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Research Article

Preparative separation of flavonoids in plant extract of *Smilacis Glabrae* Roxb. by high performance counter-current chromatography

Four flavonoids, isoastilbin, astilbin, isoengelitin, and engelitin were isolated and purified simultaneously from *Smilacis Glabrae* Roxb. for the first time by high performance counter-current chromatography using a system consisting of *n*-hexane–*n*-butanol–water (1:2:3, v/v/v). A total of 392.6 mg of astilbin, 71.4 mg of isoastilbin, 47.4 mg of engelitin, and 10.3 mg of isoengelitin were purified from 1.89 g of the ethyl acetate extract of *Smilacis Glabrae* Roxb. in six runs, each at over 94.51% purity as determined by HPLC. The structures of the four compounds were identified by their retention time, the LC-ESI-MSⁿ in the negative ion mode, and confirmed by ¹H-NMR experiments. The characteristic LC-ESI-MS fragmentation patterns of the four compounds were discussed.

Keywords: Flavonoids / High performance counter-current chromatography / LC-ESI-MS / Separation / *Smilacis Glabrae* Roxb.
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1 Introduction

Smilacis Glabrae Roxb. is widely consumed by Chinese as functional food, and has also been extensively used in traditional Chinese medicine for many efficacies, such as antioxidant activity [1], anti-inflammatory [2], immunomodulatory [3], protective against hepatocyte damage [4, 5], and anticancer [6]. The studies indicate that the major bioactive components of *Smilacis Glabrae* are flavonoids, such as astilbin, isoastilbin, engelitin, isoengelitin, and so on [7, 8].

With the above significant bioactivities, there is an increasing demand for the isolation, purification, and identification active flavonoids in the medicine herb. However, such work usually requires the use of multiple chromatographic steps for sample clean up and isolation, which usually results in low recovery rate [8–11]. In contrast, high-speed counter-current chromatography (HSCCC) has become an effective alternative to the conventional chromatographic techniques for the separation of flavonoids from some plant extracts [12–16]. Du et al. successfully purified astilbin and isoastilbin from crude astilbin by HSCCC using hexane–*n*-butanol–water (1:1:2, v/v/v) system [17]. Moreover, compared to the conventional HSCCC, HPCCC (where HPCCC stands for high performance counter-current chromatography) de-

veloped by dynamic extractions, is a new generation coil planet centrifuges with higher fields, enable higher injection and flow rates thus shortening the separation time while maintaining good resolution [18, 19]. Up to know, no report has been published on the separation and purification of four flavonoids simultaneously, isoastilbin, astilbin, isoengelitin, and engelitin from *Smilacis Glabrae* extract using HSCCC or HPCCC. There are a number of reports about determination of flavonoids in *Smilacis Glabrae* by HPLC [7], CE [1], HPLC-MS, and HPLC-¹H-NMR method [20]; however, Wang et al. only gave the molecular weights of flavonoids in *Smilacis Glabrae*.

The present paper describes a very convenient preparative separation and purification method for the four flavonoids, astilbin, isoastilbin, engelitin, and isoengelitin from the crude extract of *Smilacis Glabrae* simultaneously for the first time (Fig. 1). The optimum conditions for the HPCCC separation were obtained, which led to the successful separation of four flavonoids with the purity of each at >94.51%. Structural characterization and analysis of the four flavonoids obtained from the HPCCC were accomplished by use of HPLC coupled with ESI MS and NMR experiment. The retention time, molecular weights, and the characteristic fragment ions of the four flavonoids are presented and discussed in this paper.

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Abbreviations: HPCCC, high performance counter-current chromatography; HSCCC, high-speed counter-current chromatography

2 Experimental

2.1 Chemicals and reagents

Smilacis Glabrae was purchased from Beijing TongRenTang Medicinal Store (Changchun, China); astilbin and engelitin

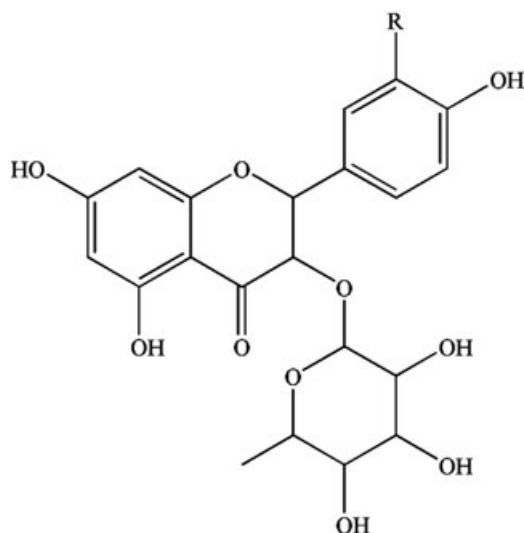


Figure 1. The structures of the compounds identified in *Smilacis Glabrae*: (1) R = OH (2R, 3R), astilbin; (2) R = OH (2R, 3S), isoastilbin; (3) R = H (2R, 3R), engelitin; (4) R = H (2R, 3S), isoengelitin.

were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). All organic solvents used for HPCCC separation were of analytical grade and purchased from Beijing Chemicals (Beijing, China); Acetonitrile and acetic acid were HPLC grade and purchased from Fisher Scientific Company. Water was purified on a Milli-Q water purification system (Millipore, France).

2.2 Sample preparation

Smilacis Glabrae (500 g) was milled to powder and extracted with 2000 mL of 80% aqueous ethanol for ten times at room temperature, each for 12 h. Each time, the extraction mixture was filtered, and the combined filtrates were concentrated to dryness in vacuo at 40°C. The extract was redissolved in 400 mL of water, which was then defatted two times each with 400 mL petroleum. The water layer was then extracted successively five times each with 400 mL of ethyl acetate. The combined ethyl acetate was concentrated to dryness in vacuo at 40°C, which yielded 7.5 g ethyl acetate extract. The ethyl acetate extract was stored at –20°C before HPCCC separation.

2.3 HPLC analysis

An Agilent Technology 1100 Series HPLC system equipped with a quaternary pump, a degasser, a thermostatic autosampler and a photodiode array detector, was used for the analysis of flavonoids in the ethyl acetate extract of *Smilacis Glabrae*, the partition coefficient (*K*) and to monitor the fractions collected from the HPCCC separation. The analysis was carried out with an Agilent Zorbax Extend C₁₈ column (250 × 4.6 mm, 5 μm). The binary mobile phase consisted of acetonitrile (solvent A) and water containing 0.5% acetic acid (solvent B). All solvents were filtered through a 0.45 μm filter prior

to use. The flow-rate was kept constant at 0.5 mL/min for a total run time of 25 min. The system was run with a gradient program: 0–12 min: 80% B to 50% B; 12–15 min, 50% B to 30%B; and 15–18 min, 30% B to 80% B. The sample injection volume was 10 μL. Peaks of interest were monitored at 291 nm by a photodiode array detector.

2.4 HPCCC separation procedure

Preparative HPCCC were carried out in a Spectrum HPCCC (DE, England). The HPCCC system was equipped with two check pumps (Smartline Pump 100, KNAUER, Germany). The revolution speed of the apparatus could be regulated between 0 and 1600 rpm. A 2500 sensitive UV detector (KNAUER, Germany) was set at 291 nm. The 132 mL coil was used for the preparative HPCCC, and the apparatus was set in the reversed phase mode. A sample injection valve with a 10 mL sample loop was used for preparative separation. The temperature was held at 35°C.

A mixture of *n*-hexane–*n*-butanol–water (1:2:3, v/v/v) was used for the HPCCC separation system. The entire coiled column was first filled with the upper phase, which serves as the stationary phase. The rotation rate of the apparatus was set at 1600 rpm, and the lower phase (mobile phase) was pumped into the column at a flow rate of 5 mL/min. A sample (ca. 300 mg) dissolved in 6 mL of the mixture of *n*-hexane–*n*-butanol–water (1:2:3, v/v/v) was loaded into the injection valve after the system reached hydrodynamic equilibrium. This biphasic solvent system was selected based on the partition coefficients (*K*) of the compounds of interest, which were 3.76, 2.09, 8.01, and 5.34 for compounds 1–4, respectively. The *K* value was the ratio of the concentrations in the top and bottom layers of the same compound as determined by HPLC. After the fraction III was eluted completely, the flow rate of the mobile phase was set at 0 mL/min, then the rotation of the apparatus was set at 0 rpm. The stationary phase, which serves as the mobile phase, was pumped into the column at a flow rate of 5 mL/min, then fraction IV was eluted. The effluent from the outlet of the column was continuously monitored by a UV detector at 291 nm and collected into test tubes with a fraction collector set at 2 min for each tube. Fractions from the HPCCC that had the same single peak as determined again by HPLC were combined and freeze-dried. The purities of the four fractions I, II, III, and IV corresponding to peaks 2, 1, 4, and 3 as determined by HPLC were 97.56, 99.89, 94.51, and 99.23%, respectively. The purified compounds were then stored at –20°C before LC-ESI-MSⁿ and NMR analyses.

2.5 LC-ESI-MS for identification

A Thermo Scientific LCQ Fleet mass spectrometer was connected to Thermo Scientific Surveyor LC Plus system via an ESI interface (ThermoFisher, USA). The operating parameters in the negative ion mode were as follows: the sheath gas and auxiliary flow rates were set at 30 and 5 (arbitrary

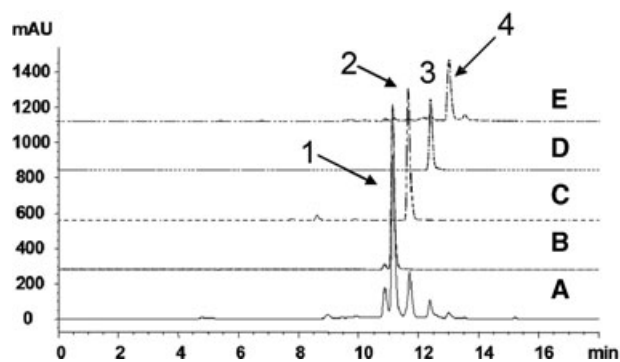


Figure 2. HPLC profiles of ethyl acetate extract of *Smilaxis Glabrae* (A), and purified compounds 1–4 (B–E): astilbin (B), isoastilbin (C), engelitin (D), isoengelitin (E).

Table 1. The K (partition coefficient) values of compounds 1–4 in different solvent systems

Solvent system (volume ration)	1	2	3	4
EtOAc–PE–EtOH–water (3:2:1:5)	0.29	0.12	0.15	0.17
EtOAc–PE–BuOH–water (4:2:1:7)	1.38	0.87	2.15	2.16
EtOAc–PE–BuOH–EtOH–water (3:1:0.2:0.5:4)	1.45	1.17	3.45	3.17
EtOAc–PE–BuOH–EtOH–water (3:1:1:0.5:5)	2.40	1.53	4.91	4.08
Hex–BuOH–water (1:2:3)	3.76	2.09	8.01	5.34

EtOAc: ethyl acetate; PE: petroleum ether; EtOH: ethanol; BuOH: *n*-butanol, hex: *n*-hexane.

unit), respectively. The capillary voltage was set at 34.98 V and its temperature was controlled at 350°C. The entrance lens voltage was fixed at –35 V and the multipole RF amplitude was set at 406 V. The ESI needle voltage was controlled at 4.5 kV. The multipole lens 1 offset was –15 V. The electron multiplier voltage was set at –980 V for ion detection.

2.6 $^1\text{H-NMR}$ for identification

$^1\text{H-NMR}$ spectra were recorded on a Bruker Avance-300 spectrometer. Compounds 1, 2, 3, and 4 were dissolved in CD_3OD .

3 Results and discussion

3.1 HPCCC separation

The ethyl acetate extract of *Smilaxis Glabrae* and the fractions corresponding to each peak isolated by HPCCC were analyzed by HPLC, and the results are given in Fig. 2A–E. Four major peaks 1–4 were separated and detected with retention times at 11.16, 11.62, 12.35, and 12.95 min, respectively.

In our experiment, we selected five series of solvent systems according to the solubility of the target compounds. The K values of these compounds were calculated from the HPLC data as aforementioned and summarized in Table 1. The first system, containing ethyl acetate–petroleum ether–ethanol–

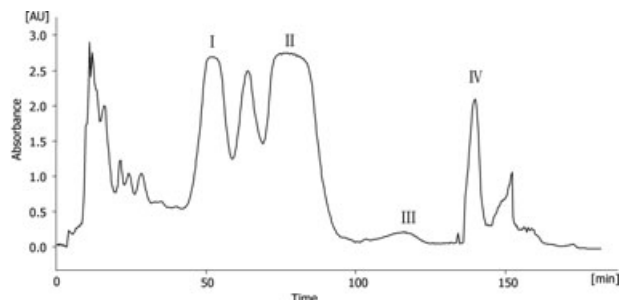
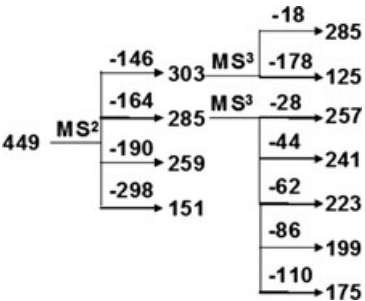
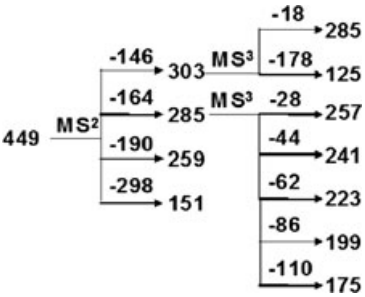
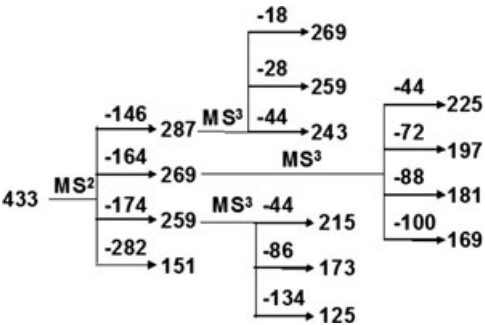
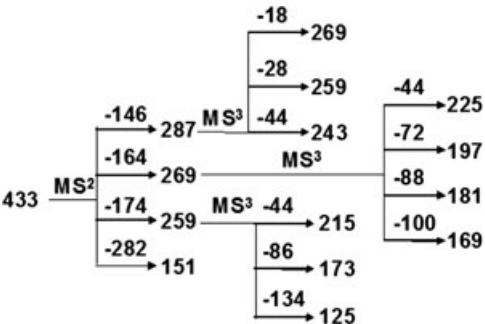


Figure 3. HPCCC chromatograms of the ethyl acetate extract of *Smilaxis Glabrae*: (I) isoastilbin, (II) astilbin, (III) isoengelitin, (IV) engelitin.

water (3:2:1:5, v/v/v/v), had very low K values for compounds 1–4, which would cause the four peaks to be eluted too close to the solvent front, leading to poor performance in separation. When ethanol was replaced by *n*-butanol and the solvents were set at the following ratios: ethyl acetate–petroleum ether–*n*-butanol–water, 4:2:1:7 by volume, had relatively long settling times and very close K values ($K_1 = 1.38$, $K_2 = 0.87$) for compounds 1 and 2, and ($K_1 = 2.15$, $K_2 = 2.16$) compounds 3 and 4, respectively, which caused the peaks 1 and 2, the peaks 3 and 4 to be eluted too close to separate. When the solvents system were set at: ethyl acetate–petroleum ether–*n*-butanol–ethanol–water (3:1:0.2:0.5:4, v/v/v/v/v), although the settling time improved significantly, the separating performance for the compounds in *Smilaxis Glabrae* remained poor due to the very close K values for compounds 1 and 2, and compounds 3 and 4 (Table 1). While when the solvents system were set at: ethyl acetate–petroleum ether–*n*-butanol–ethanol–water (3:1:1:0.5:5, v/v/v/v/v), four compounds were separated with the low resolution as the result of the close K values between compound 1 and 2, compound 3 and 4. By further modifying the solvent system containing *n*-hexane–*n*-butanol–water (1:2:3, v/v/v) turned out to be the best for separation. The K values for compounds 1–4 in this system were at 3.76, 2.09, 8.01, and 5.34, respectively. An ideal K value is around 1 for the desired compound in HSCCC separation [21]. As described above, HPCCC enables higher flow rates, and thus shortens the separation time while maintaining good resolution [18, 19]. In our previous studies [19], although a partition coefficient was 5.19, a good separation of the compounds had been achieved in short time by HPCCC. We found that K values higher than 5.34 were also acceptable for *Smilaxis Glabrae* separation in the experiment. While high K values lead to long separation times, in the case of compound 3 (IV) whose K value was 8.01 (Table 1), it would have taken a long time to be fully eluted from the column. In order to save time and solvent, the compound 3 (IV) was collected by flushing the stationary phase out. When this solvent system was applied to the prepared HPCCC separation with a sample load of 300.6 mg and flow rate at 5 mL/min, as shown in Fig. 3, the fractions I, II, III, and IV were separated within 170 min, and all fractions contained the same compound determined by HPLC were combined, and freeze-dried.

Table 2. LC-ESI-MSⁿ data obtained from the [M-H][−] ions of the four flavonol glycosides in the extract of *Smilacis Glabrae*

No.	Peak identification	<i>t</i> _R (min)	M	[M-H] [−] (<i>m/z</i>)	[2 M-H] [−] (<i>m/z</i>)	MS ² (<i>m/z</i>)	MS ³ (<i>m/z</i>)
1	Astilbin	11.16	450	449	899		
2	Isoastilbin	11.62	450	449	899		
3	Engelitin	12.35	434	433	—		
4	Isoengelitin	12.95	434	433	—		

The fractions I, II, III, and IV were confirmed as compounds 2, 1, 4, and 3 in Fig. 2. The present preparative HPCCC produced a total of 71.4 mg of I, 392.6 mg of II, 10.3 mg of III, 47.4 mg of IV from 1.89 g of the ethyl acetate extract of *Smilacis Glabrae* in six runs. The purities of the four compounds 1–4 as determined by HPLC were 99.89, 97.56, 99.23, and 94.51%, respectively.

3.2 Identification by LC-ESI-MS

The HPLC chromatogram of the ethyl acetate extract of *Smilacis Glabrae* is given in Fig. 2. A good separation was achieved within 18 min. Peaks 1–4 were separated by HPCCC and identified by HPLC-MS. Table 2 lists the information of compounds identified in this paper. As shown in Table 2,

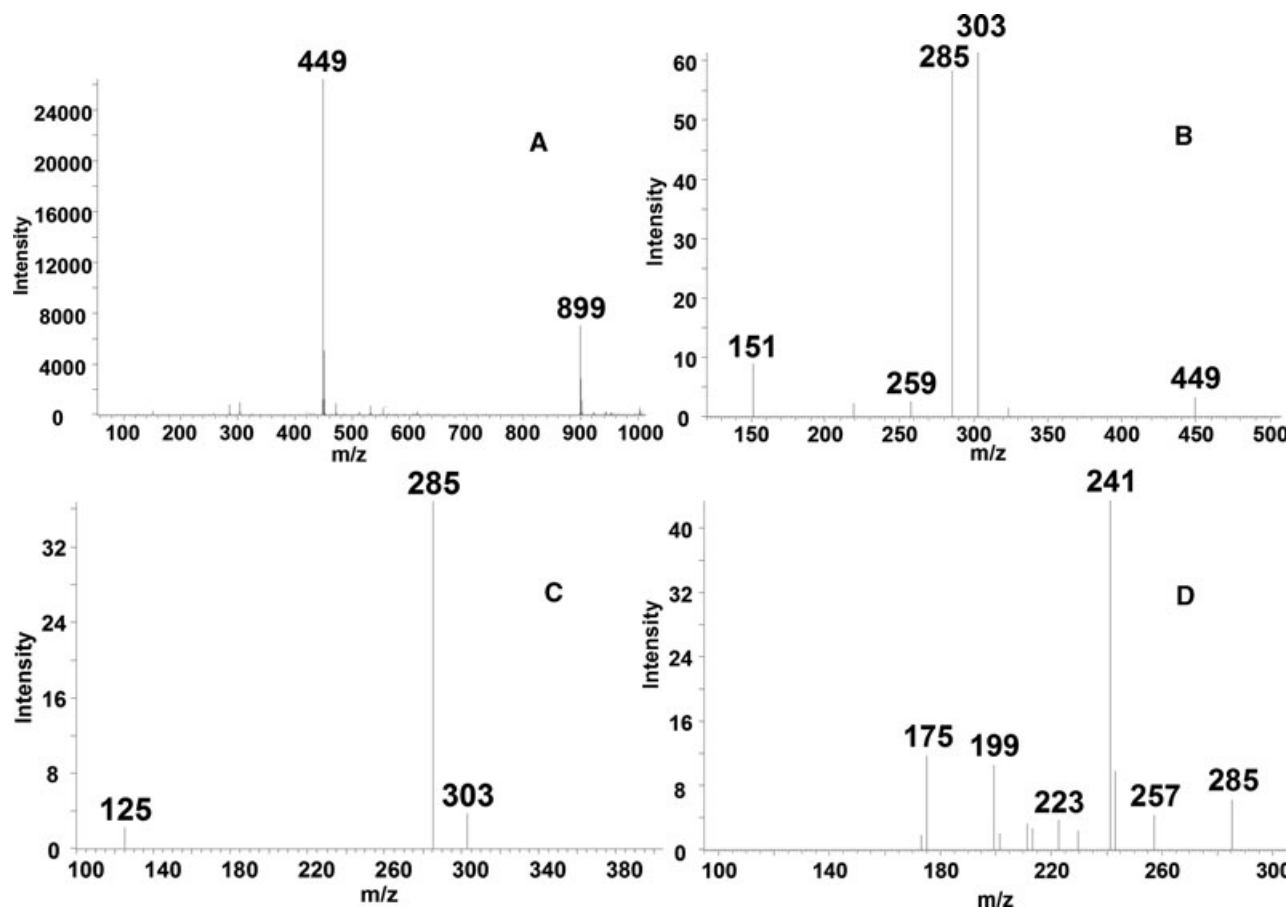


Figure 4. (A) LC-ESI-MS spectrum of m/z 499 of compound 1 in the negative mode; (B) LC-ESI-MS² spectrum of the parent ion m/z 499; (C) LC-ESI-MS³ spectrum of the fragment ion m/z 303 of compound 1; (D) LC-ESI-MS³ spectrum of the fragment ion m/z 285 of compound 1.

peaks 1 and 2 are isomers with molecular weight 450, peaks 3 and 4 are another pair isomer with molecular weight 434. By comparing the retention times with those of the authentic standards, and the molecular weight information with those found in the literatures [8–11], peaks 1 and 3 were tentatively identified as astilbin and engelitin, respectively.

To further investigate the structures of these four compounds, LC-ESI-MSⁿ experiment were attempted and the results are shown in Figs. 4 and 5 and Table 2. The nomenclature of the product ions containing intact A and B rings of flavone skeleton was adapted from the literature [22–24], and the ^{i,j}A[−] and ^{i,j}B[−] represent product ions, the superscripts *i* and *j* indicate the C-ring bonds of flavones skeleton that have been broken. As shown in Table 2, the tandem mass spectrum data display some common features, such as the neutral loss of the rhamnose moiety (146) and a molecular of water (18), the neutral loss of CO (28) and CO₂ (44) from the [M-H][−] ion.

The LC-ESI-MS of peak 1 is shown in Fig. 4A. The [M-H][−] ion at m/z 449 with high abundance and a small dimer [2M-H][−] at m/z 899 were observed in the negative mode, suggesting the molecular weight to be 450, the same as that for astilbin. Further investigation in the LC-MS²

experiment of the m/z 449 ion yielded four fragment ions at m/z 303, 285, 259, and 151 (Fig. 4B). The daughter ion at m/z 303 was produced directly from the parent ion of m/z 449 due to the neutral loss of a rhamnose moiety [M-146-H][−], which indicates that there was one terminal rhamnose residue in the structure. The fragment ion observed at m/z 285 in the LC-MS² spectrum came from the neutral loss of a rhamnose moiety and one molecular of water together from the parent ion. According to the loss of H₂O (18), the existence of two OH groups in ortho positions can be determined. The ion at m/z 259 resulted from the loss of the rhamnose moiety and CO₂ from the parent ion. The most notable ion for peak 1 is m/z 151 ion attributed to a ^{1,3}A[−] fragment, which were formed from the cleavage of C ring. The LC-MS³ spectrum of the ion at m/z 303 exhibited one base peak ion at m/z 285 and one further weak ions at m/z 125 (Fig. 4C, Table 2). The former was formed by losing a molecule of water [M-18-H][−], and the latter is another characteristic ion that attributed to a ^{1,4}A[−] fragment from the cleavage of C ring. The LC-MS³ spectra of m/z 285 gave one major ion at m/z 241 and four further weak ions at m/z 257, 223, 199, and 175 (Fig. 4C, Table 2). The fragment ions m/z 241 was produced by the ion at 285, corresponding to the loss of a CO₂ molecule. The ion

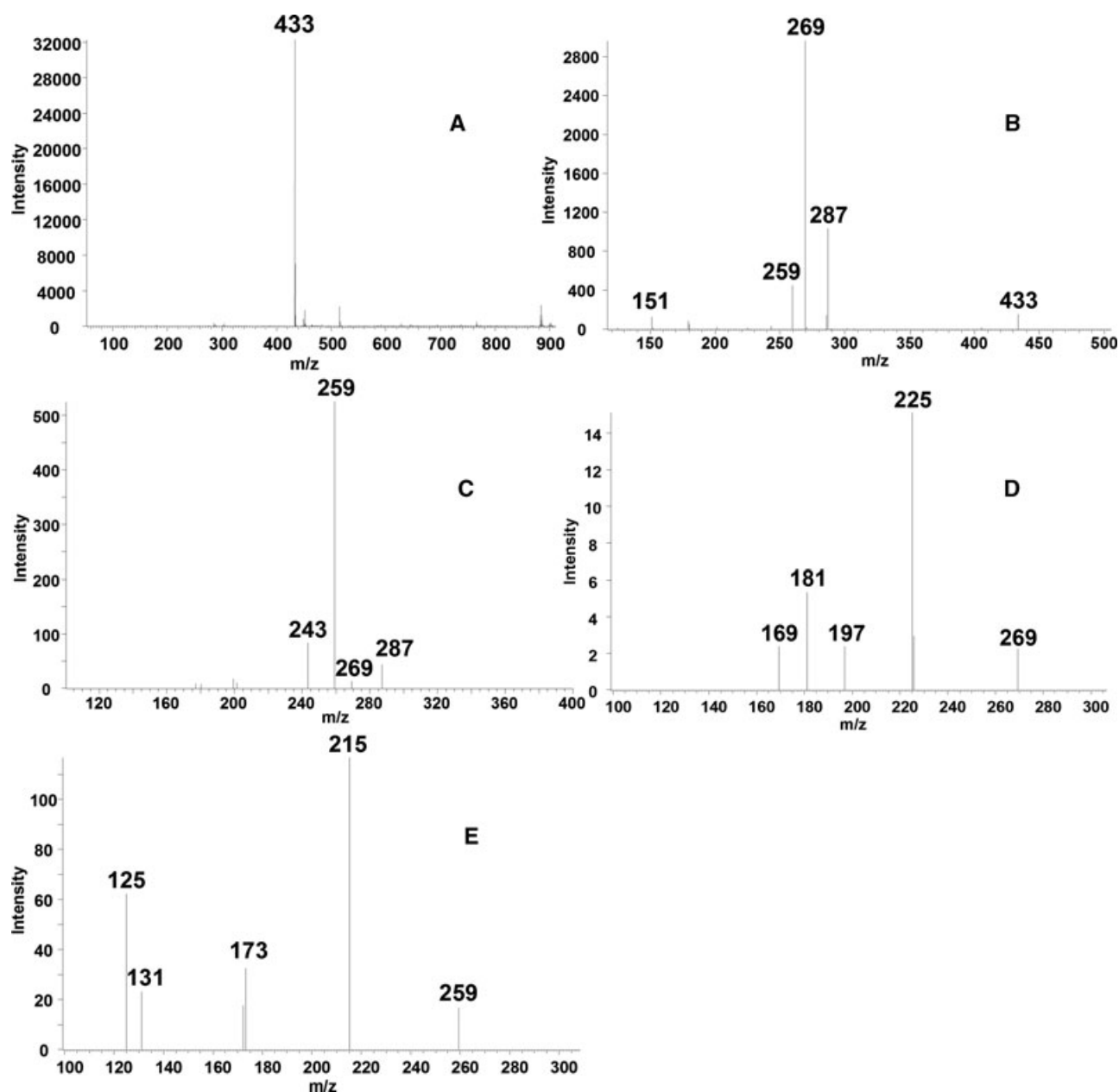
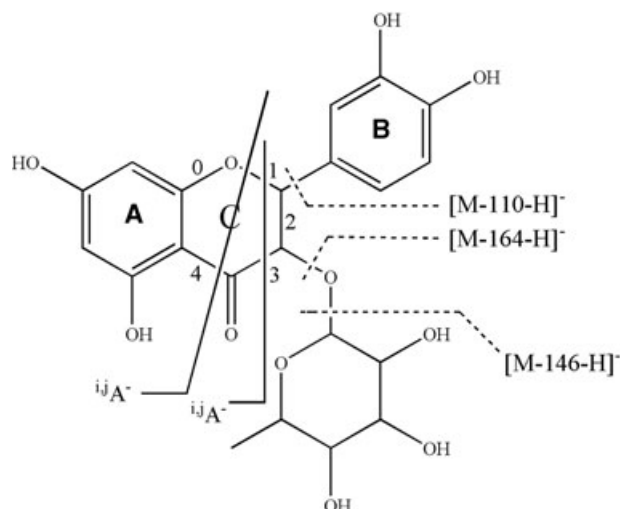


Figure 5. (A) LC-ESI-MS spectrum of m/z 433 of compound 3; (B) LC-ESI-MS² spectrum of the parent ion m/z 433 of compound 3; (C) LC-ESI-MS³ spectrum of the ion m/z 287 of compound 3; (D) LC-ESI-MS³ spectrum of the ion m/z 269 of compound 3; (E) LC-ESI-MS³ spectrum of the ion m/z 259 of compound 3.

at m/z 257 was produced by the ion at m/z 285, corresponding to the loss of a CO molecule. The ion at m/z 223 was produced by the ion at m/z 285, corresponding to the loss of a CO₂ and a molecular of water. The ion at m/z 199 was produced by the ion at 285, corresponding to the loss of a CO₂ and a C₂H₂O moiety. The ion at m/z 175 was produced by the ion at 285, corresponding to the loss of a B ring. Scheme 1 shows the fragments of the peak 1 observed in this study. Based on the LC-ESI-MSⁿ data of the standard, and the matching retention time, peak 1 was confirmed to be astilbin.

As shown in Table 2, peak 2 was an isomer of peak 1, which eluted at 11.62 min. Further experiments in the LC-ESI-MSⁿ of peaks 1 and 2 gave the same two major ions in the negative mode, i.e., the deprotonated molecular ion and its adduction product ions, and shared the same LC-ESI-MS pattern. Although the LC-ESI-MS showed that peaks 1 and 2 were eluted at different times, LC-ESI-MS alone was therefore not enough information for positive identifications of these two isomers. However, the exact structure of this compound needs to be confirmed by NMR spectroscopy.



Scheme 1. Proposed fragmentation pathway for the deprotonated molecular ion $[M-H]^-$ of astilbin.

Peaks 3 and 4, eluted at 12.35 and 12.95 min, respectively, in the HPLC profile, have been studied by LC-ESI-MSⁿ. In full scan negative ion mode MS, peaks 3 and 4 all exhibited as $[M-H]^-$ ion at m/z 433 (Fig. 5 and Table 2), indicating the molecular weights of 434. These two peaks also shared the same LC-MS pattern, and the fragment ions followed a similar pathway as the LC-MS of m/z 449 as discussed above. The LC-MS² spectra of the ion at m/z 433 showed four major ions at m/z 287, 269, 259, 151, and m/z 269 was the base peak. The fragment ion observed at m/z 287 in the LC-MS² spectrum came from the neutral loss of a rhamnose moiety $[M-146-H]^-$ from the parent ion. The m/z 269 was formed by losing rhamnose moiety and a molecule of water together. The ion at m/z 433 lost rhamnose moiety and CO to gave m/z 259. The ion at m/z 151 attributed to a $^{1,3}A^-$ fragment, which was formed from the cleavage of C ring. The fragment ions in the LC-ESI-MS³ of the ion at m/z 287, 269, and 259 followed the same fragmentation pathway as the LC-ESI-MS³ of m/z 449 as discussed above. Comparing the retention time, and the LC-ESI-MSⁿ data of the standard with literatures [20], peak 3 at m/z 433 can be identified as engelitin compared with the retention time and MSⁿ data of standard. Peak 4 followed the same fragmentation pathway as engelitin; however, the exact structure of this compound needs to be confirmed by NMR spectroscopy.

3.3 Identification by ¹H-NMR

Further studies in ¹H-NMR experiments of these four compounds showed that all ¹H-NMR data of compounds 1–4 matched with the reported data for astilbin, isoastilbin, engelitin, and isoengelitin [8–11, 20, 25]. Based on all available data, we therefore conclude that compounds 1, 2, 3, and 4 are astilbin, isoastilbin, engelitin, and isoengelitin, respectively.

Compound 1: Astilbin, ¹H-NMR (300 MHz, CD₃OD) δ 5.71 (s, 1H), 5.58 (s, 2H), 4.66 (s, 1H), 4.65 (s, 1H), 3.85 (s,

1H), 3.81 (s, 2H), 3.35 (s, 4H), 3.01 (s, 3H), 2.80 (s, 2H), 2.42 (d, $J = 9.9$ Hz, 3H), 2.29 (s, 4H), 1.83 (s, 1H), 0.92 (s, 1H), –0.05 (s, 4H).

Compound 2: Isoastilbin, ¹H-NMR (300 MHz, CD₃OD) δ 5.75 (s, 1H), 5.59 (s, 1H), 5.56 (s, 1H), 5.54 (s, 1H), 4.71 (s, 1H), 4.68 (s, 1H), 4.09 (s, 1H), 3.38 (s, 4H), 2.98 (s, 2H), 2.82 (s, 2H), 2.41 (s, 4H), 1.81 (d, $J = 8.6$ Hz, 0H), 0.91 (s, 1H), 0.06 (s, 1H), –0.10 (s, 3H), –0.12 (s, 2H).

Compound 3: Engelitin, ¹H-NMR (300 MHz, CD₃OD) δ 5.75 (s, 1H), 5.59 (s, 1H), 5.56 (s, 1H), 5.54 (s, 1H), 4.71 (s, 1H), 4.68 (s, 1H), 4.09 (s, 1H), 3.38 (s, 4H), 2.98 (s, 2H), 2.82 (s, 2H), 2.41 (s, 4H), 1.81 (d, $J = 8.6$ Hz, 0H), 0.91 (s, 1H), 0.06 (s, 1H), –0.10 (s, 3H), –0.12 (s, 2H).

Compound 4: Isoengelitin, ¹H-NMR (300 MHz, CD₃OD) δ 3.38 (s, 1H), 2.30 (s, 2H), 1.81 (d, $J = 8.5$ Hz, –1H), 0.91 (s, 0H), 0.73 (s, 1H), 0.04 (d, $J = 8.4$ Hz, 0H), –0.34 (d, $J = 5.0$ Hz, 0H).

4 Conclusions

The present paper describes the first successful attempt of using preparative HPLC for the isolation and purification of isoastilbin, astilbin, isoengelitin, and engelitin from the crude extract of *Smilaxis Glabrae* continuously. Structural characterization and analysis of the four flavonoids obtained from the HPLC were accomplished by use of HPLC coupled with ESI MS, and confirmed by ¹H-NMR experiment. The retention time, molecular weights, and the characteristic fragment ions of the four flavonoids are presented and discussed in this paper.

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The authors have declared no conflict of interest.

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