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Rapid and reliable method for analysis of raw and honey-processed *astragalus* by UPLC/ESI-Q-TOF-MS using HSS T3 columns

Manshan Xiao,[a] Hongyuan Chen,*[b] Zhongfeng Shi,[a] Yifan Feng[a] and Wen Rui*[a]

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A novel method has been developed for rapid analysis of the major constituents in *Radix Astragali* and honey-processed *Astragalus* within 23 minutes using ultra-performance liquid chromatography coupled with electrospray ionization quadrupole time-of-flight mass spectrometry (UPLC/ESI-Q-TOF-MS). Separation analysis was performed on HSS T3 columns with a gradient elution of acetonitrile-water containing 0.1% formic acid at a flow rate of 0.3 mL min^{-1}. Methodology validation showed that the established method had good repeatability (RSD < 8.07%), intra-day precisions (RSD < 4.20%), and stability (RSD < 7.78%). Accurate molecular weight and characteristic fragment ions obtained from Q-TOF-MS provided a reliable criterion for the structural characterization of the major compounds in raw and honey-processed *Astragalus*. 35 major constituents, including flavonoids, isoflavan, pterocarpan, and saponins, were identified or tentatively characterized by comparing their retention times and MS spectra with those of authentic standards and literature data. Furthermore, quantitative analysis revealed that the content changes of chemical compositions could cause the tonic effect differences between raw and honey-processed *Astragalus*. The established method is rapid and reliable for simultaneous analysis of constituents in an extract of *Radix Astragali*. Moreover, this method will simplify further studies of metabolism and pharmacodynamics and will encourage the use of HSS T3 columns in similar traditional Chinese medicine research.

1. Introduction

Radix Astragali, which is the dried root of *Astragalus membranaceous (Fisch.) Bge.* and *Astragalus membranaceous (Fisch.) Bge.* var. *mongolicus (Bge.) Hsiao* (known as HuangQi in China), has been widely used as a tonic in traditional Chinese medicine for thousands of years.[1,2] It is specified to tonify Qi (vital energy), strengthen the exterior, induce diuresis to alleviate edema, and promote pus discharge and tissue regeneration.[3,4] Honey-processed *Astragalus* is a product of *Radix Astragali* mixed with honey by a traditional Chinese medicine processing method, which strengthens the tonic effect. In certain traditional Chinese medicine prescriptions, honey-processed *Astragalus* is used instead of *Radix Astragali* to achieve fewer side effects and improved efficacy in tonifying Qi. However, there is limited specialized research that elucidates the differences in tonic effects between raw and honey-processed *Astragalus*.

Phytochemical studies have shown that *Radix Astragali* contains various active ingredients, including isoflavonoids, saponins, and polysaccharides (APS).[5] Modern pharmacological studies of these active ingredients have revealed that *Radix Astragali* contains hepatoprotective,[6] antiviral, antioxidant,[7] cardiovascular-protective,[8] and immunostimulant properties.[9,10] Compared to raw *Radix Astragali*, honey-processed *Astragalus* is more effective in improving erythrocyte deformability and immunity[11] but is less effective in its antioxidant activity.[12,13] Therefore, it is critical to explore the pharmacological changes that occur after honey processing in *Radix Astragali*.

Existing methods for analyzing the chemical composition of honey-processed *Astragalus* have relied on conventional column chromatographic and spectroscopic procedures such as liquid chromatography with a UV detector.[14] However, these methods are very time-consuming and require large amounts of samples and solvents. Modern instrumental techniques, such as UPLC/Q-TOF-MS, and Internet-based analytical methods combine the efficient separation of liquid chromatography and the high sensitivity of mass spectrometry to offer remarkable advantages for the study of traditional Chinese herbs.[15,16] The high sensitivity of UPLC/Q-TOF-MS could facilitate the detection and analysis of the trace chemical components, which are difficult to obtain by conventional isolation methods.[17–19] The accurate molecular weight of the components provided by the high-resolution mass spectrometer can be used for peak

[a]*Centre Laboratory, Guangdong Pharmaceutical University, Guangzhou 510006, P. R. China. E-mail: r_wing@163.com*

[b]*Department of Immunology & Microbiology, Guangdong Pharmaceutical University, Guangzhou 510006, P. R. China. E-mail: HCHEN31@PARTNERS.ORG; Tel: +86-020-39352523*

identification and structural characterization.[20] The sensitivity, speed, and decreased amount of resources required for this technique make it a convenient and powerful method for analyzing the complex components of Chinese medicinal herb extracts.[21]

Previous experiments in our laboratory with UPLC/Q-TOF-MS on C18 columns showed that polar compounds could not be adequately separated on BEH C18 columns and that the co-elution peaks complicated and disrupted the analytical process.[22] Therefore, we aimed to simplify the separation and analytical processes, and we found that HSS T3 columns utilize the Waters Corporation's innovative and proprietary T3 bonding, which contains a trifunctional C18 alkyl phase bonded at a ligand density that promotes polar compound retention and aqueous mobile-phase compatibility. This study describes a novel, rapid, and sensitive method for analyzing and characterizing the constituents in aqueous extracts of *Radix Astragali* and honey-processed *Astragalus* by UPLC/ESI-Q-TOF-MS using HSS T3 columns, and this method allows us to control the quality of *Radix Astragali* and its honey-processed product. We also investigated the changes in *Radix Astragali* after honey-processing using this novel method.

2. Experimental

2.1. Materials and reagents

HPLC-grade acetonitrile, methanol, and isopropanol were acquired from Merck (Darmstadt, Germany). Formic acid of HPLC grade was purchased from CNW Technologies GmbH (Germany). The water was purified by a water purification system (18.2 MΩ, Sartorius, Germany).

Reference standards of calycosin-7-*O*-β-D-glucoside (111 920–201 203, purity > 97.3% by HPLC) and astragoloside IV (110 781–200 613, purity > 98% by HPLC) were obtained from the Guangdong Institute for Food and Drug Control (Guangzhou, China).

Astragalus membranaceus (Fisch.) Bge. Var. *mongholicus (Bge.) Hsiao* was purchased from Qingping Market (Guangzhou, China). The species was properly identified by Professor Jizhu Liu from Guangdong Pharmaceutical University. All the samples were preserved in our laboratory.

2.2. Standard solutions and sample preparation

The reference standards were accurately weighed and dissolved in methanol to prepare the stock mixture of reference compounds. The concentration of astragoloside IV and calycosin-7-*O*-β-D-glucoside in the stock solution were 22.25 μg mL^{-1} and 5.4 μg mL^{-1}, respectively, and the stock mixture was kept at 4 °C.

The same batch of medicinal herbs was divided into two parts: one for the analysis of raw *Radix Astragali*, and another for preparing the honey-processed *Astragalus* using the method described in the 2010 edition of Chinese Pharmacopoeia.[1]

The *Radix Astragali* and honey-processed *Astragalus* were crushed, accurately weighed (2 g), and extracted by refluxing with 20 mL water thrice for 1 hour each time. The three filtrates were consolidated and desiccated. The desiccate was redissolved to 0.25 g mL^{-1} (equivalent to the dry weight of the raw materials). The sample solutions were then centrifuged for 10 minutes at 12 000 rpm at 25 °C, and the supernatant was injected into the UPLC/Q-TOF-MS system.

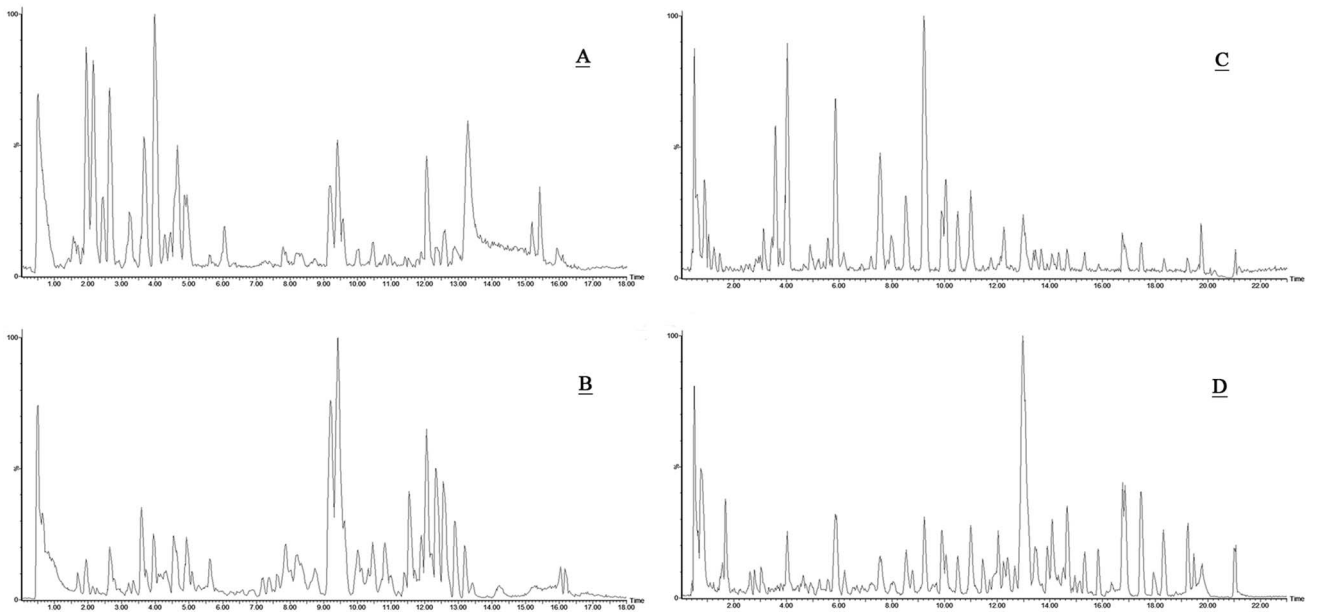


Fig. 1 The comparative chromatograms of water extract of *Radix Astragali*: (A) positive and (B) negative ion modes obtained by BEH C18 column; (C) positive and (D) negative ion modes obtained by HSS T3 column.

2.3. UPLC/Q-TOF-MS/MS conditions

UPLC/Q-TOF-MS/MS analysis was performed using an ACQUITY UPLC/Q-TOF microsystem (Waters Co., MA, USA). The UPLC separation was performed at 25 °C using an ACQUITY UPLC HSS T3 column (2.1 mm × 50 mm, 1.8 μm, Waters Co., MA, USA) with an ACQUITY UPLC HSS T3 VanGuard Pre-Column (2.1 mm × 5 mm, 1.8 μm, Waters Co., MA, USA). The mobile phase consisted of acetonitrile (A) and water with 0.1% (v/v) formic acid (B) with a gradient elution: 0–3 min, 5–20% A; 3–7 min, 20–20% A; 7–18 min, 20–50% A; 18–19 min, 50–100% A; 19–20 min, 100–100% A; 20–21 min, 100–5% A; 21–23 min, 5–5% A. The injection volume was 2 μL at a flow rate of 0.3 mL min^{-1}.

Mass detection was performed in the full scan mode at the *m*/*z* range from 100 to 1500 using both positive and negative ionization modes. The nebulizer gas (N_2) and the desolvation gas (N_2) were at flow rates of 50 L h^{-1} and 500 L h^{-1}, respectively. The source and desolvation temperatures were set to 100 °C and 350 °C, respectively. The capillary voltages were set to 3.0 kV, and the cone voltages were set to 30 V. The MS/MS experiments were performed using variable collision energy (15–45 eV), which was optimized for each individual compound.

Leucine enkephalin was used as the lock mass ($[M - H]^-$ at *m*/*z* 554.2615, $[M + H]^+$ at *m*/*z* 556.2771) to ensure mass accuracy and reproducibility at a flow rate of 0.01 mL min^{-1}. The lock spray frequency was set at 10 seconds.

All data acquisition and processing were conducted with Masslynx 4.1 software incorporated in the instrument.

2.4. UPLC method validation

We validated our method after the conditions of UPLC were optimized. The standard mixture solution was analyzed six times a day under the optimum conditions for intra-day variation to estimate precision and accuracy. Five different solutions, prepared as described in Section 2.2 by the same sample, were detected to check the repeatability.

The stability test was carried out at six different time points (0, 1, 2, 4, 6, and 8 h) with the same sample solution. The sample was prepared by the method described in Section 2.2. Stability tests were conducted to ensure whether the sample was stable during the analysis.

3. Results and discussion

3.1. Optimization of UPLC conditions

The chromatographic separation was performed on a UPLC system, which is based on the ACQUITY UPLC™ HSS T3 column (50 mm × 2.1 mm i.d., 1.8 μm.). A series of preliminary experiments was carried out on different mobile phases,

Table 1 Statistical results of the intra-day precision of the reference standards using the method ($n = 6$)

	Intra-day precision	
Investigated compounds	Relative retention time (RSD, %)	Relative peak area (RSD, %)
Calycosin-7-*O*-β-D-glucoside	0.26	4.20
Astragaloside IV	0.06	4.20

Table 2 Statistical results of the repeatability and stability test of the method

	Repeatability test ($n = 5$)		Stability test ($n = 6$)	
Investigated compounds	Relative retention time (RSD, %)	Relative peak area (RSD, %)	Relative retention time (RSD, %)	Relative peak area (RSD, %)
Calycosin-7-*O*-β-D-glucoside	0	2.32	0	2.32
Pratensein-7-*O*-glucoside	0.19	5.71	0.29	5.71
Calycosin-7-*O*-β-D-glucoside-6″-*O*-malonate	0.08	6.90	0.49	6.90
Ononin	0.06	0.43	0.12	0.43
9,10-Dimethoxypterocarpan-3-*O*-β-D-glucoside-2′-*O*-β-D-xylopyranosyl	0.19	4.55	0.10	4.55
9,10-Dimethoxypterocarpan-3-*O*-β-D-glucoside-2′-*O*-β-D-xylopyranosyl isomer	0.13	4.12	0.09	4.12
9,10-Dimethoxypterocarpan-3-*O*-β-D-glucoside	0.23	3.20	0.08	3.20
2′-Hydroxy-3′,4′-dimethoxy isoflavan-7-*O*-β-D-glucoside	0.14	6.95	0.09	6.95
Formononetin-7-*O*-β-D-glucoside-6″-*O*-malonate	0.09	7.90	0.08	7.90
(6aR,11aR)-9,10-dimethoxy pterocarpan-3-*O*-β-D-glucoside-6‴-*O*-malonate	0.13	1.89	0.05	1.89
Astragaloside IV	0.12	8.07	0.10	8.07
Astragaloside II	0.10	7.96	0.18	7.96
Astragaloside I	0.09	4.11	0.06	4.11
Isoastragaloside I	0.10	7.25	0.08	7.25

including acetonitrile–water, methanol–water, acetonitrile–0.1% formic acid, methanol–0.1% formic acid, and acetonitrile-isopropanol–water. The results showed that the mobile phase with a small amount of acid could accelerate the ionization of components to improve the peak shape and peak response intensity and restrain peak tailing in both the positive and

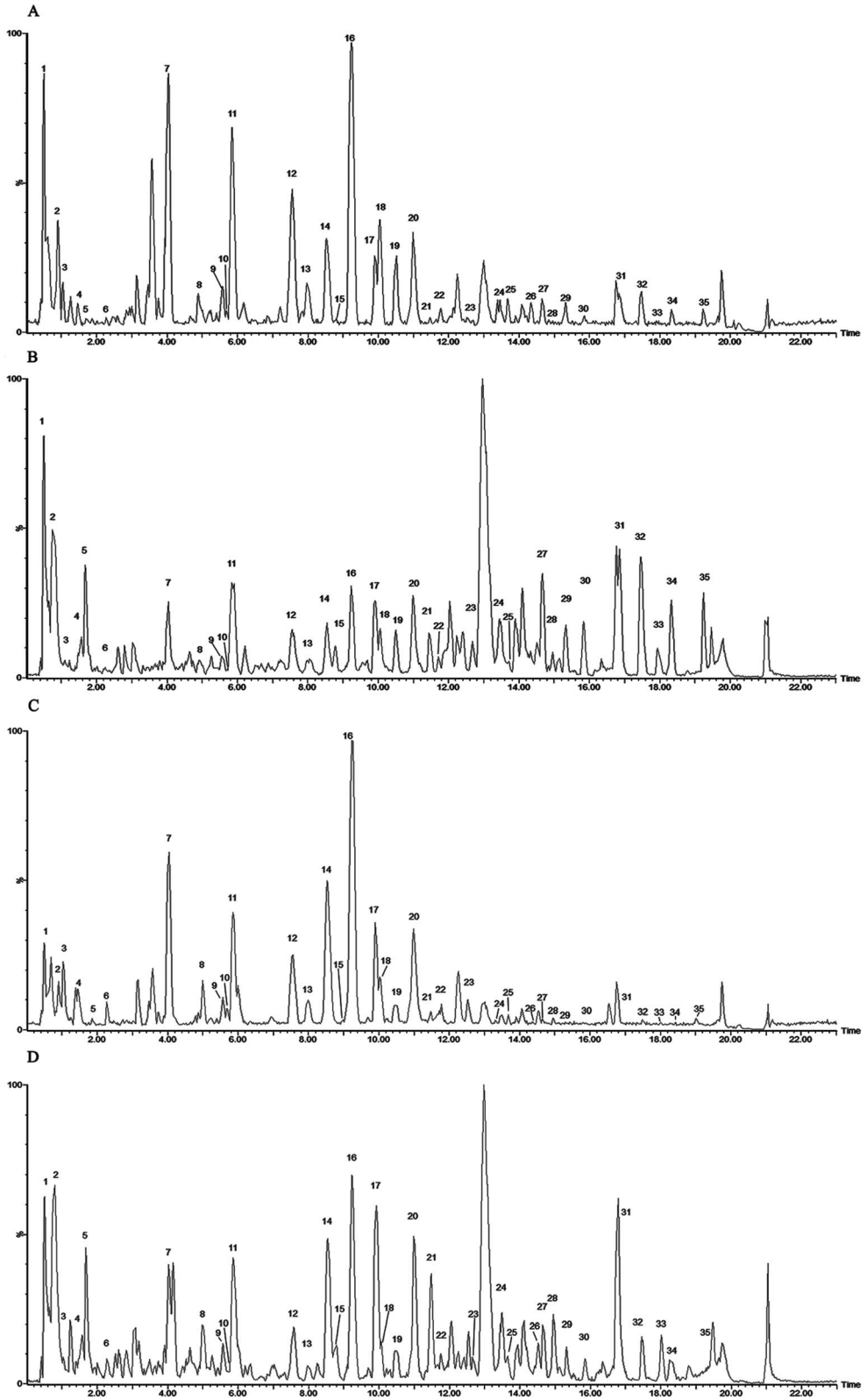


Fig. 2 The BPI chromatograms of water extract of *Radix Astragali*: (A) positive and (B) negative ion modes; and honey-processed *Astragalus*: (C) positive and (D) negative ion modes.

Table 3 Identification of the chemical constituents in water extracts of *Radix Astragali* and honey-processed *Astragalus* by UPLC–Q-TOF-MS/MS in positive and negative ion modes

No.	t_R (min)	Adduct ion	Measured value (*m*/*z*)	Calculated value (*m*/*z*)	Error (mDa)	Fragments	Formula	Identification
1	0.51	$[M + Na]^+$	365.1055	365.1060	−0.5	145	$C_{12}H_{22}O_{11}Na$	Sucrose[20]
		$[M - H]^-$	341.1044	341.1084	−4	179	$C_{12}H_{21}O_{11}$	
2	0.89	$[M + H]^+$	268.1006	268.1046	−4	136, 119	$C_{10}H_{14}N_5O_4$	Adenosine[20]
3	1.05	$[M + H]^+$	284.1036	284.0995	4.1	152	$C_{10}H_{14}N_5O_5$	Guanosine[20]
		$[M - H]^-$	282.0817	282.0838	−2.1	150, 133	$C_{10}H_{12}N_5O_5$	
4	1.57	$[M + Na]^+$	457.1327	457.1322	0.5	—	$C_{18}H_{26}O_{12}Na$	Markhamioside F[20]
		$[M - H]^-$	433.1312	433.1346	−3.4	301, 139	$C_{18}H_{25}O_{12}$	
5	1.65	$[M + Na]^+$	455.1161	455.1165	−0.4	—	$C_{18}H_{24}O_{12}Na$	Asperulosidic acid
		$[M - H]^-$	431.1197	431.119	0.7	—	$C_{18}H_{23}O_{12}$	
6	2.26	$[M + H]^+$	625.1757	625.1769	−1.2	463, 301	$C_{28}H_{33}O_{16}$	Complanatuside[23]
		$[M - H]^-$	623.1639	623.1612	2.7	461	$C_{28}H_{31}O_{16}$	
7	4.04	$[M + H]^+$	447.1277	447.1291	−1.4	285, 270, 253, 225, 137	$C_{22}H_{23}O_{10}$	Calycosin-7-*O*-β-D-glucoside[a] (ref. 4)
		$[M + COOH]-$	491.1151	491.119	−3.9	283, 239	$C_{23}H_{23}O_{12}$	
8	4.99	$[M + Na]^+$	649.2134	649.2108	2.6	303	$C_{29}H_{38}O_{15}Na$	Isomucronulatol-7,2′-di-*O*-glucoside[15]
		$[M - H]^-$	625.2127	625.2132	−0.5	301, 463	$C_{29}H_{37}O_{15}$	
9	5.56	$[M + H]^+$	463.1212	463.124	−2.8	301, 286	$C_{22}H_{23}O_{11}$	Pratensein-7-*O*-β-D-glucoside[20]
		$[M + COOH]^-$	507.1129	507.1139	−1	299, 284	$C_{23}H_{23}O_{13}$	
10	5.7	$[M + Na]^+$	471.1293	471.1267	2.6	287	$C_{22}H_{24}O_{10}Na$	Licoagroside D[10]
		$[M - H]^-$	447.1281	447.1291	−1	285	$C_{22}H_{23}O_{10}$	
11	5.84	$[M + H]^+$	533.1309	533.1295	1.4	285, 270, 253, 225	$C_{25}H_{25}O_{13}$	Calycosin-7-*O*-β-D-glucoside-6″-*O*-malonate[3]
		$[2M - H]^-$	1063.2401	1063.2356	4.5	283, 268	$C_{50}H_{47}O_{26}$	
12	7.56	$[M + H]^+$	431.1316	431.1342	−2.6	269, 226, 198, 137	$C_{22}H_{23}O_9$	Ononin[4,19]
		$[M + COOH]^-$	475.1238	475.124	−0.2	267, 252	$C_{23}H_{23}O_{11}$	
13	7.98	$[M + H]^+$	489.1417	489.1397	2	285, 270, 268, 225	$C_{24}H_{25}O_{11}$	Calycosin-7-*O*-β-D-glucoside-6″-*O*-acetate[17,24]
		$[M + COOH]^-$	533.1343	533.1295	4.8	283	$C_{25}H_{25}O_{13}$	
14	8.53	$[M + Na]^+$	617.187	617.1846	2.4	463, 301	$C_{28}H_{34}O_{14}Na$	(6aR,11aR)9,10-Dimethoxypterocarpan-3-*O*-β-D-glucoside-2′-*O*-β-D-xylopyranosyl[5]
		$[M - H]^-$	593.1876	593.187	0.6	299	$C_{28}H_{33}O_{14}$	
15	8.78	$[M + Na]^+$	617.1877	617.1846	3.1	301	$C_{28}H_{34}O_{14}Na$	(6aR,11aR)9,10-Dimethoxypterocarpan-3-*O*-β-D-glucoside-2′-*O*-β-D-xylopyranosyl isomer[5]
		$[M - H]^-$	593.1863	593.187	−0.7	299	$C_{28}H_{33}O_{14}$	
16	9.22	$[M + Na]^+$	485.1436	485.1424	1.2	301	$C_{23}H_{26}O_{10}Na$	(6aR,11aR)9,10-Dimethoxypterocarpan-3-*O*-β-D-glucoside[2]
		$[M + COOH]^-$	507.1502	507.1503	−0.1	299	$C_{24}H_{27}O_{12}$	
17	9.88	$[M + Na]^+$	487.1591	487.158	1.1	303	$C_{23}H_{28}O_{10}Na$	7,2′-Dihydroxy-3′,4′-dimethoxyisoflavan-7-*O*-β-D-glucoside[2]
		$[M - H]^-$	463.1613	463.1604	0.9	301	$C_{23}H_{27}O_{10}$	
18	10.06	$[M + H]^+$	285.0745	285.0763	−1.8	270, 253, 241, 225, 214, 137, 185	$C_{16}H_{13}O_5$	Calycosin[4]
		$[M - H]^-$	283.0608	283.0606	0.2	268, 239, 212	$C_{16}H_{11}O_5$	
19	10.51	$[M + H]^+$	517.1323	517.1346	−2.3	269, 267	$C_{25}H_{25}O_{12}$	Formononetin-7-*O*-β-D-glucoside-6″-*O*-malonate[3]
20	10.99	$[M + Na]^+$	571.1438	571.1428	1	301	$C_{26}H_{28}O_{13}Na$	(6aR,11aR)9,10-Dimethoxypterocarpan-3-*O*-β-D-glucoside-6″-*O*-malonate[17,24]
		$[2M - H]^-$	1095.302	1095.298	3.7	299	$C_{52}H_{55}O_{26}$	
21	11.45	$[M + Na]^+$	573.1591	573.1584	0.7	—	$C_{26}H_{30}O_{13}Na$	2′-Hydroxy-3′,4′-dimethoxyisoflavan-7-*O*-β-D-glucoside-6″-*O*-malonate[17,24]
		$[M - H]^-$	1099.327	1099.33	−2.4	301	$C_{52}H_{59}O_{26}$	
22	11.77	$[M + H]^+$	947.5251	947.5216	3.5	785, 653, 473, 455, 437	$C_{47}H_{79}O_{19}$	Astragaloside V/VI/VII[16,17]
		$[M + COOH]^-$	991.5161	991.5114	4.7		$C_{48}H_{79}O_{21}$	
23	12.67	$[M + H]^+$	989.5339	989.5321	1.8	457, 439	$C_{49}H_{81}O_{20}$	Agroastragaloside IV[16,17]
		$[M + COOH]^-$	1033.526	1033.522	3.8	—	$C_{50}H_{81}O_{22}$	
24	13.45	$[M + Na]^+$	807.4521	807.4507	1.4	605, 587, 473, 455, 437, 419	$C_{41}H_{68}O_{14}Na$	Astragaloside IV[b] (ref. 14, 16, 17 and 20)
		$[M + COOH]^-$	829.4623	829.4586	3.7	—	$C_{42}H_{69}O_{16}$	
25	13.71	$[M + H]^+$	269.081	269.0814	−0.4	254, 225, 197, 137	$C_{16}H_{13}O_4$	Formononetin[4,21]
		$[M - H]^-$	267.0653	267.0657	−0.4	252, 223, 195, 135	$C_{16}H_{11}O_4$	
26	14.42	$[M + H]^+$	1031.542	1031.543	−0.7	825, 651, 633, 871, 695	$C_{51}H_{83}O_{21}$	Agroastragaloside III[16]
		$[M + COOH]^-$	1075.5325	1075.5325	0	—	$C_{52}H_{83}O_{23}$	

Table 3 (*Contd.*)

No.	t_R (min)	Adduct ion	Measured value (*m*/*z*)	Calculated value (*m*/*z*)	Error (mDa)	Fragments	Formula	Identification
27	14.65	$[M + Na]^+$	849.4622	849.4612	1	647, 629, 611, 473, 455, 437, 419	$C_{43}H_{70}O_{15}Na$	Astragaloside II[14,16,17,20]
		$[M + COOH]^-$	871.4686	871.4691	−0.5	—	$C_{44}H_{71}O_{17}$	
28	14.93	$[M + H]^+$	943.5277	943.5266	1.1	797, 781, 763, 635, 617, 599	$C_{48}H_{79}O_{18}$	Soyasaponin I[16,17]
		$[M - H]^-$	941.5063	941.511	−4.7	—	$C_{48}H_{77}O_{18}$	
29	15.33	$[M + Na]^+$	849.4569	849.4612	−4.3	647, 629, 611, 473, 455, 437, 419	$C_{43}H_{70}O_{15}Na$	Isoastragalosides II[16,17]
		$[M + COOH]^-$	871.4686	871.4691	−0.5	—	$C_{44}H_{71}O_{17}$	
30	15.85	$[M + Na]^+$	849.4569	849.4612	−4.3	809, 647, 629, 611, 473, 455, 437, 419	$C_{43}H_{70}O_{15}Na$	Astragaloside II isomer[16]
		$[M + COOH]^-$	871.4704	871.4691	1.3	—	$C_{44}H_{71}O_{17}$	
31	16.86	$[M + Na]^+$	891.4721	891.4718	0.3	851, 833, 689, 671, 653, 473, 455, 437, 419	$C_{45}H_{72}O_{16}Na$	Astragaloside I[16,17]
		$[M + COOH]^-$	913.4798	913.4797	0.1	—	$C_{46}H_{73}O_{18}$	
32	17.47	$[M + Na]^+$	891.4761	891.4718	4.3	851, 833, 689, 671, 653, 455, 437, 419	$C_{45}H_{72}O_{16}Na$	Isoastragaloside I[16,17]
		$[M + COOH]^-$	913.4817	913.4797	2	—	$C_{46}H_{73}O_{18}$	
33	17.94	$[M + Na]^+$	977.4759	977.4722	3.7	775, 757, 739	$C_{48}H_{74}O_{19}Na$	Malonylastragalosides I[16]
		$[M - H]^-$	953.4776	953.4746	3	—	$C_{48}H_{73}O_{19}$	
34	18.32	$[M + Na]^+$	891.4739	891.4718	2.1	851, 689, 671, 653, 635, 617, 473, 455, 437, 419	$C_{45}H_{72}O_{16}Na$	Astragaloside I isomer[16]
		$[M + COOH]^-$	913.4798	913.4797	0.1	—	$C_{46}H_{73}O_{18}$	
35	19.22	$[M + Na]^+$	933.4854	933.4824	3	893, 731, 713, 695, 677, 455, 437, 419	$C_{47}H_{74}O_{17}Na$	Acetylastragaloside[16]
		$[M + COOH]^-$	955.4871	955.4903	−3.2	—	$C_{48}H_{75}O_{19}$	

[a] Were used as reference standards. [b] The sequence numbers in the table correspond to the label of each peak in Fig. 2.

negative ion modes. A mobile phase containing acetonitrile could obtain improved peak shape. The best separation was obtained by using acetonitrile–water with 0.1% formic acid by gradient elution. The comparative chromatograms obtained by using BEH C18 columns and HSS T3 columns were shown in Fig. 1. As observed from Fig. 1, HSS T3 columns showed better separation effect.

3.2. Precision, repeatability, and stability

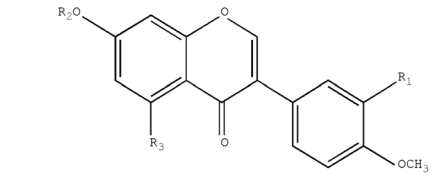
The RSD of relative retention time and relative peak area was used as the criterion for the validation of this method. The intra-day precision was assessed by six consecutive injections of reference standards. The RSD of relative retention time and relative peak area for intra-day precision was less than 0.26% and 4.20%, respectively. The repeatability was assessed by detecting the five different solutions prepared by the same sample with the same method. **14** compounds, which were chosen by the criterion of peak-to-peak amplitude values greater than 200, were used to evaluate the stability and repeatability. The RSD of relative retention time and relative peak area for repeatability was less than 0.23% and 8.07%, and he RSD of relative retention time and relative peak area for stability was less than 0.49% and 8.07%. These results indicated that the method was reliable and useful for analysis of *Radix Astragali* and honey-processed *Astragalus*. The results are shown in Tables 1 and 2.

3.3. UPLC/Q-TOF-MS/MS analysis

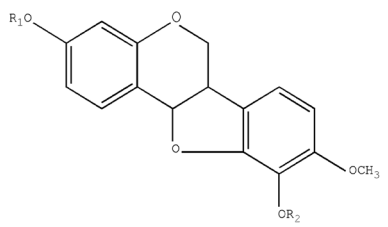
The constituents of *Radix Astragali* and honey-processed *Astragalus* were analyzed by UPLC/Q-TOF-MS using an ESI ion source. As a "soft" ionization technique, ESI generally generates molecular ions, adduct ions, and rarely produces fragment ions. ESI-TOF-MS could provide accurate molecular mass, which would aid in the identification of the components. The raw data files were handled with MassLynx 4.1 software, which provided the elemental compositions and the mass errors. The elemental compositions were accepted only when the deviation was within 5 mDa.

The base peak intensity (BPI) chromatograms, both in positive and negative ion modes of *Radix Astragali* and honey-processed *Astragalus*, are presented in Fig. 2. The reference compounds were initially analyzed to obtain the retention time and characteristic fragmentation pathway data prior to observing the samples. We then identified the compounds of *Radix Astragali* and honey-processed *Astragalus* by comparing the retention time and mass spectra data with those of the standards and the literature data, and the results are shown in Table 3. The chemical structures of compounds identified in *Radix Astragali* and honey-processed *Astragalus* are shown in Fig. 3.

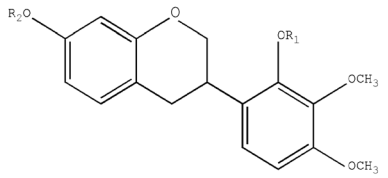
35 compounds, primarily including isoflavonoids, isoflavan, pterocarpan, and saponins, were identified in both of the extracts of *Radix Astragali* and honey-processed *Astragalus*. Fragment data from the MS/MS spectra were used to further confirm the components of the compounds.



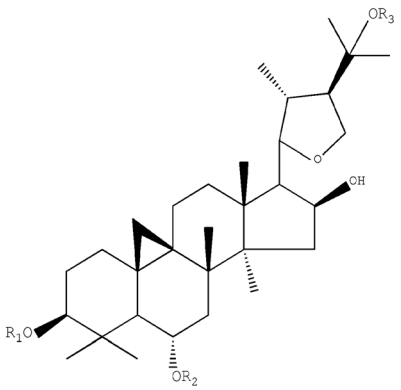
Calycosin-7-O-β-D-glucoside:R_1=OH,R_2=β-D-glc,R_3=H
Calycosin-7-O-β-D-glucoside-6"-O-malonate:R_1=OH,R_2=6"-O-malonyl-β-D-glc,R_3=H
Calycosin-7-O-β-D-glucoside-6"-O-acetate:R_1=OH,R_2=6"-O-acetyl-β-D-glc,R_3=H
Calycosin:R_1=OH,R2=,R_3=H
Ononin:R1=H,R2=β-D-gl,R3=H
Formononetin-7-O-β-D-glucoside-6"-O-malonate:R_1=H,R_2=6"-O-malonyl-β-D-glc,R_3=H
Formononetin:R_1=H,R_2=,R3=H
Pratensein-7-O-glucoside:R_1=OH,R_2=β-D-glc,R_3=OH



Licoagroside D:R_1=β-D-glc,R_2=H
9,10-dimethoxypterocarpan-3-O-β-D-glucoside-2'-O-β-D-xylopyranosyl:R_1=2'-O-β-D-xylopyranosyl-β-D-glc,R_2=CH_3
9,10-dimethoxypterocarpan-3-O-β-D-glucoside: R_1=β-D-glc,R_2=CH_3
(6aR,11aR)-3-hydroxy-9,10-dimethoxypterocarpan-3-O-β-D-glucoside-6"-O-malonate: R_1= 6"-O-malonyl-β-D-glc,R_2=CH_3



Isomucronulatol-7,2'-di-O-glucoside:R_1=β-D-glc,R_2=β-D-glc
2'-hydroxy-3',4'-dimethoxyisoflavan-7-O-β-D-glucoside: R_1=H, R_2=β-D-glc
7,2'-dihydroxy-3',4'-dimethoxyisoflavan-7-O-β-D-glucoside-6"-O-malonate: R_1=H, R_2=6"-O-malonyl-β-D-glc



Astragaloside IV:R_1=β-D-xyl,R_2=β-D-glc,R_3=H
Astragaloside II:R_1=2'-O-Ac-β-D-xyl, R_2=β-D-glc, R_3=H
Astragaloside I:R_1=2',3'-di-O-Ac-β-D-xyl, R_2=β-D-glc, R_3=H
Isoastragaloside I:R_1=2',4'-di-O-Ac-β-D-xyl, R_2=β-D-glc, R_3=H
Acetylastragaloside:R_1=2',3',4'-tri-O-Ac-β-D-xyl, R_2=β-D-glc, R_3=H
Agroastragaloside III: R_1=2',3'-di-O-Ac-β-D-xyl, R_2=β-D-glc, R_3=β-D-glc
AgroastragalosideIV: R_1=2'-O-Ac-β-D-xyl, R_2=β-D-glc, R_3=β-D-glc

Fig. 3 The chemical structures of compounds identified in *Radix Astragali* and honey-processed *Astragalus*.

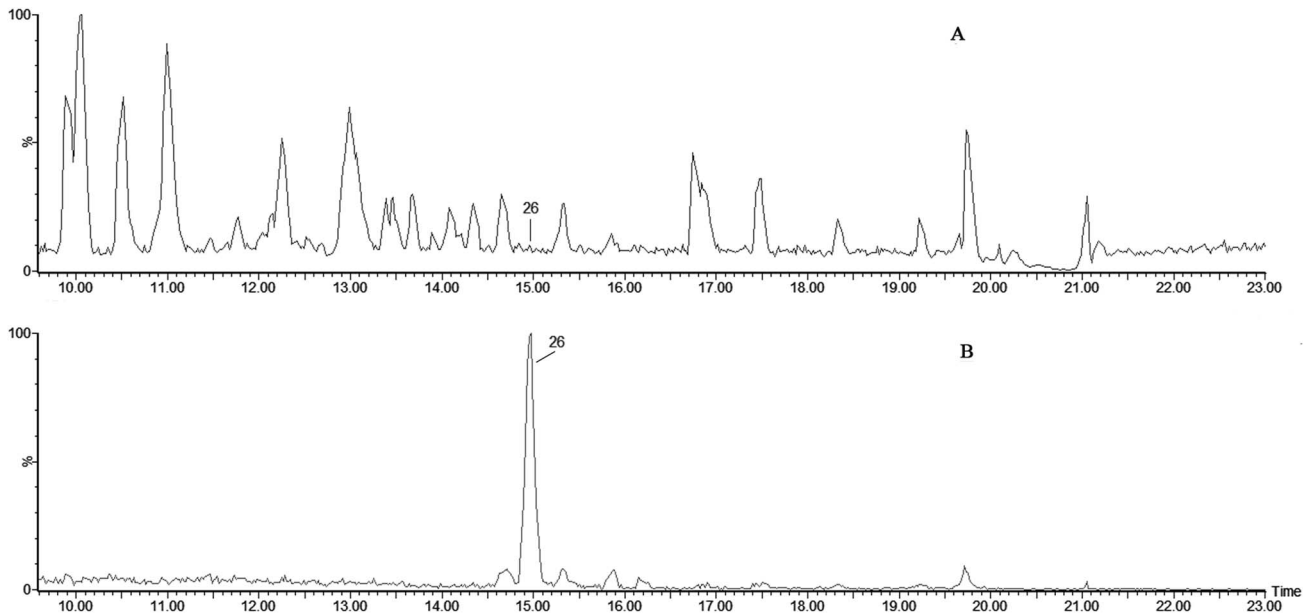


Fig. 4 The (A) BPI and (B) EIC of peak 26 in positive ion mode.

3.3.1 Identification of main isoflavonoids (*e.g.*, isoflavones, astraisoflavans and pterocarpans). The mass spectral characteristics of isoflavonoids have been extensively studied. Flavonoids usually contain *O*-glycoside and methylated phenol groups. They often lose a glycoside (162 Da), malonylglucosyl (248 Da), or acetylglucosyl (204 Da) group as the initial fragments. Some neutral fragments such as CH_3 (15 Da), CO (28 Da), H_2O (18 Da), and CO_2 (44 Da) were found in succession in MS/MS spectra. The Ret-Diels–Alder (RDA) fragmentation mechanism was found in almost all of the flavonoids and isoflavonoids detected.[20,24,25]

Compound **7** was definitely identified as calycosin-7-*O*-β-D-glucoside by its retention time and the MS data compared with the authentic standard. The product ion at *m*/*z* 285 ($C_{16}H_{13}O_5$) in the positive ion mode corresponded to the neutral loss of a hexose residue (162 Da), and then the fragment ions at *m*/*z* 270 and *m*/*z* 253 *via* the neutral loss of a CH_3 (15 Da) and CO (28 Da), respectively. Similarly, compound **11** showed the molecular ion at *m*/*z* 533 in the positive ion mode and the product ion at *m*/*z* 285 ($C_{16}H_{13}O_5$), which corresponded to a loss of malonylglucosyl (248 Da). Then, the *m*/*z* 285 ($C_{16}H_{13}O_5$) ion generated the same product ions as that of calycosin; thus, compound **11** was tentatively identified as calycosin-7-*O*-β-D-glucoside-6″-*O*-

Table 4 The percentage of relative peak areas of the identified components in *Radix Astragali* and honey-processed *Astragalus* in the positive (+) ion mode

Peak no.	Formula	Identification	Classification	Peak area in *Radix Astragali* (%)	Peak area in honey-processed *Astragalus* (%)
1	$C_{12}H_{22}O_{11}$	Sucrose	—	1.158	2.139
2	$C_{10}H_{13}N_5O$	Adenosine	—	2.480	4.242
3	$C_{10}H_{13}N_5O$	Guanosine	—	0.993	2.624
4	$C_{18}H_{26}O_{12}$	Markhamioside F	—	0.348	0.342
5	$C_{18}H_{24}O_{12}$	Asperulosidic acid	—	0.219	0.217
6	$C_{28}H_{32}O_{16}$	Complanatuside	Isoflavone	0.797	0.864
7	$C_{22}H_{22}O_{10}$	Calycosin-7-*O*-β-D-glucoside	Isoflavone	29.796	37.927
8	$C_{29}H_{38}O_{15}$	Isomucronulatol-7,2′-di-*O*-glucoside	Isoflavan	0.532	0.656
9	$C_{22}H_{22}O_{11}$	Pratensein-7-*O*-glucoside	Isoflavone	1.848	1.383
10	$C_{22}H_{24}O_{10}$	Licoagroside D	Pterocarpan	0.413	0.499
11	$C_{25}H_{24}O_{13}$	Calycosin-7-*O*-β-D-glucoside-6″-*O*-malonate	Isoflavone	18.660	0.921
12	$C_{22}H_{22}O_9$	Ononin	Isoflavone	7.619	10.206
13	$C_{24}H_{24}O_{11}$	Calycosin-7-*O*-β-D-glucoside-6″-*O*-acetate	Isoflavone	5.309	5.134
14	$C_{28}H_{34}O_{14}$	9,10-Dimethoxypterocarpan-3-*O*-β-D-glucoside-2′-*O*-β-D-xylopyranosyl	Pterocarpan	1.446	2.082
15	$C_{28}H_{34}O_{14}$	9,10-Dimethoxypterocarpan-3-*O*-β-D-glucoside-2′-*O*-β-D-xylopyranosyl isomer	Pterocarpan	0.459	0.522
16	$C_{23}H_{26}O_{10}$	9,10-Dimethoxypterocarpan-3-*O*-β-D-glucoside	Pterocarpan	3.564	4.784
17	$C_{23}H_{28}O_{10}$	2′-Hydroxy-3′,4′-dimethoxyisoflavan-7-*O*-β-D-glucoside	Isoflavan	1.665	2.068
18	$C_{16}H_{12}O_5$	Calycosin	Isoflavone	9.129	8.763
19	$C_{25}H_{24}O_{12}$	Formononetin-7-*O*-β-D-glucoside-6″-*O*-malonate	Isoflavone	4.341	3.178
20	$C_{26}H_{28}O_{13}$	(6aR,11aR)-3-hydroxy-9,10-dimethoxypterocarpan-3-*O*-β-D-glucoside-6‴-*O*-malonate	Pterocarpan	1.391	1.352
21	$C_{26}H_{30}O_{13}$	7,2′-Dihydroxy-3′,4′-dimethoxyisoflavan-7-*O*-β-D-glucoside-6″-*O*-malonate	Isoflavan	0.743	0.645
22	$C_{47}H_{78}O_{19}$	Astragaloside V/VI/VII	Saponin	1.119	1.914
23	$C_{49}H_{80}O_{20}$	AgroastragalosideIV	Saponin	0.189	0.291
24	$C_{41}H_{68}O_{14}$	Astragaloside IV	Saponin	0.288	0.342
25	$C_{16}H_{12}O_4$	Formononetin	Isoflavone	1.500	1.971
26	$C_{51}H_{82}O_{21}$	Agroastragaloside III	Saponin	0.109	0.123
27	$C_{43}H_{70}O_{15}$	Astragaloside II	Saponin	0.465	0.588
28	$C_{48}H_{78}O_{18}$	Soyasaponin I	Saponin	0.964	1.087
29	$C_{43}H_{70}O_{15}$	Isoastragalosides II	Saponin	0.238	0.297
30	$C_{43}H_{70}O_{15}$	Astragaloside II isomer	Saponin	0.223	0.228
31	$C_{45}H_{72}O_{16}$	Astragaloside I	Saponin	0.636	0.913
32	$C_{45}H_{72}O_{16}$	Isoastragaloside I	Saponin	0.757	0.978
33	$C_{48}H_{74}O_{19}$	Malonylastragalosides I	Saponin	0.074	0.048
34	$C_{45}H_{72}O_{16}$	Astragaloside I isomer	Saponin	0.312	0.374
35	$C_{47}H_{74}O_{17}$	Acetylastragaloside	Saponin	0.215	0.345
			Total saponins	5.591	7.479
			Total isoflavonoids	78.999	70.348
			Total isoflavan	2.940	3.369

malonate. Analogous MS/MS data appeared in compound **13**, which was identified as calycosin-7-*O*-β-D-glucoside-6″-*O*-acetate.

3.3.2 Identification of saponins. Most of the saponins in *Radix Astragali* and honey-processed *Astragalus* are tetracyclic triterpenoids with a 20,24-epoxy-9,19-cyclolanostane-3,6,16,25-tetrol skeleton. A saponin often contains more than one sugar molecule. Almost all saponins only generated typical adducts $[M - H + HCOOH]^-$ or $[M - H]^-$ with high intensity and rarely fragmented even at a high collision voltage of 45 V in the negative ion mode, while in the positive ion mode, abundant characteristic fragment ions were presented at a low collision voltage of 15 V. These characteristic ions were used for the identification of saponins. Because they contain the same skeleton, saponins presented a series of characteristic fragment ions, including the residues at *m*/*z* 473, 455, 437, and 419, which were produced from the simultaneous loss of a xylose and hexose residue and the sequential loss of H_2O.[16,24] Other saponins were identified or characterized with the data of the reference compounds, literature, and fragmentation patterns.

For example, compound **24** was definitely identified as Astragaloside IV by comparison with the authentic standard. The $[M + Na]^+$ at *m*/*z* 807 and the $[M - H + HCOOH]^-$ at *m*/*z* 829 determined the formula $C_{41}H_{68}O_{14}$. The fragment ions at *m*/*z* 473, 455, 437 and 419 in the positive ion mode were due to the successive losses of several H_2O molecules from the aglycone moiety produced by the losses of xylose and hexose residues.

3.3.3 The retention regularity of compounds on T3 chromatographic columns. The compounds retained on the HSS T3 columns presented certain regularity (Table 3). Flavonoids were eluted from the columns prior to the saponins, and the flavonoids and saponins retained on the columns were quite different because the number of polar groups on the parent nucleus of the flavonoids is larger than that of the saponins, and the number of carbon rings of the parent nucleus of the saponins is more than that of the flavonoids. These factors resulted in the polarity of flavonoids being larger than saponins. The greater the polarity of the compound was, the weaker was that reserved on the chromatographic column. However, formononetin was an exception because it was eluted with saponins, and our results were consistent with those in the literature.[14]

3.4. Quantitative analysis of the constituents in raw and honey-processed *Radix Astragali*

The relative peak area was used as the criterion for the quantitative analysis of the constituents. The relative peak area is the peak area ratio of each single compound to all the 35 constituents identified, which could reflect the relative contents of each compound. Although seen from the base peak intensity (BPI) chromatograms, some of the 35 compounds were not well baseline separated, the quantification is automatically integrated by the extracted ion chromatogram (EIC). For example, the peak 26 was not base-line separated in the BPI chromatogram, whereas it was well separated in extracted ion chromatogram (EIC). The contrasting result was shown in Fig. 4.

The relative peak areas of the identified constituents are shown in Table 4. The variation in the content of each compound was characterized by comparing the relative peak areas of compounds in the water extracts of *Radix Astragali* and honey-processed *Astragalus*. The results indicated that the content of isoflavonoids was decreased, whereas those of sucrose, pterocarpan, isoflavan and saponins were increased slightly after honey-processing. The content of calycosin-7-*O*-β-D-glucoside-6″-*O*-malonate was dramatically decreased, whereas calycosin-7-*O*-β-D-glucoside increased after processing. Honey-processing is a heating procedure, which increases the elimination of malonyl, and causes a reduction in the content of calycosin-7-*O*-β-D-glucoside-6″-*O*-malonate after the procedure.[26]

4. Conclusion

We developed a novel, sensitive, and reliable UPLC/ESI-Q-TOF-MS method for the rapid analysis of *Radix Astragali* and honey-processed *Astragalus*. Our experiments showed that, in contrast to separation on BEH C18 columns, the HSS T3 columns provided enhanced peak shapes and resolutions. The established method presented excellent repeatability, precision, and stability in the application of sample analysis. We detected and identified 35 compounds in the aqueous extracts of *Radix Astragali* and honey-processed *Astragalus* with this method. Furthermore, quantitative analysis revealed that the content of isoflavonoids was reduced, whereas saponins were slightly increased after honey-processing. The pharmacological activities of honey-processed *Astragalus* are linked to the changes of its chemical compositions. It has been reported that saponins in *Radix Astragali* could enhance the immune functions of macrophages in a dose-dependent manner.[27] Therefore, the increase in the content of saponins after honey-processing may contribute to enhanced immunity. The theory of traditional Chinese medicine believes that the Qi-tonifying effect is associated with improved immune function, including the enhanced immunity of mucous membranes, activation of T- and B-lymphocytes, and regulation of the innate immune response.[28] The *in vitro* antioxidant activities of *Radix Astragali* are predominantly associated with the flavonoids.[29] The reduction of the content of total flavonoids in honey-processed *Radix Astragali* may decrease its antioxidant benefits. Our results support the rationale for clinical applications of raw and honey-processed *Astragalus* in traditional Chinese medicine prescriptions. This novel method is a highly useful technique to identify constituents and control the quality of *Radix Astragali* and honey-processed *Astragalus* and offers incredible advantages, including speed, simplicity, sensitivity, selectivity, and a reduction in solvent consumption.

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