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Online identification of the antioxidant constituents of traditional Chinese medicine formula Chaihu-Shu-Gan-San by LC–LTQ-Orbitrap mass spectrometry and microplate spectrophotometer

Zhi-Heng Su[a], Guo-An Zou[a], Alfred Preiss[b], Hong-Wu Zhang[a], Zhong-Mei Zou[a,*]

[a] Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100193, PR China
[b] Fraunhofer Institut für Toxikologie und Experimentelle Medizin, Hannover 30625, Germany

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ABSTRACT

Chaihu-Shu-Gan-San (CSGS), a traditional Chinese medicine (TCM) formula containing seven herbal medicines, has been used in treatment of gastritis, peptic ulcer, irritable bowel syndrome and depression clinically. However, the chemical constituents in CSGS had not been studied so far. To quickly identify the chemical constituents of CSGS and to understand the chemical profiles related to antioxidant activity of CSGS, liquid chromatography coupled with electrospray ionization hybrid linear trap quadrupole orbitrap (LC–LTQ-Orbitrap) mass spectrometry has been applied for online identification of chemical constituents in complex system, meanwhile, antioxidant profile of CSGS was investigated by the fraction collecting and microplate reading system. As a result, 33 chemical constituents in CSGS were identified. Among them, 13 components could be detected both in positive and in negative ion modes, 20 constituents were determined only in positive ion mode and 2 components were only detected in negative ion mode. Meanwhile, the potential antioxidant profile of CSGS was also characterized by combination of 96-well plate collection of elutes from HPLC analysis and microplate spectrophotometer, in which the scavenging activities of free radical produced by DPPH of each fraction could be directly investigated by the analysis of microplate reader. This study quickly screened the contribution of CSGS fractions to the antioxidant activity and online identified the corresponding active constituents. The results indicated that the combination of LC–MS^n and 96-well plate assay system established in this paper would be a useful strategy for correlating the chemical profile of TCMs with their bioactivities without isolation and purification.

1. Introduction

Traditional Chinese Medicines (TCMs) have been proved to have a significant effect in treatment of chronic and systematic diseases with fewer side effects. In Chinese herbal therapy, the most widely used medicines are combined by many herbs and prepared according to TCM formulation concepts. It is acknowledged that complex interactions could produce synergistic effects and reduce possible side effects from some of the herbs. However, the extreme complexity of TCM formulas containing many poorly characterized chemical constituents makes standardization of herbal products and understanding of their action mechanisms challenging. In order to discern the chemical compositions of TCM formulas, many techniques, such as GC–MS [1], LC–MS [2,3] and LC–NMR [4] have been used to develop specific analytical methods for comprehensively describing and identifying the chemical components of TCMs. As a powerful analytical tool, liquid chromatography coupled with electrospray ionization tandem mass spectrometry (LC–ESI-MS^n) has been widely applied to directly identify known compounds and recognize unknown compounds from the complex mixtures [5,6].

Chaihu-Shu-Gan-San (CSGS) is one of the most widely used TCM formulas in China for treatment of gastritis, peptic ulcer, irritable bowel syndrome and depression. Pharmacological studies have proved that CSGS had prominent effects in kinds of anti-inflammatory, antidepression, anti-ulcer and prevention of liver injury [7]. CSGS involves seven commonly used Chinese herbs, i.e. the roots of *Bupleurum chinense* DC. (*Chai-Hu*), the pericarps of *Citrus reticulata* Blanco (*Chen-Pi*), the roots of *Paeonia lactiflora* Pall. (*Bai-Shao*), the fruits of *Citrus aurantium* L. (*Zhi-Qiao*), the roots of *Cyperus rotundus* L. (*Xiang-Fu*), the roots of *Ligusticum chuanxiong* Hort. (*Chuan-Xiong*) and the roots of *Glycyrrhiza uralensis* Fisch. (*Gan-Cao*). Major constituents in these single herbs have been well studied, for instance, *Chai-Hu* and *Gan-Cao* mainly contain triterpenoid saponin compounds such as saikosaponin A, saikosaponin D [8], glycyrrhizic acid and licorice-saponinG_2 [9]. Different kinds of flavonoids such as naringin, narirutin, hesperidin and neohes-

* Corresponding author. Tel.: +86 10 62899756; fax: +86 10 62899756.
E-mail address: zmzou@implad.ac.cn (Z.-M. Zou).

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peridin are found in *Chen-Pi* [10] and *Zhi-Qiao* [11]. Monoterpene glycosides such as paeoniflorin, benzoylpaeoniflorin and lactiflorin are usually recorded as the active substances of *Bai-Shao* [12]. However, to the best of our knowledge, the profile of chemical constituents in CSGS has not been investigated so far.

In the present study, LC–ESI-MS^n analysis was developed to identify the main constituents of CSGS, which gave the accurate molecular weights by orbitrap analyzer and the fragmentation patterns acquiring from multi-stage mass fragmentation in linear trap quadrupole (LTQ) for comprehensive understanding of chemical structures in complex mixture. Our previous study indicated that the antioxidant activity of CSGS may play a key role for its antidepressive effect [13]. To explore the active fractions responsible for antioxidant activity of CSGS, the antioxidant profile of CSGS was investigated by combination of 96-well plate collection of elutes from HPLC analysis and microplate spectrophotometer, in which the scavenging activities of free radical produced by DPPH of each fraction could be directly investigated by the analysis of microplate reader.

2. Experimental

2.1. Solvents and chemicals

The HPLC grade acetonitrile and methanol from Fisher (NJ, USA) were used for chromatography. Analytical-grade ethanol was purchased from Beijing Reagent Company (Beijing, China). Water was purified by Milli-Q academic water purification system (Millipore, France).

Synephrine, ferulic acid, naringin, hesperidin, neohesperidin, saikosaponin A, and glycyrrhizic acid were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Paeoniflorin (isolated and purified from *Paeonia lactiflora* Pall.), nobiletin and tangeretin (isolated and purified from pericarps of *Citrus aurantium* L.) were provided by our group. Saikosaponin A was detected with purity of 95.0% and others were determined with purity more than 98%. DPPH (1,1-diphenyl-2-picrylhydrazyl) was purchased from Sigma–Aldrich (Shanghai, China).

All raw herbs were purchased from Beijing Tongren Tang Pharmaceutical Co. Ltd. (Beijing, China) and identified as the roots of *Bupleurum chinense* DC., the roots of *Paeonia lactiflora* Pall., the pericarps of *Citrus aurantium* L., the fruits of *Citrus reticulata* Blanco, the roots of *Cyperus rotundus* L., the roots of *Ligusticum chuanxiong* Hort. and the roots of *Glycyrrhiza uralensis* Fisch. by Associate Professor Yulin Lin of the Institute of Medicinal Plant Development (IMPLAD), Chinese Academy of Medical Sciences and Peking Union Medical College. The voucher specimens are deposited in our laboratory of IMPLAD.

2.2. Sample preparations

The CSGS extract was prepared based on the traditional method used in TCM practice. Briefly, 8.4 g of mixed crude herbs, *Chai-Hu*, *Chen-Pi*, *Bai-Shao*, *Zhi-Qiao*, *Xiang-Fu*, *Chuan-Xiong* and *Gan-Cao* in the proportions of 4:4:3:3:3:3:1 by weight were crushed into small pieces. The mixture of the herbs was soaked together in 200 ml of water for 1 h at room temperature and thereafter refluxed for 2 h. The filtrate was collected and the residues were then refluxed twice in 200 ml of water for 1.5 h. The three filtrates were combined and concentrated under vacuum to give 0.655 g extract. The extracts of the individual herbs, *Chai-Hu* (**1**, 0.144 g), *Chen-Pi* (**2**, 0.12 g), *Bai-Shao* (**3**, 0.12 g), *Zhi-Qiao* (**4**, 0.156 g), *Xiang-Fu* (**5**, 0.096 g), *Chuan-Xiong* (**6**, 0.084 g) and *Gan-Cao* (**7**, 0.10 g) were prepared using procedures identical to that for CSGS. 66.5 mg of CSGS extract (equivalent to 0.84 g of raw herbs in the proportions listed above) was dissolved in 10 ml of deionized water. 10 μl of the resulting solution was injected into the HPLC system for LC–MS^n analysis. An amount of extract of the single herb equal to the same amount of raw herb in the 66.5 mg of CSGS extract was prepared and analyzed identically to CSGS. All samples were analyzed in triplicate.

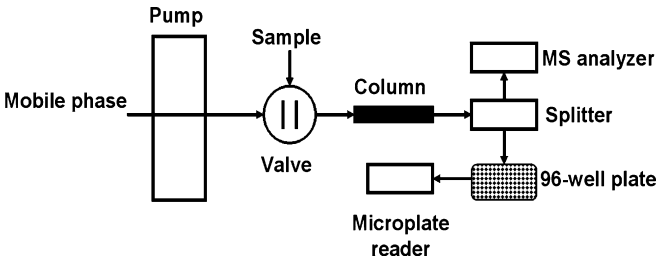

Fig. 1. Schematic diagram of high-performance liquid chromatography coupled to orbitrap analyzer and analytical system of antioxidant activities profiling.

2.3. Chromatography

The LC system consisted of a Finnigan Surveyor LC system with a built-in degasser and autosampler. HPLC analysis was performed on a Waters SunFire™ (2.1 mm × 150 mm, 5 μm) C_{18} column together and the column temperature was set at 30 °C. A mixture of aqueous with 0.1% formic acid (A) and acetonitrile (B) was used as the mobile phase. Gradient chromatography was performed in linear gradient (8:92 at 0–3 min, 8:92–31:79 at 3–30 min, 31:69–95:5 at 30–50 min and 95:5–100:0 at 50–60 min, v/v). Re-equilibration duration was 10 min between individual runs and the flow rate was 0.2 ml/min.

2.4. Mass spectrometry

Mass spectra were analyzed on a Finnigan LTQ-Orbitrap XL instrument with an ESI source (Thermo Electron, Bremen, Germany). Nitrogen and helium were used as the sheath and auxiliary gas and the collision gas, respectively. Values of auxiliary gas flow rate and capillary voltage were set at 5 arbitrary units and 40 V in positive ion mode and 8 arbitrary unit and −45 V in negative ion mode, respectively.

The scan event cycle used a full scan mass spectrum at resolution of 15,000 (at *m/z* 400) and three corresponding data-dependent MS/MS events acquired at a resolving power of 7500. The most intense ions detected in full scan MS were selected for data-dependent scanning. MS/MS activation parameters were set at isolation width of 2 Da, normalized collision energy of 35%, and an activation time of 30 ms. An external calibration for mass accuracy was performed the day before the test. The mass spectrometric data was collected from *m/z* 100 to 1000 in positive and negative ion mode.

2.5. Characterization of antioxidant profile by investigating scavenging activity of CSGS on DPPH radicals in 96-well plates

The fractions eluted from chromatographic column were splitted at a ratio of 1:1 (same length of pipelines between splitter to MS detector and splitter to 96-well plate), in which 50% was flew into MS detector and another 50% was collected in a 96-well plate (COSTAR, Corning Inc.) with time interval of 1 min (Fig. 1). 200 μl of a DPPH solution in 70% methanol (0.06 mM) was added directly to each well while totally 60 fractions were gathered and placed in the dark at room temperature for 40 min. The absorbance was measured with Microplate Spectrophotometer (MQX200 uQuant,

Table 1
The retention time, precursor ion and main fragment ions of the reference compounds in positive ion mode of MS^n analysis.

Authentic compounds	RT (min)	Quasi-molecular ion (measured)	Error (ppm)	Formula	MS^n data (measured)
Synephrine	1.48	168.1021 [M+H]+	0.979	$C_9H_{13}NO_2$	150.0916 → 135.0680, 119.0492
Paeoniflorin	17.74	503.1500 [M+Na]+	−4.757	$C_{23}H_{28}O_{11}$	381.1138 → 219.0619
Naringin	24.29	581.1852 [M+H]+	−2.138	$C_{27}H_{32}O_{14}$	435.1288, 419.1341 → 383.1120
Hesperidin	24.98	611.1961 [M+H]+	−1.534	$C_{28}H_{34}O_{15}$	465.1393, 449.1443 → 413.1220
Neohesperidin	25.93	611.1966 [M+H]+	−0.732	$C_{28}H_{34}O_{15}$	465.1393, 449.1443 → 413.1224
Saikosaponin A	38.63	781.4719 [M+H]+	−4.299	$C_{42}H_{68}O_{13}$	605.3998, 455.3498
Glycyrrhizic acid	39.01	823.4097 [M+H]+	−1.619	$C_{42}H_{62}O_{16}$	647.3786, 453.3354
Nobiletin	40.15	403.1377 [M+H]+	−2.591	$C_{21}H_{22}O_8$	388.1150 → 373.0917
Tangeretin	41.81	373.1274 [M+H]+	−2.063	$C_{20}H_{20}O_7$	358.1044 → 343.0811

Bio-Tek) at 515 nm. The radical scavenging activities of each fraction were expressed as scavenging rate (SR), which was calculated using following formula:

$$SR\% = \frac{(A_c - A_s)}{A_c} \times 100$$

where A_c is the absorbance of control (Sample solution was replaced by 70% methanol) and A_s is the absorbance of sample reaction solution. Samples were run in triplicate and the antioxidant profile was characterized by SR of 60 fractions on DPPH radicals in 96-well plate. SR plot was drawn by Origin software.

3. Results and discussion

3.1. Optimization of LC and MS conditions

Several mobile phase systems included acetonitrile-aqueous, methanol-aqueous, acetonitrile-aqueous with 0.1% formic acid and methanol-aqueous with 0.1% formic acid were selected to optimize the chromatographic conditions. As a result, acetonitrile-aqueous with 0.1% formic acid on the optimized gradient mode gave a good separation and abundant signal response both in positive and in negative ion scan mode.

For the MS conditions, flow rate of sheath gas, spray voltage, capillary temperature and voltage of tube lens were the main parameters affected the signal of ion intensity. Therefore, these parameters were optimized with the flow rate of sheath gas at 30, 20, and 15 arbitrary units, spray voltage at 5, 4.5, 4 in positive ion mode and −5, −4.5, −4 in negative ion mode, voltage of tube lens at 80, 100, 120 in positive ion mode and −80, −100, −110 in negative ion mode, capillary temperature at 250, 275, 300 °C. As a results, the optimized parameters were set as follows: for positive ion mode, sheath gas at 30 arbitrary units, spray voltage at 5 kV, capillary temperature at 275 °C, tube lens at 100 V; for negative ion mode, sheath gas at 30 arbitrary units, spray voltage at −4.5 kV, capillary temperature at 275 °C, tube lens at −100 V.

3.2. HPLC–MS^n analysis of authentic compounds

The MS^n spectra of 10 authentic compounds, i.e. synephrine, paeoniflorin, naringin, hesperidin, neohesperidin, nobiletin, tangeretin, ferulic acid, saikosaponin A, and glycyrrhizic acid, were determined by direct infusion. All compounds except ferulic acid exhibited quasi-molecular ion $[M+H]^+$ and/or adducted ions $[M+Na]^+$ in positive ion mode and all compounds except synephrine, nobiletin and tangeretin showed $[M-H]^-$ and/or $[M+HCOOH-H]^-$ in negative ion mode. Ferulic acid could only be detected in negative ion mode and synephrine, nobiletin and tangeretin only in positive ion mode. The elemental composition of their quasi-molecular ions and the fragment ions with exact mass were obtained using orbitrap analyzer, which was useful to identify those constituents in CSGS. The MS^n spectra of all authentic compounds were analyzed through LC injection using the same LC–MS/MS conditions as for CSGS. The same MS behavior as in direct infusion was observed for all compounds. The retention time (RT), together with the *m/z* values of ions, MS data and their main fragments in MS^n spectra with high resolution data (Tables 1 and 2) were the most important parameters for the identification the constituents in CSGS.

3.3. HPLC–MS^n analysis of constituents in CSGS

3.3.1. HPLC–MS^n analysis of constituents in CSGS in positive ion mode

The CSGS and its single herb extracts were analyzed using LC/MS/MS under the same conditions. 43 peaks were observed and 31 of them were identified in the liquid chromatography profile of CSGS in the positive ion mode. Corresponding quasi-molecular ions and their fragment ions in the MS/MS spectra were summarized in Table 3. By comparing individual peak retention times and the online MS spectra with those of authentic compounds, peaks **1**, **7**, **16**, **17**, **18**, **28**, **29**, **30** and **33** were identified as synephrine (**1**), paeoniflorin (**7**), naringin (**16**), hesperidin (**17**), neohesperidin (**18**), saikosaponin A (**28**), glycyrrhizic acid (**29**), nobiletin (**30**) and tangeretin (**33**), respectively.

Similarly, by comparing individual peak retention times and the online MS spectra with those of peaks in each single herbal extract, most peaks in the chromatographic profile of CSGS were found to be correlated with individual herbs. The contributions of each herb were listed in last column of Table 3. The identification of peaks **5** [14], **14** [15], **22** [16], **26** [14], **27** [16] and **32** [17] (Table 3) based on the structural information from MS^2 and MS^3 spectra and comparison of their *m/z* values and fragment ions with data from the literatures.

Table 2
The retention time, precursor ion and main fragment ions of the reference compounds in negative ion mode of MS^n analysis.

Authentic compounds	RT (min)	Quasi-molecular ion (measured)	Error (ppm)	Formula	MS^n data (measured)
Paeoniflorin	17.54	525.1611 $[M+HCOOH^-H]^-$	1.641	$C_{23}H_{28}O_{11}$	449.1457 → 327.1092
Ferulic acid	22.83	193.0505 $[M-H]^-$	4.789	$C_{10}H_{10}O_4$	178.0276, 149.0612 → 134.0374
Naringin	24.16	579.1715 $[M-H]^-$	1.169	$C_{27}H_{32}O_{14}$	459.1126 → 357.0834
Hesperidin	24.90	609.1821 $[M-H]^-$	1.203	$C_{28}H_{34}O_{15}$	301.0719 → 286.0487
Neohesperidin	25.84	609.1823 $[M-H]^-$	1.400	$C_{28}H_{34}O_{15}$	301.0720 → 286.0488
Saikosaponin A	38.46	825.4639 $[M+HCOOH^-H]^-$	0.934	$C_{42}H_{68}O_{13}$	779.4586 → 617.4065
Glycyrrhizic acid	39.89	821.3954 $[M-H]^-$	−0.028	$C_{42}H_{62}O_{16}$	645.3648, 351.0574 → 193.0356

Table 3
Identification of the chemical constituents of CSGS formula by LC–MS^n analysis in positive ion mode.

Peak no.	RT (min)	Quasi-molecular ion (measured)	Formula	Error (ppm)	MS^n data (measured)	Identification	Crude herb[a]
1	1.45	168.1021	$C_9H_{13}NO_2$	0.979	150.0916 → 135.0682, 119.0493	Synephrine	**2**
2	2.87	328.1391	$C_{15}H_{21}NO_7$	0.430	120.0808 → 103.0543	unidentified	**2**
3	3.84	427.2077	$C_{20}H_{30}N_2O_8$	0.415	248.1283 → 177.0547	unidentified	**4**
4	15.26	595.1634	$C_{27}H_{30}O_{15}$	−3.961	577.1533 → 457.1110	unidentified	**2**, **4**
5	15.56	503.1503	$C_{23}H_{28}O_{11}$	−4.200	341.0979 → 175.0720	Albiflorin[b]	**3**
6	16.25	765.2184	$C_{33}H_{42}O_{19}$	−3.673	603.1655 (−4.853) → 483.1078	Glucosyl-narirutin/glucosyl-naringin[b]	**2**, **4**
7	17.16	503.1503	$C_{23}H_{28}O_{11}$	−4.200	381.1139 → 219.0619	Paeoniflorin	**3**
8	19.52	475.3223	$C_{30}H_{44}O_3$	4.063	457.3132 → 439.3036, 361.2592	Glyyunnansapogenin H[b]	**7**
9	20.03	597.1799	$C_{27}H_{32}O_{15}$	−2.588	451.1223, 289.0699	Eriocitrin[b]	**2**, **4**
10	20.51	567.1126	$C_{28}H_{22}O_{13}$	−1.248	405.0594, 323.0874	unidentified	**3**
11	20.93	419.1327	$C_{21}H_{22}O_9$	−2.230	147.0432 → 119.0483	Isoliquiritin/Neoisoliquiritin[b]	**7**
12	21.86	597.1801	$C_{27}H_{32}O_{15}$	−2.256	451.1230, 289.0703	Neoeriocitrin[b]	**4**
13	22.34	249.1092	$C_{12}H_{18}O_4$	−2.090	231.1368, 203.1418, 195.3364	Senkyunolide J or Senkyunolide N[b]	**6**
14	22.84	581.1852	$C_{27}H_{32}O_{14}$	−2.242	435.1285, 419.1337 → 383.1120	Narirutin[b]	**2**, **4**
15	23.32	779.2332	$C_{34}H_{44}O_{19}$	−4.724	633.1760, 477.1555 → 331.0982	Rhamnosyl-hesperidin/Rhamnosyl-neohesperidin[b]	**4**
16	23.88	581.1856	$C_{27}H_{32}O_{14}$	−1.502	435.1284, 419.1335 → 383.1119	Naringin	**4**
17	24.52	611.1962	$C_{28}H_{34}O_{15}$	−1.463	465.1393, 449.1444 → 413.1224	Hesperidin	**2**, **4**
18	25.56	611.1966	$C_{28}H_{34}O_{15}$	−0.749	465.1399, 449.1448 → 413.1225	Neohesperidin	**4**
19	26.27	247.0937	$C_{12}H_{16}O_4$	−1.702	229.1210, 207.1005	Senkyunolide H/Senkyunolide I[b]	**6**
20	27.19	579.2193	$C_{30}H_{36}O_{10}$	−1.328	301.1030 → 283.0926	unidentified	**2**, **4**
21	28.08	419.1327	$C_{21}H_{22}O_9$	−2.230	257.0811 → 239.0706, 147.0440	Isoliquiritin/Neoisoliquiritin[b]	**7**
22	30.47	257.0806	$C_{15}H_{12}O_4$	−0.917	239.0703, 147.0439 → 119.0489	Liquiritigenin[b]	**7**
23	31.21	595.2014	$C_{28}H_{34}O_{14}$	−1.198	433.1471 → 397.1260	Neoponcirin[b]	**2**, **4**
24	32.17	595.2018	$C_{28}H_{34}O_{14}$	−0.492	433.1469 → 397.1259	Poncirin[b]	**4**
25	34.87	725.2272	$C_{33}H_{40}O_{18}$	−2.167	419.1338 → 404.1098, 389.0864	unidentified	**2**, **4**
26	35.13	607.1758	$C_{30}H_{32}O_{12}$	−4.773	485.1398, 341.0983, 219.0619	Benzoylpaeoniflorin[b]	**3**
27	38.22	839.4043	$C_{42}H_{62}O_{17}$	−1.999	663.3702, 487.3387 → 405.3129	Licorice-saponinG_2[b]	**7**
28	38.59	781.4713	$C_{42}H_{68}O_{13}$	−2.584	605.3998, 455.3498	Saikosaponin A	**1**
29	39.93	823.4097	$C_{42}H_{62}O_{16}$	−1.619	647.3776, 453.3349	Glycyrrhizic acid	**7**
30	40.11	403.1373	$C_{21}H_{22}O_8$	−3.583	388.1149 → 373.0916	Nobiletin	**2**, **4**
31	40.66	235.1687	$C_{15}H_{22}O_2$	−2.410	217.1585 → 199.1478, 161.0958	α-Rotunol or β-rotunol[b]	**5**
32	41.09	433.1482	$C_{22}H_{24}O_9$	−2.653	418.1239 → 403.1003	3-Methoxynobiletin[b]	**2**, **4**
33	41.79	373.1272	$C_{20}H_{20}O_7$	−2.706	358.1045 → 343.0806	Tangeretin	**2**, **4**
34	41.96	193.1218	$C_{12}H_{16}O_2$	−2.623	175.1113, 147.1164 → 119.0851, 105.0694	Senkyunolide A[b]	**6**
35	43.64	217.1792	$C_{12}H_{24}O_3$	−3.000	199.1683, 111.1162	Hydroxydodecanoic acid[b]	**6**
36	45.96	231.1583	$C_{12}H_{22}O_4$	−3.227	175.0961 → 119.0337, 101.0230	Dihydroxyl-en-dodecanoic acid[b]	**5**, **6**
37	47.33	245.1740	$C_{13}H_{24}O_4$	−2.800	171.1011, 115.0385 → 87.0436	Dihydroxyl-di-en-tridecylic acid[b]	**5**
38	49.53	228.2324	$C_{14}H_{29}NO$	1.002	172.1696, 158.1535, 102.0910	Myristamide[b]	**5**
39	50.44	254.2472	$C_{16}H_{31}NO$	−2.602	237.2210 → 219.2105	Long chain unsaturated amides	**5**
40	51.43	280.2628	$C_{18}H_{33}NO$	−2.396	263.2368 → 245.2264	Long chain unsaturated amides	**5**, **6**
41	53.35	256.2627	$C_{16}H_{33}NO$	−3.089	200.2008, 186.1850, 102.0909	Palmitamide[b]	**6**
42	54.04	282.2783	$C_{18}H_{35}NO$	−3.123	265.2524 → 247.2421	Long chain unsaturated amides	**6**
43	57.75	284.2950	$C_{18}H_{37}NO$	0.663	266.2568, 248.2460, 102.0913	Stearamide[b]	**5**, **6**

[a] Crude herbs are labeled as in Fig. 4.
[b] Structures were tentatively identified.

For example, a quasi-molecular ion at *m/z* 503.1503 ($[C_{23}H_{28}O_{11}Na]^+$, calcd. 503.1524) was observed for peak **5**, and a peak with same retention time and *m/z* value could be found in *Bai-Shao* extract. The quasi-molecular ion and the produced fragment ion at *m/z* 341.0979 ($[C_{17}H_{18}O_6Na]^+$, calcd. 341.0995) by the loss of a molecule of glucose were similar to paeoniflorin (Table 3, peak **7**). It suggested that the constituent in peak **5** has same formula with similar structure as paeoniflorin. Albiflorin was the only reported component in *Bai-Shao* [14] having same molecular weight as paeoniflorin with the difference of a carbonyl group at C_9 instead of C–O bridged bond in paeoniflorin. Further, it can be found that ratio of ion intensity between *m/z* 341.0979 ([M+Na-glucosyl]$^+$) and *m/z* 381.1138 ([M+Na-benzoyloxy]$^+$) was nearly 15:1 in MS^2 of peak **5** (Fig. 2), whereas the ratio between the ions at *m/z* 341.0981 and at *m/z* 381.1139 was nearly 1:1 in MS^2 of paeoniflorin. Additionally, in the MS^2 spectra of paeoniflorin, the relative intensity of ion at *m/z* 219.0619 ($[C_{10}H_{12}O_4Na]^+$, calcd. 219.0628, [M+Na-benzoyloxy-glucosyl]$^+$) was higher than that in the MS^2 spectra of peak **5** (ion intensity of $[C_{10}H_{12}O_4Na]^+$ was 34% in paeoniflorin and 7% in peak **5**), these findings are in accordance with the carbonyl group at C_9 in albiflorin, which made the fragment ions form a stable positive radical more easily than C–O bridged bond in paeoniflorin when a molecule of glucose or even a molecule of benzoic acid was lost from the quasi-molecular ion. As a result, it is reasonable to identify peak **5** as albiflorin (Fig. 3).

Though some constituents were hardly discriminated in their mass spectra when they had same aglycone and components of sugars but difference in connection of saccharides, their retention times were different sometimes. For instance, two chemical constituents with same molecular formula and fragment ions were recognized in peaks **9** and **12** (Table 3). Obviously, it was not possible to differentiate them by MS/MS spectra, but their retention times were different when analyzed on a reversed phase column eluted by acetonitrile-aqueous system [18,19], so peaks **9** and **12** could be tentatively identified as eriocitrin and neoeriocitrin, respectively. Similarly, peaks **23** and **24** were identified as neoponcirin and poncirin [20].

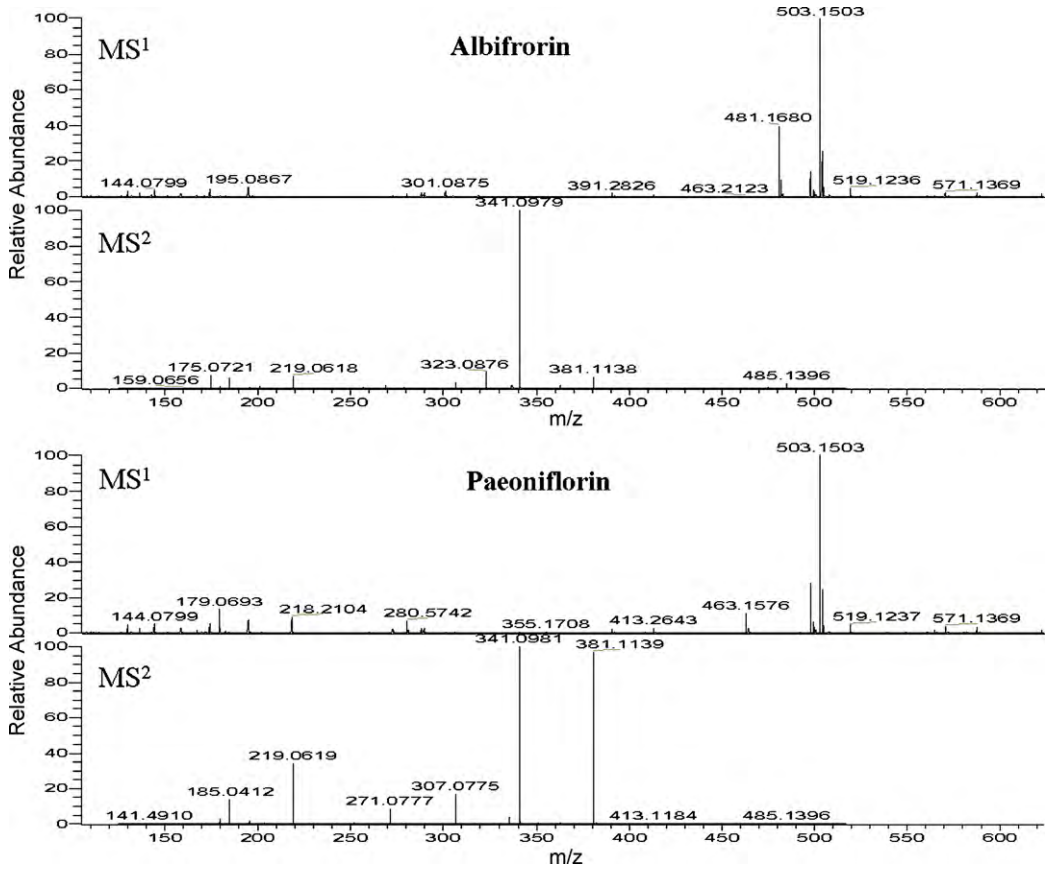

Fig. 2. Spectra of ion fragments in MSn analysis of albiflorin and paeoniflorin in positive ion mode.

Even if some constituents could not unambiguously be identified in this survey, they could be associated with the individual herbs in CSGS by their retention times, accurate molecular weights and fragmentation patterns in the positive ion mode (Fig. 4). For example, peak **6** with quasi-molecular ion at *m/z* 765.2184 ($[C_{33}H_{42}O_{19}Na]^+$, calcd. 765.2212) gave an ion at *m/z* 603.1655 ($[C_{27}H_{32}O_{14}Na]^+$, calcd. 603.1684) in MS^2 spectrum presumed as glucosyl-narirutin or glucosyl-naringin could be assigned to *Chen-Pi* and *Zhi-Qiao*. Peak **15**, contributed by *Zhi-Qiao*, was tentatively identified as rhamnosyl-hesperidin or rhamnosyl-neohesperidin.

3.3.2. HPLC–MS^n analysis of constituents in CSGS in negative ion mode

In negative ion mode, series of ions $[M-H]^-$ and/or adducted ions ($[M+HCOOH-H]^-$) appeared as quasi-molecular ions. Quasi-molecular ions and fragment ions in the MS/MS spectra were summarized in Table 4. As a result, 15 compounds were identified, 13 of them were already detected in positive ion mode. The chemical structures of constituents identified by analysis of both positive and negative ion mode were shown in Fig. 5.

Peaks **12a** and **19a** could not be detected in positive ion mode but exhibited well ionization and abundant signal responses in negative ion mode. Peak **12a** showed quasi-molecular ion at *m/z* 193.0504 ($[C_{10}H_9O_4]^-$, calcd. 193.0495), which generated fragment ions at *m/z* 178.0269 ($[C_9H_6O_4]^-$, calcd. 178.0261) and 149.0606 ($[C_9H_9O_2]^-$, calcd. 149.0597) by the losses of CH_3 group and CO_2 group in MS^2 spectrum. By comparing the fragmentation pattern and retention time with the authentic compound, peak **12a** was assigned to be ferulic acid. Peak **19a** was deduced as benzoic acid, which had quasi-molecular ion at *m/z* 121.0295 ($[C_7H_5O_2]^-$, calcd. 121.0284) and typical fragment ion at *m/z* 77.0398 ($[C_6H_5]^-$, calcd. 77.0386) generated by the loss of carboxyl group in MS^2 spectrum.

3.3.3. Characterization of antioxidant profile by investigating scavenging activity of CSGS on DPPH radicals

The activities of chemical constituents scavenging DPPH radicals in CSGS were determined with the fraction collecting and microplate reading systems. The antioxidant profile of CSGS (Fig. 6) indicated that fractions eluted in 11–18 min had higher SRs than other fractions and the containing compounds could be considered as the contributors to the *antioxidant* effect. LC–MS showed four peaks identified as albiflorin (15.56 min, **5**), glucosyl-narirutin or glucosyl-naringin (16.25 min, **6**), and paeoniflorin (17.16 min, **7**). Fractions from 19 to 29 min with SRs of no less than 20% should make contributions to antioxidative activity of CSGS too. These fractions could be easily associated with series of components identified in positive ion scan mode, including eriocitrin (20.03 min, **9**), neoeriocitrin (21.86 min, **12**), narirutin (22.84 min, **14**), naringin (23.88 min, **16**), hesperidin (24.52 min, **17**), and neohesperidin (25.56 min, **18**). Among them, naringin, hesperidin, and neohesperidin, had been reported having the bioactivities of antioxidation [21,22], and anti-inflammatory [5], especially hesperidin, which had been reported as the inhibitor of COX-2 and NOS [23]. As a result, it suggested that the antioxidant activity of CSGS was the integrated effects of chemical constituents in this formula. By characterization of antioxidant profile of CSGS, we can easily find the contribution of antioxidant activity of each fraction, conveniently, the chemical information of corresponding fractions could be simultaneously correlated too.

The qualitative and quantitative composition of natural product extracts from plants depends on various factors such as the climate in the cultivation region, geographical factors, different subspecies of the same plant family, and conditions of extraction and storage. The developed analytical approach is therefore not only suited to identify the biological active components but also to

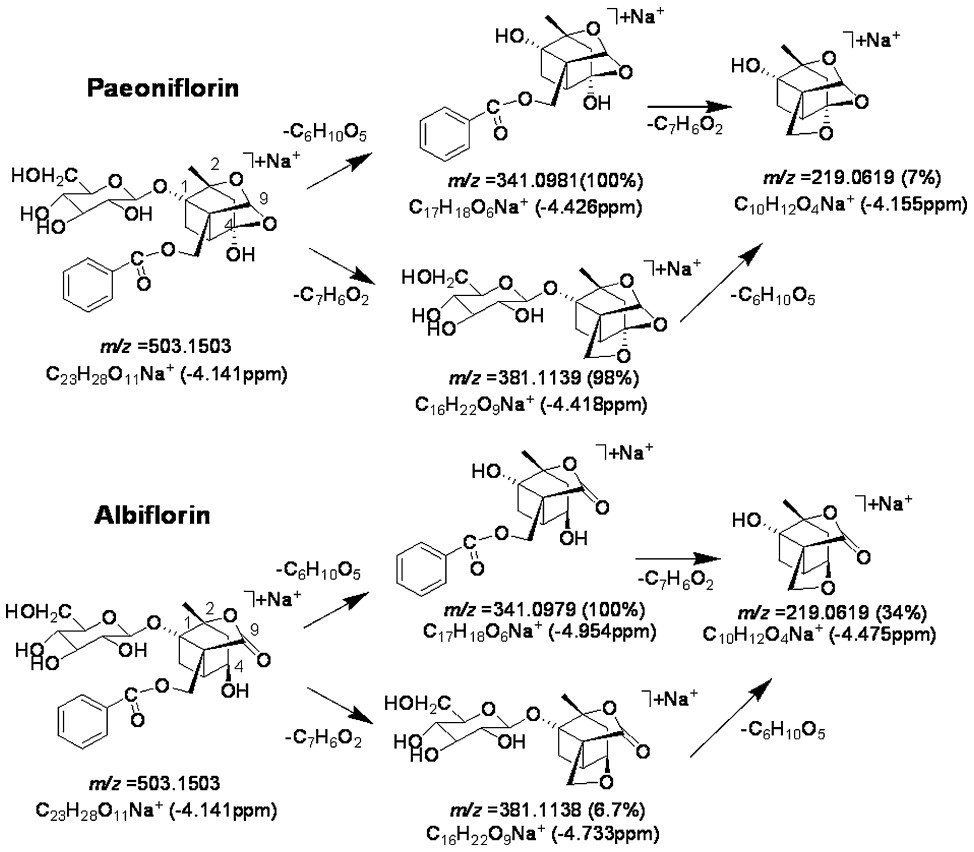

Fig. 3. Fragmentation pathway of paeoniflorin and albiflorin in positive ion mode.

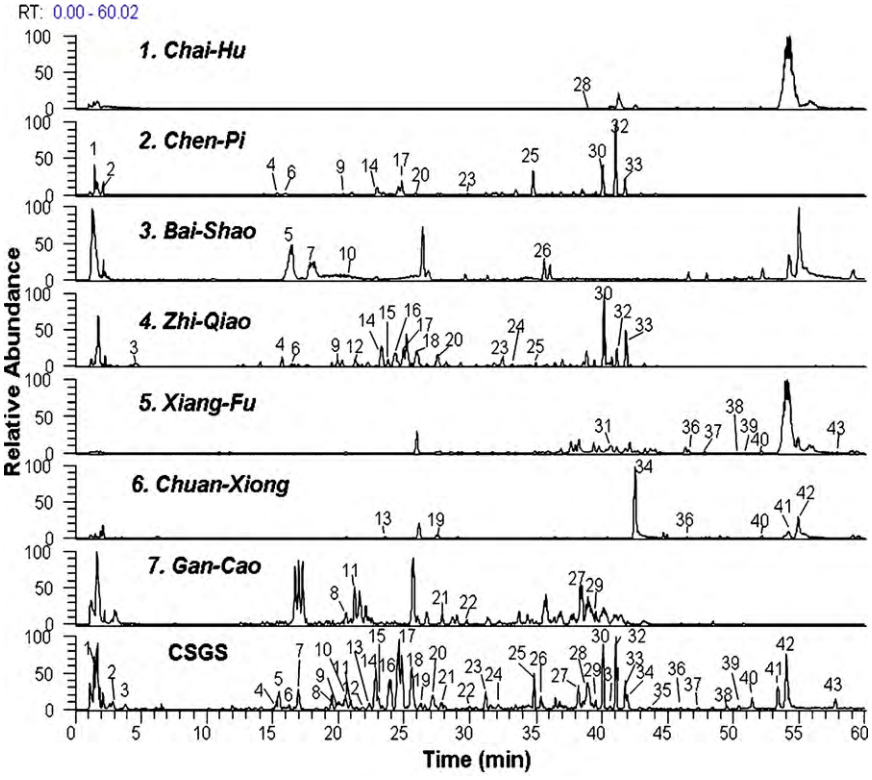

Fig. 4. Base peak spectra of CSGS and its single herbs profiled in positive ion mode by LC-LTQ-Orbitrap.

Table 4
Identification of the chemical constituents of CSGS formula by LC–MS^n analysis in negative ion mode.

Peak no.	RT (min)	Quasi-molecular ion (measured)	Formula	Error (ppm)	MS^n data (measured)	Identification
5a	15.59	593.1496	$C_{27}H_{30}O_{15}$	−0.838	473.1018 → 353.0660	Unidentified
5	16.25	525.1603	$C_{23}H_{28}O_{11}$	0.023	479.1546 → 357.1187, 283.0821	Albiflorin[a]
7	17.89	525.1600	$C_{23}H_{28}O_{11}$	−0.567	449.1444 → 327.1081, 283.0821	Paeoniflorin
9	20.50	595.1653	$C_{27}H_{32}O_{15}$	−0.467	287.0555 → 151.0038, 135.0452	Eriocitrin[a]
11a	21.11	549.1600	$C_{26}H_{30}O_{13}$	−0.433	255.0657 → 135.0087	Unidentified
11	21.32	417.1183	$C_{21}H_{22}O_9$	0.745	255.0657 → 135.0086	Isoliquiritin/neoisoliquiritin[a]
12	21.85	595.1650	$C_{27}H_{32}O_{15}$	−0.707	459.1138 → 441.1028, 357.0820	Neoeriocitrin[a]
12a	22.20	193.0504	$C_{10}H_{10}O_4$	4.582	178.0269, 149.0606 → 134.0373	Ferulic acid
14	23.21	579.1707	$C_{27}H_{32}O_{14}$	−0.195	459.1134 → 357.0822	Narirutin[a]
16	24.28	579.1707	$C_{27}H_{32}O_{14}$	−0.195	459.1145 → 357.0822	Naringin
17	24.87	609.1810	$C_{28}H_{34}O_{15}$	−0.702	301.0714 → 286.0478	Hesperidin
18a	25.17	187.0974	$C_9H_{16}O_4$	4.995	125.0973 → 97.0660	Unidentified
18	25.44	609.1812	$C_{28}H_{34}O_{15}$	−0.406	301.0716 → 286.0478	Neohesperidin
19a	26.08	121.0295	$C_7H_6O_2$	1.144	77.0398	Benzoic acid[a]
23a	30.71	543.1172	$C_{19}H_{28}O_{18}$	−3.574	421.0817 → 301.0391	Unidentified
23	31.53	639.1913	$C_{28}H_{34}O_{14}$	−0.682	593.1862 → 285.0764	Neoponcirin[a]
24	32.43	639.1915	$C_{28}H_{34}O_{14}$	−0.492	593.1855 → 285.0766	Poncirin[a]
28	38.64	825.4635	$C_{42}H_{68}O_{13}$	0.486	779.4570 → 617.4062	Saikosaponin A
29	39.93	821.3952	$C_{42}H_{62}O_{16}$	−0.259	645.3643, 351.0574 → 193.0354	Glycyrrhizic acid

[a] Structures were tentatively identified.

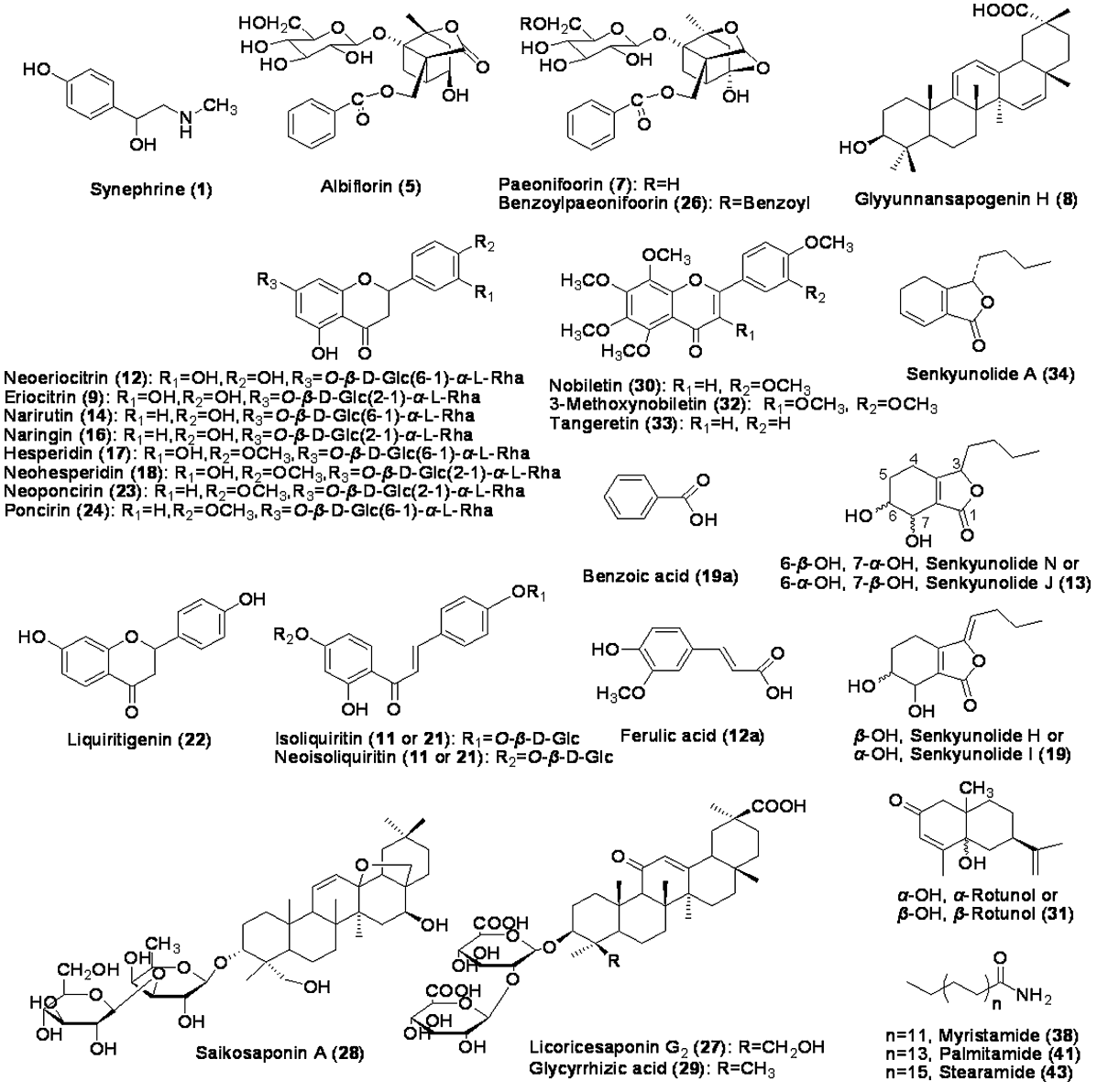

Fig. 5. The chemical structures of constituents identified in positive and negative scan ion mode.

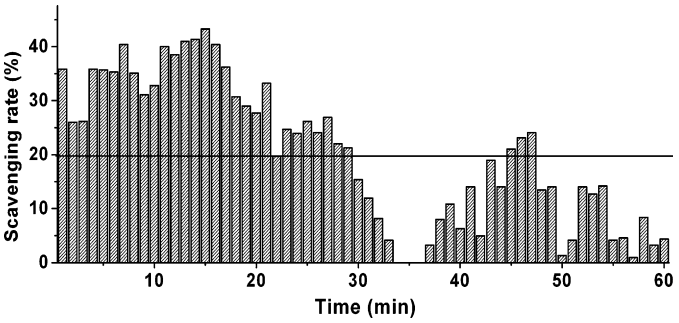

Fig. 6. Antioxidant profile of CSGS HPLC fractions by investigating scavenging activity on DPPH radicals.

detect qualitative variations in the composition of the extracts and to interpret them. This is very important for ensuring a consistent quality of TCM formula such as CSGS. On the basis of the identified compounds this analytical approach can be further developed to correlate the antioxidative activity with the contents of the active ingredients.

4. Conclusion

A simple and effective method employing liquid chromatography coupled with linear trap quadrupole (LTQ) and the high resolution mass analyzer-orbitrap (LC–LTQ-Orbitrap) was developed for online identification of chemical constituents of CSGS without traditional isolation and purification. 33 components were identified based on the fragmentation pattern information obtained by LC–MS^n and accurate molecular data from LTQ-Orbitrap, 31 of them were observed in positive mode and 15 constituents in negative mode. By the combination of fraction collecting system and microplate reading system, the contributions of antioxidant activities of fractions were interpreted. This study provided an intuitionistic result on the contribution of CSGS fractions to the antioxidant activity and a technique for online identification of the corresponding active constituents. The combination of LC–MS^n and 96-well plate assay system established in this paper would be a useful strategy for correlating the chemical profile of TCMs with their bioactivities without isolation and purification.

Acknowledgements

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