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Journal of Pharmaceutical and Biomedical Analysis 46 (2008) 418–430
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
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Systematic screening and characterization of flavonoid glycosides in *Carthamus tinctorius* L. by liquid chromatography/UV diode-array detection/electrospray ionization tandem mass spectrometry

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Received 24 July 2007; received in revised form 22 October 2007; accepted 25 October 2007
Available online 20 December 2007

Abstract

The traditional Chinese medicine (TCM) is a complex system, which always consists of numerous compounds with significant difference in the content and physical and chemical properties. In this paper, a screening method based on target molecular weights was developed to characterize the flavonoid glycosides in the flower of *Carthamus tinctorius* L. The screening tables of aglycone and glycan were designed, respectively, in order to select and combine freely. The multiple reaction monitoring (MRM) scan mode with higher sensitivity and selectivity was adopted in the screening, which benefit the characterization for the minor components. Seventy-seven flavonoid glycosides were screened out finally, and their structures were characterized by tandem mass spectrometric method in both positive and negative ion modes. The glycosylation mode, aglycone, sequence and/or the interglycosidic linkages of the glycan portion and glycosylation position were elucidated by the fragmentation rule in the MS. Numerous compounds screened out with this method showed the structure variety in secondary plant metabolites, and the purposeful screening systemically and subsequent structure characterization offered more information about the chemical constitutions of TCM.
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Keywords: Screening; Characterization; Flavonoid glycosides; *Carthamus tinctorius* L.; HPLC/DAD/ESI-MS^n

1. Introduction

The traditional Chinese medicine (TCM) is a complex sample, which always includes kinds of components, with numerous compounds showing large variation in the content and physical and chemical properties. The complexity of TCM presents a considerable challenge to the research on its chemical constituents. It is necessary to develop an effective and systematic method for characterizing a complex sample. Advances in chromatographic and spectroscopic techniques permit the isolation and structural analysis of numerous compounds in the TCM. The hyphenated techniques combine the advantage of HPLC (high selectivity and separation efficiency) and MS (structural information and further increase in selectivity) [1], so it facilitates the identification of structurally related compounds in complex mixtures. Many characterization for TCM have been carried out by LC/MS to obtain more information [2,3], especially for the flavonoids [4–6]. The fragmentation rule in mass spectrum offers us the ability to identify the related unknown compounds [7–9].

The flower of *Carthamus tinctorius* L. is an important medicinal material in the prescriptions used for cardiovascular disease. Flavonoid glycoside are main homologous constituents in flower of *C. tinctorius* L. [10–15], some acetylenic glucosides are also reported [16]. Although the separation and identification with phytochemistry methods have been developed, the preliminary analytical results showed that numerous compounds have not been investigated yet. Characterizing flavonoid glycosides in the flower of *C. tinctorius* L. has great significance to the research on active components and quality control.

In order to characterize the flavonoid glycosides by MS, the characteristics of their structures were introduced firstly. Flavonoids is one of the most important groups of secondary plant metabolites, which exists in both the free aglycones and the glycoside forms. More than 6500 known flavonoids existing

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doi:10.1016/j.jpba.2007.10.036

in many medicinal species show diverse structure [17] and broad range of biological activities [18–20]. ‘Flavonoids’ is a collective noun which includes several classes of compounds with similar structure having a common C6-C3-C6 flavone skeleton. The major classes are flavones, isoflavones, flavonols, anthocyanins, flavanones, catechins, chalcones and aurones. They are differentiated on the degree of unsaturation and oxidation of the three-carbon segment [21]. Within different subclasses, further differentiation is possibly based on the number and nature of substituent groups attached to the rings. It is well known that the flavonoid glycosides have many isomers with the same molecular weight but different aglycone and sugar conjugating at multiple linkage positions. In this paper, the screening of flavonoid glycosides in a complex sample was achieved by designing the screening table based on the structure characteristic. The subsequent structure characterization was carried out by the tandem mass spectrometric method.

2. Experimental

2.1. Materials

Standards of quercetin, kaempferol, rutin, and hydroxysafflor yellow A were purchased from the Chinese Authentication Institute of Material Medical and Biological Products (Beijing, China); 6-hydroxykaempferol-3,6,7-triglucoside was isolated from the *C. tinctorius* L. and confirmed by NMR. The flower of *C. tinctorius* L. was used for investigation, which was purchased from Xinjiang province and identified by Xiyuan Hospital.

2.2. Extraction and hydrolysis

The water extract fraction of *C. tinctorius* L. was the standard fraction of Key Project of Knowledge Innovation Program of Chinese Academy of Sciences (KGCX2-SW-213). The standard fraction was processed as follows: 100 kg herb of *C. tinctorius* L. was extracted by 1000 l water twice for 2 h and 1.5 h, respectively. The decoction was collected and concentrated until its relative density reached to 1.15–1.20 at 60 °C. The decoction was dried to fine powder by spray dryer. Inlet and outlet temperature of spray drying were 120 and 115 °C, respectively, and feed velocity was set at 1000 ml/min. The fine powder will be pretreated according to the aim of investigation. In this experiment, an aliquot of 0.5 g fine powder was weighed, and 20 ml 75% ethanol were added, then the extraction was processed in an ultrasonic water bath for 10 min. The solution was filtered to removal deposition which included impurity having low solubility at 75% ethanol, such as oligosaccharides and oligopeptides and so on. The filtrate was diluted to 25 ml accurately, and then was filtered through 0.45 μm membranes before analysis. 5 μl of the sample solution was injected for HPLC-MS analysis.

The acid hydrolysis experiment was performed to identify the aglycone firstly. Acid hydrolysis was achieved by adding 5 ml of 2N HCl to 5 ml sample solution and keeping them reflux for 20 min under nitrogen protection. The solution was filtered through 0.45 μm membranes before analysis. 5 μl of the filtrate was then injected for HPLC-MS analysis.

2.3. LC/UV-DAD/ESI-MS^n analyses

The LC/DAD/ESI-MS^n analysis was performed on an Agilent HPLC 1100 Series instrument equipped with a diode-array detector and a mass detector in series (Agilent Technologies, Waldbronn, Germany). The HPLC system consisted of a quaternary pump, an autosampler, a degasser, a photodiode-array detector, an automatic thermostatic column compartment and a computer with the Chemstation software (Agilent, Rev. B 10.02). The mass detector was an ion-trap mass spectrometer equipped with an ESI interface and was controlled by LC/MSD software (Agilent, Rev. 5.3). Nitrogen above 98% purity was used as nebulising gas at pressure of 35 psi and the flow was adjusted to 8 l/min. The temperature of drying gas was maintained at 325 °C and the voltage was set at 3.5 kV. The full scan covered the mass range from *m/z* 50 to 1000. Collision-induced dissociation (CID) experiments were performed in the ion trap using helium as the collision gas. The screening was working on the multiple reaction monitoring (MRM) mode. Ten ions were set to be detected in one experiment and mass width was set as 2 Da, and six MRM experiments were needed in all. The structure characterization and validation was carried out at auto MS^n mode with voltage ramping cycles from 0.3 up to 2 V and the MS data was acquired in negative and positive ion modes, respectively.

HPLC were running on a Phenomenex Luna C18 (2) column (250 mm × 4.6 mm i.d.; 5 μm particle size). The mobile phase was composed of water containing 0.1% (v/v) formic acid(A) and methanol(B). The linear gradient started from 5% B and reached to 30% B in 40 min, then reached to 65% B in 60 min. The flow rate was 1 ml min^{-1} and split out 200 μl min^{-1} to MS. Spectral data for all peaks were accumulated in the range 200–600 nm.

2.4. The design of screening table

The screening table of flavonoid glycosides was designed to separate screening table of aglycone and glycan. The structures of flavone, flavanone, isoflavone, isoflavanone, chalcone and dihydrochalcone have three molecular weights 222, 224 and 226 (Fig. 1). The molecular weights of flavonol and flavanonol were 238 and 240, respectively, which are same as the molecular weight of flavone and flavanoe with a hydroxyl substitute. Hence, three molecular weights 222, 224 and 226 could include the molecular weights of eight aglycone nucleus, and they were used to construct the screening table for aglycone by adding the substitutent groups. The hydroxyl and methoxyl were chosen as substitutent groups and the number of them was limited to six. Hence, the screening table, which was designed by arranging hydroxyl and methoxyl at three molecular weights from one to six positions, included 81 molecular weights in which 30 data were repeated (Table 1).

The familiar monosaccharides, disaccharides and trisaccharides linking with aglycone were listed in Table 2. Although the sugars have different configurations, they have specific neutral loss in the tandem mass spectrometry [22]. Nine monosaccharides produced four neutral losses (132, 146, 162 and 176); in the

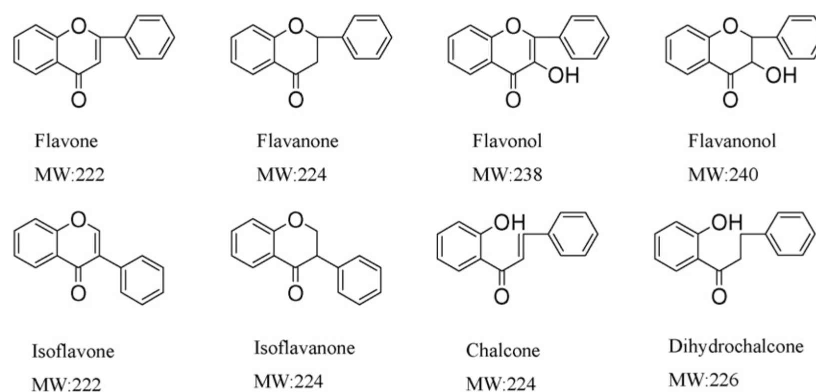


Fig. 1. The chemical structures of flavonoids.

same way, 10 disaccharides could produce three neutral losses (294, 308 and 324), and 12 trisaccharides could produce three neutral losses (454, 470 and 486). Thereby the neutral loss could be adopted as the descriptor of sugars. It is not enough to describe all the possibilities of glycan only using the neutral losses of familiar sugar moieties. For example, a flavonoid-diglycoside may include a disaccharide or two monosaccharides in the different positions. Some possibilities of the latter would be neglected if we only consider the disaccharides, so we permuted and combined the monosaccharide to give the possible neutral losses of glycan with two sugars. However, the permutation and combination of three or four sugars was rather complicated. Based on the structure of the familiar flavonoid glycosides [23], the screening table of glycan with three sugars was designed by adding the neutral losses of disaccharides and monosaccharides. In the same way, the screening table of glycan with four sugars was designed by adding the neutral losses of trisaccharides and monosaccharides. The screening table for glycan was seen in Table 2.

Table 1
The screening table of aglycone constructed by the arrangement of hydroxyl and methoxyl from one to six positions at aglycone nucleus

	Substituent groups	The molecular weights of aglycone nucleus		
		+222	+224	+226
One substituent group	OH	238	240	242
	OCH_3	252	254	256
Two substituent groups	2 × OH	**254**	**256**	258
	OCH3 + OH	268	270	272
	2 × OCH_3	282	284	286
Three substituent groups	3 × OH	**270**	**272**	274
	2 × OH + OCH_3	**284**	**286**	288
	OH + 2 × OCH_3	298	300	302
	3 × OCH_3	312	314	316
Four substituent groups	4 × OH	**286**	**288**	290
	3 × OH + OCH_3	**300**	**302**	304
	2 × OH + 2×OCH_3	**314**	**316**	318
	OH + 3 × OCH_3	328	330	332
	4 × OCH_3	342	344	346
Five substituent groups	5 × OH	**302**	**304**	306
	4 × OH + OCH_3	**316**	**318**	320
	3 × OH + 2 × OCH_3	**330**	**332**	334
	2 × OH + 3 × OCH_3	**344**	**346**	348
	OH + 4 × OCH_3	358	360	362
	5 × OCH_3	372	374	376
Six substituent groups	6 × OH	**318**	**320**	322
	5 × OH + OCH_3	**332**	**334**	336
	4 × OH + 2 × OCH_3	**346**	**348**	350
	3OH + 3 × OCH_3	**360**	**362**	364
	2 × OH + 4 × OCH_3	**374**	**376**	378
	OH + 5 × OCH_3	388	390	392
	6 × OCH_3	402	404	406

Note: 222, 224 and 226 are molecular weights for eight familiar subclasses flavonoids without substitutent groups. The ions shown in bold have the same nominal molecular weights as the others which were used to construct the screening table of flavonoid glycosides.

3. Results and discussion

3.1. Method validation

The reproducibility of separation was validated by five successive injections. The chromatogram at 280 nm is too complex to integrate accurately. So the chromatogram at 403 nm which are characteristic absorption of chalcone was selected to validate the reproducibility of separation. Twelve peaks were marked, and relative standard deviations (R.S.D.) of their retention time were less than 0.5% in the five experiments, and R.S.D. of area% were less than 4%. Flavonoid glycoside has good MS signal in both negative and positive ion modes. The screening for a target molecular weight in two modes gave further validation. The screening for *m/z* 625 and 627 in negative and positive ion modes, respectively, showed the same screening result (Fig. 2). The peaks in the extracted ion chromatogram (EIC) had the identical retention time.

3.2. Identification of aglycone produced by the hydrolysis

The hydrolysis experiment was adopted to find aglycone firstly. The experiment condition for hydrolysis was optimized to obtain complete hydrolysis for most flavonoid glycosides. C-glycosyl flavonoids formed by linkage of carbon–carbon bond are resistant to acid hydrolysis, while the O-glycosyl flavonoids

Table 2
The familiar monosaccharides, disaccharides and trisaccharides and the screening table of glycan constructed by arrangement of their neutral losses in the tandem mass spectrometry

		Neutral losses of monosaccharides			
		+132	+146	+162	+176
Familiar monosaccharides and their neutral losses		Neutral losses of glycan with two sugars[a]			
L-Arabinose = D-xylose	(132)	264	278	294	308
L-Rhamnose = D-apiose	(146)	278	292	308	322
D-Glucose = D-galactose = D-mannose = D-allose	(162)	294	308	324	338
D-Glucuronic acid	(176)	308	322	338	352
Familiar disaccharides and their neutral losses		Neutral losses of glycan with three sugars[b]			
Vicianose = lathyrose = sambubiose	(294)	426	440	456	470
Rutinose = neohesperidose = rungiose = robinobiose	(308)	440	454	470	484
Sophorose = laminaribiose = gentiobiose	(324)	456	470	486	500
Familiar trisaccharides and their neutral losses		Neutral losses of glycan with four sugars[c]			
2′-Rhamnosyl rutinose = rhamninose = sugar of alaternin = 2^{Gal}-rhamnosylrobinbiose = 4^{G}-rhamnosylneohesperidose	(454)	586	600	616	630
2^{G}-Glucosylrutinose = 2^{G}-rhamnosylgentiobiose = 3^{G}-glucosylnohesperidose	(470)	602	616	632	646
2′-Glucosylgentiobiose = sophorotriose = sorborose = 2^{G}-glucosylrobinobiose	(486)	618	632	648	662

Note: the ions shown in bold were the same as others which were used to construct the screening table of flavonoid glycosides.
[a] Neutral losses of glycan with two sugars were achieved by permutation and combination of neutral losses of the monosaccharide.
[b] Neutral losses of glycan with three sugars were achieved by adding the neutral losses of disaccharides and monosaccharides.
[c] Neutral losses of glycan with four sugars were achieved by adding the neutral losses of trisaccharides and monosaccharides.

formed by linkage of carbon–oxygen bond are acid labile. C-glycosyl flavonoids were easy to be differentiated by MS characterization.

In the hydrolysis experiment, the screening for the aglycone was processed under full scan mode. After screening with the screening table of aglycone, six aglycones showing three molecular weights were detected obviously (Fig. 3). The structures of aglycones were identified at both positive and negative ion modes, and some structures were further validated with standards. Peaks 1 and 6 were identified as kaempferol and quercetin. In positive ion mode, these two compounds showed typical neutral losses (18, 28, 46, 56 and 74 Da) and common

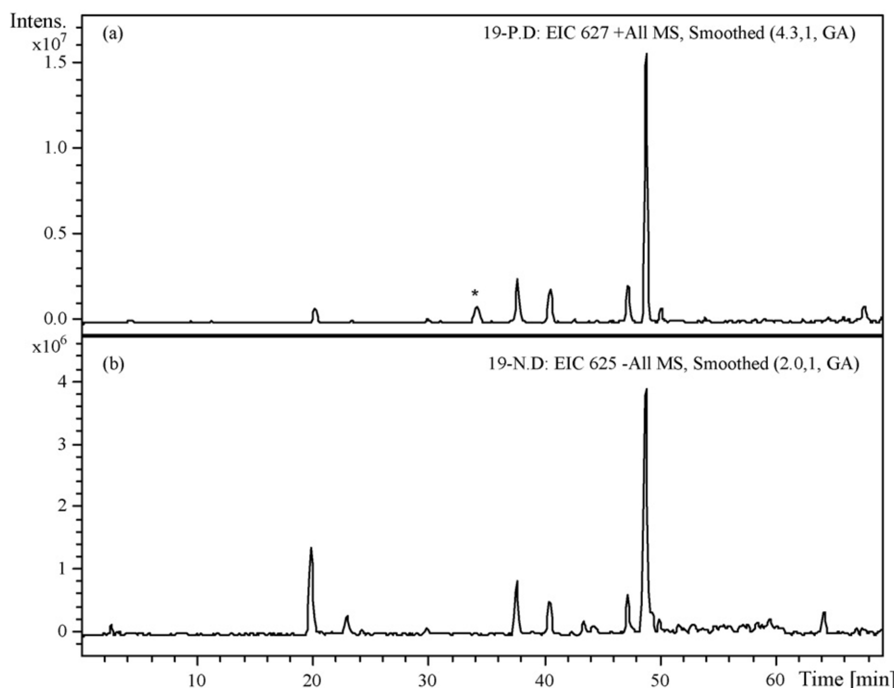


Fig. 2. The extracted ion chromatograms (EICs) of *m*/*z* 627 in positive ion mode (a) and *m*/*z* 625 in negative ion mode (b). The deprotonated molecular of peak marked with asterisk was *m*/*z* 789, and *m*/*z* 627 was its fragment ion.

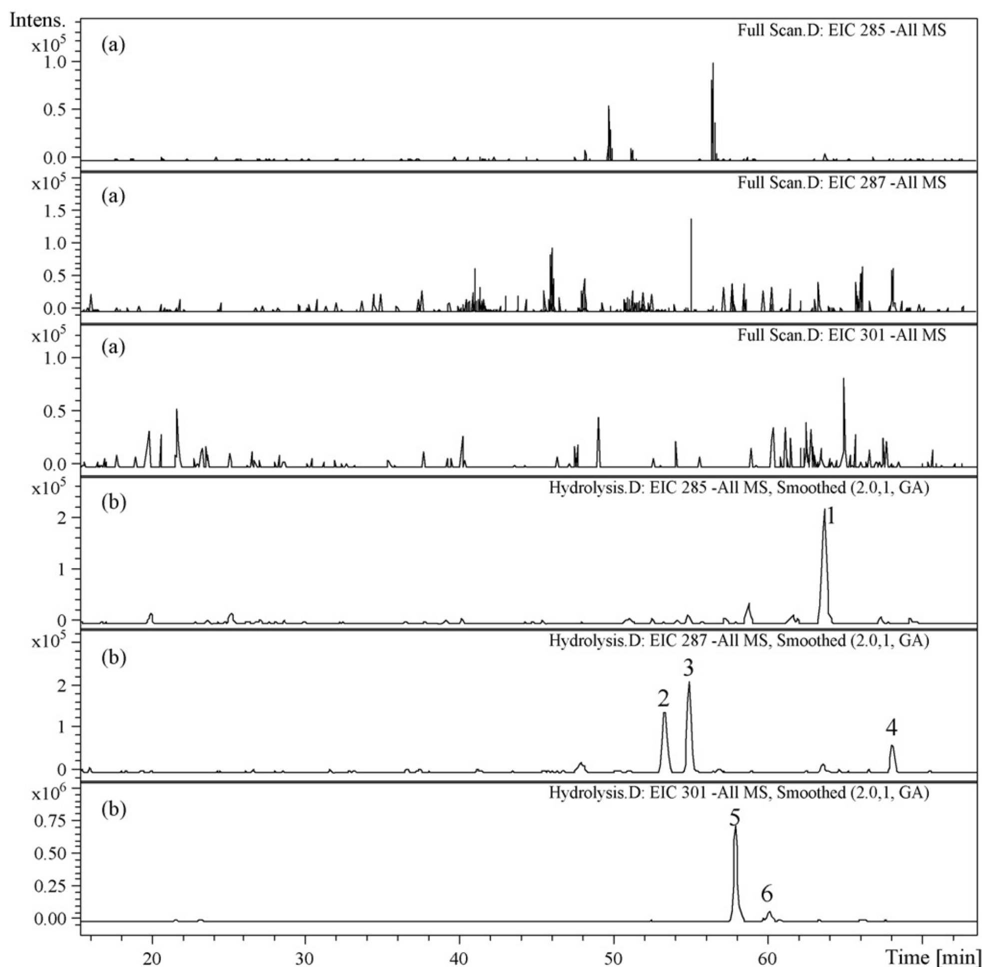
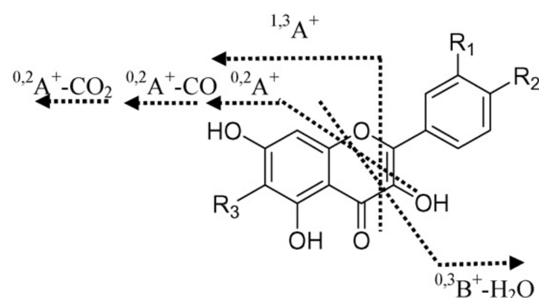


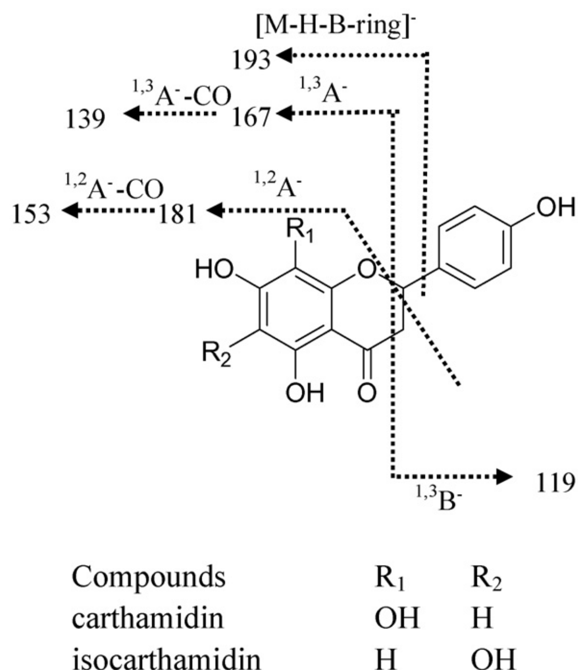
Fig. 3. Screening result of aglycone before (a) and after (b) hydrolysis. (1) Kaempferol, (2 and 3) the isomer of carthamidin or isocarthamidin, (4) unknown, (5) 6-hydroxykaempferol and (6) quercetin.

fragment ions containing intact A-rings, such as $^{0,2}A^+$ (*m*/*z* 165), $^{1,3}A^+$ (*m*/*z* 153), $^{0,2}A^+$-CO (*m*/*z* 137), and $^{0,2}A^+$-CO_2 (*m*/*z* 121). The $^{0,3}B^+$-H_2O fragment ions were *m*/*z* 149 and 133 for qucercetin and kaempferol, respectively, for more a hydroxyl at 3′-position of quercetin (Scheme 1). Peak 5 was identified as 6-hydroxykaempferol by fragmentation rule of quercetin and kaempferol. It showed same neutral losses as kaempferol and quercetin. The *m*/*z* 181, 169, 153 were due to the fragmentation at $^{0,2}A^+$, $^{1,3}A^+$, $^{0,2}A^+$-CO, which were 16 Da higher than the corresponding fragmentations of qucercetin and kaempferol. This judgment was verified with the isolation of 6-hydroxykaempferol-3,6,7-triglucoside. The fragmentation of qucercetin and kaempferol in negative ion mode were consistent with the literature [24]. $^{1,2}A^-$ (*m*/*z* 179) and $^{1,2}A^-$-CO (*m*/*z* 151) were the main fragmentations of the qucercetin, and the special neutral loss of 30 Da (reasonably attributed to HCHO-formaldehyde) was observed by the loss of the carbonyl at C3 position [25]. Peaks 2 and 3 showed the same molecular weight and fragment ions. Their structures were deduced to carthamidin and isocarthamidin isomers. The base fragment ion was $^{1,2}A^-$ (*m*/*z* 181), and other fragment ions at *m*/*z* 153, 167, 139 and 193 were attributed to $^{1,2}A^-$-CO, $^{1,3}A^-$, $^{1,3}A^-$-CO, and [M−H-B-ring]$^-$, respectively. The $^{1,3}B^-$ (*m*/*z* 119) was



Compounds	R_1	R_2	R_3
quercetin	OH	OH	H
kaempferol	H	OH	H
6-hydroxykaempferol	H	OH	OH

Scheme 1. Chemical structures of flavonol aglycone and the proposed fragmentation pathway in positive ion mode.



Scheme 2. Chemical structures of carthamidin or isocarthamidin and the proposed fragmentation pathway in negative ion mode.

also observed (Scheme 2). The structure of peak 4 could not be confirmed only by its MS data. The screening table of flavonoid glycosides for characterizing flower of *C. tinctorius* L. was produced by adding the neutral losses of glycan to three mass charge ratios of aglycone in negative ion mode. 58 data were included in the screening table besides 35 repeated data (Table 3).

Table 3
The screening table of flavonoid glycosides for characterizing *Carthamus tinctorius* L. in negative ion mode

	The neutral losses of glycan[a]	Aglycone screened out in hydrolysis experiment[b] $[M-H]^-$ (*m/z*)		
		+285	+287	+301
Flavonoid glycosides with one sugar	132	417	419	433
	146	431	**433**	447
	162	**447**	449	463
	176	461	**463**	477
Flavonoid glycosides with two sugars	264	549	551	565
	278	563	**565**	579
	292	577	**579**	593
	294	**579**	581	595
	308	**593**	**595**	609
	322	607	**609**	623
	324	**609**	611	625
	338	**623**	**625**	639
	352	637	**639**	653
Flavonoid glycosides with three sugars	426	711	713	727
	440	725	**727**	741
	454	739	**741**	755
	456	**741**	743	757
	470	**755**	**757**	771
	484	769	**771**	785
	486	**771**	773	787
	500	**785**	**787**	801
Flavonoid glycosides with four sugars	586	871	873	887
	600	885	**887**	901
	602	**887**	889	903
	616	**901**	**903**	917
	618	**903**	905	919
	630	915	**917**	931
	632	**917**	**919**	933
	646	**931**	**933**	947
	648	**933**	935	949
	662	**947**	**949**	963

Note: the deprotonated molecular ions used for screening the flavonoid glycosides in negative ion mode were achieved by adding the mass charge ratios of aglycone and the neutral losses of the glycan. The ions shown in bold have the same nominal molecular weights as others used in the screening

[a] The neutral losses of glycan were the data in screening table of glycan (Table 2).

[b] The aglycones screened out in the hydrolysis experiment were used to develop the screening table of flavonoid glycosides.

3.3. Screening for flavonoid glycosides in the flower of C. tinctorius L.

Three scan modes of ion trap mass spectrometry can be applied for screening. They are full scan, auto MS^n and MRM. Full scan is the simplest way, with which only one experiment is need. The screening with auto MS^n mode could provide further structure information simultaneously. However, both full scan and auto MS^n might miss some minor components because they give lower sensitivity and selectivity than MRM mode has. In the MRM mode of the ion trap mass spectrometry, most ten ions were selected to be dissociated with collision-induced dissociation in the MS^2, and MS^2 chromatogram is recorded. In the data processing, chromatogram which records special parent ion producing special daughter ion is extracted. Multiple neutral losses from one parent ion could be extracted in the MRM mode of ion trap mass spectrometry, so the numbers of experiment is decreased. The characterization of the *C. tinctorius* L. showed numerous co-eluted compounds or minor components. Hence, the MRM mode with higher sensitivity and selectivity was selected for screening flavonoid glycosides in the flower of *C. tinctorius* L. systematically. The screening according to Table 3 finally found 77 compounds (Table 4), and their structures were elucidated by tandem mass spectrometric method.

3.4. Structure characterization of the flavonoids glycosides by MS^n

The screening made us found the target compounds, and their structures were deduced and validation. In the structure characterization, we first judged the flavonoid glycoside is a C-glycosylated flavonoid or O-glycosylated flavonoids, and then the aglycone and the glycan were identified, respectively. It is difficult to confirm the glycosylation position. Hence, even if the aglycone and glycan were all confirmed, the accurate structure of the flavonoids glycoside could not be determined because of the uncertain connecting position. The structures of some compounds were finally identified by comparison, and most compounds were left for further identification.

Table 4
The result of screening and structure characterization of flavonoid glycosides in negative ion mode

Peak number	t_R (min)	$[M-H]^-$ m/z	HPLC/ESI-MS^n m/z (%)	Aglycone		Glycan
				m/z	Attribution	
1	52.4	447	MS^2[447]: 285(100), 327(4)	285	Unknown[a]	glu/gal
2	54.5	447	MS^2[447]: 285(100), 327(6), 357(4)	285	Unknown[a]	glu/gal
3	56.3	447	MS^2[447]: 285(100), 327(6)	285	Unknown[a]	glu/gal
4	37.4	449	MS^2[449]: 287(100), 269(66), 259(63), 329(19), 345(10)	287	Unknown[a]	glu/gal
5	39.5	449	MS^2[449]: 329(100), 359(9)	287	Unknown[b]	glu/gal
6	40.2	449	MS^2[449]: 329(100), 359(6)	287	Unknown[b]	glu/gal
7	40.7	449	MS^2[449]: 287(100), 329(8)	287	Carthamidin\isocarthamidin	glu/gal
8	41.7	449	MS^2[449]: 287(65), 329(100), 359(20)	287	Carthamidin\isocarthamidin	glu/gal
9	47.7	449	MS^2[449]: 287(100), 329(3)	287	Carthamidin\isocarthamidin	glu/gal
10	51.2	449	MS^2[449]: 287(100), 329(6)	287	Carthamidin\isocarthamidin	glu/gal
11	54.9	449	MS^2[449]: 287(100), 329(5)	287	Carthamidin\isocarthamidin	glu/gal
12	50.6	463	MS^2[463]: 301(100)	301	Quercetin	glu/gal
13	51.4	463	MS^2[463]: 301(100)	301	6-Hydroxykaempferol	glu/gal
14	53.5	463	MS^2[463]: 301(100)	301	Quercetin	glu/gal
15	55.5	463	MS^2[463]: 301(100)	301	6-Hydroxykaempferol	glu/gal
16	56.8	593	MS^2[593]: 285(100)	285	Kaempferol	rut
17	25.2	609	MS^2[609]: 489(100), 519(5) MS^3[609 → 489]: 339(100)	285	Unknown[b]	2 × glu/gal
18	26.6	609	MS^2[609]: 489(100), 519(4) MS^3[609 → 489]: 339(100)	285	Unknown[b]	2 × glu/gal
19	42.9	609	MS^2[609]: 447(100), 489(23), 285(20) MS^3[609 → 447]: 284(100), 285(44), 327(18)	285	Unknown[a]	2 × glu/gal
20	48.2	609	MS^2[609]: 285(100), 447(71)	285	Unknown[a]	sop
21	49.5	609	MS^2[609]: 301(100)	301	Unknown[a]	rut
22	51.6	609	MS^2[609]: 301(100)	301	6-Hy droxykaempferol	rut
23	53.5	609	MS^2[609]: 301(100)	301	Quercetin	rut
24	54.3	609	MS^2[609]: 301(100)	301	Unknown[a]	rut
25	20.3	611	MS^2[611]: 491(100), 593(4), 473(4), 521(4) MS^3[611 → 491]: 473(100), 353(40), 323(48)	287	Unknown[b]	2 × glu/gal
26	20.9	611	MS^2[611]: 491(100), 593(12), 473(6), 521(3) MS^3[611 → 491]: 473(100), 353(14), 323(18), 429(28), 341(60)	287	Unknown[b]	2 × glu/gal
27	34.5	611	MS^2[611]: 491(100), 593(15), 521(60), 551(5) MS^3[611 → 491]: 329(100)	287	Unknown[c]	2 × glu/gal
28	37.2	611	MS^2[611]: 491(100), 593(17), 521(57), 551(4) MS^3[611 → 491]: 329(100)	287	Unknown[c]	2 × glu/gal
29	41.1	611	MS^2[611]: 491(100), 593(3), 473(7), 521(4), 403(9), 325(14)	287	4′,5′,6′ 4-Tetrahydroxy-quinochalcone	2 × glu/gal
30	40.8	611	MS^2[611]: 449(100), 287(14) MS^3[611 → 449]: 287(100), 329(6)	287	Carthamidin\isocarthamidin	2 × glu/gal
31	47.9	611	MS^2[611]: 449(100), 287(10), 521(12), 431(5) MS^3[611 → 449]: 287(100), 329(4)	287	Carthamidin\isocarthamidin	2 × glu/gal
32	43.3	623	MS^2[623]: 447(100) MS^3[623 → 447]: 284(100), 285(46), 327(9)	285	Kaempferol	glu/gal + glu A
33	46.0	623	MS^2[623]: 447(100), 301(13) MS^3[623 → 447]: 301(100)	301	Unknown[a]	glu A + rha
34	49.4	623	MS^2[623]: 447(100) MS^3[623 → 447]: 285(100)	285	Unknown[a] (not flavanol)	glu/gal + glu A
35	49.8	623	MS^2[623]: 447(100) MS^3[623 → 447]: 285(100)	285	Unknown[a] (not flavanol)	glu/gal + glu A
36	18.9	625	MS^2[625]: 505(100) MS^3[625 → 505]: 355(100), 427(15), 397(16), 343(24)	301	Unknown[b]	2 × glu/gal

Table 4 (*Continued*)

Peak number	t_R (min)	$[M-H]^-$ m/z	HPLC/ESI-MS^n m/z (%)	Aglycone		Glycan
				m/z	Attribution	
37	22.0	625	MS^2[625]: 505(100) MS^3[625 → 505]: 355(100), 427(6), 397(7), 343(35)	301	Unknown[b]	2 × glu/gal
38	37.2	625	MS^2[625]: 463(100), 301(36), 505(10) MS^3[625 → 463]: 301(100)	301	Quercetin	2 × glu/gal
39	40.0	625	MS^2[625]: 463(100), 301(6) MS^3[625 → 463]: 301(100)	301	6-Hydroxykaempferol	2×glu/gal
40	42.8	625	MS^2[625]: 449(100) MS^3[625 → 449]: 287(100), 329(9)	287	Carthamidin/isocarthamidin	glu/gal + glu A
41	43.6	625	MS^2[625]: 449(100) MS^3[625 → 449]: 287(100), 329(4)	287	Carthamidin/isocarthamidin	glu/gal + glu A
42	46.8	625	MS^2[625]: 463(100), 301(8) MS^3[625 → 463]: 301(100)	301	6-Hydroxykaempferol	2 × glu/gal
43[d]	48.9	625	MS^2[625]: 463(100), 301(39), 449(31) MS^3[625 → 463]: 301(100)	301	6-Hydroxykaempferol	2 × glu/gal
44[e]	48.9	625	MS^2[625]: 463(100), 301(39), 449(31) MS^3[625 → 449]: 287(100), 329(6)	287	Carthamidin/isocarthamidin	glu/gal + glu A
45	63.9	625	MS^2[625]: 449(100), 607(20), 563(30), 505(8) MS^3[625 → 449]: 287(100), 431(89)	287	Chalcone	glu/gal + glu A
46	64.6	625	MS^2[625]: 449(100), 607(18), 563(19) MS^3[625 → 449]: 287(100), 431(75)	287	Chalcone	glu/gal + glu A
47	37.0	639	MS^2[639]: 463(100), 301(5), 505(7) MS^3[639 → 463]: 301(100), 343(14)	301	Quercetin	glu/gal + glu A
48	41.5	639	MS^2[639]: 463(100) MS^3[639 → 463]: 301(100)	301	6-Hydroxykaempferol	glu/gal + glu A
49	48.1	639	MS^2[639]: 463(100) MS^3[639 → 463]: 301(100)	301	Unknown[a]	glu/gal + glu A
50	44.3	755	MS^2[755]: 593(100) MS^3[755 → 593]: 285(100)	285	Unknown[a]	glu/gal + rut
51	34.1	757	MS^2[757]: 595(100) MS^3[757 → 595]: 433(100), 463(12), 301(11)	301	Unknown[a]	2 × glu/gal + ara/xyl
52	35.1	757	MS^2[757]: 595(100), 463(14), 301(4) MS^3[757 → 595]: 433(10), 432(33), 463(100), 301(23)	301	Unknown[a]	2 × glu/gal + ara/xyl
53	39.2	757	MS^2[757]: 595(100) MS^3[757 → 595]: 433(100), 475(17)	301	Unknown[a]	2 × glu/gal + ara/xyl
54	42.7	757	MS^2[757]: 449(100), 595(37), 287(19) MS^3[757 → 449]: 287(100), 329(4)	287	Carthamidin	glu/gal + rut
55	44.4	757	MS^2[757]: 449(29), 595(100), 287(9) MS^3[757 → 595]: 287(100), 329(10)	287	Carthamidin	glu/gal + rut
56[f]	47.0	757	MS^2[757]: 595(100) MS^3[757 → 595]: 301(100)	301	Unknown[a]	glu/gal + ara/xly (1 → 6)
57	49.0	757	MS^2[757]: 449(100), 595(26), 287(18) MS^3[757 → 449]: 287(100), 329(6)	287	Carthamidin	glu/gal + rut
58	32.0	771	MS^2[771]: 609(100) MS^3[771 → 609]: 429(60), 447(21), 285(100)	285	Unknown[a]	glu/gal + sop
59	35.0	771	MS^2[771]: 595(100) MS^3[771 → 595]: 433(100), 301(55), 463(5)	301	Unknown[a]	glu/gal + glu A + ara/xly

Table 4 (*Continued*)

Peak number	t_R (min)	$[M-H]^-$ m/z	HPLC/ESI-MS^n m/z (%)	Aglycone		Glycan
				m/z	Attribution	
60	36.1	771	MS^2[771]: 595(100) MS^3[771 → 595]: 463(100), 433(8), 301(12)	301	Unknown[a]	glu/gal + glu A + ara/xly
61	38.4	771	MS^2[771]: 609(100), 463(8), 301(6) MS^3[771 → 609]: 447(76), 463(55), 301(100)	301	Quercetin	2 × glu/gal + rha
62	41.5	771	MS^2[771]: 609(100), 463(16) MS^3[771 → 609]: 301(100)	301	Unknown[a]	glu/gal + rut
63	43.2	771	MS^2[771]: 609(100) MS^3[771 → 609]: 447(100), 489(7), 463(17), 301(16)	301	Unknown[a]	2 × glu/gal + rha
64	48.9	771	MS^2[771]: 609(100), 463(2), 301(5) MS^3[771 → 609]: 301(100)	301	6-Hydroxykaempferol	glu/gal + rut
65	39.7	785	MS^2[785]: 609(100) MS^3[785 → 609]: 447(73), 301(100), 463(71)	301	6-Hydroxykaempferol	glu/gal + gluA + rha
66	25.0	787	MS^2[787]: 697(11), 667(100), 625(23), 505(42), 535(6)	301	Unknown[c]	3 × glu/gal
67	30.6	787	MS^2[787]: 625(100) MS^3[787 → 625]: 463(100), 301(6)	301	Unknown[a]	3 × glu/gal
68	33.7	787	MS^2[787]: 625(100), 667(2), 607(5) MS^3[787 → 625]: 463(100), 301(38)	301	6-Hydroxykaempferol	3 × glu/gal
69	43.8	787	MS^2[787]: 625(100) MS^3[787 → 625]: 607(21), 479(24), 359(10), 317(100)	317	Unknown[a]	glu/gal + neo
70	44.1	787	MS^2[787]: 625(100)	301	Unknown[a]	glu/gal + sop
71	45.3	787	MS^2[787]: 625(100), 301(6), 463(2) MS^3[787 → 625]: 301(100)	301	Unknown[a]	glu/gal + gen
72	46.1	787	MS^2[787]: 625(100), 463(3) MS^3[787 → 625]: 301(100)	301	Unknown[a]	glu/gal + gen
73	30.9	801	MS^2[801]: 625(100) MS^3[801 → 625]: 463(100), 301(12)	301	Unknown[a]	2 × glu/gal + ara/xly
74	35.0	801	MS^2[801]: 625(100), 667(8) MS^3[801 → 625]: 463(100), 301(43)	301	6-Hydroxykaempferol	2 × glu/gal + gluA
75	25.9	933	MS^2[933]: 771(19), 651(86), 813(100), 843(17)	301	Unknown[c]	2 × glu/gal + rut
76	35.5	933	MS^2[933]: 771(100), 625(13) MS^3[933 → 771]: 609(100), 301(8), 463(6) MS^4[933 → 771 → 609]: 301(100)	301	6-Hydroxykaempferol	2 × glu/gal + rut
77	33.6	963	MS^2[963]: 787(100), 625(21) MS^3[963 → 787]: 625(10), 301(20), 667(4), 463(7)	301	Unknown[a]	2 × glu/gal + sop

Abbreviation: glu: glucose, gal: galactose, glu A: glucuronic acid, rha: rhamnose, ara: arabinose, xyl: xylose, rut: rutinose, neo: neohesperidose, sop: sophorose, gen:gentiobiose.

[a] The intensity of the aglycone is too low to be identified accurately.

[b] The aglycone of the C-glycosyl flavonoid was not detected in the MS^n.

[c] The aglycone of the C, O-mixed glycosyl flavonoid was not detected in the MS^n.

[d] The compound 43 and 44 were co-eluted, so the m/z 449 was the fragment ions of compound 44.

[e] The m/z 463 and 301 were the fragment ions of compound 43.

[f] A glu or gal was linked to ara or xly with 1 → 6 linkage mode.

3.4.1. Identification of the glycosylation mode of flavonoids glycosides

The C-glycosylation and O-glycosylation were two main glycosylation modes of the flavonoid glycosides. The great difference in fragmentation could be observed in MS^n, which made them be distinguished easily. The carbon–carbon bond of C-glycosyl flavonoids is resistant to rupture, so the main cleavages are at the bonds in the sugar [26,27]. On the contrary, the sugars of O-glycosyl flavonoids linking at phenolic hydroxyl groups are easily to loss sugar residue by neutral loss.

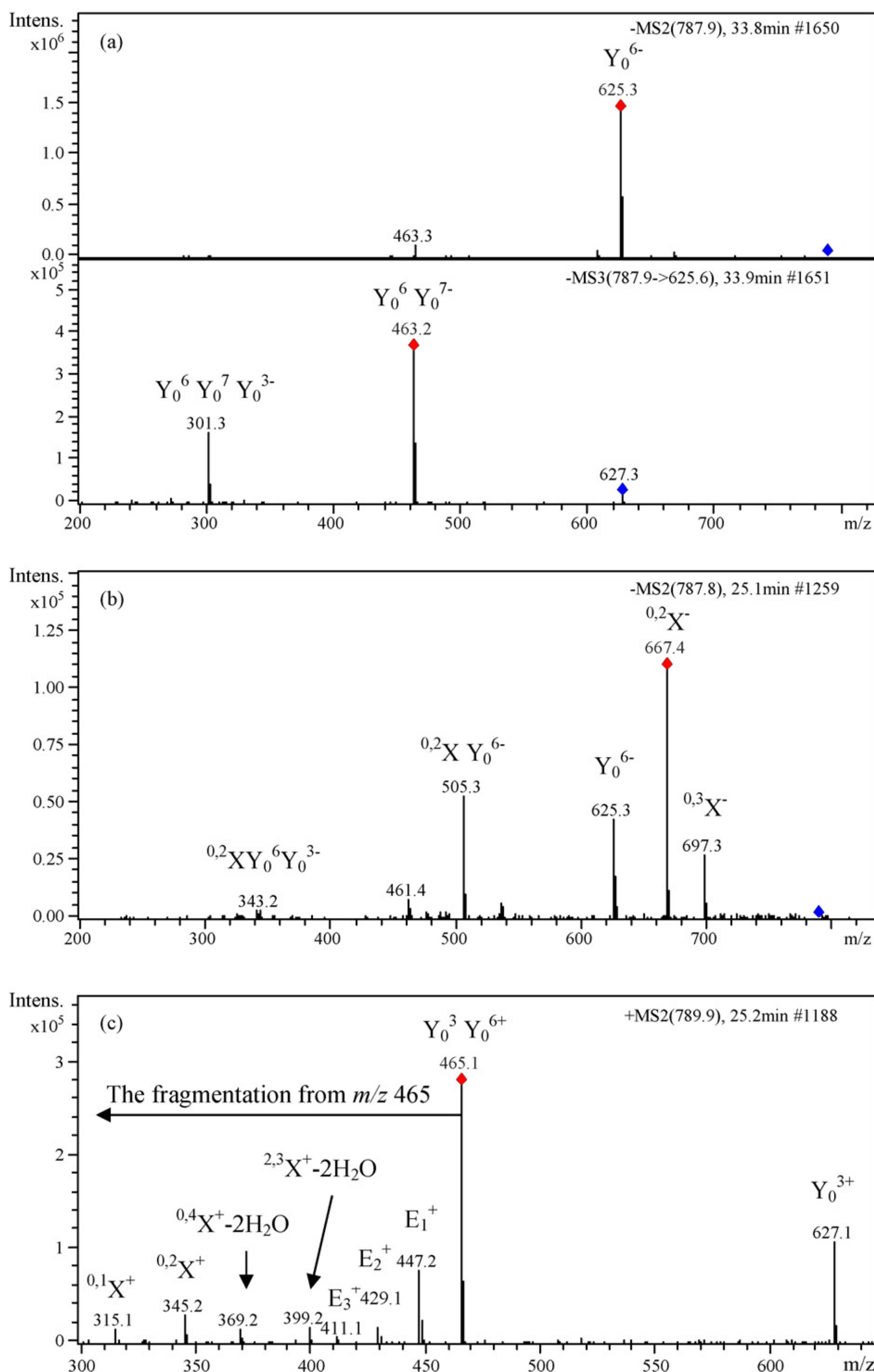


Fig. 4. ESI-MS^n mass spectra of compound 68 (6-hydroxykaempferol-3,6,7-O-triglucoside) and compound 66 (6-hydroxykaempferol-3,6-di-glucosyl-8-C-glucoside). (a) ESI-MS^n spectra of $[M-H]^-$ generated from compound 68 (*m/z* 787), (b) ESI-MS^2 spectrum of $[M-H]^-$ generated from compound 66 (*m/z* 787) and (c) ESI-MS^2 spectrum of $[M+H]^+$ generated from compound 66 (*m/z* 789).

Compound 68 was isolated and identified as 6-hydroxykaempferol-3,6,7-triglucoside belonging to O-glycosyl flavonoid. The deprotonated molecular of compound 68 was *m/z* 787, and the characteristic UV absorption at 338 nm obviously showed the 3-position was glycosylated [28]. In Fig. 4(a), the MS^2 and MS^3 spectra showed the typical neutral loss of O-glycosyl flavonoids. The $[M-H\text{-glu}]^-$ (*m/z* 625, $Y_0{}^{6-}$) in the MS^2 was formed by a neutral loss of a glucose. MS^3 spectra of compounds 68 was similar to MS^2 spectra of compound 38 (quercetin-3,7-diglucoside) and compound 43 (6-hydroxykaempferol-3,7-*O*-diglucoside). A base peak at *m/z* 463 ($Y_0{}^6\ Y_0{}^{7-}$) and *m/z* 301 ($Y_0{}^6\ Y_0{}^7\ Y_0{}^{3-}$) in 38% relative abundance were detected in MS^3. The deprotonated flavonol with substitutes at 3-O and 7-O positions may lose the substitute at 7-O position more easily than that at 3-O position in negative ion mode [29], i.e., the analysis of kaempferol 3,7-*O*-diglucoside

by LC-MS showed the high ratio of $Y_0{}^7$ $Y_0{}^{3-}$ to $Y_0{}^{7-}$ (23%) in the MS^2 spectrum [30]. So we deduced that the sequence of neutral loss in 6-hydroxykaempferol-3,6,7-triglucoside was 6-O, 7-O and 3-O position in turn.

In Fig. 4(b) and Fig. 4(c), compound 66 showed the *m/z* 787 in negative ion mode and *m/z* 789 in positive ion mode, which had the same molecular weight and the similar UV spectrum of 6-hydroxykaempferol-3,6,7-triglucoside but earlier retention time. In MS^2 of negative ion mode, compound 66 showed the main product ions of $^{0,3}X^-$[M−H-90]$^-$, $^{0,2}X^-$[M−H-120]$^-$, $Y_0{}^{6-}$[M−H-162]$^-$, $^{0,2}XY_0{}^{6-}$[M−H-282]$^-$ and $^{0,2}XY_0{}^6$ $Y_0{}^{3-}$[M−H-444]$^-$ (Fig. 4(b)). The fragmentation was different from the pure O-glycosyl flavonoids, such as 6-hydroxykaempferol-3,6,7-triglucoside. In positive ion mode, the MS^2 mainly showed the typical fragmentation of O-glycoside. The loss of a glucose from [M+H]$^+$ ion resulted in the product ion at *m/z* 627, while *m/z* 465 was formed by successive losing another glucose residue. The product ions obtained from *m/z* 465 had no aglycone ion, but typical fragment ions of C-glycosyl flavonoids. $E_1{}^+$[M+H-H_2O]$^+$, $E_2{}^+$[M+H-2H_2O]$^+$, $E_3{}^+$[M+H-3H_2O]$^+$ were the result of successive losses of H_2O, and $^{2,3}X^+$-2H_2O[M+H-66]$^+$, $^{0,4}X^+$-2H_2O[M+H-96]$^+$, $^{0,2}X^+$[M+H-120]$^+$, $^{0,1}X^+$[M+H-150]$^+$ were due to the bond cleavages in the glucose residue (Fig. 4(c)). Thus it can be seen compounds 66 was a C, O-mixed glycosylation. It was tentatively identified as 6-hydroxykaempferol-3,6-diglucosyl-8-C-glucoside according to the fragmentation rule of C-glycosyl flavonoids [26,27].

3.4.2. Identification of the aglycone of the flavonoids glycosides

The structure identification of the aglycones was based on the fragmentation rule concluded above. For example, four compounds showing *m/z* 463 in negative ion mode were screened out, and they all produced the base peak at *m/z* 301 in MS^2, which indicated a glucose or agalactose linking to the aglycone. The fragmentation of *m/z* 301 of four compounds showed different MS spectra. The aglycone of compounds 12 and 14 was identified as quercetion, while the aglycone of compounds 13 and 15 was attributed to the 6-hydroxykaempferol (Table 4). However, the fragment ions of aglycone usually were obtained in MS^4 or MS^5 because of multiple sugars connection. The intense of the fragment ions decreased in multi-stage mass spectrometry greatly so that the fragment ions were not very stable. The sensitivity in the MS^n brought difficulty to deduce the structure accurately, which was one of important reasons why many compounds were left unidentified. Moreover, the C-glycosylated or the C, O-mixed glycosylated flavonoids could not give the fragment ions of the aglycone, so the structure of their aglycones was difficult to deduce.

3.4.3. Identification of the number and category of sugar, the sequence and/or the interglycosidic linkages of the glycan portion

It is easy to identify the number of sugar and the category of parts of sugars by the stepwise neutral loss of glycan obviously in negative ion mode. Fragmentation rules have been concluded to identify the sequence and/or the interglycosidic linkage effectively [31,32]. Compounds 64 and 58 were taken as an example to illustrate the analysis of the glycan portion. The deprotonated molecular of compound 64 was at *m/z* 771, and the cleavages of a glucose unit yielded the fragment ion at *m/z* 609 as the base peak in MS^2. The fragmentation of *m/z* 609 in MS^3 only yielded the aglycone ion. The absence of intermediary ions with relevant abundance resulting from the rupture of the interglycosidic linkage was similar to the fragmentation of rutinosides reported by Cuyckens et al. [32]. The fragmentation of interglycosidic bond $1 \rightarrow 6$ linkage was special in the positive ion mode. The loss of 146 Da was related to a terminal rhamnose unit and the fragment ion at *m/z* 627 was yielded, while an additional loss of 162 Da or the direct loss of the rutinose residue resulted in the fragment ion at *m/z* 465. The rather irregular Y^* ion was corresponding to the loss of the inner glucose residue, which is firstly described by Kovacik et al. [33] (Fig. 5). According to the same fragmentation as report at both positive and negative ion mode, the glycan of compound 64 include a rutinose (rhamnosyl-($1 \rightarrow 6$)-glucose) and a glucose, and they connected to aglycone at two glycosylation positions. Compound 58 produced same fragment ions as those of compound 64 in MS^2. Compared with the fragmentation of the interglycosidic bond $1 \rightarrow 6$ linkage, more fragment ions of compound 58 were observed in MS^3. The fragmentation produced the fragment ions at *m/z* 429(Z^-) with high relative abundance (60%). The characterization of the $1 \rightarrow 2$ interglycosidic linkage was defined by the high relative abundance of

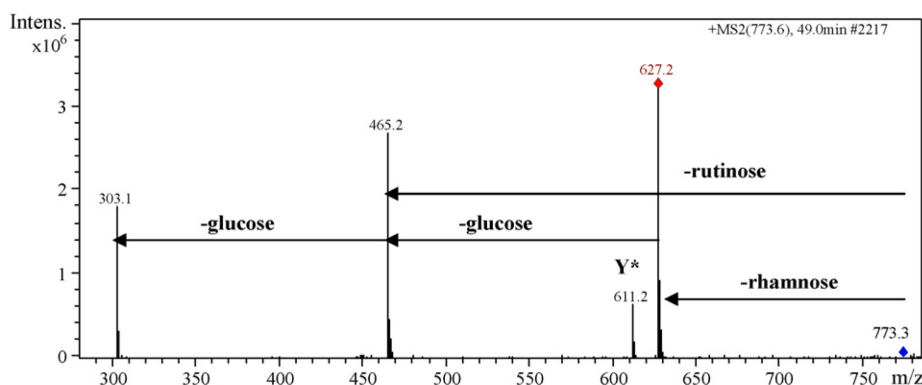


Fig. 5. The MS^2 spectrum of [M+H]$^+$ generated from compound 64(*m/z* 773).

the Y^- and/or Z^- ions [26]. So the glycan of compound 58 included a sophorose (glucosyl-(1 → 2)-glucose). Seven compounds at *m/z* 771 were screened out in all. Their glycan was composed of four sugars with the different categories and/or interglycosidic linkage (Table 4).

3.4.4. Identification of the glycosylation positions of flavonoids glycosides

The O-glycosyl flavonoids always have multi-hydroxyl positions to be glycosylated. It is difficult to determine glycosylation position by MS characterization. In this study, we tried to deduce the glycosylation positions by comparison of some compounds. Compounds 38, 39, 42 and 43 were tentatively identified as quercetin-3,7-diglucoside, 6-hydroxykaempferol-3,6-*O*-diglucoside, 6-hydroxykaempferol-6,7-*O*-diglucoside, and 6-hydroxykaempferol-3,7-*O*-diglucoside, respectively, according to the following deduction. 6-hydroxykaempferol is easy to be glycosylated at three positions (3, 6 and 7-positon). The 6-hydroxykaempferol-6, 7-*O*-diglucoside could be distinguished from UV spectrum. Its maximal UV band I was at 368 nm which is specific for 3-hydroxyl substituent. The 6-hydroxykaempferol-3,7-*O*-diglucoside had the same fragmentation as quercetin-3,7-*O*-diglucoside. The fragment ions of them in MS^2 showed the high ratio of $[M-H-324]^-$ ($Y_0{}^7\ Y_0{}^{3-}$) to $[M-H-162]^-$($Y_0{}^{7-}$), while the 6-hydroxykaempferol-3,6-*O*-diglucoside only yield the $[M-H-162]^-$ peak in MS^2 (Table 4). Compared with the fragmentation of the 6-hydroxykaempferol-3,6,7-O-triglucoside, it was deduced that the glucose at 6-position lost preferentially, and the rupture at 3-position occurred subsequently.

3.5. The structure variety of the flavonoids glycosides in Carthamus tinctorius L.

Total 77 flavanoid glycosides were found with the screening method. Target screening makes us found more co-eluted compounds and minor components. Some compounds having same molecular weight showed the structure variety produced in secondary plant metabolites. For example, 11 compounds whose retention times ranged from 18.9 to 64.6 min were found by screening for *m/z* 625 in negative ion mode (Table 4). They were classified into C-glycosyl flavonoids (compounds 36 and 37) and O-glycosyl flavonoids (compounds 38–46). The aglycone of 9 O-glycosyl flavonoids included three subclasses of flavonoid, namely flavonol, flavanone and chalcone. The aglycone of compound 38 was quercetin, while that of compounds 39, 42 and 43 was 6-hydroxykaempferol. The aglycones of compounds 40, 41 and 44 were carthamidins or isocarthamidins which belong to the flavanones. Compounds 45 and 46 showed the characteristics of chalcone in the UV absorption. The glycan of all 11 compounds only contained two kinds of sugars, which were the glucose or galactose and glucuronise acid, thus the variety of glycan was mainly caused by the different glycosylation position.

Although most familiar flavonoid glycosides have been included in the screening table, some flavonoid skeleton such as phenylchromone and anthocyanidins and some substituent groups such as the isopropenyl were not included. Practically it is not realistic to include all possible structures in the screening, so the screening table should be designed reasonably according to the certain analytical task.

4. Conclusions

The screening method for characterizing flavonoid glycosides was proved to be useful for systematic and rapid characterization of chemical components in the flower of *C. tinctorius*. Totally 77 flavonoid glycosides were screened out, and the structure characterization in tandem mass spectrometry was carried out to validate the screening result. The result of characterization proved the structure variety adequately. 77 flavonoid glycosides in the flower of *C. tinctorius* showed various aglycone, the number and category of sugar, the sequence and the interglycosidic linkage mode of glycan, the glycosylation position and the glycosylation mode. Numerous compounds were discovered for the first time as much as we known, especially those minor components. The screening and characterization by high-performance liquid chromatography combined with electrospray ionization tandem mass spectrometry was important part of systematic research on chemical components, which lead to purposeful preparative separation in order to identify their accurate structures.

Acknowledgments

This work was supported by the “Key Project of Knowledge Innovation Program of Chinese Academy of Sciences (KGCX2-SW-213)”.

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