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Identification and quantification of 32 bioactive compounds in Lonicera species by high performance liquid chromatography coupled with time-of-flight mass spectrometry

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ABSTRACT

Flos Lonicerae, referred to the flower buds of several medicinal *Lonicera* species, is a commonly used traditional Chinese herbal medicine. A multi-component-assay quality control method, using high performance liquid chromatography coupled with electrospray ionization time-of-flight mass spectrometry (HPLC-ESI/TOF MS), has been developed for the simultaneous identification and quantification of 32 bioactive compounds in Flos Lonicerae. The limits of detection (LOD) and quantification (LOQ) were in the range of 0.002–0.089 and 0.006–0.355 μg/ml, respectively. All calibration curves showed good linear regression ($r^2 \geq 0.99$) within the test ranges. The overall intra- and inter-day precisions of analytes were less than 3.47% for peak area and 0.38% for retention time. The recoveries were from 85.4% to 101.6%. The validated method was applied to assay of 32 compounds in 8 medicinal *Lonicera* species. Furthermore, six unknown chromatographic peaks were tentatively characterized. It was demonstrated that the HPLC-ESI/TOF MS method was suitable for quality control of *Lonicera* species, owing to the advantages of accurate mass analysis, resolving power, enhanced selectivity and high sensitivity.

1. Introduction

Flos Lonicerae, derived from the flower buds of several medicinal *Lonicera* species, is a commonly used traditional Chinese medicine (TCM) for the treatment of sores, carbuncles, furuncles, swelling and affections caused by exopathogenic wind-heat or epidemic febrile diseases at the early stage [1]. A number of compounds including organic acids, flavonoids, iridoid glycosides and saponins have been isolated from *Lonicera* species [2–5], which were proven to be responsible for the various biological activities such as hepatoprotective, cytoprotective, antimicrobial, antioxidative, antiviral and anti-inflammatory effects of the herbal remedy [6–9].

As a consequence of the potential medicinal value of Flos Lonicerae several methods have been published for its quality control. High performance liquid chromatography (HPLC) coupled with evaporative light scattering detection (ELSD) was used for the determination of iridoid glycosides [10] and saponins [11], capillary zone electrophoresis (CZE) with diode array detection (DAD) was developed for qualitative and quantitative analysis of flavonoids [12], capillary electrophoresis with electrochemical detection (CE-ED) was employed to analyze four polyphenolic components [13,14], capillary HPLC with electrospray ionization mass spectrometry (capillary HPLC-ESI/MS) was proposed for identification and quantification of iridoids [15]. Nevertheless, the above reports concerned mostly with one types of metabolites. Recently, an HPLC with DAD and electrospray ionization mass spectrometry (ESI-MS) for the determination of 13 compounds including 4 iridoids, 4 organic acids and 5 flavanoids [16], and a capillary HPLC-DAD/ESI-MS for quantification of 9 flavonoids, 8 iridoid glucosides and 7 saponins [17] in Flos Lonicerae were described. However, saponins and organic acids were still neglected in these two reports, respectively.

It has been well demonstrated that in most cases TCMs exert their efficacies from the synergistic actions of multi-components. Therefore, a multi-component-assay including organic acids, flavonoids, iridoids and saponins might be a rational strategy to elucidate the overall outcomes and comprehensively control the quality of Flos Lonicerae. Very recently, an HPLC-DAD-ELSD was successively employed for simultaneous characterization of 20 compounds in Flos Lonicerae [18]. Although this method really took the multi-component-assay into consideration, due to the

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chemical complexity of the plant extract encountered, it had some limitations: firstly, some minor compounds, especially the saponins such as hederagenin-28-*O*-[*β*-D-glucopyranosyl-(1 → 6)-*β*-D-glucopyranosyl] ester, macranthoidin B, macranthoidin A, 3-*O*-[*α*-L-rhamnopyranosyl-(1 → 2)-*α*-L-arabinopyranosyl] hederagenin, were not accurately quantified because of the relatively low sensitivity of ELSD; secondly, without the aid of MS detection, the HPLC-DAD-ELSD method suffered from the risk arising from chromatographic peak identification only by retention times.

Nowadays, HPLC coupled with various MS detectors for analysis of phytomedicines has become an attractive approach. Particularly, HPLC connected to MS with time-of-flight (TOF) analyzer is showing its unique advantages in providing accurate mass analysis, resolving power, enhanced selectivity and high sensitivity for analysis of complex matrixes such as herbal samples [19–23]. These advantages allow unequivocal identification of low levels of ingredients, as well as the possibility of quantitation at low levels using extracted ion chromatograms (XICs). Consequently, in the present study, a highly comprehensive HPLC method coupled with TOF/MS was developed for the systemic quantitative analysis of 4 groups of bioactive metabolites (including 6 organic acids, 7 iridoid glycosides, 10 flavonoids and 9 saponins) for profiling and evaluating the different botanical origins of Flos Lonicerae. This method was also successively applied to structural characterization of six unknown chromatographic peaks.

2. Experimental

2.1. Chemical reagents and references

HPLC-grade acetonitrile and methanol were supplied by Merck (Darmstadt, Germany) and water provided by a Millipore water purification system (Millipore, Bedford, MA, USA). Formic acid purchased from Nanjing Chemical Factory (Nanjing, China) was of analytical grade.

Thirty-two reference substances including 6 organic acids, i.e. chlorogenic acid (C1), caffeic acid (C2), 3-*O*-caffeoylquinic acid methyl ester (C3), 3, 5-di-*O*-caffeoylquinic acid (C4), 3,4-di-*O*-caffeoylquinic acid (C5) and 3-*O*-caffeoylquinic acid butyl ester (C6); 7 iridoid glycosides, i.e. loganin (IG1), secoxyloganin (IG2), 7-epi-vogeloside (IG3), sweroside (IG4), dimethyl-secoxyloganoside (IG5), centauroside (IG6) and secoxyloganin 7-butyl ester (IG7); 10 flavonoids, i.e. rutin (F1), hyperoside (F2), quercetin-3-*O*-*β*-D-glucoside (F3), luteolin-7-*O*-*β*-D-glucoside (F4), lonicerin (F5), tricin-7-*O*-*β*-D-glucoside (F6), luteolin (F7), quercetin (F8), diosmetin (F9) and cupressuflavone (F10); 9 saponins,

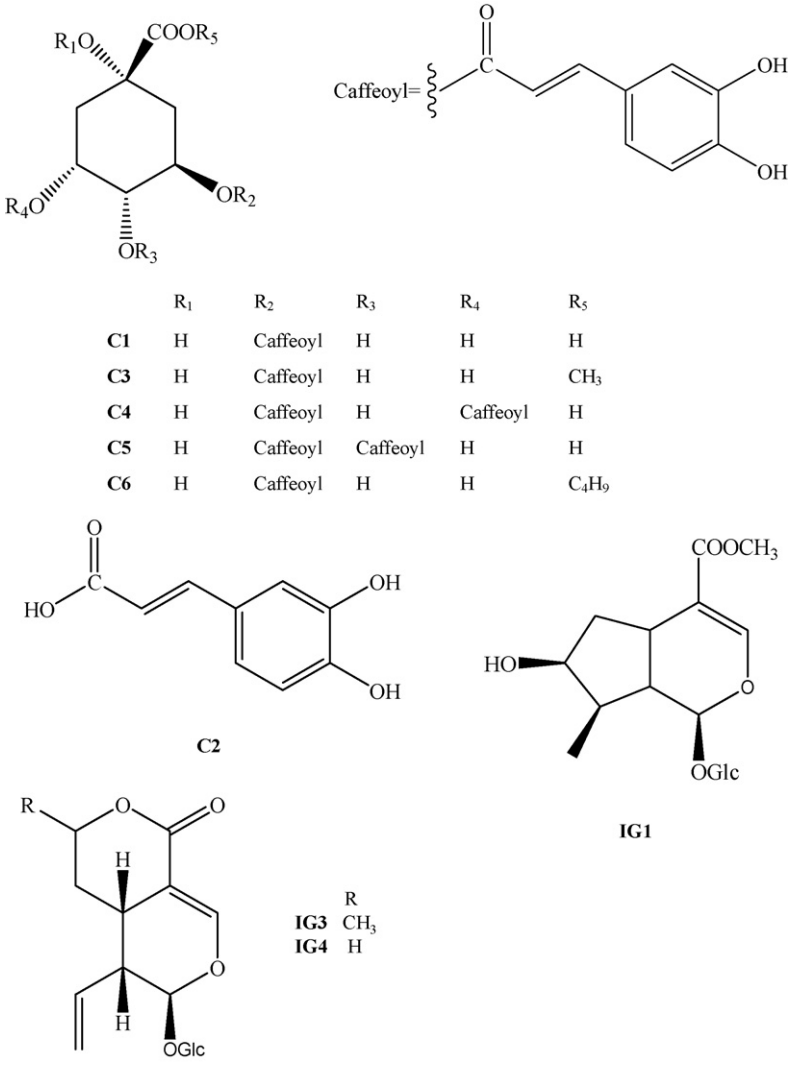
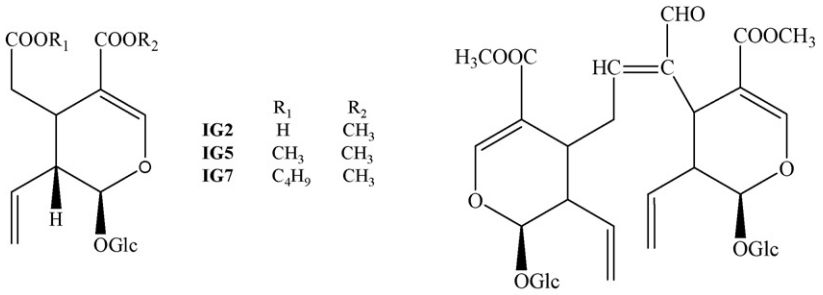
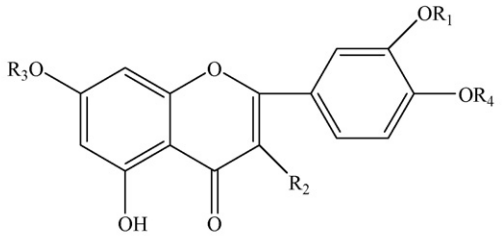


Fig. 1. Chemical structures of organic acids (C1–C6), flavonoides (F1–F10), iridoid glycosides (IG1–IG7), saponins (S1–S9).



IG6



	R_1	R_2	R_3	R_4
F1	H	-O-Glc-Rha	H	H
F2	H	-O-Gal	H	H
F3	CH_3	-O-Glc	H	H
F4	H	H	-Gal	H
F5	H	OH	-Rha-Glc	H
F6	CH_3	H	-Glc	H
F7	H	H	H	H
F8	H	OH	H	H
F9	H	H	H	CH_3

Fig. 1. (*Continued*).

i.e. akebiasaponin F (S1), macranthoidin B (S2), macranthoidin A (S3), dipsacoside B (S4), akebiasaponin D (S5), hederagenin-28-*O*-[β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranosyl] ester (S6), macranthoside B (S7), macranthoside A (S8), and 3-*O*-[α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl] hederagenin (S9) (Fig. 1), were isolated from *L. japonica*, *L. confuse*, *L. macranthoides* and *L. fulvotomentosa* [24–29] in our laboratory. Their structures were elucidated based on spectral (NMR and MS) analyses and the purities were over 98% by LC analysis. Ginsenoside R_1, using as internal standard (IS), was obtained from the National Institute for the Control of Pharmaceutical and Biological Product.

2.2. Plant samples

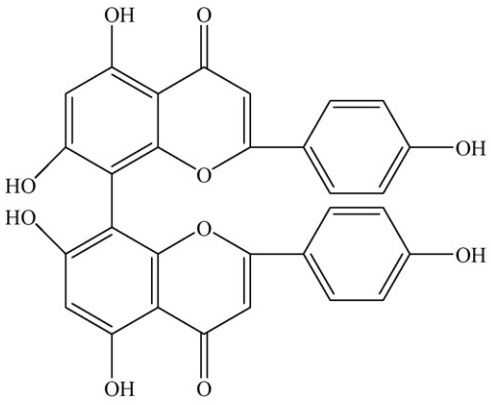
The flower buds from eight *Lonicera* species, including *L. japonica* (A) from Fengqiu, Henan Province, *L. macranthoides* (B) from Chongqing, *L. confusa* (C) from Xupu, Hunan Province, *L. hypoglauca* (D) from Jiujiang, Jiangxi Province, *L. fulvotomentosa* (E) from Anlong, Guizhou Province, *L. similes* (F) from Bazhong, Sichuan Province, *L. dasystyla* (G) from Nanning, Guangxi Province and *L. syringantha* (H) from Xining, Qinghai Province, were authenticated by Professor Ping Li. The voucher specimens were deposited in Department of Pharmacognosy, China Pharmaceutical University, Nanjing, China.

2.3. Liquid chromatography

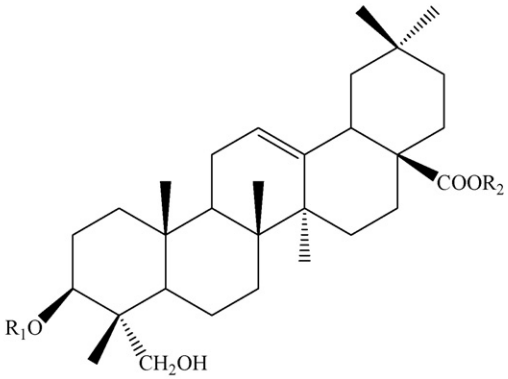
Liquid chromatography was carried out on an Agilent 1100 HPLC system (Agilent Technologies, Waldbronn, Germany) equipped with a binary pump, an online degasser, an auto sampler and a thermostatically controlled column compartment. The chromatographic separation was performed on an Agilent Zorbax Extend C_{18} column (4.6 mm × 150 mm, 5 μm) at 25 °C. A mixture of solvent A (0.1% aqueous formic acid) and solvent B (acetonitrile–methanol (5:1, v/v) containing 0.05% formic acid) was used as the mobile phase at a flow rate of 1.0 ml/min. The gradient elution program was: 0–20 min, 10–17% B; 20–32 min, 17–21% B; 32–40 min, 21-26% B; 40–49 min, 26–36% B; 49–54 min, hold on 36% B; 54–59 min, 36–37% B; 59–65 min, 37–58% B; 65–70 min, 58–61%; 70–75 min, 61–65%. The sample volume injected was 10 μl.

2.4. Mass spectrometry

The LC system was coupled to an orthogonal time-of-flight mass spectrometer (Agilent Corp., Santa Clara, CA, USA) equipped with an electrospray ionization (ESI) source. TOF MS analysis was performed in negative mode using full scan mode and the mass range was set at 100–3000 Da. The conditions of the ESI source were as follows: drying gas (N_2) flow rate, 10.0 l/min;



F 10



	R_1	R_2
S1	Ara	Glc(1→6)Glc
S2	Glc(1→4) Glc(1→3) Rha(1→2)Ara	Glc(1→6)Glc
S3	Glc(1→3) Rha(1→2)Ara	Glc(1→6)Glc
S4	Rha(1→2)Ara	Glc(1→6)Glc
S5	Ara(2-1)glc	Glc(1→6)Glc
S6	H	Glc(1→6)Glc
S7	Glc(1→4) Glc(1→3) Rha(1→2)Ara	H
S8	Glc(1→3) Rha(1→2)Ara	H
S9	Rha(1→2)Ara	H

Fig. 1. (*Continued*).

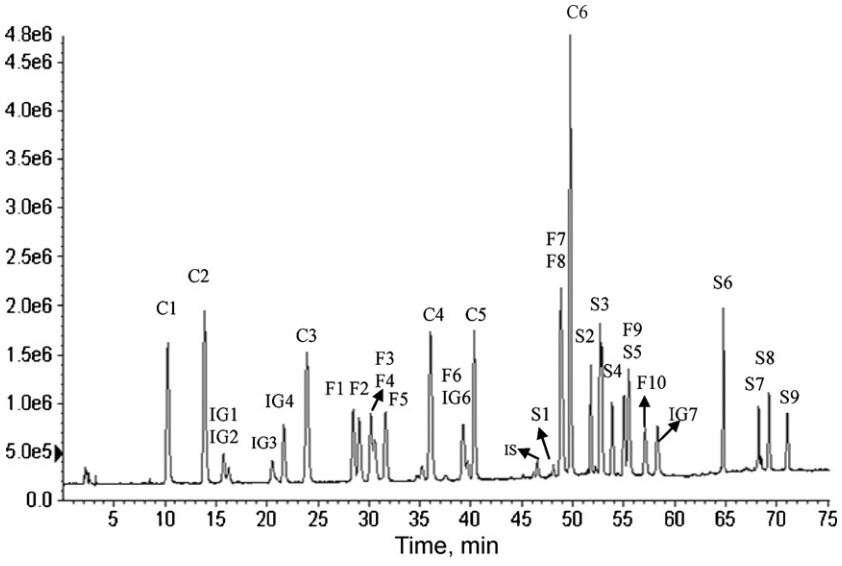


Fig. 2. TIC of reference substances and internal standard. (Peak assignments are same as described in Section 2.1.)

Table 1
LC-ESI/TOF-MS accurate mass measurements for the 32 analytes.

Analytes	t_R (min)	ESI/TOF MS ions (m/z)			
		Proposal ions	Measured mass (Da)	Elemental composition	Error (ppm)
C1	10.88	$[M-H]^-$	353.0883	$C_{16}H_{17}O_9$	1.399
C2	14.68	$[M-H]^-$	179.0351	$C_9H_7O_4$	0.6567
C3	24.74	$[M-H]^-$	367.1038	$C_{17}H_{19}O_9$	0.9367
C4	37.13	$[M-H]^-$	515.1186	$C_{25}H_{23}O_{12}$	−1.7475
C5	41.46	$[M-H]^-$	515.1185	$C_{25}H_{23}O_{12}$	−1.9416
C6	50.24	$[M-H]^-$	409.1512	$C_{20}H_{25}O_9$	1.9397
IG1	16.18	$[M+HCOO]^-$	435.1507	$C_{18}H_{27}O_{12}$	−0.2305
IG2	16.76	$[M-H]^-$	403.1245	$C_{17}H_{23}O_{11}$	−0.2121
IG3	21.11	$[M+HCOO]^-$	433.1353	$C_{18}H_{25}O_{12}$	0.3457
IG4	22.17	$[M+HCOO]^-$	403.1246	$C_{17}H_{23}O_{11}$	0.0358
IG5	35.93	$[M+HCOO]^-$	463.1455	$C_{19}H_{27}O_{13}$	−0.4641
IG6	40.11	$[M+HCOO]^-$	803.2612	$C_{35}H_{47}O_{21}$	−0.4144
IG7	64.59	$[M+HCOO]^-$	505.1930	$C_{22}H_{33}O_{13}$	0.6626
F1	29.51	$[M-H]^-$	609.1471	$C_{27}H_{29}O_{16}$	1.6268
F2	31.46	$[M-H]^-$	463.0882	$C_{21}H_{19}O_{12}$	0
F3	30.31	$[M-H]^-$	463.0881	$C_{21}H_{19}O_{12}$	−0.2159
F4	31.60	$[M-H]^-$	447.0928	$C_{21}H_{19}O_{11}$	−1.0856
F5	32.50	$[M-H]^-$	593.1511	$C_{27}H_{29}O_{15}$	−0.159
F6	40.00	$[M-H]^-$	491.1195	$C_{23}H_{23}O_{12}$	−0.0003
F7	49.75	$[M-H]^-$	285.0406	$C_{15}H_9O_6$	0.4849
F8	49.97	$[M-H]^-$	301.0355	$C_{15}H_9O_7$	0.4105
F9	56.58	$[M-H]^-$	299.0562	$C_{16}H_{11}O_6$	0.2947
F10	58.26	$[M-H]^-$	537.0835	$C_{30}H_{17}O_{10}$	1.451
S1	48.31	$[M+HCOO]^-$	1133.5398	$C_{54}H_{85}O_{25}$	1.1089
S2	51.97	$[M-H]^-$	1397.6610	$C_{65}H_{105}O_{32}$	1.1116
S3	52.90	$[M-H]^-$	1235.6065	$C_{59}H_{95}O_{27}$	−0.0992
S4	54.15	$[M+HCOO]^-$	1119.5600	$C_{54}H_{87}O_{24}$	0.6445
S5	55.37	$[M+HCOO]^-$	973.5017	$C_{48}H_{77}O_{20}$	0.3395
S6	65.86	$[M+HCOO]^-$	841.4601	$C_{43}H_{69}O_{16}$	1.1758
S7	68.49	$[M-H]^-$	1073.5545	$C_{53}H_{85}O_{22}$	0.6529
S8	69.55	$[M-H]^-$	911.5026	$C_{47}H_{75}O_{17}$	1.7822
S9	71.46	$[M+HCOO]^-$	795.4549	$C_{42}H_{67}O_{14}$	1.5950

Table 2
Calibration curves, test ranges, LODs and LOQs for the 32 analytes.

Analytes	Calibration curves	r^2	Test ranges (μg/ml)	LODs (μg/ml)	LOQs (μg/ml)
C1	$y = 0.0105x - 9.22$	0.9957	0.220–220	0.025	0.100
C2	$y = 0.0158x - 0.1901$	0.9994	0.204–204	0.009	0.036
C3	$y = 0.0102x + 0.25$	0.9902	0.198–69.3	0.005	0.014
C4	$y = 0.0051x + 0.256$	0.9940	0.320–112	0.032	0.130
C5	$y = 0.0059x + 1.1758$	0.9992	0.204–71.4	0.019	0.077
C6	$y = 0.0151x + 0.2612$	0.9948	0.268–134	0.002	0.006
IG1	$y = 0.0333x + 0.0125$	0.9975	0.024–12.0	0.006	0.060
IG2	$y = 0.0037$x-0.193	0.9990	0.024–12.0	0.006	0.012
IG3	$y = 0.004x + 0.1782$	0.9998	0.028–14.0	0.007	0.070
IG4	$y = 0.1277x + 0.1259$	0.9923	0.024–12.0	0.006	0.012
IG5	$y = 0.0051x - 0.5729$	0.9930	0.020–10.0	0.005	0.050
IG6	$y = 0.0025x + 0.1526$	0.9986	0.024–12.0	0.012	0.120
IG7	$y = 0.0191x + 0.4151$	0.9986	0.026–13.0	0.013	0.130
F1	$y = 0.0046x - 2.1246$	0.9912	0.128–12.8	0.003	0.032
F2	$y = 0.0058x - 1.8058$	0.9926	0.128–12.8	0.003	0.128
F3	$y = 0.0058x - 1.3178$	0.9956	0.132–13.2	0.003	0.033
F4	$y = 0.0018x + 0.0591$	0.9964	0.104–10.4	0.013	0.104
F5	$y = 0.0036x - 1.3818$	0.9971	0.114–7.98	0.029	0.057
F6	$y = 0.003x - 0.2193$	0.9947	0.194–19.4	0.005	0.049
F7	$y = 0.0197x - 0.2118$	0.9988	0.168–11.8	0.004	0.042
F8	$y = 0.0139x - 2.2679$	0.9916	0.104–8.20	0.002	0.021
F9	$y = 0.0133x + 2.6918$	0.9982	0.138–9.66	0.003	0.069
F10	$y = 0.0066x - 2.0357$	0.9900	0.068–6.80	0.017	0.068
S1	$y = 0.0001x + 0.0045$	0.9906	0.126–8.82	0.038	0.265
S2	$y = 0.0013x - 0.1668$	0.9902	0.182–18.2	0.055	0.182
S3	$y = 0.0014x - 0.4361$	0.9936	0.196–19.6	0.059	0.196
S4	$y = 0.0006x - 0.0072$	0.9941	0.174–174	0.052	0.174
S5	$y = 0.0004x - 0.0066$	0.9999	0.100–10.0	0.003	0.006
S6	$y = 0.0069x - 0.0164$	0.9915	0.088–8.80	0.026	0.088
S7	$y = 0.0026x - 0.4109$	0.9980	0.104–36.4	0.030	0.208
S8	$y = 0.004x - 1.942$	0.9928	0.142–14.2	0.089	0.355
S9	$y = 0.005x - 1.1808$	0.9940	0.102–10.2	0.003	0.008

drying gas temperature, 320 °C; nebulizing gas (N_2) pressure, 241 kPa (35 psig); capillary voltage, 4000 V; fragmentor of 120 V applied for quantitative analysis and 300 V for qualitative analysis; skimmer voltage, 60 V; octapole DC1, 37 V; octopole RF, 250 V. Reference masses consisted of fluorinated compound furnished by the manufacturer with empirical formulas. The instrument performed automatic autotuning using a dual nebulizer electrospray source with an automated calibrant delivery system, which introduced a constant flow (100 μl/min) of calibrating solution containing the internal standard masses (*m*/*z* 112.9856, 301.9981, 601.9790, 1033.9881, 1333.9689, 1633.9498, 1933.9306, 2233.9115, 2533.8923, 2833.8731). XICs for the $[M-H]^-$ ions or $[M+HCOO]^-$ ions of the target compounds were used for peak area determination and subsequent quantification. All the operation, acquisition and analysis of data were controlled by Agilent LC-MS TOF Software Ver. A.01.00 (Agilent Technologies, USA) and Applied Biosystems/MDS-SCIEX Analyst QS Software (Frankfurt, Germany).

2.5. Preparation of sample solution

Approximately 0.5 g pulverized plant samples were accurately weighed and extracted by refluxing for 1.5 h with 30 ml of methanol, and cooled at room temperature. Methanol was added to compensate for the lost weight. The methanol solution was filtered, and 5 ml of the filtrate was evaporated under vacuum. The residue was made up to exactly 5 ml with methanol containing a final concentration of 75 μg/ml ginsenoside R_1 (IS), and finally, the resultant solution was filtered through a 0.22 μm PTFE filter for HPLC-ESI/TOF MS analysis.

2.6. Calibration curves and limits of detection and quantification

Methanol stock solutions containing the 32 reference compounds were prepared and diluted to 6 appropriate concentrations for the construction of the calibration curves. The concentration of ginsenoside R_1 as the internal standard was 75 μg/ml for all the analysis. The calibration curves were constructed by plotting the peak area ratio (peak area of the analyte/peak area of the internal standard) versus the concentration of each analyte. The LODs and LOQs under the present chromatographic conditions were determined at signal-to-noise ratios (S/N) of 3 and 10, respectively.

2.7. Precision and accuracy

The precision was evaluated by calculating intra-day and inter-day variations at the intermediate concentration of standard solution. For the intra-day variability test, the standard solution was analyzed six times within one day; while for the inter-day variability test, the standard solution was examined in triplicate on three consecutive days. The relative standard deviations of the retention time (t_R) and peak area ratio (P_a) were taken as the measures of precision.

The recovery test was used to evaluate the accuracy of the method. Accurate amounts of mixed references were added to approximately 0.25 g of flower buds of *L. japonica*, *L. macranthoides* and *L. syringantha*, respectively, then extracted and analyzed as described in Section 2.5. The mean recovery was calculated on three assays.

3. Results and discussion

3.1. Separation optimization

The previous chromatographic conditions for determination of 20 compounds in *Lonicera* extracts by HPLC-DAD-ELSD [19] were used as the basis for mobile phase selection and optimization. Unfortunately, insufficient separation was obtained when the reported gradient of mobile phase was applied to the separation of 32 compounds and poor peak shapes were obtained for F3–F10. Under this circumstance, three parameters including mobile phase modifier, the type of solvent and the gradient program were studied by using univariate test. Eventually, after the adjustment of the chromatographic conditions (the additive was changed into formic acid, the eluting solvent was replaced with a mixture of water–acetonitrile–methanol system, and a new gradient program was selected), the resolutions as well as the peak shapes were much improved.

As shown in Fig. 2, under the optimized conditions, 32 compounds were separated within 75 min except for 6 couples of compounds (IG1 and IG2, F1 and F2, F3 and F4, IG6 and F6, F7 and F8, F9 and S5) remained unresolved. To overcome this post-separation issue, the choice of MS detector by using XIC experiment was preferred. Through XIC mode, qualitative and quantitative analysis could be achieved because the overlapped peaks produced different *m*/*z* values.

3.2. HPLC-ESI/TOF MS analysis of 32 reference compounds

In order to obtain information about retention times (t_R) and MS spectral data, 32 reference compounds were analyzed and the ESI/MS conditions were optimized firstly. Although positive and negative ion analyses were complementary, the best analytical selectivity and sensitivity for the compounds of interest were obtained by acquiring spectrum in negative ion mode.

The typical negative ESI mass spectra of 32 compounds studied in the experiment are shown in Fig. 2. The retention times, proposal ions and assignment of the chromatographic peaks are summarized in Table 1. Since formic acid was used as additive in the mobile phase, the base ions of most iridoid glycosides including IG1 and IG3–IG7, and some saponins like S1 and S4–S6 corresponded to the typical adduct ions $[M+HCOO]^-$, on the other hand, due to the free carboxyl and/or phenolic hydroxyl group in the skeletons, compounds like C1–C6, IG2, F1–F10, S2, S3, and S7–S9 gave pesudomolecular ions $[M-H]^-$ as the base peak ions (Fig. 1). These MS data observed were in good agreement with our previous reports [15,17]. Thus, XICs for the $[M+HCOO]^-$ or $[M-H]^-$ ions with a 0.01 Da mass window were selected for peak area determination and the peak area ratios of the analytes/IS were used for subsequent quantification.

It is worth noting that in this study, a daily calibration for the mass axis was performed prior to sample analysis by post-column infusion technique. After mass calibration, the effect of mass shift (less than 3 ppm) was negligible on the accuracy and precision of analysis within one day.

3.3. Method validation

Validation results for the established method are reported in Table 2. In general, the linearity of the analytical response within the test range was excellent, with correlation coefficients (r^2) higher than 0.99. The developed method also provided satisfactory sensitivity for all analytes with LODs less than 0.089 μg/ml. Compared with our previous studies, values of instrumental LODs obtained by LC/TOF MS were considerably improved, between 14- and 400-fold more sensitive than those reported previously by other methods, for example, for the 7 saponins S2, S3, S4, S6 S7, S8 and S9, the present values (0.055, 0.059, 0.052, 0.026, 0.030, 0.089 and 0.003 μg/ml, respectively) versus the literature values obtained by HPLC-DAD/ESI-MS (1.45, 1.91, 0.75, 1.40, 1.17, 0.42 and 1.0 μg/ml, respectively) [17] and the literature values obtained by HPLC-DAD-

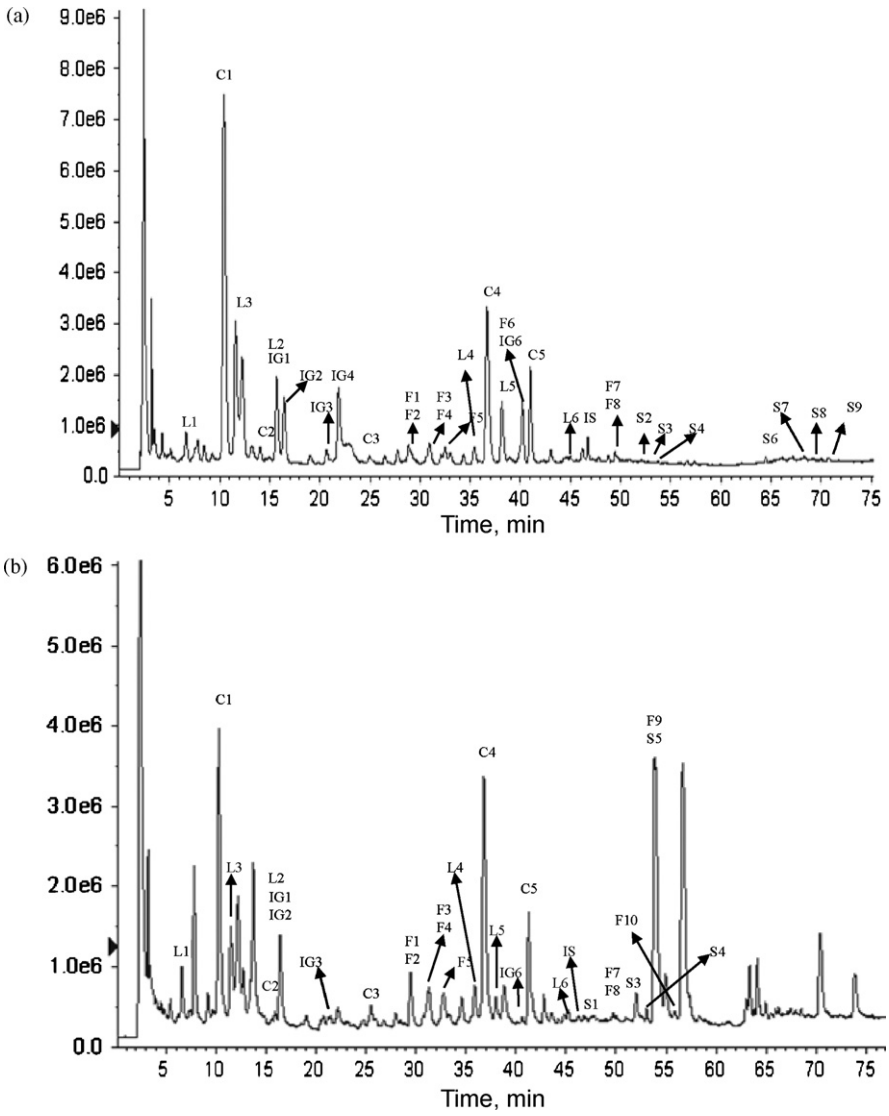


Fig. 3. (a) LC-ESI/TOF-MS TIC of *L. japonica*. (b) LC-ESI/TOF-MS TIC of *L. syringantha*. (Peak assignments are same as described in Section 2.1.)

ELSD (16.4, 11.4, 7.2, 10.6, 9.7, 10.2 and 7.8 μg/ml, respectively) [18].

The overall intra-day variations of P_a and t_R were less than 1.58% and 0.13%, respectively, and the overall inter-day variations of P_a and t_R were less than 3.47% and 0.38%, respectively. These precisions offered by TOF MS were nearly comparable with those obtained by selected ion monitoring (SIM) mode of single quadrupole MS [17]. The overall recoveries for all the constituents analyzed were between 85.4% and 101.6%. The above results were considered to be satisfactory for subsequent analysis of all samples.

We want to mention here that mainly due to the limited amounts of standards, the comparison of detector responses between the individual standards and the mixed standards was not performed, this is to say, the extent of ionization suppression effects related to co-eluting was not evaluated in this study.

3.4. Analysis of 32 bioactive constituents in Lonicera species by HPLC-ESI/TOF MS

The newly developed HPLC-MS assay was applied to simultaneous qualitative and quantitative analysis of the 32 compounds in 8 *Lonicera* species. Unequivocal peak identifications in the herbal matrixes were performed by means of accurate mass measurements (within 3 ppm error) and retention time match.

The representative total ion chromatogram (TIC) of *L. japonica* and *L. syringantha* are shown in Fig. 3. In general, *Lonicera* species contained the highest concentration of organic acids, followed by iridoid glycosides and saponins, and lowest concentration of flavonoids (Table 3), this chemical trend was similar to our previous work [17,18]. Primarily, according to the chemical distribution, eight species could be divided into three groups: group I contained *L. japonica*, characterized as relatively higher concentration of iridoid glycosides IG1–IG5, and relatively lower concentration of saponins; group II included *L. macranthoides*, *L. confusa*, *L. hypoglauca*, *L. fulvotomentosa*, *L. similes* and *L. dasystyla*, represented by higher level of saponins; group III covered *L. syringantha*, in which flavonoids F9 and F10, saponins S1 and S5 distinctively occurred. This division seemed to be consistent with the geographic characteristics of the species: *L. japonica* is a widespread species, *L. macranthoides*, *L. confusa*, *L. hypoglauca*, *L. fulvotomentosa*, *L. similes* and *L. dasystyla* are limited south-west China such as Hunan, Sichuan, and Guangxi Province, while the species *L. syringantha* is indigenous to north-west China, such as Qinghai and Gansu Province.

Table 3
Contents of the 32 analytes in eight *Lonicera* species (μg/g, $n = 3$).

Analyte	A	B	C	D
C1	12450.11 ± 388.44	15340.59 ± 478.61	12540.23 ± 391.25	3880.35 ± 121.06
C2	65.21 ± 2.33	31.82 ± 1.14	20.82 ± 0.75	10.25 ± 0.37
C3	11.59 ± 0.35	11.46 ± 0.30	3.38 ± 0.09	9.83 ± 0.26
C4	5441.23 ± 337.89	11540.10 ± 716.63	9876.59 ± 613.30	5102.38 ± 316.83
C5	3364.58 ± 147.34	5105.78 ± 223.60	2440.32 ± 106.87	1201.35 ± 52.60
C6	TR[a]	TR	TR	TR
IG1	2790.29 ± 64.17	103.57 ± 2.38	103.54 ± 2.38	200.14 ± 4.60
IG2	2551.00 ± 79.59	4.32 ± 0.14	3.11 ± 0.10	180.33 ± 5.62
IG3	100.60 ± 5.37	TR	TR	TR
IG4	3668.01 ± 158.46	7482.45 ± 323.22	5002.35 ± 216.09	302.15 ± 13.05
IG5	TR	TR	ND	ND
IG6	112.94 ± 6.63	40.56 ± 2.38	57.43 ± 3.37	9.37 ± 0.55
IG7	TR	TR	ND	ND
F1	179.27 ± 8.28	31.92 ± 1.48	34.08 ± 1.57	32.73 ± 1.51
F2	120.63 ± 6.16	21.36 ± 1.09	23.72 ± 1.21	18.94 ± 0.97
F3	70.21 ± 2.25	21.87 ± 0.70	61.75 ± 1.98	20.33 ± 0.65
F4	221.02 ± 9.54	58.08 ± 2.51	57.29 ± 2.50	2.77 ± 0.12
F5	631.65 ± 33.60	23.22 ± 1.24	32.25 ± 1.72	13.49 ± 0.72
F6	10.08 ± 0.22	38.06 ± 0.81	24.01 ± 0.51	16.21 ± 0.35
F7	151.32 ± 5.22	5.44 ± 0.19	6.31 ± 0.22	5.670 ± 0.20
F8	21.05 ± 1.20	10.44 ± 0.59	8.45 ± 0.48	9.83 ± 0.56
F9	ND	ND	ND	ND
F10	ND	ND	ND	ND
S1	ND	ND	ND	ND
S2	10.02 ± 0.56	6438.05 ± 361.82	6505.37 ± 365.58	3001.26 ± 168.66
S3	19.98 ± 0.87	6901.32 ± 300.88	5803.49 ± 253.01	TR
S4	40.26 ± 1.44	6338.12 ± 227.53	5811.29 ± 208.62	2500.49 ± 89.75
S5	ND	ND	ND	ND
S6	0.49 ± 0.05	44.70 ± 2.34	156.31 ± 8.19	3998.25 ± 209.50
S7	9.78 ± 0.43	316.35 ± 13.79	1100.42 ± 47.96	101.13 ± 4.41
S8	29.16 ± 1.34	116.32 ± 5.34	229.14 ± 10.52	20.50 ± 0.94
S9	10.92 ± 0.59	105.72 ± 5.67	96.04 ± 5.15	120.25 ± 6.44

Analyte	E	F	G	H
C1	9680.26 ± 302.02	15004.43 ± 468.00	1500.39 ± 46.80	4502.45 ± 140.46
C2	59.21 ± 2.12	15.99 ± 0.57	1003.42 ± 35.91	800.34 ± 28.65
C3	21.58 ± 0.56	10.56 ± 0.27	50.62 ± 1.31	30.29 ± 0.78
C4	8433.36 ± 523.69	6501.26 ± 403.71	1325.26 ± 82.28	4999.56 ± 310.50
C5	8060.35 ± 353.03	5000.45 ± 219.00	1254.32 ± 54.93	2507.11 ± 108.81
C6	ND[b]	ND	ND	ND
IG1	2222.32 ± 51.11	TR	600.14 ± 13.80	1600.68 ± 36.82
IG2	4.58 ± 0.14	39.96 ± 1.25	40.23 ± 1.26	2001.14 ± 62.43
IG3	TR	TR	51.64 ± 2.76	31.54 ± 1.68
IG4	8664.32 ± 374.28	TR	1005.43 ± 43.42	TR
IG5	TR	ND	TR	ND
IG6	687.12 ± 40.33	ND	98.52 ± 5.78	98.23 ± 5.77
IG7	ND	ND	ND	ND
F1	439.24 ± 20.29	261.13 ± 12.06	150.14 ± 6.94	79.98 ± 3.70
F2	19.56 ± 1.00	40.55 ± 2.07	106.34 ± 5.43	50.01 ± 2.56
F3	125.83 ± 4.04	55.38 ± 1.78	61.23 ± 1.97	14.79 ± 0.48
F4	140.63 ± 6.07	100.53 ± 4.34	203.14 ± 8.77	205.43 ± 8.87
F5	117.52 ± 0.40	19.86 ± 1.06	498.74 ± 26.53	600.09 ± 31.92
F6	TR	10.59 ± 0.23	91.10 ± 1.95	TR
F7	150.62 ± 5.20	101.24 ± 3.49	82.53 ± 2.85	142.34 ± 4.91
F8	10.02 ± 0.57	90.57 ± 5.14	60.59 ± 3.44	25.09 ± 1.42
F9	ND	ND	ND	2.64 ± 0.18
F10	ND	ND	ND	27.15 ± 0.97
S1	ND	ND	ND	5.23 ± 0.29
S2	64.20 ± 3.61	31.02 ±1.74	15.11 ± 0.85	TR
S3	4411.26 ± 192.32	20.32 ± 0.89	10.26 ± 0.45	5700.36 ± 248.52
S4	6501.32 ±233.39	60.88 ± 2.19	31.26 ± 1.12	5000.89 ± 179.50
S5	ND	ND	ND	6003.46 ± 313.98
S6	234.24 ± 12.27	302.62 ± 15.86	120.09 ± 6.29	795.63 ± 41.69
S7	5.32 ± 0.23	ND	21.30 ± 0.93	ND
S8	116.39 ± 5.34	ND	31.01 ± 1.42	TR
S9	508.73 ± 24.32	ND	ND	ND

[a] Trace.
[b] Not detected.

Table 4
Identification of unknown chromatographic peaks in *Lonicera* species.

Peak	t_R (min)	ESI/TOF MS ions (*m*/*z*)	Measured mass (Da)	Elemental composition	Error (ppm)	Identity
L1	6.57	$[M-H]^-$	353.0868	$C_{16}H_{17}O_9$	−2.8492	5-*O*-Caffeoyl quinic acid
		$[M-caffeoyl]^-$	191.0559	$C_7H_{11}O_6$	−1.1088	
		$[caffeoyloxy]^-$	179.0345	$C_9H_7O_4$	−2.6945	
		$[caffeoyloxy-CO_2]^-$	135.0447	$C_8H_7O_2$	−3.3554	
L2	15.51	$[M-H]^-$	353.0888	$C_{17}H_{19}O_9$	2.8151	4-*O*-Caffeoyl quinic acid
		$[M-caffeoyl]^-$	191.0566	$C_7H_{11}O_6$	2.5549	
		$[caffeoyloxy]^-$	179.0352	$C_9H_7O_4$	−2.6937	
L3	11.45	$[M-H]^-$	389.1094	$C_{16}H_{21}O_{11}$	1.1938	Secologanoside
		$[M-CO_2-H]^-$	345.1198	$C_{15}H_{21}O_9$	2.0103	
		$[M-glc-CO_2-H]^-$	183.0663	$C_9H_{11}O_4$	0.0951	
		$[M-glc-CO_2-H_2O-H]^-$	165.0557	$C_9H_9O_3$	−0.1082	
L4	35.59	$[M-H]^-$	515.1197	$C_{25}H_{23}O_{12}$	0.3879	4,5-di-*O*-Caffeoyl quinic acid
		$[M-caffeoyl]^-$	353.0883	$C_{16}H_{17}O_9$	1.399	
		$[M-caffeoyl-H_2O]^-$	335.0772	$C_{16}H_{15}O_8$	−0.1232	
		$[M-2C_9H_6O_3-H]^-$	191.0560	$C_7H_{11}O_6$	−0.5854	
		$[caffeoyloxy]^-$	179.0352	$C_9H_7O_4$	1.2152	
		$[M-2C_9H_6O_3-H_2O-H]^-$	173.0458	$C_7H_9O_5$	1.4602	
		$[caffeoyloxy-CO_2]^-$	135.0451	$C_8H_7O_2$	−0.3934	
L5	38.3	$[M-H]^-$	515.119	$C_{25}H_{23}O_{12}$	−0.9709	1,5-di-*O*-Caffeoyl quinic acid
		$[M-caffeoyl]^-$	353.0879	$C_{16}H_{17}O_9$	0.2661	
		$[M-caffeoyl-H_2O]^-$	335.0769	$C_{16}H_{15}O_8$	−1.1085	
		$[M-2C_9H_6O_3-H]^-$	191.0561	$C_7H_{11}O_6$	−0.062	
		$[caffeoyloxy]^-$	179.0350	$C_9H_7O_4$	0.0981	
		$[M-2C_9H_6O_3-H_2O-H]^-$	173.0456	$C_7H_9O_5$	0.3054	
		$[caffeoyloxy-CO_2]^-$	135.0451	$C_8H_7O_2$	−0.3934	
L6	45.23	$[M-H]^-$	529.1352	$C_{26}H_{25}O_{12}$	0.094	Methyl-di-*O*-caffeoylquinate
		$[M-caffeoyl]^-$	367.1040	$C_{17}H_{19}O_9$	1.4815	
		$[M-caffeoyl-CH_3]^-$	353.0882	$C_{16}H_{17}O_9$	1.1158	
		$[M-2C_9H_6O_3-H]^-$	191.0561	$C_7H_{11}O_6$	−0.062	
		$[caffeoyloxy]^-$	179.0356	$C_9H_7O_4$	3.4494	
		$[M-2C_9H_6O_3-H_2O-H]^-$	173.0455	$C_7H_9O_5$	−0.2724	
		$[caffeoyloxy-CO_2]^-$	135.0451	$C_8H_7O_2$	−0.3934	

3.5. Identification of six unknown chromatographic peaks in Lonicera species by HPLC-ESI/TOF MS

As for the "soft" ionization technique of electrospray ionization interface, the structure information about the target peaks was insufficient. The in-source collision-induced dissociation (CID) technique could partly compensate this disadvantage. The six chromatographic peaks L1–L6, lacking authenticated standards, were structurally characterized based on their MS fragmentation behavior obtained under the fragmentor voltage of 300 V.

Table 4 summarizes the MS data of the six compounds. It could be easily found that the compounds L1–L6 belonged to chlorogenic acid derivatives, since they all produced a series of diagnostic ions $[M-caffeoyl]^-$, $[caffeoyloxy]^-$, and$[caffeoyloxy-CO_2]^-$. L1 and L2, exhibiting identical fragment ions, were assigned to be isomers of chlorogenic acid (C1). Similarly, L4 and L5 were isomers of 3,5-di-*O*-caffeoyl quinic acid (C4). According to their polarity and eluting order on reversed column [30], L1, L2, L4 and L5 were tentatively identified as 5-*O*-caffeoylquinic acid, 4-*O*-caffeoylquinic acid, 4,5-di-*O*-caffeoyl quinic acid and 1,5-di-*O*-caffeoyl quinic acid, respectively. These four chlorogenic acid derivatives had ever been isolated from *L. macranthoides* [25] and *L. fulvotomentosa* [26], respectively. Compared with L4, L6 exhibited $[M-H]^-$ ion at *m*/*z* 529, strongly indicating an additional methyl group in the molecular structure. By retrieving the published phytochemical reports on *Lonicera*, L6 was tentatively identified as methyl-di-*O*-caffeoylquinate [25].

Compound L3 generated $[M-H]^-$ ion at *m*/*z* 389, corresponding to molecular formula of $C_{16}H_{21}O_{11}$. The fragment ions at *m*/*z* 345, 183 and 165 were attributed to successive loss of CO_2, glucose and water. By analyzing the above information combined with the previous phytochemical literature, L3 was tentatively identified as secologanoside [31].

4. Conclusion

An HPLC-ESI/TOF MS method for the simultaneously qualitative and quantitative determination of 32 constituents in *Lonicera* species was reported. At the same time, six unknown chromatographic peaks were structurally characterized through accurate mass measurement of TOF MS and CID experiment. Compared with our previous studies, this multi-component-assay method seemed more sensitive and informative for the quality control of Flos Lonicerae.

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