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Analysis of multiple constituents in a Chinese herbal preparation Shuang-Huang-Lian oral liquid by HPLC-DAD-ESI-MS^n

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Abstract

A high-performance liquid chromatography/mass spectrometry (HPLC–MS) method for the quality control of Shuang-Huang-Lian oral liquid, an antimicrobial and antipyretic herbal preparation, has been developed. Pure compounds are subjected to tandem mass spectrometry (MS^n) analysis to clarify their fragmentation rules. Then, the sample of Shuang-Huang-Lian was analyzed by on-line LC–MS^n. A total of 27 compounds, including seven phenylethanoid glycosides, three lignans, seven quinic acids, six saponins and four flavonoids, in the extract of Shuang-Huang-Lian oral liquid have been identified or tentatively characterized. It is expected to develop a comprehensive quality control method of this commonly used herbal preparation.
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Keywords: HPLC-DAD-ESI-MS^n; Multiple constituents; Chinese herbal preparation; Shuang-Huang-Lian oral liquid

1. Introduction

Due to the effective treatment with minimum side effects in many diseases [1–3], traditional Chinese medicine (TCM) has attracted considerable attention worldwide in recent years. However, the chemical components of TCM still remained unclear since they are a complex mixture containing hundreds of different chemical constituents [4], which hindered the use of TCM in clinical practice and retarded the process of TCM modernization. According to the Chinese medical theory, the effects of herbal medicine attributed to the combination of the multiple components in the herbs. Hence, development of practical and reliable methods for the identification of multi-components in the herbal mixture seems to be the first step for the comprehensive quality control of herbal products.

High-performance liquid chromatography (HPLC) is currently the most frequently used separation method to deal with complex chemical mixtures including the crude extracts of herbal medicines. HPLC fingerprinting now is a widely accepted technique which could cover most of the chemical constituents and is valuable for the quality control of herbal medicines. Certainly, a valuable and convincing HPLC fingerprinting should have most of its peaks assigned, especially the peaks corresponding to the effective constituents and toxic ingredients. It is, however, usually difficult toward this end owing to the lack of reference standards necessary for the structure identification. Mass spectrometry (MS) is a rapid and sensitive technique for structural elucidation. HPLC coupled with MS could facilitate informative and high-throughput screening of chemical constituents in TCM, especially those trace components which are difficult to obtain by conventional isolation means [5].

Shuang-Huang-Lian oral liquid (SHL) is a combined herbal remedy comprised of three herbs: Radix Scutellariae, Flos Lonicerae and Fructus Forsythiae. It is often used to treat acute upper respiratory tract infection caused by virus or bacteria. Its preparation was recorded in Chinese Pharmacopoeia [6] as follows: 375 g of Radix Scutellariae, 375 g of Flos Lonicerae and 750 g of Fructus Forsythiae were decocted, concentrated, extracted with ethanol, adjusted pH value with HCl and NaOH, distilled to eliminate solvent and the residue were dissolved and diluted with water to 1000 ml in volume. Baicalin, chlorogenic acid and forysthin are the marker compounds representing Radix Scutellariae, Flos Lonicerae and Fructus Forsythiae, respectively, for the quality control of this formula.

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Several published papers have reported the determination of major active components in SHL [7–9]. Apparently, only identification and quantitative determination of these major reference standards are not sufficient for the comprehensive quality control since TCM is a complex system containing tens or even hundreds of different chemical constituents which are responsible for the therapeutic effects [10]. Recently, there was a published paper [11] reported the development of characteristic HPLC-UV fingerprints of Radix Scutellariae, Flos Lonicerae, Fructus Forsythiae and their pharmaceutical preparation-SHL, but the "common peaks", existed in all chromatograms of different batches of the same samples, were not identified.

In this paper, an assay method to screen and identify the main constituents in SHL by LC/ESI-MS technique was described. With this method, 27 compounds in the formula, including seven phenylethanoid glycosides and three lignans contained in Fructus Forsythiae, seven quinic acids and six saponins contained in Flos Lonicerae, four flavonoids contained in Radix Scutellariae have been identified or tentatively characterized. Traditionally, quality control of a herbal medicine involves qualitative and quantitative analyses of one or several of the major compounds. Concerning the Shuang-Huang-Lian formula, determination of baicalin, chlorogenic acid and forysthin has been used in its quality control for a long time. While the less abundant compounds commercially unavailable were usually neglected. It is well known that one essential difference of traditional medicines from chemical drugs is that their effects owe to the joint contribution of multi-components, not only the major ones. Thus, it is expected to provide solid scientific evidence for the comprehensive quality control of Shuang-Huang-Lian oral liquid in the future.

2. Materials and methods

2.1. Chemicals and materials

Standards of baicalin, wogonin and chlorogenic acid were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (NICPBP) (Beijing, China). Suspensaside, suspensaside methyl ether, suspensaside A, forsythoside A, pinoresinol-4′-*O*-β-D-glucopyranoside, epipinoresinol-4″-*O*-β-D-glucopyranoside, phillyrin, forsythoside B were isolated from the fruits of *Forsythia suspense* by the authors. Their structures were unambiguously identified by NMR techniques, and their purities were above 98% as determined by HPLC. Shuang-Huang-Lian oral liquid was provided by a pharmaceutical company in China.

HPLC grade acetonitrile (CH_3CN) (Fisher, Fair Lawn, NJ, USA) and ultra-pure water were used for all analyses. The methanol used for extraction of sample was AR grade, purchased from Beijing Chemical Corporation (Beijing, China).

2.2. Sample preparation

A 2.0 ml SHL oral liquid were diluted with 70% methanol aqueous solution to 50 ml in a volumetric flask, and then filtered through a 0.45 μm membrane before LC–MS analysis.

2.3. HPLC conditions

An Agilent series 1100 HPLC instrument (Agilent, Waldbronn, Germany) equipped with a quaternary pump, a diode-array detector, an autosampler and a column compartment was used for analyses. The sample was separated on a Zorbax XDB-C_{18} column (5 μm, φ 4.6 mm × 250 mm, Agilent). The mobile phase were (A) CH_3CN and (B) water-acetic acid (100:0.2, v/v). A gradient program was used as follows: 12% A in the first 10 min, 20% A at 11–20 min, 25% A at 21–30 min, linearly gradient to 35% A at 40 min, then linearly gradient to 60% A at 41 min and hold for 10 min. The mobile phase flow rate was 0.8 ml/min; the chromatogram was recorded at 280 nm and spectral data for all peaks were accumulated in the range of 190–400 nm. Column temperature was controlled at 25 °C.

2.4. Mass spectrometric conditions

A Finnigan LCQ Advantage ion trap mass spectrometer (ThermoFinnigan, San Jose, CA) was connected to the Agilent 1100 HPLC instrument via ESI interface for HPLC/MS analysis. Ultrahigh pure helium (He) was used as the collision gas and high purity nitrogen (N_2) as the nebulizing gas. The optimized parameters in the negative ion mode were as follows: ion spray voltage, 4.5 kV; sheath gas, 40 arbitrary units; auxiliary gas, 15 units; capillary temperature, 320 °C in the whole analytical times. Capillary voltage, −5 V in the first 30 min; −35 V in the last 30 min. For full scan MS analysis, the spectra were recorded in the range of *m/z* 100–2000. The isolation width of precursor ions was 3.0 units. MS^n data were acquired in the automatic data-dependant mode.

3. Results and discussion

3.1. Tandem mass spectrometry of authentic compounds

Negative ion mode was selected for ESI-MS analysis in this study, as it provided extensive structural information via collision-induced dissociation (CID).

The ESI-MS^n data and fragmentations of the 11 standard compounds were listed in Table 1. Most of the authentic compounds exhibit $[M–H]^-$ ions of sufficient abundance that they could be subjected to MS^n analysis. While epipinoresinol-4″-*O*-β-D-glucopyranoside and phillyrin produced $[M + CH_3COO]^-$ ions at *m/z* 579 and 593, respectively.

Suspensaside A showed $[M–H]^-$ ion at *m/z* 621. And its MS^2 spectrum produced ions at *m/z* 459 and 441, resulted from the successive loss of caffeoyl moiety and H_2O group. CID of ion at *m/z* 459 gave a prominent peak at *m/z* 151, corresponding to the losses of a rhamnose (Δm = 146 u) and hexose (Δm = 162 u). These fragmentations were similar to those of forsythoside B. On the other hand, neutral loss of 134 Da (*m/z* 487) was observed due to the special ether ring in its structure, which yielded from the α-cleavage of the ether ring accompanying H transformation. Ions of *m/z* 427 and 397 were characteristic of hexose [12].

Table 1
MS^n product ions and fragmentations obtained from authentic compounds in this study

Compounds	$[M–H]^-$	MS^n ions m/z [fragments][a]
Phenylethanoid glycosides		
Forsythoside B	755	MS^2[755]: 593 $[M–H-caffeoyl]^-$; MS^3[593]: 461 $[593-api]^-$, 447 $[593-rha]^-$, 429 $[593-rha-H_2O]^-$, 315 $[593-rha-api]^-$; MS^4[447]: 315 $[447-api]^-$, 135 $[447-api-hexose-H_2O]^-$
Forsythoside A	623	MS^2[623]: 477 $[M–H-rha]^-$, 461 $[M–H-caffeoyl]^-$; MS^3[461]: 315 $[461-rha]^-$, 135 $[461-rha-hexose-H_2O]^-$
Suspensaside A	621	MS^2[621]: 487 $[M–H-134]^-$, 469 $[487-H_2O]^-$, 459 $[M–H-caffeoyl]^-$, 441 $[M–H-caffeoyl-H_2O]^-$, 427 $[487-60]^-$; MS^3[487]: 397 $[487-90]^-$, 179 $[glc-H]^-$
Suspensaside	639	MS^2[639]: 621 $[M–H-H_2O]^-$; MS^3[621]: 487 $[621-134]^-$, 469 $[487-H_2O]^-$, 459 $[621-caffeoyl]^-$, 441 $[621-caffeoyl-H_2O]^-$, 179 $[glc-H]^-$; MS^3[459]: 151$[459-rha-hexose]^-$
Suspensaside methyl ether	653	MS^2[653]: 621 $[M–H-OCH_4]^-$; MS^3[621]: 487 $[621-134]^-$, 469 $[487-H_2O]^-$, 459 $[621-caffeoyl]^-$, 441 $[621-caffeoyl-H_2O]^-$, 179 $[glc-H]^-$; MS^4[469]: 179 $[glc-H]^-$, 161 $[179-H_2O]^-$; MS^4[459]: 151 $[459-rha-caffeoyl]^-$
Lignans		
Pinoresinol-4′-*O*-β-D-glucopyranoside	519	MS^2[519]: 357 $[M–H-glc]^-$; MS^3[357]: 342 $[357-CH_3]^{-\bullet}$, 327 $[357-2CH_3]^-$, 151 $[357-206]^-$, 136 $[151-CH_3]^{-\bullet}$; MS^4[151]: 136 $[151-CH_3]^{-\bullet}$
Epipinoresinol-4″-*O*-β-D-glucopyranoside	579[b]	MS^2[579]: 519 $[M–H]^-$, 357 $[M–H-glc]^-$; MS^3[519]: 357 $[M–H-glc]^-$; MS^4[357]: 151 $[357-206]^-$
Phillyrin	593[b]	MS^2[593]: 533 $[M–H]^-$, 371 $[M–H-glc]^-$; MS^3[371]: 356 $[371-CH_3]^{-\bullet}$, 326 $[371-3CH_3]^{-\bullet}$; MS^4[356]: 341 $[356-CH_3]^-$, 177 [tetrahydrofuran ring cleavage], 135 $[341-206]^-$, 121$[135-CH_2]$
Flavonoids		
Baicalin	445	MS^2[445]: 269 $[M-GluA]^-$; MS^3[269]: 251 $[269-H_2O]^-$, 241 $[269-CO]^-$, 225 $[269-CO_2]^-$, 223 $[269-H_2O-CO]^-$, 197 $[269-CO_2-CO]^-$; MS^4[251]: 223 $[251-CO]^-$
Wogonin	283	MS^2[283]: 268 $[M–H-CH_3]^{-\bullet}$; MS^3[268]: 240 $[268-CO]^{-\bullet}$, 239 $[268-COH^\bullet]^-$, 223 $[268-CO_2H^\bullet]^-$, 212 $[268-2CO]^{-\bullet}$, 196 $[268-CO_2-CO]^-$, 163 $[^{0,2}A^-]$
Quinic acid		
Chlorogenic acid	353	MS^2[353]: 191 $[quinic acid-H]^-$, 179 $[caffeic acid-H]^-$; MS^3[191]: 127 $[191-CO-2H_2O]^-$

[a] gluA: glucuronic acid; rha: rhamnose; api: apinose; glc: glucose.
[b] $[M+CH_3COO]^-$ ion.

All of the three lignan standards belong to the furofuran type. The characteristic fragmentation of this type of lignans is the cleavage of a tetrahydrofuran ring as proposed in literature [13], leading to the occurrence of ions at m/z 176, 151 or 136.

Wogonin is a methoxylated flavone. It exhibited a significant radical anion $[M–H–CH_3]^{-\bullet}$ as the base peak. In MS^3 spectra, we observed the significant ion at m/z 239 $[M–H–CH_3{}^{-\bullet}–COH^\bullet]^-$, and the low signal intensity ions at m/z 163 ($^{0,2}A^-$, Nomenclature proposed by Fabre and Rustan [14]), 212 $[M–H–CH_3{}^\bullet–2CO]^{-\bullet}$, 223 $[M–H–CH_3{}^\bullet–CO_2H^\bullet]^-$, 240 $[M–H–CH_3{}^\bullet–CO]^{-\bullet}$, which is identical with the previous report [15].

3.2. HPLC-DAD-ESI-MS^n analysis of the Shuang-Huang-Lian oral liquid extract

The HPLC-UV and total ion current (TIC) profiles of the extract of Shuang-Huang-Lian oral liquid are given in Fig. 1.

More than 30 peaks were detected in Shuang-Huang-Lian oral liquid, and 27 of them were characterized (Table 2). Their chemical structures were provided in Fig. 2. Compounds **4**, **6**, **7**, **8**, **10**, **11**, **13**, **15**, **17** and **27** were unambiguously identified by comparing their HPLC retention times and UV spectra with the reference standards. For other constituents, the structures were tentatively characterized based on their retention times, UV spectra and MS^n fragmentation behaviors. Among these 27 compounds, there were seven phenylethanoid glycosides and three lignans derived from Fructus Forsythiae, seven quinic acids and six saponins from Flos Lonicerae, four flavonoids from Radix Scutellariae.

3.2.1. Identification of phenylethanoid glycosides

By comparing the retention times, UV spectra and MS^n fragmentation pattern with those of the reference compounds, the chemical identities corresponding to peaks **6**, **7**, **8**, **10** and **11** were identified as suspensaside, forsythoside B, suspensaside methyl ether, forsythoside A and suspensaside A, respectively.

Due to the lack of reference compounds, the other 2 peaks were tentatively identified as phenylethanoid glycosides according to their HPLC/MS^n analysis. Peak 2 exhibited a $[M–H]^-$ ion at m/z 461. And its MS^2 produced ion at m/z 315, suggesting the presence of a rhamnose moiety. The ions of m/z 205, 163 and 135 were also observed, which were identical with the MS^3 spectrum of forsythoside A, while its molecular mass was 162 Da less than that of forsythoside A. Peak 9 gave a $[M–H]^-$ ion at m/z 623, an isomer of forsythoside A. By examining the known phenylethanoid glycosides in Fructus Forsythiae, compounds 2 and 9 were tentatively characterized as forsythoside E [16] and acteoside [17,18], respectively.

Table 2
Characterization of compounds in Shuang-Huang-Lian oral liquid by HPLC-DAD-ESI-MS

Peak no.	Retention time (t_R, min)	UV λ_{max} (nm)	$[M–H]^-$ m/z	HPLC-ESI-MS^n m/z [fragments][b] (% base peak)	Identification
1	2.94	202, 222	401[a]	MS^2[401]: 341 $[M–H]^-$(100); MS^3[341]: 179 $[M–H-glc]^-$ (100), 161 $[M–H-glc-H_2O]^-$ (25)	Caffeic acid glucoside
2	5.46	200, 222	461	MS^2[461]: 315 $[M–H-rha]^-$(65), 205(70), 163(25), 135(100)	Forsythoside E
3	5.95	222, 306, 326	353	MS^2[353]: 191(100), 179 [caffeic acid-H]$^-$ (70), 135 [caffeic acid-H-CO_2]$^-$ (10)	3-Caffeoylquinic acid
4*	8.97	222, 306, 326	353	MS^2[353]: 191 [quinic acid-H]$^-$ (100), 179 [caffeic acid-H]$^-$ (2); MS^3[191]: 127 [191-CO-2H_2O]$^-$ (100)	Chlorogenetic acid
5	9.83	222, 306, 326	353	MS^2[353]:191(7), 179(85), 173 [quinic acid-H_2O-H]$^-$ (100), 135(5); MS^3[173]: 111 [173-CO_2-H_2O]$^-$ (100)	4-Caffeoylquinic acid
6*	15.37	204, 218	639	MS^2[639]: 621 $[M–H_2O]^-$(100), 529(5); MS^3[621]: 469 [621-134-H_2O]$^-$ (100)	Suspensaside
7*	18.03	226	755	MS^2[755]: 593 [M–H-caffeoyl]$^-$ (100); MS^3[593]: 461 [593-api]$^-$(53), 447 [593-rha]$^-$ (100), 429 [593-rha-H_2O]$^-$ (18), 315 [593-rha-api]$^-$ (3)	Forsythoside B
8*	18.41	222, 306, 326	653	MS^2[653]: 621[M–H-OCH_4]$^-$ (100); MS^3[621]: 469[487-H_2O]$^-$ (100), 459 [621-caffeoyl]$^-$ (75), 441 [621-caffeoyl-H_2O]$^-$ (15), 179 [glc-H]$^-$ (20)	Suspensaside methyl ether
9	18.68	222, 306	623	MS^2[623]: 461(100), 443(10), 203(2)	Acteoside
10*	19.16	222, 328	623	MS^2[623]: 461 [M–H-caffeoyl]$^-$ (100), 443 [461-H_2O]$^-$(10), 179 [glc-H]$^-$ (5); MS^3[461]: 315 [461-rha]$^-$ (20), 205 [315-dihydroxyphynely]$^-$(100), 135[461-rha-hexose-H_2O]$^-$ (60)	Forsythoside A
11*	22.37	222, 306, 324	621	MS^2[621]: 487 [M–H-134]$^-$ (100), 469 [487-H_2O]$^-$ (80), 459 [M–H-caffeoyl]$^-$ (60)	Suspensaside A
12	22.93	224, 302, 324	515	MS^2[515]: 353 [M–H-caffeoyl]$^-$(100); MS^3[353]: 179(100), 173(70), 191(40), 135(5)	3,4-Dicaffeoylquinic acid
13*	23.30	226	519	MS^2[519]: 357 [M–H-glc]$^-$ (100); MS^3[357]: 311(4), 151 [357-206]$^-$ (100), 136 [151-CH_3]$^{-\bullet}$(5)	Pinoresinol-4′-*O*-β-D-glucopyranoside
14	25.32	222, 306, 326	515	MS^2[515]: 353 [M–H-caffeoyl]$^-$ (100); MS^3[353]: 191(100), 179(70)	3,5-Dicaffeoylquinic acid
15*	25.73	228	579[a]	MS^2[579]: 519 $[M–H]^-$ (100), 357 [M–H-glc]$^-$ (80); MS^3[519]: 357 [M–H-glc]$^-$ (100), 151 [357-206]$^-$ (95)	Epipinoresinol-4″-*O*-β-D-glucopyranoside
16	26.69	222, 326	515	MS^2[515]: 353 [M–H-caffeoyl]$^-$ (100); MS^3[353]: 173(100), 179(80), 191(20), 135(10)	4,5-Dicaffeoylquinic acid
17*	29.18	222, 276, 316	445	MS^2[445]: 269 [M-GluA]$^-$ (100); MS^3[269]: 251[269-H_2O]$^-$ (25), 241 [269-CO]$^-$ (5), 197 [269-CO_2-CO]$^-$ (3)	Baicalin
18	32.86	230	593[a]	MS^2[593]: 533 $[M–H]^-$ (30), 371 [M–H-glc]$^-$ (100); MS^3[371]: 356 [371-CH_3]$^{-\bullet}$(100); MS^4[356]: 121(100)	3′,4′,5′-trimethoxyl-4″-hydroxyllignan-*O*-glucoside
19	36.71	228, 270, 306	459	MS^2[459]: 283 [M-GluA]$^-$ (100); MS^3[283]: 268 [283-CH_3]$^{-\bullet}$(100), 239 [268-CO-H]$^-$(2); MS^4[268]: 239 [268-CO-H]$^-$ (25), 223 [268-CO_2-H]$^-$(15)	Oroxylin A-7-*O*-glu acid
20	39.22	228, 274	459	MS^2[459]: 283 [M-GluA]$^-$ (100); MS^3[283]: 268 [283-CH_3]$^{-\bullet}$(100); MS^4[268]: 239 [268-CO-H]$^-$ (25), 163 [$^{0,2}A^-$] (22)	Wogonoside
21	39.81	–	1457[a]	MS^2[1457]: 1397 $[M–H]^-$(80), 1133 [M + CH_3COO-2glc]$^-$(100)	Macranthoidin B
22	40.79	–	1295[a]	MS^2[1295]: 1235 $[M–H]^-$(100)	Macranthoidin A
23	41.90	–	1133[a]	MS^2[1133]: 1073 $[M–H]^-$(100)	Dipsacoside B
24	44.73	–	911	MS^2[911]: 749(100), 603 (70), 471 (2); MS^3[749]: 603 (100), 585 (10), 471(5); MS^4[603]: 471 (100)	Hederagenin-28-*O*-ara-rha-glc ester
25	45.40	–	1073	MS^2[1073]: 911 [M–H-glc]$^-$(100), 893 [M–H-glc-H_2O]$^-$ (10), 749 [M–H-2glc]$^-$ (60), 603 [M–H-2glc-rha]$^-$ (50); MS^3[911]: 749 [911-glc]$^-$ (100), 603 [911-glc-rha]$^-$ (50), 471 [911-glc-rha-ara]$^-$ (5)	Macranthoside B
26	45.64	–	911	MS^2[911]: 749 [M–H-glc]$^-$ (100), 603 [M–H-glc-rha]$^-$ (80), 471 [A-H]$^-$ (5); MS^3[749]: 603 [M–H-glc-rha]$^-$ (100), 585 [M–H-glc-rha-H_2O]$^-$ (5), 471 [A-H]$^-$ (10); MS^4[603]: 471 [603-ara]$^-$ (100)	Macranthoside A
27*	47.97	226, 274	283	MS^2[283]: 268 (100); MS^3[268]: 239(60), 212(15), 163(30)	Wogonin

*Compared with standards.
[a] $[M + CH_3COO]^-$ ion.
[b] glc: glucose; ara: arabinose; rha: rhamnose; GluA: glucuronic acid.

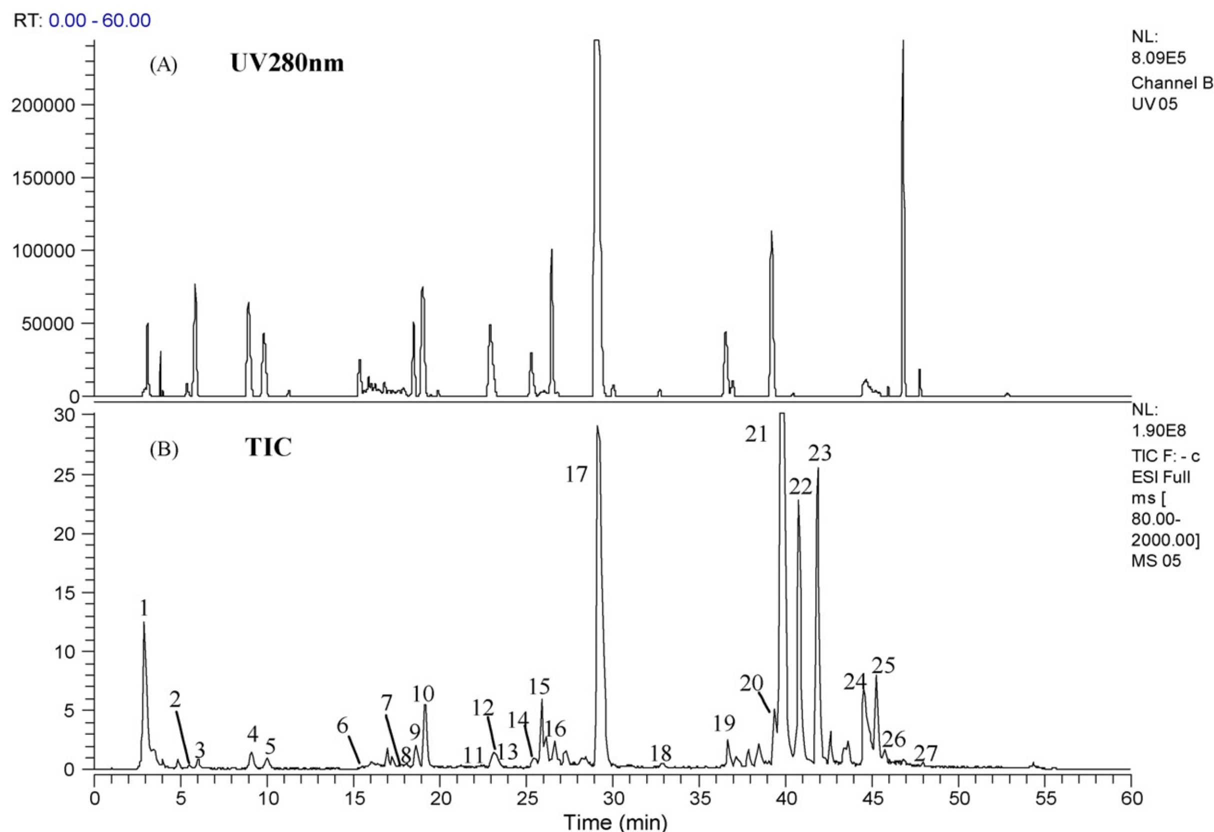


Fig. 1. HPLC-DAD-ESI-MS^n analysis of the methanolic extract of Shuang-Huang-Lian oral liquid. (A) HPLC-UV chromatogram monitored at 280 nm. (B) LC-negative ion ESI-MS total ion current (TIC).

3.2.2. Identification of lignans

Peaks 13 and 15 were identified as pinoresinol-4′-*O*-β-D-glucopyranoside and epipinoresinol-4″-*O*-β-D-glucopyranoside, respectively, by comparing with the standard compounds. The retention time of peak 18 was almost the same as phillyrin, but their MS^4 spectrum (as shown in Fig. 3) were greatly different. Except a significant base ion at *m/z* 121, no other fragments in the MS^4 spectrum of peak 18 were observed, while in the MS^4 spectrum of phillyrin, ions of *m/z* 177 and 341, accompanying the ion at *m/z* 121 displayed. By referring the recent literature [19], the peak 18 was thus tentatively identified as 3′,4′,5′-trimethoxyl-4″-hydroxyllignan-*O*-glucoside.

3.2.3. Identification of flavonoids

Peaks 17 and 27 were identified as baicalin and wogonin, respectively, by comparing with the standards. And peak 17 is the main constituent in SHL oral liquid. Peaks 19 and 20 are a pair of isomers. Both of them gave a $[M–H]^-$ ion at *m/z* 459. Their MS^2 spectrum gave the ion at *m/z* 283 (−176), involving the loss of a glucuronic acid, then *m/z* 283 yielded ion at *m/z* 268 (−15), suggesting the presence of a $–CH_3$ group. By examining the known flavonoids in *S. baicalensis*, there were two flavones, named oroxylin A-7-*O*-glucuronide and wogonoside, consistent with the above data. According to the content difference and the retention time in HPLC reported before [20], compounds 19 and 20 were plausibly identified as oroxylin A-7-*O*-glucuronide and wogonoside, respectively.

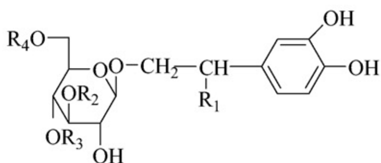
3.2.4. Identification of quinic acid derivatives

Chlorogenic acid (5-caffeoylquinic acid) has been previously reported as the chemical marker for the quality control of Flos Lonicerae, owing to its antipyretic and antibiotic property as well as its high content in the herb. In this paper several other quinic acid derivatives were identified by HPLC/MS^n.

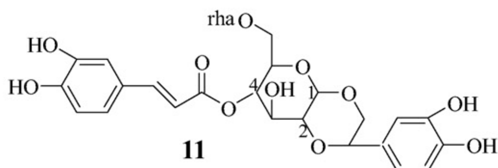
It was reported that the linkage position of acyl groups on quinic acid could be determined on the basis of the MS^2 fragmentation [21]. Generally, when the acyl group was linked to 3-OH or 5-OH, the $[\text{quinic acid–H}]^-$ ion at *m/z* 191 was the base peak, and the $[\text{caffeic acid–H}]^-$ ion at *m/z* 179 was more significant for 3-*O*-caffeoylquinic acids. While the $[\text{quinic acid–H}_2\text{O–H}]^-$ ion at *m/z* 173 was the prominent peak when the acyl group connected to 4-OH.

Chlorogenic acid (peak 4, t_R = 8.97 min) was identified by comparing with the standard. Compounds 3 and 5 both displayed $[M–H]^-$ ion at *m/z* 353, the same as chlorogenic acid. But these two isomers exhibited different MS^2 spectra. In the MS^2 spectrum of compound 3, the *m/z* 191 was the base peak, while *m/z* 173 $[\text{quinic acid–H}_2\text{O–H}]^-$ was the base peak for compound 5. Their MS spectra were shown in Fig. 4. By referring to literature data [13], compounds 3 and 5 were identified as 3-caffeoylquinic acid and 4-caffeoylquinic acid, respectively. Compounds 12, 14 and 16 all gave the $[M–H]^-$ ions at *m/z* 515 and the $[M–H–162]^-$ ions at *m/z* 353. However, their MS^3 spectra were significantly different. Compound 14 produced base peak ion at *m/z* 191, as reviewed above, it was identified as

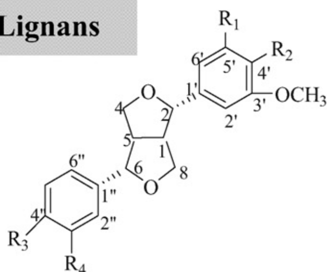
Phenylethanoid glycosides



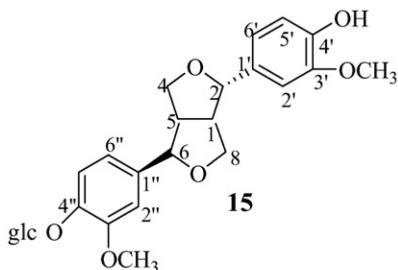
Compounds	R_1	R_2	R_3	R_4
2	H	H	H	rha
6	OH	H	caffeoyl	rha
7	H	rha	caffeoyl	api
8	OCH_3	H	caffeoyl	rha
9	H	rha	caffeoyl	H
10	H	H	caffeoyl	rha



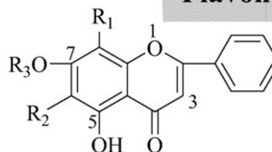
Lignans



Compounds	R_1	R_2	R_3	R_4
13	H	Oglc	OH	OCH_3
18	OCH_3	OCH_3	Oglc	H

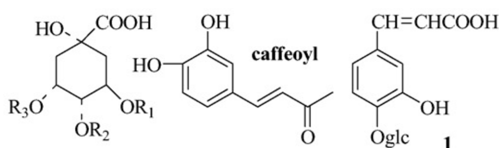


Flavonoids



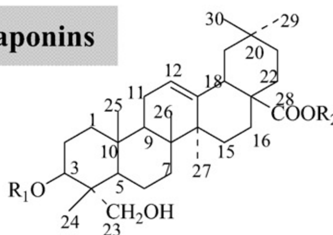
Compounds	R_1	R_2	R_3
17	H	H	glu acid
19	H	OCH_3	glu acid
20	OCH_3	H	glu acid
27	OCH_3	H	H

Quinic acid derivatives



Compounds	R_1	R_2	R_3
Qunic acid	H	H	H
3	H	H	caffeoyl
4	caffeoyl	H	H
5	H	caffeoyl	H
12	H	caffeoyl	caffeoyl
14	caffeoyl	H	caffeoyl
16	caffeoyl	caffeoyl	H

Saponins



Compounds	R_1	R_2
21	ara(2-1)rha(3-1)glc(4-1)glc	glc(6-1)glc
22	ara(2-1)rha(3-1)glc	glc(6-1)glc
23	ara(2-1)rha	glc(6-1)glc
24	H	ara(2-1)rha (3-1)glc
25	ara(2-1)rha(3-1)glc(4-1)glc	H
26	ara(2-1)rha(3-1)glc	H

Fig. 2. Chemical structures of compounds identified in Shuang-Huang-Lian oral liquid. glc: glucosyl; ara: arabinose; rha: rhamnosyl; api: apiosyl.

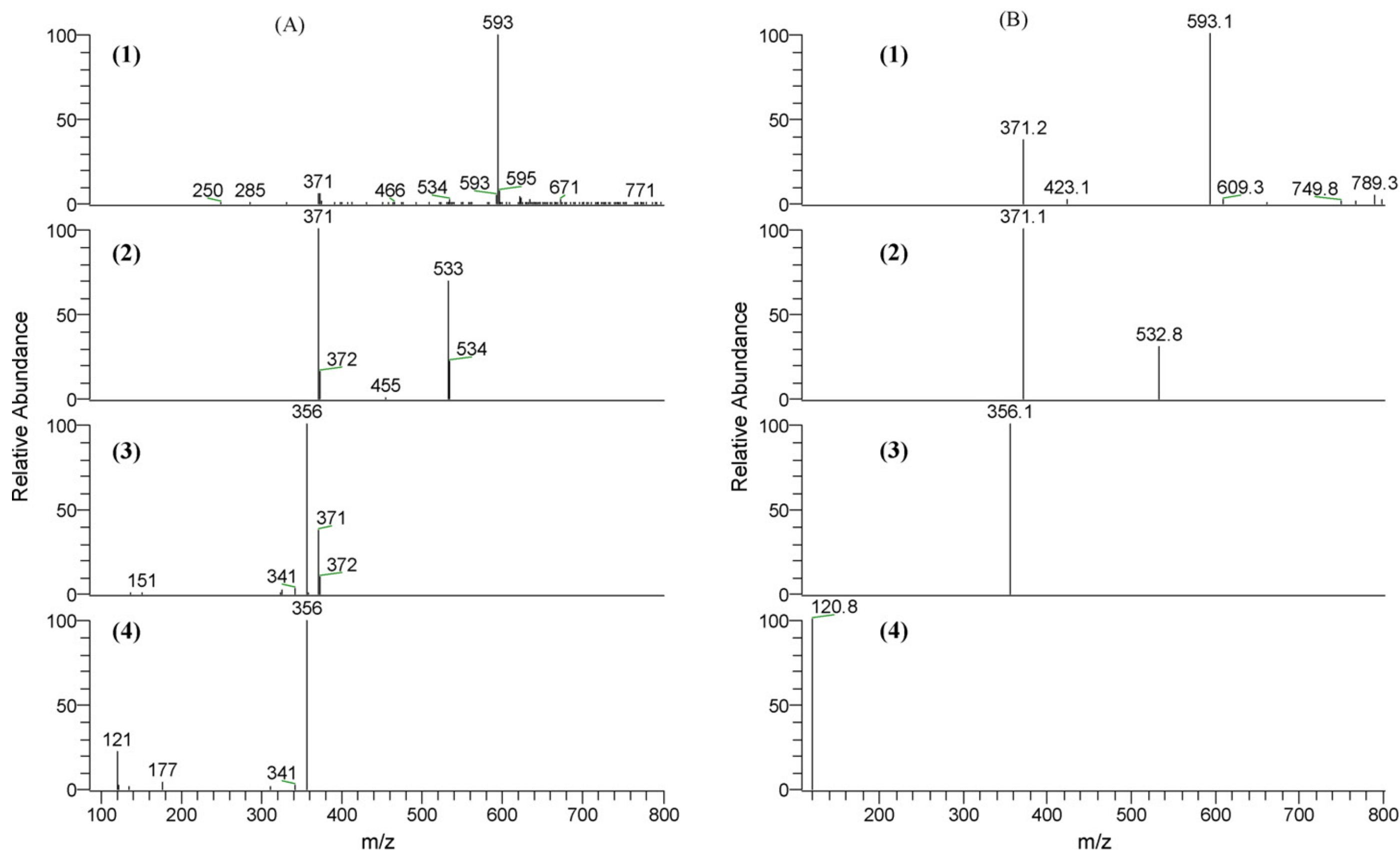


Fig. 3. Mass spectra of phillyrin (A) and 3′,4′,5′-trimethoxy-4″-hydroxyl lignan-*O*-glucoside (B). (1) MS spectrum; (2) MS^2 spectrum of the $[M+Ac]^-$ ion at *m/z* 593; (3) MS^3 spectrum of the ion at *m/z* 371; (4) MS^4 spectrum of the ion at *m/z* 356.

3,5-dicaffeoylquinic acid, which was consistent with the previous report [13]. Other two compounds produced base peak at *m/z* 179 or 173, hence they were identified as 4-substituted quinic acids. According to the literature [22], 3,5-dicaffeoylquinic acid was more easily eluted from the reverse-phase column when compared with 4,5-dicaffeoylquinic acid. Thus, compound 16 was identified as 4,5-dicaffeoylquinic acid, and the compound 12 was identified as 3,4-dicaffeoylquinic acid.

Peak 1 exhibited $[M+CH_3COO]^-$ ion at *m/z* 401, and further CID analysis produced $[M–H]^-$ ion at *m/z* 341, 179 and 161. Thus, it was tentatively identified as the glucoside of caffeic acid.

3.2.5. Identification of saponins

Besides the organic acids such as chlorogenic acid, saponins have also been isolated from Flos Lonicerae. And it was revealed that triterpenoidal saponins in Flos Lonicerae have the protective effect on hepatic injury [23].

Peak 26 ($t_R = 45.77$ min) exhibited $[M–H]^-$ ion at *m/z* 911. The MS^2 fragmentation showed ions at *m/z* 749 $[M–H–Glc]^-$, 603 $[M–H–Glc–Rha]^-$ and 471 $[A–H]^-$, suggesting the sugar sequence and the aglycone ion of the saponin. In its MS^3 spectrum, ion at *m/z* 585 $[M–H–Glc–Rha–H_2O]^-$ and 471 appeared. Further MS^4 analysis of *m/z* 603 produced ion at *m/z* 471, attributed to the loss of arabinose moiety ($\Delta m = 132$ u). By examining the known saponins in Flos Lonicerae, it was plausibly characterized as macranthoside A [24]. Compound 24 ($t_R = 44.73$ min) also exhibited $[M–H]^-$ ion at *m/z* 911, and it showed similar CID fragmentation with macranthoside A. It appeared that the saponins with sugar moieties at the C-28 position eluted easily than that with sugar moieties at the C-3 position in RP-HPLC [25]. Thus the compound 24 was tentatively identified as hederagenin-28-*O*-ara-rha-glc ester, which was reported in Flos Lonicerae for the first time. In the MS spectrum of compounds 21 ($t_R = 39.81$ min), 22 ($t_R = 40.79$ min), 23 ($t_R = 41.90$ min) and 25 ($t_R = 45.40$ min), ions of $[M–H]^-$ and their adduct ions $[M+CH_3COO]^-$ with high abundance were found. As a result, the molecular weights of these four compounds could be given as 1398, 1236, 1074 and 1074, respectively. Based on the information of their retention times in HPLC and referred to the previous reported literature [25], the compounds corresponding to peaks 21, 22, 23 and 25 were therefore tentatively characterized as macranthoidin B, macranthoidin A, dipsacoside B and macranthoside B, respectively.

Difficulties in detection of saponins by UV detector encouraged the method development using MS in characterization, confirmation and determination of saponins in plant extracts. A number of comprehensive researches have been performed on ginseng saponins in *Radix Gingseng* [26–28] and soyasaponins in soybean [29,30]. These studies, however, clearly showed that original standards are essential for successful application of LC/ESI-MS technique in the identification and determination of saponins. In this paper, owing to the lack of reference saponins, these peaks were subjected to LC/MS^n analysis. However, under

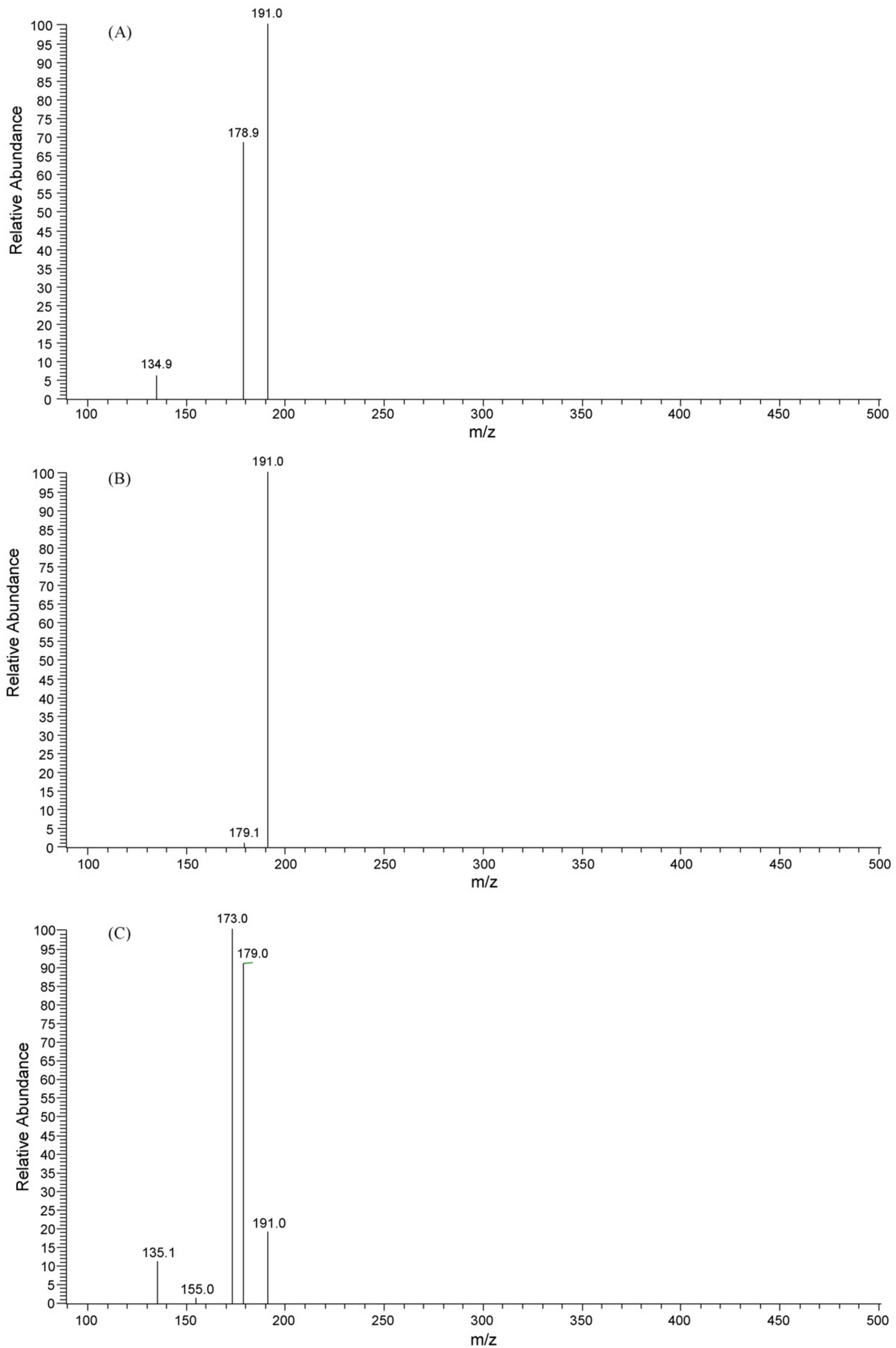


Fig. 4. On-line mass spectra of 3-caffeoylquinic acid (3), chlorogenetic acid (4), 4-caffeoylquinic acid (5). (A) MS^2 spectrum of 3-caffeoylquinic acid; (B) MS^2 spectrum of chlorogenetic acid (5-caffeoylquinic acid); (C) MS^2 spectrum of 4-caffeoylquinic acid.

this condition, except for compound 26 (macranthoside A), other compounds were difficult to produce MS^n fragmentation, while they were easy to gave MS^2 fragments of $[M–2 \times Glc]^-$. This could be attributed to their high molecular weight and relatively less contents in the sample.

4. Conclusions

In the present study, 27 compounds including seven phenylethanoid glycosides, three lignans, seven quinic acids, six saponins and four flavonoids, in the extracts of Shuang-Huang-Lian oral liquid have been identified or tentatively characterized by LC-DAD-ESI-MS^n method. Based on their retention times in RP-HPLC, UV spectra and MS^n information, the components derived from each herb in preparations were rapidly assigned, which demonstrated that LC–MS is a valuable technique to analyze the multi-component herbal formulae. Our present method could comprehensively evaluate the quality of Shuang-Huang-Lian samples and extracts covering majority of the chemical constituents, and reduce the necessity to isolate each individual component utilizing tedious conventional procedures. It set a good example for the rapid identification of bioactive constituents in complex herbal preparations and made it possible to fulfill the requirements for a modern drug characterized as safe, effective and quality controllable.

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