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Characterization of flavonoids in the traditional Chinese herbal medicine-Huangqin by liquid chromatography coupled with electrospray ionization mass spectrometry

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Abstract

The root of *Scutellaria baicalensis*, called Huangqin in Chinese, is one of the most commonly used traditional Chinese medicines for the treatment of hepatitis, tumors, diarrhea, and inflammatory diseases. The major chemical constituents of Huangqin are flavonoids. In the present paper, HPLC-DAD-ESI-MS^n was used to analyze flavonoids in the roots of *S. baicalensis*. A total of 26 flavonoids were identified or tentatively characterized, including 5 C-glycosides, 12 O-glycosides, and 9 free aglycones. Two C-glycosides, apigenin-6-C-glucyl-8-C-arabinoside and chrysin-6,8-di-C-glucoside, together with some O-glycosides, are reported from *S. baicalensis* for the first time. This method is simple, reliable and sensitive, and could be used for the quality control of Huangqin and its related preparations.
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Keywords: Huangqin; Flavonoids; HPLC/DAD/ESI-MS^n; Chinese medicine

1. Introduction

Huangqin (*Radix Scutellariae*) is an important traditional Chinese medicine prepared from the roots of *Scutellaria baicalensis* Georgi (Labiatae family) [1]. Traditionally, it was employed for detoxication and relief of fever. Currently, Huangqin is widely used for the treatment of various inflammatory diseases, hepatitis, tumors and diarrhea in East Asian countries. It acts as a key ingredient in a number of formulae such as Shuanghuanglian oral liquid, Compound Huangqin granule, Yinhuang Tablet, etc. The chemical constituents of Huangqin have been investigated in some detail, mostly involving flavonoids like baicalein, baicalin, wogonin and wogonoside. More than 60 flavonoids have been reported from Huangqin, and most of them occur as glucuronoids [2]. These compounds showed significant biological activities, and are critical for the quality control of Huangqin.

For quality control of Huangqin, previous reports involved the analysis of few major flavonoids by HPLC coupled to UV detection. Although LC/MS has recently been used for the analysis of Huangqin, these reports only focused on known compounds [3,4]. Little effort has been contributed to global analysis of flavonoids in *S. baicalensis*, especially those new minor ones.

Liquid chromatography coupled with mass spectrometry (LC/MS) is a powerful tool for the rapid identification of chemical constituents in plant extracts. The high sensitivity of MS as an LC detector facilitates to discover new minor constituents, which are difficult to obtain by classical means.

The tandem mass spectrometric fragmentation behavior of flavonoids has been investigated extensively, which allows the characterization of unknown compounds even without the reference standards [5–7]. A study on 14 flavonoids, monohydroxy- to pentahydroxy-substituted, has been carried out using HPLC-ESI-MS by Hughes et al. [8]. Wu et al. [9] studied the fragmentation pathways of nine flavonoid compounds by ESI-MS and observed some diagnostic neutral losses for the identification of the functional groups in the structures. Also, it was demonstrated that ions $[M-H-60]^-$, $[M-H-90]^-$,

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$[M - H - 120]^-$ were characteristic for C-glycoside flavonoids. Eleven naturally occurring flavonoid aglycones, belonging to different types, were determined by HPLC-ESI-MS/MS [10]. Kite et al. [11] described an ion trap LC–MS/MS method for the analysis of C-glycosylflavone O-glycosides in crude methanolic extracts of plants.

In the present study, HPLC-DAD-ESI-MS^n was used to analyze flavonoids in the roots of *S. baicalensis*. A total of 26 flavonoids were identified or tentatively characterized, including 5 C-glycosides, 12 O-glycosides, and 9 free aglycones. Two C-glycosides, apigenin-6-C-glucyl-8-C-arabinoside and chrysin-6, 8-di-C-glucoside, together with some O-glycosides, are reported from *S. baicalensis* for the first time.

2. Experimental

2.1. Chemicals and materials

Baicalin, baicalein and wogonin were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (NICPBP) (Beijing, China). Their structures are shown in Fig. 1.

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

2.2. Plant materials and samples preparation

The roots of *S. baicalensis* were collected from different regions around China (see Table 1) and identified by the authors.

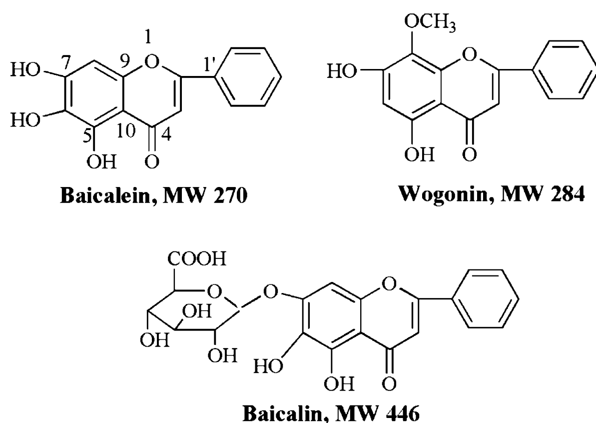

Fig. 1. Chemical structures of three standard flavones in *Scutellaria baicalensis*.

Table 1
The source of *Radix Scutellariae*

	Sample number					
	1#	2#	3#	4#	5#	6#
Growth region	Shanxi	Shanxi	Hebei	Heilongjiang	Neimenggu	Beijing

The materials were pulverized and an aliquot of 1 g was extracted with 50 mL of 70% methanol aqueous solution by ultrasonication for 30 min and then filtered. The filtrate was evaporated to dryness and the residue was dissolved in 1 mL of methanol. This solution was then filtered through a 0.45 μm membrane before use and 10 μl was injected into the HPLC instrument for 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 × 250 mm, Agilent). The mobile phase consisted of CH_3CN (A) and water containing 0.2% acetic acid (B). A gradient program was used as follows: 20% A in the first 10 min, 25% A at 11–25 min, 35% A at 26–41 min, linearly gradient to 55% A at 45 min, then linearly gradient to 60% A at 55 min and hold for 5 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; capillary voltage, −5 V; tube lens offset voltage, 40 V. For full scan MS analysis, the spectra were recorded in the range of *m/z* 100–1000. The isolation width of precursor ions was 3.0 U. The relative collision energy was adjusted to 45% of maximum. MS^n data were acquired in the automatic data-dependant mode.

2.5. Method validation

The repeatability was assessed by analyzing six independently prepared samples of Huangqin. The relative standard deviation (R.S.D.) of the peak retention times for each of the main characterized flavonoids in the extracts was calculated.

The method precision was evaluated by analyzing the same sample for five consecutive times. The R.S.D. of the peak retention times for each of the main characterized flavonoids in the extracts was calculated.

The sample stability test was determined with one sample during two days. In this period, the solution was stored at room temperature, and analyzed at 0, 12, 24 and 48 h, respectively.

Table 2
MS^n product ions obtained from standard flavonoids in this study

Compound	$[M-H]^-$	MS^n ions m/z (% base peak)
Baicalein	269	MS^2[269]: 251(100), 241(40), 225(40), 223(40); MS^3[251]: 223(100)
Baicalin	445	MS^2[445]: 269(100), 175(20); MS^3[269]: 251(100), 241(25), 225(10), 223(20), 197(10); MS^4[251]: 223(100)
Wogonin	283	MS^2[283]: 268(100); MS^3[268]: 240(100), 239(90), 223(20), 212(20), 196(20), 163(80)

3. Results and discussion

3.1. Tandem mass spectrometry of pure standards

Negative ion mode was selected for ESI-MS analysis in this study, as it provided extensive structural information via collision-induced dissociation (CID). Nomenclature proposed by Fabre et al. was used in this context for flavonoid fragments [10].

ESI-MS^n spectral data of baicalin, baicalein and wogonin are shown in Table 2. The $[M-H]^-$ ion of baicalein at m/z 269 produced an ion at m/z 251, which should result from the loss of H_2O. The ion at m/z 241 was due to the loss of CO, and the m/z 223 ion was due to the successive loss of H_2O and CO (Fig. 2).

Baicalin is an O-glucuronide. Upon CID, the glycosidic bond was easily cleaved to generate an ion at m/z 269, which resulted from the neutral loss of a glucuronic acid ($\Delta m = 176$u). In the MS^3 experiment, ion at m/z 269 produced the same ions as baicalein described above.

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 ($^{0,2}A^-$), 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 [9].

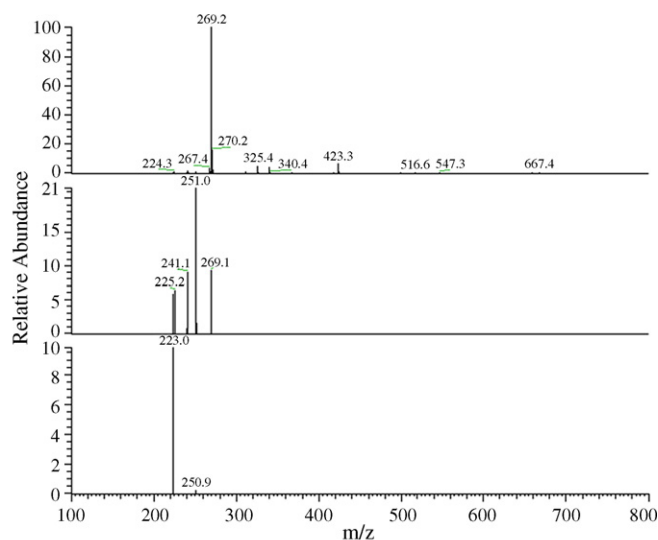

Fig. 2. Full scan MS^n spectra for Baicalein. (a) MS spectrum of the Baicalein. (b) MS^2 spectrum of the $[M-H]^-$ ion at m/z 269. (c) MS^3 spectrum of the ion at m/z 251.

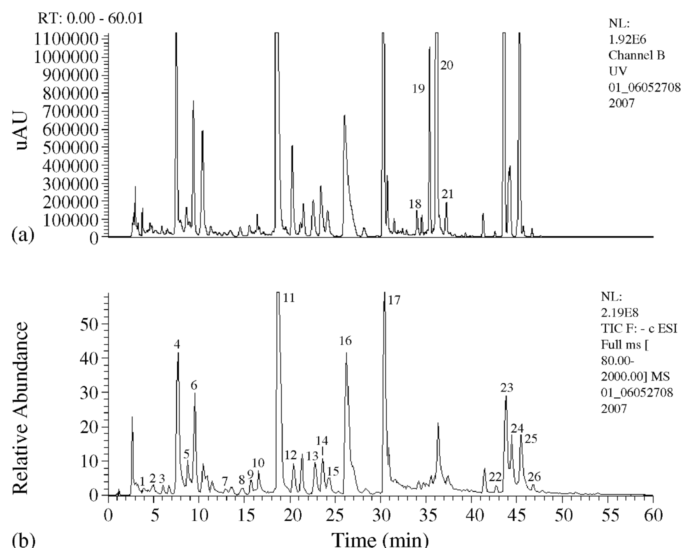

Fig. 3. A representative HPLC-ESI-MS^n analysis of the extract of Huangqin. (a) HPLC-UV chromatogram monitored at 280 nm (b) LC-negative ion ESI-MS total ion current (TIC) profile.

3.2. HPLC-DAD-ESI-MS^n analysis of the plant extract

Fig. 3 showed the HPLC-UV and TIC profiles of the extract of *S. baicalensis*. The retention behavior of flavonoids on the reversed-phase HPLC column was remarkably affected by the pH of the mobile phase. Therefore, water containing 0.2% (v/v) CH_3COOH was used to achieve a good base line and complete resolution of the major peaks.

The HPLC peaks were preliminary identified as flavonoids according to their on-line UV spectra. Flavonols and their glycosides exhibited two maximum absorptions at 330–360 nm and 250–270 nm, originating from their B and A rings, respectively. The structures were further characterized mainly based on their MS fragmentation behaviors (Table 3). A number of flavonoids have been reported from *S. baicalensis* previously. The aglycones belong to several subtypes, flavone, flavonol, flavanone, flavanonol, flavan and chalcone. The sugar moieties included glucose, glucuronic acid and arabinose.

3.2.1. Method validation

Considering the features of fingerprints, the R.S.D. of the retention times of the 26 “common peaks” identified in the extracts of Huangqin were used to evaluate the developed method.

The repeatability was evaluated by analyzing six independently prepared samples of Huangqin. The relative standard

Table 3
Characterization of flavonoids by HPLC-DAD-ESI/MS from *Scutellaria baicalensis*

Peak no.	Retention time (t_R min)	UV λ_{max} (nm)	$[M-H]^-$ m/z	HPLC-ESI-MS^n m/z (% base peak)	Identification
1	4.14	222, 388	577	MS^2[577]: 487(2), 457(100), 415(2), 295(15); MS^3[457]: 295(100), 337(10); MS^4[295]: 267(100)	Chrysin-7-O-glu-8-C-glu
2	5.47	222, 388	563	MS^2[563]: 545(5), 503(35), 473(85), 443(100), 383(40), 353(45); MS^3[443]: 353(100), 383(15); MS^4[353]: 325(100); MS^5[325]: 297(100)	Apigenin-6-C-glu-8-C-ara (schaftoside)
3	6.06	222, 388	577	MS^2[577]: 559(10), 517(5), 487(35), 457(100), 367(15), 337(35); MS^3[457]: 367(10), 337(100); MS^4[337]: 309(100), 281(25); MS^5[309]: 281(100)	Chrysin-6, 8-di-C-glu
4	7.69	224, 272, 314	547	MS^2[547]: 529(30), 487(80), 457(100), 427(55), 367(50), 337(40); MS^3[457]: 337(100); MS^3[427]: 367(10), 337(100); MS^4[337]: 309(100), 281(20); MS^5[309]: 281(100)	Chrysin-6-C-ara-8-C-glu
5	8.77	220, 290, 330	623	MS^2[623]: 461(100); MS^3[461]: 315(90), 135(100), 161(35)	Isorhamnetin-7-O-rha-glu
6	9.42	220, 274, 316	547	MS^2[547]: 529(10), 487(5), 457(60), 427(100), 367(10), 337(15); MS^3[457]: 367(100), 337(55); MS^3[427]: 337(100), 367(10); MS^4[337]: 309(100), 281(10); MS^5[309]: 281(100)	Chrysin-6-C-glu-8-C-ara
7	12.88	222, 264	621	MS^2[621]: 445(100); MS^3[445]: 430(100), 283(90), 268(65); MS^4[430]: 267(100); MS^5[267]: 239(100)	Wogonin-7-O-glu-glu acid
8	14.39	222, 254, 302(sh), 340	491	MS^2[491]: 329(100); MS^3[329]: 314(100), 299(10), 261(5); MS^4[314]: 299(100); MS^5[299]: 271(100), 165(75)	3′,4′-Dihydroxy-5,6-dimethoxy-7-O-glucoside flavone
9	15.72	220, 274	637	MS^2[637]: 461(100); MS^3[461]: 315(90), 135(100), 161(80)	Isorhamnetin-7-O-rha-glu acid
10	16.55	222, 270, 332	951	MS^2[951]: 475(100), 299(15); MS^3[475]: 299(100); MS^4[299]: 284(100)	5,2′-Dihydroxy-6′-methoxy-7-O-glu acid flavone
11	18.57	222, 276, 316	445	MS^2[445]: 269(100); MS^3[269]: 251(25), 241(5), 197(3)	Baicalin
12	20.38	218, 278, 318	431	MS^2[431]: 269(100); MS^3[269]: 251, 241, 197	Baicalein-7-O-glu
13	22.75	220, 264	447	MS^2[447]: 271(100); MS^3[71]: 253(50), 243(100), 227(20), 199(5), 167(5)	5,6-Dihydroxy-7-O-glu acid flavanone
14	23.55	220, 280	891	MS^2[891]: 445(100), 269(15); MS^3[445]: 241(15), 225(35), 197(15), 183(2); MS^4[269]: 239(100)	Galengin-7-O-glu acid
15	24.05	222, 270	445	MS^2[445]: 430(100), 283(95), 268(75); MS^3[430]: 267(100)	Wogonin-5-O-glu
16	26.09	218, 272, 312	919	MS^2[919]: 459(100), 283(15), 268(5); MS^3[459]: 283(100), 175(4); MS^4[283]: 268(100)	Oroxylin A-7-O-glu acid
17	30.61	222, 272	919	MS^2[919]: 459(100); MS^3[459]: 283(100); MS^4[283]: 268(100)	Wogonoside
18	34.12	226, 276, 294, 326	299	MS^2[299]: 284(100); MS^3[284]: 256(10), 228(20), 137(5)	5,7,3-Trihydroxy-4′-methoxy flavone
19	35.48	220, 276, 322	269	MS^2[269]: 251(60), 241(10), 169(5)	Norwogonin
20	36.17	220, 276, 322	269	MS^2[269]: 251(100), 241(20), 223(20), 169(5)	Baicalein
21	37.44	218, 270, 334	299	MS^2[299]: 284(100); MS^3[284]: 267(10), 256(10), 166(100), 138(60)	5,7,4′-Trihydroxy-8-methoxy flavone
22	42.70	264	343	MS^2[343]: 328(100), 313(15); MS^3[328]: 313(100), 273(10); MS^4[313]: 297(10), 285(100), 269(10), 165(15)	5,2′-Dihydroxy-7,8,6′-trimethoxy flavone (Skullcapflavon I)
23	43.71	226, 274	283	MS^2[283]: 268(100); MS^3[268]: 239(60), 212(15), 163(30)	Wogonin
24	44.45	224, 272	373	MS^2[373]: 358(100), 343(25); MS^3[358]: 343(100); MS^4[343]: 328(100), 300(10), 285(10), 169(5); MS^5[328]: 300(100), 272(15), 228(5)	5,2′-Dihydroxy-6,7,8,6′-tetramethoxy flavone (Skullcapflavon II)
25	45.42	222, 270, 318	283	MS^2[283]: 268(100); MS^3[268]: 239(35), 212(15), 163(5)	5, 7-Dihydroxy-6-methoxy flavone (Oroxylin A)
26	46.87	278	343	MS^2[343]: 328(100); MS^3[328]: 313(100); MS^4[313]: 298(100), 285(5), 270(10)	5,2′-Dihydroxy-6,7,8-trimethoxy flavone (Tenaxin I)

Note: glu: glucose, ara: arabinose, rha: rhamnose, glu acid: glucuronic acid.

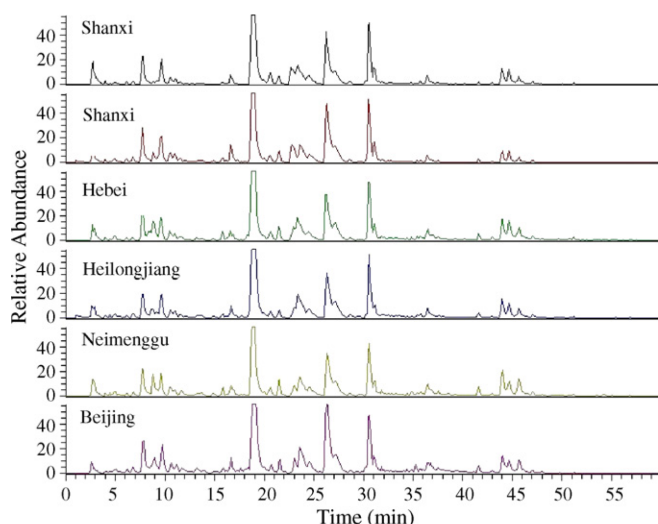

Fig. 4. HPLC-ESI-MS TIC profiles of the Huangqin collected from different regions.

deviation of the retention times for each of the 26 "common peaks" was less than 2.0% (Table A.1 and Fig. 4).

The method precision was measured by analyzing the same sample for five consecutive times. The R.S.D. of the common peak retention times were less than 1.2% (Table A.2).

To test the stability of the analytes, the same sample was analyzed in two days. Peak retention time R.S.D. for all of the 26 common peaks were less than 1.0% (Table A.3), suggesting that the sample remained stable within 2 days.

All of above results of repeatability, precision and stability test indicated that this method was sensitive, reliable and applicable.

3.2.2. Identification of flavone glycosides

Seventeen flavone glycosides were plausibly identified. Seven of them have previously been reported from *S. baicalensis*. The other ten compounds were reported for the first time and were tentatively identified on the basis of their tandem mass spectra.

3.2.2.1. C-glycosides. Two compounds (peaks 4 and 6 in Fig. 3) in the HPLC chromatogram exhibited $[M-H]^-$ ions at *m/z* 547. Their UV spectra showed maximum absorption bands at 224, 272 and 314 (sh) nm. Their MS/MS spectra gave ions of $[M-H-60]^-$ at *m/z* 487, $[M-H-90]^-$ at 457 and $[M-H-120]^-$ at 427, consistent with the characteristic ions of C-glycoside. It had been demonstrated that flavonoid C-6 and C-8 glycosides could be distinguished by the relative abundance of ions $[M-H-60]^-$ and $[M-H-120]^-$ [12,13]. Similar difference was observed for MS/MS spectra of these two compounds (Table 4). Therefore, compounds 4 and 6 were identified as the known compounds, chrysin-6-C-arabinosyl-8-C-glucoside and chrysin-6-C-glucosyl-8-C-arabonoside, respectively [9,12].

Compound 2 ($t_R = 5.47$ min) exhibited a $[M-H]^-$ at *m/z* 563, 16 Da greater than that of compound 6. Its MS/MS spectrum showed ions of $[M-H-60]^-$ at *m/z* 503, $[M-H-90]^-$ at 473 and $[M-H-120]^-$ at 443, which were characteristic ions of C-glycosides demonstrated by Becchi and Fraisse [14]. The structure of compound 2 was proposed to be apigenin-6-C-glucosyl-8-C-arabinoside (schaftoside).

Compound 3 ($t_R = 6.06$ min) yielded a $[M-H]^-$ ion at *m/z* 577, 30 Da greater than that of compound 4. Its MS/MS spectra produced prominent ions of $[M-H-60]^-$ at *m/z* 517, $[M-H-90]^-$ at 487, $[M-H-120]^-$ at 457, $[M-H-210]^-$ at 367 and $[M-H-240]^-$ at 337, all of which are the characteristic ions of di-C-hexosyl flavones [15], suggesting the presence of chrysin (254) + hexose (162) + hexose (162) (MW 578). In previous studies, all of the hexose moiety in flavonoids in *S. baicalensis* were identified as glucosides. Thus, compound 3 was tentatively characterized as chrysin-6,8-di-C-glucoside.

3.2.2.2. O-glycosides. Compound 5 ($t_R = 8.77$ min) displayed a $[M-H]^-$ ion at *m/z* 623. The MS^2 and MS^3 spectra gave ions at *m/z* 461 and 315, suggesting sequential losses of glucosyl (162 Da) and rhamnosyl (146 Da) residues. Compound 5 was thus tentatively identified as isorhamnetin-7-O-rhamnosylglucoside. The signals at *m/z* 135 and 161 were the ions of $^{1,2}B^-$ and $[^{1,2}A^- - 18]^-$, respectively. Alycone such as isorhamnetin was first reported in *S. baicalensis*, although it is commonly occurred in natural flavonoids.

Compound 7 ($t_R = 12.88$min) gave a $[M-H]^-$ ion at *m/z* 621. CID of this compound produced an ion of $[M-H-176]^-$ at *m/z* 445, which resulted from the neutral loss of a glucuronic acid residue. In the MS^3 of *m/z* 445, fragments of *m/z* 430 and 283 indicated the presence of a methoxyl group and a hexosyl residue, respectively. Compound 7 was thus proposed as wogonin-7-O-glucosyl-glucuronide, which was identified in this plant for the first time.

Compound 10 ($t_R = 16.55$min) exhibited $[2M-H]^-$ ion at *m/z* 951 and $[M-H]^-$ signal at *m/z* 475. The MS^2 spectrum of *m/z* 951 produces ion at *m/z* 475. It has been reviewed that the peak at the highest *m/z* ration is not always the molecular ion species, because molecular complexes can be generated [6]. Thus we presumed that compound 10 was a molecular complex. The MS^3 spectrum of 475 produced base peak at *m/z* 299, attributed to the loss of 176 Da, suggesting the presence of a glucuronic acid. Then MS^4 of *m/z* 299 resulted in the ion of *m/z* 284 (−15 Da), suggesting the existence of a methoxy group. In ref-

Table 4
Relative intensity of ions in ESI-MS/MS of the compounds 4 and 6

Peak	$[M-H-60]^-$ (%)	$[M-H-90]^-$ (%)	$[M-H-120]^-$ (%)	$[M-H-180]^-$ (%)	$[M-H-210]^-$ (%)
4	80	100	55	50	40
6	5	60	100	10	15

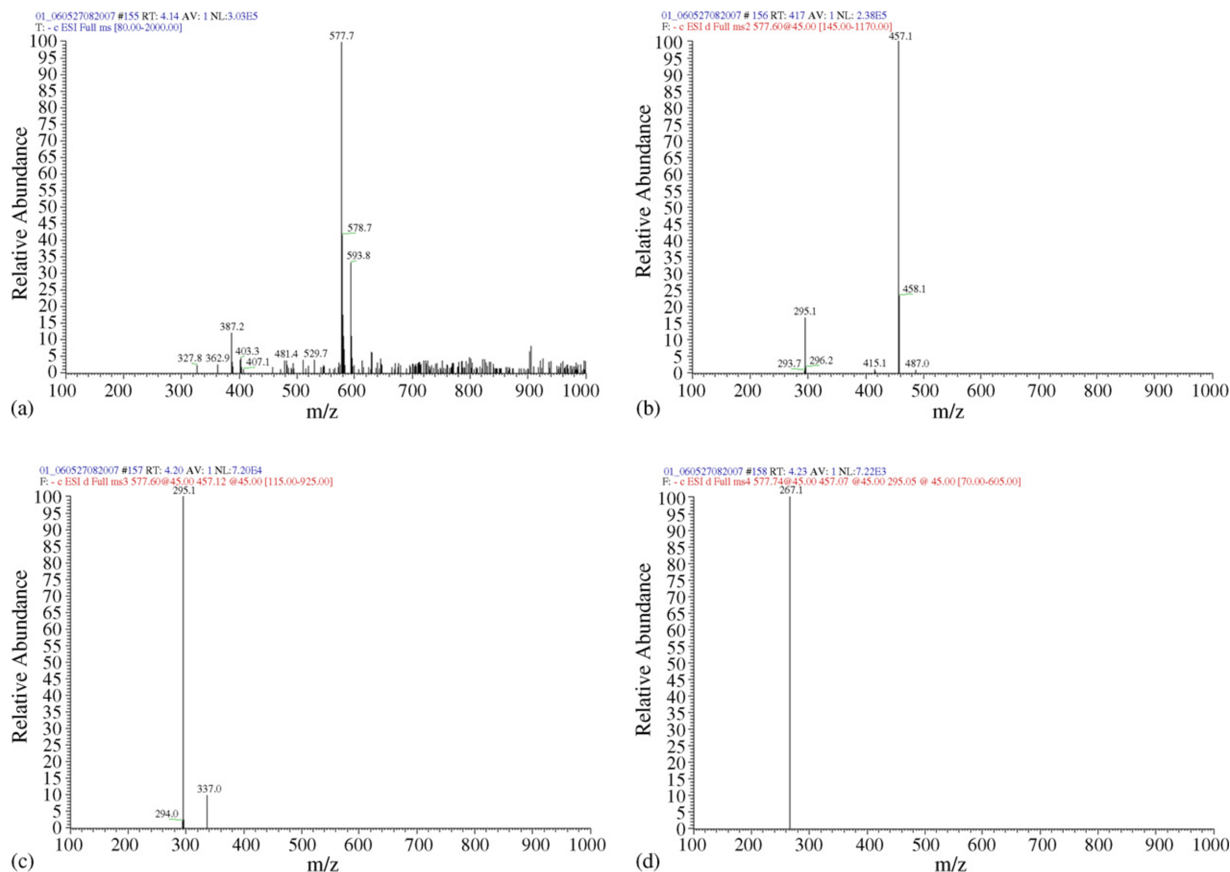

Fig. 5. ESI-MS spectra for compound 1: (a) MS spectrum; (b) MS^2 spectrum of the $[M-H]^-$ at *m/z* 577; (c) MS^3 spectrum of *m/z* 457; (d) MS^4 spectrum of *m/z* 295.

erence to the previous report [16], compound 10 was plausibly characterized as 5, 2′-dihydroxy-6′-methoxy-7-O-glucuronate flavone.

Compound 11 (t_R = 18.57min) represented a main constituent of *S. baicalensis*. It exhibited $[M-H]^-$ ion at *m/z* 445, and its CID gave the ions consistent with baicalin. Thus, compound 11 was identified as baicalin and this identification was confirmed by comparison with a pure standard.

Compound 13 (t_R = 22.75min) exhibited $[M-H]^-$ ion at *m/z* 447. Its MS^2 produced an ion at *m/z* 271 $[M-H-176]^-$, indicating the existence of a glucuronic acid. Its MS^3 yielded ion at *m/z* 253, resulted from the loss of H_2O group, suggesting the presence of two OH in *ortho* positions. The ion at *m/z* 167 was attributed to the $^{1,3}A^-$, proving the occurrence of three OH groups in A ring. In addition, the molecular mass of the compound 13 was 2 Da greater than baicalin, it was thus identified as 5,6-dihydroxy-7-glucuronic acid flavanone.

Compound 14 (t_R = 23.55min) gave a $[2M-H]^-$ ion at *m/z* 891, another strong peak at *m/z* 445 $[M-H]^-$ was also observed. The MS^2 spectrum of *m/z* 891 produced a base peak at *m/z* 445, thus compound 14 was assumed to be a complex of flavone, too. The MS^2 spectrum of *m/z* 445 yielded an ion at *m/z* 269, loss of 176 Da, suggesting the presence of a glucuronic acid. The *m/z* 269 ion was further subjected to MS^3 analysis, ions of *m/z* 241 (−28 Da), 225 (−44 Da), 197 (−72 Da) and 183 (−86 Da) were observed, which were consistent with the data of galengin reported by Fabre and Rustan [10]. Accordingly, compound 14 was tentatively identified as galengin 7-O-glucuronide.

Compounds 16 (t_R = 26.09 min) and 17 (t_R = 30.61 min) are a pair of isomers. Both of them gave a $[2M-H]^-$ ion at *m/z* 919 and significant $[M-H]^-$ signal at *m/z* 459. And *m/z* 459 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 [17], compounds 16 and 17 were plausibly identified as oroxylin A-7-O-glucuronide and wogonoside, respectively.

3.2.2.3. C-glycosylflavone O-glycoside. Compound 1 exhibited a $[M-H]^-$ ion at *m/z* 577. Its MS^2 spectrum (Fig. 5) gave the ions at *m/z* 487 $[M-H-90]^-$ and base peak 457 $[M-H-120]^-$, indicating that it was a C-glycoside. In the MS^2 spectrum $[M-H-60]^-$ did not appear, thus it was concluded that the sugar moiety is a hexsose, but not a pentose. Interestingly, ion of $[M-H-162]^-$ at *m/z* 415, characteristic ions of O-glycoside, was also observed. Thus it was plausibly identified as chrysin-7-O-glucosyl-8-C-glucoside, which was firstly identified in *Scutellaria* species.

3.2.3. Aglycone

Nine aglycones were characterized or tentatively characterized. Seven of them were known constituents previously identified in *S. baicalensis*.

Compound 18 ($t_R = 34.12$ min) and compound 21 ($t_R = 37.44$ min) are isomers, both yielding $[M-H]^-$ at *m/z* 299. The MS of compound 18 indicated the existence of a $-CH_3$ group. And the ion at *m/z* 228 represented the ion of $[M-H-2CO]^-$, which was only observed in flavonols, reported by Fabre and Rustan [10]. Thus, it was tentatively identified as 5,7,3-trihydroxy-4′-methoxy flavone. While MS^3 spectrum of compound 21 exhibited an ion at *m/z* 267 originated from ion at *m/z* 284, corresponding to the loss of H_2O group, which indicated the existence of two –OH groups in *ortho* positions. Moreover, its MS spectrum was similar to that of wogonin, only an –OH group difference. Based on these information, compound 21 was identified as 5,7,4′-trihydroxy-8-methoxy flavone, which has been identified in *S. baicalensis* [18] and *S. amoena* C.H. Wright [19].

Compound 19 ($t_R = 35.48$ min) and compound 20 ($t_R = 36.17$ min) both displayed the $[M-H]^-$ ion at *m/z* 269. Compared with the standard, compound 20 was confirmed as baicalein. The MS fragmentation of compound 19 was extremely similar with that of compound 20. By searching the known flavones which MW was 270 in *S. baicalensis*, compound 19 was tentatively characterized as norwogonin.

Compound 22 ($t_R = 42.70$ min) and compound 26 ($t_R = 46.87$ min) both gave the $[M-H]^-$ ion at *m/z* 343. By analyzing their CID, it was concluded that both of them contain three $-OCH_3$ groups since the ions of *m/z* 328, 313 and 298 have been observed. However, their MS^4 spectra of *m/z* 313 were significantly different. Compound 22 showed a base peak at *m/z* 285, and the intensity of ion at *m/z* 298 was very weak; on the contrary, compound 26 showed base peak at *m/z* 298 and a very weak intensity at *m/z* 285. Based on these data and by the knowledge on the flavones in *S. baicalensis*, compounds 22 and 26 were identified as skullcapflavon I and tenaxin I, respectively.

Compound 23 ($t_R = 43.71$ min) and compound 25 ($t_R = 45.42$ min) both yielded an $[M-H]^-$ ion at *m/z* 283. The MS fragments and retention time in HPLC of compound 23 were consistent with the known wogonin, and allowed its identification. The MS spectra of compound 25 were almost identical to those of compound 23. However, its retention time supported its possible identification as oroxylin A [17].

Compound 24 ($t_R = 44.45$ min) gave the $[M-H]^-$ ion at *m/z* 373. Its CID yielded ions at 358 $[M-H-CH_3]^{-\bullet}$, 343 $[M-H-2CH_3]^-$, 328 $[M-H-3CH_3]^{-\bullet}$, 300 $[M-H-3CH_3-CO]^{-\bullet}$, 285 $[M-H-3CH_3-CO-CH_3]^-$, 272 $[M-H-3CH_3-2CO]^{-\bullet}$ and 228 $[M-H-3CH_3-CO-CO_2]^{-\bullet}$. These ions should come from four $-OCH_3$ groups and the flavone skeleton. By examining the known flavones in *S. baicalensis*, it was tentatively identified as skullcapflavone II.

4. Conclusion

In this study, a simple, reliable and sensitive method has been established for the screening of main flavonoids in *S. baicalensis* by HPL*C*-DAD-/ESI-MS^n. Using negative ion mode and applying the MS fragmentation rules of flavonoids reported before, 26 flavonoids, involving 17 flavonoid glycosides and 9 free aglycones, were identified or tentatively identified in methanol extracts of *S. baicalensis*. Only two C-glycosides have been previously reported in *S. baicalensis*. However, several new analogues were identified in the present study, which proved that HPLC-MS is a powerful and rapid method to discover new constituents in medicinal herbs.

The major reason that traditional Chinese medicines could not be accepted by the western world is the ambiguous chemical components of it, which limits the modernization and globalization of traditional medicine. Our present method could comprehensively evaluate the quality of *S. baicalensis* samples and extracts covering most of the chemical constituents, and reduce the necessity to isolate each individual flavonoid from the herb utilizing tedious conventional phytochemical procedures. It set a good example for the rapid identification of bioactive constituents in plant extracts and made it possible to fulfil the requirements for a modern drug with characters of safety, efficacy and stability.

Appendix A

Table A.1
The retention time in HPLC-MS fingerprints of *Radix Scutellariae* (repeatability)

Peak number	Sample number						R.S.D. (%)
	1#	2#	3#	4#	5#	6#	
1	4.11	4.08	4.08	4.22	4.09	4.16	1.24
2	5.20	5.10	4.94	5.03	4.93	4.97	1.91
3	6.04	6.06	6.13	6.17	6.07	6.11	0.73
4	7.71	7.60	7.78	7.71	7.70	7.77	0.76
5	8.77	8.92	8.79	8.86	8.82	9.00	0.90
6	9.56	9.64	9.64	9.64	9.58	9.65	0.36
7	13.36	13.48	13.26	13.19	13.4	12.98	1.23
8	14.64	14.55	14.54	14.60	14.59	14.81	0.62
9	15.77	15.84	15.86	15.84	15.85	15.85	0.19
10	16.60	16.57	16.71	16.66	16.74	16.66	0.35
11	18.84	18.77	18.77	18.92	18.92	18.99	0.43
12	20.52	20.55	20.51	20.63	20.63	20.70	0.33
13	22.76	22.76	22.92	23.04	23.04	22.91	0.50
14	23.55	23.67	23.37	23.39	23.51	23.67	0.51
15	24.04	24.25	24.06	24.07	24.33	24.23	0.46
16	26.19	26.27	26.27	26.27	26.32	26.27	0.15
17	30.56	30.59	30.48	30.55	30.45	30.57	0.17
18	34.35	34.31	34.25	34.26	34.80	34.91	0.78
19	35.60	35.64	35.62	35.79	35.79	35.80	0.25
20	36.37	36.46	36.41	36.45	36.42	36.59	0.19
21	37.59	37.56	37.50	37.55	37.60	37.52	0.09
22	42.87	42.92	42.95	42.84	42.86	42.87	0.09
23	43.97	43.9	43.97	43.87	43.87	44.05	0.15
24	44.60	44.76	44.66	44.70	44.60	44.69	0.13
25	45.79	45.61	45.57	45.67	45.65	45.65	0.15
26	46.85	46.89	47.03	46.94	46.94	47.10	0.18

Table A.2
The retention time in HPLC-MS fingerprints of *Radix Scutellariae* (injection precision)

Peak number	Injection times					R.S.D. (%)
	1	2	3	4	5	
1	4.11	4.15	4.14	4.16	4.1	0.56
2	5.34	5.39	5.30	5.32	5.31	0.60
3	6.06	5.99	5.90	6.06	6.00	0.98
4	7.62	7.66	7.45	7.69	7.50	1.23
5	8.69	8.72	8.50	8.77	8.60	1.10
6	9.44	9.45	9.27	9.54	9.50	0.98
7	12.94	12.91	12.99	13.03	12.98	0.32
8	14.34	14.42	14.35	14.39	14.35	0.21
9	15.66	15.69	15.71	15.67	15.66	0.12
10	16.57	16.51	16.51	16.55	16.52	0.15
11	18.65	18.65	18.72	18.68	18.69	0.14
12	20.38	20.39	20.28	20.27	20.28	0.26
13	22.70	22.71	22.69	22.75	22.70	0.09
14	23.53	23.56	23.61	23.55	23.60	0.13
15	24.19	24.19	24.22	24.19	24.20	0.05
16	26.11	26.18	26.22	26.21	26.20	0.15
17	30.41	30.43	30.47	30.40	30.42	0.08
18	34.14	34.24	34.20	34.24	34.20	0.11
19	35.48	35.57	35.60	35.59	35.58	0.12
20	36.31	36.31	36.36	36.42	36.41	0.13
21	37.39	37.38	37.40	37.44	37.40	0.05
22	42.68	42.79	42.72	42.83	42.80	0.13
23	43.77	43.76	43.79	43.81	43.80	0.04
24	44.43	44.42	44.52	44.45	44.46	0.08
25	45.50	45.51	45.43	45.42	45.50	0.09
26	46.90	46.91	46.93	46.87	46.89	0.04

Table A.3
The retention time in HPLC-MS fingerprints of *Radix Scutellariae* (stability)

Peak number	Times				R.S.D. (%)
	0 h	12 h	24 h	48 h	
1	4.16	4.14	4.06	4.10	0.93
2	4.97	5.02	5.04	5.00	0.52
3	6.11	6.06	6.03	6.00	0.67
4	7.77	7.69	7.65	7.75	0.62
5	9.00	8.86	8.86	8.90	0.64
6	9.65	9.54	9.44	9.55	0.78
7	12.98	13.03	13.09	12.99	0.33
8	14.81	14.54	14.64	14.70	0.67
9	15.85	15.67	15.71	15.68	0.46
10	16.66	16.55	16.52	16.55	0.32
11	18.99	18.68	18.65	18.70	0.73
12	20.70	20.35	20.38	20.40	0.69
13	22.91	22.87	22.80	22.88	0.18
14	23.67	23.55	23.53	23.60	0.23
15	24.23	24.31	24.27	24.25	0.12
16	26.27	26.21	26.13	26.25	0.20
17	30.57	30.40	30.44	30.40	0.23
18	34.15	34.19	34.24	34.00	0.26
19	35.80	35.48	35.48	35.60	0.37
20	36.59	36.38	36.32	36.40	0.28
21	37.52	37.44	37.44	37.50	0.10
22	42.87	42.83	42.81	42.80	0.06
23	44.05	43.91	43.79	44.00	0.22
24	44.69	44.45	44.43	44.50	0.23
25	45.65	45.42	45.41	45.50	0.21
26	47.10	47.00	46.95	47.00	0.12

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