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Identification of prenyl flavonoid glycosides and phenolic acids in *Epimedium koreanum* Nakai by Q-TOF-MS combined with selective enrichment on "click oligo (ethylene glycol)" column

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ABSTRACT

Prenyl flavonoid glycosides and phenolic acids are constituents of the medicinal plant *Epimedium koreanum* Nakai (EK). An efficient method was developed to enrich these compounds and identify them, using ultra performance liquid chromatography combined with Q-TOF-MS, and a "click oligo (ethylene glycol)" (Click OEG) column. Using this method, 51 prenyl flavonoid glycosides and 18 phenolic acids were identified or tentatively identified. Of these, 11 prenyl flavonoid glycosides and 4 phenolic acids were new compounds, and 7 phenolic acids were newly identified in EK. Therefore, MS combined with selective enrichment provided a powerful means for analyzing prenyl flavonoid glycosides and phenolic acids.

1. Introduction

Herba Epimedii is a well-known botanical supplement used for menopausal symptoms and bone health that contains essential oil [1], icarisides [2] and flavonoids [2–4]. The major active compounds are thought to be prenyl flavonoid glycosides (PFG), in particular, 3-O, 7-O or 3,7-di-O-glycosides. The sugar moieties of the glycosides are usually glucose, rhamnose, xylose or their corresponding mono- or di-acetyl sugars [2,3]. Studies of Herba Epimedii have focused on purification of PFG by reversed phase liquid chromatography (RPLC) [5] or high-speed counter-current chromatography [6], quantitative determination by HPLC-UV [7] or capillary zone electrophoresis combined with UV [8]. However, data is lacking on the phenolic acids of Herba Epimedii. Phenolic acids are aromatic secondary plant metabolites that are phenols with one carboxylic acid. They are rarely found in free form, but are commonly esterified to other small molecules or bonded to cellulose, proteins and lignin [9,10]. Chlorogenic acids (CGA), a family of esters generated from trans-cinnamic acids and quinic acids, were reported to be the most common acids in plants [11,12]. Phenolic acids (PA) have attracted attention because their biological activities include antioxidative, anti-inflammatory, antimutagenic and anticarcinogenic properties[13]. For this reason, we investigated PA in Herba Epimedii.

HPLC combined with ESI-IT-MS has also been used to detect phenolic compounds [3], PFG [3,14] and PFG metabolites [15,16] in Herba Epimedii. Compared to IT-MS, Q-TOF-MS obtains relatively accurate mass/charge ratio (*m/z*) for molecular ions and their corresponding product ions (operated in collision-induced dissociation mode). Knowing the accurate *m/z* potentially allows the element composition of target compounds and product ions to be determined. Knowing these element compositions facilitates identification of structures [17]. Although Q-TOF-MS combined with ultra performance liquid chromatography (UPLC) or rapid-resolution liquid chromatography has improved sensitivity and selectivity, the comprehensive identification of natural products is still restricted by low concentrations and overlapping peaks. Therefore, SPE or SPME was combined with MS to enrich and simplify samples of natural products [18].

Recently, stationary phases C_{18}, C_8 and diol were also used in SPE or SPME [19], but these methods were not effective at distinguishing phenolic compounds, or enriching them with high selectivity. A type of stationary phase with polar substituents – "click oligo (ethylene glycol)" (Click OEG) – was prepared by our lab [20,21]. We used

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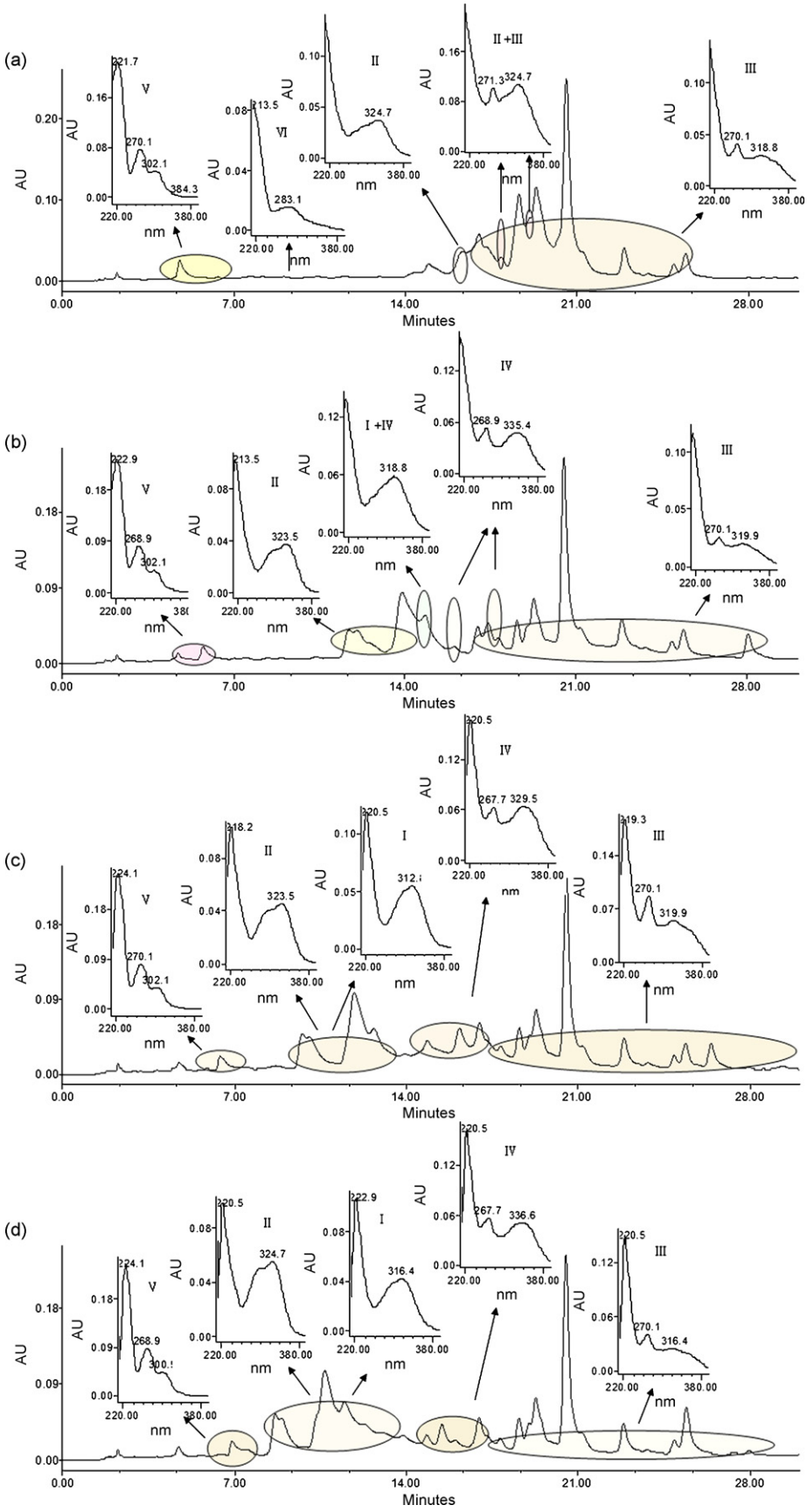


Fig. 1. The chromatograms of *Epimedium koreanum* aqueous extract at 320 nm on Click OEG column. Chromatographic conditions: Click OEG column; mobile phase, acetonitrile (A), water (B), and 200 mM ammonium formate aqueous solution (pH 3.5, C); flow-rate, 1.0 mL/min. The linear gradient was adopted as 2–30% acetonitrile at 0–30 min. (a) 5% C; (b) 10% C; (c) 15% C; (d) 20% C.

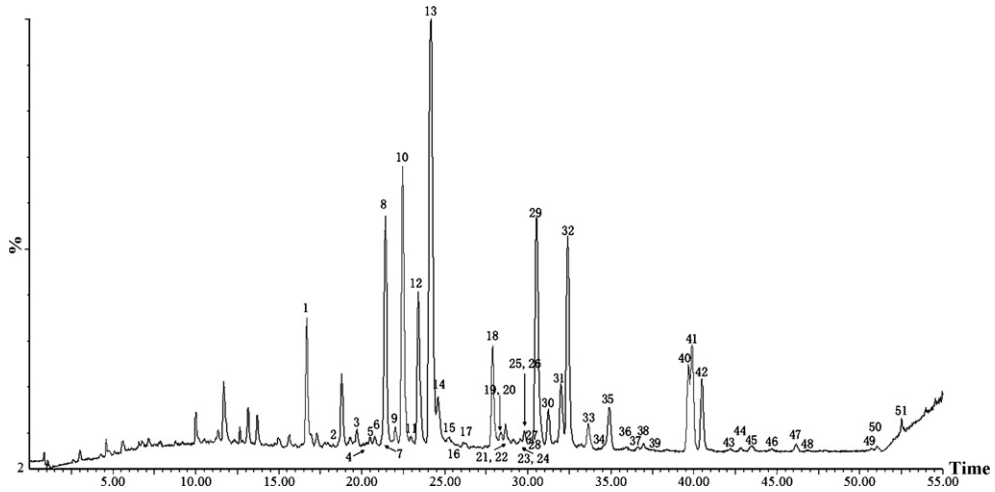


Fig. 2. Total ion current profile of fraction II in ESI-Q-TOF-MS. Chromatographic conditions: RP18 column; mobile phase, acetonitrile (A) and water (0.3% formic acid, B); flow-rate 0.25 mL/min. The linear gradient was adopted as follows: 2 to 12% A at 0–5 min, 12 to 20% A at 5–10 min, 20 to 35% A at 10–50 min, 35 to 100% A at 50–55 min.

Click OEG to selectively enrich simple phenylpropanoids and lignans, which are phenolic compounds from *Forsythia suspensa* [22]. Here we used Click OEG to separate aqueous extracts of *Epimedium koreanum* Nakai (EK, an official source using for Herba Epimedii). The two common phenolic components PFG and PA were selectively enriched and fractionated using optimized conditions. We also used UPLC combined with Q-TOF-MS to identify PFG and PA.

2. Experimental

2.1. Plant materials and reagents

The aerial part of *E. koreanum* Nakai (EK) was purchased from Liaoning Province. Acetonitrile of HPLC grade was obtained from Fisher (USA). Formic acid and ammonium formate of HPLC grade were purchased from Acros (Geel, Belgium). Water was purified by a Milli-Q water-purification system (USA). Trans-5-O-caffeoylquinic acid was obtained from Sigma (USA); Epimedin A, epimedin B, epimedin C and icariin were prepared by us.

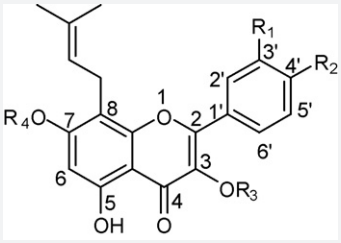
2.2. Extraction

The aerial part of EK was pulverized and dried to constant weight before being extracted. An aliquot of 10 g materials was weighed and extracted with 200 mL water in an ultrasonic water bath for 1 h. The extraction was centrifuged for 6 min at 4500 rpm, the supernatant was transferred and made into powder (625 mg) by freeze drying. An aliquot of 30 mg powder was dissolved in 1.5 mL 25% methanol aqueous solution, the sample solution was filtered through 0.22 μm membranes before analysis.

2.3. Enrichment on Click OEG column

The chromatographic system was composed of a Waters 2695 HPLC pump, an autosampler and a Waters 2996 diode-array detector (Waters, USA). Separation was carried out on Click OEG column (150 × 4.6 mm i.d., 5 μm, laboratory-made). The column temperature was maintained at 30 °C. Mobile phases consisted of acetonitrile (A), water (B), and 200 mM ammonium formate aqueous solution (pH 3.5, C). The linear gradient was adopted as follows: 2–30% A, at 0–30 min. And concentration of the additive was maintained by the change of mobile phase C. Flow rate was 1.0 mL/min. The injection volume was 10 μL for separation and 100 μL for fractionation. The two fractions were collected on 15% C from 9.3 to 14 min (fraction I) and 17.2 to 30 min (fraction II) following with being concentrated to 50 μL.

Table 1
The structures and formula of $[M-H]^-$ ions of aglycone and standards.



Compounds	Calculated of $[M-H]^-$	Formula	R_1	R_2	R_3	R_4
Aglycone A	353.1025	$C_{20}H_{17}O_6$	H	OH	H	H
Aglycone B	367.1182	$C_{21}H_{19}O_6$	H	OCH3	H	H
Aglycone C	383.1131	$C_{21}H_{19}O_7$	OH	OCH3	H	H
Epimedin A	837.2817	$C_{39}H_{49}O_{20}$	H	OCH3	Rha-Glu	Glu
Epimedin B	807.2712	$C_{38}H_{47}O_{19}$	H	OCH3	Rha-Xyl	Glu
Epimedin C	821.2868	$C_{39}H_{49}O_{19}$	H	OCH3	Rha-Rha	Glu
Icariin	675.2289	$C_{33}H_{39}O_{15}$	H	OCH3	Rha	Glu

Table 2
Identification of PFG from fraction II.

Peak No.	RT (min)	[M−H]⁻	Cal	Error (ppm)	Formula	MS/MS (relative intensity)	Agl	Substituents		Compounds
								3 Position	7 Position	
1	16.55	661.2133	661.2132	0.2	$C_{32}H_{37}O_{15}$	[661]:661(22), 515(18), 514(27), 499(12), 395(8), **353.0989**(100)	A	Rha-Glu		Icarisoside B or its isomer [3]
2	17.88	865.2784	865.2766	2.1	$C_{40}H_{49}O_{21}$	[865]:865(22), 703(100), 685(5), 515(12), **353.1094**(18)	A	Glu-Rha(Ac)	Glu	
3	19.71	865.2773	865.2766	0.8	$C_{40}H_{49}O_{21}$	[865]:865(11), 703(100), 685(5), 515(8), **353.1007**(33)	A	Glu-Rha(Ac)	Glu	
4	20.67	807.2712	807.2712	0.0	$C_{38}H_{47}O_{19}$	[807]:513(100), **367.1230**(50)	B	Rha	Xyl- Glu	
5	20.80	691.2213	691.2238	−3.6	$C_{34}H_{41}O_{18}$	[737]:529(100), 409(30), **367.1215** (18) [737–529]:529(64), 367(48), 366(100), 351(18), 323(16)	B	Glu	Glu	
6[a]	21.11	818.2877	818.2817	0.7	$C_{39}H_{48}NO_{18}$	[818]:818(13), 675(52), 656(24), 529(11), 513(7), 409(6), **367.1206**(100)	B	Rha-$C_6H_9NO_3$	Glu	
7[b]	21.33	837.2808	837.2817	−1.1	$C_{39}H_{49}O_{20}$	[837]:675(100), 513(2), 367(1) [837–675]:675(100), **367.1208**(28)	B	Rha-Glu	Glu	Epimedin A [3]
8[a]	22.02	805.2570	805.2555	1.9	$C_{38}H_{45}O_{19}$	[805]:805(70), 661(8), 643(24), 515(30), 395(10), **353.1057**(100), 289(12)	A	Glu-Rha-$C_6H_9O_4$		
9[b]	22.57	807.2712	807.2712	0.0	$C_{38}H_{47}O_{19}$	[807]:645(100) [807–645]:675(100), **367.1159**(40)	B	Xyl-Rha	Glu	Epimedin B [3]
10	23.33	821.2879	821.2868	1.3	$C_{39}H_{49}O_{19}$	[821]:659(100), 409(1), 366(1) [821–659]:659(100), **367.1183**(11)	B	Rha-Rha	Glu	Epimedin C [3]
11[b]	23.53	907.2883	907.2872	1.2	$C_{42}H_{51}O_{22}$	[907]:907(19), 745(100), 703(4), 666(11), 515(12), **353.1065** (28), 233(6)	A	Glu(Ac)-Rha(Ac)	Glu	
12[b]	23.96	721.2318	721.2344	−3.6	$C_{34}H_{41}O_{17}$	[721]:529(16), 513(100), 409(14), **367.1168**(78)	B	Rha	Rha	Icariin [3]
13	24.59	821.2889	821.2868	2.6	$C_{39}H_{49}O_{19}$	[821]:659(100), 587(2), **367.1225**(1)	B	Rha-Rha	Glu	Isomer of Epimedin C [3]
14	24.75	717.2374	717.2395	−2.9	$C_{35}H_{41}O_{16}$	[717]:529(20), 513(100), 409(21), **367.1207**(85)	B	Rha (Ac)	Glu	
15	25.44	937.2962	937.2978	−1.7	$C_{43}H_{53}O_{23}$	[775]:775(78), **383.1212**(100)	C	Glu(Ac)-Rha(Ac)	Glu	
16	25.74	515.1553	515.1553	0.0	$C_{26}H_{27}O_{11}$	[515]:515(78), **353.0998**(100), 309(45)	A	Glu		[3]
17[a]	26.29	835.2694	835.2661	4.0	$C_{39}H_{47}O_{20}$	[835]:835(80), 673(24), 529(40), 409(6), **367.1154**(100), 305(11)	B	Glu-$C_6H_9O_5$-Glu		
18	27.79	879.2935	879.2923	1.4	$C_{41}H_{51}O_{21}$	[879]:717(100), 529(2), **367.1197**(100) [879–717]:717(15), 367(100)	B	Rha (Ac)-Glu	Glu	[3]
19[a]	28.1	851.2641	851.2610	3.6	$C_{39}H_{47}O_{21}$	[851]:851(30), 689(12), 555(18), 513(18), 321(8), **367.1197**(8), 175(22), 131(100)	B	Rha -$C_6H_9O_6$	Glu	
20[a]	28.38	851.2644	851.2610	4.0	$C_{39}H_{47}O_{21}$	[851]:851(46), 689(15), 555(12), 513(18), 529(7), **367.1236**(8), 175(16), 131(100)	B	Rha-$C_6H_9O_6$	Glu	
21[a]	28.66	851.2596	851.2610	−1.6	$C_{39}H_{47}O_{21}$	[851]:851(50), 689(17), 555(12), 513(22), 529(8), **367.1203**(6), 175(16), 131(100)	B	Rha-$C_6H_9O_6$	Glu	
22	28.82	979.3115	979.3083	3.3	$C_{45}H_{55}O_{24}$	[979]:979(6), 817(100), 775(12), 757(4), 545(8), **383.1171**(14)	C	Glu(2Ac)-Rha(Ac)	Glu	
23	29.12	979.3122	979.3083	4.0	$C_{45}H_{55}O_{24}$	[979]:817(100), 775(2), 545(10), **383.1166**(14)	C	Glu(2Ac)-Rha(Ac)	Glu	
24[a]	29.4	835.2681	835.2661	2.4	$C_{39}H_{47}O_{20}$	[835]:835(36), 715(12), 673(100), 655(3), 529(3), **367.1206** (22), 513(4)	B	Glu-$C_6H_9O_4$	Glu	
25	29.58	949.3010	949.2978	3.4	$C_{44}H_{53}O_{23}$	[949]:949(60), 787(100), 745(12), 515(12), **353.0989** (13)	A	Glu(2Ac)-Rha(Ac)	Glu	[3]
26	29.88	949.3022	949.2978	4.6	$C_{44}H_{53}O_{23}$	[949]:949(40), 787(100), 745(11), 515(13), **353.1024** (27), 395(5)	A	Glu(2Ac)-Rha(Ac)	Glu	[3]
27[a]	29.95	835.2667	835.2661	0.7	$C_{39}H_{47}O_{20}$	[835]:835(45), 715(12), 673(100), 513(8), **367.1181** (32), 305(8), 159(8)	B	Rha-$C_6H_9O_5$	Glu	
28	30.12	921.3043	921.3028	1.6	$C_{43}H_{53}O_{22}$	[921]:759(100), 717(38), 571(5), 529(6), **367.1235**(35) [759]:759(30), 367(100)	B	Glu(Ac)-Rha(Ac)	Glu	Epimedokoreanoside I or its isomers [3]
29	30.36	819.2691	819.2712	−2.6	$C_{39}H_{47}O_{19}$	[819]:819(54), 657(23), 639(5), 529(38), 409(8), **367.1125**(100), 289(10)	B	Rha-$C_6H_9O_4$	Glu	[3]
30[a]	31.23	835.2628	835.2661	−4.0	$C_{39}H_{47}O_{20}$	[835]:835(15), 749(3), 673(12), 569(5), 529(4), 513(25), **367.1214**(18), 305(36), 159(37), 73(100)	B	Rha-$C_6H_9O_5$	Glu	
31[a]	31.89	821.2493	821.2504	−1.3	$C_{38}H_{45}O_{20}$	[821]:821(22), 659(7), 641(4), 569(4), 529(4),**367.1210**(8),291(52),145(100)	B	Rha-$C_5H_7O_5$	Glu	
32	32.41	921.2988	921.3028	−4.3	$C_{43}H_{53}O_{22}$	[921]:759(100), 529(2), **367.1198**(17) [921–759]:759(24), 367(100)	B	Glu(Ac)-Rha(Ac)	Glu	Epimedokoreanoside I or its isomers [3]
33	33.59	921.3057	921.3028	3.1	$C_{43}H_{53}O_{22}$	[921]:759(100), 529(2), **367.1182** (24) [759]:759(30), 367(100)	B	Glu(Ac)-Rha(Ac)	Glu	Epimedokoreanoside I or its isomers [3]
34	34.50	515.1550	515.1553	−0.6	$C_{26}H_{27}O_{11}$	[515]:515(23), **353.1043**(100), 325(9), 309(14), 297(6), 219(15)	A	Glu		Epimedoside C [3]
35	34.65	529.1701	529.1710	−1.7	$C_{27}H_{29}O_{11}$	[529]:529(58), 459(8), **383.1213**(23), 312(100), 297(11), 269(6)	C	Rha		Caohuoside C [3]
36	34.93	921.3042	921.3028	1.5	$C_{43}H_{53}O_{22}$	[921]:759(100), **367.1151**(15) [921–759]:759(48), 367(100), 571(10)	B	Glu(Ac)-Rha(Ac)	Glu	Epimedokoreanoside I or its isomers [3]
37[a]	35.67	828.2711	828.2715	−0.5	$C_{40}H_{46}NO_{18}$	[828]:666(43), 513(100), 529(15), 409(18), **367.1026**(40), 152(45)	B	Rha-$C_7H_8NO_3$	Glu	
38	36.54	717.2414	717.2395	2.6	$C_{35}H_{41}O_{16}$	[763]:555(100), 529(20), 409(22), **367.1140**(78)	B	Rha(Ac)	Glu	[3]
39	36.98	745.2377	745.2344	4.4	$C_{36}H_{41}O_{17}$	[745]:625(4), 583(100), 529(5), **367.1212**(100), 409(6)	B	Xyl(2Ac)	Glu	[3]
40	39.68	963.3157	963.3134	2.4	$C_{45}H_{55}O_{23}$	[963]:801(100), 759(4), 571(1), 409(2), **367.1198**(6)	B	Glu(2Ac)-Rha(Ac)	Glu	Epemedin L or its isomers [3]
41	39.88	963.3101	963.3134	-3.4	$C_{45}H_{55}O_{23}$	[963]:801(100), 759(12), **367.1185**(20) [976–801]:801(40), 367(100), 741(2)	B	Glu(2Ac)-Rha(Ac)	Glu	Epemedin L or its isomers [3]
42	40.58	963.3171	963.3134	3.8	$C_{45}H_{55}O_{23}$	[963]: 801(100), 759(16), 571(2), 529(4), 409(2), **367.1169**(20)	B	Glu(2Ac)-Rha(Ac)	Glu	Epemedin L or its isomers [3]
43	42.25	631.2044	631.2027	2.7	$C_{31}H_{35}O_{14}$	[631]:631(100), 481(5), 352(85), **353.1091**(30)	A	Rha-Xyl		Icarisoside F [2]
44	42.75	963.3153	963.3134	2.0	$C_{45}H_{55}O_{23}$	[963]:801(100), 759(15), **367.1286** (12)	B	Glu(2Ac)-Rha(Ac)	Glu	Epemedin L or its isomers [3]
45	43.58	963.3120	963.3134	−1.5	$C_{45}H_{55}O_{23}$	[963]:801(100), 759(8), 529(2), **367.1291**(12)	B	Glu(2Ac)-Rha(Ac)	Glu	Epemedin L or its isomers [3]
46	44.73	499.1599	499.1604	−1.8	$C_{26}H_{27}O_{10}$	[499]:499(8), **353.1050**(100), 352(60)	A		Rha	Icarisoside A [2]
47	46.19	675.2321	675.2289	4.7	$C_{33}H_{39}O_{15}$	[675]:675(100), 495(1), **367.1164**(36)	B	Rha-Glu		Baohuoside VII [2]
48	46.96	529.1704	529.1710	−1.1	$C_{27}H_{29}O_{11}$	[529]:**367.1181**(100), 352(8)	B		Glu	Icariin I [2]
49	50.7	659.2367	659.2340	4.1	$C_{33}H_{39}O_{14}$	[659]:659(100), **367.1157**(29), 366(42)	B	Rha-Rha		[3]
50	51.05	645.2198	645.2183	2.3	$C_{32}H_{37}O_{14}$	[645]:645(100), **367.1181**(47), 366(30)	B	Rha-Xyl		Sagittatoside B [2]
51	52.53	513.1736	513.1761	−4.9	$C_{27}H_{29}O_{10}$	[513]:513(34), 366(100), 351(23), 323(14) **367.1141**	B		Rha	Icariin II [2]

The bold values indicate the *m/z* of aglycones.

[a] New compounds from EK.

[b] Compared with reference standards. Glu, Rha, Xyl represented glucosyl, rhamnosyl and xylosyl, respectively; Ac: acetyl; Cal: calculated mass; Agl: aglycone. (a) MS/MS of a; (a–b) MS^3 of a, MS/MS of b.

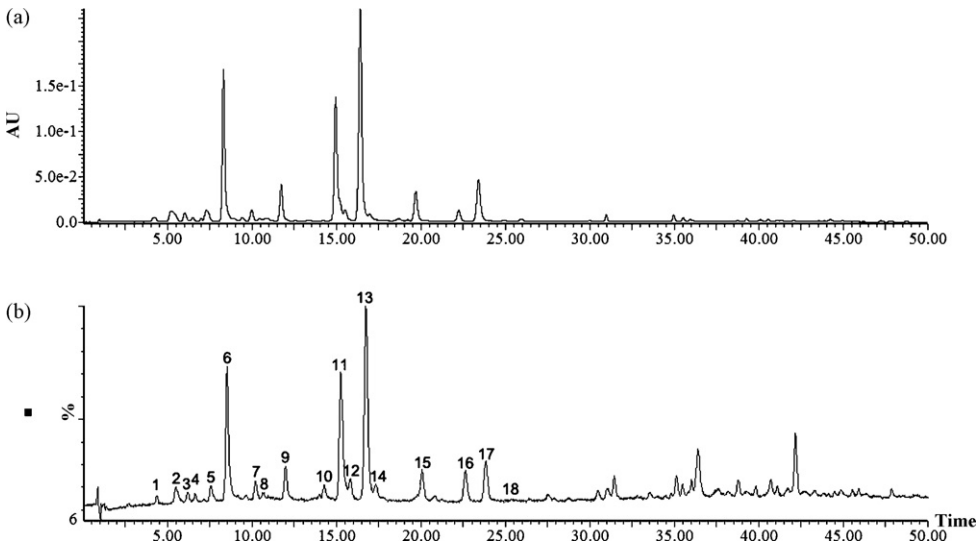


Fig. 3. UPLC-UV chromatogram and total ion current profile of fraction I in ESI-Q-TOF-MS. Chromatographic conditions: RP18 column; mobile phase, acetonitrile (A) and water (0.3% formic acid, B); flow-rate 0.25 mL/min. The linear gradient was adopted as follows: 2–20% A at 0–50 min. (a) UV chromatogram at 320 nm; injection volume, 10 μL. (b) Total ion current profile in ESI-Q-TOF-MS; injection volume, 2 μL.

2.4. Identification by Q-TOF-MS

UPLC was performed by a Waters ACQUITY UPLC™ system (Waters, USA). Separation was enforced on a RP18 column (100 × 2.1 mm i.d., 1.7 μm). The column temperature was maintained at 35 °C. The mobile phases of UPLC were composed of acetonitrile (A) and water (0.3% formic acid, B). The linear gradient for analyzing fraction II was adopted as follows: 2 to 12% A at 0–5 min, 12 to 20% A at 5–10 min, 20 to 35% A at 10–50 min, 35 to 100% A at 50–55 min; and for separating fraction I was adopted as follows: 2 to 20% A at 0–50 min. Flow rate was 0.25 mL/min. The injection volume was 2 μL for MS analysis and 10 μL for MS/MS analysis.

Mass spectrometry was performed on a Q-TOF Premier™ (Waters, UK) operated in ESI- mode. The nebulization gas was controlled to 800 L/h at 350 °C, the cone gas was controlled to 50 L/h, and the source temperature was controlled to 120 °C. The capillary voltage was controlled to 2.5 kV and the cone voltage was controlled to 35 V. Samples were analyzed by collision-induced dissociation (CID) in MS/MS. The collision energy of CID was 25 V. Argon was used as collision gas. Data were collected in centroid mode. The accuracy and reproducibil-

Table 3
Identification of phenolic acids from fraction I.

No	t_R (min)	$[M-H]^-$ (*m/z*)		Elemental composition	Error (mDa)	MS/MS (%base peak)	Proposed structure
		Measured	Calculated				
1[a]	4.29	371.0647	371.0614	$C_{15}H_{15}O_{11}$	−3.3	[371]:209(12), 191(100), 85(47)	Caffeoyl-hexaric acid or its isomers
2[a]	5.47	371.0611	371.0614	$C_{15}H_{15}O_{11}$	−0.3	[371]:209(42), 191(18), 133(16), 85(100)	Caffeoyl-hexaric acid or its isomers
3[a]	6.19	371.0595	371.0614	$C_{15}H_{15}O_{11}$	−1.9	[371]:209(28), 191(22), 85(100)	Caffeoyl-hexaric acid or its isomers
4[b]	6.64	353.0856	353.0873	$C_{16}H_{17}O_9$	−1.7	[353]:191(100), 179(43), 135(68), 85(7)	*cis*-3-O-caffeoylquinic acid [23,24]
5[a]	7.58	371.0590	371.0614	$C_{15}H_{15}O_{11}$	−2.4	[371]:209(35), 191(32), 85(100)	Caffeoyl-hexaric acid or its isomers
6	8.36	353.0847	353.0873	$C_{16}H_{17}O_9$	−2.6	[353]:191(100), 179(46), 135(72), 85(5)	*trans*-3-O-caffeoylquinic acid [23,24]
7[b]	10.20	337.0907	337.0923	$C_{16}H_{17}O_8$	−1.6	[337]:191(49), 163(64), 119(100)	*cis*-3-O-*p*-coumaroylquinic acid [24]
8	10.65	353.0857	353.0873	$C_{16}H_{17}O_9$	−1.6	[353]:191(94), 179(74), 173(78), 135(100), 111(12), 93(26)	*cis*-4-O-caffeoylquinic acid [23,24]
9[b]	11.98	337.0896	337.0923	$C_{16}H_{17}O_8$	−2.7	[337]:191(20), 163(69), 119(100)	*trans*-3-O-*p*-coumaroylquinic acid [24]
10[b]	14.01	707.1832	707.1823	$C_{32}H_{35}O_{18}$	0.9	[707]: 707(75), 353(46), 191 (100)	Dimer of 5-O-caffeoylquinic acid
11	15.10	353.0841	353.0873	$C_{16}H_{17}O_9$	−3.2	[353]:191(83), 179(52), 173(74), 111(12), 135(100), 93(26)	*trans*-4-O-caffeoylquinic acid [23,24]
12[b]	15.81	337.0902	337.0923	$C_{16}H_{17}O_8$	−2.1	[337]:191(27), 173(100), 163(22), 119(52), 111(20), 93(48)	*cis*-4-O-*p*-coumaroylquinic acid [24]
13[c]	16.51	353.0845	353.0873	$C_{16}H_{17}O_9$	−2.8	[353]:191(100), 85(5)	*trans*-5-O-caffeoylquinic acid [23,24]
14[b]	17.34	353.0845	353.0873	$C_{16}H_{17}O_9$	−2.8	[353]:191(100), 85(6)	*cis*-5-O-caffeoylquinic acid [23,24]
15[b]	19.95	337.0892	337.0923	$C_{16}H_{17}O_8$	−3.1	[337]:191(5), 173(100), 163(26), 119(41), 111(15), 93(45)	*trans*-4-O-*p*-coumaroylquinic acid [24]
16	22.76	337.0907	337.0923	$C_{16}H_{17}O_8$	−1.6	[337]:191(100), 163(5), 93(8)	*trans*-5-O-*p*-coumaroylquinic acid [24]
17	23.74	337.0892	337.0923	$C_{16}H_{17}O_8$	−3.1	[337]:191(100), 163(12), 93(26)	*cis*-5-O-*p*-coumaroylquinic acid [24]
18	25.36	367.1003	367.1029	$C_{17}H_{19}O_9$	−2.6	[367]:193(12), 191(100), 134(18), 93(33)	5-O-feruloylquinic acid [24]

[a] New compounds from EK.
[b] Compounds firstly reported from EK.
[c] Compared with reference standards.

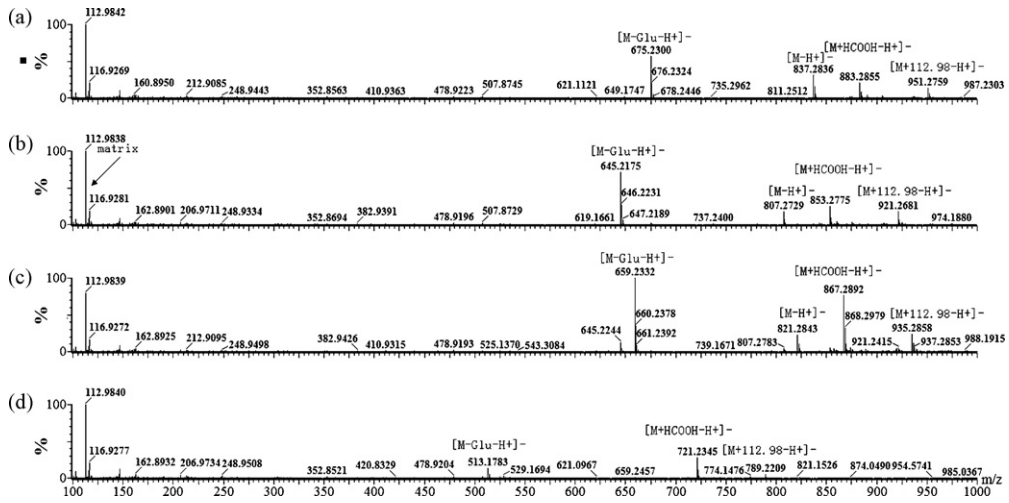


Fig. 4. MS spectra of the four standards. (a) epimedin A; (b) epimedin B; (c) epimedin C; (d) icariin.

ity of all analyses was guaranteed by using the lock spray. Leucine-enkephalin, generating $[M-H]^-$ ions at 554.2615 Da in ESI- mode, was employed as the lock mass at a concentration of 50 pg/mL and a flow rate of 10 μL/min. The raw data were processed by MassLynx Applications Manager 4.1 (Waters, UK).

3. Results and discussion

3.1. Enrichment by Click OEG column

The Click OEG stationary phase allows polar interactions through its four ether bonds, a hydroxyl group and a triazole,

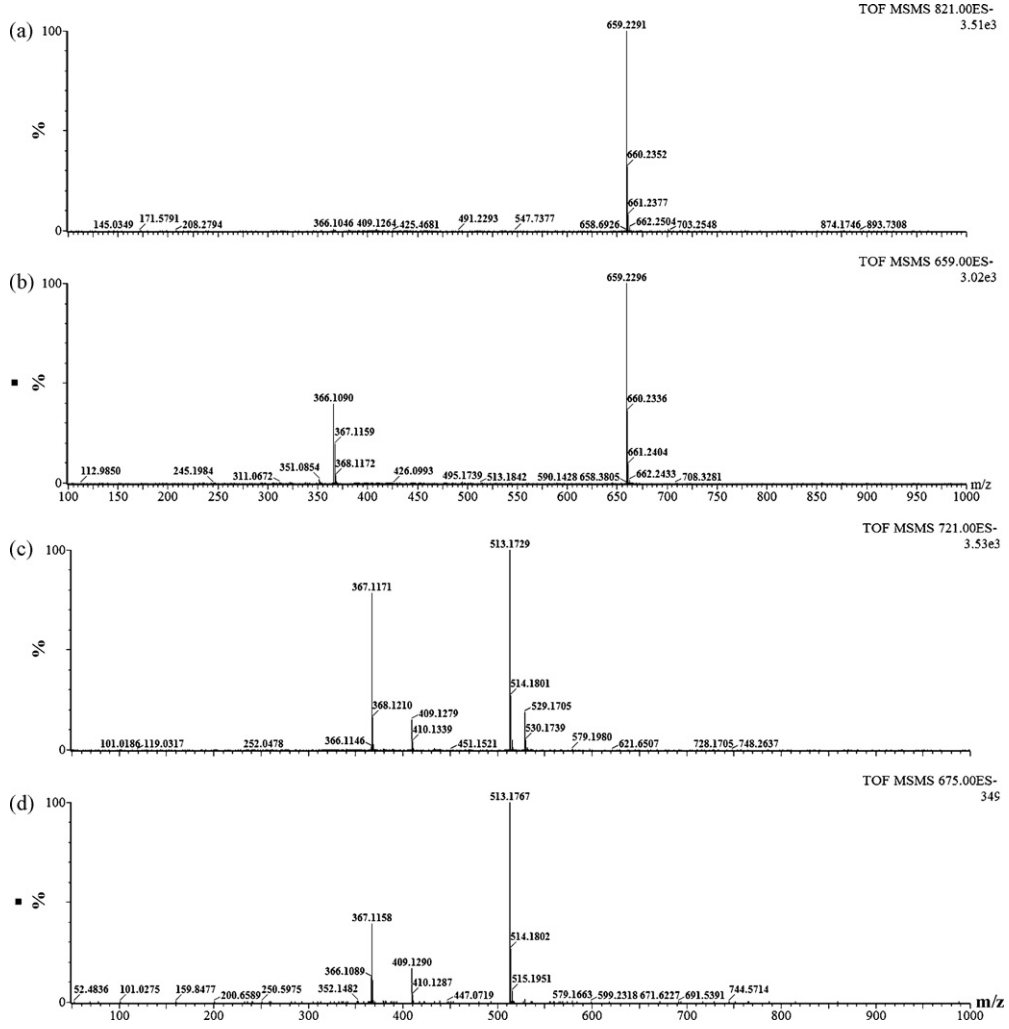


Fig. 5. MS^n spectra of epimedin C and icariin. (a) MS^2 of epimedin C; (b) MS^3 of epimedin C (821–659); (c) MS^2 of icariin ($[M+HCOOH-H^+]^-$); (d) MS^2 of icariin ($[M-H^+]^-$).

but mobile phases must be optimized to enrich based on these interactions. An ammonium formate aqueous solution (pH 3.5) of 200 mM was used as a stock solution for adjusting the separation selectivity, and selectivity under different ammonium formate concentrations was investigated. We found that retention times of PA changed, while those of PFG and others components remained constant (Fig. 1). UV spectra were used to identify the different phenolic compounds. PFG (UV_{max} approximately 270 and 320 nm; Fig. 1, type III) were the main constituents of EK, and CGA were the most common PA, with a UV_{max} at approximately 323 nm (caffeoyl or feruloyl; Fig. 1, type II), or at approximately 315 nm (coumaroyl; Fig. 1, type I). Three other kinds of phenolic compounds were also observed (Fig. 1, type IV, V and VI).

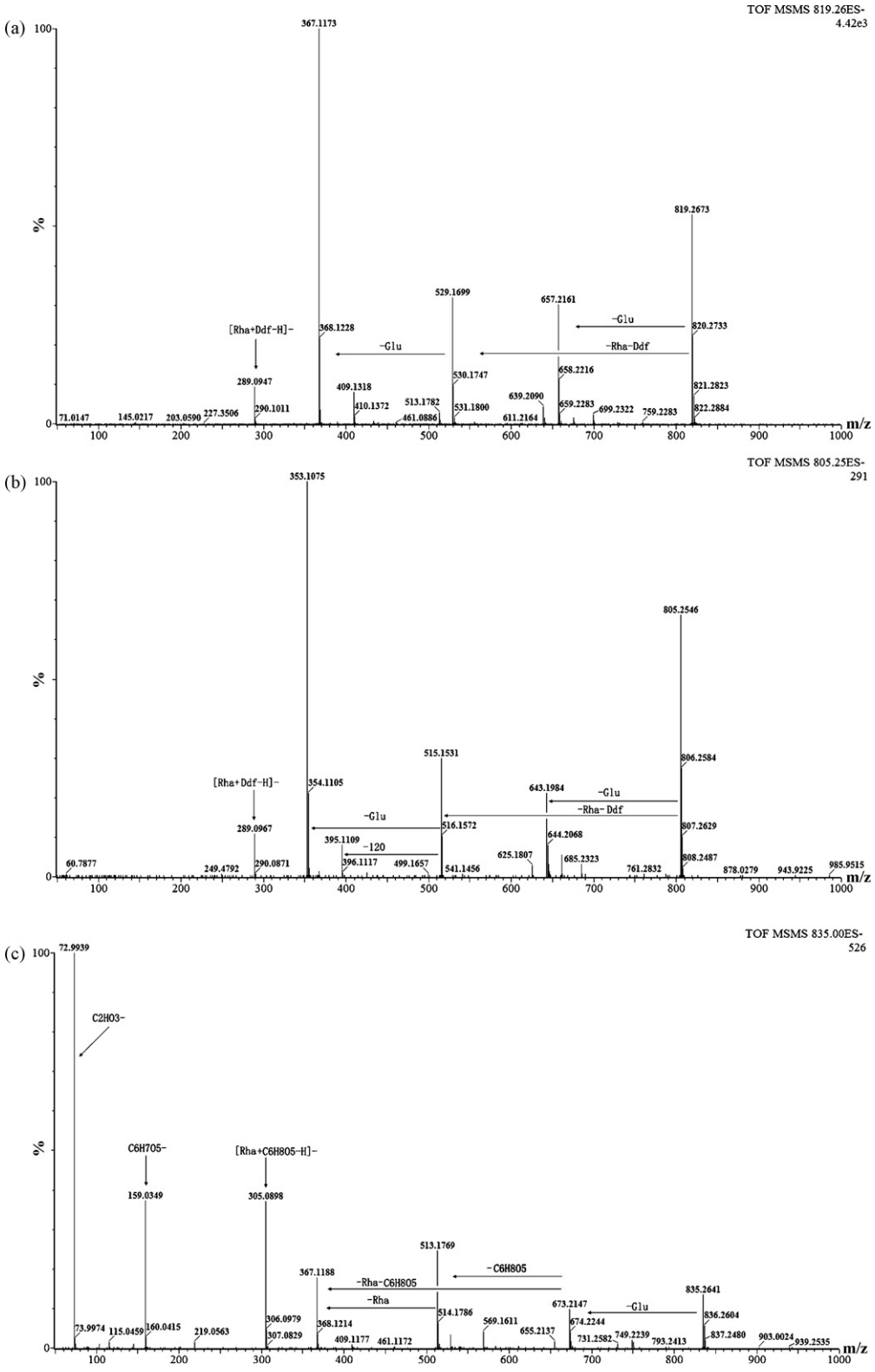


Fig. 6. MS^2 spectra of peak 29, 8 and 30. (a) Peak 29; (b) peak 8; (c) peak 30.

At 5% ammonium formate, PA and PFG co-eluted (Fig. 1a), and several peaks were observed at retention times from 9 to 14 min. If PA eluted in this range, selective enrichment of PA and PFG would be possible. At 10% ammonium formate, the retention times of PFG and other phenolic compounds showed no obvious changes, and those of PA became shorter (Fig. 1b). At 15% ammonium formate, PA eluted in the anticipated range and could be distinguished from other peaks (Fig. 1c). At 20% ammonium formate, some PA co-eluted with other phenolic compounds before 9 min (Fig. 1d). Therefore, selectively enrichment of PFG and PA was carried out at 15% ammonium solution on the Click OEG column, with fraction I containing PA and fraction II containing PFG. The two fractions were reanalyzed by UPLC. Most compounds in fraction II could be identified as PFG, and the majority of compounds in fraction I were confirmed as PA by their UV spectra. To further identify PFG and PA, the two fractions were further analyzed by UPLC combined with Q-TOF-MS.

3.2. Identification by Q-TOF-MS

51 compounds showed UV characteristic spectra of PFG in fraction II and 18 compounds showed UV characteristic spectra of CGA in fraction I. These peaks were labeled in total ion current profile in ESI-Q-TOF-MS (Figs. 2, 3b), and structures of corresponding compounds were further identified by MS. The structures of PFG and PA were identified by accurate *m/z* and MS/MS fragmentation behavior (Tables 2 and 3). The errors of measured masses were within 5 ppm for PFG and 4 mDa for PA. In total, 40 PFG and 14 PA were identified, and 11 new PFG and four new PA were tentatively identified (Tables 2 and 3).

3.2.1. Identification of PFG in fraction II by Q-TOF-MS

3.2.1.1. The fragmentation rules of standards. Epimedin A, epimedin B, epimedin C and icariin were used as standards for Q-TOF-MS in negative mode. The standards all have an aglycone B (*m/z* 367.1182) and a glucose substituent at the 7 position, but different sugars at the 3 position, with a monosaccharide for icarrin and a disaccharide for the others (Table 1). The peak of matrix was observed at an *m/z* of 112.9841 in our conditions. All three standards with a disaccharide at the 3 position showed fragmentation pathways with $[M-H^+]^-$ ions, $[M+HCOOH-H^+]^-$ ions, $[M+112.98-H^+]^-$ ions and abundant $[M-Glu-H^+]^-$ ions by MS (Fig. 4a–c). All three standards showed lost sugars at the 7 position as a base peak in MS^2, and successive loss of sugars at the 3 position in MS^3, with $[M-H^+]^-$ of aglycone B ($C_{21}H_{19}O_6$) or the free radical of aglycone B (MS^2 and MS^3 spectra of epimedin C, Fig. 5a,b). Icarrin with a monosaccharide at the 3 position showed a different MS behaviors, with descending order intensities of $[M+HCOOH-H^+]^-$ ions, $[M-Glu-H^+]^-$ ions and $[M+112.98-H^+]^-$ ions, and very low intensities of $[M-H^+]^-$ ions (Fig. 4d). $[M+HCOOH-H^+]^-$ ions at *m/z* 721 yielded fragment ions at *m/z* 513, 529 and 367 from loss of formic acid and Glu; formic acid and Rha; or all the three moieties simultaneously. We also observed $^{0,2}X$ fragmentation of sugar (Fig. 9l; *m/z* 409, Fig. 5c, d).

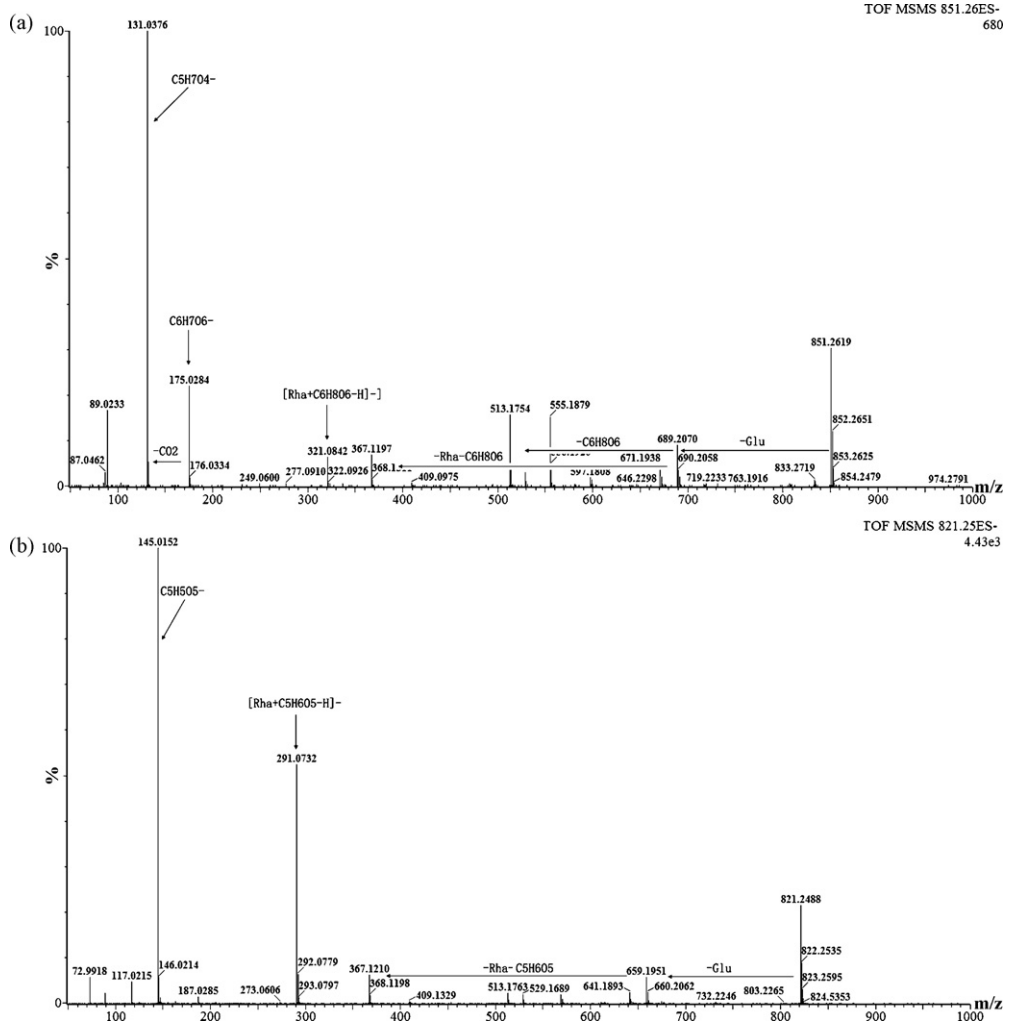


Fig. 7. MS^2 spectra of peak 19 and 31. (a) Peak 19; (b) peak 31.

Generally, PFG with the structures shown in Table 1 had different acidities, based on the phenolic hydroxyl (7,4′ -dihydroxyl > 7- or 4′-hydroxyl > 3-hydroxyl > 5-hydroxyl). Thus, in negative mode, PFG with aglycone B tended to lose the 7-sugar, creating a major fragment and base peak in MS/MS.

PFG with three major aglycones have been observed [2,3]. The three aglycones at C-3′ and C-4′ in the B ring have different substituents, with hydrogen and hydroxyl for aglycone A (calculated $[M-H^+]^-$ at *m/z* 353.1025), hydrogen and methoxyl for aglycone B (calculated $[M-H^+]^-$ at *m/z* 367.1182) and hydroxyl and methoxyl for aglycone C (calculated $[M-H^+]^-$ at *m/z* 383.1131) (Table 1). Based on the literature [3], if 7 or 4′ position is hydroxyl, aglycones or [M-7-sugar-H^+]$^-$ would appear as a base peak in MS/MS. These fragmentation behaviors, combined with the use of standards, could be used to identify PFG. The calculated *m/z* of [aglycone–H^+]$^-$ could be used to identify aglycones using our Q-TOF-MS conditions, and the 3-O, 7-O, or 3,7-diO position of the sugar might be identified by the abundance of the characteristic fragment.

3.2.1.2. Identification of PFG in fraction II. After selective enrichment, 51 compounds were identified as PFG in EK by Q-TOF-MS, with 11 PFG containing aglycone A, 36 containing aglycone B and 4 containing aglycone C (Fig. 2, Table 2). Of these, 11 compounds were new PFG, 9 containing acidity substituents and 2 containing nitrogen. Under Q-TOF-MS conditions, some neutral fragments or their forming ions might be observed, so some fragments formed by neutral losses [22] were also detected and new substituents were confirmed based on the fragments formed by neutral losses. Four known substituents (Fig. 9a–d) of PFG were identified, and four new substituents (Fig. 9e–h) of PFG were found. For identification of known PFG might base on the criteria described above, inference of new PFG would be emphasized.

Peak 29 (Fig. 2) gave $[M-H^+]^-$ ions at *m/z* 819.2691, corresponding to $C_{39}H_{47}O_{19}$. This allowed identification as a PFG with aglycone B, Rha-(1-2)dideoxyfuranose at the 3 position and Glu at the 7 position. The MS/MS data were consistent with the literature [3], except for fragment ions at *m/z* 289.0947 (Fig. 6a). This might result from neutral loss of Rha-(1-2)dideoxyfuranose (Rha-Ddf) at the 3 position, in which loss of a proton from Rha-Ddf generated ions at *m/z* 289.0947. Peak 8 (Fig. 2) was a new PFG that gave $[M-H^+]^-$ ions at *m/z* 805.2570, corresponding to $C_{38}H_{45}O_{19}$. This was identified as a PFG with aglycone A (Fig. 6b), and had similar fragmentation behavior to peak 29, except for the aglycone. In addition to identification as a PFG with aglycone A, we determined a Rha-Ddf at the 3 position and Glu at the 7 position. Peaks 17, 24, 27 and 30 (Fig. 2) were also new PFG, with $[M-H^+]^-$ ions at *m/z* 835.266, corresponding to $C_{39}H_{47}O_{20}$. The MS/MS spectrum of peak 30 was shown in Fig. 6c. This peak produced successively neutral losses of Glu, $C_6H_8O_5$, Rha or Glu, Rha-$C_6H_9O_5$, yielding aglycone B. $C_6H_8O_5$ might be obtained after neutral loss of $C_6H_{10}O_6$, so the probable $C_6H_{10}O_6$ compound

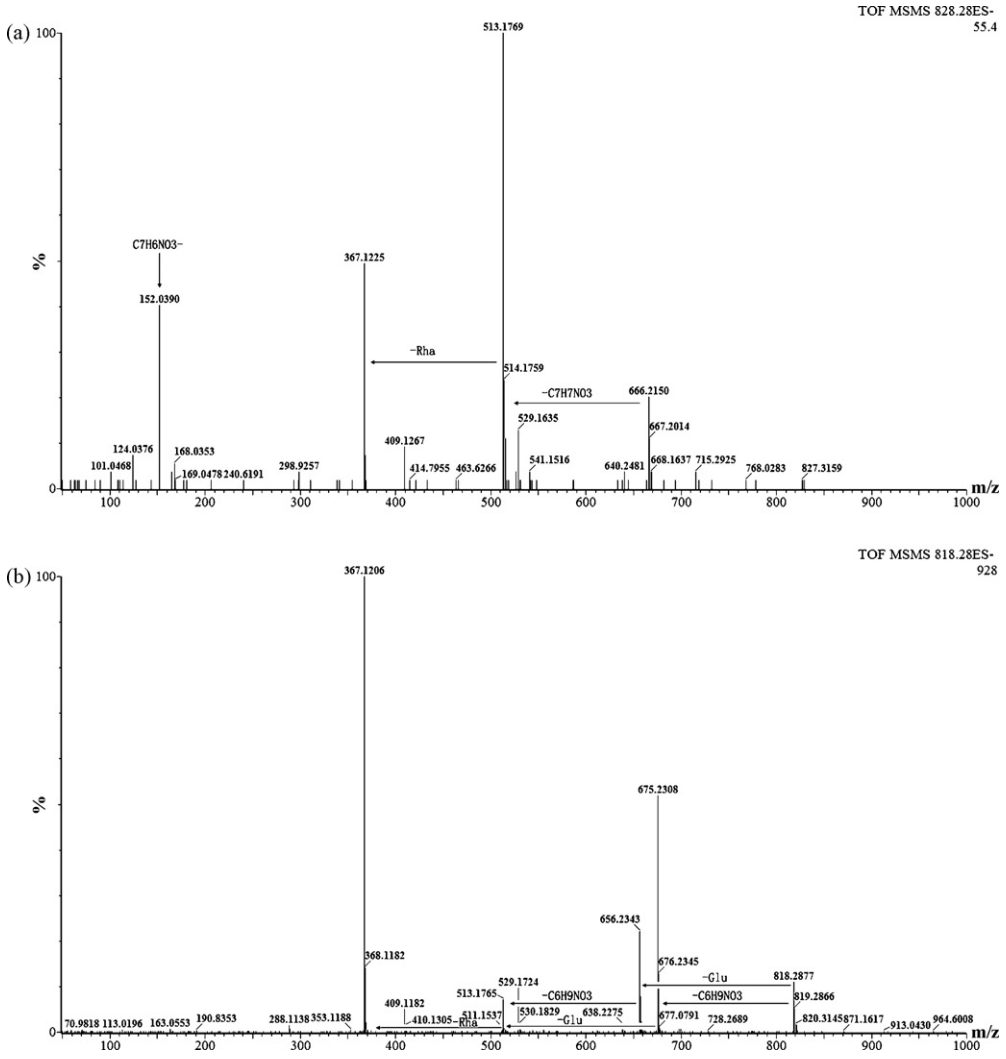


Fig. 8. MS^2 spectra of peak 37 and 6. (a) Peak 37; (b) peak 6.

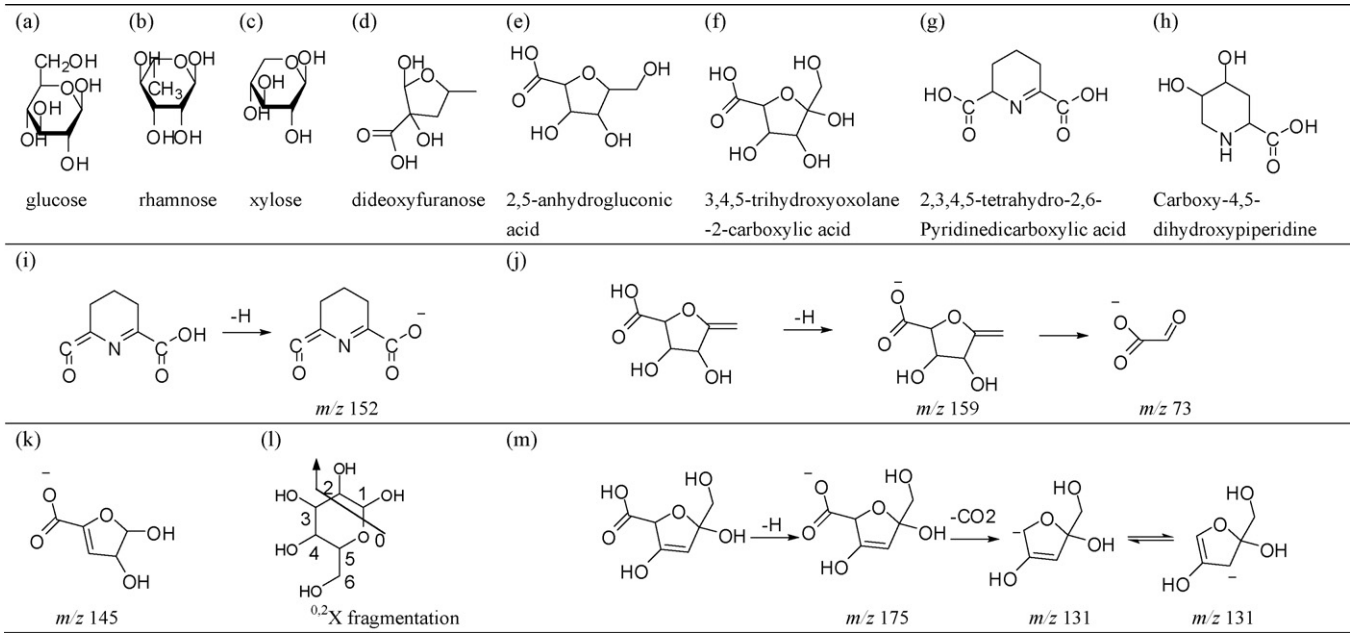


Fig. 9. Compounds forming substituents of PFG and probable fragmentation behavior of some neutral fragments.

was 2,5-anhydrogluconic acid (Aga, Fig. 9e). $C_6H_8O_5$ might lose a proton and yield ions at *m/z* 159.0390, yielding a stable fragment ion at *m/z* 72.9939 (Fig. 9j). Thus, the compound was deduced to be a PFG with aglycone B, Rha-Aga at the 3 position and Glu at the 7 position. Peaks 17, 24 and 27 might be isomers, and the different fragment intensities with *m/z* 305, 159 and 73 might result from different positions of connection. Similarly, peak 19, 20 and 21 (Fig. 2) gave $[M-H^+]^-$ ions at *m/z* 851.261, corresponding to $C_{39}H_{47}O_{21}$. These were distinguished from peak 30 because of more oxygen in the acidic substituent (Fig. 7a). This substituent produced $C_6H_8O_6$ by neutral loss, and was deduced to be fructuronate (Fru) based on an element composition of $C_6H_{10}O_7$. The neutral fragment $C_6H_8O_6$ might lose a proton to give ions at *m/z* 175.0284, then lose carbondioxide, yielding ions at *m/z* 131.0376 (Fig. 9m). Therefore, the three compounds were inferred to be PFG with aglycone B, Rha-Fru at the 3 position and Glu at the 7 position. Peak 31 (Fig. 2) gave $[M-H^+]^-$ ions at *m/z* 821.2493, corresponding to $C_{38}H_{45}O_{20}$. This produced successively neutral losses of Glu, $C_5H_6O_5$, Rha or Glu, Rha-$C_5H_6O_5$, yielding aglycone B. $C_5H_6O_5$ was formed by neutral losses of $C_5H_8O_6$. $C_5H_8O_6$ was deduced as 3,4,5-trihydroxyoxolane-2-carboxylic acid (Fig. 9f) based on the deprotonated ions at *m/z* 145.0152 (Fig. 9k).

Peak 37 and peak 6 (Fig. 2) were nitrogen derivatives of icariin. Peak 37 gave $[M-H^+]^-$ ions at *m/z* 828.2711, corresponding to $C_{40}H_{46}NO_{18}$, which had more of the $C_7H_8NO_3$ substituent than icariin. This compound produced successively neutral losses of Glu, $C_7H_7NO_3$ and Rha, and yielded aglycone B (Fig. 8a). $C_7H_7NO_3$ might form from $C_7H_9NO_4$ from neutral loss, so $C_7H_9NO_4$ was inferred as 2,3,4,5-tetrahydro-2,6- pyridinedicarboxylic acid (Fig. 9g). Furthermore, $C_7H_7NO_3$ could lose a proton and form the conjugative structure (Fig. 9i). Indeed, $[M-H^+]^-$ ions from $C_7H_7NO_3$ were observed at *m/z* 152.0390. Peak 6 gave $[M-H^+]^-$ ions at *m/z* 818.2877, corresponding to $C_{39}H_{48}NO_{18}$, which had more of the $C_6H_9NO_3$ substituent than icariin. This compound might yield neutral losses of $C_6H_9NO_3$ (Fig. 8b) in MS/MS, but if it had no $C_6H_9NO_3$, its fragment would be similar to icariin (Fig. 5d). The MS/MS spectrum showed that the difference between peak 6 and icariin was $C_6H_9NO_3$, which might be from $C_6H_{11}NO_4$. We determined the probable structure to be carboxy-4,5-dihydroxypiperidine (Fig. 9h).

3.2.2. *Identification of phenolic acids in fraction I by Q-TOF-MS*

Q-TOF-MS identified 18 compounds as PA in EK. Four were new compounds, and the others were CGA or their isomers. The fragmentation behaviors of monoacyl CGA is reported in detail [23,24]. We found that 5-monoacyl CGA gave a base peak at *m/z* 191 accompanied by a weak peak of [cinnamic acid–H^+]$^-$, while 4-monoacyl CGA yielded a base peak of [quinic acid–H_2O–H^+]$^-$ (*m/z* 173) accompanied by a peak of [cinnamic acid–H^+]$^-$ (relative intensity > 50%) and [quinic acid–H^+]$^-$ (*m/z* 191). The compound 3-monoacyl CGA produced either a base peak of [cinnamic acid–H^+]$^-$ or a base peak at *m/z* 191, accompanied by a peak of [cinnamic acid–H^+]$^-$ (relative intensity > 50%). Although the fragment intensities were slightly different when different MS instruments were used, the fragment ions at *m/z* 191, *m/z* 173, [cinnamic acid–H^+]$^-$ and their intensities could be used to identify monoacyl CGA. Three kinds of [cinnamic acid–H^+]$^-$ were observed by MS/MS (*m/z* 179 for [caffeic acid–H^+]$^-$, *m/z* 163 for [coumaric acid–H^+]$^-$ and *m/z* 193 for [ferulic acid–H^+]$^-$). The *trans* and *cis* isomers of monoacyl CGA showed similar fragmentation behaviors in MS/MS, but different retention behaviors on RPLC [25,26]. *Cis*-5-monoacyl CGA eluted later than the corresponding *trans* isomers, since they formed more hydrogen bonds, while the reverse was found for *cis*-3-monoacyl CGA and *cis*-4-monoacyl CGA. Therefore, CGA and their cis isomers could be identified by fragmentation behavior and RPLC retention times.

Peak 13 (Fig. 3b) was identified as trans-5-O-caffeoylquinic acid by comparison to standards. Peak 10 was identified as dimer of 5-O-caffeoylquinic acid based on $[M-H^+]^-$ ions at *m/z* 707.1832 (molecular formula $C_{32}H_{35}O_{18}$), and a base peak at *m/z* 191 by MS/MS (Table 3). Five isomers (peaks 4, 6, 8, 11 and 14) of *trans*-5-O-caffeoylquinic acid were also observed. Peaks 7, 9, 12, 15, 16 and 17 gave the same $[M-H^+]^-$ ions at *m/z* 337.0902, which corresponded to the molecular formula $C_{16}H_{17}O_8$ (*p*-coumaroylquinic acids). Peak 18 gave $[M-H^+]^-$ ions at *m/z* 367.1003 corresponding to the molecular formula $C_{17}H_{19}O_9$ (feruloylquinic acids). Thus, the 12 monoacyl CGA (peaks 4, 6–9, 11, 12, 14–18) were preliminarily identified based on their MS/MS behaviors. Peak 18 was identified as 5-O-feruloylquinic acid, peaks 4 and 6 as 3-O-caffeoylquinic acids, peaks 8 and 11 as 4-O-caffeoylquinic acids and peak 14 as 5-O-caffeoylquinic acid.

Similarly, peaks 7 and 9 were 3-O-*p*-coumaroylquinic acids, peaks 12 and 15 were 4-O-*p*-coumaroylquinic acids, and peaks 16 and 17 were 5-O-*p*-coumaroylquinic acids. We noted stronger [cinnamic acid–CO_2–H^+]$^-$ ions than that reported in the literature, including [caffeic acid–CO_2–H^+]$^-$ (*m/z* 135) and [coumaric acid–CO_2–H^+]$^-$ (*m/z* 119). Biosynthesis yields *trans* isomers, but *cis* isomers of CGA form from *trans* isomers after irradiation by ultraviolet light [25]. *E. koreanum* Nakai is a perennial herb plant, so it is irradiated by sunlight, which could generate *cis* isomers. These *cis* and *trans* isomers were identified by RPLC retention times (Table 3), and the main structures were found in Figures from the report [27].

The UV spectra of peaks 1, 2, 3 and 5 were consistent with *trans*-caffeic acid (UV_{max} at 323 nm). They gave [M−H^+]$^-$ ions at *m/z* 371.0647, corresponding to the molecular formula $C_{15}H_{15}O_{11}$, and produced peaks at *m/z* 209, 191 and 85 in MS/MS. They were thus inferred to be caffeoyl-hexaric acid. Monocaffeoyl-hexaric acids were rarely been reported, but dicaffeoyl-hexaric acids and tricaffeoyl-hexaric acid were identified in Yacon roots [28].

4. Conclusion

PFG and PA were selectively enriched by a Click OEG column under optimized conditions. Fraction I contained mainly PA, and fraction II contained mainly PFG. The two fractions were successfully identified by UPLC-UV combined with Q-TOF-MS. UPLC-Q-TOF-MS identified or tentatively identified 51 PFG from fraction II and 18 PA from fraction I. Three major aglycones were found in PFG, of which PFG with aglycone B were the major component. Two PFG with nitrogen substituents, and nine with acidic substituents were found as new compounds, and their probable structures were inferred by MS/MS fragmentation behavior. Four new PA were deduced, and seven PA were first reported in EK. Furthermore, CGA, along with their *cis* isomers and dimers were found to be the main PA in EK. Therefore, UPLC-Q-TOF-MS identification combined with selective enrichment was shown to be an efficient method for analyzing PFG and PA from complex samples.

Acknowledgements

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