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Original Article

Simultaneous Quantitative Determination of Six Triterpenoid Saponins in *Ardisia japonica* Collected from Different Regions of China through LC-ESI-MS

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ABSTRACT

Background: The whole plants of *Ardisia japonica* (Myrsinaceae) are used in traditional Chinese medicine for the treatment of phlegm, cough, jaundice, edema, and bruise, and triterpenoid saponins are the main bioactive components. However, triterpenoid saponins from this plant have not yet been simultaneously quantitatively determined and reported. **Objectives:** The present study aimed to establish a fast, simple, and reliable LC-ESI-MS analysis method for simultaneous quantization of the major triterpenoid saponins in *A. japonica* for the first time. **Materials and Methods:** Six major triterpenoid saponins from the plant species of *Ardisia* were selected as the reference compounds. Five batches of *A. japonica* were gathered from five distinct regions of P. R. China. Quantitative analysis was developed and validated by LC-ESI-MS in SIM mode. The separation conditions were optimized on a RP C_{18} Column with ACN and 0.1% FA (*v/v*) at a flow rate of 0.2 ml/min and column temperature of 35°C. **Results:** The validation investigation found that the analysis method was accurate, recoverable, and sensitive for quantification of the six triterpenoid saponins. All of the calibration curves revealed acceptable linear regression (R^2>0.9975). The contents of six triterpenoid saponins in *A. japonica* from five regions varied considerably. **Conclusion:** The present study established a highly efficient analytical technique to quantify the triterpenoid saponins in *A. japonica*, and it could be further applied in the quality control of *A. japonica*.

Keywords: LC-MS, Quantitative analysis, Triterpenoid saponin, *Ardisia japonica*.

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INTRODUCTION

Ardisia japonica (Thunb.) Blume is a sub-shrub of the family Myrsinaceae, and distributed widely in Southeast Asia and North America.[1] The whole plants of *A. japonica* are listed in the Chinese Pharmacopoeia (2020 version) as a traditional Chinese medicine to cure phlegm, cough, jaundice, edema, and bruise.[2-4] Previous studies have demonstrated *A. japonica* possessing various biological effects, such as anti-HIV,[5] antioxidant,[6,7] and anticancer activities.[4,8,9] The chemical constituents have been reported benzoquinones, phenols, flavonoids, chromones, triterpenoids, and triterpenoid saponins, among which triterpenoid saponins were the main bioactive components.[4] However, to the best of our knowledge, simultaneously quantitative analysis of the triterpenoid saponins in this plant have not yet been reported. Even though some analytical methods such as HPLC-UV,[10] HPLC-PDA,[11] and HPLC-ELSD,[12] have been adopted for the quantitative analysis of triterpenoid saponins, LC-MS techniques have emerged as one of the most powerful tools for the quantitative analysis of triterpenoid saponins with the advantage of high selectivity and sensitivity.[13,14] ESI represents a commonly used ionization mode in mass spectrometers, which allows thermolabile and high-molecular-weight compounds (>1000 Da), such as triterpenoid saponins, to be ionized and moved into gas phase.[15] In the present study, a useful LC-ESI-MS approach was developed to simultaneously quantify six major triterpenoid saponins in *A. japonica*. Moreover, the proposed method was used to quantify these saponins in five samples taken from different regions in P. R. China.

MATERIALS AND METHODS

Chemicals, materials, and reagents

Six triterpenoid saponins: ardisiacrenoside A (**1**), cyclamine (**2**), ardisiacrispin A (**3**), ardisiacrispin B (**4**), 3β-*O*-(α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl-

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(1→4)-*α*-L-arabinopyranosyl)-cyclamiretin A (**5**) and 3*β*-*O*-*β*-D-glucopyranosyl-(1→2)-[*α*-L-rhamnopyranosyl-(1→2)-*β*-D-glucopyranosyl-(1→4)]-*α*-L-arabinopyranosyl-13*β*,28-epoxy-16 *α*-hydroxyoleanane (6), were selected as the reference compounds, and were obtained in our previous phytochemical work (Figure 1).[4] Their purity > 98% were confirmed by the ^{1}H-NMR spectra and HPLC analysis. Ginsenoside Rg1 (Sigma-Aldrich Corp., St. Louis, MO, USA) was used as an internal standard.

Five batches of the aerial parts of *A. japonica* were collected from five different regions in June 2012: Guangxi (sample 1), Hunan (sample 2), Hubei (sample 3), Yunnan (sample 4), and Guizhou (sample 5) provinces, P. R. China (Figure 2). Plant identification was done by Dr. Wei Li, and the voucher specimens (accession number: AJ-01, AJ-02, AJ-03, AJ-04, AJ-05, respectively) were stored in the Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Toho university.

ACN, MeOH, EtOH, FA, and TFA (MS grade) were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Ultra-pure water (18 mΩ) was produced by a Milli-Q water filtration system (Millipore Co., Ltd., Milford, MA, USA).

Instrument and chromatographic conditions

A Shimadzu ultrafast liquid chromatography system (Shimadzu, Japan) with an auto-sampler and a binary solvent delivery module was used for the chromatographic analysis. Chromatographic peaks were separated on a Zorbax Eclipse Plus C_{18} column (10 cm × 2.1 mm,1.8 μm, Agilent Technology, Germany) at a flow rate 0.2 mL/min at 35°C. The mobile phase was consisted of eluent A (H_2O with 0.1% FA) and eluent B (ACN with 0.1% FA), and was programmed as follows: linear gradient 20-30% B (0-4 min); 30-38% B (4-10 min); 38% B (10-15 min); 38-65% B (15-18 min); 65-100% B (18-25 min); 100% B (25-30 min). The column was re-equilibrated at 20% B for 20 min at the original conditions before the next injection. The injection volume was 1μL for each analysis.

MS was performed by using a quadrupole mass spectrometer system with an ESI source (Shimadzu, Japan) in the positive- and

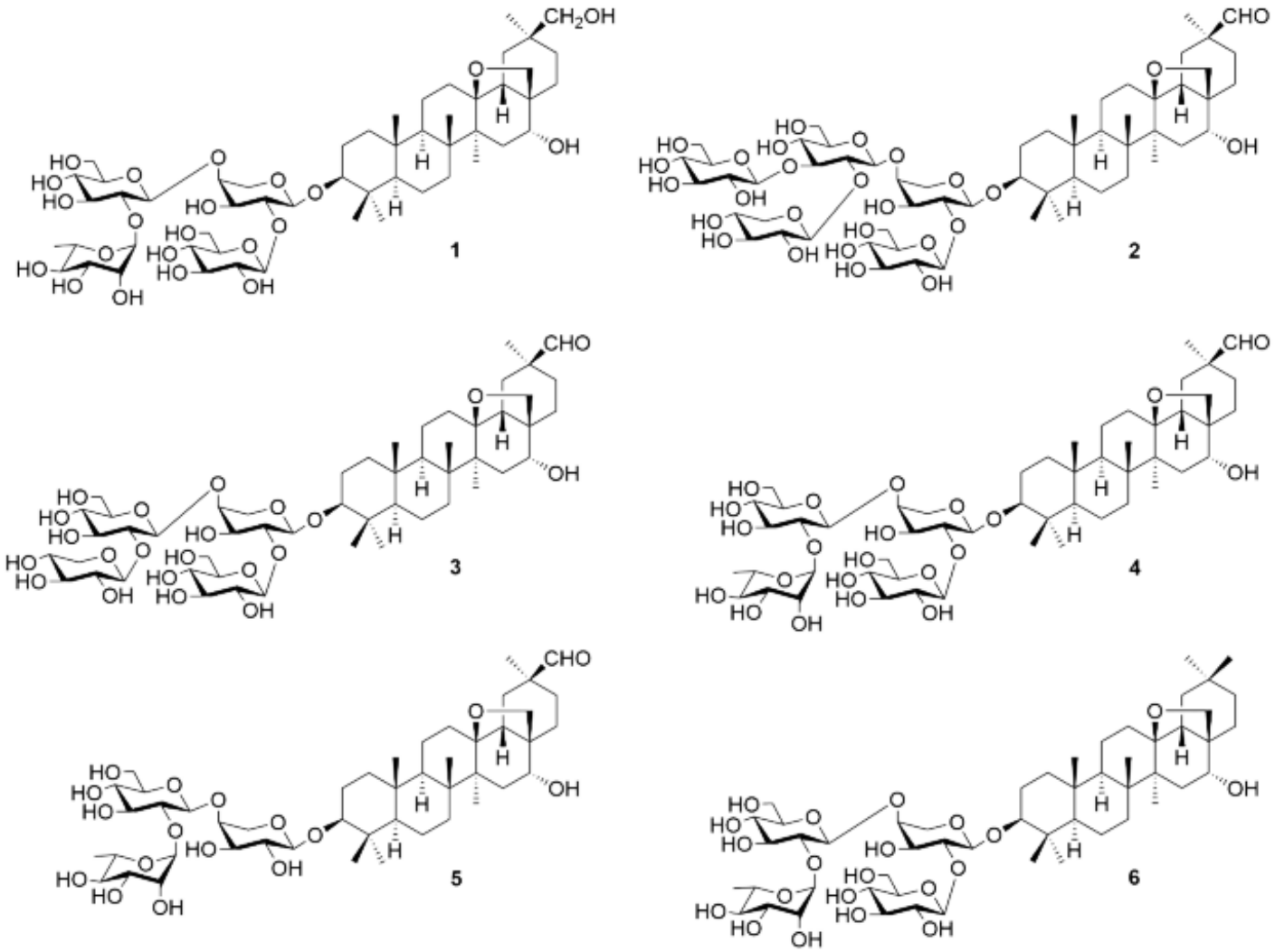

Figure 1: Structures of reference compounds 1–6.

negative-ion scan modes. The interface, CDL, and heat block were set at temperatures of 250°C, 250°C, and 200°C, separately. The nebulizing gas was fixed to a flow rate of 1.5 L/min. In the positive-ion mode, the voltage settings were as follows: the interface with 4.5 kV, Q-array DC with 30 V, Q-array RF with 150 V, CDL with 30 V, and the detector with 1.6 kV. In the negative-ion mode, the voltage settings were as follows: the interface with -3.5 kV, the Q-array DC with -30 V, Q-array RF with 150 V, CDL with -25 V, and the detector with -1.6 kV. The scan range was set at *m/z* 100 to 2000 for both positive- and negative-ion MS scan mode. SIM data-acquisition mode was performed in negative-ionization mode. LCMS-2010EV evolution software was used for data processing.[16]

Standard solutions preparation

The stock standard solution (400 μg/mL) was prepared by accurately weighed reference compounds 1–6 dissolved in H_2O-ACN (50:50, *v/v*). Diluting the stock solutions of 1–6 with H_2O-ACN (50:50, *v/v*) obtained the working solutions. Dilution of standard stock solution of 1–6 with MeOH produced a range of concentrations for calibration curve construction. An internal standard stock solution of Ginsenoside Rg1 was prepared with H_2O-ACN (50:50, *v/v*). The internal standard solution was transformed into an internal standard spiking solution (40 μg/mL) by diluting with MeOH. Ginsenoside Rg1 was undetected in the crude extract of *A. japonica*. All solutions were kept at 4°C for analysis.[16]

Preparation of sample solutions

Five batches of the aerial parts of *A. japonica* from different origins were dried and ground into a homogenous powder (40 mesh). Each powder was weighed (ca. 0.80 g) and extracted with MeOH (20 ml) at room temperature using ultrasonication at 40 kHz for 20 min. During the extraction, MeOH was used to compensate for the weight lost. After the solutions were thoroughly mixed and centrifuged at 13,000 rpm for 10 min, the supernatants (1mL, 400 μg/mL) of compounds 1–6 and the internal standard solution of ginsenosides Rg1 (1mL, 40μg/mL) were added to a 25-ml volumetric flask, and MeOH was added to fill the flask. Prior to LC-MS analysis, all solutions were filtered through a 0.22 μm filter and kept at 4°C.

Method Validation

Calibration curves, LOD, and LOQ

The normalized peak area ratios of analytes (the corresponding analyte peak area divided by the internal standard peak area) against their concentrations were used to construct the calibration curves. Six concentrations were used to perform each calibration curve in duplicate. The R^2 was used to judge the linearity and

Figure 2: Samples 1–5 collected from five different regions of China.

Table 1: Precision and recovery of reference compounds 1–6.

Compd. no.	Precision (RSD, %)		Mean recovery (%, *n*=6)				
	Intra-day (*n*=6)	Inter-day (*n*=6)	Original (*μg*)	Spiked (*μg*)	Found (*μg*)	Recovery (%)	RSD (%)
1	0.49	5.49	1.8971	27.1967	28.5045	97.77	3.06
2	2.55	5.70	38.6627	53.7600	91.3228	97.75	5.47
3	2.45	2.94	9.7060	21.3300	31.7071	102.59	7.84
4	2.87	4.22	73.8437	58.0500	132.3425	100.60	7.19
5	1.53	5.81	4.8497	20.3657	24.6377	97.09	7.87
6	2.82	3.23	3.7783	19.7227	24.4567	104.73	6.89

showed a linear response with $R^2 \geq 0.9975$ for all compounds. LODs and LOQs for compounds 1–6 were established at *S*/*N* values of three and ten, respectively.

Precision, repeatability, stability, and accuracy

Intra-day and inter-day variations were chosen to assess the accuracy of the method (Table 1). Six replicates of the standard solutions were analyzed in the intra-day variability test. The solutions were tested in duplicate as part of the inter-day variability test for three consecutive days. Six sample solutions from sample 1 were examined to establish repeatability. The stability of one sample solutions was evaluated during 24 hr at various time intervals (0, 2, 4, 8, 12, 24) at 20°C. The relative standard deviations were used to express all of these variations (RSDs). The accuracy was assessed by a recovery test, adding the corresponding marker compounds to sample 1 (ca. 0.80 g), at high, intermediate, and low levels. The spiked samples were then extracted, processed, and measured using the methods described above, with triplicate tests done at each level. The following formula was used to estimate average recoveries: recovery (%) = [(amount found – original amount)/amount added] × 100%.

RESULTS

Optimization of extraction conditions

To maximize extraction efficiency, optimisation of procedure parameters such as solvent, solvent volume, and extraction time were carried out. Firstly, several kinds of solvents were tested, including H_2O, 50, 70, and 100% MeOH, and 70% EtOH. The results revealed that methanol was the best extraction solvent, afforded highest yields of the six marker saponins. Extraction times of 10, 20, 30, and 60 min, as well as solvent volumes of 10, 20, and 30 mL were also tested, revealing that the amounts of compounds 1–6 in samples 1–5 increased with the extension of extraction time, reaching a maximum at 20 min, but no substantial increase even further increasing of the extraction time. Given the solvent volume had a minor effect on extraction performance, samples 1–5 were extracted with MeOH (20 mL). Based on the aforementioned findings, the extraction procedures were determined: each sample was sonicated for 20 min. with MeOH (20 mL), which was sufficient and suitable for LC-MS analysis.

Optimization of LC conditions

Column temperature, mobile phase, and gradient program were preliminarily adjusted to obtain optimal separation and peak shape in a condensed analysis time with minimal peak tailing. Among the column temperatures of 25°C, 35°C, and 40°C, the best peak resolutions was obtained at35°C. Various mobile-phase systems were also compared, such as H_2O (A)-MeOH (B), H_2O (A)-ACN (B), H_2O with 0.05% FA (A)-ACN (B), H_2O with 0.1% TFA (A)-ACN (B), and H_2O with 0.1% FA (A)-ACN with 0.1% FA (B).The mobile phase composed of eluents A (H_2O with 0.1% FA) and B (ACN with 0.1% FA), afforded acceptable peak forms and noise. The gradient elution procedure was improved using six reference compounds (1–6) and an *A. japonica* methanol extract (sample 1), as follows: linear gradient 20-30% B (0-4 min); 30-38% B (4-10 min); 38-38% B (10-15 min); 38-65% B (15-18 min); 65-100% B (18-25 min); 100% B (25-30 min). These conditions afforded acceptable peak resolutions for all of the compounds.

Optimization of MS conditions

The MS conditions were optimized to obtain the best *S/N* ratio, higher responses, and improved detection, which including the interface voltage, the CDL voltage, the nebulizing gas, and the CID by varying the Q-array DC voltages. The negative-ion mode was chosen for the quantitative analysis since the adjusted LC-MS settings revealed that it had higher sensitivity than the positive-ion mode. The format adduct ion $[M+HCOO]^-$ was used for identification of 1–6. Comparing the *S/N* value of the target peaks in LC-ESI-TIC with that in LC-ESI-SIM-TIC revealed the SIM mode was more sensitive than the full scan mode. The characteristic chromatograms of six saponins in the SIM mode was shown in Figure 3.

Table 2: Quantitation ion, calibration curve, linear range, LOD and LOQ of reference compounds 1–6.

Compd. no.	Quantitation ion (*m/z*) $[M+HCOO]^-$	Linear regression			LOD (ng/mL)	LOQ (ng/mL)
		Calibration curve [a] (*n* = 6)	R^2	Linear range (μg/mL)		
1	1121.35	$y = 0.1077x - 0.0073$	0.9994	0.03980-31.84	39.80	143.28
2	1267.50	$y = 0.0528x + 0.0412$	0.9979	0.08640-69.12	86.40	311.04
3	1105.30	$y = 0.1481x + 0.0230$	0.9991	0.04740-37.92	18.96	94.80
4	1119.30	$y = 0.1177x + 0.0116$	0.9991	0.04300-34.40	34.40	137.60
5	957.30	$y = 0.3937x - 0.0208$	0.9994	0.02160-17.28	15.12	43.20
6	1105.55	$y = 0.0824x + 0.0015$	0.9975	0.04128-33.02	41.28	165.12

[a] *y*: peak area, *x*: concentration of each compound.

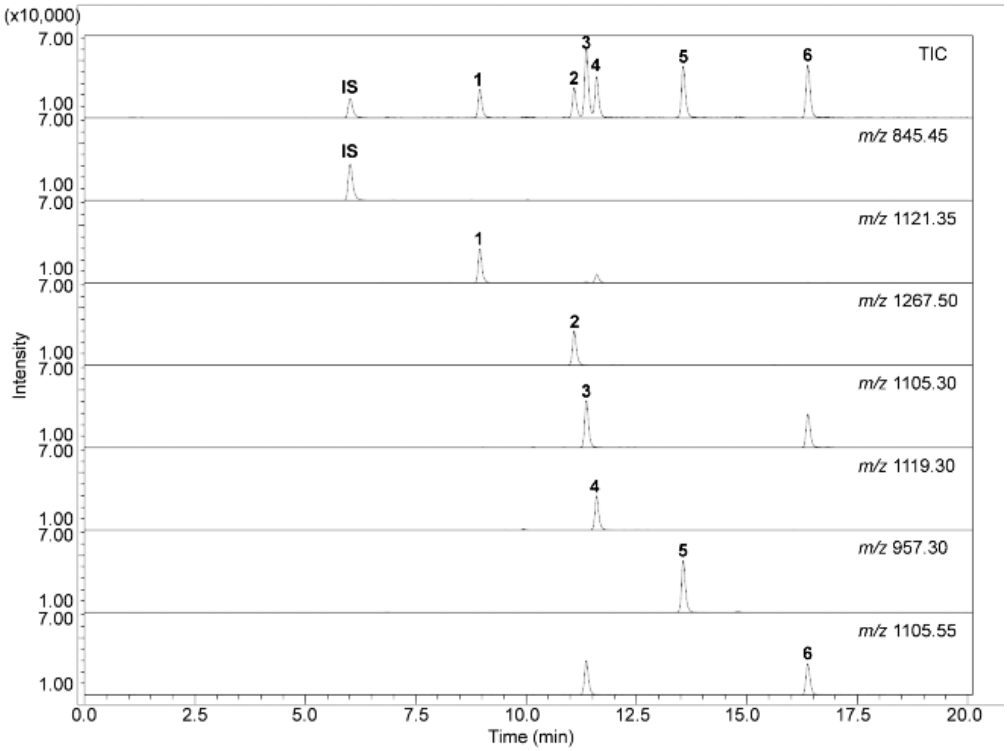

Figure 3: The total ion chromatograms of reference compounds 1–6 and internal standard (IS) in SIM mode.

Table 3: ESI-MS data of reference compounds 1–6 from *A. japonica*.

Compd.no.	*R*t(min)	Molecular formula	Molecular weight	$[M+Na]^+$	$[Aglycone–H_2O+H]^+$	$[M–H]^-$	$[M+HCOO]^-$
1	8.941	$C_{53}H_{88}O_{22}$	1077.25	1101.10	457.20	1076.10	1121.35
2	11.124	$C_{58}H_{94}O_{27}$	1223.10	1245.95	455.20	1221.55	1267.50
3	11.402	$C_{52}H_{84}O_{22}$	1061.21	1083.85	455.15	1060.10	1105.30
4	11.635	$C_{54}H_{88}O_{21}$	1075.24	1097.95	455.15	1073.60	1119.30
5	13.593	$C_{47}H_{76}O_{17}$	912.85	935.55	455.15	911.00	957.30
6	16.388	$C_{53}H_{88}O_{21}$	1061.15	1083.85	423.10	1059.30	1105.55

Table 4: Contents (mg/kg) of reference compounds 1–6 in five *A. japonica* samples from five different origins (RSD[a] %, *n*=3).

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
1	12.24±2.38	26.68±4.31	118.83±2.11	19.12±3.28	38.56±2.78
2	246.71±1.91	19.92±0.61	467.70±3.55	c	22.46±2.80
3	63.39±2.80	44.23±1.24	116.37±2.70	33.63±2.89	17.97±4.16
4	478.31±1.44	582.11±4.60	2916.09±4.82	319.45±2.20	536.75±2.67
5	31.45±4.71	c	109.64±0.95	b	10.87±0.10
6	24.48±3.65	40.90±6.56	124.59±2.38	108.79±2.95	55.36±6.14

[a]RSD (%) = (SD/mean) × 100.b Under the limit of detect.c Out of linear range.

Method validation

The linearity, LODs, LOQs, intra-day and inter-day precision, stability, and accuracy of the LC-MS method for the quantitation of six saponins 1–6 in *A. japonica* were determined (Table 2). The result indicated excellent correlations between the concentrations of references 1–6 and their peak areas within the test ranges. The internal standard method was used for quantification. Ginsenoside Rg1 was employed as an internal standard because it was chemically comparable to reference compounds1–6 and had a similar ionization pattern in the negative ion mode but is not contained in *A. japonica*. The LODs and LOQs ranged from 15.12 to 86.40 ng/mL and from 43.20 to 311.04 ng/mL, respectively.

The intra-day and inter-day variations of compounds 1–6 were in the ranges of 0.49 to 2.87% and 2.94 to 5.81%, respectively. The recoveries were between 97.09 to 104.73% with the RSD between 3.06 and 7.87%. These results demonstrated the sensitivity, reproducibility, and accuracy of the established LC-MS approach for the quantitative study of triterpenoid saponins 1–6.

DISCUSSION

The established LC-ESI-MS method was applied to five samples (1–5) of *A. japonica* for the simultaneous quantitative analysis of six triterpenoid saponins (1–6). Saponins (1–6) were identifiedby comparing their retention times and MS spectra with those of the reference saponins (Figure 2, Table 3). Among these compounds, 3 and 4 are a pair of saponins with similar retention times, the structures of which differed only in the terminal sugar, which was a xylopyranosyl or a rhamnopyranosyl moiety, respectively. The retention time of 3 with the xylopyanosyl moiety was shorter than 4 with the rhamnopyranosyl moiety, which was consistent with our prior work.[16] The contents of triterpenoid saponins (1–6) were calculated from the corresponding calibration curve. Three parallel analyses of each sample's mean values and standard deviations were summarized (Table 4). The results exhibited that the contents of 1–6 in plants samples from different regions of China varied considerably, which might be due to the different plant origins and growth circumstances. All compounds 1–6 could be detected in samples 1, 3, and 5, and the content of compound 4 is highest. Compound 5 was out of the linear range in sample 2 and not detected in sample 4, while compound 2 was out of the linear range in sample 4.

CONCLUSION

Six triterpenoid saponins in *A. japonica* were simultaneously quantified in the present study utilizing a feasible LC-ESI-MS technique in SIM mode. The precision, repeatability, stability, and accuracy of the established method were verified. Using this method, the content of six triterpenoid saponins in *A. japonica* collected from five distinct origins was investigated. The contents of triterpenoid saponins in *A. japonica* from different regions of P. R. China differed significantly. The proposed method could be further utilized in the quality control of *A. japonica*.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

ACN: Acetonitrile; **CID:** Collision-induced dissociation; **ESI:** Electrospray ionization; **EtOH:** Ethanol; **FA:** Formic acid; **HPLC-ELSD:** High-performance liquid chromatography-evaporative light scattering detection; **HPLC-UV:** High-performance liquid chromatography-ultraviolet detection; **HPLC-PDA:** High-performance liquid chromatography-photodiode array detection; **LC-MS:** High-performance liquid chromatography-mass spectrometry; **LOD:** Limit of detection; **LOQ:** Limit of quantitation; **R^2:** Coefficient of correlation; **RSD:** Relative standard deviation; **RP:** Reversed phase; **MeOH:** Methanol; **TFA:** Trifluoroacetic acid; **TIC:** Total-ion chromatogram; **SIM:** Selected ion monitor; **S/N:** Signal-to-noise ratios.

SUMMARY

A quick, easy, and reliable method was constructed and validated by LC-ESI-MS in SIM mode for the simultaneous quantitative analysis of six triterpenoid saponins (1–6) in *A. japonica*. The validation study discovered that the developed method was accurate, precise, recoverable, and sensitive for quantifying the six compounds. The established method was further successfully applied to quantify these saponins in plant samples from five distinct regions of P. R. China. The present study developed a highly efficient analytical technique for quantifying triterpenoid saponins in *A. japonica*, which could be used in the quality control of *A. japonica* from various regions.

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