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Simultaneous determination of five triterpenoid saponins in different parts of *Lonicera macranthoides* by RRLC-MS/MS method

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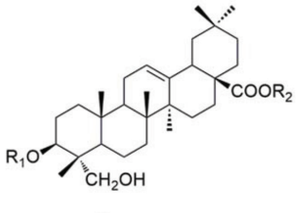
A rapid resolution liquid chromatography-tandem mass spectrometry (RRLC-MS/MS) method was developed and validated for the determination of five major saponins (macranthoidin B, macranthoidin A, dipsacoside B, akebiasaponin D, and dipsacoside A) in the flower bud, stem, and leaf parts of *Lonicera macranthoides*. Chromatographic separation was performed on a ZORBAX SB-C_{18} column (2.1 × 50 mm, 1.8 μm). Acetonitrile and 0.1% aqueous formic acid were adopted as mobile phase. Detection was carried out on a triple quadrupole mass spectrometer in the negative ion mode using an electrospray source. Multiple reaction monitoring (MRM) mode was employed. The established method showed good linearity ($r^2 \geq 0.9994$) for all the analytes within the test ranges and the recoveries were 95.19–103.28% . Desirable intra-day and inter-day precision as well as repeatability were obtained with relative standard deviations (RSDs) less than 5%. The method was simple, sensitive, accurate and performed well in application to the sample determination within a short analysis time of 15 min. The saponin profiles of different parts of *Lonicera macranthoides* were obtained based on the quantitative data, showing that the flower bud contained much higher level of saponins than the stem and leaf by several orders of magnitude, and that the quantity ratios varied remarkable between these three part. The conclusions might provide scientific evidences for the reasonable application of *Lonicera macranthoides*, and the proposed RRLC-MS/MS method might be useful for the quality control of this medicinal plant.

1. Introduction

Lonicera macranthoides Hand.-Mazz. (Caprifoliaceae) is an important medicinal plant in China. It is widely distributed in the southwest areas of China such as Hunan, Guizhou and Sichuan Provinces. The flower bud combined with those of other three *Lonicera* species, i.e. *L. hypoglauca* Miq., *L. confusa* DC. and L. *fulvotomentosa* Hsu et S.C.Cheng, are recorded in the Chinese Pharmacopoeia (ChP) as Flos Lonicerae (FL, Shanyinhua in Chinese), which is used for treating exopathogenic wind-heat, carbuncles, boils, erysipelas, and some infectious diseases (Chinese Pharmacopoeia Committee 2015). However, the stem and leaf are not officially recognized as active parts and ultimately discarded by the herbal drug market.

Chemical investigations into *L. macranthoides* mainly disclosed four types of constituents: organic acids, iridoids, flavonoids, and triterpenoid saponins (Chen et al. 2008, 2012; Jia et al. 2008; Liu et al. 2012, 2014; Sun et al. 2012). Among them, saponins were found to possess various pharmacological effects, such as anti-inflammatory (Guan et al. 2014), antitumor (Guan et al. 2012; Wang et al. 2009), antioxidant (Guan et al. 2013), and hepatoprotective activities (Shi and Liu 1996). Several analytical methods have been published for the quantification of saponins in FL, including HPLC coupled with evaporative light scattering detector (ELSD, Chai et al. 2005; Chen et al. 2007) or time-of-flight (TOF) MS (Ren et al. 2008), capillary HPLC with single quadrupole MS (Chen et al. 2007), and so on. Due to the high content and significant bioactivity, two major saponins macranthoidin B and dipsacoside B were used as chemical markers for the quality control of FL (Chinese Pharmacopoeia Committee 2015). However, little is known about the distribution of saponins in the stem and leaf of *L. macranthoides*.

The aim of the present study was to develop an advanced rapid resolution liquid chromatography-tandem mass spectrometry (RRLC-MS/MS) method for the simultaneous identification and determination of five saponins (macranthoidin B, macranthoidin A, dipsacoside B, akebiasaponin D, and dipsacoside A) in batches of flower bud, stem and leaf samples of *L. macranthoides*. Their structures are shown in Fig. 1. Here, a triple quadrupole MS with multiple reaction monitoring (MRM) mode was employed to enhance the sensitivity and selectivity of the method and, by that, reliability of the results. Jujuboside A was used as internal standard (IS). Furthermore, the saponin profiles of different parts of *L. macranthoides* were compared so as to provide scientific evidences for reasonable application of this medicinal plant.



	R_1	R_2
Macranthoidin B	Glc(1→4)Glc(1→3)Rha(1→2)Ara	Glc(1→6)Glc
Macranthoidin A	Glc(1→3)Rha(1→2)Ara	Glc(1→6)Glc
Dipsacoside B	Rha(1→2)Ara	Glc(1→6)Glc
Akebiasaponin D	Ara	Glc(1→6)Glc
Dipsacoside A	Rha(1→2)Ara	H

Fig.1: Chemical structures of the five investigated saponins in *L. macranthoides*

2. Investigations, results and discussion

2.1. Optimization of extraction conditions

To obtain satisfactory extraction efficiencies, the extraction conditions (extraction methods, extraction solvents, and extraction time) were optimized. The results indicated that ultrasonic extraction was simpler and more effective than reflux extraction. It was also found that among the tested different concentrations (25%, 50%, 75%, and 100%, v/v) of methanol, 50% methanol was the most efficient extraction solvent. In addition, the samples were extracted for different times (30, 45, 60, and 90 min), and it was demonstrated that the target compounds could be extracted completely within 45 min. Therefore, the sample solutions were prepared by ultrasonic extraction with 50 mL 50% methanol for 45 min.

2.2. Optimization of chromatographic conditions

In order to achieve a rapid and efficient analysis, a short column (Agilent ZORBAX SB-C_{18}, 2.1 × 50 mm) with 1.8 μm particle packing was employed in the RRLC system. Various mobile phase systems (methanol–water, acetonitrile–water, methanol–acid aqueous solution, and acetonitrile–acid aqueous solution) were tested in order to obtain suitable chromatographic behavior and appropriate ionization. The result showed that acetonitrile–formic acid aqueous solution was better. Moreover, the concentrations of formic acid (0.1%, 0.2%, and 0.3%, v/v) were also investigated. Finally, acetonitrile–0.1% aqueous formic acid was chosen as the eluting solvent system as it offered good separation efficiency and ionization intensity within a run time of 15 min.

2.3. Optimization of mass spectrometric conditions

Mass spectra were studied in both positive and negative modes. The negative ion mode was selected because it resulted in higher ionization efficiencies of the compounds. For data acquisition, the MRM mode was eventually adopted as it had great advantages in reducing interference and enhancing sensitivity. Fragmentor voltage (FV) and collision energy (CE), which played important roles in precursor and product ion responses respectively, were optimized for each analyte to achieve the most abundant, specific, and stable transitions. The retention time (RT) and MS information of the investigated saponins are shown in Table 1.

2.4. Method validation

2.4.1. Calibration curves, limit of detection (LOD), and limit of quantification (LOQ)

For the calibration curves, at least five concentrations of standard solutions were injected in triplicate. All calibration curves were constructed from the peak area ratio of the tested saponins to that of IS *versus* their concentrations. The LODs and LOQs of each analyte were determined at signal-to-noise values (S/N) of 3 and 10, respectively. As a result, the calibration curves exhibited good linearity ($r^2 \geq 0.9994$) within the test ranges, and the LODs and LOQs were less than 4.0 ng/mL and 8.0 ng/mL, respectively (Table 2).

2.4.2. Precision

The precision of the method was evaluated in terms of intra- and inter-day variations. For the intra-day test, the mixed standard solutions

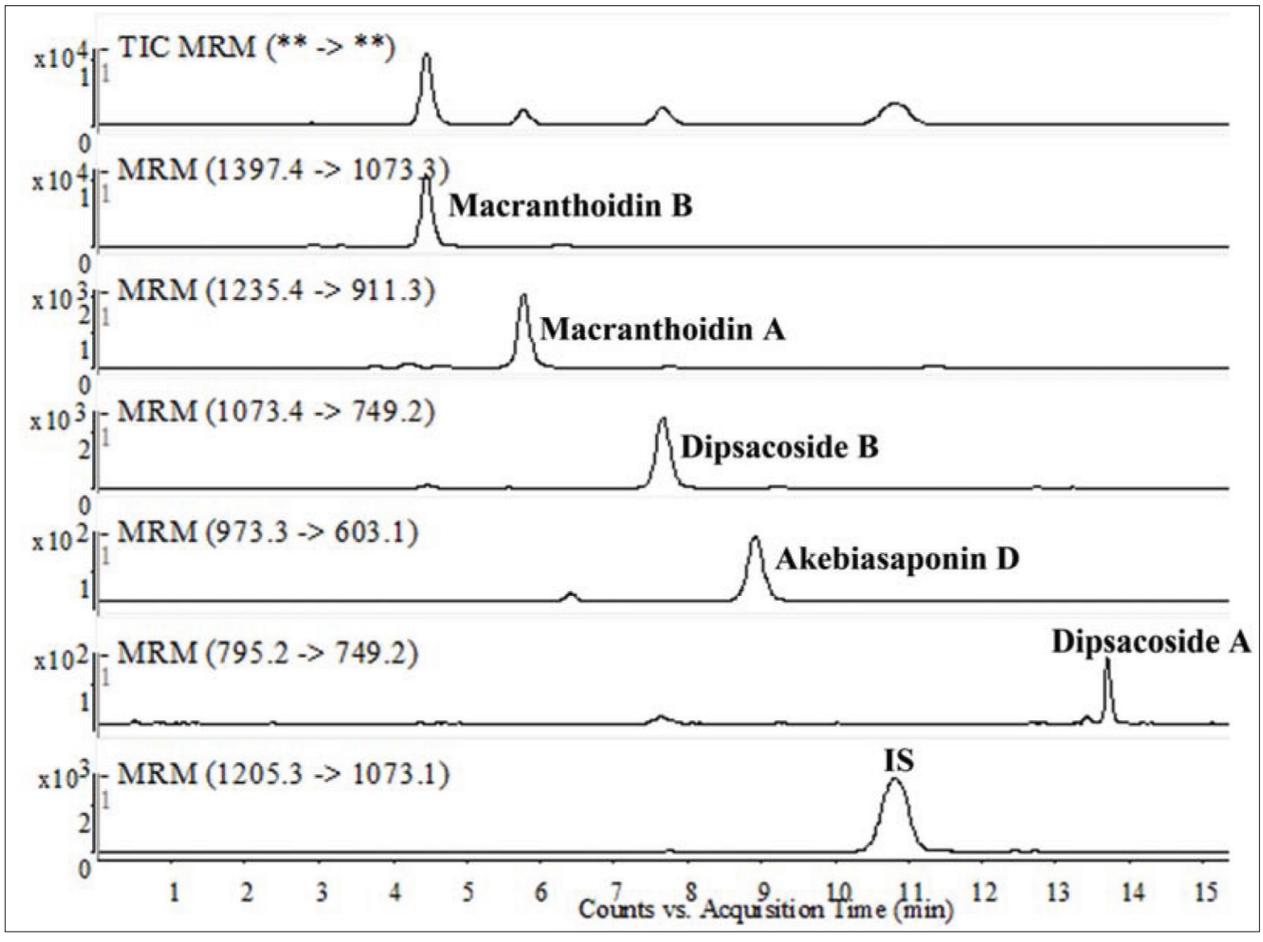


Fig.2: MRM chromatograms of the five saponins in *L. macranthoides* sample solution (FL-11). The concentration of IS was 100 μg/mL.

Table 1: Retention time and related MS information of the target compounds

Compounds	RT (min)	Precursor ion (*m/z*)	Product ion (*m/z*)	FV (V)	CE (V)
Macranthoidin B	4.45	1397.4 $[M - H]^-$	1073.3	140	53
Macranthoidin A	5.78	1235.4 $[M - H]^-$	911.3	185	35
Dipsacoside B	7.66	1073.4 $[M - H]^-$	749.2	166	44
Akebiasaponin D	8.92	973.3 $[M + HCOO]^-$	603.1	146	55
Dipsacoside A	13.70	795.2 $[M + HCOO]^-$	749.2	135	23
Jujuboside A (IS)	10.79	1205.3 $[M - H]^-$	1073.1	135	20

Table 2: Regression equation, LOD, and LOQ of the five investigated saponins

Compounds	Regression equation	r^2	Linear range (ng/mL)	LOD (ng/mL)	LOQ (ng/mL)
Macranthoidin B	y = 0.1159x + 0.0039	0.9997	8.0–20034	4.0	8.0
Macranthoidin A	y = 0.2282x + 0.0027	0.9999	3.9–3900	1.9	3.9
Dipsacoside B	y = 0.4264x + 0.0099	0.9995	3.7–9238	1.8	3.7
Akebiasaponin D	y = 0.6240x – 0.0003	0.9994	1.8–1835	0.72	1.8
Dipsacoside A	y = 1.1062x – 0.0005	1.0000	0.8–203	0.32	0.81

Table 3: Precision, stability, and repeatability

Compounds	Precision RSD (%)		Stability RSD (%, n = 6)	Repeatability (%, n = 6)
	Intra-day (n = 6)	Inter-day (n = 3)		
Macranthoidin B	1.05	2.00	1.99	3.43
Macranthoidin A	2.15	3.44	2.26	2.75
Dipsacoside B	0.86	1.90	2.14	1.73
Akebiasaponin D	1.46	3.21	3.02	3.57
Dipsacoside A	1.99	3.58	3.79	4.03

Table 4: Recovery of the five investigated saponins (n=3)

Compounds	Original (mg)	Spiked (mg)	Detected (mg)	Recovery (%)	RSD (%)
Macranthoidin B	16.723	13.097	29.119	97.65	2.11
	16.459	16.371	33.266	101.33	3.35
	16.317	19.645	34.822	96.83	1.20
Macranthoidin A	1.622	1.262	2.772	96.12	3.58
	1.596	1.578	3.129	98.58	2.64
	1.582	1.893	3.442	99.05	3.02
Dipsacoside B	1.469	1.241	2.777	102.47	2.28
	1.446	1.551	3.021	100.81	3.44
	1.434	1.861	3.403	103.28	2.95
Akebiasaponin D	0.151	0.130	0.272	96.80	3.72
	0.149	0.163	0.297	95.19	2.09
	0.147	0.195	0.333	97.37	3.85
Dipsacoside A	0.0162	0.0131	0.0298	101.71	2.98
	0.0160	0.0164	0.0313	96.60	1.65
	0.0158	0.0197	0.0340	95.77	2.43

were analyzed six times within one day; while for the inter-day test, the standard solutions were examined in triplicate on three consecutive days. The relative standard deviations (RSDs) for the intra- and inter-day assays were less than 2.15% and 3.58%, respectively (Table 3).

2.4.3. Stability and repeatability

The stability was tested using a sample solution, which was stored at room temperature and analyzed at 0, 2, 4, 8, 12, and 24 h. To confirm the repeatability, six replicates of the same sample were extracted and analyzed. The concentration of each solution was determined by a calibration curve produced on the same day. The stability RSDs ranged from 1.99% to 3.79% within 24 h, and the repeatability RSDs were between 1.73% and 4.03% (Table 3).

2.4.4. Accuracy

The accuracy was determined by recovery test. The proposed method was applied to the samples blended with standard solutions at low, middle and high concentration levels (approximately

Table 5: Contents of five investigated saponins in flower bud, stem, and leaf samples of *L. macranthoides*

Sample code	Part	Source	Content (μg/g)				
			Macranthoidin B	Macranthoidin A	Dipsacoside B	Akebiasaponin D	Dipsacoside A
FL-1	Flower bud	Changsha, Hunan	63671.12	5234.93	5949.09	245.02	26.71
FL-2	Flower bud	Zhongfang, Hunan	61864.35	5724.53	4981.00	192.00	25.11
FL-3	Flower bud	Longhui, Hunan	73635.08	6525.43	7646.81	446.21	30.55
FL-4	Flower bud	Longhui, Hunan	49909.44	5448.32	6506.84	686.89	210.00
FL-5	Flower bud	Zunyi, Guizhou	73368.53	6898.33	8377.76	424.27	37.87
FL-6	Flower bud	Zunyi, Guizhou	53153.94	9870.86	7051.68	409.01	105.48
FL-7	Flower bud	Suiyang, Guizhou	88030.8	9511.29	12739.87	618.39	22.51
FL-8	Flower bud	Chengdu, Sichuan	82753.66	9608.94	11803.83	264.29	134.45
FL-9	Flower bud	Wulong, Chongqing	72995.59	6329.58	6752.63	244.15	29.14
FL-10	Flower bud	Xiushan, Chongqing	88846.37	7734.14	8255.08	505.13	45.23
FL-11	Flower bud	Xiushan, Chongqing	64367.25	6241.73	5655.01	582.73	62.51
FL-12	Flower bud	Yongjia, Zhejiang	72790.44	6254.77	8028.10	341.68	20.66
FL-13	Flower bud	Ziyuan, Guangxi	80506.1	5882.55	8011.67	700.07	60.71
FL-14	Flower bud	Yulin, Guangxi	61823.61	5547.10	6288.62	579.96	103.69
SL-1	Stem	Longhui, Hunan	0.088	0.039	0.107	0.034	0.070
SL-2	Stem	Longhui, Hunan	0.072	0.12	0.16	0.025	0.18
SL-3	Stem	Zunyi, Guizhou	0.060	0.041	0.20	0.10	2.90
SL-4	Stem	Zunyi, Guizhou	0.15	0.087	0.22	0.32	0.44
SL-5	Stem	Xiushan, Chongqing	0.065	0.043	0.34	0.15	0.53
SL-6	Stem	Xiushan, Chongqing	0.033	0.050	0.15	0.078	0.16
LL-1	Leaf	Longhui, Hunan	50.03	6.97	4.82	0.95	0.20
LL-2	Leaf	Longhui, Hunan	0.29	0.143	0.085	0.014	0.048
LL-3	Leaf	Zunyi, Guizhou	0.11	0.048	0.054	0.018	0.030
LL-4	Leaf	Zunyi, Guizhou	10.03	1.06	0.34	0.048	0.071
LL-5	Leaf	Xiushan, Chongqing	0.060	tr[a]	0.38	0.033	0.41
LL-6	Leaf	Xiushan, Chongqing	0.079	tr	0.075	0.024	0.13
LL-7	Leaf	Xiushan, Chongqing	1.33	0.16	0.21	0.029	0.038

[a] Less than the LOQ

equivalent to 0.8, 1.0 and 1.2 times the concentration in the sample, respectively) in three replicates. The average recoveries were calculated by the formula: Recovery (%) = (detected amount – original amount) / spiked amount × 100%. The recoveries for all the analytes varied between 95.19% and 103.28%, with RSDs less than 3.85% (Table 4).

These results indicated that the validated method is precise, accurate and sensitive enough for the simultaneous quantification of all of the samples.

2.5. Quantitative analysis of samples

The developed method was subsequently applied to the determination of *L. macranthoides* samples collected from different regions in China: 14 batches of flower bud samples, 6 batches of stem samples, and 7 batches of leaf samples. The contents of each analyte were calculated by internal standard method using the respective calibration curves. The typical MRM chromatograms are shown in Fig. 2, and the quantitative data are listed in Table 5. It was observed that all the saponins examined were abundantly present in the flower bud samples with the total contents in the range of 62761.49–110922.86 μg/g, which were several orders of magnitude higher than those in stem (0.34–3.30 μg/g) and leaf (0.26–62.97 μg/g) samples. And that the quantity ratios of these saponins in the three parts were markedly different. For example, the flower bud part contained higher level of macranthoidin B (mean value: 70551.16 μg/g), followed by dipsacoside B (mean value: 7717.71 μg/g) and macranthoidin A (mean value: 6915.18 μg/g). While, in the stem part, dipsacoside A and dipsacoside B were relatively abundant, and the mean values were 0.71 and 0.20 μg/g, respectively. However, the contents of saponins in the leaf part varied substantially among samples, and there was not significant regularity. Since the composition and contents of bioactive compounds influence the pharmacological activities of herbal medicine directly, it is reasonable to some extent to use the flower bud as an active part of *L. macranthoides* rather than the stem and leaf parts based on the results of present study.

2.6. Conclusions

As an important type of components, triterpenoid saponins contribute significantly to the bioactivity of *L. macranthoides*. But their distribution variations in different parts of the medicinal plant have been unknown. Here, we reported a RRLC-MS/MS method for simultaneous determination of five major saponins in several flower bud, stem and leaf samples of *L. macranthoides*. It was found that there were great differences in the contents and quantity ratios of these saponins between the three parts, which might provide scientific evidence for the reasonable application of this medicinal plant. Since the proposed method was sensitive, accurate, reliable, and took shorter analysis time than the previously reported methods, we expect that it might be used for the standardization and quality control of *L. macranthoides*.

3. Experimental

3.1. Reagents and materials

The reference compounds of macranthoidin B, macranthoidin A, dipsacoside B, akebiasaponin D, dipsacoside A, and jujuboside A (IS) were supplied from the National Institutes for Food and Drug Control (Beijing, China). HPLC grade acetonitrile, methanol, and formic acid were purchased from Fisher Scientific Products (Fair Lawn, NJ, USA). Water was prepared using a Milli-Q academic water purification system (Millipore, Bedford, MA, USA). The flower bud, stem and leaf samples of *L. macranthoides* were collected from different regions in China and authenticated by Professor Ji Zhang, Institute for Control of Chinese Traditional Medicine and Ethnic Medicine, National Institutes for Food and Drug Control (Beijing, China).

3.2. Instruments and conditions

The analysis was performed on an Agilent series 1200 RRLC system (Agilent, USA) equipped with a degasser, quaternary pump, autosampler and thermostatted column compartment. Chromatographic separation was carried out at 30 °C on an Agilent ZORBAX SB-C_{18} column (2.1 × 50 mm, 1.8 μm). The mobile phase consisted of 0.1% formic acid aqueous solution (A) and acetonitrile (B) using a gradient elution of 25–25% B at 0–10 min, 25–60% B at 10–12 min, and 60–60% B at 12–15 min. The flow rate was set at 0.3 mL/min, and the injection volume was 5 μL. All MS experiments were conducted on a 6410B triple quadrupole mass spectrometer (Agilent, USA) in the negative mode using an ESI source. The ionization source conditions were as follows: desolvation temperature, 350 °C; desolvation gas flow, 600 L/h; nebulizer pressure, 30 psi; capillary voltage, 4000 V. Detection was carried out in multiple reaction monitoring (MRM) mode. All data were processed using MassHunter Workstation software (V.7.0 Quantitative Analysis; Agilent, USA).

3.3. Procedures for standard and sample solution preparations

3.3.1. Standard solutions

A stock standard solution containing macranthoidin B (50.08 μg/mL), macranthoidin A (9.75 μg/mL), dipsacoside B (9.24 μg/mL), akebiasaponin D (3.67 μg/mL), and dipsacoside A (1.02 μg/mL) were prepared using 50% methanol as solvent. The stock solution was appropriately diluted by 50% methanol to obtain a series of working solutions, to which jujuboside A (IS) was added to give a final concentration of 100 μg/mL. All the solutions were stored at 4 °C and brought to room temperature before use.

3.3.2. Sample solutions

The plant materials were powdered and passed through a 40-mesh sieve. Each powder (0.5 g) was accurately weighed and ultrasonically extracted with 50% methanol (50 mL) for 45 min, and then cooled to room temperature. Fifty percent methanol was added to compensate for the loss of weight, and then the solution was filtered. For the analysis of flower bud samples, 100 μL of the filtrate was diluted by 50% methanol in a 10 mL volumetric flask and jujuboside A (IS) was added to get a final concentration of 100 μg/mL. For the analysis of stem and leaf samples, 20 mL of the filtrate was concentrated to dryness *in vacuo* at 45 °C, and the residue was dissolved with 50% methanol in a 10 mL volumetric flask, and the final concentration of IS was 100 μg/mL. All resultant solutions were filtered through a 0.22 μm Nylon filter before RRLC-MS/MS analysis.

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