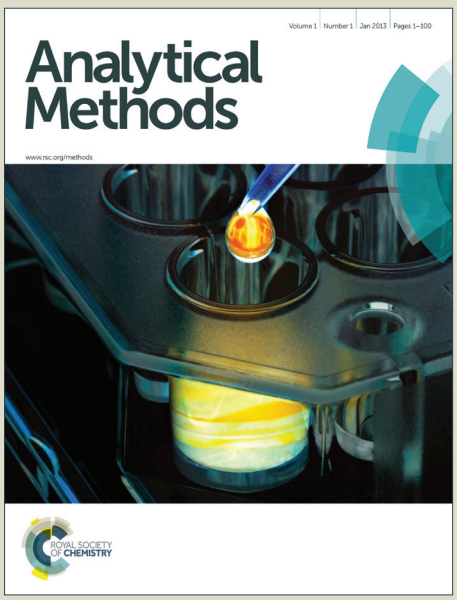
Analytical Methods

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/methods

Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

ARTICLE TYPE

Study on the chemical markers of Caulis Lonicerae Japonicae for quality control by HPLC-QTOF/MS/MS and chromatographic fingerprints combined with chemometrics methods

Yi-Da Zhang, Xi Huang, Fang-Li Zhao, Yuan-Lin Tang and Lian Yin*,

Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX
DOI: 10.1039/b000000x

Caulis Lonicerae Japonicae (CLJ) has been used in China for centuries. In order to achieve the chemical markers that can reflect the inherent quality of it, a simple and reliable HPLC-fingerprint combined with chemometrics method has been developed. 36 batches of CLJ samples collected with different deposited years, at different harvesting season and from various cultivation locations were analyzed under the optimized HPLC conditions. First, to obtain the more convincing chemical makers, the differences on chemical compositions between CLJ and Flos Lonicerae Japonicae (FLJ) were analyzed by HPLC-QTOF-MS/MS mass spectrometry. A total of 42 major constituents were screened and identified with no doubt, and the fragmentation patterns of some iridoid glycosides, like L-phenylalaninosecologanin, grandifloroside, etc, are explained for the first time. After that, based on these fingerprints data of CLJ, different chemometrics analysis methods were applied to evaluate the intrinsic quality of it and achieve the most responsible chemical markers on the discrimination of the different qualities. Finally, through the comprehensive analysis from the different perspectives, seven chemical compounds, 5-caffeoylquinic acid, 1, 3-O-dicaffeoylquinic acid, epi-loganic acid, sweroside, loganin vogeloside and grandifloroside have been taken as chemical markers to control the quality of Caulis Lonicerae Japonicae. Our study revealed an approach that has helped in guiding for the quality control of Caulis Lonicerae Japonicae, as well as traditional Chinese medicine.

1. Introduction

Traditional Chinese medicine (TCM) has been used in China for thousands of years and during the recent decades, TCMs also have been gradually accepted by western societies as alternative medicines or health products for their good safety and low toxicity. However, owing to the fact that there are hundreds of complex active components in TCMs and most of them are unidentified, uncharacteristic and undetectable, how to evaluate the efficacy and safety of botanical drugs has always been an intensely focused issue all over the world.[1,2] To conquer the uprising problem of TCMs, the World Health Organization, the European Medicine Agency, the American Food and Drug Administration, and the Chinese State Food and Drug Administration have accepted fingerprint technology as a methodology for the assessment of natural products.[3-5] As we know, fingerprint chromatograms are complex multivariate data sets due to the complexity of herbal medicines, so minor difference between very similar chromatograms might be missed and it would be hard to find the index component which plays an important role on the quality control of TCM. Thus, the chemical pattern recognition methods, such as similarity analysis (SA), hierarchical cluster analysis (HCA), principal component analysis (PCA) and partial least squares discrimination analysis (PLS-DA) etc, should be taken into the consideration for quality control and analytical markers exploration of the TCMs.[6,7]

Lonicera japonica Thunb. (LJT) is a commonly used medicinal plant that plays an important role on the clinic of TCM. Flos Lonicerae Japonicae (FLJ, named Jinyinhua in Chinese), the dried flower bud of LJT, has been used for the treatment of the affects of exopathogenic wind-heat or epidemic febrile diseases, sores and furuncles in hundreds of years. Likewise, Caulis Lonicerae Japonicae (CLJ), derived from the dried caulis of LJT, has also long been used for the treatment of acute fever, headache, respiratory infection and epidemic diseases with Chinese name Rendongteng (Chinese Pharmacopoeia, 2010). Since they are from the different parts of the same plant, phytochemical investigations have discovered that phenolic acids and iridoid glycosides were the common constituents in both of CLJ and FLJ, like chlorogenic acid, di-caffeoylquinic acids, loganin, sweroside, etc, while most flavonoids were observed only in FLJ and hardly detected in CLJ, like lutin, cynaroside, hyperoside, and so on.[8] Although they are partly similar on the types of chemical composition, they have different pharmacological effects. Modern pharmacological studies have elucidated that FLJ possesses wide pharmacological actions, such as anti-bacterial, anti-inflammatory, anti-viral, hepatoprotective, anti-tumor, anti-hyperlipidemic, anti-thrombotic etc,[9] while CLJ also has good properties of anti-inflammatory, anti-edema, anti-tumor, etc and its anti-edema activity is better than that of FLJ.[10-13]

In recent years, to control the quality of TCMs, the method of simultaneously determinate multiple compounds and chromatographic fingerprint analysis has gained prominence. Up to now, FLJ has been widely studied. Many bioactive components, like chlorogenic acid, caffeic acid, 3, 5-di-O-caffeoylquinic acid, loganin, sweroside, logaic acid, rutin, hyperoside, cynaroside, akebiasaponin F, macranthoidin B, macranthoidin A, etc. were used as the index components to comprehensively control the quality of FLJ.[14-17] And chemical pattern recognition method from the data of chromatographic fingerprints was also developed to classify and identify the FLJ samples from the confused plantspecies, different origins and so on.[18-20] Nevertheless, the research about quality control of CLJ is relatively less. In the Chinese Pharmacopoeia (2010 version), only two components (chlorogenic acid and loganin) were selected as analytical markers to control the quality of CLJ, and chlorogenic acid was also the characterized component to evaluate the quality of FLJ. Thus, measuring these two particular compounds for quality

Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

ARTICLE TYPE

control of CLJ remains insufficient and unreasonable. Until now, the chemical compounds used as markers to control the quality of CLJ were similar to those for FLJ, such as chlorogenic acid, loganin, sweroside, rutin, etc.[21] Owing to the different clinical efficacy and the overall property between them, the analytical markers which could truly reflect the quality of CLJ were still not clear. So finding out the chemical markers that different from FLJ and can also comprehensively reflect the overall property of CLJ is the precondition of controlling the quality of CLJ.

In the present study, to achieve the more convincing chemical makers for quality control of CLJ, the differences on chemical compositions between CLJ and FLJ were analyzed by HPLC-QTOF-MS/MS mass spectrometry. After that, a simple and facile HPLC-fingerprint method of CLJ was developed. Then based on these fingerprint data, SA and PCA were applied to overview the distribution of all 36 batches of CLJ samples and PLS-DA was applied to construct a discrimination model of them. In addition, the characteristic components which have the most influence on separation among different samples of CLJ from diverse quality were found out with the help of loadings plots. They could be used as main chemical markers for quality control of CLJ in the future.

2. Experimental

2.1 Chemical reagents and plant materials

Methanol and acetonitrile (HPLC grade) were supplied by Tedia Company Inc. (Fairfield, USA). HPLC-grade acetic acid and water were purchased from Merck Company (Darmstadt, Germany) and C' estbon Company (Kunshan, China), respectively. Ultra Pure Water was prepared using an EPED water purification system (EPED, NJ, China). All other reagents were of analytical grade. Thirty-six batches of CLJ samples were collected from six provinces of China and with various storage time (supporting information shows in Table 1), and one FLJ sample was collected from Shandong province (batch number: 130731). All samples were authenticated by Professor De-Kang Wu and the voucher specimens were deposited in Nanjing University of Chinese Medicine, Nanjing 210038, PR China.

2.2 Preparation of samples

Approximately 1.0 g pulverized plant samples, accurately weighed, were extracted with 25 mL of 50% methanol (v/v) for 30 min by ultrasonication and cooled to room temperature; 50% methanol was added to compensate for the lost weight. After the methanol solution was filtered, the filtrate was stored at 6 °C and then the solution was subjected to HPLC-QTOF-MS analysis and the HPLC-PAD analysis after centrifugation at 10,000 rpm for 10 min.

2.3 Preparation of standard solutions

Authentic standards, Chlorogenic acid (Lot: 110753-200413) and Rutin (Lot: 100080-200707) were obtained from China National Institutes for Food and Drug Control. Caffeic acid (YLS0174) was purchased from ShangHai Yilin Bio-technology CO., LTD. And besides, the other five standards including Loganic acid (MUST-13042501), Loganin (MUST-13052813), Isochlorogenic acid A (MUST-12101101), Astragaline (MUST-12092001) and Isochlorogenic acid C (MUST-13081401) were purchased from ChengTu MUST Bio-technology CO., LTD. The purity of the 8 standards was >98% by HPLC analysis based on the normalization of peak areas. Then, the mixture standard solution was prepared by dissolving them into 50% methanol at the

Table 1 Thirty-six batches of Caulis Lonicerae Japonicae samples with different cultivation areas and at different harvest time used in the study

No.	Origin and batch number	Similarity	No.	Origin and batch number	Similarity
S1	Shandong 140225	1	S19	Shandong 130410	0.998
S2	Shandong 140309	0.954	S20	Jiangsu 130324	0.921
S3	Shandong 131113	0.903	S21	Hunan 130115	0.917
S4	Huhei 131101	0.898	S22	Anhui 121120	0.646
S5	Shandong 131022	0.847	S23	Shandong 121110	0.956
S6	Shandong 131020	0.930	S24	Shandong 120713	0.738
S7	Huhei 131020	0.902	S25	Henan 111108	0.806
S8	Shandong 131018	0.868	S26	Shandong 110111	0.627
S9	Anhui 131016	0.843	S27	Hubei 110102	0.799
S10	Huhei 131005	0.884	S28	Henan 101226	0.766
S11	Jiangsu 130801	0.995	S29	Hubei 101216	0.795
S12	Henan 130727	0.915	S30	Henan 101216	0.752
S13	Shandong 130721	0.887	S31	Henan 101207	0.813
S14	Shandong 130701	0.876	S32	Henan 101111	0.798
S15	Shandong 130615	0.903	S33	Hubei 101106	0.767
S16	Henan 130602	0.857	S34	Shandong 101106	0.77
S17	Shandong 130601	0.994	S35	Shandong 100918	0.784
S18	Shandong 130519	0.988	S36	Shandong 100826	0.767

Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

ARTICLE TYPE

concentration of 1.15, 0.68, 0.315, 1.50, 1.23, 0.42, 0.16 and 0.58 mg ml^{-1}. This mixed reference solution was used for qualitative analysis to identify compounds in HPLC-QTOF-MS analysis and chromatographic fingerprint.

2.4 Instrumentation and Chromatographic Conditions

2.4.1 HPLC analysis. The HPLC system (Waters 2695 Alliance HPLC system, Waters, Milford, MA, USA) consisted of a quaternary pump, an online-degasser AF, a 20 µl loop manual injector and a photodiode array detector (Waters 2998). The chromatographic separation was performed on a Thermo C_{18} ODS HYPERSIL column (4.6 mm × 150 mm, 5 µm) at 35 °C. A mixture of solvent A (1.5% glacial acetic acid; v/v) and solvent B (acetonitrile) was used as the mobile phase at a flow rate of 0.8 mL min^{-1}. The gradient elution program was: 0–15 min, 5–10% B; 15–20 min, 10% B; 20–35 min, 10–16% B; 35–55 min, 16–20% B; 55–60 min, 20–80% B; 60–70 min, 80% B; 70–75 min, 80–5% B. Then this was followed by 15 min equilibrium period prior to the injection of next sample. The injection volume was 10 µL. The PDA spectra were recorded from 210 to 400 nm, and the chromatograms were monitored at 254 nm.

2.4.2 HPLC/ESI-MS/MS. For LC/ESI-MS/MS analysis, a LC-20a Shimadzu HPLC system was coupled to the orthogonal AB SCIEX Triple TOFTM 5600 mass spectrometry equipped with electronic spray ionization (ESI) source. The HPLC conditions used for HPLC-ESI-MS analysis were the same conditions in Section 2.4.1. ESI-MS spectra were acquired in negative ion mode for the full-scan MS analysis, the spectra was recorded in the range of *m/z* 50 to 1500. The conditions of MS analysis were designed as follows: capillary voltage, 2800 V; the source temperature 100 °C; the cone voltage, 20 V; Data collected mode, Dynamic Background Deduction and Information Dependent Acquisition; MCR detection voltage, 2100 V; collision energy, 10 V; Spray voltage, 20 V; Nebulizer, 55 psi; Aux Gas Pressure, 60 psi; Curtain Gas, 40 psi; IS 20 V; Desolvation Gas Flow 450 (L/HR); Desolvation Temp 250 °C; Injection volume, 10 µL; Detector, Time Of fly; Declisting potential, –70 V.

2.5 Data processing

In this study, the Mass spectrometry was performed using Analyst TF 1.6 software and the analysis of data was controlled by Peakview software. Each chromatogram was exported to the form of AIA (*.cdf) files from Waters Empower 1 HPLC workstation (Waters, Milford, U. S. A). Then, all these AIA (*.cdf) files were introduced to professional software named *Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine* composed by Chinese Pharmacopoeia Committee (Version 2004 A) (Beijing, China). PCA and PLS-DA were performed on SIMACA-P+ 11.5 Demo (Umetrics AB, Sweden) software.

3. Results and discussion

3.1 HPLC fingerprints of CLJ

3.1.1 Optimization of HPLC conditions. In order to establish an accurate, valid and optimal HPLC fingerprint of CLJ, different HPLC parameters including mobile phase, elution gradient, column temperature and detection wavelength were investigated in this study. Different elution conditions (methanol–water–acetic acid, acetonitrile–water and different concentrations of acetic acid in water) were firstly studied to get the most suitable mobile phase. The results showed that acetonitrile–water/acetic acid (1.5%; v/v) system was the optimal mobile phase system with best baseline and the most satisfactory resolution of major peaks. Meanwhile, the linear gradient (sees Section 2.4.1) was applied in HPLC procedure considering the weakness (having long retention times and poor resolution) of isocratic elution mode. Varying the ratios of water/acetic acid and acetonitrile in the mobile phase provided a significant improvement in separation by yielding narrow and high-resolved peaks. The effects of temperature and flow rate were also investigated, and the results indicated that 35 °C and 0.8 ml min^{-1} were found to be optimal parameters. Photodiode array detector (PAD) was applied to select the optimum wavelength. In consideration of the number of detectable peaks and baseline of chromatogram, 254 nm was selected as the optimum detection wavelength by investigating the spectra of all characteristic peaks. The optimal HPLC condition was shown in Section 2.4.1.

3.1.2 Validation of methodology. Reproducibility was verified by replicating HPLC injections of the same sample solution 6 times. The relative standard deviations (RSD) of retention time (t_R) and peak area (PA) of common peaks for replicated injections were lower than 0.57% and 2.76%, respectively. Precision of sample stability was determined with measurements from a single sample solution stored at room temperature for 0, 1.5, 3, 6, 12 and 24 h. And the RSDs of t_R and PA of common peaks were estimated to be no more than 0.52% and 2.77%, respectively. Finally, the method precision test was determined by analyzing six independently prepared samples. The results showed that the RSDs of t_R and PA of all the common peaks were less than 0.52% and 4.96%, respectively. Thus, all results indicated that the conditions of HPLC for the fingerprint analysis were stable and satisfactory.

3.1.3 Establishment of chromatographic fingerprint. In this study, to establish the chromatographic fingerprints of CLJ, 36 batches of CLJ samples (Table 1) were analyzed under the optimized HPLC condition (Fig. 1). In the all acquired chromatograms, 34 peaks existing in all batches of samples were assigned as common peaks (Fig. 2(a)), and they are labeled based on their elution order. Peak 18 (loganin) was selected as the reference peak because it was indicated with higher content among others, good resolution and located near the middle of the chromatogram.

3.2 The distinctive constituents of CLJ

3.2.1 Identification of the compounds in CLJ and FLJ. In order to qualitatively analyze the differences on chemical compounds between CLJ and FLJ, the CLJ (S13) sample that used for HPLC/MS analysis was selected having the same habitat, harvesting season and storage time with FLJ. Then compounds were analyzed and identified by using ESI-MS/MS technique. As most of the compounds in CLJ and FLJ are classified into phenolic acids, iridoids and flavonoids, the best analytical selectivity and sensitivity was obtained by acquiring spectrum in

Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

ARTICLE TYPE

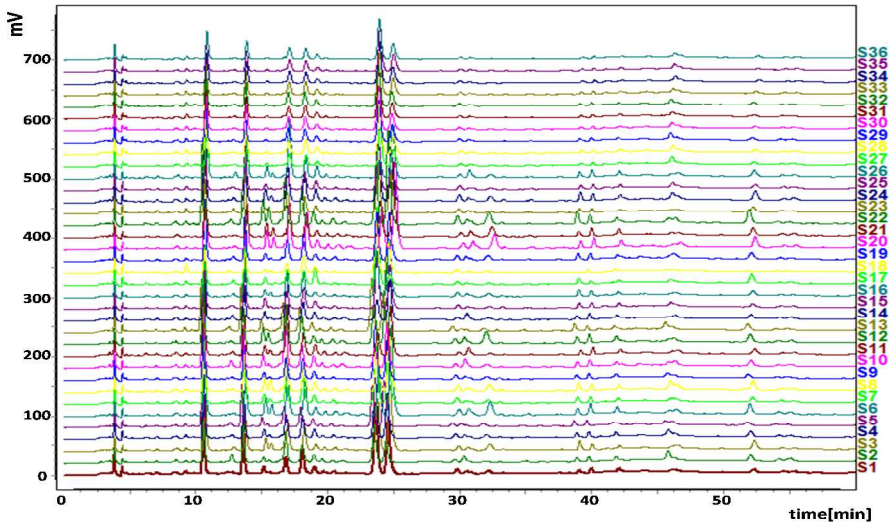


Fig. 1. The chromatographic fingerprints of 36 batches of Caulis Lonicerae Japonicae samples. The samples were coded as mentioned in Table 1

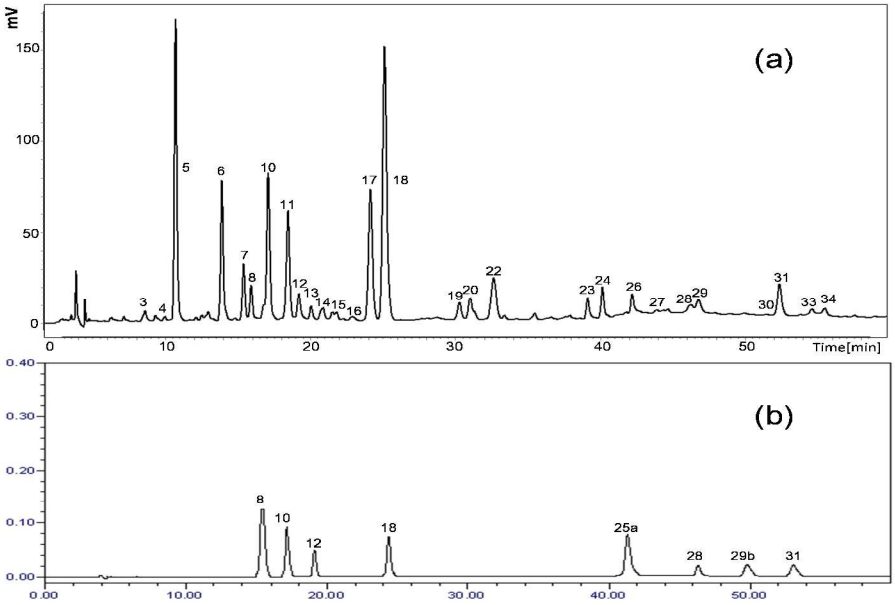


Fig. 2. (a) Representative HPLC fingerprint of Caulis Lonicerae Japonicae at 254nm (b) The chromatogram of the reference standards: (8) loganic acid; (10) 3-O-caffeoylquinic acid ; (12) caffeic acid; (18) loganin; (25a) rutin; (28) 3, 5-O-dicaffeoylquinic acid; (29b) astragalin; (31) 4, 5-O-dicaffeoylquinic acid

the negative ionizaton mode.

The total ion current (TIC) in the negative mode profiles of CLJ and FLJ extracts are presented in Fig. 3(a) and Fig. 3(b). Some compounds were characterized by comparing their HPLC retention time, UV spectra with references and the MS/MS spectra with literatures. And most compounds which did not get with reference standards were identified based on the data that reported in the literature or according to the proposed MS fragmentation mechanisms. Table 2 summarized the theoretical molecular formula of these compounds, their retention time, characteristic fragment ions, and so on. Generally, in negative ion mode of MS analysis, the deprotonated molecular ion $[M - H]^-$ corresponds to the most intense peak in the MS1 spectra. Note that in addition to the $[M - H]^-$ ions, adduct ions such as $[M + Cl]^-$ and $[M + HAc - H]^-$ were also observed in ESI-MS spectra making it much easier to determine the molecular weights of detected compounds. The specific structures of these compounds were provided in Fig. 4.

Identification of phenolic acids. In general, the structures of phenolic compounds consist of one or more caffeic acid substituent bound to a quinic acid moiety. This class of compounds showed similar UV absorptions maxima with two

bands at 230–240 nm and 320–330 nm and a shoulder at 290–300nm. Firstly, most of the detected phenolic compounds are more likely to produce the deprotonated molecular ions, $[M - H]^-$, of high abundance that allowed for MS/MS analysis. Besides, their MS/MS spectra were characterized by the collision-induced dissociation of caffeic acid moiety and quinic acid moiety, resulting in a base peak at $[M - H - \text{caffeoyl}]^-$, a marked fragment ion at $[M - H - \text{caffeoyl} - H_2O]^-$. In addition to this, the fragmentation of phenolic components and caffeic components in the TOF-MS/MS experiment tends to produce some characteristic fragment ions, such as $[\text{quinic acid} - H]^-$ at *m/z* 191, $[\text{quinic acid} - H_2O - H]^-$ at *m/z* 173, $[\text{caffeic acid} - H]^-$ at *m/z* 179, $[\text{caffeic acid} - CO_2 - H]^-$ at *m/z* 135, etc.[22]

An extracted ion chromatogram (EIC) for the molecular ions of monocaffeoylquinic acid (CQA, Mr 354) showed three distinct components (see Table 2) in both CLJ and FLJ extracts. They all gave a $[M - H]^-$ ion at *m/z* 353. Meanwhile, their MS^2 spectrums were also very similar with showing a fragment ion at *m/z* 191 as base peak. Among monocaffeoylquinic acids, compound **10** was unambiguously identified as chlorogenic acid (3-CQA) by comparison with commercial standard. Based on their characteristic fragment ions and the reference,[23,24] compound **4** and **15** were characterized as 5-CQA and 4-CQA, respectively.

In addition to the monocaffeoylquinic acids isomers, a total of five dicaffeoylquinic acids (diCQA, Mr 516) were found in the extracts of CLJ and FLJ. Compounds **5**, **27a**, **28**, **31** and **35** were identified as dicaffeoylquinic acids with the same pseudomolecular ions at *m/z* 515 and characteristic of secondary fragment ions at *m/z* 353, 191, 173, 179, 135, etc. By comparison with the standard substances and the reference,[25] compounds **28** and **31** were identified as 3, 5-O-diCQA and 4, 5-O-diCQA, respectively. According to the interpretation of fragmentation patterns about dicaffeoylquinic acids in the reference,[26,27] 1, 3-diCQA, 3, 4-diCQA and 1, 4-diCQA were the candidates for compound **5, 27a** and **35**, respectively. It is important to note that compound **50** (1, 4-diCQA) was not reported in CLJ before.

Compound **1** showed a $[M - H]^-$ ion at *m/z* 191 and its MS2 fragmentation gave fragment ions at *m/z* 173, 111 characteristic of quinic acid.[28] So it was identified as quinic acid. Compound **7** showed a $[M + HAc - H]^-$ ion at *m/z* 401, which indicates an acetate adduct that the pseudo-molecular ion was at *m/z* 341, and it dissociated to give ion at *m/z* 179 by losing a hexose sugar. Then it was tentatively identified as caffeic acid hexoside.[29] Caffeic acid (Compound **12**) was also identified by comparison of its retention time with authentic standard and the MS/MS spectra. Compound **23** displayed the deprotonated molecular ion at *m/z* 677 and its MS^2 fragmentation showed three consecutive losses of caffeoyl moieties (162 Da) at *m/z* 515, 353 and 191, which are consistent with the 3, 4, 5-triCQA in terms of literature report.[28] Compound **29** exhibited a $[M - H]^-$ ion at *m/z* 193 which suggested it would be ferulic acid or its isomer methyl caffeate. Comparing with caffeic acid, the same type of compound, it occurred at a much longer retention time. And its MS2 fragmentation showed a fragment ion $[M - COOCH_3 - H]^-$ at *m/z* 133 as base peak instead of $[M - CO_2 - H]^-$ ion at *m/z* 149. So compound **29** was acknowledged as methyl caffeate, and its MS/MS fragmentations were coincident with the reference, too.[30] According to TOF-MS data and diagnostic ions, Compound **34b**

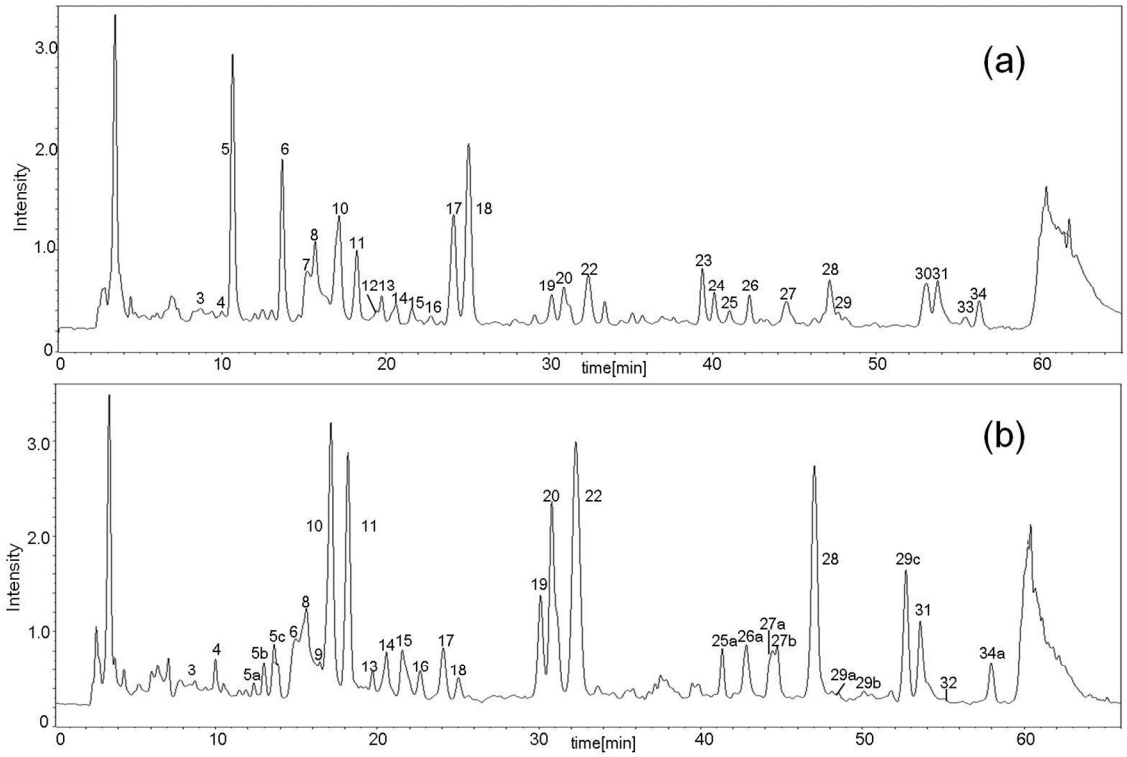


Fig.3. (a) The extract total ion chromatogram of Caulis Lonicerae Japonicae; (b) The extract total ion chromatogram of Flos Lonicerae Japonicae

Table 2 Identified results of the constituents of Caulis Lonicerae Japonicae and Flos Lonicerae Japonicae. "+" stands for the detectable compound, "–" stands for the undetectable compound.

No.	t_R /min	Molecular formula	ESI⁻ MS (*m/z*)	ESI⁻ MS/MS	Identification	CLJ	FLJ

1	4.58	$C_7H_{12}O_6$	191[M – H]$^-$	191,173,154,117	Quinic acid	+	+
2	4.65	$C_4H_6O_5$	133[M – H]$^-$	133,115,71	(±)-Malic acid	+	+
2a	5.19	$C_{16}H_{24}O_{12}$	407[M – H]$^-$	407,363,209,157	lamalbidic acid	–	+
3	8.10	$C_{17}H_{26}O_{10}$	389[M – H]$^-$	227,183,165,121	Scandoside	+	+
4	10.01	$C_{16}H_{18}O_9$	353[M – H]$^-$	191,179,135	5-O-Caffeoylquinic acid	+	+
5	10.64	$C_{25}H_{24}O_{12}$	515[M – H]$^-$	353,191,179,173	1,3-O-dicaffeoyl quinic acid	+	–
5a	12.37	$C_{16}H_{22}O_{10}$	373[M – H]$^-$	211,167,149,123	Secologanate	–	+
5b	13.01	$C_{16}H_{18}O_9$	352[M–2H]$^-$	339,229,191,157	Unidentifed	–	+
			705[2M–H]$^-$				
5c	13.61	$C_{17}H_{24}O_{11}$	463[M + HAc – H]$^-$	241,197,179,139	Kingiside	–	+
6	13.68	$C_{16}H_{24}O_{10}$	375[M – H]$^-$	213,169,151,119	8-epiLoganic acid	+	+
			411[M + Cl]$^-$				
7	15.30	$C_{16}H_{22}O_8$	401[M + HAc – H]$^-$	179,164,161,146	Caffeic acid hexoside	+	–
7a	15.45	$C_{17}H_{26}O_{11}$	465[M + HAc – H]$^-$	243,155,141,101	Morroniside	–	+
			405[M – H]$^-$				
8	15.71	$C_{16}H_{24}O_{10}$	375[M – H]$^-$	195,151,121,119	Loganic acid	+	+
9	16.90	$C_{16}H_{22}O_{11}$	389[M – H]$^-$	345,191,183,165	Secologanoside	+	+
10	17.14	$C_{16}H_{18}O_9$	353[M – H]$^-$	191,161	3-O-Caffeoylquinic acid	+	+
			707[2M – H]$^-$				
11	18.24	$C_{16}H_{22}O_{10}$	373[M – H]$^-$	193,149,119	Secologanic acid	+	+
12	19.50	$C_9H_8O_4$	179[M – H]$^-$	179,135	Caffeic acid	+	–
13	19.77	$C_{28}H_{28}O_9$	507[M – H]$^-$	507,357,327,283	Syringetin hexoside	+	+
14	20.60	–	697[M – H]$^-$	535,355,341,179	Unidentifed	+	+
			733[M + Cl]$^-$				
15	21.64	$C_{16}H_{18}O_9$	353[M – H]$^-$	191	4-O-Caffeoylquinic acid	+	+
16	22.65	$C_{17}H_{24}O_{10}$	447[M + HAc – H]$^-$	225,123,101	7-ketologanin	+	+
			423[M + Cl]$^-$				
17	24.15	$C_{16}H_{22}O_9$	417[M + HAc – H]$^-$	195,125,89	Sweroside	+	+
18	25.08	$C_{17}H_{26}O_{10}$	449[M + HAc – H]$^-$	227,127,101	Loganin	+	+
19	30.17	$C_{17}H_{24}O_{10}$	447[M + HAc – H]$^-$	225,155,111	Vogeloside	+	+
			423[M + Cl]$^-$				
20	30.91	$C_{17}H_{24}O_{11}$	403[M – H]$^-$	371,223,165,121	Secoxyloganin	+	+
21	31.24	$C_{17}H_{24}O_{10}$	447[M + HAc – H]$^-$	225,155,111	epi-vogeloside	+	+
			423[M + Cl]$^-$				
22	32.45	$C_{17}H_{24}O_{10}$	447[M + HAc – H]$^-$	225,179,155,123	Secologanin (Loniceroside)	+	+
			423[M + Cl]$^-$				
23	39.38	$C_{34}H_{30}O_{15}$	677[M – H]$^-$	515,353,191,179	3,4,5-Tricaffeoylquinic acid	+	–
24	40.10	$C_{30}H_{40}O_{18}$	687[M – H]$^-$	525,329,167	7-O-(4β-D-glucopyranosyloxy-3-	+	–
			723[M + Cl]$^-$		methoxylbenzoyl)secologanolic acid		
25	41.06	$C_{24}H_{26}O_{13}$	581[M + HAc – H]$^-$	359,341,329	Iridin	+	–
			557[M + Cl]$^-$				
25a	41.34	$C_{27}H_{30}O_{16}$	609[M – H]$^-$	609,301,271	Rutin	–	+
26	42.28	$C_{36}H_{30}O_{16}$	717[M – H]$^-$	555,359,197	Unidentifed	+	–

26a	42.75	$C_{21}H_{20}O_{12}$	463[M – H]$^-$	463,300,271	Hyperoside	–	+
26b	42.93	$C_{21}H_{20}O_{11}$	447[M – H]$^-$	447,285	Cynaroside	–	+
27	44.50	$C_{28}H_{30}O_6$	521[M + HAc – H]$^-$	521,461	Unidentifed	+	–
			497[M + Cl]$^-$				
27a	44.55	$C_{25}H_{24}O_{12}$	515[M – H]$^-$	353,335,191,179	3,4-O-dicaffeoyl quinic acid	–	+
27b	44.78	–	743[M – H]$^-$	511,479,467	Unidentifed	–	+
28	47.18	$C_{25}H_{24}O_{12}$	515[M – H]$^-$	353,191,179,135	3,5-O-dicaffeoyl quinic acid	+	+
29	47.69	$C_{10}H_{10}O_4$	193[M – H]$^-$	161,133	Methyl caffeate	+	–
29a	48.50	$C_{27}H_{30}O_{15}$	593[M – H]$^-$	593,285	Lonicerin	–	+
29b	50.31	$C_{21}H_{20}O_{11}$	447[M – H]$^-$	447,284,255	Astragalin	–	+
29c	52.70	$C_{34}H_{46}O_{19}$	757[M – H]$^-$	595,525,493,179	(E)-Aldosecologanin	–	+
			817[M + HAc – H]$^-$				
30	53.18	$C_{21}H_{24}O_{10}$	435[M – H]$^-$	273,179,167,123	Phloridzin	+	–
			471[M + Cl]$^-$				
31	53.76	$C_{25}H_{24}O_{12}$	515[M – H]$^-$	353,191,179,173	4,5-O-dicaffeoyl quinic acid	+	+
32	55.14	$C_{26}H_{35}NO_{11}$	536[M – H]$^-$	356,312,272,164	L-phenylalaninosecologanin	+	+
33	55.48	$C_{31}H_{26}O_8$	525[M – H]$^-$	167,123	Vanillic acid derivatives	+	–
34	56.30	$C_{25}H_{30}O_{13}$	537[M – H]$^-$	375,179,161,135	Grandifloroside	+	–
34a	57.99	$C_{34}H_{46}O_{19}$	757[M – H]$^-$	595,525,493,179	(Z)-Aldosecologanin	–	+
			817[M + HAc – H]$^-$				
34b	59.96	$C_{26}H_{26}O_{12}$	529[M – H]$^-$	367,353,191,179	1,3- or 3,5- or 1,5-O-dicaffeoylquinic acid methyl ester	–	+
35	60.22	$C_{25}H_{24}O_{12}$	515[M – H]$^-$	353,191,179,173	1,4-O-dicaffeoyl quinic acid	+	+
36	60.89	$C_{15}H_{10}O_6$	285[M – H]$^-$	285,151,133	Luteolin	+	–
37	61.50	$C_{18}H_{16}O_6$	327[M – H]$^-$	291,229,211,183	2-(3,4-dimethoxyphenyl)-5-hydroxy-7-methoxy-chromone	+	+
38	61.81	$C_{17}H_{14}O_7$	329[M – H]$^-$	229,211,183,171	tricin	+	+

could be tentatively identified as methylated dicaffeoylquinic acid, but the methylation position was unknown. Compound **33** exhibited [M – H]$^-$ ion at *m/z* 525, and the fragment ions at *m/z* 167 and 123 were insufficient to explain the interpretation of its fragmentation pattern. So it was temporarily authenticated as vanillic acid derivative.

Identification of flavonoids. Flavonoids were firstly characterized by two major absorption bands in the UV region: band I absorption occurring in the 330–351 nm range and band II in the 254–272 nm range. In the negative ion mode, the base peak in a full MS spectrum was assigned as the [M – H]$^-$ ion. As for the MS^2 spectrum, the glycosidic bond of O-glycosides is easily cleaved in the collision cell to generate a product ion of [M – H – 162]$^-$ corresponding to loss of a hexose sugar, or [M – H – 308]$^-$ corresponding to loss of a rutinose unit. And dehydration, successive losses of CO owing to the presence of phenolic hydroxyl groups and ketone group, Retro-Diels-Alder (RDA) fragmentation, C-ring fragmentation and loss of CHO• are also the most possible fragmentation pathways for flavonoids.[22]

In this study, seven flavonoid glycosides and three flavonoid aglycons had been discovered in total. Among the 9 flavonoids, rutin (compounds **25a**) and astragalin (compounds **29b**) were identified by comparing the t_R, UV λmax with the standard substances. The rest of flavonoid glycosides including compounds **26a**, **26b** and **29a** were then identified on the basis of their fragmentation patterns. Compounds **26a** displayed a [M – H]$^-$ ion at *m/z* 463 and its MS^2 spectra showed a fragment ion at *m/z* 300, as base peak, due to the homolytic cleavage at glycosidic bond indicating [M – glc – H]$^-$. A weak ion at *m/z* 271 was also detected (ca.19% of base peak) which corresponds to the loss of a CO (28 Da) from the aglycone part. This compound was identified as hyperoside. The same is true for compounds **26b** and **29a** and they were characterized as cynaroside and lonicerin by the deprotonated ions at *m/z* 447, 593 and their corresponding fragment ions [M – H – 162]$^-$, [M – H – 308]$^-$ in MS^2 spectrum.[31] Compounds **25** showed a [M + HAc – H]$^-$ ion at *m/z* 581, and according to its fragment ions at *m/z* 359 and 329, it was assigned as iridin.[32]

Compound **36** showed *m/z* 285 [M – H]$^-$ in the MS spectra. The fragment ions at *m/z* 151 and 133 that are specific to the RDA reaction pathway leading to the A ring and B ring. So the compound was identified as luteolin.[31,33] Compound **30**, which was just discovered in CLJ extract, provided a quasi-molecular ion peak [M – H]$^-$ at *m/z* 435. The fragment ions at *m/z* 273, 167 and 123 were exactly same with the report in the reference.[34] Thus, this compound was the identified as phloridzin and it was

Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

ARTICLE TYPE

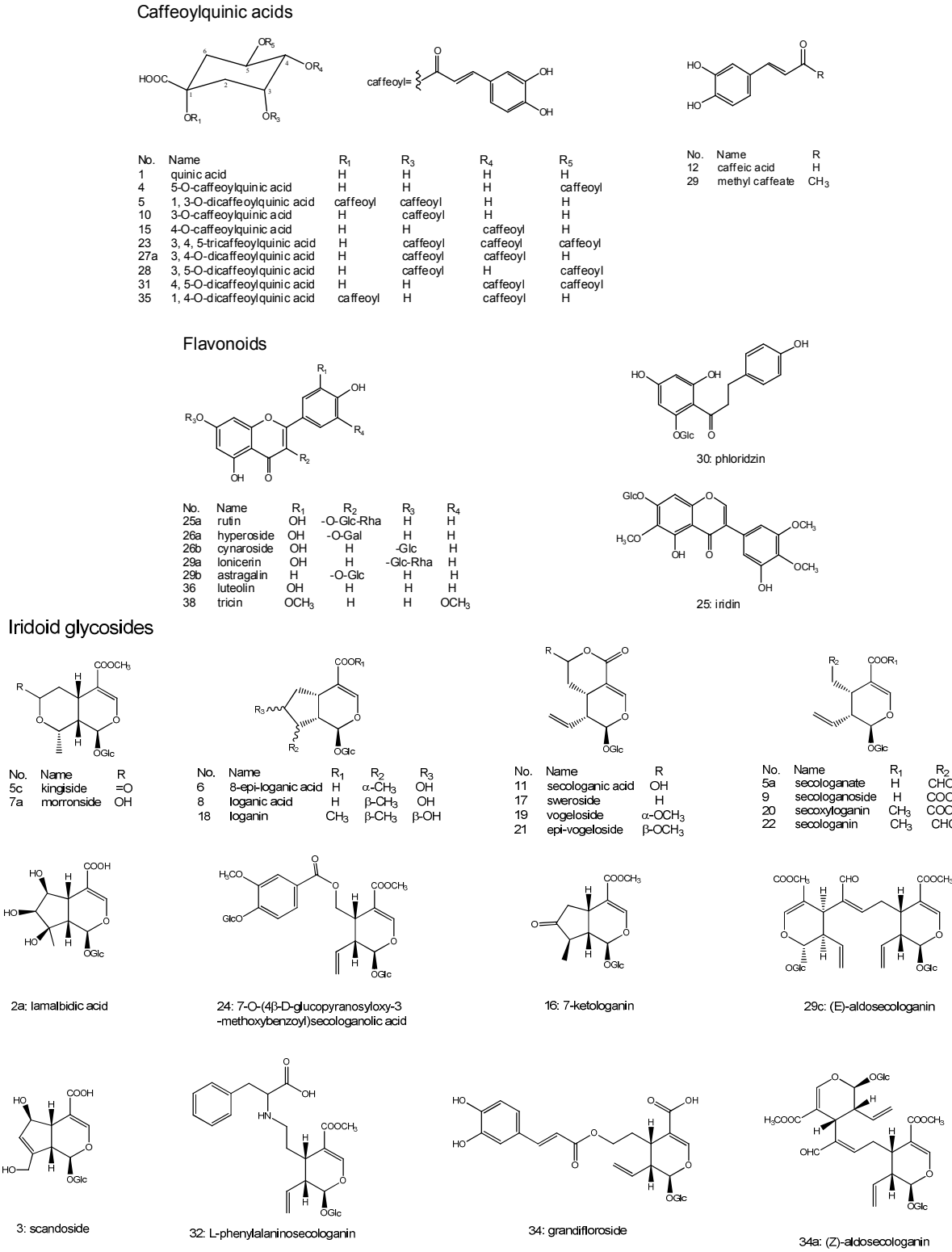


Fig. 4. Chemical structures of compounds detected in Caulis Lonicerae Japonicae and Flos Lonicerae Japonicae

Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

ARTICLE TYPE

worth noting that as a common compound in many plant, phloridzin has never been reported in *Lonicera Linn*.

Identification of iridoid glycosides (IGs). IGs are a large group of monoterpenes characterized by the presence in their skeleton of a cyclopentanoid residue.[35,36] A great number of IGs has been isolated from Lonicera species plants. This category of compounds was primarily characterized by its typical PAD spectra showing maximum absorption at 240 nm. IGs showed characteristic product ions due to cleavage at the glycosidic linkage, and subsequent losses of H_2O, CH_3OH, CO and RDA cleavage were also generally observed in methoxylated most IGs with carboxyl in its structure showed highly abundant deprotonated molecule $[M - H]^-$ and relatively low abundant adduct ions. On contrary, IGs without carboxyl showed that the high tendency for adduct formation replaced the formation of protonated molecules thus the acetic acid adducts $[M + HAc - H]^-$ at *m/z* $[M + 59]^-$ or chlorinated ions $[M + Cl]^-$ at *m/z* $[M + 35]^-$ was fairly abundant in the spectra, which was selected as the precursor ions (showed in Table 2).

A total of 18 compounds were screened and identified as IGs from CLJ and FLJ samples with their UV and TOF-MS data shown in Table 2. Compound **8** and compound **18** were definitely assigned as loganic acid and loganin by comparing these pieces of information about UV and retention times with reference compounds. Compound **3** and compound **9** yielded the same deprotonated molecule $[M - H]^-$ at *m/z* 389, which was corresponding to the molecular formula of $C_{17}H_{26}O_{10}$. According to the literature report,[35] it was possible to characterise compound **3** as scandoside. The MS^2 spectrum of compound **9** gave a fragment ion $[M - CO_2 - H]^-$ at *m/z* 345, a $[M - glc - H_2O - H]^-$ at *m/z* 191, a $[M - glc - CO_2 - H]^-$ at *m/z* 183 and a $[M - glc - CO_2 - H_2O - H]^-$ at *m/z* 165. It was assigned as secologanoside.[17] Meanwhile, compound **6** showed the same $[M - H]^-$ ion with compound **8** (loganic acid) at *m/z* 375, too. The MS^2 spectrum gave some fragment ions at *m/z* 113, 119, 151, 169 and 213, which were also very similar with loganic acid. Thus, it was identified as 8-epi-loganic acid.[37]

Compound **5a** and compound **11** also displayed the same deprotonated molecule $[M - H]^-$ at *m/z* 373, corresponding to the molecular formula of $C_{16}H_{22}O_{10}$. Compound **5a** was characterized as secologanate based on the fragment ions $[M - glc - H]^-$ at *m/z* 211, $[M - glc - CO_2 - H]^-$ at *m/z* 167 and $[M - glc - CO_2 - H_2O - H]^-$ at *m/z* 149 in MS^2 spectrum. The MS^2 spectrum of compound **11** gave several fragment ions at *m/z* 193,149 and 119. These fragment ions were quite similar with the report.[22] So it was ascribed as secologanic acid. Compound **20** showed the quasi-molecular ion peak $[M - H]^-$ at *m/z* 403. The fragment ions at *m/z* 371 and 223 were correspondent with $[M - CO_2 - H]^-$ and $[M - glc - H_2O - H]^-$. Fragment ion at *m/z* 165 was formed by loss of $COOCH_2$ at C-4 from $[M - glc - H_2O - H]^-$. And then the highest abundance fragment ion at *m/z* 121 was confirmed by follow-up loss of CO_2 from the fragment at *m/z* 165. So compound **20** was identified as secoxyloganin.[31]

Compound **17** displayed high-abundant acetic acid adduct $[M + HAc - H]^-$ at *m/z* 417, suggesting its structure might not contains carboxyl moiety. Its molecular formula was determined to be $C_{16}H_{22}O_9$ by accurate elemental composition from TOF-MS. Neutral loss of a glucose unit (162Da) generated the aglycone ion $[M - glc - H]^-$ at *m/z* 195. The fragment ion at 125, as base peak in product ion scan, proved that it was originated from RDA cleavage. Compound **17** was tentatively characterised as sweroside.[31] Compound **16, 19, 21** and **22** all gave high-abundant acetic acid adduct $[M + HAc - H]^-$ at *m/z* 447 and low-abundant chlorinated adduct $[M + Cl]^-$, suggesting their molecular formula might be $C_{17}H_{24}O_{10}$. Four compounds were found in *Lonicera Linn.* with its molecular weight at 388,[38] and they were 7-ketologanin, vogeloside, epi-vogeloside and secologanin. Compound **19** showed the exactly same fragmentation pattern with **21** and it was interesting to find that their daughter ions one minor peak $[M - glc - H]^-$ at *m/z* 225 and a base peak at 155 were both 30 Da more than those of sweroside. This discovery was more evidenced that the structure of compound **19** and **21** were quite similar with sweroside. According to the reference,[39] these compounds were identified as vogeloside and epi-vogeloside, respectively. Since compound **22** had fragment ions $[M - glc - H]^-$ at *m/z* 225, base peak at *m/z* 155 and a fragment ions $[M - glc - CH_3OH - H]^-$ at *m/z* 179, so it was indicated to be secologanin which can also give daughter ion at 155 by RDA cleavage. Then compound **16**, the last one, was considered as 7-ketologanin. Compound **29c** and **34a** showed the same deprotonated molecule $[M - H]^-$ at *m/z* 757, and adduct ion $[M + HAc - H]^-$ at *m/z* 817. An obvious fragment ion $[M - glc - H]^-$ at *m/z* 595 was characterized by loss of a neutral glucose unit of mass 162 Da. The highest abundance of the characteristic fragment ion at *m/z* 525 originated from RDA cleavage. These compounds which were only found in FLJ were identified as (E)-Aldosecologanin and (Z)-Aldosecologanin, respectively.[8,40] Likewise, compound **7a** presented $[M - H]^-$ at *m/z* 405 and $[M + HAc - H]^-$ at *m/z* 465.And the main fragment ions at *m/z* 243, 143 were coincident with the literature.[41] So it was characterized as morroniside.

Compound **24** gave $[M - H]^-$ ion at *m/z* 687 and chlorinated adduct $[M + Cl]^-$ ion at *m/z* 723 with the molecular formula $C_{30}H_{40}O_{18}$. And the fragmentation of this compound yielded the fragment ions at *m/z* 525, 329 and 167. The first fragment ion was extracted after the neutral loss of $C_6H_{12}O_5$ corresponding to the glucityl group, while fragment ion at *m/z* 167 was attributed to isovanillic acid moiety and ion at *m/z* 329 was corresponding to the fragment caused by cleavage at C-6. According to the literature review study,[38] it was tentatively authorized as 7-O-(4β-D-glucopyranosyloxy-3-methoxylbenzoyl) secologanolic acid. Compound **32** with the pseudo-molecular ion at *m/z* 536 and the molecular formula $C_{26}H_{35}NO_{11}$ was tentatively identified as N-contained iridoid glycosides. This compound provided several fragment ions, among which the fragment ion at *m/z* 272 was formed by the cleavage of RDA, while the fragment ions at *m/z* 356 and 312 corresponded to $[M - glc - H_2O - H]^-$ and $[M - glc - H_2O - CO_2 - H]^-$. Thus, compound **32** was identified as L-phenylalaninosecologanin.[39] Compound **34** gave a $[M - H]^-$ ion at *m/z* 537 with the molecular formula $C_{25}H_{30}O_{13}$. In its MS/MS spectrum, fragment ion $[M - glc - H]^-$ at *m/z* 375 was characterized by loss of a neutral glucose unit of mass 162 Da. Other fragment ions were detected at *m/z* 179, 161 and 135, corresponding to caffeic acid, further water loss and carboxyl loss, respectively. So grandifloroside was the proposed compound for it.[42]

3.2.2 Comparative analysis the components between CLJ and FLJ. A total of 53 compounds were found in CLJ and FLJ extracts through LC/MS/MS analysis, of which 38 were

discovered in CLJ, 40 in FLJ (sees Fig. 3). There were 25 compounds that detected in both of CLJ and FLJ, and most of them belonged to phenolic acids and IGs. Among these common compounds, the content of most of them in FLJ was much higher than in CLJ, like 3-CQA, secologanic acid, vogeloside, secologanin, 3, 5-diCQA, 4, 5-diCQA, etc. But still there were some which had higher intensity in CLJ, like 8-epiloganic acid, sweroside and loganin. Hence, it should be worthy of more attention that they may be the distinctive constituents of CLJ.

Relatively, components that discovered in only one of these two herbs were defined as typical chemical components. Note that kingiside, 3, 4-diCQA, aldosecologanin and most of flavonoid glycosides were just detected in FLJ. This result was agreed with the previous report.[43] There were 13 typical components detected in CLJ, which were attributed to phenolic acids and IGs (sees Table 2). Among them, 1, 3-diCQA, 3, 4, 5-triCQA, 7-O-(4β-D-glucopyranosyloxy-3-methoxylbenzoyl) secologanolic acid, phloridzin, grandifloroside etc, which were with relatively high contents should be considered as major typical components of CLJ.

3.3 Characteristic constituents influenced by storage time

3.3.1 Similarity analysis. It is necessary that the chromatographic fingerprint should be evaluated by their similarities, which come from the calculation on the correlative coefficient of original data. Thus to evaluate the similarity of CLJ profiles, the correlation coefficients of each chromatogram with the standard chromatogram (SC) were calculated by γ2 and/or angle cosine values. According to the guideline from Chinese pharmacopoeia, the herbal material from the geo-authentic habitats, with short deposited time, etc. was superior to the others. So in this paper, the fingerprint from herb (S1) was considered as SC.

Similarities among the fingerprints of the 36 batches of CLJ samples (Fig. 1) were calculated using the similarity evaluation system. From Table 1, the correlation coefficients of 36 samples with the SC were in a range of 0.627 to 1. And the samples from S1 to S21 with collected from 2013 and 2014 had the bigger correlation coefficient (>0.8), whereas most of the rest samples which were collected from 2010 to 2012 were less than 0.8. The higher the similarity values of a sample, the more the similarity between its fingerprint and SC, and the more the desirable quality consistency and stability of the sample. The correlation coefficients of the samples collected from 2013 and 2014 were similar, illustrating that the intrinsic qualities of these samples were more similar. On the other hand, samples collected from 2010 to 2012 were clearly differentiated with SC, based on the lower correlation coefficient. Therefore, these results indicated that the samples with low correlation coefficients of similarities may have the inferior qualities and storage time of CLJ has a great influence on its quality.

3.3.2 Principal component analysis. PCA is a popular method in applied statistical work and data analysis, and the principle of this method is to generate new principal components (PCs) which are independent of the original variables but shows linear combinations of them, and simultaneously capture most features of the original data. In the PCA scores plot, the samples with diverse quality will be divided into the groups according to their similarity. So in order to confirm the impact of storage time on the quality of CLJ, PCA was used to further research. In the present study, the relative peak areas (RPAs) of all the 34 common peaks of S1 was used as the variables for PCA in SIMCA-P software to analyze the similarities of the 36 batches of CLJ samples.

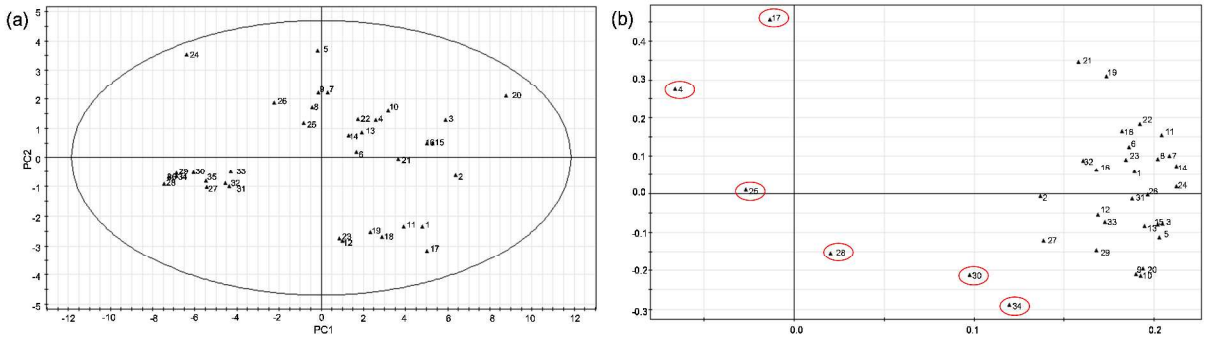


Fig.5. – (a) 2D projection plot of two principal components (scores) from principal component analysis (PCA) for the 36 batches of Caulis Lonicerae Japonicae samples. (b) Corresponding loadings plot from principal component analysis (PCA) of 36 batches of Caulis Lonicerae Japonicae samples; possible markers has been picked out

PCA scatter plots were utilized to describe general variations in concentrations of the characteristic constituents of samples. On the basis of eigenvalues >1, four PCs accounting for 82.6% of the total variance were considered significant. And fig. 5 (a) showed the score plot of the first two PCs, which provided the highest variation of data objects (61.4% and 9.6% of the variation). As shown in the score plot of PC1 (x-axis) and PC2 (y-axis), it could be easily seen that the samples could be classified into two big domains. The samples from 2010 and 2011 (S25 to S36) were comparatively concentrated on the left of the X axis, while the others with comparatively better quality were distributed in the right side. And it was interesting to find that to some extent, the result of PCA was consistent with that of similarity analysis except the samples collected from 2012 (S22, S23 and S24). This was mainly because samples collected from 2012 were relatively less that could not reflect the integrity of whole samples. This result further confirmed that the impact of storage time had a great influence on the quality of CLJ.

Generally, the loading of a variable on a PC reflects not only how much the variable contributed to that PC, but also how well that PC takes into account that variable's variation over the data points. Therefore, if the scores plot can discriminate the different quality of samples, the loadings plot can partly express the influence of variables (chemical markers) on separation between them. These variables which have the greatest influence on separation are those furthest away from the main cluster of variables. In order to find the possible chemical markers that had important influence on the discrimination of the samples, the loadings plot of PCA was performed. Fig. 5 (b) indicated that peak 4 (5-CQA), 17 (sweroside), 25 (iridin), 28 (3, 5-diCQA), 30

(phloridzin), 34 (grandifloroside) might have had a significant influence on the classification of the samples. This result was predominantly contributed to that the differences on the contents of compounds in CLJ were the main factors that affect the quality of CLJ caused by storage time.

3.4 Characteristic constituents influenced by harvesting season

3.4.1 Principal component analysis. Since the traditional Chinese medicines (TCMs) were cultivated in different habitats, harvested at different seasons and so on, the contents of chemical constituents in TCMs are largely different, which would affect the clinical therapeutic effects. Based on the reports about CLJ in Chinese Pharmacopoeia (2010), its best harvesting season is autumn/winter. So for further study the impact of harvesting time on the quality of the CLJ, the samples with similar correlation coefficient collected from 2013 and 2014 (S1-S21) were researched in the next.

Four principal components accounting for 85.1% of the total variance were considered significant, and the score plot of the first two principal components was illustrated in Fig. 6 (a). From the scatter points, the samples could be classified into three groups, and it was worth noting that the most samples in group I were harvested at fall and winter except S14 which was harvested at July in 2013, while the samples from other harvesting season were assigned to group II and III. The classification of these groups shows that the discrimination of CLJ samples from different harvesting time using PCA method was effective. To find the peaks which were most responsible for the distribution of the samples in scores plot, loadings plot (Fig. 6(b)) was generated. The loadings plot indicated that peak 2, 4, 6, 17, 19, 21, 25 and 28 might have had the most significant influence on the classification of the samples. From the above ESI-MS/MS analysis in section 3.4.1, the compounds in these peaks were malic acid, 5-CQA, 8-epiLoganic acid, sweroside, vogeloside, epi-vogeloside, iridin, 3,5-diCQA. Therefore, these components can be considered as characteristic components that affect the quality of CLJ which was harvested at the best harvesting season.

3.4.2 Partial least squares-discrimination analysis. PLS-DA, a supervised method, is used for classification purposes. It is a regression extension of PCA that takes advantage of class information to attempt to maximize the separation between groups of observations. The principle of PLS-DA consists of the

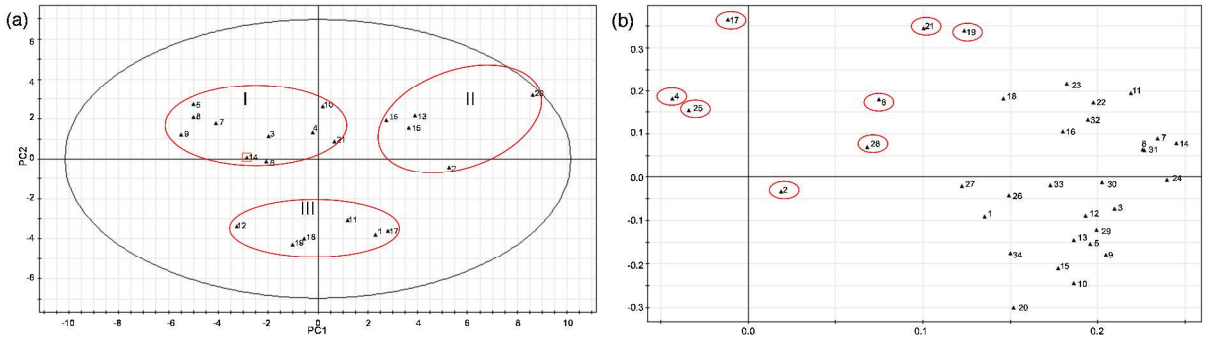


Fig.6. – (a) 2D projection plot of two principal components (scores) from principal component analysis (PCA) for the 21 batches of Caulis Lonicerae Japonicae samples. (b) Corresponding loadings plot from principal component analysis (PCA) of 21 batches of Caulis Lonicerae Japonicae samples; possible markers has been picked

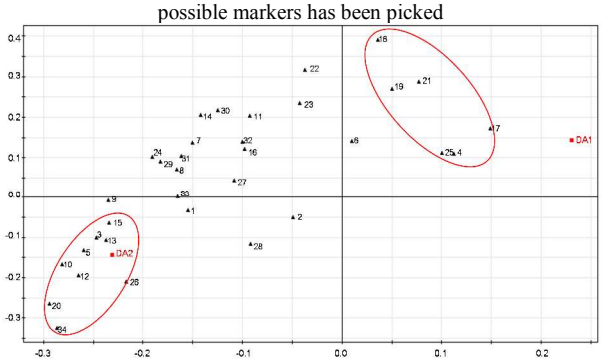


Fig. 7. –The corresponding loadings plot of PLS-DA for 21 batches of Caulis Lonicerae Japonicae samples; possible markers has been picked out.

separation of priori given classes of objects. In this part, to further confirm the result of study above, PLS-DA was employed to establish a discrimination model of two groups which one group of samples was collected at the best harvesting season and while the other was not, using the RPAs of the 34 characteristic common compounds of SC as input data. The recognition ability, according to the posteriori probabilities, was 100% for each class. The leave-one-out method was used as cross-validation procedure to evaluate the classification performance. The prediction abilities were 100% for all classes.

In the loading plot of PLS-DA (Fig. 7), DA1 was the group with all samples collected at the autumn or winter, while DA2 was the group which did not, and the important chemical markers situated far from the origin could classify DA1 and DA2. Therefore, the loadings plot of PLS-DA demonstrated that peak 4 (5-CQA), 17 (sweroside), 18 (loganin), 19 (vogeloside), 21 (epi-vogeloside) and 25 (iridin) might be the chemical markers for discrimination of group of DA1. Meanwhile, it was indicated that peaks 3, 5, 10, 12, 13, 15, 20, 26 and 34, which were considered as scandoside, 1, 3diCQA, 3-CQA, caffeic acid, syringetin hexoside, 4-CQA, secoxyloganin and grandifloroside may have more influence on the discrimination of DA2.

PCA is an unsupervised multivariate data analysis approach, while PLS-DA, a supervised method, is used to build a predictive model of the group membership based on observed characteristics of each case. In this paper, with the complementation of using

both of them, the chemical marker obtained from PCA and PLS-DA was more convincible. Through the above analysis by PCA and PLS-DA, the result indicated that 5-CQA, sweroside, vogeloside, epi-vogeloside and iridin were the most responsible chemical markers to control the quality of the CLJ samples that collected in autumn/winter.

4. Conclusion

Caulis Lonicerae Japonicae (CLJ) is widely used in the clinic of Chinese medicine. It is generally known that the quality of TCMs was affected by many factors, such as deposited time, the harvesting season, cultivation location, etc. And the differences in relative contents of main chemical compounds caused by these factors were important indicators that would affect the quality of TCMs.[43,44] In order to control the quality of CLJ, it is necessary to find out these index compounds. Compared with conventional analytical approaches, the fingerprint technique emphasizes the integral characterization of a complex system with a quantitative degree of reliability. So in order to find out the chemical markers that can holistically reflect the intrinsic quality of CLJ, a HPLC fingerprint combined with chemometrics method has been developed by using HPLC-UV-MS/MS, and thirty-six batches of CLJ samples collected with different deposited years, at different harvest time and from various cultivation locations were analyzed under the optimized HPLC conditions. This evaluation approach can overcome the deficiencies of tradition methods and effectively reveal the complexity and synergistic effects of the ingredients of samples.

With the purpose of finding out the chemical markers, it has been studied from the different factors that could impact the quality of CLJ. Because the chemical markers that used to control the quality of CLJ were similar to those for FLJ, the extracts of CLJ and FLJ are both studied by HPLC-QTOF-MS/MS mass spectrometry to achieve the more characteristic compounds through comparative analysis between them. Compared with the latest research[8], which was just identified 22 compounds from aerial parts of the plant, a total of 42 major constituents were rapidly screened and identified with no doubt. And it was also worth to notice that phloridzin, as a common compound in many plant, has never been reported in *Lonicera Linn* and the fragmentation patterns of some iridoid glycosides, like L-phenylalaninosecologanin, grandifloroside, etc, are explained for the first time. The result of comparative analysis between CLJ and FLJ suggested that 1, 3-diCQA, epi-loganic acid, sweroside, loganin, 3, 4, 5-triCQA, 7-O-(4β-D-glucopyranosyloxy-3-methoxylbenzoyl) secologanolic acid, phloridzin and grandifloroside were found as characterized components in CLJ that different from FLJ and with relatively high contents.

To find out the factors that may affect the inherent quality of CLJ and the chemical markers, different chemical pattern recognition methods, SA, PCA and PLS-DA are utilized. First, SA and PCA were applied to overview the distribution of 36 batches of CLJ samples. The result showed that the samples with long deposited time are well separated from the samples with short deposited time. Thus, it is suggested that the storage time has a huge influence on the quality of CLJ. And with the help of loadings plot of PCA, we could know that 5-CQA, sweroside, iridin, 3, 5-diCQA, phloridzin, grandifloroside may have significant impact on the discrimination caused by storage time. Second, to find out the other factors that may also impact the quality of CLJ and the chemical indictors, the data obtained from CLJ samples which have similar correlation coefficient with SC was further analyzed by PCA and PLS-DA. The CLJ samples collected at different harvesting time are well separated in scores plot of PCA. And with the help of loading plot, 5-CQA, sweroside, vogeloside, epi-vogeloside and iridin were considered as the most responsible chemical markers to control the quality of CLJ samples that collected in autumn/winter.

In this study, the selection of chemical markers to overall control the quality of CLJ was from different perspectives. Finally, through the comprehensive analysis of all the factors, 5-CQA, sweroside, vogeloside, 1, 3-diCQA, epi-loganic acid, loganin and grandifloroside were taken as chemical markers for control the quality of CLJ. Among them, 1, 3-diCQA, epi-loganic acid, loganin and grandifloroside were the representative components for CLJ that different from FLJ and with relatively high contents. 5-CQA, sweroside, vogeloside and grandifloroside were obtained as index components by study on the factor of both storage time and harvesting season of CLJ. So controlling the content of these ingredients may be more persuasive for the quality of CLJ. Overall, this paper has helped in guiding for the quality control of CLJ to some extent, as well as TCM. Multivariate statistical analysis combined with chromatographic fingerprint is presented to analyze the factors that may influence the quality of CLJ from different perspectives, and the chemical compositions whose relative content were influenced by these factors were also discovered.

Acknowledgements

This study is supported by Natural Science Foundation of Jiangsu Province, China (NO.BK2002033), Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions, China and Research Foundation of Nanjing University of Traditional Chinese Medicine, China.

Conflict of interest

All authors have no financial or scientific conflict of interest with regard to the research described in this manuscript.

Notes and references

Xianlin road No. 138, Xianlin University town, Nanjing, 210023, P.R. China. E- mail: njxlyl2002@163.com

1 S.P. Li, J. Zhao and B. Yang, J. Pharm. Biomed. Anal., 2011, **55**, 802–809.
2 D. Normile, Science, 2003, **299**, 188–190.
3 Y. Jiang, B. David, P. F. Tu, et al., Anal. Chim. Acta, 2010, **657**, 9–18.
4 B. Xie, T. Gong, M. H. Tang, et al., J. Pharm. Biomed. Anal., 2008, **48**, 1261–1266.
5 L. J. Ma, X. Z. Zhang, H. P. Zhang, et al., J. Chromatogr. B: Anal. Technol. Biomed. Life Sci., 2007, **846**, 139–146.
6 C. Tistaert, B. Dejaegher and Y. V. Heyden, Anal. Chim. Acta, 2011, **690**, 148–161.
7 S. Q. Song, X. M. Zhang, K. Hayat, et al., J. Chromatogr. A, 2013, **1278**, 145–152.
8 J. Ye, J. Su, K. Y. Chen, et al., J. Anal. Chem., 2014, **69**, 777–784.
9 X. F. Shang, H. Pan, M. X. Li, et al., J. Ethnopharmacol., 2011, **138**, 1–21.
10 Y. Sato, S. Itagaki, T. Kurokawa, et al., Int. J. Pharm., 2011, **403**, 136–138.
11 B. Dai, Z. Xiao, L. Liu, et al., Tradit. Chin. Drug Res. Pharmacol., 2007, **6**, 448–450.
12 C. Y. Yao and Q. Wu, Chin. J. Laser Med. Surg. , 2006, **6**, 361–364.
13 L. Li, H. J. Wang and J. Tong, Tradit. Chin. Drug Res.

Pharmacol., 2000, **5**, 274–276.

14 H. J. Li, P. Li and W. C. Ye, J. Chromatogr. A, 2003, **1008**, 167–172.
15 X. Y. Chai, S. L. Li and P. Li, J. Chromatogr. A, 2005, **1070**, 43–48.
16 J. Chen, Y. Song and P. Li, J. Chromatogr. A , 2007, **1157**, 217–226.
17 M. T. Ren, J. Chen,, Y. Song, et al., J. Pharm. Biomed. Anal., 2008, **48**, 1351–1360.
18 W. Gao, H. Yang, L. W. Qi, et al., J. Chromatogr. A , 2012, **1245**, 109–116.
19 F. Li, B. Yuan, Z. L. Xiong, et al., Biomed. Chromatogr., 2006, **20**, 634–641.
20 C. Y. Chen, L. W. Qi, H. J. Li, et al., J. Sep. Sci., 2007, **30**, 3181–3192.
21 Z. M. Qian, H. J. Li, P. Li, et al., Biomed. Chromatogr., 2007, **21**, 649–654.
22 L. W. Qi, C. Y. Chen and P. Li, Rapid Commun. Mass Spectrom., 2009, **23**, 3227–3242.
23 W. Zhou, S. J. Liu, W. Z. Ju, et al., J. Pharm. Biomed. Anal.,2013, **86**, 189–197.
24 E. Liu, Q. Liu, C, Chu, et al., J. Sep. Sci., 2011, **34**, 2566–2575.
25 M. N. Clifford, K. L. Johnston, S. Knight, et al., J. Agric. Food Chem., 2003, **51**, 2900−2911.
26 M. N. Clifford, S. Knight and N. Kuhnert, J. Agric. Food Chem., 2005, **53**, 3821−3832.
27 S. Gouveia and P. C. Castilho, Food Chem., 2011, **129**, 333–344.
28 S. C. Gouveia and P. C. Castilho, Rapid Commun. Mass Spectrom., 2009, **23**, 3939–3953.
29 A. K. Sandhu and L. Gu, J. Agric. Food Chem., 2010, **58**, 4681–4692.
30 H. Chen, Q. Zhang, X. Wang, et al., Phytochem. Anal., 2011, **22**, 247–257.
31 X. Li, X. Sun, S. Cai, et al., Acta Pharm. Sin., 2009, **44**, 895−904.
32 J. Li, W. Li, W. Huang, et al., J. Chromatogr. A, 2009, **1216**, 2071–2078.
33 Y. Wang, Z. Wang, L. Lin, Chin. J. Chin. Mater. Med., 2008, **8**, 969−972.
34 P. Hilt, A. Schieber, C. Yildirim, et al., J. Agric. Food Chem., 2003, **51**, 2896−2899.
35 C. M. Li, X. L. Zhang, X. Y. Xue, et al., Rapid Commun. Mass Spectrom., 2008, **22**, 1941–1954.
36 A. K. Yadav, N. Tiwari, P. Srivastava, et al., J. Pharm. Biomed. Anal., 2008, **47**, 841–846.
37 C. Li, Y. Dai, J. B. Zhang, et al., Chin. Tradit. Herb Drugs, 2013, **21**, 2951−2954.
38 R. F. Ji, S. X. Liu, W. Q. Wang, et al., Chin. Tradit. Herb Drugs, 2012, **6**, 1226−1232.
39 R. Kakuda, M. Imai, Y. Yaoita, et al., Phytochemistry, 2000, **5**, 879−881.
40 K. Machida, H. Sasaki, T. Iijima, et al., Chem. Pharm. Bull., 2002, **50**, 1041−1044.
41 G. Cao, H. Cai, Y. Zhang, et al., J. Pharm. Biomed. Anal., 2011, **56**, 118−125.
42 R. Ma, Z. Q. Yin, C. Zhang., et al., J. China Pharm. Univ., 2010, **41**, 333−336.
43 Y. B. Teng, Y. Chen, Y. Y. Zhao, et al., J. Chin. Med. Mater., 2012, **4**, 586−588.
44 P. Ma, L. Y. Li and Y. Zhang, Chin. J. Chin. Mater. Med., 2014, **6**, 981−991.