samples were extracted with 5 mL of 50% methanol by sonication for 10 min through 70 min. Finally, sonication with 0.5 mL of 50% methanol internal standard solution and 4.5 mL of 50% methanol at room temperature for 60 min were selected as the conditions used to prepare the sample solution for HPLC analysis.

3.2. HPLC-DAD analysis

3.2.1. Optimization of chromatographic separation conditions

Since chromones and coumarins have similar structures and physicochemical properties, good resolution of the two types of compounds is a prerequisite for the simultaneous quantification of the 11 targeted compounds in Radix Saposhnikoviae. Different types of chromatographic columns were tested in an effort to optimize the separation of the compounds found in Radix Saposhnikoviae, using mixtures of standards of the 11

bioactive components. When the peak tailing factors and peak heights among four different columns were compared, the Supelco Ascentis Express C18 column packed with thin porous shell particles of high-purity solid silica core showed sharper peaks and less peak tailing than the other columns and allowed for the detection of the 11 compounds at lower concentrations (Supplementary Table S1 and Fig. S1). Thus, the Supelco Ascentis Express C18 column was finally selected for the HPLC-DAD analysis.

Various compositions of mobile phase were investigated with Radix Saposhnikoviae extract. As 10% (v/v) aqueous acetonitrile and 90% (v/v) acetonitrile gradient elution provided good resolu-

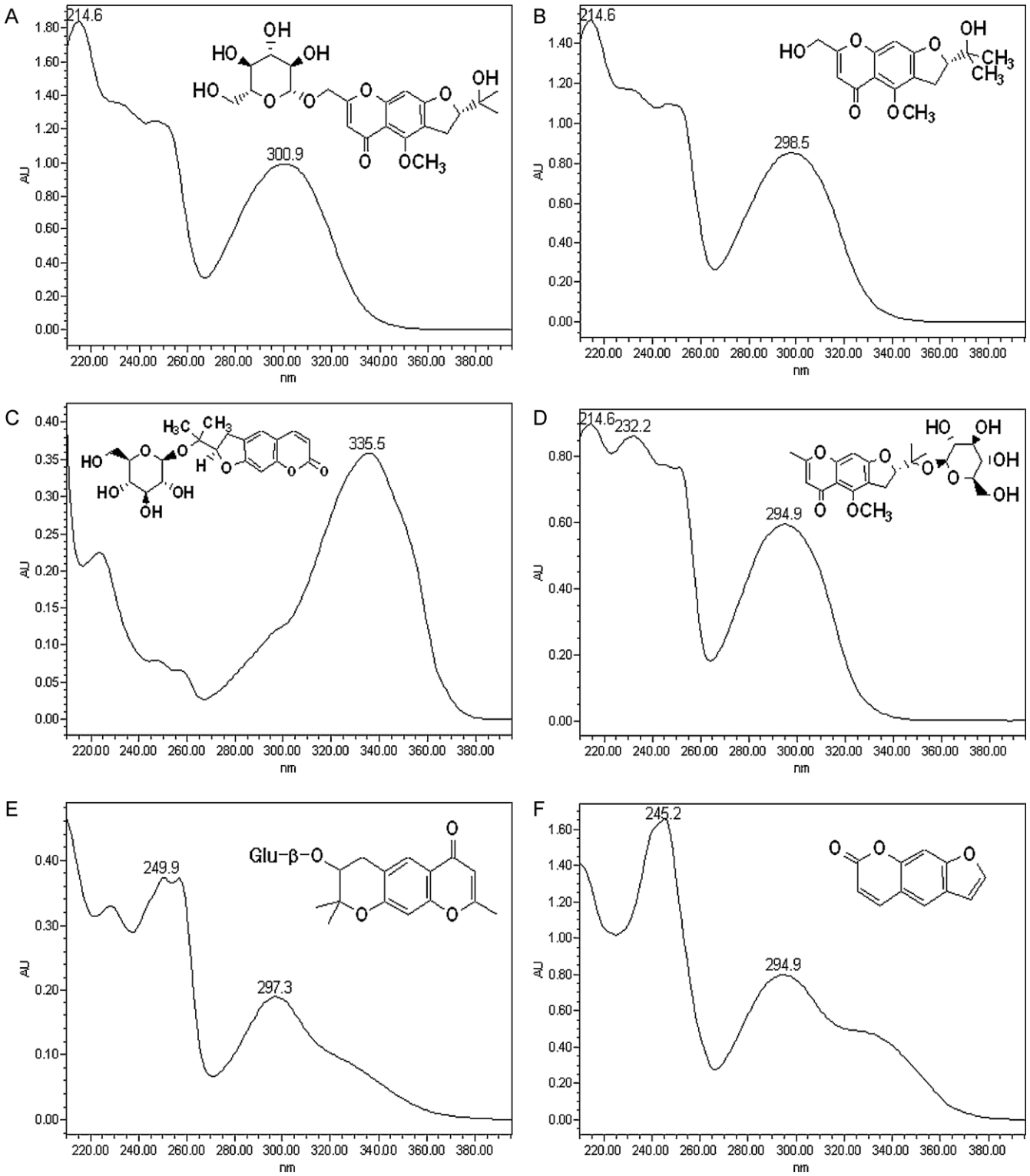

Fig. 1. HPLC-DAD spectra of Radix Saposhnikoviae. (A) prim-*O*-glucosylcimifugin, (B) cimifugin, (C) nodakenin, (D) 4′-*O*-β-D-glucosyl-5-*O*-methylvisamminol, (E) sec-*O*-glucosylhamaudol, (F) psoralen, (G) bergapten, (H) imperatorin, (I) phellopterin, (J) 3′-*O*-angeloylhamaudol, (K) anomalin, and (L) byakangelicol (internal standard, 50 μg/mL).

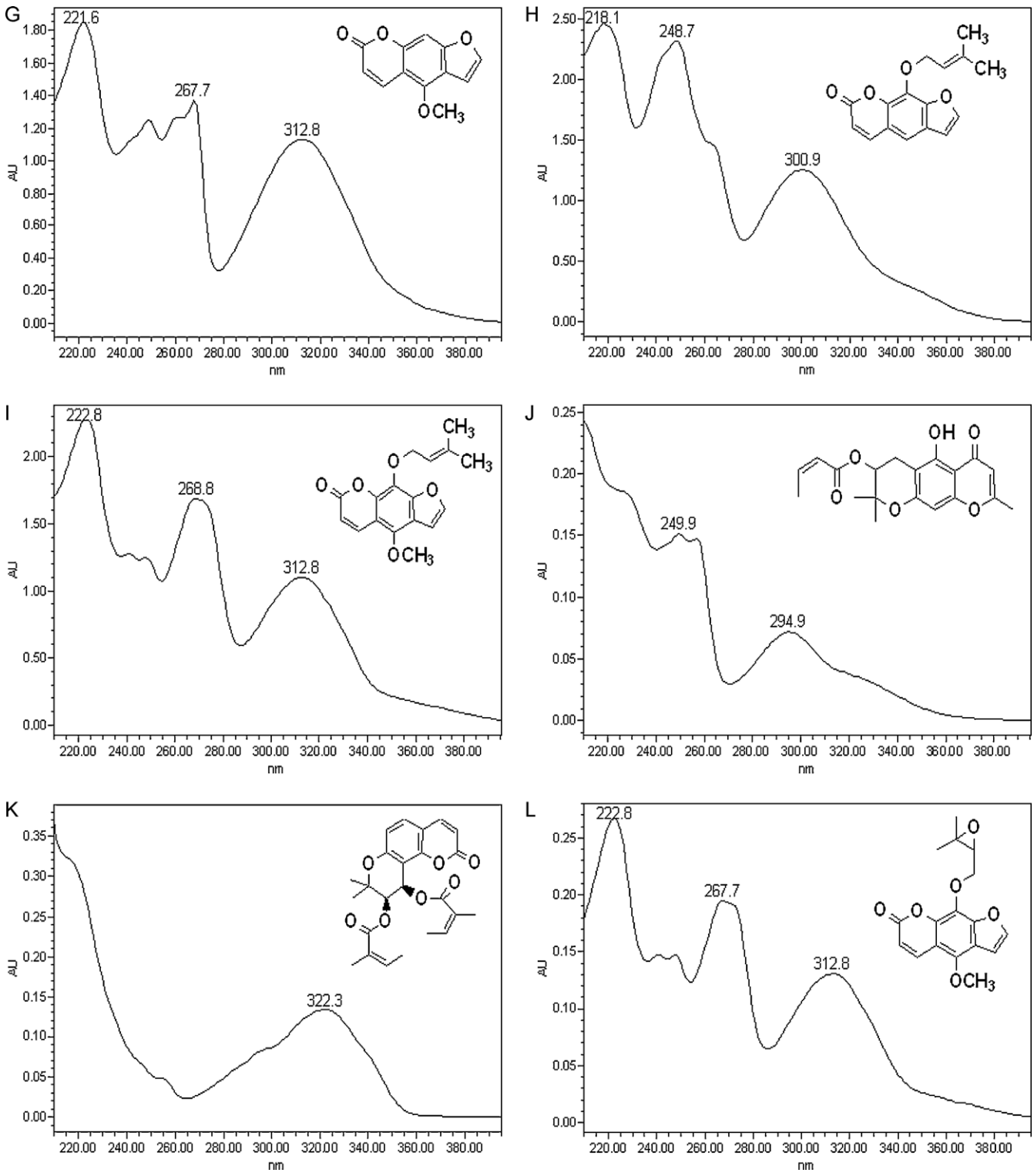

Fig. 1. *Continued.*

tion, mobile phase modifiers, such as acetic acid and formic acid, were added to test their impacts on separation. However, the use of mobile phase with additive led to advent of unresolved or newly emerged peaks (Supplementary Fig. S2). Consequently, 10% aqueous acetonitrile and 90% acetonitrile with no acid modifier were chosen to produce the desired separation and favorable peak tailing factor.

The column temperature was also optimized to achieve better separation from potential interfering molecules found in Radix Saposhnikoviae extract. When four different temperatures, 25 °C, 30 °C, 35 °C, and 40 °C, were compared, baseline separation of the 11 markers was achieved at 25 °C, but the separation of some target analytes was unsatisfactory at elevated temperatures up to 40 °C (Supplementary Fig. S3). Accordingly, 25 °C was

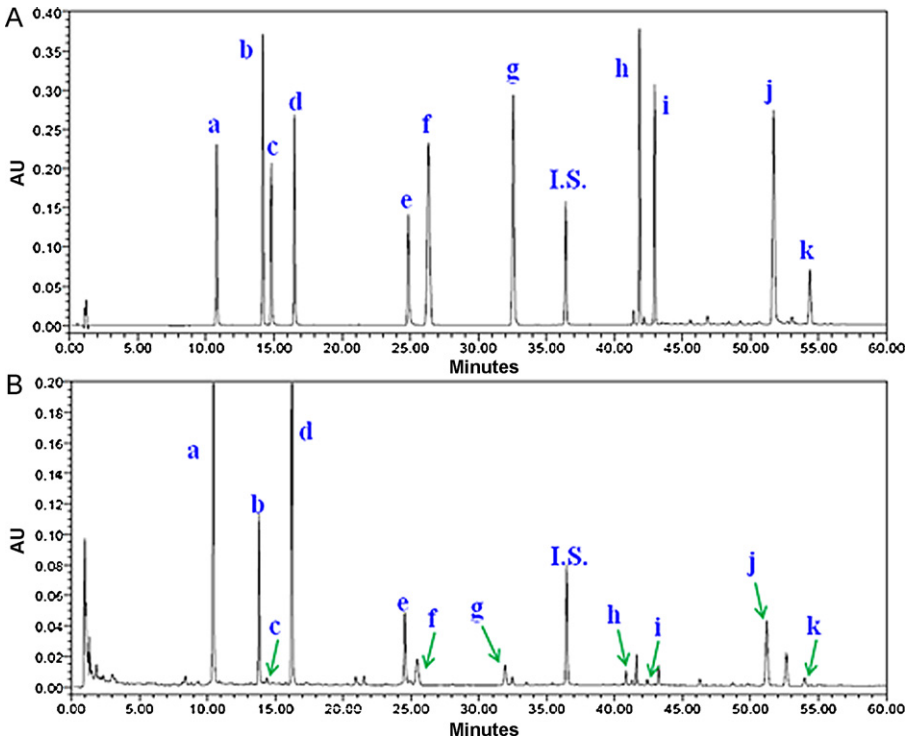

Fig. 2. HPLC-DAD chromatograms of (A) standard solution and (B) 50% methanol extract of Radix Saposhnikoviae. prim-*O*-glucosylcimifugin (a, 100 μg/mL), cimifugin (b, 50 μg/mL), nodakenin (c, 20 μg/mL), 4′-*O*-β-D-glucosyl-5-*O*-methylvisamminol (d, 100 μg/mL), sec-*O*-glucosylhamaudol (e, 20 μg/mL), psoralen (f, 50 μg/mL), bergapten (g, 50 μg/mL), imperatorin (h, 50 μg/mL), phellopterin (i, 50 μg/mL), 3′-*O*-angeloylhamaudol (j, 10 μg/mL), anomalin (k, 100 μg/mL), and byakangelicol (internal standard, 50 μg/mL).

selected as the optimal column temperature for the HPLC-DAD analysis.

The 300 nm wavelength was selected for quantification of the 11 marker compounds, according to the maximum absorptions of the compounds on the UV spectra obtained from DAD as shown in Fig. 1.

3.2.2. HPLC-DAD method validation: specificity, linearity, limit of quantification (LOQ), intra- and inter-day precision and accuracy, recovery, and robustness

To improve quantification precision and accuracy, byakangelicol, a member of the coumarins, was introduced as an internal standard (IS). The specificity of the method was determined by comparing the chromatograms of extracts from Radix Saposhnikoviae with spectra of standards containing the 11 tested bioactive components. The representative chromatograms of a standard mixture and 50% methanol extract of Radix Saposhnikoviae are given in Fig. 2. Eleven bioactive components and IS (byakangelicol) were well separated without apparent interference from the other constituents of Radix Saposhnikoviae.

Each calibration curve was obtained with six different concentrations of the 11 bioactive markers using linear regression analysis. All compounds showed good linearity ($r^2 > 0.999$) over wide concentration ranges. The limits of quantification (LOQs) of the 11 bioactive markers ranged from 0.5 to 10 μg/mL. The results are given in Table 1.

The precision of the method was evaluated using both intra- and inter-day precision, which were determined by testing a mixed standard solution in five replicates during a single day and also repeating the tests on five separate days. The intra-day precision for 11 bioactive markers ranged from 0.2% to 9.0%, and the inter-day precision ranged from 0.2% to 8.1%. The intra-day accuracy ranged from 96.3 to 106.8%, and the inter-day accuracy varied between 96.8 and 110.6% (Supplementary Table S2). The overall precision and accuracy values were sufficient to allow the quantification of the 11 markers in Radix Saposhnikoviae.

The recoveries were determined by spiking samples of Radix Saposhnikoviae with known amounts of the 11 markers. Accurate amounts of the 11 analytes at three different concentration levels were added to 250 mg of Radix Saposhnikoviae and then extracted, prepared, and analyzed with the developed method. The recoveries for the 11 markers were as follows: 91.0–97.9% for prim-*O*-glucosylcimifugin (10–50 μg/mL); 93.3–100.9% for cimifugin (5–25 μg/mL); 83.3–97.7% for nodakenin (2–10 μg/mL); 88.7–100.6% for 4′-*O*-β-D-glucosyl-5-*O*-methylvisamminol (10–50 μg/mL); 85.4–97.3% for sec-*O*-glucosylhamaudol (2–10 μg/mL); 83.1–86.4% for psoralen (5–25 μg/mL); 95.6–99.0% for bergapten (5–25 μg/mL); 84.9–91.0% for imperatorin (5–25 μg/mL); 82.7–85.7% for phellopterin (5–25 μg/mL); 90.1–95.1% for 3′-*O*-angeloylhamaudol (1–5 μg/mL); 82.0–93.4% for anomalin (10–50 μg/mL). Overall, recoveries for each of the analytes at three different concentrations were all higher than 80%. In spite of the simultaneous quantification of major and minor compounds and the complex matrix of Radix Saposhnikoviae, the recoveries were satisfactory. These results are shown in Table 2.

The robustness of the HPLC-DAD method was also evaluated by varying several chromatographic parameters, such as column temperature, flow rate, and C18 column manufacturer. All chromatograms obtained showed that peak shape, peak area precision, and resolution were satisfactory in all tested conditions, which indicated that the robustness of this method was sufficient (Supplementary Table S3).

Table 1
Regression equations, correlation coefficients, linearity ranges, and limit of quantification (LOQ) for the HPLC-DAD and HPLC–MS/MS method.

Compound	HPLC-DAD				HPLC–MS/MS			
	Regression equation	r^2	Linearity range (μg/mL)	LOQ (μg/mL)	Regression equation	r^2	Linearity range (ng/mL)	LOQ (ng/mL)
Prim-*O*-glucosylcimifugin	$y = 0.018x + 0.014$	0.999	1–500	1	$y = 0.00159x - 0.000728$	0.997	20–1000	10
Cimifugin	$y = 0.032x + 0.017$	0.999	0.5–250	0.5	$y = 0.00716x - 0.0199$	0.995	10–500	10
Nodakenin	$y = 0.012x$	1	1–100	1	$y = 0.00287x - 0.03389$	0.994	40–2000	40
4′-*O*-β-D-glucosyl-5-*O*-methylvisamminol	$y = 0.019x + 0.021$	0.999	1–500	1	$y = 0.0104x - 0.04109$	0.990	10–500	10
Sec-*O*-glucosylhamaudol	$y = 0.016x - 0.006$	0.999	1–100	1	$y = 0.0063x - 0.0075$	0.993	10–500	5
Psoralen	$y = 0.045x - 0.003$	1	0.5–250	0.5	$y = 0.03823x - 0.00708$	0.995	2–100	2
Bergapten	$y = 0.039x + 0.002$	1	0.5–250	0.5	$y = 0.0179x - 0.01598$	0.997	2.5–250	2.5
Imperatorin	$y = 0.032x + 0.006$	1	0.5–250	0.5	$y = 0.0228x + 0.00188$	0.995	1–100	1
Phellopterin	$y = 0.022x + 0.007$	1	0.5–250	0.5	$y = 0.0147x + 0.00497$	0.998	2–100	2
3′-*O*-angeloylhamaudol	$y = 0.016x - 0.002$	0.999	1–50	1	$y = 0.0481x + 0.00532$	0.999	1–100	1
Anomalin	$y = 0.002x - 0.001$	1	10–500	10	$y = 0.00259x + 0.0051$	0.997	15–750	15

Table 2
Recoveries of bioactive compounds in Radix Saposhnikoviae by the HPLC-DAD method.

Compound	Spiked quantity (μg/mL)	Measured quantity (μg/mL)	Recovery (%)
Prim-*O*-glucosylcimifugin	10	9.5	94.6
	25	24.5	97.9
	50	45.5	91.0
Cimifugin	5	4.7	93.3
	12.5	12.2	97.8
	25	25.2	100.9
Nodakenin	2	1.5	83.3
	5	4.9	97.7
	10	9.5	95.1
4′-*O*-β-D-glucosyl-5-*O*-methylvisamminol	10	10.1	100.6
	25	22.2	88.7
	50	50.3	100.5
Sec-*O*-glucosylhamaudol	2	1.7	85.4
	5	4.4	87.7
	10	9.7	97.3
Psoralen	5	4.3	86.4
	12.5	10.4	83.1
	25	21.2	84.9
Bergapten	5	5.0	99.0
	12.5	12.0	95.6
	25	24.4	97.7
Imperatorin	5	4.4	87.1
	12.5	10.6	84.9
	25	22.8	91.0
Phellopterin	5	4.1	82.7
	12.5	10.3	82.7
	25	21.4	85.7
3′-*O*-angeloylhamaudol	1	1.0	95.1
	2.5	2.3	90.6
	5	4.5	90.1
Anomalin	10	8.8	88.3
	25	23.4	93.4
	50	41.0	82.0

3.3. HPLC–MS/MS analysis

3.3.1. Optimization of chromatographic separation conditions

Several C18 columns were compared according to peak separation and sharpness to select the column with the best performance. The Thermo Hypersil GOLD C18 column exhibited the best performance in terms of chromatographic separation and sensitivity for HPLC–MS/MS.

Acid modifiers, including formic acid and acetic acid, were added to the mobile phase in an attempt to improve the ionization efficiency and thus produce better separation with sharper peaks than might be seen in the mobile phase with no acid. Acetic acid (0.1%) was selected as an additive, which provided better sensitivity than 0.1% formic acid and 0.05% acetic acid. Finally, 0.1% (v/v) aqueous acetic acid–acetonitrile was used in the HPLC–MS/MS method, which gave both good separation and high signal intensities for the 11 markers.

For the MS/MS analysis, electrospray ionization (ESI) provided better sensitivity than APCI (atmospheric pressure chemical ionization). In addition, the positive ion detection mode showed better sensitivity than the negative ion detection mode. Parameters such as gas flow, capillary voltage, cone voltage, extractor voltage, entrance voltage, RF lens voltage, exit voltage, desolvation temperature, and collision energy were optimized to obtain the highest signal response for the precursor and product ions of

the 11 bioactive components. The precursor-to-product ion pair (Fig. 3), cone voltage and collision energy optimized for each compound are described in Table 3. Using the developed HPLC–MS/MS method satisfactory separation of the 11 bioactive components and the internal standard were achieved within 15 min (Supplementary Fig. S4).

3.3.2. Validation of the analytical method: linearity, limit of quantification (LOQ), and intra- and inter-day precision and accuracy

Validation of HPLC–MS/MS was performed using the same procedure as for HPLC-DAD method validation. All target compounds showed good linearity ($r^2 > 0.990$) over wide concentration

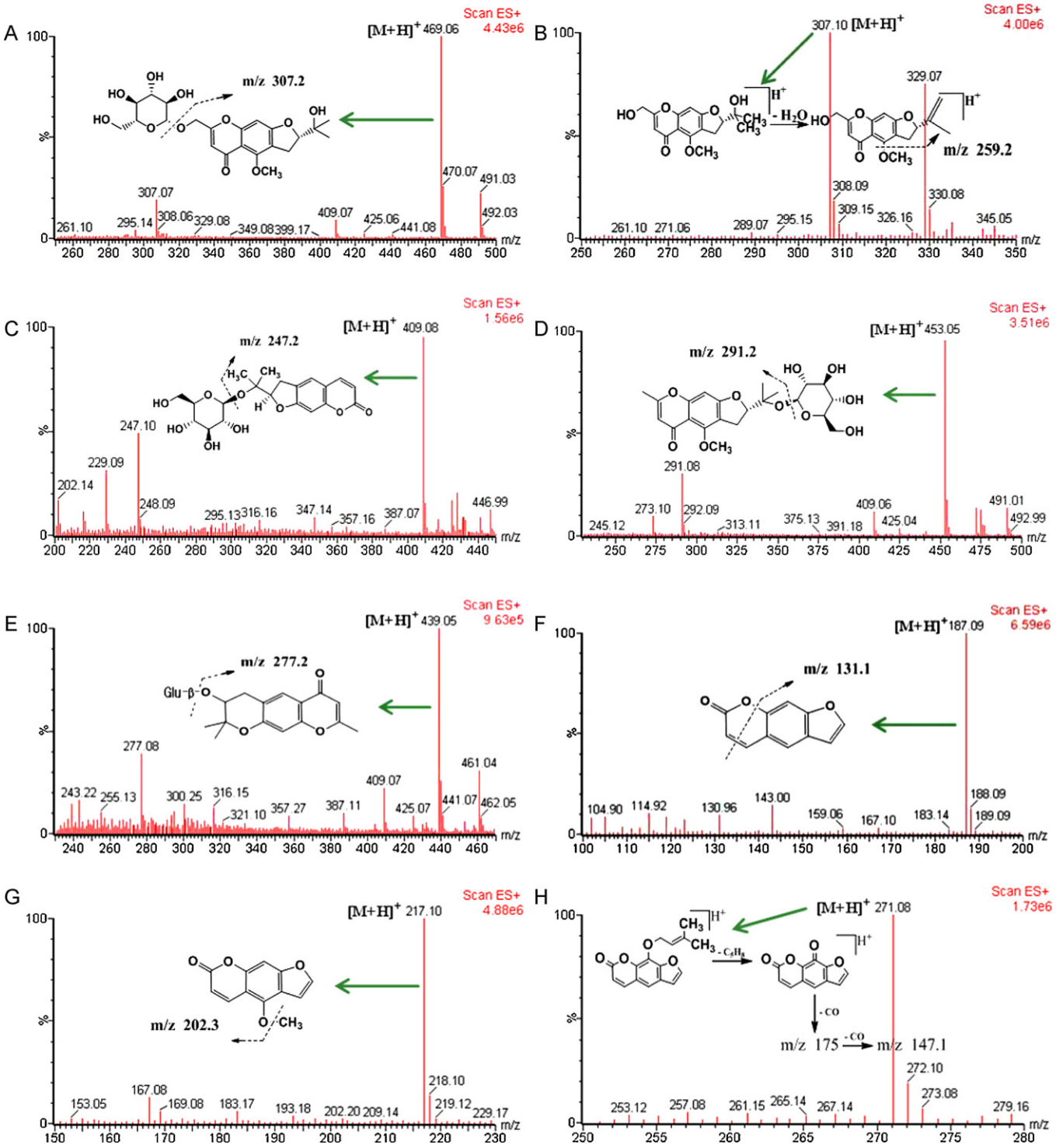

Fig. 3. MS spectra of prim-*O*-glucosylcimifugin (A), cimifugin (B), nodakenin (C), 4′-*O*-β-D-glucosyl-5-*O*-methylvisamminol (D), sec-*O*-glucosylhamaudol (E), psoralen (F), bergapten (G), imperatorin (H), phellopterin (I), 3′-*O*-angeloylhamaudol (J), anomalin (K), and byakangelicol (internal standard) (L).

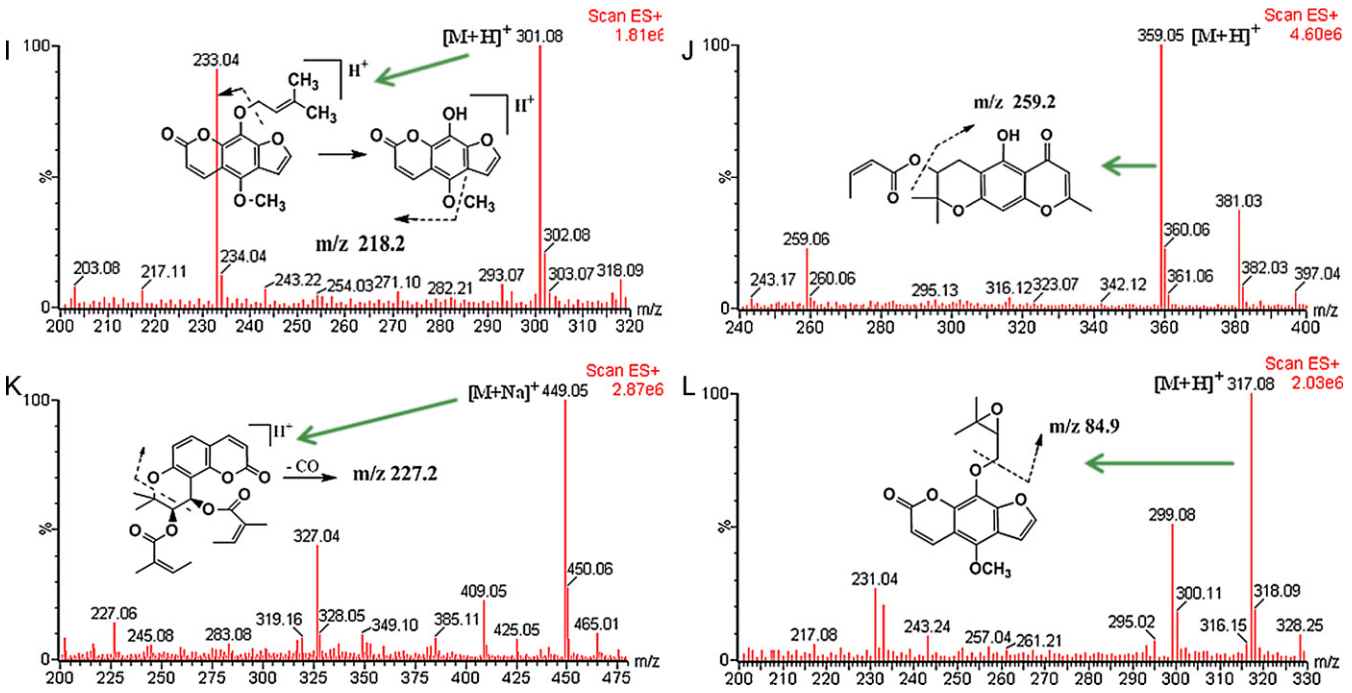

Fig. 3. *continued.*

ranges. The limits of quantification (LOQs) of the 11 markers ranged from 1 to 40 ng/mL (Table 1). The intra-day precision ranged from 1.8% to 6.7%, and the inter-day precision ranged from 1.2% to 9.0%. The intra-day accuracy was in the range of 89.2–109.4%, and the inter-day accuracy varied between 86.7 and 109.1% (Supplementary Table S4). All of the measured precision and accuracy values were found to be acceptable for the identification and quantification of the 11 components in Radix Saposhnikoviae.

3.4. Sample analysis

The established HPLC-DAD method was applied to comprehensive analysis and quality evaluation of 51 commercial Radix Saposhnikoviae samples from various geographic origins (4 samples collected from Korea and 47 samples collected from China). The 11 bioactive components were determined without any apparent interference from the other constituents in Radix Saposhnikoviae. Quantification of each analyte in the samples was calculated using the peak area ratio (peak area of analyte versus peak area of internal standard) based on the calibration curve of each individual standard. As tabulated in Table 4, the results demonstrated the successful application of the HPLC-DAD method for the quantification of both major chromones and minor coumarins with a wide dynamic range in various samples of Radix Saposhnikoviae. The findings also showed that different samples may differ in their quantities of chromones and coumarins, which could result in different qualities and efficacies for the samples. Among samples 1 through 47, which were collected from Inner Mongolia, Hebei, Northeast, Hunan, Guangxi, Anhui, Gansu, and Yunnan regions in China, the total content of the five chromones examined ranged from 0.003 to 1.187 μg/g, whereas the maximum total amount of coumarins was 0.099 μg/g. Samples from Inner Mongolia contained the richest amounts of bioactive components, while samples from Gansu had the least amount of marker compounds. Variation in the levels of bioactive marker compounds may affect the therapeutic efficacy of the medicine in clinical practice. These results demonstrated that simultaneous quantification of major and minor compounds may be important for comprehensive quality evaluation of Radix Saposhnikoviae samples.

Table 3
The retention time, MS/MS fragment ions, cone voltage and collision energy for the HPLC–MS/MS method.

Compound	Retention time (min)	Precursor ion (*m/z*)	Product ion (*m/z*)	Cone voltage (V)	Collision energy (eV)
Prim-*O*-glucosylcimifugin	2.0	469.1 $[M+H]^+$	307.2	55	30
Cimifugin	5.4	307.1 $[M+H]^+$	259.2	45	30
Nodakenin	5.4	409.1 $[M+H]^+$	247.2	20	15
4′-*O*-β-D-glucosyl-5-*O*-methylvisamminol	5.5	453.1 $[M+H]^+$	291.2	46	23
Sec-*O*-glucosylhamaudol	7.6	439.1 $[M+H]^+$	277.2	24	15
Psoralen	10.1	187.1 $[M+H]^+$	131.1	43	25
Bergapten	11.0	409.1 $[M+H]^+$	247.2	30	22
Imperatorin	12.2	271.1 $[M+H]^+$	147.2	25	33
Phellopterin	12.3	301.1 $[M+H]^+$	218.2	22	30
3′-*O*-angeloylhamaudol	13.3	359.1 $[M+H]^+$	259.2	31	20
Anomalin	13.4	449.1 $[M+Na]^+$	227.2	45	35
Byakangelicol (IS)	11.4	317.1 $[M+H]^+$	84.9	25	15

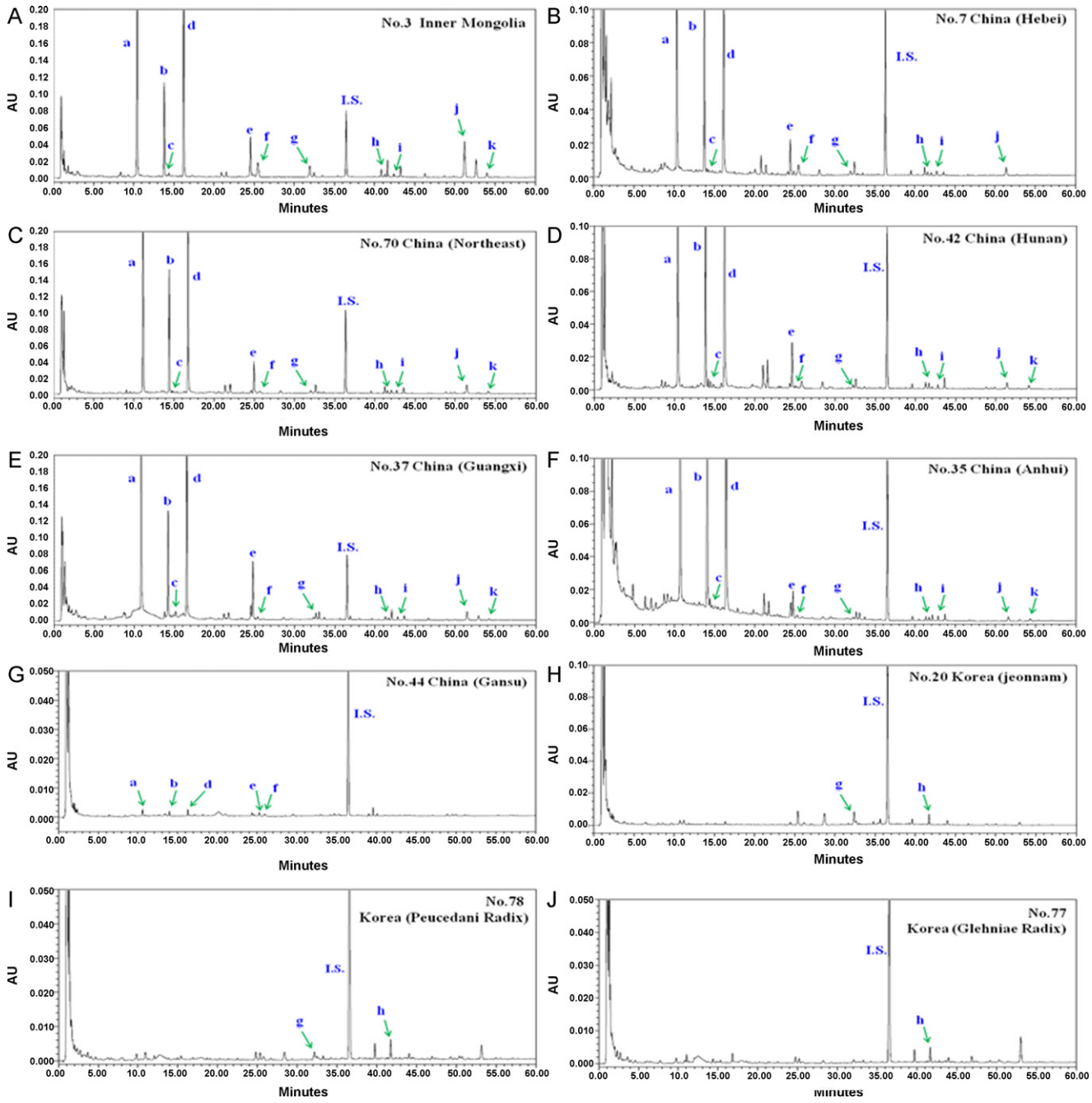

Fig. 4. Representative HPLC-DAD chromatograms of Radix Saposhnikoviae samples. prim-*O*-glucosylcimifugin (a), cimifugin (b), nodakenin (c), 4′-*O*-β-D-glucosyl-5-*O*-methylvisamminol (d), sec-*O*-glucosylhamaudol (e), psoralen (f), bergapten (g), imperatorin (h), phellopterin (i), 3′-*O*-angeloylhamaudol (j), anomalin (k), and byakangelicol (internal standard).

Fig. 4 shows the representative chromatograms of commercial Radix Saposhnikoviae samples from eight different collection sites, Radix Glehniae, and Radix Peucedani. The plant samples from Korea had no chromones and small amounts of bergapten or imperatorin, and showed similar chromatograms to those of Radix Glehniae and Radix Peucedani. Therefore, samples from Korea were assumed to be Radix Glehniae or Radix Peucedani. These results showed that this HPLC-DAD method could be used for the discrimination of Radix Saposhnikoviae from its substitutes.

Harvesting medicinal plants from different geographic regions could result in products with drastically different constituents [28]. Typically, quality control of herbal medicines has been performed using identification and content determination of only one or two markers. However, the characteristic multi-target and synergistic actions of traditional herbal medicines come from their multiple constituents [29]. A comprehensive method that reflects the variation of constituents in herbal medicines is necessary. Therefore, the multi-component quantification method established in this study could be used for the quality evaluation of Radix Saposhnikoviae.

Table 4
Contents (μg/g) of 11 bioactive components in Radix Saposhnikoviae samples.

No.	Collection site	Five major chromones						Six minor coumarins						
		A	B	D	E	J	Total[a]	C	F	G	H	I	K	Total[b]
1	Northeast	0.242	0.090	0.188	0.048	0.011	0.579	0.002	0.001	0.001	0.001	0.001	0.018	0.024
2		0.004	0.001	0.003	0.003	–[c]	0.011	0.004	0.001	–	–	–	–	0.005
3		0.310	0.074	0.285	0.050	0.013	0.732	0.004	0.001	0.001	0.002	0.002	0.029	0.039
4		–	–	–	0.003	–	0.003	–	0.001	–	–	–	–	0.001
5		0.308	0.073	0.302	0.051	0.020	0.754	0.004	0.002	0.002	0.002	0.003	0.043	0.056
6		0.319	0.090	0.297	0.064	0.027	0.797	0.007	0.001	0.002	0.002	0.003	0.045	0.060
7	Inner Mongolia	0.445	0.122	0.294	0.135	0.033	1.029	0.004	0.001	0.001	0.001	0.001	0.079	0.087
8		0.258	0.051	0.304	0.072	0.022	0.707	0.005	0.002	0.003	0.001	0.002	0.025	0.038
9		0.316	0.134	0.347	0.085	0.031	0.913	0.007	0.003	0.003	0.005	0.003	0.064	0.085
10		0.372	0.182	0.451	0.148	0.034	1.187	0.007	0.001	0.001	0.001	0.002	0.087	0.099
11		0.197	0.207	0.156	0.051	0.012	0.623	0.003	0.004	0.001	0.001	0.001	0.015	0.025
12		0.450	0.072	0.296	0.095	0.028	0.941	0.004	0.001	0.001	0.002	0.005	0.050	0.063
13		0.239	0.044	0.226	0.063	0.011	0.583	0.004	0.001	0.001	0.001	0.002	0.023	0.032
14		0.470	0.123	0.348	0.164	0.022	1.127	0.005	0.002	0.002	0.003	0.001	0.058	0.071
15		0.356	0.192	0.314	0.144	0.029	1.035	0.004	0.004	0.001	0.001	0.001	0.073	0.084
16		0.311	0.051	0.306	0.065	0.015	0.748	0.002	0.002	0.002	0.001	0.001	0.026	0.034
17		0.287	0.046	0.271	0.065	0.015	0.684	0.003	0.001	0.002	0.001	0.001	0.035	0.043
18		0.287	0.075	0.175	0.103	0.012	0.652	0.002	0.002	0.001	0.006	0.002	0.026	0.039
19		0.577	0.087	0.317	0.095	0.034	1.110	0.027	0.002	0.004	0.001	0.002	0.056	0.092
20		0.391	0.057	0.298	0.106	0.039	0.891	0.003	0.001	0.001	0.001	0.001	0.060	0.067
21	Gansu	0.002	0.001	0.002	0.002	–	0.007	–	0.001	–	–	–	–	0.001
22		–	0.001	–	0.002	–	0.003	–	–	–	–	–	–	–
23		0.001	0.001	–	0.001	0.001	0.004	–	–	–	–	–	–	–
24		0.054	0.001	–	0.001	–	0.056	–	0.001	–	–	–	–	0.001
25		–	0.001	–	0.002	–	0.003	–	–	–	–	–	–	–
26		–	–	–	0.003	–	0.003	–	–	–	–	–	–	–
27	Guangxi	0.346	0.080	0.264	0.084	0.027	0.801	0.013	0.002	0.004	0.002	0.002	0.031	0.054
28		0.502	0.094	0.342	0.115	0.025	1.078	0.023	0.002	0.002	0.002	0.004	0.033	0.066
29	Hebei	0.257	0.080	0.281	0.025	0.009	0.652	0.002	0.005	0.002	0.001	0.002	–	0.012
30		0.185	0.260	0.329	0.104	0.027	0.905	0.015	0.001	0.002	0.002	0.002	0.056	0.078
31		0.127	0.019	0.084	0.018	0.003	0.251	–	0.001	0.001	0.001	0.002	–	0.005
32		0.232	0.038	0.244	0.023	0.008	0.545	0.002	0.008	0.003	0.001	0.001	–	0.015
33		0.223	0.222	0.338	0.113	0.03	0.926	0.019	0.001	0.001	0.002	0.002	0.056	0.081
34		0.242	0.075	0.272	0.023	0.005	0.617	0.003	0.002	0.001	0.001	0.002	–	0.009
35		0.150	0.033	0.077	0.018	0.004	0.282	–	0.001	0.004	0.001	0.001	–	0.007
36		0.159	0.027	0.104	0.023	0.004	0.317	–	0.003	0.001	0.002	0.001	0.011	0.018
37		0.152	0.042	0.101	0.022	0.006	0.323	–	–	–	0.004	0.001	0.009	0.014
38		0.155	0.044	0.044	0.025	0.005	0.273	–	0.024	0.008	0.001	–	–	0.033
39		0.261	0.041	0.291	0.025	0.009	0.627	0.006	0.003	0.001	0.001	0.001	0.010	0.022
40		0.248	0.236	0.370	0.118	0.032	1.004	0.014	0.002	0.002	0.002	0.002	0.072	0.094
41	Anhui	0.273	0.171	0.219	0.022	0.008	0.693	0.006	0.001	0.003	0.001	0.002	0.016	0.029
42		0.438	0.211	0.435	0.02	0.005	1.109	0.004	0.001	0.001	0.001	0.002	0.020	0.029
43		0.179	0.088	0.236	0.039	0.010	0.552	0.005	0.001	–	0.003	0.002	0.011	0.022
44		0.170	0.073	0.164	0.031	0.007	0.445	0.003	0.002	0.002	0.001	0.001	0.005	0.014
45	Hunan	0.230	0.064	0.416	0.034	0.007	0.751	0.006	0.004	0.001	0.002	0.002	0.029	0.044
46		0.266	0.068	0.422	0.036	0.009	0.801	0.006	0.002	0.001	0.002	0.002	0.038	0.051
47	Yunnan	0.014	0.004	0.002	0.003	–	0.023	0.011	0.002	0.001	–	–	–	0.014
48	Korea	–	–	–	–	–	–	–	–	0.001	0.003	–	–	0.004
49		–	–	–	–	–	–	–	–	0.001	0.002	–	–	0.003
50		–	–	–	–	–	–	–	–	0.003	0.001	–	–	0.004
51		–	–	–	–	–	–	–	–	–	0.001	–	–	0.001

[a] The sum of prim-*O*-glucosylcimifugin (A), cimifugin (B), 4′-*O*-β-D-glucosyl-5-*O*-methylvisamminol (D), sec-*O*-glucosylhamaudol (E), and 3′-*O*-angeloylhamaudol (J).
[b] The sum of nodakenin (C), psoralen (F), bergapten (G), imperatorin (H), phellopterin (I), and anomalin (K).
[c] Not detected or below LOQ.

4. Conclusions

In this work, HPLC-DAD and HPLC–MS/MS methods were established for the simultaneous determination of 11 bioactive components from Radix Saposhnikoviae. These methods were fully validated in terms of selectivity, linearity, precision, accuracy, and limit of quantification (LOQ). Robustness and recovery were also favorable for the HPLC-DAD method. The applicability of the HPLC-DAD method was successfully demonstrated by analyzing 51 batches of commercial samples for the simultaneous quantification of major chromones and minor coumarins in Radix Saposhnikoviae. The results showed that the quantification of both major chromones and minor coumarins can better differentiate the batch-to-batch consistency of Radix Saposhnikoviae harvested from different geographic origins. These HPLC-DAD and HPLC–MS/MS methods will be very helpful for comprehensive quality control of Radix Saposhnikoviae, as well as for discrimination of this plant from its substitutes such as Radix Glehniae and Radix Peucedani.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.06.103.

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