

Article

## Qualitative and Quantitative Analysis of *Rhizoma Smilacis glabrae* by Ultra High Performance Liquid Chromatography Coupled with LTQ Orbitrap<sup>XL</sup> Hybrid Mass Spectrometry

Shao-Dan Chen <sup>1,2</sup>, Chuan-Jian Lu <sup>1,2,\*</sup> and Rui-Zhi Zhao <sup>1,2</sup>

<sup>1</sup> The Second College of Clinic Medicine, Guangzhou University of Chinese Medicine, Guangzhou 510000, China

<sup>2</sup> Guangdong Provincial Hospital of Chinese Medicine, Guangzhou 510000, China

\* Author to whom correspondence should be addressed; E-Mail: luchuanjian888@vip.sina.com; Tel./Fax: +86-20-8188-7233.

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**Abstract:** *Rhizoma Smilacis glabrae*, a traditional Chinese medicine (TCM) as well as a functional food, has been commonly used for detoxification treatments, relieving dampness and as a diuretic. In order to quickly define the chemical profiles and control the quality of *Smilacis glabrae*, ultra high performance liquid chromatography coupled with electrospray ionization hybrid linear trap quadrupole orbitrap mass spectrometry (UHPLC-ESI/LTQ-Orbitrap-MS) was applied for simultaneous identification and quantification of its bioactive constituents. A total of 56 compounds, including six new compounds, were identified or tentatively deduced on the basis of their retention behaviors, mass spectra, or by comparison with reference substances and literature data. The identified compounds belonged to flavonoids, phenolic acids and phenylpropanoid glycosides. In addition, an optimized UHPLC-ESI/LTQ-Orbitrap-MS method was established for quantitative determination of six marker compounds from five batches. The validation of the method, including linearity, sensitivity (LOQ), precision, repeatability and spike recoveries, was carried out and demonstrated to be satisfied the requirements of quantitative analysis. The results suggested that the established method would be a powerful and reliable analytical tool for the characterization of multi-constituent in complex chemical system and quality control of TCM.

**Keywords:** *Rhizoma Smilacis glabrae*; UHPLC-ESI/LTQ-Orbitrap-MS; qualitative analysis; quantitative analysis

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## 1. Introduction

The rhizome of *Smilacis glabrae* Roxb (family Smilacaceae) is a well-known traditional Chinese medicine (TCM) with great medicinal values. It is officially listed in the Chinese Pharmacopoeia and has been widely used for detoxification treatments, relieving dampness and as a diuretic [1]. It was also consumed as a functional food. People in China like to use it to boil soup or tea for clearing damp. Besides, it is one of the main ingredients of turtle jelly (Gui-ling-gao), a traditional functional food popular in Southern China and Hong Kong. Phytochemical studies have shown the presence of abundant compounds in *S. glabrae*, such as flavonoids, phenolic acids and phenylpropanoid glycosides [2,3], among which flavonoids were considered to be the primary bioactive constituents of the herbal medicine. Astilbin, neoastilbin, isoastilbin, neoisoastilbin, engeletin and isoengeletin were considered as marker constituents included in *S. glabrae*. These six flavonoids were reported to possess various biological activities, involving anti-inflammatory, antioxidative, antibacterial and antitumor properties [4–10]. Some analytical methods have been used for qualitative or quantitative analysis of some of these bioactive constituents in *S. glabrae*. Li *et al.* identified the main constituents in *Rhizoma Smilacis glabrae* by means of UHPLC-DAD-MS [3]. Chen *et al.* established an HPLC method for determination of five compounds in *Rhizoma Smilacis glabrae* [11]. Although these methods have made significant contributions to the studies of the quality control of *Smilacis glabrae*, they have limitations, such as taking a long time to perform or being either qualitative or quantitative. Less effort has been dedicated to further characterize minor new components or the rapid determination of active components, so a new method is required to address the limitations of the previous techniques.

The present work aimed at developing a rapid and simple UHPLC-ESI-MS method for analyzing and discovering minor new constituents, and quantifying the active components in *Smilacis glabrae*. The advantages of this method comprised high-speed detection, excellent peak shapes, and less solvent usage. With the new method it took less than 10 min to detect 56 compounds of *Smilacis glabrae*, including six new compounds. Further, six marker flavonoids were quantitatively determined in negative ionization mode and five batches of *Smilacis glabrae* were analyzed for assessment of quality consistence. This is the first time for determination of multiple components in *Smilacis glabrae* using UHPLC-ESI/LTQ-Orbitrap-MS.

## 2. Results and Discussion

### 2.1. Optimization of Chromatographic Conditions

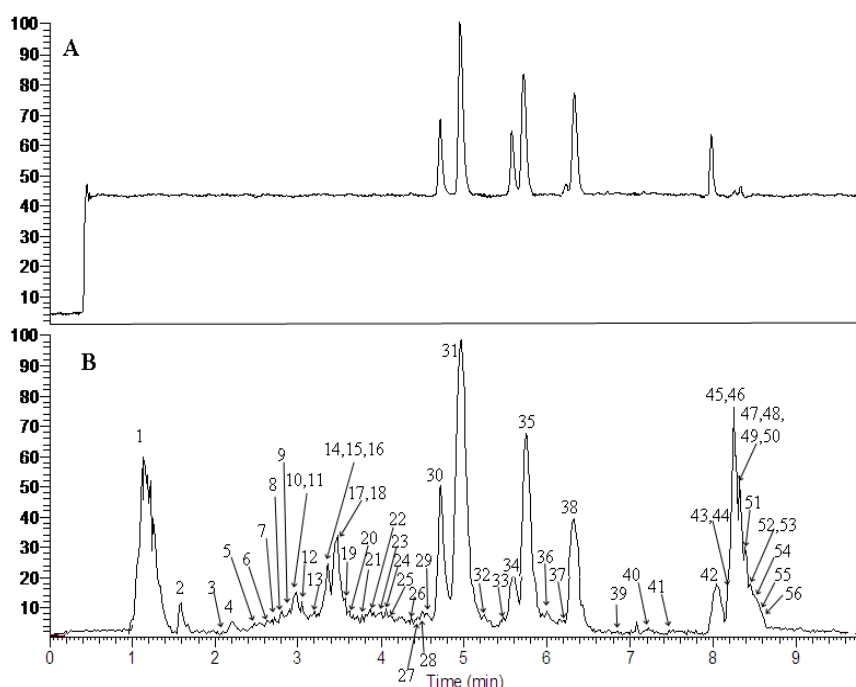
To improve the resolution and sensitivity of the analysis but reduce the analytical time, the mobile phase system was optimized. To inhibit ionization of the acidic ingredients in *Smilacis glabrae* extract, formic acid was added to the mobile phase. Two mobile phase systems, methanol-aqueous solution and acetonitrile-aqueous solution were compared. Both negative and positive modes were examined.

Generally, in positive mode, low abundance of  $[M+H]^+$ ,  $[M+NH_4]^+$  ions and few product ions were observed, while, in negative ion mode, a series of  $[M-H]^-$  ions and/or adduct ions ( $[M+HCOOH-H]^-$ ) appeared with sufficient abundance. Thus the negative ion mode was chosen and the  $[M-H]^-/([M+HCOOH-H]^-)$  ions were further subjected to LC-MS<sup>n</sup> analysis.

## 2.2. Identification of Chemical Constituents in *Smilacis glabrae* Extract

The reference standards and *Smilacis glabrae* sample were analyzed by using the optimized UHPLC-ESI-MS<sup>n</sup> method. The TIC chromatograms of the six reference standards and the extract of *Smilacis glabrae* in negative ESI mode were shown in Figure 1. Fifty six peaks were observed. The MS data showed high precision with all the mass accuracies within 5 ppm. For most of the constituents, a  $[M-H]^-$  peak was observed. Due to the use of formic acid in mobile phase, there were additional ions of  $[M+46-H]^-$  corresponding to  $[M+HCOOH-H]^-$  in negative ion mode. These results provided valuable information for confirming accurate molecular weights and composition of the constituents. The 56 compounds including six new ones were tentatively identified on the basis of their retention behaviors, accurate molecular weight and MS<sup>n</sup> fragment data, or by comparison with reference standards or literature data (chemical structures of the compounds corresponding to the peaks shown in Figure 1 below can be found in Figure 1 in the Supplementary). The corresponding quasimolecular ions and their fragment ions in the MS<sup>n</sup> spectra are listed in Table 1.

**Figure 1.** UHPLC(-) ESI-MS total ion chromatograms of a mixture of six standards (A) and the extract of *Smilacis glabrae* (B).



The identified compounds can be classified into three classes, namely flavonoids, phenolic acids and phenylpropanoid glycosides. Four flavanone isomers (compounds **30**, **31**, **34** and **35**) were unambiguously identified by the same deprotonated ions at  $m/z$  449 ( $C_{21}H_{21}O_{11}$ ) and the same product ions at  $m/z$  303 and  $m/z$  285, and they could be distinguished through their UV absorption and elution

order when compared to reference standards. Neoastilbin (**30**) with 2*S*,3*S* configuration and astilbin (**31**) with 2*R*,3*R* configuration had the same  $UV_{max}$  absorption at 290 nm, while neoisoastilbin (**34**) with 2*S*,3*R* configuration and isoastilbin (**35**) with 2*R*,3*S* configuration had the same UV absorption at 295–296 nm (see Figure 2 in the Supplementary), the latter caused a red shift of 5–6 nm, and the elution order of the four flavanonol isomers were 2*S*,3*S* > 2*R*,3*R* > 2*S*,3*R* > 2*R*,3*S*. The four flavanonols were the main constituents of *S. glabrae*. To our surprise, compounds **19**, **21**, **25** and **29** had the same deprotonated ions at  $m/z$  629 ( $C_{30}H_{29}O_{15}$ ) and the same fragment ions (Figure 2), which demonstrated they were also diastereomers. In the MS<sup>2</sup> spectra, the product ions at  $m/z$  449 [M-H-C<sub>9</sub>H<sub>8</sub>O<sub>4</sub>] and  $m/z$  303 [M-H-C<sub>9</sub>H<sub>8</sub>O<sub>4</sub>-rhamnose] suggested the four diastereomers were the derivatives of the four configurationally different astilbins. In addition, two prominent MS<sup>2</sup> product ions were observed at  $m/z$  475 and  $m/z$  483, respectively, for the neutral loss of CO<sub>2</sub> + C<sub>6</sub>H<sub>6</sub>O<sub>2</sub> and for the loss of a rhamnose, which indicated they had the same substituent group and substituent site. The four isomers could also be distinguished through their UV absorption. Compounds **19** and **21** had the same UV absorption at 289 nm, while compounds **25** and **29** had the same UV absorption at 295 nm (see Figure 2 in the Supplementary), which indicated that compounds **19** and **21** had the 2*S*,3*S* or 2*R*,3*R* configuration, while compounds **25** and **29** had the 2*S*,3*R* or 2*R*,3*S* configuration. As the elution order was 2*S*,3*S* > 2*R*,3*R* > 2*S*,3*R* > 2*R*,3*S*, thus compounds **19**, **21**, **25** and **29** were tentatively identified as 8-[β-(3,4-dihydroxyphenyl)-α-carboxyl-3-oxopropyl]-substituted neoastilbin, 8-[β-(3,4-dihydroxyphenyl)-α-carboxyl-3-oxopropyl]-substituted astilbin, 8-[β-(3,4-dihydroxyphenyl)-α-carboxyl-3-oxopropyl]-substituted neoisoastilbin and 8-[β-(3,4-dihydroxyphenyl)-α-carboxyl-3-oxopropyl]-substituted isoastilbin, respectively. Similarly, compounds **38** and **42** were unambiguously identified as engeletin (**38**) and isoengeletin (**42**) based on reference standards, and compounds **24** and **28** were tentatively identified as 8-[β-(3,4-dihydroxyphenyl)-α-carboxyl-3-oxopropyl]-substituted engeletin and 8-[β-(3,4-dihydroxyphenyl)-α-carboxyl-3-oxopropyl]-substituted isoengeletin, respectively (Figure 3 in the Supplementary). Compounds **19**, **21**, **24**, **25**, **28** and **29** were identified as new compounds, but their absolute configurations could not be determined.

### 2.3. Method Validation of the Quantitative Analysis

The calibration curves, linear ranges, limit of quantification (LOQ) and repeatability of six analytes were performed using the above-developed UHPLC-ESI-MS method (Table 2). Reasonable correlation coefficient values ( $r^2 \geq 0.9981$ ) indicated good correlations between investigated standards concentrations and their peak areas within the ranges tested. The ranges of LOQ for all the analytes were from 0.011 to 0.067 µg/mL, respectively. The repeatability present as RSD ( $n = 6$ ) was between 1.77% and 2.37% of the 6 analytes. The overall intra- and inter-day precisions (RSD) of the six analytes were in the range from 1.03% to 3.19%, and 0.76% to 3.91% (Table 2), respectively. The developed method had good accuracy with the RSD of the recoveries were between 1.49% and 4.73% (Table 2). Therefore, the results demonstrated that the UHPLC-ESI-MS method was sensitive, precise, and accurate enough for quantitative evaluation of *Smilacis glabrae*.

**Table 1.** Identification of the chemical constituents of *Smilacis glabrae* by UHPLC-ESI-MS<sup>n</sup> analysis.

Peak No.	<i>t</i> <sub>R</sub> (min)	Selected Ion	Observed Mass ( <i>m/z</i> )	Calculated Mass ( <i>m/z</i> )	Formula	MS/MS Fragmentation Patterns	Identification
1 <sup>a</sup>	1.15	[M-H] <sup>-</sup>	173.0457	173.0450	C <sub>7</sub> H <sub>9</sub> O <sub>5</sub>	173→155, 129, 111	shikimic acid
2	1.62	[M-H] <sup>-</sup>	117.0195	117.0188	C <sub>4</sub> H <sub>5</sub> O <sub>4</sub>	117→99, 73	succinic acid
3	2.32	[M-H] <sup>-</sup>	359.0984	359.0978	C <sub>15</sub> H <sub>19</sub> O <sub>10</sub>	359→197, 182	syringic acid-4- <i>O</i> -β-D-glucopyranoside
4	2.34	[M+COOH] <sup>-</sup>	255.0512	255.0505	C <sub>11</sub> H <sub>11</sub> O <sub>7</sub>	255→209, 193, 179, 165	3,4-dihydroxy-5-methoxycinnamic acid
5	2.47	[M+COOH] <sup>-</sup>	345.1191	345.1186	C <sub>15</sub> H <sub>21</sub> O <sub>9</sub>	345→299	rhodioloside
6 <sup>a</sup>	2.70	[M-H] <sup>-</sup>	153.0194	153.0188	C <sub>7</sub> H <sub>5</sub> O <sub>4</sub>	153→109	protocatechuic acid
7	2.91	[M+COOH] <sup>-</sup>	197.0458	197.0450	C <sub>9</sub> H <sub>9</sub> O <sub>5</sub>	197→153	syringic acid
8	2.97	[M-H] <sup>-</sup>	387.1296	387.1291	C <sub>17</sub> H <sub>23</sub> O <sub>10</sub>	387→207, 177	3-(β-D-glucopyranosyloxy)-1-(4-hydroxy-3,5-dimethoxyphenyl)-1-propanone
9	3.08	[M-H] <sup>-</sup>	577.1346	577.1346	C <sub>30</sub> H <sub>25</sub> O <sub>12</sub>	577→559, 451, 425, 407, 289	procyanidin B
10 <sup>a</sup>	3.13	[M-H] <sup>-</sup>	289.0720	289.0712	C <sub>15</sub> H <sub>13</sub> O <sub>6</sub>	289→271, 245, 205, 179, 151	catechin
11	3.19	[M-H] <sup>-</sup>	239.0564	239.0556	C <sub>11</sub> H <sub>11</sub> O <sub>6</sub>	239→221, 195, 179, 177, 149	syringic acid acetate
12	3.36	[M-H] <sup>-</sup>	315.1074	315.1080	C <sub>14</sub> H <sub>19</sub> O <sub>8</sub>	315→153	3,4-dihydroxyphenethyl glucoside
13 <sup>a</sup>	3.45	[M-H] <sup>-</sup>	469.1141	469.1135	C <sub>24</sub> H <sub>21</sub> O <sub>10</sub>	469→315, 289	(2 <i>R</i> ,3 <i>S</i> )-8-[β-(3,4-dihydroxyphenyl)-α-carboxyl-3-oxopropyl]-substituted catechin
14 <sup>a</sup>	3.55	[M-H] <sup>-</sup>	335.0777	335.0767	C <sub>16</sub> H <sub>15</sub> O <sub>8</sub>	335→291, 179, 135	3- <i>O</i> -caffeoylshikimic acid
15 <sup>a</sup>	3.58	[M-H] <sup>-</sup>	561.1397	561.1397	C <sub>30</sub> H <sub>25</sub> O <sub>11</sub>	561→543, 435, 289	3',4',5,7-tetrahydroxyflavan
16 <sup>a</sup>	3.61	[M-H] <sup>-</sup>	289.0722	289.0712	C <sub>15</sub> H <sub>13</sub> O <sub>6</sub>	289→271, 245, 205, 179, 151	(4→8)-3,3',4',5,7-pentahydroxyflavan epicatechin
17 <sup>a</sup>	3.74	[M-H] <sup>-</sup>	335.0777	335.0767	C <sub>16</sub> H <sub>15</sub> O <sub>8</sub>	335→291, 179, 135	4- <i>O</i> -caffeoylshikimic acid
18 <sup>a</sup>	3.76	[M-H] <sup>-</sup>	179.0350	179.0344	C <sub>9</sub> H <sub>7</sub> O <sub>4</sub>	179→161, 135	caffeic acid

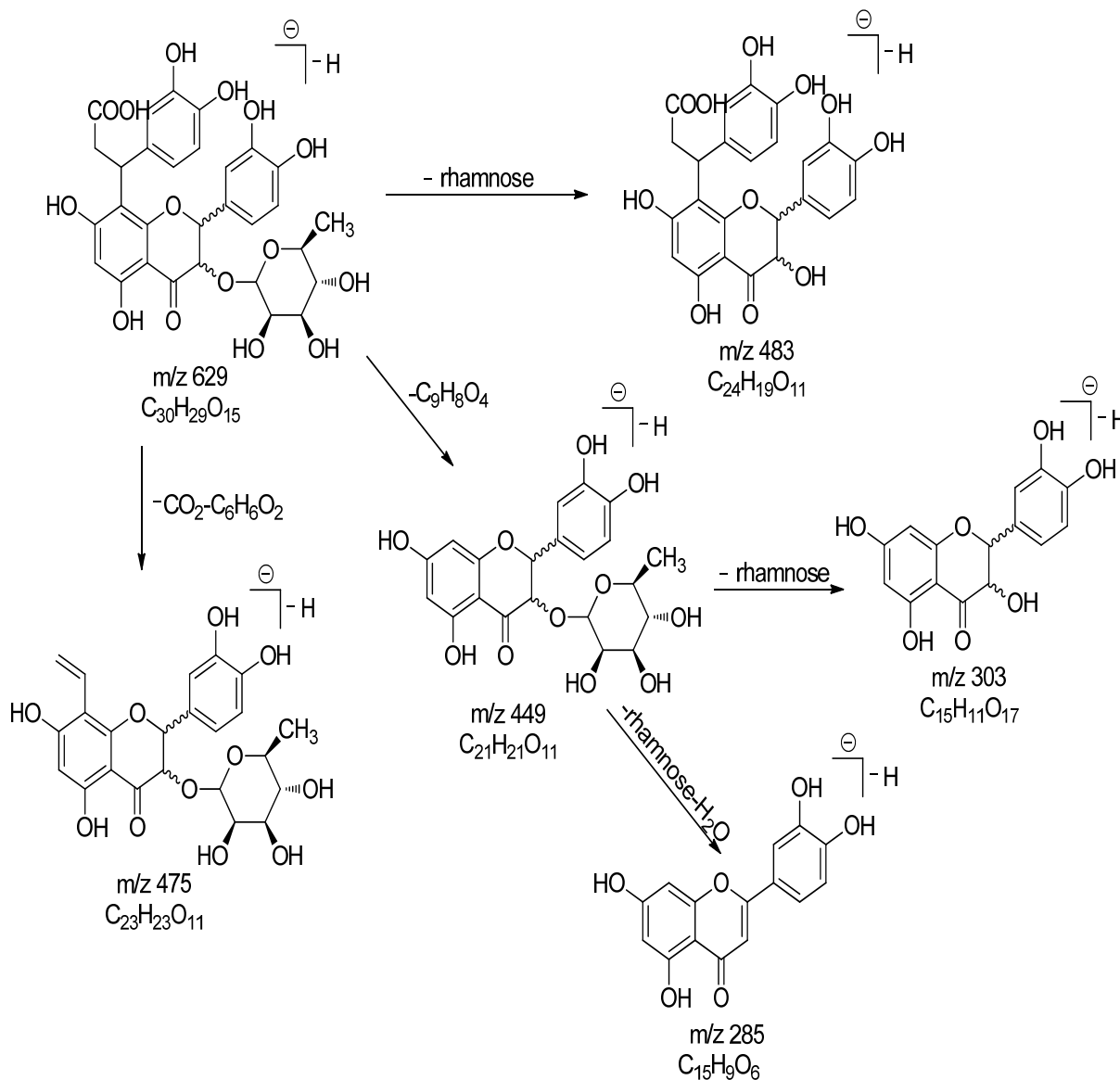
Table 1. Cont.

Peak No.	$t_R$ (min)	Selected Ion	Observed Mass (m/z)	Calculated Mass (m/z)	Formula	MS/MS Fragmentation Patterns	Identification
19 <sup>b</sup>	3.93	[M-H] <sup>-</sup>	629.1514	629.1506	C <sub>30</sub> H <sub>29</sub> O <sub>15</sub>	629→483, 475, 449, 303, 285	8-[β-(3,4-dihydroxyphenyl)-α-carboxyl-3-oxopropyl]-substituted neoastilbin
20	4.04	[M-H] <sup>-</sup>	465.1041	465.1033	C <sub>21</sub> H <sub>21</sub> O <sub>12</sub>	465→421, 297	4-O-β-D-(6-O-gentisoylglucopyranosyl)-vanillic acid
21 <sup>b</sup>	4.20	[M-H] <sup>-</sup>	629.1514	629.1506	C <sub>30</sub> H <sub>29</sub> O <sub>15</sub>	629→483, 475, 449, 303, 285	8-[β-(3,4-dihydroxyphenyl)-α-carboxyl-3-oxopropyl]-substituted astilbin
22	4.23	[M-H] <sup>-</sup>	339.0721	339.0716	C <sub>15</sub> H <sub>15</sub> O <sub>9</sub>	339→193	smiglanin
23 <sup>a</sup>	4.39	[M-H] <sup>-</sup>	335.0777	335.0767	C <sub>16</sub> H <sub>15</sub> O <sub>8</sub>	335→291, 179, 135	5-O-caffeoylshikimic acid
24 <sup>b</sup>	4.44	[M-H] <sup>-</sup>	613.1565	613.1557	C <sub>30</sub> H <sub>29</sub> O <sub>14</sub>	613→467, 459, 433, 287	8-[β-(3,4-dihydroxyphenyl)-α-carboxyl-3-oxopropyl]-substituted engeletin
25 <sup>b</sup>	4.56	[M-H] <sup>-</sup>	629.1514	629.1506	C <sub>30</sub> H <sub>29</sub> O <sub>15</sub>	629→483, 475, 449, 303, 285	8-[β-(3,4-dihydroxyphenyl)-α-carboxyl-3-oxopropyl]-substituted neoisoastilbin
26	4.84	[M-H] <sup>-</sup>	301.0354	301.0348	C <sub>15</sub> H <sub>9</sub> O <sub>7</sub>	301→283, 255, 215, 175, 151	quercetin
27	4.97	[M+COOH] <sup>-</sup>	435.1297	435.1291	C <sub>21</sub> H <sub>23</sub> O <sub>10</sub>	435→389, 227, 195	polydatin
28 <sup>b</sup>	5.06	[M-H] <sup>-</sup>	613.1565	613.1557	C <sub>30</sub> H <sub>29</sub> O <sub>14</sub>	613→467, 459, 433, 287	8-[β-(3,4-dihydroxyphenyl)-α-carboxyl-3-oxopropyl]-substituted isoengeletin
29 <sup>b</sup>	5.12	[M-H] <sup>-</sup>	629.1514	629.1506	C <sub>30</sub> H <sub>29</sub> O <sub>15</sub>	629→483, 475, 449, 303	8-[β-(3,4-dihydroxyphenyl)-α-carboxyl-3-oxopropyl]-substituted isoastilbin
30 <sup>c</sup>	5.29	[M-H] <sup>-</sup>	449.1099	449.1084	C <sub>21</sub> H <sub>21</sub> O <sub>11</sub>	449→303, 285	neoastilbin
31 <sup>c</sup>	5.63	[M-H] <sup>-</sup>	449.1099	449.1084	C <sub>21</sub> H <sub>21</sub> O <sub>11</sub>	449→303, 285	astilbin
32 <sup>a</sup>	5.72	[M-H] <sup>-</sup>	193.0511	193.0501	C <sub>10</sub> H <sub>9</sub> O <sub>4</sub>	193→178, 161, 134	ferulic acid
33 <sup>a</sup>	6.10	[M-H] <sup>-</sup>	303.0513	303.0505	C <sub>15</sub> H <sub>11</sub> O <sub>7</sub>	303→285, 177, 125	taxifolin
34 <sup>c</sup>	6.55	[M-H] <sup>-</sup>	449.1099	449.1084	C <sub>21</sub> H <sub>21</sub> O <sub>11</sub>	449→303, 285	neoisoastilbin
35 <sup>c</sup>	6.81	[M-H] <sup>-</sup>	449.1099	449.1084	C <sub>21</sub> H <sub>21</sub> O <sub>11</sub>	449→303, 285	isoastilbin
36	6.86	[M-H] <sup>-</sup>	243.0665	243.0657	C <sub>14</sub> H <sub>11</sub> O <sub>4</sub>	243→225, 201, 199, 175	piceatannol

Table 1. Cont.

Peak No.	$t_R$ (min)	Selected Ion	Observed Mass ( $m/z$ )	Calculate d Mass ( $m/z$ )	Formula	MS/MS Fragmentation Patterns	Identification
37	7.27	[M-H] <sup>-</sup>	433.1149	433.1135	C <sub>21</sub> H <sub>21</sub> O <sub>10</sub>	433→287, 269	neoengeletin
38 <sup>c</sup>	7.43	[M-H] <sup>-</sup>	433.1149	433.1135	C <sub>21</sub> H <sub>21</sub> O <sub>10</sub>	433→287, 269	engeletin
39 <sup>a</sup>	7.49	[M-H] <sup>-</sup>	359.0771	359.0767	C <sub>18</sub> H <sub>15</sub> O <sub>8</sub>	359→341, 291, 239, 197	rosmarinic acid
40	7.53	[M-H] <sup>-</sup>	433.1149	433.1135	C <sub>21</sub> H <sub>21</sub> O <sub>10</sub>	433→287,269	neoisoengeletin
41	8.16	[M-H] <sup>-</sup>	693.2029	693.2031	C <sub>32</sub> H <sub>37</sub> O <sub>17</sub>	693→517, 337	helonioside A
42 <sup>c</sup>	8.20	[M-H] <sup>-</sup>	433.1149	433.1135	C <sub>21</sub> H <sub>21</sub> O <sub>10</sub>	433→287,269	isoengeletin
43 <sup>a</sup>	8.23	[M-H] <sup>-</sup>	451.1038	451.1029	C <sub>24</sub> H <sub>19</sub> O <sub>9</sub>	451→341	cinchonain Ia
44	8.25	[M-H] <sup>-</sup>	693.2029	693.2031	C <sub>32</sub> H <sub>37</sub> O <sub>17</sub>	693→357	securoside A
37	7.27	[M-H] <sup>-</sup>	433.1149	433.1135	C <sub>21</sub> H <sub>21</sub> O <sub>10</sub>	433→287, 269	neoengeletin
45 <sup>a</sup>	8.30	[M-H] <sup>-</sup>	451.1035	451.1029	C <sub>24</sub> H <sub>19</sub> O <sub>9</sub>	451→341	cinchonain Ib
46	8.32	[M-H] <sup>-</sup>	227.0717	227.0708	C <sub>14</sub> H <sub>11</sub> O <sub>3</sub>	227→209,185, 183, 159, 157, 143	resveratrol
47 <sup>a</sup>	8.35	[M-H] <sup>-</sup>	809.2293	809.2293	C <sub>40</sub> H <sub>41</sub> O <sub>18</sub>	809→767, 663, 633	smilaside G
48 <sup>a</sup>	8.36	[M-H] <sup>-</sup>	839.2408	839.2398	C <sub>41</sub> H <sub>43</sub> O <sub>19</sub>	839→797, 693, 663, 517	smilaside J
49 <sup>a</sup>	8.38	[M-H] <sup>-</sup>	869.2502	869.2504	C <sub>42</sub> H <sub>45</sub> O <sub>20</sub>	869→827, 693, 675	smilaside L
50	8.40	[M-H] <sup>-</sup>	777.2248	777.2242	C <sub>36</sub> H <sub>41</sub> O <sub>19</sub>	777→735, 717, 601, 559	(3,6-di-O-feruloyl)-β-D-fructofuranosyl-(3,6-di-O-acetyl)-α-D-glucopyranoside
51	8.42	[M-H] <sup>-</sup>	819.2354	819.2348	C <sub>38</sub> H <sub>43</sub> O <sub>20</sub>	819→777, 643, 601, 513	smilaside C
52	8.44	[M-H] <sup>-</sup>	923.2604	923.2610	C <sub>45</sub> H <sub>47</sub> O <sub>21</sub>	923→881, 863, 747, 601, 483	smilaside E
53	8.45	[M-H] <sup>-</sup>	953.2712	953.2715	C <sub>46</sub> H <sub>49</sub> O <sub>22</sub>	953→911, 777, 735, 717, 289	smilaside B
54 <sup>a</sup>	8.48	[M-H] <sup>-</sup>	271.0614	271.0606	C <sub>15</sub> H <sub>11</sub> O <sub>5</sub>	271→177, 151	naringenin
55	8.52	[M-H] <sup>-</sup>	965.2719	965.2715	C <sub>47</sub> H <sub>49</sub> O <sub>22</sub>	965→923, 905, 789, 747, 483	smilaside D
56	8.55	[M-H] <sup>-</sup>	995.2829	995.2821	C <sub>48</sub> H <sub>51</sub> O <sub>23</sub>	995→953, 819, 777, 513	smilaside A

<sup>a</sup> Compared with reference [3]; <sup>b</sup> Identified as new compound; <sup>c</sup> Compared with reference standards.

**Figure 2.** Proposed fragmentation pathways for compounds **19**, **21**, **25** and **29**.

#### 2.4. Quantitative Analysis

The newly established analytical method was subsequently applied to determine the six compounds of *Smilacis glabrae*. The target compounds were identified based on comparison of retention time and mass information obtained from UHPLC-ESI-MS analysis of the reference standards. Table 3 showed the content determined for each compound. The results indicated that the amount of most components determined was similar in the five different batches.



**Table 2** Summary of calibration curves, linear range, LOQ, repeatability, intra-day and inter-day precisions and recoveries for six analytes analyzed with the LC-MS system

Analyte	Linear Range (µg/mL)	Calibration Curve (n = 7)	r <sup>2</sup>	LOQ (µg/mL)	Repeatability RSD (%)	Intra-day (RSD, %) (n = 6)	Inter-day (RSD, %) (n = 3)	Recoveries (n = 3)				
								Initial (µg)	Spiked (µg)	Detected (µg)	Recoveries (%)	RSD (%)
Neoastilbin	0.82–32.8	$y = 8593.3x + 281942$	0.9993	0.016	2.37	3.19	3.05	3.470	2.628	5.872	96.27	3.51
									3.284	6.890	101.99	3.16
									3.940	7.085	95.63	1.65
Astilbin	3.10–124.1	$y = 8921.6x + 16423$	0.9991	0.062	1.86	1.03	0.76	13.677	9.932	22.963	97.27	2.57
									12.416	25.468	97.60	3.86
									14.900	27.916	97.69	2.66
Neoisostilbin	0.33–13.3	$y = 8299.7x + 165713$	0.9988	0.067	1.91	2.43	2.49	1.517	1.064	2.342	90.77	4.73
									1.340	2.426	91.94	3.39
									1.606	3.205	102.65	3.83
Isoastilbin	1.78–71.2	$y = 8479.3x + 161354$	0.9981	0.018	2.15	1.07	0.79	7.188	5.702	12.485	96.86	1.80
									7.128	14.153	98.86	2.83
									8.555	15.011	95.35	2.30
Engeletin	0.86–34.4	$y = 4620.5x - 107846$	0.9992	0.017	1.77	2.00	3.91	4.110	2.756	7.038	102.51	2.14
									3.444	7.300	96.59	1.49
									4.132	8.271	100.39	2.37
Isoengeletin	0.28–11.1	$y = 4472.8x - 12397$	0.9991	0.011	1.94	2.83	2.86	1.237	0.896	2.152	100.95	2.34
									1.120	2.368	100.37	3.09
									1.134	2.506	97.18	4.50

**Table 3.** Contents of the six compounds in different batches of *Smilacis glabrae*.

Analyte	Content ( $\mu\text{g/g}$ )				
	Batch 1	Batch 2	Batch 3	Batch 4	Batch 5
Neoastilbin	2173.1	2735.9	2356.9	2537.4	2253.7
Astilbin	8548.2	8996.1	9262.1	10,962.2	9988.6
Neoisostilbin	948.3	1046.4	971.2	1188.7	1097.3
Isoastilbin	4493.2	4189.5	4257.9	2800.9	3461.3
Engeletin	2587.2	2494.3	2682.1	1821.6	2047.6
Isoengeletin	771.6	727.6	834.9	594.3	488.5

### 3. Experimental Section

#### 3.1. Chemicals and Materials

HPLC grade acetonitrile and methanol were purchased from Fisher Chemicals (Fairlawn, NJ, USA). Formic acid of HPLC grade was purchased from Sigma Aldrich (St. Louis, MO, USA). Water (18.2 M $\Omega$ ) was from a Milli-Q water system (Millipore, Bedford, MA, USA). Neoastilbin (**30**), astilbin (**31**), neoisoastilbin (**34**), isoastilbin (**35**), engeletin (**38**) and isoengeletin (**42**) were provided by Dr. Lixiong from the Guangdong Provincial Hospital of Chinese Medicine. Three batches of *Smilacis glabrae* originating from Guangdong Province, China were supplied by Kangmei Pharmaceutical Co. Ltd. (Puning, China). Two batches of *Smilacis glabrae* from the Hunan and Guangxi provinces of China were purchased from Er-tian-tang Pharmacy (Guangzhou, China). Voucher samples were deposited in the Laboratory of Chinese Materia Medica Preparation, Second Affiliated Hospital, Guangzhou University of Traditional Chinese Medicine.

#### 3.2. Standard Solutions and Sample Preparation

The standard solution mixture of the six flavonoids was prepared by dissolving the reference substances in methanol to final concentration of 32.8  $\mu\text{g/mL}$  for neoastilbin, 124.1  $\mu\text{g/mL}$  for astilbin, 13.3  $\mu\text{g/mL}$  for neoisoastilbin, 71.2  $\mu\text{g/mL}$  for isoastilbin, 34.4  $\mu\text{g/mL}$  for engeletin and 11.1  $\mu\text{g/mL}$  for isoengeletin, respectively. Then, the standard solution mixture was diluted to 80%, 60%, 40%, 20%, 10%, 5% and 2.5% of the concentration of the original solution. All the standard solutions were stored at 4 °C.

The dried rhizome (0.2 g, 60 mesh) was accurately weighed and ultrasonically extracted by infusion with 25 mL water for 30 min. The extracted solution was centrifuged at 10,000 rpm for 10 min, and then filtered through a 0.22  $\mu\text{m}$  nylon membrane filter prior to injection for UHPLC-MS analysis.

#### 3.3. Analytical System

Chromatographic separation was performed on an Accela™ ultra high pressure liquid chromatography (UHPLC) system (Thermo Fisher Scientific, San Jose, CA, USA) comprising a UHPLC pump, a PDA detector, scanning from 200 to 400 nm, and an autosampler settled to 30 °C. The LC conditions were as follows: column: Agilent Eclipse Plus C18 (100 mm  $\times$  3.0 mm, 1.7  $\mu\text{m}$ ); mobile phase: acetonitrile (A) and water (B) both containing 0.1% (v/v) formic acid; gradient: 0 min,

10: 90; 1 min, 20: 80; 3–6.5 min, 23: 77; 7 min, 80: 20; 9–10 min, 100: 0 (A: B, v/v); flow rate: 0.3 mL/min; injection volume: 10  $\mu$ L.

### 3.4. Qualitative Characteristic of Chemical Constituents

Identification of chemical constituents in *Smilacis glabrae* extract was performed by UHPLC-ESI-MS<sup>n</sup> analysis. MS analysis was performed using an LTQ Orbitrap<sup>XL</sup> hybrid mass spectrometer (Thermo Fisher Scientific), fitted with an ESI source, and operated in negative ion mode, with a mass range of 100–1500 with resolution set at 30000 using the normal scan rate.

The data-dependent MS/MS events were always performed on the most intense ions detected in full scan MS. The MS/MS isolation width was 1 amu, and the normalized collision energy was 35% for all compounds. Nitrogen was used as sheath gas and helium served as the collision gas. The key optimized ESI parameters were as follows: source voltage: 3.8 kV; sheath gas (nitrogen): 50 L/min; auxiliary gas flow: 10 L/min; capillary voltage: –35.0 V; capillary temperature: 300.0 °C; tube lens: –110.0 V. The ion injection time used was 50.0 ms. MS scan functions and HPLC solvent gradients were controlled by the Xcalibur data system (Thermo Fisher Scientific). Data was collected and analyzed with Xcalibur 2.0.7 software (Thermo Fisher Scientific). The Orbitrap mass analyzer was calibrated according to the manufacturer's directions using a mixture of caffeine, methionine-arginine-phenylalanine-alanine-acetate (MRFA), sodium dodecyl sulfate, sodium taurocholate and Ultramark 1621 in an acetonitrile-methanol-water solution containing 1% acetic acid by direct injection at a flow rate of 5  $\mu$ L/min in negative mode before analysis.

### 3.5. Validation of the Quantitative Analysis

A calibration curve was used to determine the calculated concentration of the samples. The calibration curve of each compound was performed with at least six appropriate concentrations. The limit of quantification (LOQ) under the present chromatographic conditions was determined at signal-to-noise ratios (S/N) of 10.

Intra- and inter-day variations were chosen to determine the precision of the developed method. The precision was examined by five repetitive injections in the same day and in three consecutive days, respectively. The relative standard deviation (R.S.D.) was considered as the measure of precision.

The accuracy was evaluated by calculating the mean recoveries of six reference standards from the spiked standard solutions. A known amount of *Smilacis glabrae* sample was spiked with the standard solution at three different concentration levels. The high spiked amount was 1.2 times of the known amount sample, the middle spiked amount was 1.0 times of the known amount sample and the low spiked amount was 0.8 times of the known amount sample. The recovery percentages were calculated using the following equation:  $(\text{total detected amount} - \text{original amount}) / \text{added amount} \times 100\%$ .

## 4. Conclusions

In this study, a total of 56 compounds, including six minor new ones, were simultaneously detected and identified by UHPLC-LTQ-Orbitrap-MS. Based on the qualitative analysis, a rapid method was established for quantitative analysis of six marker components in *Smilacis glabrae* extract. This is the first report on the

comprehensive determination of chemical constituents in *S. glabrae* by UHPLC-LTQ-Orbitrap-MS. The results would provide the chemical support for the further pharmacokinetic studies and for the improvement of quality control of *Smilacis glabrae* and its preparations. The study also suggested that UHPLC-ESI/LTQ-Orbitrap mass spectrometry would be a powerful and reliable analytical tool for the characterization of chemical profile in complex chemical system, such as TCM preparations.

### Supplementary Materials

Supplementary materials can be accessed at: <http://www.mdpi.com/1420-3049/19/7/10427/s1>.

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### Author Contributions

C.-J. Lu and R.-Z. Zhao designed the experiments and provided critical advice on operation of the analytical equipment. S.-D. Chen was responsible for performing most of the experiment and analysis, and preparing the draft of the manuscript.

### Conflicts of Interest

The authors declare no conflict of interest.

### References

1. Chinese Pharmacopoeia Commission. *Pharmacopoeia of People's Republic of China*; China Medical Pharmaceutical Science and Technology Publishing Press: Beijing, China, 2010; Volume 1, p. 15.
2. Xu, S.; Shang, M.Y.; Liu, G.X.; Xu, F.; Wang, X.; Shou, C.C.; Cai, S.Q. Chemical constituents from the rhizomes of *Smilax glabra* and their antimicrobial activity. *Molecules* **2013**, *18*, 5265–5287.
3. Li, X.; Zhang, Y.F.; Yang, L.; Feng, Y.; Deng, Y.H.; Liu, Y.M.; Zeng, X. Chemical profiling of constituents of *Smilacis glabrae* using ultra-high pressure liquid chromatography coupled with LTQ Orbitrap mass spectrometry. *Nat. Prod. Commun.* **2012**, *7*, 181–184.
4. Huang, H.Q.; Cheng, Z.H.; Shi, H.M.; Xin, W.B.; Wang, T.T.Y.; Yu, L.L. Isolation and characterization of two flavonoids, engeletin and astilbin, from the leaves of *Engelhardia roxburghiana* and their potential anti-inflammatory properties. *J. Agric. Food. Chem.* **2011**, *59*, 4562–4569.

5. Haraguchi, H.; Mochida, Y.; Sakai, S.; Masuda, H.; Tamura, Y.; Mizutani, K. Protection against oxidative damage by dihydroflavonols in *Engelhardtia chrysolepis*. *Biosci. Biotechnol. Biochem.* **1996**, *60*, 945–948.
6. Igarashi, K.; Uchida, Y.; Murakami, N.; Mizutani, K.; Masuda, H. Effect of astilbin in tea processed from leaves of *Engelhardtia chrysolepis* on the serum and liver lipid concentrations and on the erythrocyte and liver antioxidative enzyme activities of rats. *Biosci. Biotechnol. Biochem.* **1996**, *60*, 513–515.
7. Nia, R.; Adesanya, S.A.; Okeke, I.N.; Illoh, H.C.; Adesina, S.K. Antibacterial constituents of *Calliandra haematocephala*. *Niger. J. Natur. Prod. Med.* **1999**, *3*, 58–60.
8. Mizutani, K.; Kambara, T.; Masuda, H.; Tamura, Y.; Tanaka, O.; Tokuda, H.; Nishino, H.; Kozuka, M. In *Food Factors for Cancer Prevention*; Springer: Tokyo, Japan, 1995; pp. 607–612.
9. Wirasathien, L.; Pengsuparp, T.; Suttisri, R.; Ueda, H.; Moriyasu, M.; Kawanishi, K. Inhibitors of aldose reductase and advanced glycation end-products formation from the leaves of *Stelechocarpus cauliflorus* R.E. Fr. *Phytomedicine* **2007**, *14*, 546–550.
10. Ruangnoo, S.; Jaiaree, N.; Makchuchit, S.; Panthong, S.; Thongdeeying, P.; Itharat, A. An *in vitro* inhibitory effect on RAW 264.7 cells by anti-inflammatory compounds from *Smilax corbularia* Kunth. *Asian Pac. J. Allergy Immunol.* **2012**, *30*, 268–274.
11. Chen, L.; Yin, Y.; Yi, H.W.; Xu, Q.; Chen, T. Simultaneous quantification of five major bioactive flavonoids in *Rhizoma Smilacis Glabrae* by high-performance liquid chromatography. *J. Pharm. Biomed. Anal.* **2007**, *43*, 1715–1720.

*Sample Availability:* Samples of the compounds **30**, **31**, **34**, **35**, **38** and **42** are available from the authors.

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