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Chun-Yan Niu[a]; Cai-Sheng Wu[a]; Yu-Xin Sheng[a]; Jin-Lan Zhang[a]
[a] Key Laboratory of Bioactive Substances and Resources Utilization of Chinese Herbal Medicine, Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing, China

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Identification and characterization of flavonoids from semen zizyphi spinosae by high-performance liquid chromatography/linear ion trap FTICR hybrid mass spectrometry

Chun-Yan Niu, Cai-Sheng Wu, Yu-Xin Sheng and Jin-Lan Zhang*

Key Laboratory of Bioactive Substances and Resources Utilization of Chinese Herbal Medicine, Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100050, China

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Semen zizyphi spinosae (SZS) has been used to treat insomnia and anxiety for thousands of years. In this paper, a novel high-performance liquid chromatography coupled with the photodiode array detector/linear ion trap-MS^n (HPLC-PDA/LTQ-MS^n) method was established to separate and identify flavonoids from the extract of SZS. Separation was performed on an HYPERSIL C_{18} column by gradient elution using CH_3CN/H_2O-CH_3COOH as the mobile phase at a flow rate of 0.8 ml/min. UV spectral data, accurate molecular weights, and multi-stage MS/MS fragmentation information were obtained. Electrospray ionization/MS/MS fragmentation patterns were proposed. Nineteen flavonoid glycosides were identified or tentatively characterized based on their retention time, UV spectral data, accurate molecular weights, and mass fragmentation behavior. The method was useful for separation and identification of the flavonoid components from SZS and could be applied to other complex samples, especially for minor constituents.

Keywords: semen zizyphi spinosae; HPLC-PDA/LTQ-MS^n; flavonoid glycosides; multi-stage mass fragmentation; constituent identification

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1. Introduction

Semen zizyphi spinosae (SZS), the mature seed of *Zizyphus jujuba* var. *spinosa* (Bunge) Hu.et H.f. Chou, is a well-known traditional Chinese medicine for the treatment of insomnia and anxiety [1]. Previous chemical investigations resulted in the isolation of bioactive components, including flavonoids, saponins, and fatty acids [2,3]. Modern pharmacological studies have demonstrated that flavonoids from SZS are the main bioactive compounds responsible for the sedative and hypnotic effects [4–6]. Structural elucidation and analysis of the flavonoids from SZS are important for its quality control and active mechanism.

Several groups have reported the isolation and identification of various compounds from SZS [2,7–9]. Thirteen flavonoids have been isolated and identified (Figure 1). High-performance liquid chromatography (HPLC), coupled with mass spectrometry (LC/MS), has been used to analyze three of the main flavonoids in SZS [4,10,11]. However, reports on the mass characterization and fragmentation of the flavonoids in the extract of SZS by LC/MS/MS are rare. In this paper, HPLC was carried out

*Corresponding author. Email: zhjl@imm.ac.cn

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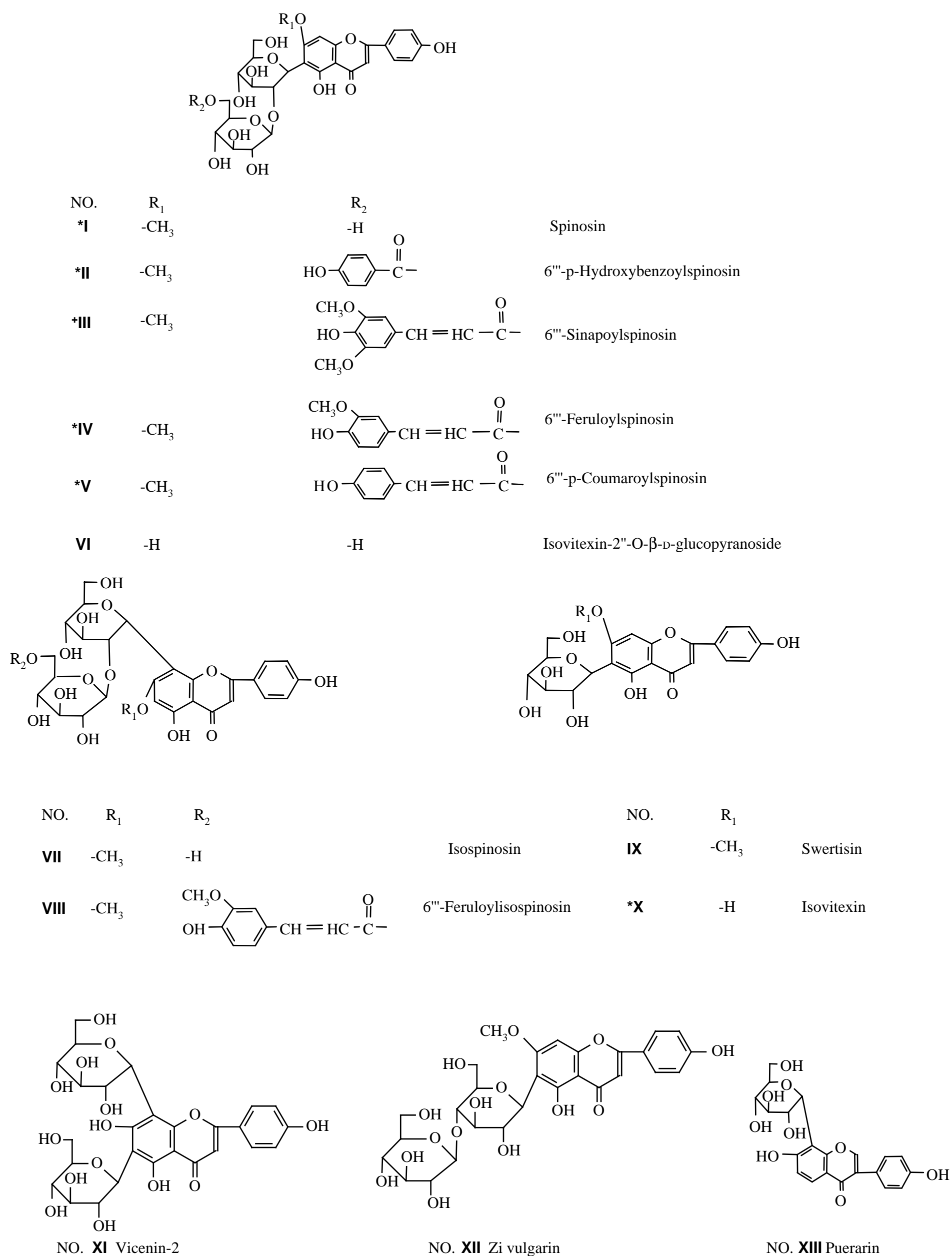

Figure 1. Chemical structures of 13 flavonoids from SZS. '*' denotes compounds **I**, **II**, **IV**, **V**, and **X**, isolated and identified from SZS. ' + ' denotes compound **III**, identified from SZS.

to separate the flavonoids from the extract of SZS. The separated flavonoids were then analyzed by a linear ion trap FTICR hybrid mass spectrometer equipped with an electrospray ionization (ESI) ion source to rapidly acquire their MS information. UV spectra, pseudo-molecular weights with highest mass accuracies less than 3 ppm in most cases, and multi-stage MS/MS fragmentation information were obtained for the flavonoids from the extract of SZS. The mass fragmentation rules of

known SZS flavonoids were summarized and used to identify other flavonoids from the extract of SZS. A total of 19 flavonoids were detected from the extract of SZS. Ten flavonoids were identified based on comparison with reference data and standards and one of them has possibly not been identified before. Nine compounds were tentatively characterized as flavonoids that have not been reported previously.

2. Results and discussion

2.1 The representative HPLC/FTICR-MS chromatogram of flavonoids in the SZS extract

To achieve good separation and a strong total ion current (TIC), a mobile phase consisting of acetonitrile and 0.1% aqueous acetic acid was chosen as the eluting solvent system. The addition of acetic acid also improved efficiency and selectivity of main peaks.

The representative HPLC/FTICR-MS chromatograms of the SZS extract and five authentic compounds are presented in Figure 2. Nineteen peaks were detected and they showed two absorption maxima at 220–280 and 300–400 nm in the UV spectra. Pseudo-molecular weights with highest mass accuracy of less than 3 ppm and multi-stage MS/MS fragmentation information were obtained for these 19 compounds (Tables 1 and 2). Five peaks of these compounds (**4**, **5**, **8**, **13**, and **14**) had similar retention behavior, similar UV spectra, and accurate mass data to five authentic compounds. So, these compounds could be confirmed as spinosin (peak **4**), isovitexin (peak **5**), 6‴-*p*-hydroxybenzoylspinosin (peak **8**), 6‴-feruloylspinosin (peak **13**), and 6‴-*p*-coumaroylspinosin (peak **14**).

2.2 Characteristic mass spectrometry and the fragmentation patterns of five authentic compounds

In order to obtain the fragmentation patterns and identify the other compounds in the SZS extract, the high accuracy mass spectra and multi-stage MS/MS fragmentation information for five authentic compounds were carefully analyzed. Five authentic compounds had similar structural skeletons with different substituting groups at C-6‴. According to reference [12], only isovitexin was exclusively flavonoid C-glycoside with one saccharide group, while the other four compounds were *O*-glycosyl-*C*-glycosyl flavonoids.

*2.2.1 HPLC/LTQ-MS^n investigation of peak **5***

Isovitexin (peak **5**) gave an $[M+H]^+$ ion at *m/z* 433.1130 ($C_{21}H_{21}O_{10}$, calculated $[M+H]^+$ 433.1129, error 0.23 ppm). In the MS^2 spectrum, the highest product ion at *m/z* 415 was formed by the neutral loss of H_2O and the ions at *m/z* 367, 337, and 283 were formed by the loss of CH_4O_2, $C_2H_6O_3$, and $C_5H_8O_4$, respectively, from the saccharide group attached to the C-6 position of the aglycone. The fragment ions at *m/z* 397 and 379 were produced by the neutral loss of H_2O and $2H_2O$, respectively, from the *m/z* 415 ion. The fragment ion at *m/z* 313 ($[^{0,2}X+H]^+$) was observed due to internal cleavage of the sugar at C-6 by the loss of a $C_4H_8O_4$ residue (120 Da), which is the characteristic loss of the sugar moiety from C-glycosylation [12]. In the MS^3 spectrum of the ion at *m/z* 415, the product ions at *m/z* 367 (base peak), 337, 397, 379, and 283 were also observed due to similar fragmentation to the MS^2 spectrum.

*2.2.2 HPLC/LTQ-MS^n investigation of peaks **4, 8, 13, and 14***

Spinosin (peak **4**), with two saccharide groups at C6, exhibited an $[M+H]^+$ ion at *m/z* 609.1799 ($C_{28}H_{33}O_{15}$, calculated $[M+H]^+$ 609.1814, error −2.46 ppm). In the MS^2 spectrum of spinosin, the fragment ion at *m/z* 429 ($[Z_1+H]^+$) had the most abundant intensity and

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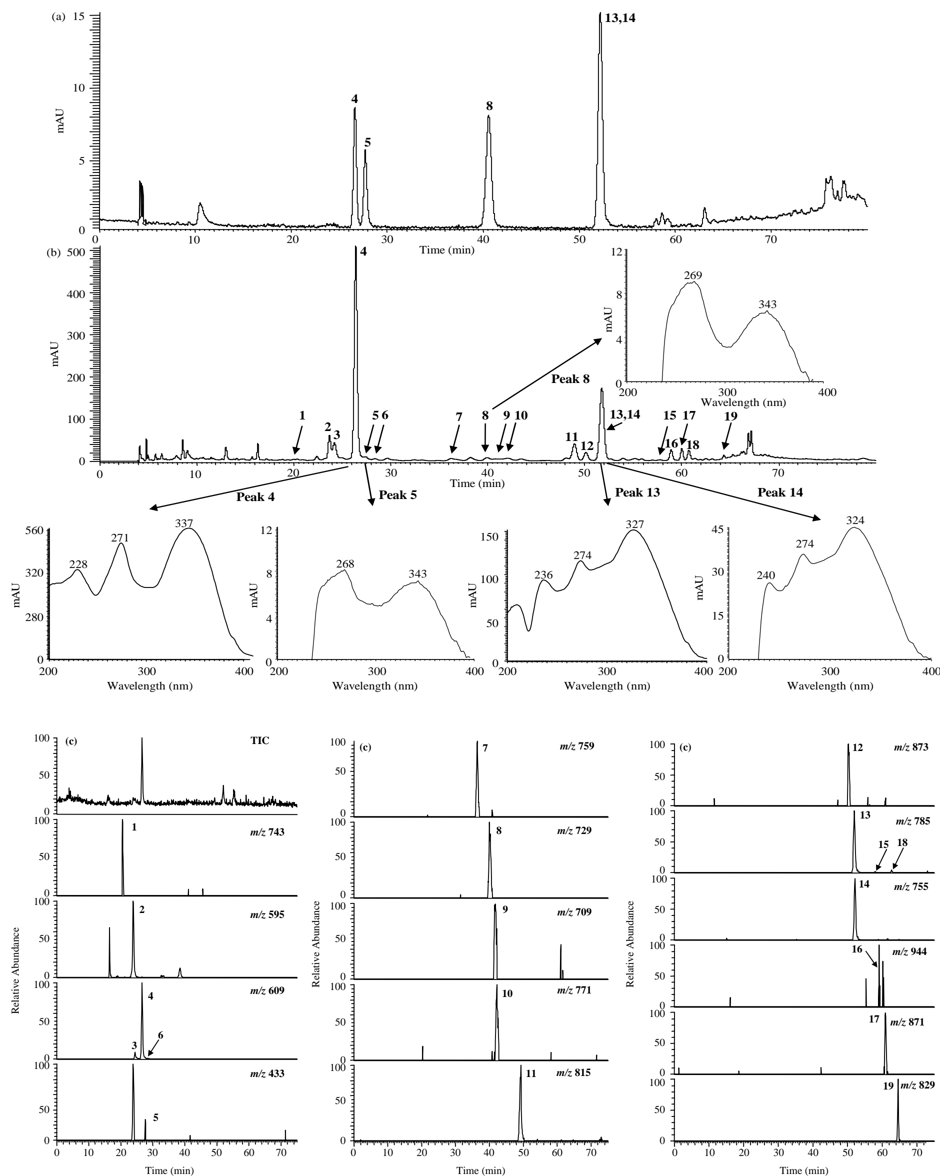

Figure 2. HPLC/LTQ-MS^n chromatograms of the flavonoid standards and the SZS extract: (a) LC-UV chromatogram at 270 nm of the flavonoid standards; (b) LC-UV chromatogram at 270 nm of the SZS extract; and (c) TIC chromatogram and extracted ion current chromatogram of the SZS extract.

corresponded to the loss of glucose from the protonated molecule. The ion at *m/z* 327 ($[^{0,2}X+H]^+$) with the second abundant intensity was observed due to the loss of glucose from the protonated molecule, followed by the loss of a $C_4H_8O_4$ residue from the glucose attached to the C-6 position of the aglycone. The product ion at *m/z* 489 ($[M+H-120]^+$) in the MS^2 spectrum probably could be attributed to the loss of a $C_4H_8O_4$ residue from the glucose attached to the C-6 position of the

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Table 1. Mass data for the 19 compounds detected in the SZS extract by HPLC/FTICR-MS.

No.	RT[a] (min)	$[M+H]^+$	Proposed formula	RDB[b]	Calculated mass (*m/z*)	Error[c] (ppm)	Possible compound
1	20.7	743.2007	$C_{32}H_{39}O_{20}^+$	13.5	743.2029	−2.96	Unknown
2	23.8	595.1641	$C_{27}H_{31}O_{15}^+$	12.5	595.1658	−2.86	Saponarin
3	24.5	609.1796	$C_{28}H_{33}O_{15}^+$	12.5	609.1814	−2.95	Isocytisoside-7-*O*-glucoside or flavocommelin
4	26.7	609.1798	$C_{28}H_{33}O_{15}^+$	12.5	609.1814	−2.63	Spinosin
5	27.8	433.1124	$C_{21}H_{21}O_{10}^+$	11.5	433.1129	−1.15	Isovitexin
6	28.6	609.1806	$C_{28}H_{33}O_{15}^+$	12.5	609.1814	−1.31	4′-*O*-Glucosylisoswertisin or 7 -glucosyl-4′-methoxyvitexin
7	36.2	759.2113	$C_{36}H_{39}O_{18}^+$	17.5	759.2131	−2.37	Unknown
8	40.0	729.2007	$C_{35}H_{37}O_{17}^+$	17.5	729.2025	−2.47	6‴-*p*-Hydroxylbenzoylspinosin
9	41.6	709.2320	$C_{33}H_{41}O_{17}^+$	13.5	709.2338	−2.54	Unknown
10	42.2	771.2111	$C_{37}H_{39}O_{18}^+$	18.5	771.2131	−2.59	Unknown
11	49.2	815.2384	$C_{39}H_{43}O_{19}^+$	18.5	815.2393	−1.10	6‴-Sinapoylspinosin
12	50.4	873.3166	$C_{43}H_{53}O_{19}^+$	17.5	873.3176	−1.15	Unknown
13	51.9	785.2265	$C_{38}H_{41}O_{18}^+$	18.5	785.2287	−2.80	6‴-Feruloylspinosin
14	52.5	755.2266	$C_{37}H_{38}O_{17}^+$	18.5	755.2182	−1.11	6‴-Coumaroylspinosin
15	57.5	785.2278	$C_{38}H_{41}O_{18}^+$	18.5	785.2287	−1.15	Unknown
16	58.8	944.2731	$C_{47}H_{42}O_{15}N_7^+$	30.5	944.2734	−0.31	Unknown
17	60.6	871.3000	$C_{43}H_{51}O_{19}^+$	18.5	871.3019	−2.07	Unknown
18	62.5	785.2271	$C_{38}H_{41}O_{18}^+$	18.5	785.2287	−2.04	7-*O*-(6″-*O*-Feruloylglucosyl)-isocytisoside or 4′-*O*-(6″-*O*-Feruloylglucosyl)-swertisin
19	64.3	829.2537	$C_{40}H_{45}O_{19}^+$	18.5	829.2550	−1.57	Unknown

Notes: [a] RT, retention time.
[b] RDB displays the ring and double-bond equivalents calculated for each of the formulas. The value is calculated by the following formula: $D = 1 + ([\sum_i^{i_{max}} N_i(V_i - 2)]/2)$, where D is the value for the RDB, i_{max} is the total number of different elements in the composition, N_i is the number of atoms of element i, and V_i is the valence of atom i.
[c] The value is calculated by the following formula: Error = (Experimental mass − Calculated mass)/Experimental mass.

Table 2. MS^n data and online UV spectral data for the 19 compounds detected in the SZS extract.

No.[a]	MS^1	MS^n	UV λ_{max} (nm)
1	743.20074	MS^2 [743] 303 (100), 465 (21), 611 (12)	238, 270, 341
2	595.16412	MS^2 [595] 433 (100), 415 (23), 367 (12), 313 (8), 397 (5), 379 (4) MS^3 [433] 415 (100), 367 (52), 313 (24), 397 (21), 379 (15), 283 (2)	227, 270, 337
3	609.17957	MS^2 [609] 447 (100), 429 (10), 327 (9), 351 (8), 489 (6), 591 (0.35) MS^3 [447] 327 (100), 297 (72), 285 (35), 429 (20), 411 (24)	227, 268, 341
4	609.17981	MS^2 [609] 429 (100), 327 (95), 447 (67), 381 (48), 351 (45), 411 (25), 393 (20), 297 (15), 489 (12), 591 (5) MS^3 [429] 351 (100), 381 (85), 393 (30), 297 (9), 411 (42)	228, 270, 335
5	433.11243	MS^2 [433] 415 (100), 367 (45), 337 (28), 397 (20), 313 (18), 379 (17), 269 (17), 283 (8) MS^3 [415] 367 (100), 337 (79), 397 (58), 379 (25), 283 (9)	268, 343
6	609.18060	MS^2 [609] 447 (100), 327 (95), 429 (92), 381 (40), 351 (40), 411 (20), 393 (16), 489 (15), 297 (10), 591 (3) MS^3 [447] 429 (100), 381 (24), 411 (11), 327 (10), 351 (5)	240, 270, 338
7	759.21130	MS^2 [759] 327 (100), 429 (42), 351 (33), 447 (17), 639 (14), 381 (14), 411 (13), 297 (13) MS^3 [327] 309 (100), 281 (30)	238, 270, 341
8	729.20068	MS^2 [729] 327 (100), 429 (39), 351 (30), 297 (16), 381 (15), 609 (14), 393 (13), 447 (10), 411 (10), 283 (1) MS^3 [327] 309 (100), 281 (41)	269, 343
9	709.23199	MS^2 [709] 327 (100), 429 (32), 351 (22), 297 (12), 589 (11), 411 (11), 381 (10), 447 (10) MS^3 [327] 309 (100), 281 (23)	235, 272, 329
10	771.21106	MS^2 [771] 433 (100), 415 (52), 313 (48), 283 (38), 367 (36), 337 (36), 379 (29), 609 (21), 397 (19), 651 (5) MS^3 [433] 415 (100), 367 (68), 337 (35), 313 (29), 313 (29), 397 (23), 379 (19)	237, 271, 328
11	815.23841	MS^2 [815] 327 (100), 429 (57), 351 (46), 297 (31), 393 (29), 381 (20), 411 (18), 447 (15), 609 (4), 369 (4) MS^3 [327] 309 (100), 281 (29)	235, 272, 331

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Table 2 – *continued*

No.[a]	MS^1	MS^n	UV λ_{max} (nm)
12	873.31659	MS^2 [873] 855 (100), 327 (50), 351 (27), 447 (25), 393 (24), 429 (20), 297 (16), 411 (15), 381 (12), 735 (11), 591 (9), 675 (5) MS^3 [855] 327 (100), 393 (34), 351 (28), 429 (28), 411 (15), 735 (14), 297 (13), 381 (12), 675 (5), 447 (5), 783 (3)	240, 272, 340
13	785.22650	MS^2 [785] 327 (100), 429 (52), 351 (32), 393 (27), 297 (25), 411 (20), 381 (19), 447 (14), 767 (11), 609 (3) MS^3 [327] 309 (100), 281 (29)	227, 273, 330
14	755.22661	MS^2 [755] 327 (100), 429 (51), 351 (34), 393 (21), 381 (19), 447 (17), 297 (16), 411 (15), 609 (4), 309 (2) MS^3 [327] 309 (100), 281 (29)	229, 273, 328
15	785.22778	MS^2 [785] 327 (100), 429 (33), 351 (33), 297 (24), 381 (20), 411 (17), 393 (17), 447 (15), 609 (5), 339 (4) MS^3 [327] 309 (100), 281 (16)	239, 272, 335
16	944.27307	MS^2 [944] 327 (100), 764 (96), 393 (70), 782 (36), 926 (31), 602 (17) MS^3 [327] 309 (100), 281 (31)	239, 272, 335
17	871.30005	MS^2 [871] 327 (100), 429 (36), 351 (25), 297 (22), 393 (16), 447 (14), 381 (11), 411 (10), 591 (7), 751 (6) MS^3 [327] 309 (100), 281 (25)	242, 271, 337
18	785.22711	MS^2 [785] 447 (100), 327 (44), 429 (21), 351 (10), 411 (6), 393 (5), 297 (4), 339 (4) MS^3 [447] 327 (100), 297 (63), 411 (13), 429 (10), 351 (6), 393 (4)	242, 272, 326
19	829.25372	MS^2 [829] 327 (100), 429 (56), 351 (51), 297 (31), 393 (29), 381 (23), 411 (16), 709 (16), 447 (14), 649 (8) MS^3 [327] 309 (100), 281 (32)	240, 274, 323

Note: [a]The numbers assigned to peaks are the same as in Table 1.

aglycone. The MS^3 spectrum showed a series of product ions at *m/z* 411, 381, 351, and 297, which were formed by the loss of H_2O, CH_4O_2, $C_2H_6O_3$, and $C_5H_8O_4$, respectively, from the sugar group at C-6 of the fragment ion at *m/z* 429.

The 6‴-*p*-hydroxybenzoylspinosin (peak **8**), with two saccharide groups at C-6, had an $[M+H]^+$ ion at *m/z* 729.2010 ($C_{35}H_{37}O_{17}$, calculated $[M+H]^+$ 729.2025, error −2.05 ppm). The MS^2 spectrum of 6‴-*p*-hydroxybenzoylspinosin

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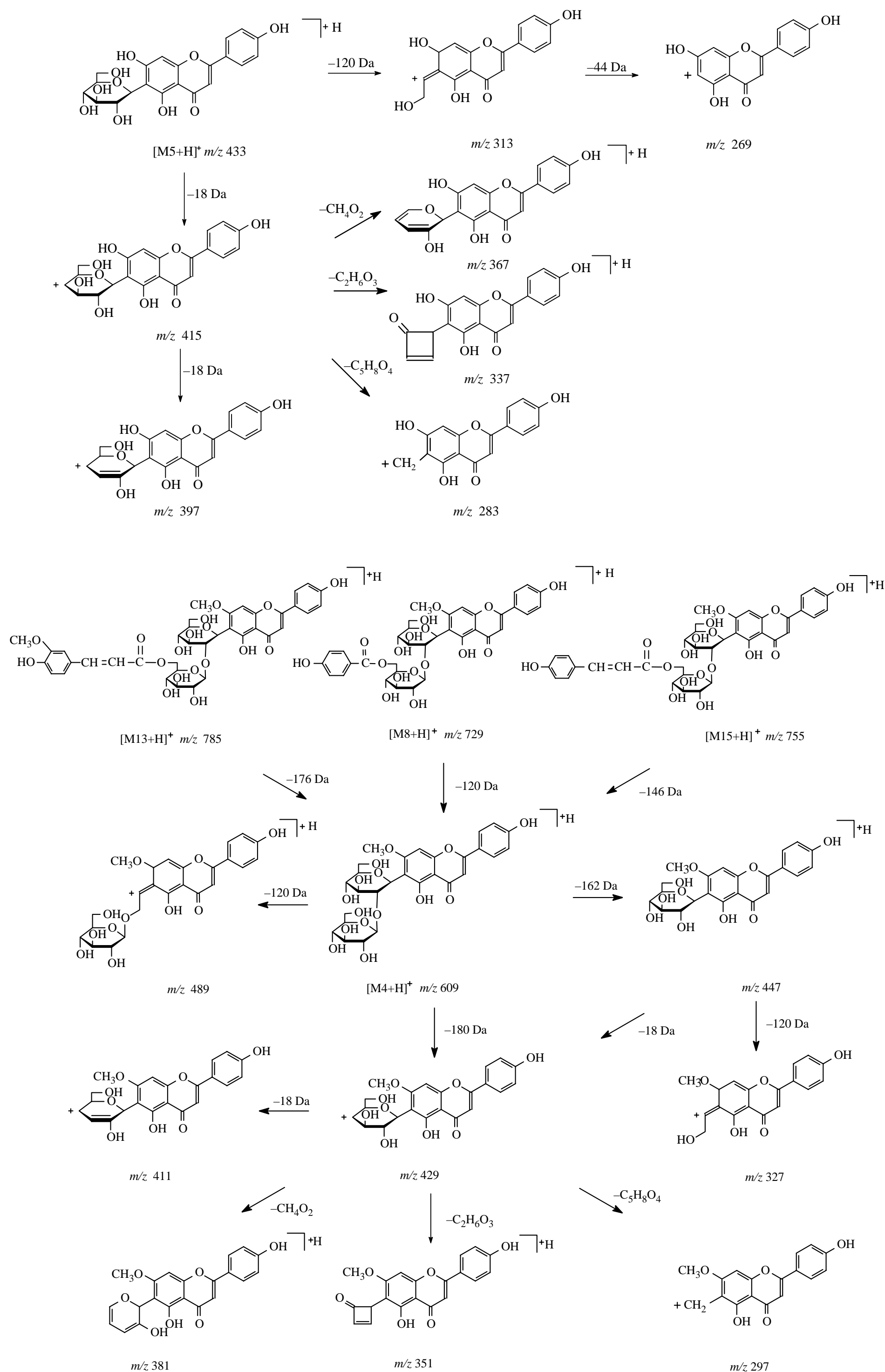

Figure 3. Proposed fragmentation pathways for the five authentic compounds.

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with one acyl group attached to C-6‴ showed similar fragmentation features to those of spinosin without the acyl group, except for the relative abundance of ions. The MS^3 spectrum of the ion at *m/z* 327 ($[^{0,2}X+H]^+$) displayed fragment ions at *m/z* 309 and 281, which were formed by the loss of H_2O and CO.

The mass spectral fragmentation patterns of 6‴-feruloylspinosin (peak **13**) ($C_{38}H_{41}O_{18}$, observed $[M+H]^+$ 785.2275, calculated $[M+H]^+$ 785.2287, error − 1.61 ppm) and 6‴-*p*-coumaroylspinosin (peak **14**) ($C_{37}H_{38}O_{17}$, observed $[M+H]^+$ 755.2170, calculated $[M+H]^+$ 755.2182, error − 1.50 ppm) were the same as those of 6‴-*p*-hydroxybenzoylspinosin (peak **8**), due to their similar structures.

The MS/MS analysis of the five authentic compounds showed that the main mass fragmentation pathways always occur in the sugar groups and the substituting group on C-6‴ (Figure 3). For the *O*-glycosyl-*C*-glycosyl flavonoids (peaks **4**, **8**, **13**, and **14**), the MS^2 data for the $[M+H]^+$ ions displayed some common features, with the fragment ions $[^{0,2}X+H]^+$ at *m/z* 327 and $[Z_1+H]^+$ at *m/z* 429. Among them, the occurrence of the $[^{0,2}X+H]^+$ ion is characteristic of the sugar moiety from C-glycosylation. By contrast, the occurrence of $[Z_1+H]^+$ produced by the loss of the sugar moiety indicates that the position of O-glycosylation is on the sugar moiety of the C-glycosylation instead of the phenolic hydroxyl groups of aglycone [13]. According to the reported data [12], the appearance of the $[Y_0+H]^+$ ion at *m/z* 447 suggested that one glucose moiety was substituted on the flavonoid skeleton through the hydroxyl groups. In addition, the loss of the substituting group on C-6‴ was easily observed in the MS^2 spectrum. For instance, in the MS^2 spectrum of peak **8**, the fragment ion at *m/z* 609 was formed and attributed to the loss of the *p*-hydroxybenzoic acid group.

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2.3 HPLC/LTQ-MS^n^ analysis of the SZS extract

On the basis of retention time, UV spectra, MS information, and the fragmentation patterns discussed above, the chemical structures of some flavonoids in the SZS extract were identified or tentatively characterized and some isomers were distinguished.

*2.3.1 HPLC/LTQ-MS^n investigation of peaks **2**, **3**, **6**, **11**, and **18***

Peak **2** had an $[M+H]^+$ ion at *m/z* 595.1641. Its most intense fragment ion occurred at *m/z* 433 ($[Y_0+H]^+$) in the MS^2 spectrum. The MS^3 spectrum of the ion at *m/z* 433 was very similar to the MS^2 spectrum of isovitexin (peak **5**). These results demonstrated that peak **2** had a similar structure to isovitexin but with an additional glucose substituted on the hydroxyl group of aglycone. This compound was identified as saponarin in consideration of the reference and mass data collected.

Peaks **3**, **4**, and **6** were isomers with the same molecular weight (609.1796, 609.1798, and 609.1806 Da). The identification of these three compounds was made mainly based on the multi-stage mass fragmentation and the relative abundance of the fragment ions. In the MS^2 spectrum of peak **3**, an abundant ion at *m/z* 447 ($[Y_0+H]^+$) was formed by the loss of one glucose. This indicated that one glucose moiety was substituted on the flavonoid skeleton. The abundant ion at *m/z* 327 ($[^{0,2}X+H]^+$) (base peak) in the MS^3 spectrum was formed by the loss of a $C_4H_8O_4$ residue, which was characteristic of C-glycosylation on the aglycone. Due to difficulty in assigning the position of O-glycosylation, peak **3** was identified as either isocytisoside-7-*O*-glucoside or flavocommelin. Similarly, the diagnostic ion at *m/z* 447 ($[Y_0+H]^+$) (base peak) was observed in the MS^2 spectrum of peak **6**. Compared with peak **3**, peak **6** had a lower

relative intensity for the diagnostic ion at *m/z* 327 ($[^{0,2}X+H]^+$) (18%) in the MS^3 spectrum. This indicated that the C-glycosylation substitution was at C-8, since the internal cleavage of the sugar moiety from C-8-glucosides is possibly more difficult than that from C-6-glucosides [14]. Thus, peak **6** was tentatively identified as either 4′-*O*-glucosylisoswertisin or 7-glucosyl-4′-methoxyvitexin.

Peak **11** displayed an $[M+H]^+$ ion at *m/z* 815.2384, in accordance with 6‴-sinapoylspinosin previously described in SZS by Woo *et al.* [15]. Both the MS^2 and MS^3 spectra of peak **11** showed the same pattern as those of peak **8**. This demonstrated that the structure of peak **11** was similar to that of peak **8**, except for different substituting groups on the C-6‴. This led to the identification of peak **11** as 6‴-sinapoylspinosin.

Peak **18** exhibited an $[M+H]^+$ ion at *m/z* 785.2271 that was very similar to that of peak **13**. The MS^2 spectrum of peak **18** presented the highest fragment ion at *m/z* 447 ($[Y_0+H]^+$). The product ions at *m/z* 609 ($[M+H-176]^+$) and 327 ($[^{0,2}X+H]^+$) in the MS^2 spectrum were formed by the loss of one ferulic acid group and internal cleavage of the sugar moiety. Consequently, peak **18** was tentatively characterized as either 7-*O*-(6″-*O*-feruloylglucosyl)-isocytisoside or 4′-*O*-(6″-*O*-feruloylglucosyl)-swertisin, neither of which have been reported previously.

2.3.2 *HPLC/LTQ-MS^n investigation of peaks **1**, **7**, **9**, **10**, **12**, **15**, **16**, **17**, and **19***

Peaks **7**, **9**, **12**, **17**, and **19** afforded $[M+H]^+$ ions at *m/z* 759.2113, 709.2320, 873.3166, 871.3000, and 829.2537, respectively. In positive ion mode, these five compounds showed typical losses (120 Da), which could be attributed to the loss of a $C_4H_8O_4$ residue from the glucose linked to A-ring at C-6. This finding suggested that the substituting groups were attached to C-2″ of the glucose. The other common product ions in the MS^2 spectrum of the five compounds, such as *m/z* 447, 429, 381, 351, and 327, were very similar to those of spinosin. This implied that they all have the same structural skeleton and one saccharide group (glucose) at C-6. However, other substituents could not be confirmed. The elemental compositions of the unknown substituents of these five compounds were $C_{14}H_{17}O_8$ (peak **7**), $C_{11}H_{19}O_7$ (peak **9**), $C_{21}H_{31}O_9$ (peak **12**), $C_{21}H_{29}O_9$ (peak **17**), and $C_{18}H_{23}O_9$ (peak **19**).

Peaks **1**, **10**, **15**, and **16** afforded $[M+H]^+$ ions at *m/z* 771.2111, 785.2278, 944.2731, and 743.2007, respectively. On the basis of MS information of these compounds, we could confirm that peak **1** contained the arabinose, rhamnose, and glucose groups. Similarly, the structure of peak **10** was similar to isovitexin but with one more $C_{16}H_{19}O_8$ residue substituted on the saccharide group at C-6. Peak **15** contained one ferulic acid group and two saccharide groups. In addition, the flavonoid skeleton of peak **16** was the same as that of peak **4**. However, because of the MS/MS fragment data, their exact chemical structures could not be confirmed.

3. Experimental

3.1 *General experimental procedures*

A Thermo Scientific Surveyor LC Plus instrument linked with a photodiode array (PDA) detector and a Thermo Scientific LTQ FTICR controlled by Xcalibur version 2.0 software and equipped with an ESI ion source was used to carry out the assay. NMR spectra were taken on a Varian Inova-600 spectrometer using TMS as the internal standard. Macroporous resin (Amberlite XAD-16) was purchased from Rohm & Haas Holding Co. Ltd (Philadelphia, PA, USA). Sephadex LH-20 was purchased from GE Healthcare Bio-Science AB Ltd (Beijing, China). Acetonitrile of LC/MS reagent grade was supplied by Mallinckrodt

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Table 3. HR-MS data, MS^n data and online UV spectral data of five flavonoids isolated and identified from SZS by HPLC/FTICR MS/MS.

Compound	$[M-H]^-$	Chemical formula	RDB	Calculated mass	Error (ppm)	MS^2	MS^3	λ_{max} (nm)
Spinosin	607.1664	$C_{28}H_{31}O_{15}^-$	13.5	607.1658	0.99	487, 445, 427	307	228, 270, 335
6‴-Feruloylspinosin	783.2156	$C_{38}H_{39}O_{18}^-$	19.5	783.2131	3.19	607, 445, 427	307	227, 273, 330
6‴-*p*-Coumaroylspinosin	753.2032	$C_{37}H_{37}O_{17}^-$	19.5	753.2025	0.93	607, 445, 427	307	229, 273, 328
6‴-*p*-Hydroxybenzoylspinosin	727.1888	$C_{35}H_{35}O_{17}^-$	18.5	727.1869	2.61	607, 445, 427	307	270, 340
Isovitexin	431.0987	$C_{21}H_{19}O_{10}^-$	12.5	431.0973	3.25	413, 341, 311	283	268, 343

Baker, Inc. (Phillipsburg, NJ, USA). All other solvents were from the Beijing Chemical Corp. (Beijing, China) and of analytical grade.

3.2 Plant material

SZS (4 kg) was purchased from Tong-Ren-Tang Co. Ltd (Beijing, China).

3.3 Extraction and isolation of five standards

The powdered sample of SZS was degreased with petroleum ether (60–90°C) three times (each time for 90 min) and the residue was refluxed with 70% ethanol twice (each time for 90 min). The pooled extract of 70% ethanol was concentrated and the residue obtained was extracted with *n*-butanol saturated with water three times. The combined *n*-butanol saturated with water extracts was chromatographed on a macroporous resin column, eluted with H_2O–EtOH (100:0, 90:10, 70:30, 50:50, 30:70, 5:95), and then a 50% EtOH fraction was chromatographed on a Sephadex LH-20 column and eluted with H_2O–MeOH (100:0, 90:10, 70:30, 50:50, 30:70, 5:95). Further purification was carried out by a semi-preparative HPLC column (9.4 × 250 mm, 5 μm, XDB-C18) to afford compounds **4**, **5**, **8**, **13**, and **14**. The structures of compounds **4**, **5**, **8**, **13**, and **14** were confirmed by UV, MS, and ^{1}H NMR data and the structure of compound **5** was confirmed by UV and MS data in accord with reference data (Tables 3 and 4). The structures of these compounds are shown in Figure 1.

3.4 Preparation of the SZS extract

Approximately 0.3 g of the dried *n*-butanol extract was accurately weighed and dissolved in 3 ml of methanol, and then centrifuged at 8497*g* for 10 min. Aliquots (25 μl) of the supernatant fluid were diluted to 500 μl with methanol. The sample solution was filtered through

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Table 4. ^{1}H NMR spectral data of five flavonoids isolated and identified from SZS (600 M, DMSO-d_6).

No.	Spinosin	6‴-Feruloylspinosin	6‴-*p*-Coumaroylspinosin	6‴-*p*-Hydroxybenzoylspinosin
3	6.78 (s)	6.77, 6.75 (s)	6.64, 6.63 (s)	6.64, 6.60 (s)
8	6.75 (s)	6.71, 6.53 (s)	6.61, 6.47 (s)	6.52, 6.48 (s)
2′, 6′	7.92 (d, *J* = 7.8 Hz)	7.81 (d, *J* = 7.8 Hz)	7.75 (d, *J* = 7.8 Hz)	7.80, 7.73 (d, *J* = 8.4 Hz)
3′, 5′	6.84 (d, *J* = 7.8 Hz)	6.90, 6.85 (d, *J* = 7.8 Hz)	6.70 (d, *J* = 7.8 Hz)	6.85, 6.82 (d, *J* = 8.4 Hz)
1″	4.67 (d, *J* = 10.2 Hz)	4.69, 4.67 (d, *J* = 9.9 Hz)	4.67, 4.66 (d, *J* = 9.6 Hz)	4.67, 4.66 (d, *J* = 9.6 Hz)
1‴	4.16 (d, *J* = 7.8 Hz)	4.28, 4.26 (d, *J* = 8.4 Hz)	4.29, 4.25 (d, *J* = 8.4 Hz)	4.29, 4.25 (d, *J* = 8.4 Hz)
2⁗, 6⁗			7.39, 7.30 (d, *J* = 8.4 Hz)	7.51, 7.45 (d, *J* = 8.4 Hz)
3⁗, 5⁗			6.75, 6.73 (d, *J* = 8.4 Hz)	6.73, 6.66 (d, *J* = 8.4 Hz)
7⁗		7.22, 7.08 (d, *J* = 16.2 Hz)	7.26, 7.19 (d, *J* = 16.2 Hz)	
8⁗		6.37, 6.17 (d, *J* = 16.2 Hz)	6.13, 6.14 (d, *J* = 16.2 Hz)	
OCH_3	3.88 (s)	3.82, 3.90 (s)	3.80, 3.90 (s)	3.80, 3.89 (s)

a 0.45 μm nylon filter film. Aliquots (20 μl) of this were injected into the LC/MS system for analysis.

3.5 Instrumentation and chromatographic condition

The chromatographic separation was performed on a HYPERSIL C_{18} column (250 × 4.6 mm, 5 μm) maintained at 25°C. A linear gradient elution of acetonitrile (A) and water containing 0.1% acetic acid (B) were used. The gradient was programmed as follows: 0–5 min, 5–10% A; 5–12 min, linear change to 15% A; 12–22 min, linear change to 15.5% A; 20–40 min, linear change to 16% A; 40–58 min, linear change to 22% A; and 58–75 min, linear change to 37% A. Then, the column was re-equilibrated with the initial condition for 10 min before the next injection. The sample injection volume was 20 μl. UV spectra were recorded between 200 and 400 nm and the detection wavelength was set at 270 nm. The solvent flow rate was 0.8 ml/min, with 30% of the eluent being split into the inlet of the mass spectrometer. Ultra high-purity He was used as the collision gas and high-purity N_2 as the nebulizing gas. The optimized parameters in the positive ion mode were: ion spray voltage, 3.5 kV; capillary temperature, 275°C; capillary voltage, 45 V; and tube lens, 94.5 V. Mass spectra were recorded in a mass range of *m/z* 90–1000 at a resolving power of 100,000 with data-dependent MS/MS analysis triggered by the most abundant ions from the parent mass list of predicted compounds (mass list: 433, 595, 609, 709, 729, 743, 755, 759, 771, 785, 815, 829, 871, 873, and 944), followed by MS/MS/MS analysis on the most abundant product ions. Collision-induced dissociation was conducted with an isolation width of 1 Da.

3.6 Nomenclature

The nomenclature used to designate fragments of flavonoid glycosides was

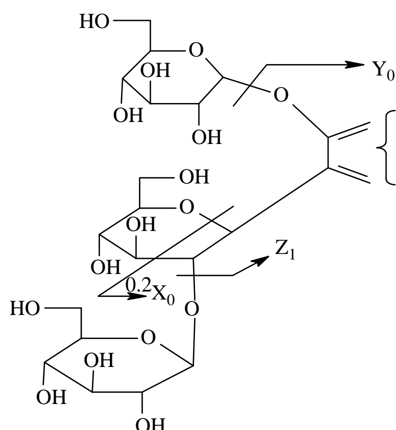

Figure 4. Ion nomenclature for the flavonoid glycosides.

proposed by Domon and Costello [16]. Ions containing the intact aglycone are labeled $^{k,l}X_j$, Y_j, and Z_j, where k and l represent the cleaved bonds of the glycosyl residue and j represents the number of the interglycosidic bond broken, counted from the aglycone. The glycosidic bond linking the glycan part to the aglycone is numbered 0 (Figure 4) [12].

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