Rapid Identification of Polyphenol C-Glycosides from *Swertia franchetiana* by HPLC–ESI-MS–MS

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Abstract

High-performance liquid chromatography coupled to positive ion electrospray ionization tandem mass spectrometry (MS) and diode array detection was employed to identify the polyphenol C-glycosides in the extract of *Swertia franchetiana*, a traditional Chinese/Tibetan herb. The neutral loss scan of the extract of *S. franchetiana* using the characteristic losses of 120 and 150 u provided a detailed profile of the polyphenol C-glycosides in the complex mixture. On-line UV spectroscopy along with MS–MS and MS–MS–MS mass spectra analysis produced with and without in-source collision induced dissociation was contributed to discriminate and identify the polyphenol C-glycosides. Three xanthone C-glycosides (i.e., mangiferin, isomangiferin, and 1,6,7-trihydroxyl-2-C-glucosexanthone) and three flavone C-glycosides (i.e., isoorientin, isovitexin, and swertisin) were tentatively identified. Isomangiferin and 1,6,7-trihydroxyl-2-C-glucosexanthone were for the first time found in this plant.

Introduction

As one group of the important secondary metabolites, polyphenols possess several aromatic rings with one or more hydroxyl or methoxyl substituents, and are widely found in our diet. Natural polyphenols have multiple structures, ranging from simple molecules to highly polymerized compounds. Flavonoids are the most common and widely distributed group of plant polyphenols, which are classified as flavones, flavonols, isoflavones anthocyanins, chalcones, etc. As a special type of flavonoid, xanthonoids are also widely distributed in the plants. Naturally, polyphenols mostly exist as O- or C-glycosides. O-Glycosides normally have one or more sugar substituents bonded to the hydroxyl groups on the aglycone by the formation of C-O-C bond, which are acid labile. In contrast, the sugars of the C-glycosides are linked to a carbon atom of the aglycone by forming a C-C bond, giving them resistance to acid hydrolysis. At present, C-glycosylation was only found at C-6 and/or C-8 position in the flavonoid nucleus, C-2 and/or C-4 in the xanthone nucleus (1).

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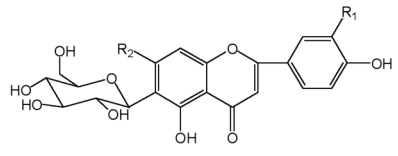
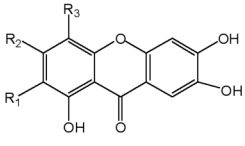
Polyphenols usually exist as complex mixtures in the plants. Most analytical methods for the analysis of polyphenols are based on high-performance liquid chromatography (HPLC) (2) or capillary electrophoresis (CE) (3) separation with UV detection. These methods are limited to the detection of a limited number of known compounds and are not applicable for the characterization of unknown polyphenols in a crude mixture. With the development of ionization techniques, electrospray ionization with multi-stage tandem mass spectrometry (ESI-MS^n) has demonstrated its great advantages in the structure analysis of polyphenols in plant extracts (4–7). The analysis of polyphenol *O*-glycosides by HPLC–MS has been widely reported (8,9), while the analysis of C-glycosides and the identification of position of C-sugar subsitutents on aglycone (C-6 or C-8 for flavonoids, C-2 or C-4 for xanthones) were more problematic since the difficult analysis of product ions (10,11). Meanwhile, neutral loss scan by triple quadrupole MS^n coupling to the HPLC became an especially useful choice for selective detection of important bioactive substitutes in plants and biological samples (12,13). For example, Kuhn et al. (12) used the atmospheric pressure ionization (API) neutral losses scan of 56 u (double CO) to discriminate and identify the isoflavone/flavone aglycones in the extract of



isoorientin, R_1=R_2=OH, MW=448
swertisin, R_1=H, R_2=OMe, MW=446
isoorientin, R_1=H, R_2=OH, MW=432



mangiferin,[a]	R_1=glu, R_2=OH, R_3=H	MW=422
isomangiferin,	R_1=H, R_2=OH, R_3=glu	MW=422
1,6,7-trihydroxyl-2-C-glucosexanthone,	R_1=glu, R_2=R_3=H	MW=406

Figure 1. The structures of polyphenol C-glycosides in *S. franchetiana* ("a" stands for standard).

Table I. UV–vis and the Product Ion Mass Spectral Data* of Polyphenol C-Glycosides Identified in *S. Franchetiana*†,‡

Peak	Compounds	t_R (min)	λ_{max} (nm)	$[M+H]^+$	E_1^+	E_2^+	E_3^+	E_4^+	$^{2,3}X^+_{-2w}$
1	Mangiferin	18.6	240, 256, 318, 362	423 (11)	405 (40)	387 (48)	369 (48)	351 (9)	357(60)
2	Isomangiferin	19.3	252, 325, 368	423 (10)	405 (14)	387 (39)	369 (23)	351 (5)	357 (5)
3	Isoorientin	22.7	256 (sh), 268, 347	449 (42)	431 (58)	413 (58)	395 (52)	377 (14)	383 (88)
4	1,6,7-Trihydroxyl-2-C-glucosexanthone	23.9	249, 325, 368	407 (8)	389 (14)	371 (52)	353 (40)	335 (20)	341 (9)
5	Isovitexin	26.9	268, 337	433 (47)	415 (51)	397 (65)	379 (47)	361 (21)	367 (66)
6	Swertisin	28.4	268, 332	447 (7)	429 (71)	411 (55)	393 (100)	375 (13)	381 (60)

* %, Relative Abundance
† Collision voltage = 20 V.

Table I. (continued) UV–vis and the Product Ion Mass Spectral Data* of Polyphenol C-Glycosides Identified in *S. Franchetiana*†,‡

Peak	$^{2,3}X^+_{-3w}$	$^{0,4}X^+_{-2w}$	$^{0,3}X^+_{-2w}$	$^{0,2}X^+$	$^{0,2}X^+_{-w}$	$^{0,1}X^+$	Y_0^+
1	339(18)	327 (56)	–‡	303 (54)	285 (8)	273 (100)	261 (5)
2	339 (16)	327 (73)	297 (6)	303 (100)	285 (6)	273 (31)	261 (2)
3	365 (6)	353 (100)	–‡	329 (93)	–‡	299 (43)	287 (6)
4	–‡	311 (84)	–‡	287 (89)	269 (8)	257 (100)	245 (9)
5	349 (8)	337 (97)	–‡	313 (100)	–‡	283 (63)	271 (4)
6	–‡	351 (97)	–‡	327 (82)	–‡	297 (85)	285 (9)

* %, Relative Abundance
† Collision voltage = 20 V.
‡ – the ion without being detected.

leaves of *Lupinus albus*. Schlosser et al. (13) analyzed the protein phosphorylation by a neutral loss scan of phosphoric acid (98 u) after elastase digestion.

Swertia franchetiana, traditional Chinese/Tibetan herb, belongs to the Gentianaceae category. Its extracts were frequently used against various diseases such as hepatitis, cholecystitis, and hypoglycemia, etc. (14,15). It has been prepared in commercial tablets or injections for public in China. Its pharmacological properties were generally attributed to its xanthones, iridoid glycosides, and flavonoids. As one of the major and important bioactive components in *S. franchetiana*, mangiferin (1,3,6,7-tetrahydroxyl-xanthone-2-C-glucose) was widely spread in natural plants, especially in Gentianaceae category and was taken as an indicative compound in quality control (16). Meanwhile, mangiferin has been reported to have diverse bioactivities, including antioxidant (17), antitumor, immu-nomodulatory and anti-HIV (18), chemopreventive, antidiabetic (19), etc. Thus, the study of *S. franchetiana* is highly relevant to chronic human diseases.

As far as we know, most of the published papers regarding MS studies of plant polyphenols pay more attention to flavonoids, especially the common flavonoid aglycones and their glycosides, which are the predominant conjugates in the polyphenols. The xanthonoids are seldom reported, and systematic MS characterizations of xanthone C-glycosides are few in the literature. To the best of our knowledge, there is no report on the MS characterization of the various polyphenol C-glycosides profiles in the extract of *S. franchetiana* until now. In this report, two kinds of polyphenol C-glydosides, xanthone C-glycosides and flavone C-glycosides (Figure 1), were determined in the extract of *S. franchetiana*. Meanwhile, in the present work, an integrated approach consisting of LC–MS^n and positive ion neutral loss-scan MS together with diode-array detection (DAD) was used to selectively separate and identify the polyphenol C-glycosides (xanthone C-glycosides and flavone C-glycosides) in the extract of *S. franchetiana*.

Experimental

Reagents and chemicals

Mangiferin was previously isolated from the plant *S. franchetiana* in our laboratory and identified by UV, IR, MS, and NMR spectroscopies (20); HPLC-grade methanol purchased from Yuwang Co. Ltd (Shangdong, China) was used to dissolve the sample; double-deionized water was obtained from a Milli-Q system (Millipore, Billerica, MA). The HPLC-grade acetonitrile was purchased from Fisher (Norcross, GA). The analytical-grade ethanol was obtained from Lianbang Reagent Company (Shenyang, China); the formic acid (99%) was purchased from Acros Organics (Fair Lawn, NJ); the polyamide was obtained from Shanghai Chemical Reagent Co. Ltd (Shanghai, China), respectively.

Apparatus

A Waters 2695 series chromatographic system (Milford, MA) with a photodiode array detector was coupled to a ThermoQest Finnigan TSQ/SSQ triple quadrupole mass spectrometer (San Jose, CA) with an electrospray interface. The UV spectroscopy was obtained by scanning from 200 to 600 nm. A Dikma Inertsil ODS-3 column (4.6 × 250 mm, 5 µm) (GL Sciences INC. Japan) with a Dikma EasyGuard C18 Kit sentry guard column (8.0 × 4.0 mm, 5 µm) was used. The mobile phase was prepared from two solutions (A and B), where A was 0.1% formic acid (v/v) in water and B was acetonitrile. The gradient profile was: 0–5 min linearly

from 5% B to 10% B; 5–50 min linearly from 10% B to 40% B; then held for 5 min. The flow rate was 1.0 mL/min, and the temperature of the column oven was kept at 35°C. The LC effluent was introduced into the ESI source with a 4:1 post-column stream splitting. For the mass spectrometer, the ESI spray voltage was set at 5.0 kV in the positive ion mode, and the heated capillary temperature was fixed at 350°C. The scan mode was positive and the isolation window width was set at 1.0 u. The instrument automatically adjusted the capillary voltage according to the ions selected in the tandem MS experiments. Nitrogen was used both as the sheath gas and the auxiliary gas with the pressure of 60 psi and 20 arbitrary units, respectively. For the collision-induced dissociation (CID) experiments, argon (Ar) was used as the collision gas (3.0 mTorr). The collision voltage was set between 20–35 V.

Preparation of samples

The *Swertia franchetiana* H. Smith was collected from Qinghai Province of China, and the extraction procedure was adapted from one described by Wang et al. (20). The pulverized sample (25 g) of *S. franchetiana* was extracted three times with methanol–water (150 mL, 4:1, v:v) under sonication at room temperature for 30 min. The extract was concentrated with rotatory evaporator under vacuum, then was dissolved with 200 mL hot water and partitioned sequentially with the petroleum ether, chloroform, and *n*-butanol. 2.0 mL of *n*-butanol fraction was loaded into a glass column (2 × 30 cm) wet-packed with 25 mL polyamide and washed with 200 mL water. It was then desorbed four times of 200 mL water–ethanol solutions in volume ratios of 4:1, 3:2, 2:3, and 5:95, successively. The eluted fraction of water–ethanol solution (4:1, v:v) was evaporated and dried. The solid residual was dissolved in 5 mL water–methanol (1:1, v:v) solution and filtered through 0.22-µm cellulose membrane before analysis.

Nomenclature

In the present paper, the nomenclature on the product ions adopted the nomenclature system proposed by Domon and Costello (21) followed by Li and Claeys (22) for glycoconjugates. For the C-glycosides, those ions from the cleavage of hexose are labeled as $^{k,l}X$, Y_j, where *j* denotes the number of the broken interglycosidic bonds, counting from the aglycone, and the cross-ring cleavage ions are described by the superscripted *k* and *l,* indicating the cleavage of the two bonds on the carbohydrate ring. The glycosidic bond linking the glycan part to the aglycone is numbered "0". E_n is labeled as the ions from the loss of water molecules from the protonated ions, where *n* is the number of the lost water molecules. For free aglycones, the nomenclature on the product ions is based on the system proposed by Ma et al. (23). The $^{i,j}A$ and $^{i,j}B$ ions refer to the product ions containing intact A and B rings, in which the superscripts *i* and *j* indicate the C-ring bonds of flavone skeleton that have been broken. The $^{i,j}A_{-W}$ and $^{k,l}X_{-W}$ mean the losses of water molecules from $^{i,j}A$ and $^{k,l}X$ ions, respectively. The product ions from the nomenclature on the C-glycosides are shown in Figure 2.

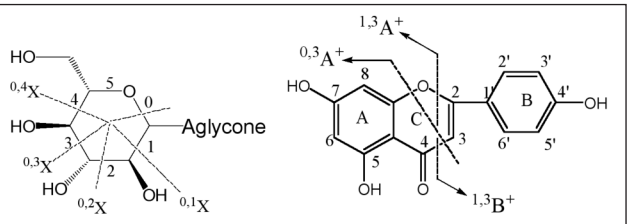


Figure 2. Carbohydrate fragmentations and the flavone aglycone nomenclature according to Domon and Costello (21), Li and Claeys (22), and Ma et al. (23).

Results and Discussion

The polyphenol C-glycosides components in *S. franchetiana* were determined by means of HPLC–DAD-ESI-MS with neutral loss scan. To avoid severe peak broadening due to the dissociation of polyphenols, various concentrations of formic acid in the mobile phase were evaluated. It has been found that a 0.1% aqueous solution of formic acid was sufficient to achieve good resolution and a low detection limit, and the difference in retention time was not greater than 0.1 min for three continuous injections. Figure 3A shows the HPLC–UV chromatogram of ethanol-eluted fraction of *S. franchetiana* extract under the optimized condition. Meanwhile, in the present study, higher sensitivity was achieved in the positive ion mode for the determination of different polyphenol C-glycosides, and the reproducibility of relative abundance of the dominating ions with RSD values of less than 4.0% was obtained for the MS–MS spectrum of protonated mangiferin. Thus, the following analysis was performed using the MS and MS^n analysis of protonated molecules.

Neutral loss scan (MS–MS) in the ethanol-eluted fraction of *S. franchetiana* extract

In the positive ion full-scan mass spectrum, the C-glycosides show only the prominent $[M+H]^+$ ion. In addition, different from O-glycosides, the C-glycosides do not generate abundant Y_0^+ aglycone ions in the CID mass spectrum, but give characteristic

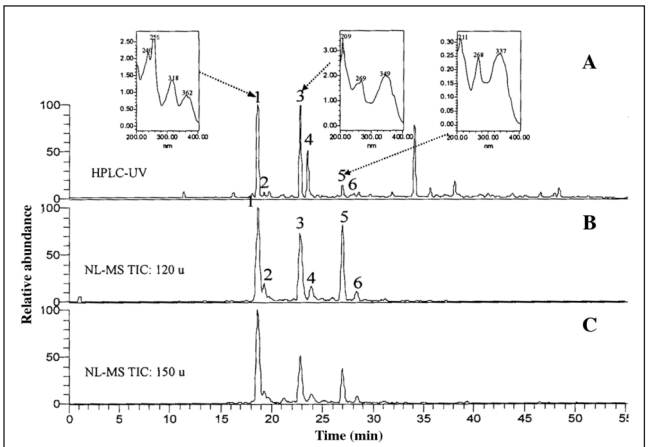


Figure 3. HPLC–UV chromatogram (A), positive ion neutral loss scan MS total ion current chromatography of *S. franchetiana* (B and C). Condition: at wavelength 254.0 nm (A); with the neutral loss of 120 u (B); with the neutral loss of 150 u (C); the inserted figures in Figure 3A are the characteristic UV–vis spectra of peaks 1, 3, and 5, respectively.

ions of the fragmentation of the C-glycoside unit itself (10,11). Losses of 120 and 150 u ($^{0,2}X^+$ [M+H–120]$^+$ and $^{0,1}X^+$ [M+H–150]$^+$) from the cross-ring cleavage of C-sugar of [M+H]$^+$ ions are relatively easy and can be directly observed with strong abundance in the product ion spectra of C-glycosides. The analysis of protonated flavone C-glycosides and xanthone C-glycosides by ESI-MS have proven that the ions of $^{0,3}X^+$ [M+H–90]$^+$, $^{0,2}X^+$ [M+H–120]$^+$, and $^{0,1}X^+$ [M+H–150]$^+$ are the characteristic product ions for polyphenol C-glycosides, and the losses of 120 and 150 u are more favorable than that of 162 u in the product ion spectra (24).

The HPLC–ESI-MS analysis of the eluted fraction extract of *S. franchetiana* extract showed that many constituents with different molecular ions in a broad range of *m/z* 200–800 are distributed in the extract, which made the direct analysis of the extract difficult. To reduce the complexity of the mass spectrum and to screen out the C-glycosides, the extract of *S. franchetiana* was subjected to positive ion neutral loss scan MS analysis. Figures 3B and 3C represent the positive ion neutral loss scan total ion current ion chromatograms of *S. franchetiana* extract by the characteristic losses of 120 and 150 u, respectively, which showed that six polyphenol C-glycosides with retention times at 18.6, 19.3, 22.7, 23.9, 26.9, and 28.4 min and molecular weights of 422, 422, 448, 406, 432, and 446 Da, respectively, existed in the extract. In order to identify their structures on the basis of their retention time (t_R) and product ions, LC–DAD-MS and LC–ESI-MS–MS experiments were performed. The chromatographic and spectral features of all the C-glycoside compounds in *S. franchetiana* extract are listed in Table I, and the structures of polyphenol C-glycosides in the extract of *S. franchetiana* are shown in Figure 1.

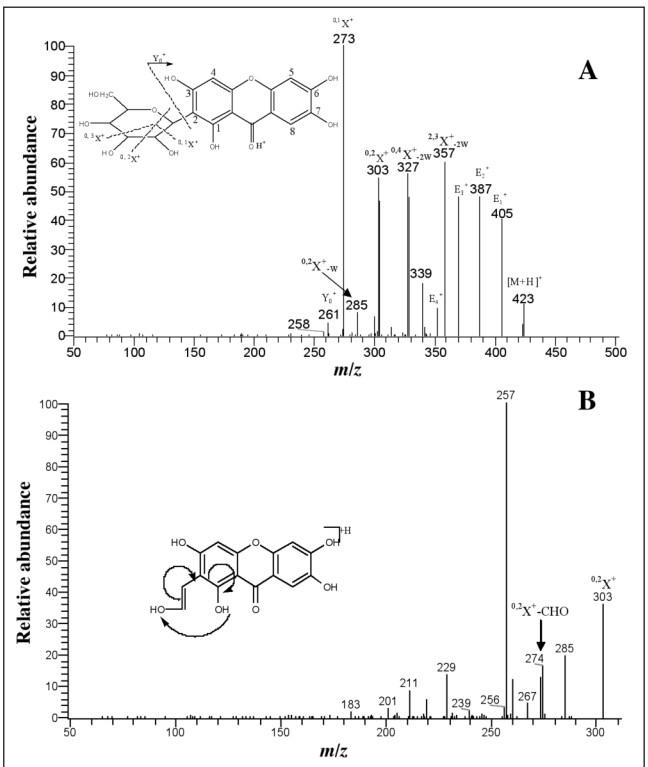


Figure 4. Product ion spectral comparison of peak 1: (A) product ion spectrum of [M+H]$^+$ at *m/z* 423; (B) product ion spectrum of parent ion $^{0,2}X^+$ [M+H–120]$^+$ at *m/z* 303 from (A).

LC–MS–MS analyses of xanthone C-glycosides from *S. franchetiana*

One of the major peaks in the neutral loss scan total ion current chromatograms (Figure 3B and 3C) is peak 1, whose UV–vis spectra showed four absorption maximas at 240, 256, 318, and 368 nm (Figure 3A). The four characteristic bands with various intensities between 200 and 400 nm in UV spectra were indicative of the xanthone structure (25). The UV–vis spectra and retention time of peak 1 were identical to those of the standard of mangiferin. The MS–MS and MS–MS–MS mass spectra allowed the confirmation of the identity of peak 1. In the product ion spectra of [M+H]$^+$ ion as shown in Figure 4A, the abundant product ions E_1^+, E_2^+, and E_3^+ at *m/z* 405, 387, and 369 were observed, and these ions can be attributed to the losses of one, two, and three water molecules, respectively, from the rearrangements of C-sugar of [M+H]$^+$. The E_4^+ ion was produced by the elimination of water between two hydroxyl groups at the ortho positions (6 and 7 positions) on the aglycone moiety. Meanwhile, these ions with high abundances, $^{0,4}X^+_{-2W}$ [M+H–96]$^+$, $^{2,3}X^+_{-2W}$ [M+H–66]$^+$, $^{0,2}X^+$ [M+H–120]$^+$, $^{0,1}X^+$[M+H–150]$^+$, which were also observed in the product ion spectra of peak 1, were the characteristic product ions of C-glycosides. These results are in good agreement with the characteristic ions of C-glycosides reported in literature (10,11). Additionally, the ion Y_0^+ with relatively weak intensity was also observed in product ion spectra.

Table II. Product Ion MS Data (%, Relative Abundance) of $^{0,2}X^+$ Ions From Xanthone C-Glycosides in *S. franchetiana

Peak	t_R (min)	$^{0,2}X^+$	–18	–29	–36	–43	–18–28	–36–28	–18–56	–36–56	Other ions
1	18.6	303 (36)	285 (19)	274 (15)	267 (4)	260 (12)	257 (100)	239 (2)	229 (13)	211 (8)	273 (12), 219 (5), 201 (3), 183 (1), 155 (1)
2	19.3	303 (36)	285 (20)	274 (17)	267 (4)	260 (10)	257 (100)	239 (2)	229 (12)	210 (5),	273 (17), 219 (4), 201 (3), 173 (1), 155 (2)
4	23.9	287 (31)	269 (27)	257 (27)	251 (< 1)	244 (12)	241 (100)	–†	213 (22)	–	257(31), 229(8), 202.8(3), 183(1), 161(1), 155(1), 153(4),135(1)

* In-source CID voltage: 35 V; CID voltage: 30 V.
† Ion without being detected.

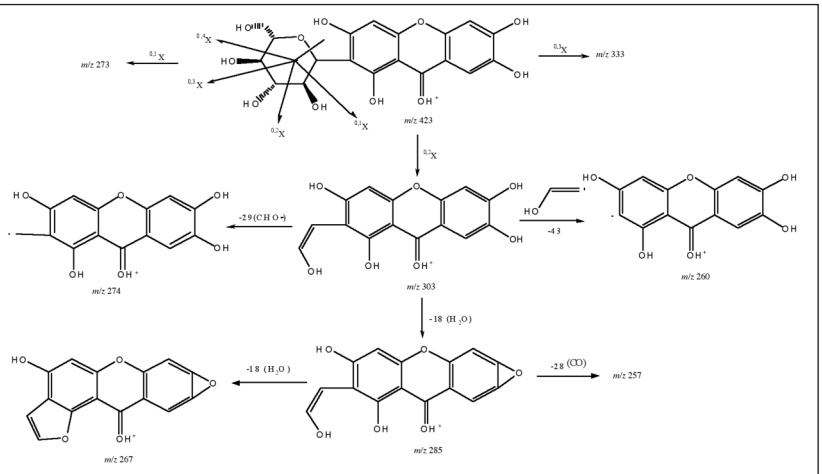


Figure 5. Proposed fragmentation mechanism of the $[M+H]^+$ ion for peak 1 (mangiferin).

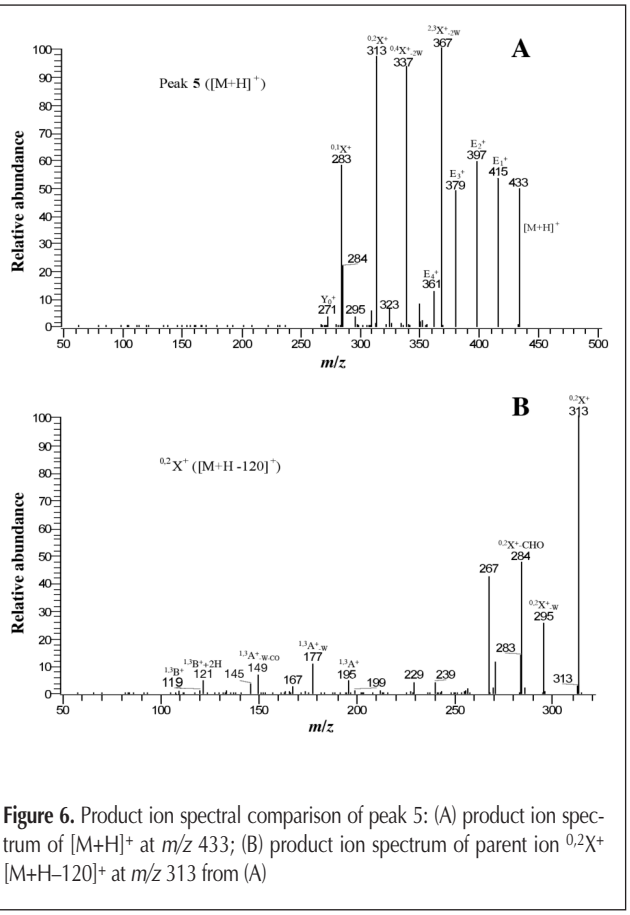


Figure 6. Product ion spectral comparison of peak 5: (A) product ion spectrum of $[M+H]^+$ at *m/z* 433; (B) product ion spectrum of parent ion $^{0,2}X^+$ $[M+H-120]^+$ at *m/z* 313 from (A)

To investigate the structure and fragmentation pathway of the aglycone moiety of polyphenol C-glycosides, the characteristic product ions were produced by in-source fragmentation of the glycosides $[M+H]^+$ (which was obtained by increasing the declustering potential in the ionization source) followed by product ion scan of the characteristic product ions. Then, the "pseudo" MS–MS–MS experiments were achieved on the present mass spectrometer. This method was set up for analyzing these polyphenol C-glycosides in the extract of *S. franchetiana*. Table II lists the data of product ions obtained in the product ion spectra of $^{0,2}X^+$ $[M+H-120]^+$ of xanthone C-glycosides. In the product ion spectra of $^{0,2}X^+$ ion of peak 1, significant $[^{0,2}X-H_2O]^+$ (*m/z* 285), $[^{0,2}X-CHO]^+$ (*m/z* 274), $[^{0,2}X-CO-H_2O]^+$ (*m/z* 257) ions with high intensity were also observed (Figure 3B). The $[^{0,2}X-H_2O]^+$ ion at *m/z* 285 was produced by the dehydration reaction of two hydroxyl groups on ortho position of the xanthone aglycone. The abundant and unusual odd-electron ion $[M+H-149]^+$ at *m/z* 274 may be produced from the i-cleavage of carbonyl group in the C6 substituent. This loss of 149 u from the protonated molecular ion was already observed for the flavone C-glycosides produced by thermospray (TSP) and fast-atom bombardment (FAB) combined MS–MS and is common in the product ion spectra of $[M+H-120]^+$ of C-glycosides (10,22). The $[^{0,2}X-H_2O-CO]^+$ (*m/z* 257), existing as a base peak, corresponded to a dehydration reaction, followed by a CO loss from the C-ring of xanthone aglycone. The relatively weak intensity of the ion at *m/z* 267, corresponded to the loss of H_2O from the *m/z* 285 ion by the similar mechanism with the E_4^+ ion in the C-glycosides mentioned earlier, implied that there was a dehydration course existing between the hydroxyl group on C6 substituent and its adjacent hydroxyl groups on the C1 or C3 on the xanthone aglycones by the H-rearrangement reaction, and this course is unfavorable in energy. The ions at *m/z* 229 and 211 were produced by the losses of two CO groups plus a H_2O and two CO groups plus two H_2O from the $[M+H]^+$ ion, respectively. On the basis of the above spectral data analysis and comparison of MS–MS data with mangiferin standard, peak 1 was identified as mangiferin. The proposed fragmentation pathway is shown in Figure 5.

Peak 2 presents a predominant molecular ion at *m/z* 423 in the full-scan mass spectrum. The UV–vis spectra recorded on-line showed a similar profile as peak 1, which indicated that peaks 1 and 2 were a pair of xanthone C-glycoside isomers. The data in the MS–MS and MS–MS–MS spectra of peak 2 can help us to confirm its structure. The abundant product ions at *m/z* 303, 273 etc., from the cross-ring cleavage of C-sugar in the product ion spectrum of peak 2, further confirmed that peak 2 was a xanthone C-glycoside. Meanwhile, the difference in the abundance of the characteristic product ions in the product ion spectra of peaks 1 and 2 indicated that both peaks were a pair of xanthone C-glycoside isomers with the difference in the position of C-sugar substituent on the aglycone (24). In the MS–MS–MS spectrometric analysis, as seen in Table II, the product ions with similar relative abundance from the same fragmentation pattern were obtained in the product ion spectra of $^{0,2}X^+$ $[M+H-120]^+$ from the peaks 1 and 2 produced by in-source CID, which indicated that the structures of the product ions $^{0,2}X^+$ in both compounds were similar except for the positions of C-sugar substituent. Thus, peak 2 was identified as isomangiferin. To the best of our knowledge, isomangiferin has not been reported in this plant.

For peak 4, the on-line UV–vis spectral analysis and positive ion full-scan mass spectrometric analysis indicated that this peak was a xanthone C-glycoside with a molecular weight of 406. In comparison with the product ion spectra of peaks 1 and 2, the higher intensity of ion $[M+H-150]^+$ at *m/z* 257 than that of

Table III. Product Ion MS Data (%, relative abundance) of $^{0,2}X^+$ Ions from Flavone C-Glycosides in *S. franchetiana

Peak	t_R (min)	$^{0,2}X^+$	–18	$^{1,3}A^+$	$^{1,3}B^+$	$^{1,3}B^+$+2H	$^{1,3}A^+$–18	$^{1,3}A^+$–46	Other ions
3	22.7	329 (100)	311 (19)	195 (2)	135 (2)	137 (3)	177 (8)	149 (9)	300 (41), 283 (37), 286 (5), 255 (6), 245 (3), 137 (3), 135 (2)
5	26.9	313 (100)	295 (14)	195(4)	119 (3)	121 (4)	177(7)	149 (16)	284 (42), 270 (7), 267 (40), 239 (8), 229 (3), 145(4)
6	28.4	327 (100)	309 (29)	209(3)	–	121 (1)	191 (15)	163 (9)	298 (23), 294 (1), 284 (21), 269 (7), 255 (3), 251 (3), 239 (3), 224 (2), 145 (8), 109 (2)

* In-source CID voltage: 35 V; CID voltage: 30 V.
† Ion without being detected.

$[M+H–120]^+$ at *m/z* 287 of peak 4, implied that peak 4 was a xanthone 2-C-glycoside (26,27). The tetradehydrated ion E_4^+ at *m/z* 335 ($[M+H–4H_2O]^+$) with relatively higher intensity indicated that two ortho hydroxyl groups existed in the aglycone moiety. In the MS–MS–MS analysis, the low abundance of the ion at *m/z* 251 produced by the loss of two molecules of water from the $[M+H–120]^+$ of peak 4, implied that there was a hydroxyl group at the ortho position to the 2-C-sugar substituent. Considering the biogenesis and metabolism of plant, as well as the longer elution time compared with peak 1, peak 4 was tentatively identified as 1,6,7-trihydroxyl-2-C-glucosexanthone.

LC–MS–MS analyses of flavone C-glycosides from *S. franchetiana*

Peaks 3, 5, and 6, eluted at 22.7, 26.9, and 28.4 min, respectively in HPLC–DAD chromatogram (Figure 2A), and exhibit the characteristic UV spectra of flavone and were studied by ESI-MS–MS. The positive ions neutral loss scans of 120 and 150 u in the extract of *S. franchetiana* have shown that the three peaks were flavone C-glycosides with the molecular weights of 448, 432, and 446, respectively. Table I lists the product ion spectral data of peaks 3, 5, and 6, and the characteristic product ions of flavone C-glycosides from the cross-ring cleavage of C-sugar were observed. In comparing these data with those reported for 6-C- and 8-C-flavone glucosides using LSI-MS (26,27) and ESI-MS (28), the higher abundance of $[M+H–120]^+$ ion than $[M+H–150]^+$ ion, as well as a wide range of neutral losses from the glycan, suggested that compounds of peaks 3, 5, and 6 were flavone 6-C-glucosides. However, the structure information on the flavone aglycone was rare in the product ion spectra of protonated molecules. The abundant ions $^{0,2}X^+$ $[M+H–120]^+$ in the full-scan mass spectra produced by in-source CID were selected as precursor ions, and the CID spectral data are shown in Table III. Figure 6 shows the product ion spectra of peak 5 and its characteristic product ion $^{0,2}X^+$ $[M+H–120]^+$. It was obvious that the specific daughter ions for the flavone aglycones existed in the CID spectrum. The ions at *m/z* 121, 195, and 119 as shown in Figure 6B could be attributed to $^{1,3}B^+$+2H, $^{1,3}A^+$, and $^{1,3}B^+$, respectively, which were formed from the RDA cleavage of C-ring of flavone aglycone (Figure 2). These ions produced from the analogical fragmentation mechanism were also observed in the product ion spectra of peaks 3 and 6 (Table III), including *m/z* 195($^{1,3}A^+$), 135($^{1,3}B^+$), and 137 ($^{1,3}B^+$+2H) for peak 3; *m/z* 209 ($^{1,3}A^+$) and 121 ($^{1,3}B^+$+2H) for peak 6, respectively. According to these ions, the position of hydroxyl group can be easily deduced, and the structures of peaks 3, 5, and 6 as shown in Figure 1 were also confirmed. In the further analysis of the product ion spectral data of peak 3, 5, and 6, the most notable ions in peaks 3 and 5 were observed at *m/z* 177 and 149, which corresponded to $^{1,3}A^+_{-W}$ and $^{1,3}A^+_{-W-CO}$ formed by the losses of one water and one water molecule plus one CO, respectively. Additionally, ions at *m/z* 191 and 163 with difference of 14 u in peaks 3 and 5 were also obtained in peak 6, which were produced by the same mechanism as the *m/z* 177 and 149 ions for peak 3 and 5. According to the product ions described by Rath et al. (17), these kinds of ions $^{1,3}A^+_{-W}$ and $^{1,3}A^+_{-W-CO}$, are the characteristic ions for the flavone 6-C-glucoside. The position of C-sugar substituent on aglycone moiety of peaks 3, 5, and 6 were further confirmed. All of the results are in good full agreement with those obtained by Rath et al. (10) and Waridel et al. (11). Meanwhile, in the reversed phase chromatography, the retention times were known to be inversely correlated to glycosylation levels, and the additional hydroxyl groups cause stronger polarity and less retention time of the analytes; however, the methoxyl groups have the contrary effect. Compared to peak 5, the shorter retention time of peak 3 was due to the additional hydroxyl substituent on the B-ring (Figure 1), and the longer retention time of peak 6 implied that there was an extra methoxyl group existing on the A-ring. Based on the described spectral data analysis and comparison of MS–MS data with those in the literature, the structures of peaks 3, 5, and 6 could be confirmed as isoorientin (luteolin-6-C-glucoside, known also as homoorientin), isovitexin, and swertisin, respectively.

Conclusion

In this study, HPLC coupled to ESI-MS–MS and DAD was used to selectively separate and identify the polyphenol C-glycosides in the extract of *S. franchetiana*. Taking advantage of the scan features of the triple quadrupole MS, neutral loss scan of *S. franchetiana* extract using the typical losses of 120 and 150 u for polyphenol C-glycosides was able to provide a specific profile of the polyphenol C-glycosides from the complex mixture. Structure identification of unknown xanthone C-glycosides and flavone C-glycosides in the extract was further addressed by comparison with the UV–vis and the product ion spectra to the known compounds, as well as the product ion spectral data in the literature. Six polyphenol C-glycosides, including three xanthone C-glycosides and three flavone C-glycosides, were tentatively identified. Meanwhile, a systematical study on the fragmentation mechanism of xanthone C-glycosides has been

given. This work also provided a rapid, precise, and accurate method for the detection and identification of polyphenol C-glycosides in the complex mixtures. It can not only achieve the good separation of compounds, but also obtain a rapid discrimination and identification of the known and unknown compounds in the natural products, including the isomers. Meanwhile, an alternative method for the study of natural products has been developed.

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