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Fragmentation patterns study of iridoid glycosides in Fructus Gardeniae by HPLC-Q/TOF-MS/MS

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ABSTRACT: Iridoid glycosides (IGs), the major constituents in Fructus Gardeniae, have demonstrated various pharmacological activities, but there is no systematic chemical profile of IGs in Fructus Gardeniae in the published literature until now. Therefore, it is imperative that a rapid and sensitive high-performance liquid chromatography coupled with quadrupole time-of-flight tandem mass spectrometry (HPLC-Q/TOF-MS/MS) method is established for comprehensive characterization of IGs in Fructus Gardeniae. Firstly, the fragmentation patterns of six known IGs were investigated and proposed and further concluded the diagnostic fragment ions and characteristic fragmentation pathways. Then, based on the summarized fragmentation patterns and the known compounds in the literatures, the other IGs in Fructus Gardeniae were identified successively. As a result, a total of 20 IGs were identified, of which three pairs of epimers were structurally characterized and differentiated. More importantly, one compound, the isoshanzhiside methyl ester, was tentatively identified as a new compound. The results of this study demonstrate the superiority of HPLC-MS with a high-resolution mass spectrometer for the rapid and sensitive structural elucidation of the multiple groups of constituents in Fructus Gardeniae. Copyright © 2014 John Wiley & Sons, Ltd.

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Keywords: Fragmentation patterns; iridoid glycosides; Fructus Gardeniae; HPLC-Q/TOF-MS/MS

Introduction

Fructus Gardeniae, from the dried mature fruit of *Gardenia jasminoides* Ellis, is one of the most commonly used herbal remedies in China. It has been used as anti-inflammatory, hepatinica, diuretic and cholagogue herbs using the Chinese name '*Zhizi*' in traditional Chinese medicine for thousands of years and has been recorded in the *Chinese Pharmacopoeia* (Chinese Pharmacopoeia Commission, 2010; Tang and Eisenbrand, 1992). Based on various chemical and pharmacological studies, iridoid glycosides (IGs) in Fructus Gardeniae, which include geniposide, gardenoside, shanzhiside, scandoside methyl ester, geniposidic acid and genipin gentiobioside, have been demonstrated to be the major bioactive ingredients in Fructus Gardeniae (Chen *et al*., 2009; Wang *et al*., 2004; Zhou *et al*., 2010b).

Because of structural complexity and low abundance, the isolation and purification all of IGs from Fructus Gardeniae using the traditional phytochemical methods, especially for trace amounts, are often time-consuming and labor-intensive, and even impossible. For herbal medicine, gaining a systematic chemical profile of traditional Chinese medicine formulae is very important for revealing the material basis of their therapeutic effects (Ye *et al*., 2007; Zheng *et al*., 2008). Recently, the development of high-performance liquid chromatography coupled with quadrupole time-of-flight tandem mass spectrometry (HPLC-Q/TOF-MS/MS), combining the advantages of HPLC with the exact mass measurement offered by TOF mass spectrometry, has demonstrated its merits in measuring the exact mass of the precursor ion and its fragmentation ions and in providing the elemental composition of the corresponding ions, which has made it a powerful analytical tool for the analysis of known compounds and the elucidation of unknown compounds in complex matrices at fairly low levels, even when standard samples are not available (Ling *et al*., 2013; Liu *et al*., 2012b; Zhang *et al*., 2010; Zhou *et al*., 2010a).

Therefore, in the present work, a HPLC-Q/TOF-MS/MS method is established as the effective analytical technique to rapidly screen and characterize IGs in Fructus Gardeniae. Firstly, the fragmentation patterns of six known IGs were investigated by HPLC-Q/TOF-MS/MS technique in negative ion mode and the diagnostic fragment ions and characteristic fragmentation

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Abbreviations used: BPC, base peak chromatogram; CE, collision energy; EIC, extracted ion chromatography; HPLC-Q/TOF-MS/MS, high-performance liquid chromatography coupled with quadrupole time-of-flight tandem mass spectrometry; IG, iridoid glycoside; RDA, Retro Diels–Alder; HRMS, high resolution mass spectrum; 1D and 2D NMR, 1 dimensional and 2 dimensional nuclear magnetic resonance.

pathways further determined. Subsequently, based on the fragmentation behaviors and retention times of reference IGs and known compounds in the literature, the other IGs from crude extract of Fructus Gardeniae were analyzed. To our knowledge, it is the first time that the systematic chemical profile of IGs in Fructus Gardeniae has been carried out, and the results demonstrate the superiority of HPLC-MS with a high-resolution mass spectrometer for separating, characterizing and predicting components in complex natural matrices.

Experimental

Acetonitrile and formic acid of LC-MS grade were purchased from Dikma Technologies Inc. (Dikma, CA, USA). All other analytical chemical reagents of analytical grade were purchased from Sinopharm Chemical Reagent Co. Ltd (Shanghai, China). Deionized water was purified using a Milli-Q system (Millipore, Billerica, MA, USA).

The reference IGs of geniposidic acid, geniposide and shanzhiside methyl ester were purchased from the Nanjing Zelang Medical Technology Co., Ltd (Nanjing, Jiangsu, China). The scandoside methyl ester, genipin gentiobioside, and 6″-*O*-*trans*-sinapoyl genipin gentiobioside were isolated previously from Fructus Gardeniae in our laboratory. Their structures were confirmed by high resolution mass spectrum (HRMS), 1 dimensional and 2 dimensional nuclear magnetic resonance (1D and 2D NMR) comparison of these data with those reported in the literature (Hamerski *et al*., 2003; Ono *et al*., 2005; Yu *et al*., 2009). Their purity was >95% by HPLC analysis.

The crude herbs of Fructus Gardeniae were purchased from Shanghai Kangqiao Traditional Chinese Medicine Co. Ltd (Shanghai, China), and identified by Professor C. G. Huang (Shanghai Institute of Meteria Medica, Chinese Academy of Sciences).

Standard solutions and sample preparation

Certain amounts of geniposidic acid, shanzhiside methyl ester, geniposide, scandoside methyl ester, genipin gentiobioside and 6″-*O*-*trans*-sinapoyl genipin gentiobioside were dissolved with methanol to get six reference compound stock solutions (about 1.0 mg/mL), and were stored at 4°C. Then certain amounts of the six reference compound stock solutions were mixed, and diluted with methanol to obtain the reference compound mixture solution (about 100 μg/mL for each compound).

A 1.0 g aliquot of crushed Fructus Gardeniae was accurately weighed and refluxed with 10 mL 50% methanol for 60 min to get the Fructus Gardeniae sample. The supernatants of the extracts were subjected to HPLC-Q/TOF-MS/MS for analysis after centrifuging at 5000***g*** for 10 min at 4°C.

HPLC conditions

The chromatography analytical procedures were performed on an Agilent 1260 Series (Agilent, Santa Clara, CA, USA) LC system equipped with a binary pump, an online degasser, an auto plate-sampler, a thermostatically controlled column compartment and a diode array detector. The columns were maintained at 35°C. The separation was carried out on an Agilent Poroshell 120 EC-C_{18} column (100 × 2.1 mm, 2.7 μm; Agilent, CA, USA). The binary gradient elution system consisted of 0.1% formic acid (solvent A) and acetonitrile (solvent B) and separation was achieved using the following optimized gradient: 3% B at 0–3 min; 3–15% B at 3–10 min; 15–20% B at 10–15 min; 20–30% B at 15–16 min; 30–45% at 16–20 min; and 45–95% at 20–30 min. The flow rate was 0.35 mL/min and the injection volume was 1.0 μL.

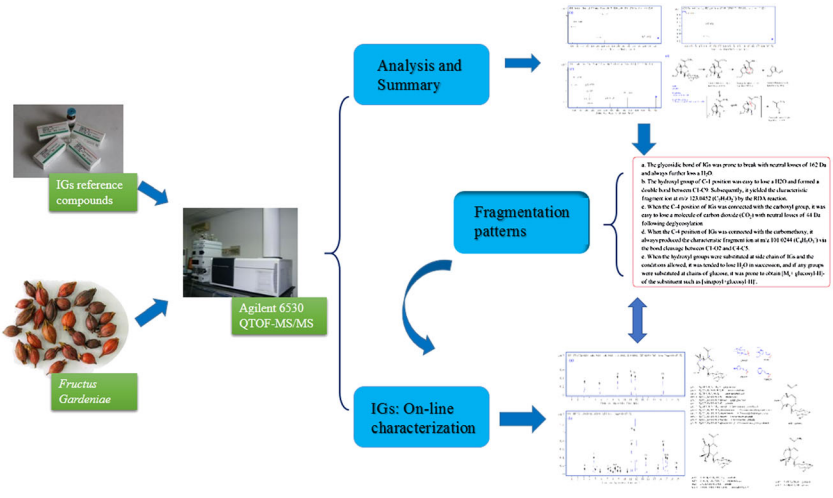
Mass spectrometry conditions

Mass spectrometry was performed using an Agilent 6530 Q-TOF mass spectrometer (Agilent, Santa Clara, CA, USA) equipped with an electrospray ionization interface, and was operated in negative ion mode with parameters set as follows: capillary voltage, 3500 V; fragmentor, 180 V; skimmer, 65 V; OCT 1 RF Vpp, 750 V; pressure of nebulizer, 35 psi; drying gas temperature, 300°C; sheath gas temperature, 350°C. Nitrogen (N_2) was used as sheath and drying gas at a flow rate of 11.0 and 5.0 L/min, respectively. The collision energy (CE) was set at values of 11, 18, 25 and 35 V. Data were collected between 0 and 20 min in centroid mode and the mass range was set at *m/z* 50–1100 using the extended dynamic range. A calibration solution was continuously sprayed in the electrospray ionization source of the Q-TOF system, employing the ions with *m/z* 112.9856 trifluoroacetic acid (TFA) and 1033.9881 (adducts of HP-0921 and TFA) to recalibrate the mass axis ensuring mass accuracy and reproducibility throughout the chromatographic run. The MassHunter Workstation software LC/MS Data Acquisition for 6500 series Q-TOF (version B.05.01) was used to control all the acquisition parameters of the HPLC-Q-TOF-MS/MS system and also to process the obtained data.

Results and discussion

Optimum conditions for HPLC-Q/TOF-MS/MS analysis

Although MS can distinguish overlapping peaks through extracted ion chromatography (EIC), the isomers have to be identified by their retention behaviors if the co-eluting components have the same *m/z* values. Considering the complex constituents of the sample, a Poroshell 120-EC18 column was selected here to get better separation. Its packing has a solid core of 1.7 μm in size with a 0.5 μm thick porous outer layer



Scheme 1. General strategy for identification of Iridoid glycosides (IGs) in the samples of Fructus Gardeniae extract using HPLC-Q/TOF-MS/MS.

and a total particle size of 2.7 μm, providing high efficiency at lower column pressures (Gritti and Guiochon, 2011). In addition, an appropriate concentration of formic acid (0.1%) was introduced into the mobile phase during HPLC to alleviate the peak tailing and increase the ionization effect. In order to obtain the most sensitive ionization method for analytes, positive and negative ion modes were investigated with the same HPLC mobile phase at a flow rate of 0.35 mL/min. As can be seen in the Supporting Information, Fig. S1, more fragmentation ion information on the structures was obtained in the negative ion mode.

For the purpose of optimizing signals and obtaining maximal structural information from the ions of interest, the MS experiments were performed at different fragmentor and CE values. For the analysis of IGs, the fragmentor values of 120, 150 and 180 V were set for the test (Supporting Information, Fig. S2). A more appropriate proportion of precursor ion and product ion was acquired at 180 V. In addition, because of the complexity of IGs in Fructus Gardeniae, four CE values of 11, 18, 25 and 35 V were applied to obtain more characteristic fragment ions and select the most suitable condition for each specific compound for structures identification.

Analytical strategy used in the present work

Traditional Chinese medicines are complicated systems containing various kinds of ingredients. Identification or elucidation of these compounds by HPLC-MS/MS can be performed to a great extent with the assistance of reference standards and previous knowledge about the chemical constituents in these plants. In the present work, firstly, the fragmentation patterns of the known IGs (the reference compounds) were investigated by HPLC-MS/MS technique and the diagnostic fragment ions and characteristic fragmentation pathways were determined. Subsequently, based on the fragmentation

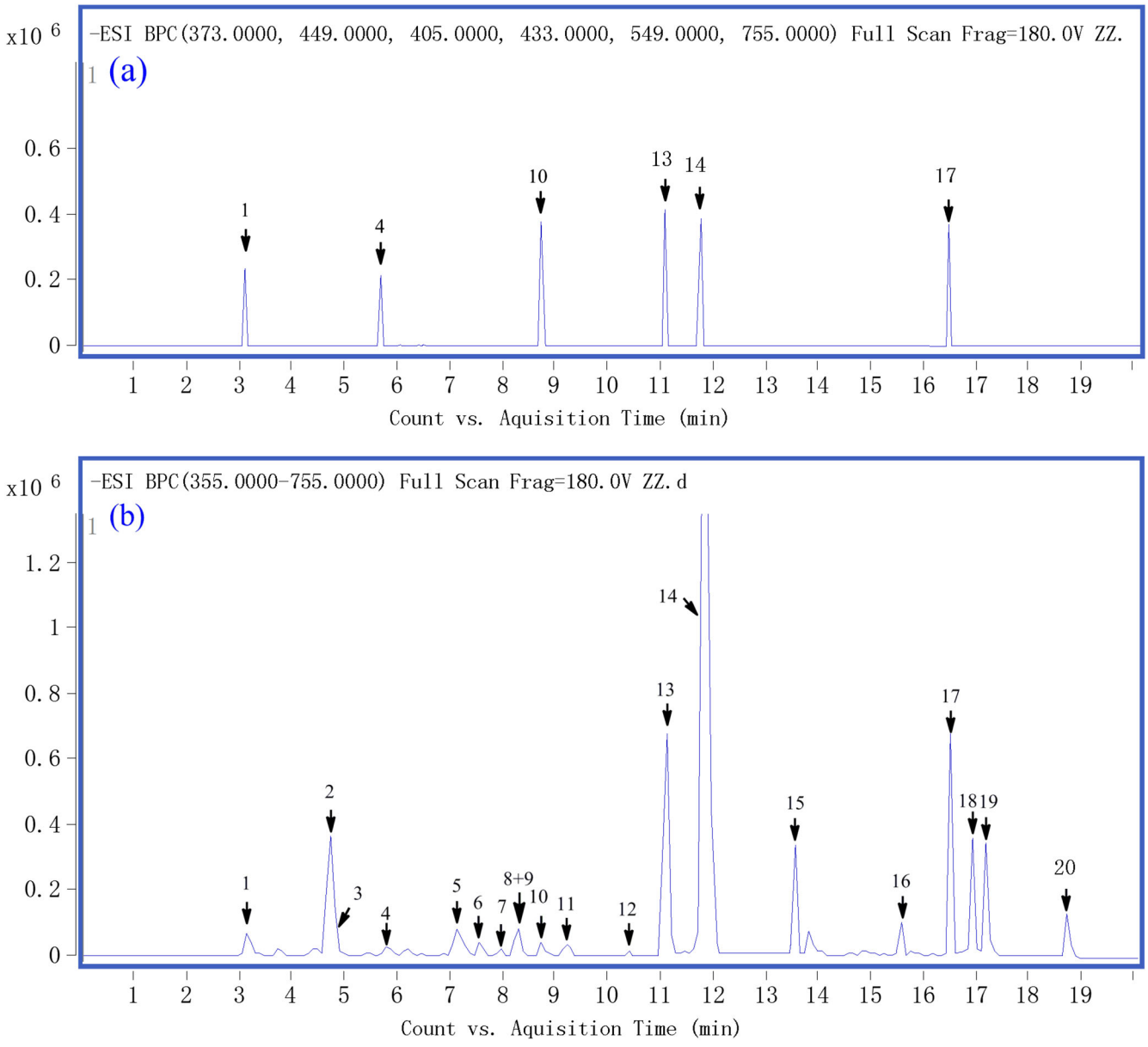


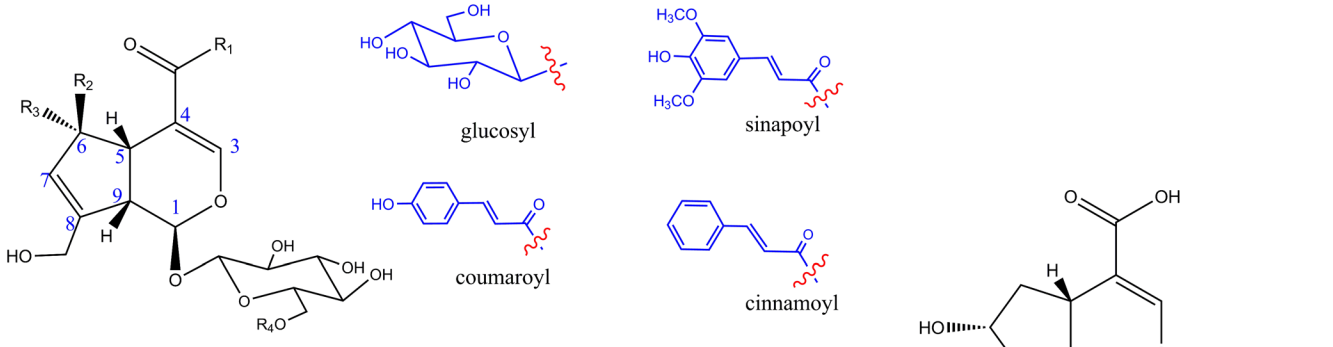
Figure 1. The base peak chromatogram (BPC) in negative ion mode: The six reference IGs (a); the extract of Fructus Gardeniae (b).

behaviors and retention times of reference IGs and known compounds in the literature, the IGs from crude extract of Fructus Gardeniae were analyzed to make a tentative identification. The process of identification used in the present work is presented in Scheme 1.

Fragmentation behavior of the IGs in Fructus Gardeniae

Authentic samples of six IGs were studied by means of QTOF-MS/MS in negative mode. The base peak chromatograms (BPCs) of the reference compounds and the Fructus Gardeniae extract are shown in Fig. 1. Peaks 1, 4, 10, 13, 14 and 17 were unambiguously assigned as geniposidic acid, scandoside methyl ester, shanzhiside methyl ester, genipin gentiobioside, geniposide and 6″-*O*-*trans*-sinapoyl genipin gentiobioside, respectively. Their structures are depicted in Fig. 2 and tandem mass data are shown in Table 1.

Fragmentation of geniposide, genipin gentiobioside and 6″-*O*-*trans*-sinapoyl genipin gentiobioside. In the first-order MS spectra of standard geniposide (peak 14) at 11.784 min, the formic acid-adducted ion $[M-H+HCOOH]^-$ at *m/z* 433.1358 and the deprotonated ion $[M-H]^-$ at *m/z* 387.1285 were observed and gave the molecular formula $C_{17}H_{24}O_{10}$. The $[M-H+HCOOH]^-$ ion was selected as the precursor ion in the MS/MS experiment to give fragmentation information (Fig. 3a). The MS/MS spectra contained an abundant fragment ion at *m/z* 225.0762 ($C_{11}H_{13}O_5^-$), formed by the loss of a glucose unit of $C_6H_{10}O_5$ (162 Da). Furthermore, the fragment ion at *m/z* 207.0660 ($C_{11}H_{11}O_4^-$) was produced by the neutral loss of H_2O, corresponding to the hydroxyl group on the aglycone moiety. In addition, ring cleavages of the aglycone were also observed in the MS/MS spectra of the $[M-H+HCOOH]^-$ ion, which provided valuable information for characterizing the basic structural skeleton. These dominating product ions could be considered as the diagnosed ions of geniposide or its skeleton analogs; those at *m/z* 123.0457



peak 1 R_1=OH, R_2=H, R_3=H, R_4=H geniposidic acid
peak 4 R_1=OCH_3, R_2=OH, R_3=H, R_4=H scandoside methyl ester
peak 6 R_1=OH, R_2=H, R_3=OH, R_4=H deacetylasperulosidic acid
peak 11 R_1=OCH_3, R_2=H, R_3=H, R_4=H geniposidic acid
peak 13 R_1=OCH_3, R_2=H, R_3=H, R_4=glucosyl genipin gentiobioside
peak 14 R_1=OCH_3, R_2=H, R_3=H, R_4=H geniposide
peak 15 R_1=OH, R_2=H, R_3=H, R_4=coumaroyl 6'-*O*-*trans*-coumaroyl geniposidic acid
peak 16 R_1=OCH_3, R_2=H, R_3=H, R_4=glucosyl-coumaroyl 6"-*O*-*trans*-coumaroyl genipin gentiobioside
peak 17 R_1=OCH_3, R_2=H, R_3=H, R_4=glucosyl-sinapoyl 6"-*O*-*trans*-sinapoyl genipin gentiobioside
peak 18 R_1=OCH_3, R_2=H, R_3=H, R_4=sinapoyl 6'-*O*-*trans*-sinapoyl geniposide
peak 19 R_1=OCH_3, R_2=H, R_3=H, R_4=coumaroyl 6'-*O*-*trans*-coumaroyl geniposide
peak 20 R_1=OCH_3, R_2=H, R_3=H, R_4=glucosyl-cinnamoyl 6"-*O*-*trans*-cinnamoyl genipin gentiobioside

peak 3 gardoside

peak 2 R_1=OH, R_2=OH, R_3=H, R_4=CH_3 shanzhiside
peak 7 R_1=OH, R_2=H, R_3=OH, R_4=CH_3 mussaenosidic acid
peak 8 R_1=H, R_2=H, R_3=H, R_4=OH ixoroside
peak 10 R_1=OCH_3, R_2=OH, R_3=OH, R_4=CH_3 shanzhiside methyl ester

peak 5 R_1=OH, R_2=CH_2OH gardenoside
peak 12 R_1=CH_2OH, R_2=OH galioside

Figure 2. The chemical structures of 19 IGs identified in the Fructus Gardeniae extract.

Table 1. Iridoid glycosides identified in an extract of Fructus Gardeniae by HPLC-Q/TOF-MS/MS

Peak no.	Retention time (min)	Collision energy (V)	$[M-H]^-$	Measured (*m/z*)	Theoretical (*m/z*)	Error[a] (ppm)	Error[b] (mDa)	MS/MS fragmentation ions (*m/z*)[c]	Proposed compounds
1	3.134	11	$C_{16}H_{21}O_{10}^-$	373.1146	373.1140	1.61	0.6	123.0453, 149.0604, 211.0612, 167.0712, 193.0502	Geniposidic acid[e]
2	4.736	25	$C_{16}H_{23}O_{11}^-$	391.1250	391.1246	1.02	0.4	149.0616, 167.0712, 185.0823, 123.0449, 141.0549, 229.0713, 211.0615	Shanzhiside
3	4.820	11	$C_{16}H_{21}O_{10}^-$	373.1138	373.1140	−0.54	−0.2	193.0509, 123.0461, 149.0611, 167.0711, 105.0344, 211.0615	Gardoside
4	5.798	11	$C_{18}H_{25}O_{13}^-$ [d]	449.1307	449.1301	1.34	0.6	241.0719, 139.0397, 101.0239, 223.0610, 403.1247	Scandoside methyl ester[e]
5	7.231	11	$C_{18}H_{25}O_{13}^-$ [d]	449.1297	449.1301	−0.89	−0.4	241.0714, 139.0399, 121.0299, 403.1249, 101.0242, 223.0615	Gardenoside
6	7.569	18	$C_{16}H_{21}O_{11}^-$	389.1088	389.1089	−0.26	−0.1	165.0551, 139.0395, 183.0667, 209.0450, 227.0566,	Deacetylasperulosidic acid
7	7.990	18	$C_{16}H_{23}O_{10}^-$	375.1303	375.1297	1.6	0.6	107.0510, 151.0770, 125.0605, 169.0877, 213.0764, 195.0660	Mussaenosidic acid
8	8.243	11	$C_{17}H_{25}O_{11}^-$ [d]	405.1411	405.1402	2.22	0.9	197.0817, 359.1343, 125.0610, 107.0496	Ixoroside
9	8.328	11	$C_{17}H_{25}O_{11}^-$	405.1406	405.1402	0.99	0.4	179.0558, 225.0769, 141.0564, 123.0447, 101.0238, 119.0348	Isoshanzhiside methyl ester[f]
10	8.749	11	$C_{17}H_{25}O_{11}^-$	405.1395	405.1402	−1.73	−0.7	101.0239, 141.0555, 179.0566, 123.0447, 225.0766, 119.0349	Shanzhiside methyl ester[e]
11	9.086	11	$C_{18}H_{25}O_{13}^-$ [d]	449.1305	449.1301	0.89	0.4	241.0710, 139.0394, 101.0248, 223.0617, 403.1246	Deacetylasperulosidic acid methyl ester
12	10.267	11	$C_{18}H_{25}O_{13}^-$ [d]	449.1307	449.1301	1.34	0.6	241.0723, 121.0299, 223.0611, 139.0397, 403.1245, 101.0239	Galioside
13	11.110	18	$C_{23}H_{33}O_{15}^-$	549.1831	549.1825	1.09	0.6	123.0451, 101.0247, 225.0761, 207.0665	Genipin Gentiobioside[e]
14	11.784	11	$C_{18}H_{25}O_{12}^-$ [d]	433.1358	433.1351	1.62	0.7	225.0762, 123.0457, 101.0244, 207.0660, 387.1285	Geniposide[e]
15	13.553	18	$C_{25}H_{27}O_{12}^-$	519.1511	519.1508	0.58	0.3	145.0299, 163.0397, 123.0445, 149.0601, 167.0716, 193.0504, 211.0606, 355.1029, 307.0824	6′-*O*-*trans*-Coumaroyl geniposidic acid
16	15.746	35	$C_{32}H_{39}O_{17}^-$	695.2199	695.2193	0.86	0.6	123.0448, 101.0247, 145.1293, 163.0406, 225.0767, 207.0658, 369.1188, 469.1344, 307.0817	6″-*O*-*trans*-Coumaroyl genipin gentiobioside
17	16.613	35	$C_{34}H_{43}O_{19}^-$	755.2412	755.2404	1.06	0.8	123.0459, 101.0240, 225.0753, 223.0608, 529.1567, 205.0502, 207.0658, 385.1133	6″-*O*-*trans*-Sinapoyl genipin gentiobioside[e]
18	16.952	25	$C_{28}H_{33}O_{14}^-$	593.1878	593.1876	0.34	0.2	205.0495, 123.0446, 101.0245, 223.0612, 367.1033, 225.0765, 207.0656	6′-*O*-*trans*-Sinapoyl geniposide
19	17.168	25	$C_{26}H_{29}O_{12}^-$	533.1659	533.1664	−0.94	−0.5	163.0406, 225.0767, 123.0455, 101.0241, 325.0926, 307.0819, 145.0287, 207.0669	6′-*O*-*trans*-Coumaroyl geniposide
20	18.821	11	$C_{33}H_{41}O_{18}^-$ [d]	725.2302	725.2298	0.55	0.4	679.2238, 225.0768, 123.0450, 147.0448, 207.0666, 101.0239, 531.1722, 453.1399	6″-*O*-*trans*-Cinnamoyl genipin gentiobioside

[a]Differences between the measured and theoretical values.
[b]Milli-Dalton, differences between the measured and theoretical values.
[c]Sequencing according to the abundance
[d]Gave $[M-H+HCOOH]^-$ as quasi-molecular ions
[e]Identified with the reference compounds.
[f]Identified as the new compound

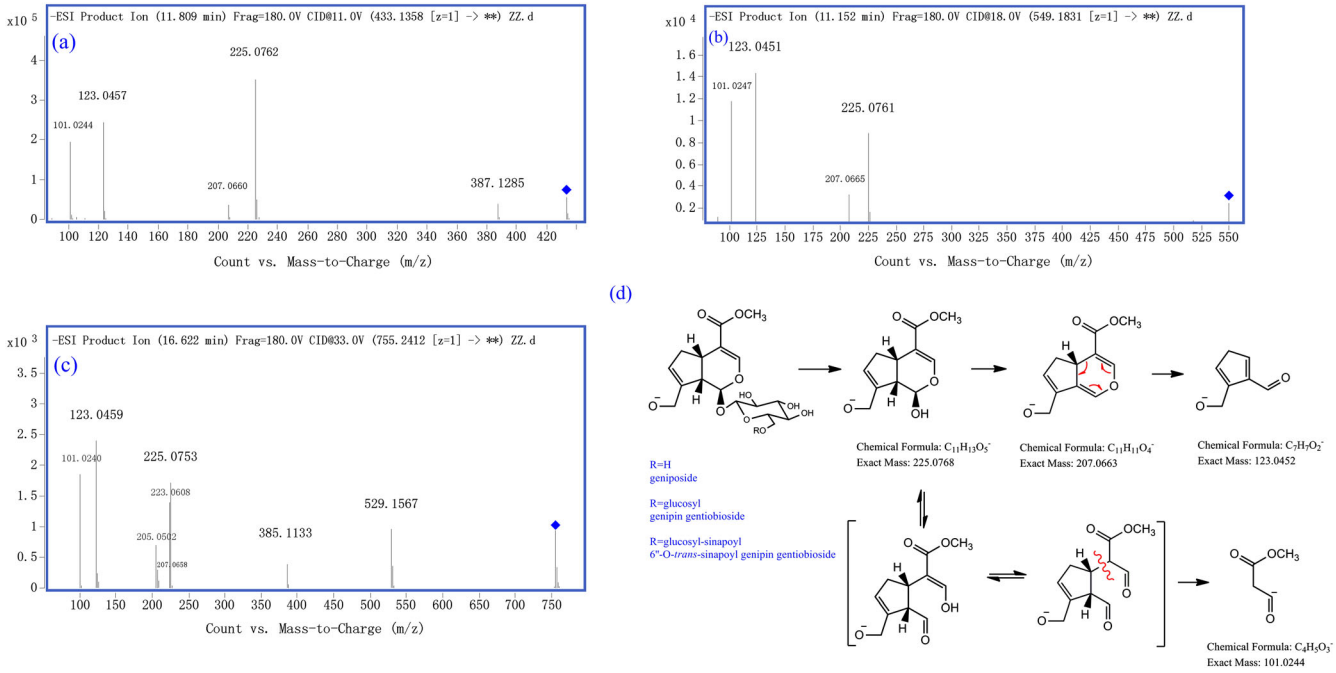


Figure 3. The MS/MS spectra of the $[M-H+HCOOH]^-$ ion at *m/z* 433.1358 for geniposide (a), the $[M-H]^-$ ion at *m/z* 549.1831 for genipin gentiobioside (b), the $[M-H]^-$ ion at *m/z* 755.2412 for 6″-*O*-*trans*-sinapoyl genipin gentiobioside (c) and the proposed fragmentation pathways of geniposide analog (d).

($C_7H_7O_2^-$) and 101.0244 ($C_4H_5O_3^-$) were characteristic fragments produced by the Retro Diels–Alder (RDA) reaction and the bond cleavage between C_1–O_2 and C_4–C_5, respectively. A possible fragmentation pathway of geniposide is shown in (Fig. 3d). Similarly, the other peak of genipin gentiobioside (peak 13) at 11.110 min gave an $[M-H]^-$ ion at *m/z* 549.1831 with the formula $C_{23}H_{34}O_{15}$, and showed almost identical MS/MS spectra to geniposide (Fig. 3b).

As for the peak 17 of 6″-*O*-*trans*-sinapoyl genipin gentiobioside (Fig. 3c), the ion at *m/z* 755.2412 was assigned to the $[M-H]^-$ ion, which was in accordance with the formula $C_{34}H_{44}O_{19}$ based on its accurate mass. Additionally, fragment ions at *m/z* 225.0753, 207.0658, 123.0459 and 101.0240 were obtained, which indicated that its basic skeleton was identical to geniposide. The ions at *m/z* 223.0608 ($C_{11}H_{11}O_5^-$) and 205.0502 ($C_{11}H_9O_4^-$) could be assigned as $[sinapoyl\text{-}H]^-$ and $[sinapoyl\text{-}H\text{-}H_2O]^-$, suggesting the presence of a sinapoyl group in the molecule. Moreover, the ions at *m/z* 529.1567 ($C_{23}H_{29}O_{14}^-$) and 385.1133 ($C_{17}H_{21}O_{10}^-$) composed of $[sinapoyl + gentiobiosyl - H - H_2O]^-$ and $[sinapoyl + glucosyl\text{-}H]^-$, respectively, could also be considered as the diagnostic fragmentation ions.

Fragmentation of geniposidic acid. For standard geniposidic acid, the $[M-H]^-$ at *m/z* 373.1146 ($C_{16}H_{21}O_{10}^-$) gave an ion at *m/z* 211.0612 ($C_{10}H_{11}O_5^-$) by the characteristic loss of a glucose unit, with several characteristic product ions at *m/z* 193.0502 (very low abundance), 167.0712, 149.0604 and 123.0453 (Fig. 4a). The product ion at *m/z* 167.0712 ($C_9H_{11}O_3^-$) formed by the neutral loss of CO_2 from the ion at *m/z* 211, as well as the product ion $C_9H_9O_2^-$ at *m/z* 149.0604 (*m/z* 193.0502 → 149.0604), owing to the presence of a carboxyl group at the C-4 position, which is the most significant difference from geniposide. Furthermore, the characteristic fragment ion at *m/z* 123.0453 ($C_7H_7O_2^-$) was also observed owing to the ring cleavages of the aglycone via the RDA reaction, as described previously.

Fragmentation of scandoside methyl ester and shanzhiside methyl ester. As for the reference compound of scandoside methyl ester, the typical $[M-H+HCOOH]^-$ ion at *m/z* 449.1307 and $[M-H]^-$ ion at *m/z* 403.1247 suggested that its molecular formula was $C_{17}H_{24}O_{11}$. By loss of a glucose unit, it produced the fragment ion at *m/z* 241.0719 ($C_{11}H_{13}O_6^-$). As it contained a 6-hydroxy residue, the fragment ions at *m/z* 139.0397 ($C_7H_7O_3^-$) and 101.0239 ($C_4H_5O_3^-$) were yielded by the skeleton characteristic fragment ions by the RDA reaction and bond cleavage between C1–O2 and C4–C5, respectively. The related mass spectra are shown in Fig. 4(b).

In the MS/MS spectra of shanzhiside methyl ester (Fig. 4c), the deprotonated molecule at *m/z* 405.1395 ($C_{17}H_{25}O_{11}^-$) yielded an ion at *m/z* 225.0766 ($C_{11}H_{13}O_5^-$), which corresponded to the successive losses of a molecular glucose and a water (180 Da). The predominant fragment ions at *m/z* 141.0555 ($C_7H_9O_3^-$) and 123.0447 ($C_7H_7O_2^-$) were obtained owing to the RDA reaction and further loss of a water. In addition, the skeleton characteristic fragment ions at *m/z* 101.0239 ($C_4H_5O_3^-$) were also acquired via the bond cleavage between C1–O2 and C4–C5, which indicated the existence of 4-carbomethoxy in the basic skeleton as well. The other ions at *m/z* 179.0566, 119.0349 and 89.0239 were assigned as the typical fragmentation ions of the glucose.

Brief summary of fragmentation patterns of IGs

The fragmentation patterns of IGs were revealed to be as follows based on the above structural identification of the standard compounds:

(1) The glycosidic bond of IGs was prone to break with neutral losses of 162 Da and always further lost an H_2O.
(2) The hydroxyl group of C-1 position was easy to lose a H_2O and formed a double bond between C1 and C9. Subsequently, it yielded the characteristic fragment ion at *m/z* 123.0452 ($C_7H_7O_2^-$) by the RDA reaction.

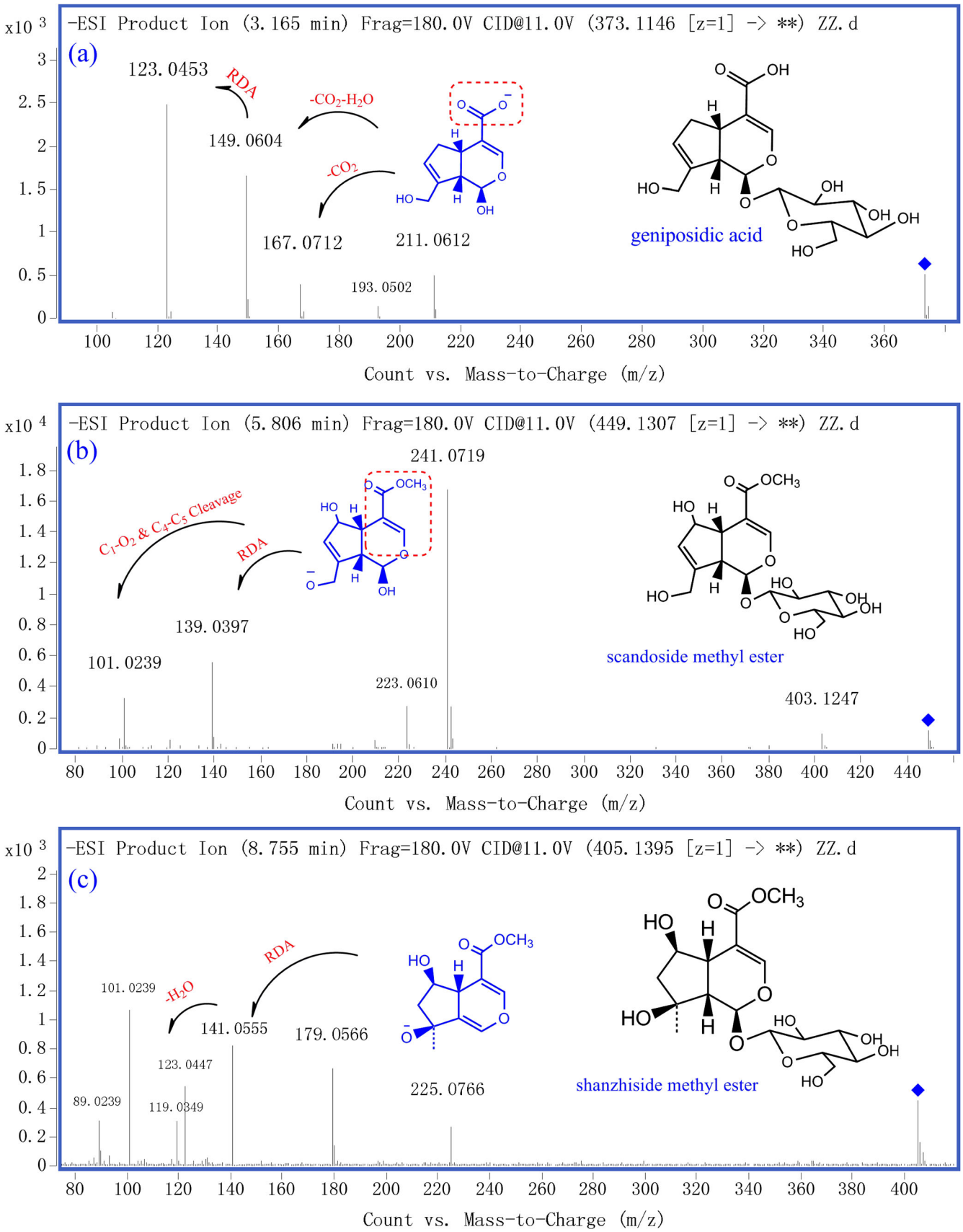


Figure 4. The MS/MS spectra of the $[M-H]^-$ ion at *m/z* 373.1146 for geniposidic acid (a), the $[M-H+HCOOH]^-$ ion at *m/z* 449.1307 for scandoside methyl ester and the $[M-H]^-$ ion at *m/z* 405.1395 for shanzhiside methyl ester (c).

(3) When the C-4 position of IGs was connected with the carboxyl group, it was easy to lose a molecule of carbon dioxide (CO_2) with neutral losses of 44 Da following deglycosylation
(4) When the C-4 position of IGs was connected TO the carbomethoxy, it always produced the characteristic fragment ion at *m/z* 101.0244 ($C_4H_5O_3^-$) via the bond cleavage between C_1–O_2 and C_4–C_5.
(5) When the hydroxyl groups were substituted at side chain of IGs and the conditions allowed, it tended to lose H_2O in succession, and if any groups were substituted at chains of glucose, it was prone to obtain $[M_s + \text{glucosyl} - H]^-$ FROM the substituent, such as $[\text{sinapoyl} + \text{glucosyl-H}]^-$.

Online characterization of IGs in an extract of Fructus Gardeniae

As shown in Fig. 1b, from the HPLC-Q/TOF-MS/MS profile of the Fructus Gardeniae extract, a total of 20 IGs were found and characterized in negative ion mode. The accurate masses and elemental compositions of the quasi-molecular ions and main product ions of the standards are shown in Table 1 and the structures of all IGs are shown in Fig. 2; the other 14 peaks except the above six standard compounds were tentatively identified as discussed below.

Identification of peak 3. Peak 3 afforded the $[M - H]^-$ ion at *m/z* 373.1138 ($C_{16}H_{21}O_{10}^-$) and the chemical formula was in line with geniposidic acid. Thus, peak 3 was considered as the isomer of geniposidic acid. The EIC of *m/z* 373 is shown in Fig. 5(a) and demonstrated that there were only two compounds with the molecular weight of 374 in the Fructus Gardeniae. Moreover, the MS/MS spectra of peak 3 (Fig. 5b) showed prominent ions at *m/z* 211.0615 ($C_{10}H_{11}O_5^-$), 193.0509 ($C_{10}H_9O_4^-$), 149.0611 ($C_9H_9O_2^-$), 167.0711 ($C_9H_{11}O_3^-$) and 123.0461 ($C_7H_7O_2^-$). These ions also represented the same fragmentation pathway as geniposidic acid. As the previous literature reported (Carmona *et al.*, 2006; Quirantes-Pine *et al.*, 2009), peak 2 could be tentatively identified as gardoside. Furthermore, the abundance of ion at *m/z* 193 of peak 3 was higher than that of geniposidic acid and the existence of fragment ion at *m/z* 105.0344($C_7H_5O^-$) indicated that the hydroxyl at C-7 position of gardoside was easier to lose while the hydroxyl of geniposidic acid was at the

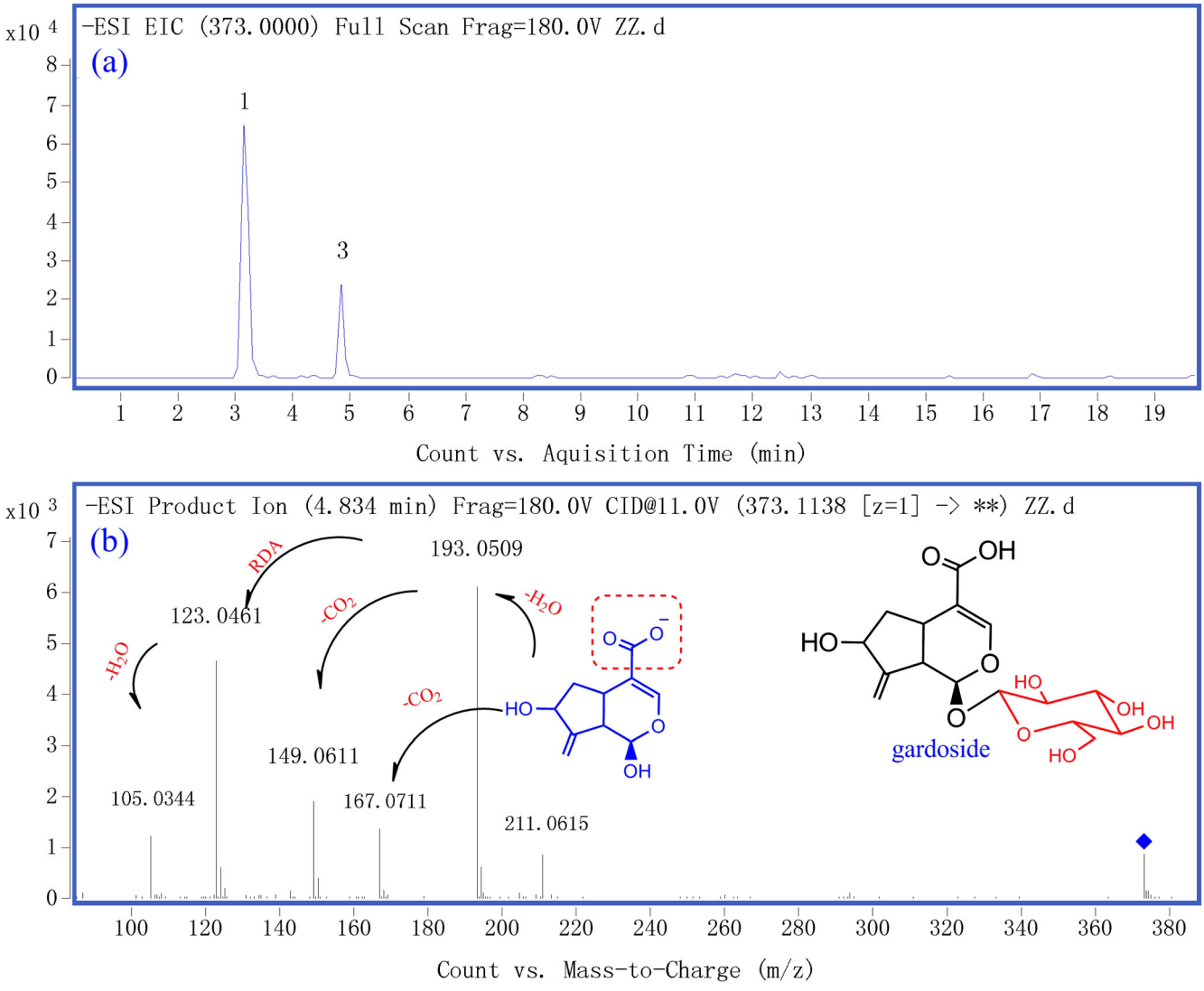


Figure 5. The extracted ion chromatography (EIC) of *m/z* 373 in negative ion mode (a) and the MS/MS spectra of the $[M - H]^-$ ion at *m/z* 373.1138 for gardoside (b).

C-11 position. The fragmentation pathways obtained above further confirmed our deductions and peak 3 could be identified as gardoside.

Identification of peaks 2, 6, 7 and 8. Peak 2 displayed the $[M-H]^-$ ion at *m/z* 391.1250, suggesting a molecular formula of $C_{16}H_{24}O_{11}$. In addition, MS/MS spectra of peak 2 also manifested characteristic ions at *m/z* 229.0713 ($C_{10}H_{13}O_6^-$), 185.0823 ($C_9H_{13}O_4^-$), 167.0712 ($C_9H_{11}O_3^-$) and 149.0616 ($C_9H_9O_2^-$) resulting from the successive losses of glucose (162 Da), CO_2 (44 Da), H_2O (18 Da) and H_2O (18 Da), respectively. Moreover, the acquisition of ion at *m/z* 123.0449 ($C_7H_7O_2^-$) also indicated the consistency of basic skeleton. The above fragments were consistent with the known shanzhiside (Fu *et al.*, 2014), which allowed the identification of peak 2. The related MS/MS spectra are shown in the Supporting Information, Fig. S3.

Peak 6 exhibited an $[M-H]^-$ ion at *m/z* 389.1088 ($C_{16}H_{21}O_{11}^-$) in the MS/MS spectra and further produced the typical fragment ions at *m/z* 227.0566 ($C_{10}H_{11}O_6^-$) and 209.0450 ($C_{10}H_9O_5^-$) by losses of a glucose and [glucose + H_2O], respectively. In addition, the product ions at *m/z* 183.0662 ($C_9H_{11}O_4^-$) and 165.0551 ($C_9H_9O_3^-$) corresponded to the loss of CO_2 (44 Da) from *m/z* 227.0566 and 209.0450, respectively. Thus, it is proposed that there is a carboxyl group in the structure of peak 6. Moreover, the characteristic ion of peak 6 at *m/z* 139.0392 ($C_7H_7O_3$) was produced according to the same path as scandoside methyl ester, as described previously. Based on the fragmentation pathways obtained above, peak 6 corresponds to the known deacetylasperulosidic acid (Liu *et al.*, 2012a; Zhou *et al.*, 2010b). The related MS/MS spectra are shown in the Supporting Information, Fig. S4.

Peak 7 in the MS/MS spectrum yielded an $[M-H]^-$ ion at *m/z* 375.1303 and matched up with the chemical formula of $C_{16}H_{24}O_{10}$. The product ion at *m/z* 213.0764 ($C_{10}H_{13}O_5^-$) corresponded to the neutral loss of a glucose unit and it further gave an ion at *m/z* 195.0660 ($C_{10}H_{11}O_4^-$) by loss of an H_2O. The fragment ions at *m/z* 169.0877 ($C_9H_{13}O_3^-$) and 151.0770 ($C_9H_{11}O_2^-$) were produced by the neutral loss of CO_2 and H_2O, respectively, suggesting the existence of carboxyl group and hydroxyl. Additionally, the fragment ion at *m/z* 125.0605 ($C_7H_9O_2^-$) produced via the RDA reaction was also observed in the MS/MS spectra and further afforded the ion at *m/z* 107.0510 ($C_7H_7O^-$) by loss of a H_2O with dehydration. These fragmentation pathways represented a similar pathway to mussaenosidic acid. Thus, peak 7 could be tentatively identified as mussaenosidic acid (Fu *et al.*, 2014). The related MS/MS spectra are shown in the Supporting Information, Fig. S5.

Identification of peaks 5, 11 and 12. Peaks 5, 11 and 12 as the isomers exhibited the same $[M-H+HCOOH]^-$ ions at *m/z* 449.1301 and $[M-H]^-$ ions at *m/z* 403.1246, which is in line with a molecular formula of $C_{17}H_{24}O_{11}$ and consistent with the standards of scandoside methyl ester. The EIC of *m/z* 449 is shown in Fig. 6(a) and peak 4 with retention time of 5.798 min was identified as scandoside methyl ester by comparison with the reference compounds. The MS/MS spectra data of other three peaks are displayed in Table 1. Based on these fragmentation behaviors, peaks 5, 11 and 12 could be tentatively identified as gardenoside, deacetylasperulosidic acid methyl ester, and galioside, respectively. Firstly, the main fragment ions of peak 11 (Fig. 6c) were almost identical to the reference of scandoside methyl ester. Thus, peak 11 was an epimer of peak 4 and tentatively identified as deacetylasperulosidic acid methyl ester (Li *et al.*, 2008; Liu *et al.*, 2012a). In addition, the MS/MS spectra of peaks 5 (Fig. 6b) and 12 (Fig. 6d) were identical and suggested that they are also epimers with the same basic skeletons. Furthermore, the special product ion at *m/z* 121.0295 ($C_7H_5O_2^-$) was observed as the main product ion in the spectra of peaks

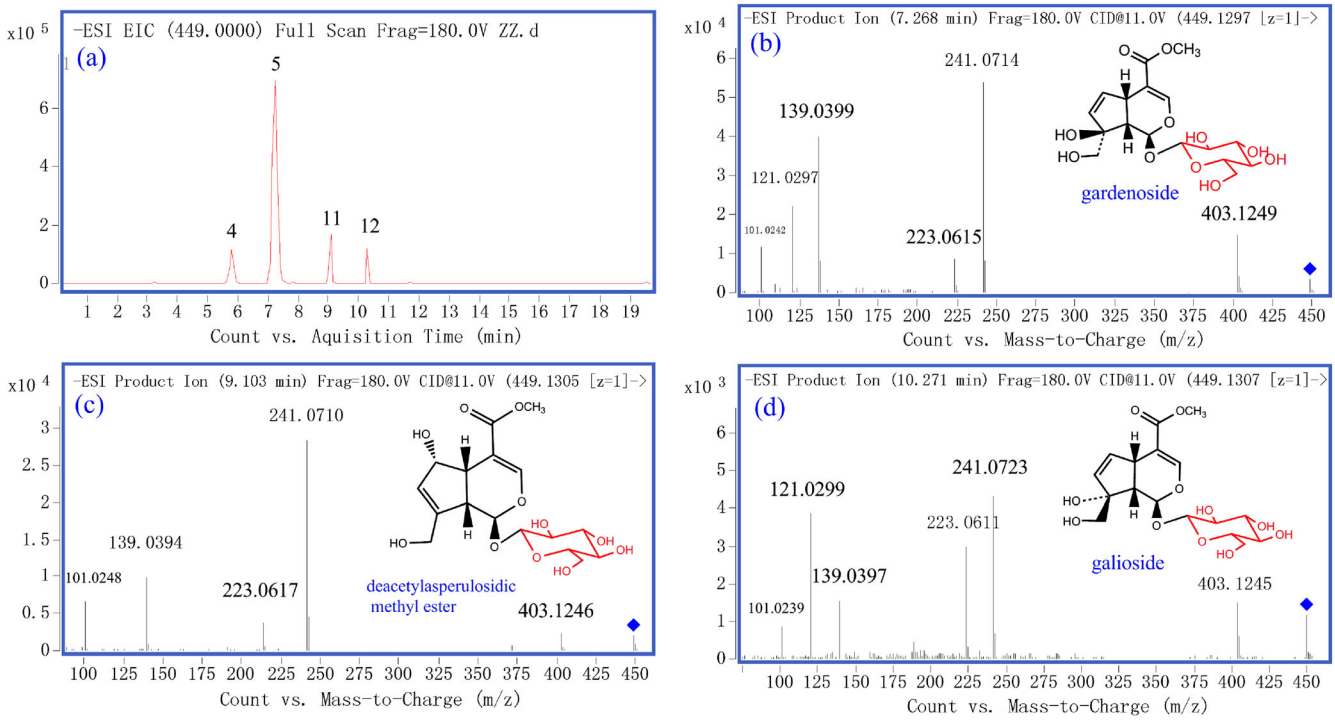


Figure 6. The EIC of *m/z* 449 in negative ion mode (a), the MS/MS spectra of the $[M-H+HCOOH]^-$ ions at *m/z* 449.1297 for gardenoside (b), *m/z* 449.1305 for deacetylasperulosidic acid methyl ester (c) and *m/z* 449.1307 for galioside (d).

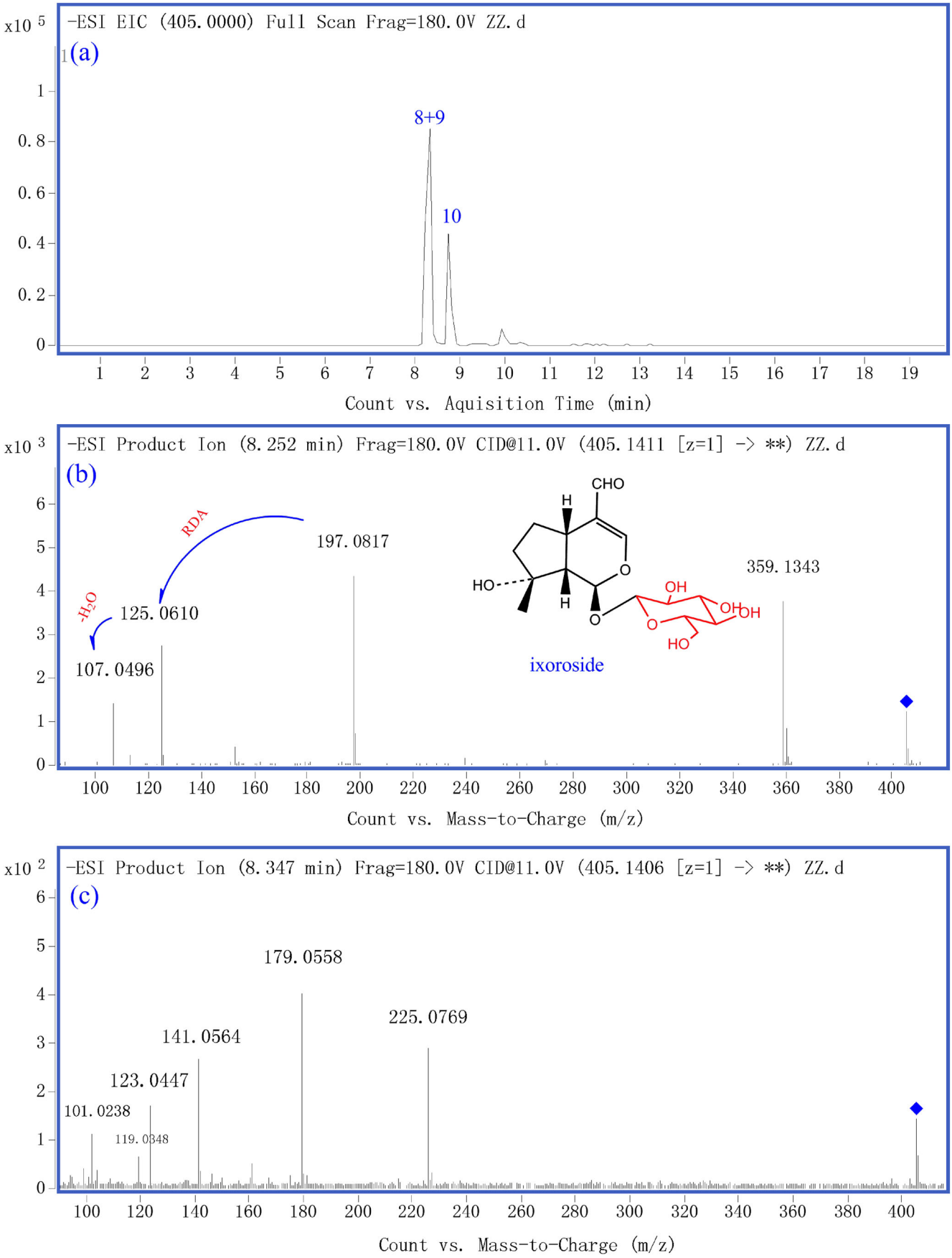


Figure 7. The EIC of *m/z* 405 in negative ion mode (a), the MS/MS spectra of the $[M-H+HCOOH]^-$ ion at *m/z* 405.1411 for ixoroside (b) and $[M-H]^-$ ion at *m/z* 405.1406 for isoshanzhiside methyl ester (c).

5 and 12 via the loss of a H_2O from *m/z* 139. This suggested that the substituted hydroxyl group of peaks 5 and 12 more easily lost an H_2O. As the previously literatures reported, the polarity of gardenoside was higher and its retention time was shorter on the reverse HPLC column than that of galioside. Therefore, peaks 5 and 12 were tentatively identified as gardenoside and galioside, respectively (Fu *et al*., 2014; Ono *et al*., 2005; Zhang *et al*., 2006).

Identification of peaks 8 and 9. The EIC of *m/z* 405 is shown in Fig. 7(a). Peaks 8 and 9 showed the same retention times of 8.3 min and they also exhibited the same quasi-molecular ions at *m/z* 405.1402 ($C_{17}H_{25}O_{11}^-$). However, their MS/MS spectra were so different that we could differentiate them easily. As Fig. 7(b and c) shows, peak 8 showed a representative $[M-H]^-$ ion at *m/z* 359.1343 ($C_{16}H_{23}O_9^-$). The prominent ions at *m/z* 197.0817 ($C_{10}H_{13}O_4^-$) resulted from the loss of a glucose unit, which was similar to the other IGs. The characteristic ions of peak 8 were at *m/z* 125.0610 ($C_7H_9O_2^-$) and 107.0496 ($C_7H_7O^-$), resulting from the RDA reaction of the basic skeleton and further loss of an H_2O, respectively. Thus, as the fragmentation pattern described above, peak 8 was tentatively identified as ixoroside (Fu *et al*., 2014).

Peak 9 presented the $[M-H]^-$ ion at *m/z* 405.1406, corresponding to the molecular formula of $C_{17}H_{26}O_{11}$. Thus, peak 9 and shanzhiside methyl ester could be considered as a pair of isomers. To the best of our knowledge, there are no reports on the isomers of shanzhiside methyl ester in Fructus Gardeniae. Compared with the fragment ions obtained in the MS/MS spectra (Fig. 7c), it was almost identical to the shanzhiside methyl ester. We tentatively deduced that it might be the epimer of shanzhiside methyl ester and was a new compound. Owing to the limitations of mass spectrometry, we could not confirm the exact structure. However, the goal-directed isolation of peak 9 is being performed by our group. Thus, we temporarily named it as isoshanzhiside methyl ester.

Identification of peaks 15, 16, 18, 19 and 20. Peak 15 produced the $[M-H]^-$ ion at *m/z* 519.1511, indicating the formula of $C_{25}H_{28}O_{12}$. The MS/MS spectra exhibited nine characteristic fragment ions at *m/z* 145.0299, 163.0397, 123.0445, 149.0601, 167.0716, 193.0504, 211.0606, 355.1029 and 307.0824 (Fig. 8a). Among them, the ions at *m/z* 355.1029, 211.0606, 193.0504, 167.0716, 149.0601 and 123.0445 were identical to the fragmentation patterns of standard compound of geniposidic acid. Additionally, the ions at *m/z* 307.0824 ($C_{15}H_{15}O_7^-$), 163.0397 ($C_9H_7O_3^-$) and 145.0299 ($C_9H_5O_2^-$) corresponded to [coumaroyl + glucosyl – $H-H_2O]^-$, [coumaroyl $-H]^-$ and [coumaroyl – $H-H_2O]^-$, respectively. Thus, the peak 15 could be tentatively identified as 6′-*O*-*trans*-coumaroyl geniposidic acid (Yu *et al*., 2009; Zhao *et al*., 2012).

As to peak 19, the typical $[M-H]^-$ ion at *m/z* 533.1659 ($C_{26}H_{29}O_{12}^-$) was displayed in the first-order MS spectra and yielded the characteristic fragment ions at *m/z* 225.0767, 207.0669, 123.0455 and 101.0241, suggesting that its basic skeleton was composed of geniposide (Fig. 8d). Moreover, the other fragment ions at *m/z* 307.0819 ($C_{15}H_{15}O_7^-$), 163.0406 ($C_9H_7O_3^-$) and 145.0287 ($C_9H_5O_2^-$) were also observed as the dominating

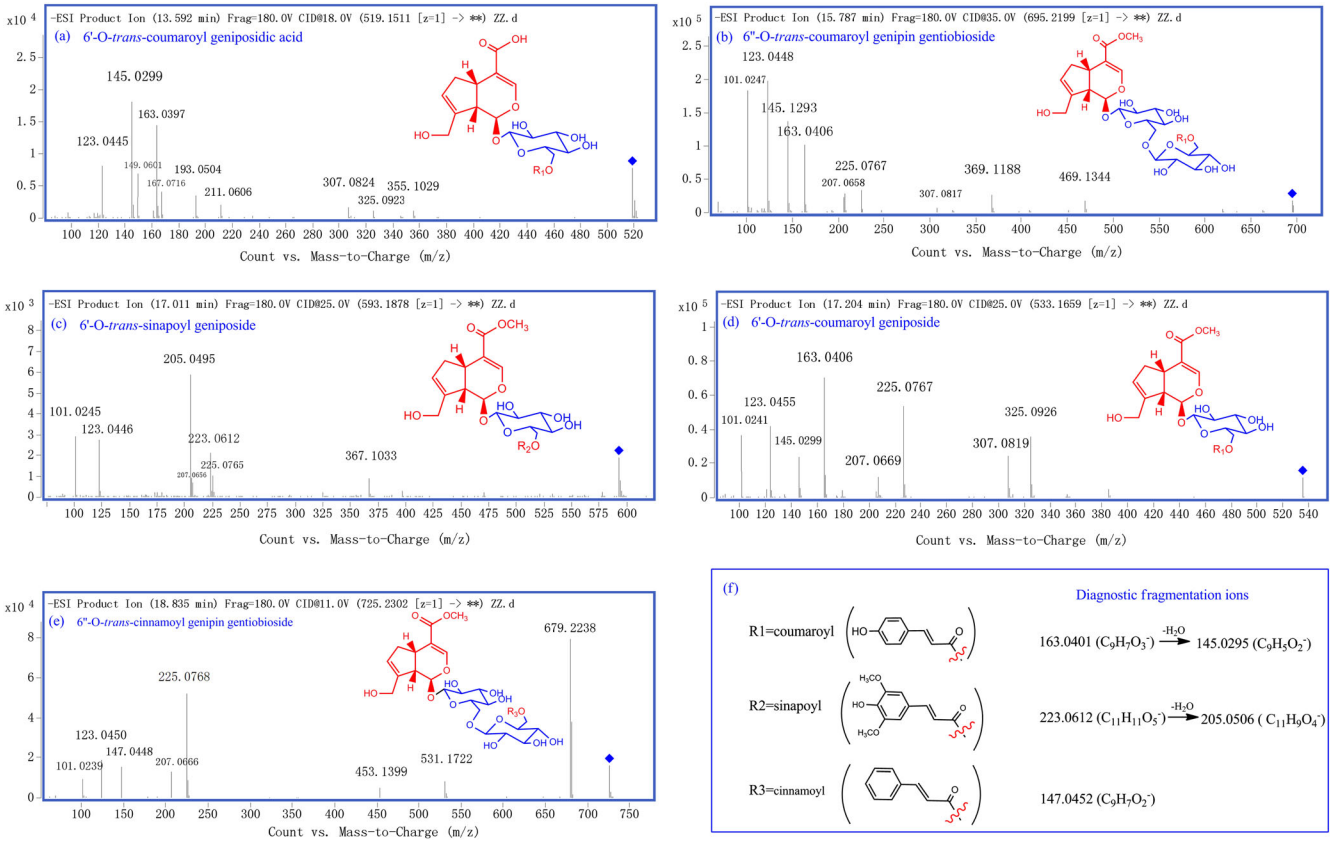


Figure 8. The MS/MS spectra of the $[M-H]^-$ ions at *m/z* 519.1511 for 6′-*O*-*trans*-coumaroyl geniposidic acid (a), *m/z* 695.2199 for 6″-*O*-*trans*-coumaroyl genipin gentiobioside (b), *m/z* 593.1878 for 6′-*O*-*trans*-sinapoyl geniposide (c), *m/z* 533.1659 for 6′-*O*-*trans*-coumaroyl geniposide (d), the $[M-H+HCOOH]^-$ ion at *m/z* 725.2302 for 6″-*O*-*trans*-cinnamoyl genipin gentiobioside (e) and the diagnostic fragmentation ions for peaks 15–20 (f).

fragments in the MS/MS spectra, which corresponded to [coumaroyl + glucosyl – H – H_2O]$^-$, [coumaroyl – H]$^-$ and [coumaroyl – H – H_2O]$^-$, respectively. Therefore, peak 19 was tentatively identified as 6′-*O*-*trans*-coumaroyl geniposide (Yu *et al*., 2009; Zhao *et al*., 2012). Compared with peak 19, peak 16 generated the [M – H]$^-$ ion at *m/z* 695.2199 ($C_{32}H_{39}O_{17}^-$), 162 Da (a glucose unit) more than that of peak 19 (Fig. 8b). According to the other MS/MS data shown in Table 1, it was almost identical to peak 19, and the ion at *m/z* 469.1344 ($C_{21}H_{25}O_{12}^-$) originating from [coumaroyl + gentiobiosyl – H – H_2O]$^-$ indicated the existence of a [coumaroyl + gentiobiosyl] group. Thus, peak 16 was tentatively identified as 6″-*O*-*trans*-coumaroylgenipin gentiobioside (Yu *et al*., 2009; Zhao *et al*., 2012).

A compound with a retention time of 16.952 min in the chromatogram was observed as peak 18. In its mass spectra, the ion at *m/z* 593.1878 were assigned to the [M – H]$^-$ ion, 162 Da less than the standard of 6″-*O*-*trans*-sinapoylgenipin gentiobioside, which is in accordance with the formula $C_{28}H_{34}O_{14}$ based on its accurate mass (Fig. 8c). Additionally, the fragment ions at *m/z* 225.0765, 123.0446, 101.0245 and 207.0656 were obtained, which indicated that its basic skeleton was identical to geniposide. Furthermore, the ions at *m/z* 223.0612 ($C_{11}H_{11}O_5^-$) and 205.0495 ($C_{11}H_9O_4^-$) could be assigned as [sinapoyl – H]$^-$ and [sinapoyl – H – H_2O]$^-$, suggesting the presence of a sinapoyl group in the molecule. Thus, peak 18 might be tentatively identified as 6′-*O*-*trans*-sinapoyl geniposide (Chen *et al*., 2009), the deglycosylation product of 6″-*O*-*trans*-sinapoylgenipin gentiobioside. In addition, the ion at *m/z* 367.1033 composed of [sinapoyl + glucosyl – H – H_2O]$^-$ also further verified our deduction.

Peak 20 showed a typical [M – H + HCOOH]$^-$ ion at *m/z* 725.2302 ($C_{33}H_{41}O_{18}^-$) and a deprotonated molecular ion [M – H]$^-$ at *m/z* 679.2238 ($C_{32}H_{39}O_{16}^-$) in negative ion mode, which yielded four diagnostic product ions at *m/z* 225.0768, 207.0666, 123.0450 and 101.0239 that were almost identical to those of the standard geniposide (Fig. 8e). More importantly, the characteristic fragment ions at *m/z* 147.0448 ($C_9H_7O_2^-$) originating from the [cinnamoyl – H]$^-$ and *m/z* 531.1722 ($C_{23}H_{31}O_{14}^-$) generating from a loss of H_2O from genipin gentiobioside were also observed. Thus, peak 20 was considered as a cinnamoyl-substituted genipin gentiobioside. As the previous publication described (Zhao *et al*., 2012), peak 20 was assigned to the 6″-*O*-*trans*-cinnamoylgenipin gentiobioside. In addition, because the cinnamoyl lacking the hydroxyl group substituted, the retention time on the reverse HPLC column was longer than those of peaks 16 and 17. The related diagnostic fragmentation ions are shown in Fig. 8(f).

Conclusions

In this work, a rapid and efficient method for the screening and identification of major IGs in Fructus Gardeniae was established and the characteristic fragmentation patterns of IGs obtained through the MS/MS data were outline systematically, which provided the best sensitivity and specificity for characterization of IGs or the analogs. Based on the fragmentation pathways concluded, 20 IGs in Fructus Gardeniae were tentatively identified in total. Importantly, during the process of structural elucidation, three pairs of epimers were structurally characterized and differentiated according to their nuances of distinctive fragmentation patterns, which were closely related to their isomeric differentiations, and the compound isoshanzhiside methyl ester was reported for the first time in Fructus Gardeniae as a new compound. The results of this study clearly demonstrated the superiority of HPLC-Q/TOF-MS/MS for the rapid and sensitive structural elucidation of the multiple groups of constituents in Fructus Gardeniae, and opened perspectives for similar studies on other medicinal herbs or preparations.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web site.