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Investigations of the fragmentation behavior of 11 isoflavones with ESI-IT-TOF-MS^n

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Abstract: The fragmentation behavior of isoflavones was studied using electrospray ionization-ion trap-time of flight mass spectrometry (ESI-IT-TOF-MS^n). It was found that the isoflavone glycoside bond was easily broken. The fragmentation occurred mostly on the C-ring, and the fragment ions of $A^{1,3+}$ produced by the RDA cracking will predict the hydroxylation replacement on A-ring or B-ring. In addition, four carbonyl groups on the C-ring were fragmented through neutral loss of 28 (-CO). A and B-rings primarily lose substituents which including a neutral losses of 32 (-CH_3OH), 16 (-CH_4), or 16 (-O), and 18 (-H_2O). A-ring in the presence of adjacent hydroxylation, also easily made to be a neutral losses of 28 (-CO) or 18 (-H_2O). It is likewise common to see methoxy replaced with a neutral losses of 16 (-CH_4) or 32 (-CH_3OH) in B-ring, also the hydroxylation on benzene ring can occasionally results with the neutral loss of 28 (-CO).
Keywords: Isoflavone, Fragmentation behavior, ESI-IT-TOF-MS^n

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1. Introduction

It is well-known that isoflavonoids are biologically active plant constituents that occur mainly in legumes and act as phytoestrogens. A diet rich in isoflavonoids can help to lower the risk of getting certain diseases including breast and prostate cancers, osteoporosis and various cardiovascular diseases. Researches also show that isoflavonoid possess anti-carcinogenic, hormone-altering, and estrogenic and anti-estrogenic properties[1,2].

Mass spectrometry is an analytic technique based on determination of *m/z*, or mass-charge ratios, of ions in the gas phase. Ion trap (IT) technology is currently one of the more mature small mass spectrometer. The technology is convenient for achievement of the measurement of multistage tandem (MS^n)[2–5]. The time of flight of the mass spectrometer (TOF) is the space quality analyzer, which can quickly and at high resolution capture positive and negative ion pieces from the ion source to ensure that a majority of the ions can reach the detector[2–5]. IT-TOF-MS^n greatly improves the sensitivity and resolution of the mass spectrum by taking advantage of the simultaneous multistage mass spectrometry analysis. The Retro Diels-Alder (RDA) cleavage can be used to predict the structure of the compounds after the ion source boom the C-ring into dienes and dienes fragments with a double-bond. The RDA occurs in structures containing a cyclohexene unit[6].

It was reported that the cleavage of the isoflavones mostly happened on the C_4 position in C-ring with a neutral loss of 28 (-CO), and formed the fragment ions of $A^{1,3+}$ by the RDA cracking in mass[2–6]. In the present study, we use high resolution mass spectra to predict the exactly formulas of the fragment ions, and then with the relative ion abundance to indicate which bond broke firstly. At the same time we compared the fragmentation pathway of isoflavones with the similar structure to observe how the substitution location influences the fragmentation behavior. Eleven isoflavonoids were chosen and their fragment ions were generated by ESI-IT-TOF-MS^n, with both positive

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ions (PI) and negative ions (NI) mode. Finally, the analysis was conducted in PI mode because fragment ions of isoflavonoids in MS provide more information than in NI mode. This study aims to study the fragmentation behavior of isoflavonoids, which will be helpful to understand the fragmentation rules of isoflavones. These investigations could likewise provide significant support for the further identification and analysis of isoflavones in plant extracts by ESI-IT-TOF-MS^n.

2. Experimental

2.1. Chemicals and materials

The following selective compounds (Fig. 1), including formononetin (**M1**), 6,7,4′-trihydroxy-isoflavonoid (**M2**), 7,4′-dihydroxy-isoflavonoid (**M3**), 7,8,-dihydroxy-4′-methoxy-isoflavonoid (**M4**), 6,7-dihydroxy-4′-methoxy-isoflavonoid (**M5**), formononetin-7-*O*-β-D-glucoside (onion) (**M6**), 6″-*O*-acetyl-onion (**M7**), calycosin (**M8**), 8-hydroxycalycosin (**M9**), 6-hydroxycalycosin (**M10**), calycosin-7-*O*-β-D-glucoside (**M11**), were isolated by the authors and their structures were confirmed based on UV, MS, and NMR data. Methanol (Merck Co., Darmstadt, Germany) was of HPLC grade.

2.2. Sample solutions preparation

Weighed amount (1.0 mg) that is carefully transferred into a 50 mL volumetric flask with methanol, respectively, and a portion of the solution (1 μL) is injected into an ESI-IT-TOF-MS^n system for analysis.

2.3. Instrument and conditions

High resolution mass spectra were recorded on an IT-TOF mass spectrometer (Shimadzu, Kyoto, Japan). Both NI and PI mode were utilized by the ESI source in operation. The full-scan mass spectra covered the range from *m/z* 100–1000 Da (MS^1), *m/z* 50–1000 Da (MS^2 and MS^3). The trifluoroacetic acid sodium solution (2.5 mM) was used to calibrate the mass range from 50 to 1000 Da. Additional parameters were configured as below: heat block and curved desolvation line temperature, 200 °C; nebulizing nitrogen gas flow, 1.5 L/min; interface voltage: (PI), 4.5 kV; (NI), −3.5 kV; detector voltage, 1.70 kV; relative collision-induced dissociation energy, 50%.

All data were recorded and processed by Shimadzu software LCMS solution version 3.60, Formula Predictor version 1.2 and Accurate Mass Calculator (Shimadzu, Kyoto, Japan).

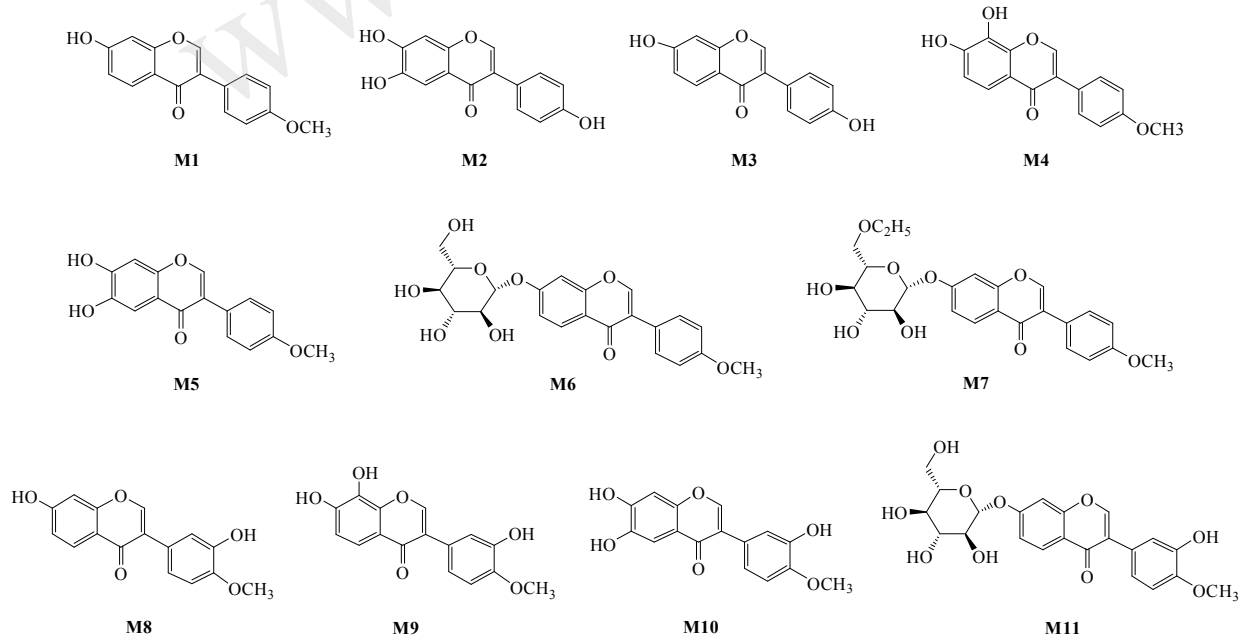

Figure 1. Chemical structures of the 11 isoflavonoids.

3. Results and discussion

3.1. The selection of MS conditions

The fragmentation pathways of 11 isoflavones were analyzed in order to facilitate structural identification. Both PI and NI modes were tested through the experiment with the goal to obtain desirable mass spectrometry chromatograms. The analysis was conducted in PI mode owing to the fact that fragment ions give more information in PI than in NI mode.

3.2. The fragment ions analysis of the 11 isoflavones

As illustrated in Figure 2, ions of isoflavones generated by the breaking of C ring in PI mode were named as $A^{0,3+}$, $B^{0,3+[3,4]}$.

The fragment ions and their abundance are shown in Table 1 and mass spectra are shown in Figure 3 (all the fragment ions were originated from precursor ions $[M+H]^+$).

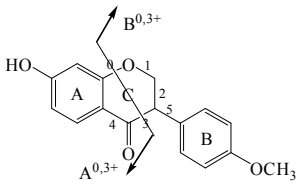

Figure 2. Nomenclature adopted for isoflavone (illustrated with formononetin).

Table 1. The fragment ions of the 11 isoflavones originated from the precursor ions $[M+H]^+$

	Fragment ions	Ion abundance (%)	The cleavage originated from the fragment ion	MW	Molecule formula
	269.0812	100.00	$[M+H]^+$, 253, 237, 213, 163	268.0736	$C_{16}H_{12}O_4$
	237.0548	26.55	–16 (CH_4), –16 (O), 209, 181, 152	236.0473	$C_{15}H_8O_3$
	213.0912	14.46	–32*2 (CO*2)	212.0837	$C_{14}H_{12}O_2$
	118.0430	11.10		117.0340	C_8H_5O
	254.0588	10.45		253.0501	$C_{15}H_9O_4$
M1	253.0500	10.77	–16 (CH_4), 225, 197, 181, 141	252.0423	$C_{15}H_8O_4$
	107.0512	8.23		106.0419	C_7H_6O
	163.0389	2.46		162.0317	$C_9H_6O_3$
	137.0233	4.34	$A^{1,3+}$	136.0160	$C_7H_4O_3$
	181.0655	0.99		180.0575	$C_{13}H_8O$
	197.0593	100.00	–18 (H_2O), –28*2 (CO*2) 169, 141, 115	196.0524	$C_{13}H_8O_2$
	272.0623	96.99		271.0606	$C_{15}H_{11}O_5$
	153.0189	92.85	$A^{1,3+}$	152.0110	$C_7H_4O_4$
	215.0694	82.93	–28*2 (CO*2)	214.0630	$C_{13}H_{10}O_3$
	145.0288	76.52		144.0211	$C_9H_4O_2$
	169.0648	66.89		168.0575	$C_{12}H_8O$
	225.0534	43.53	–18 (H_2O), –28 (CO)	224.0473	$C_{14}H_8O_3$
M2	253.0496	34.26	–18 (H_2O)	252.0423	$C_{15}H_8O_4$
	141.0705	27.06			
	243.0638	20.42	–28 (CO)	242.0579	$C_{14}H_{10}O_4$
	271.0587	17.99	$[M+H]^+$	270.0528	$C_{15}H_{10}O_5$
	181.0644	9.98		180.0575	$C_{13}H_8O$
	125.0238	6.58		124.0160	$C_6H_4O_3$
	227.0706	5.44	–16 (O), –28 (CO)	226.0630	$C_{14}H_{10}O_3$
	147.0479	5.37		146.0368	$C_9H_6O_2$
	137.0229	100.00	$A^{1,3+}$, 109, 81	136.0160	$C_7H_4O_3$
	199.0756	75.81	–28*2 (CO*2)	198.0681	$C_{13}H_{10}O_2$
	255.0645	39.63	$[M+H]^+$	254.0579	$C_{15}H_{10}O_4$
	181.0650	29.12		180.0575	$C_{13}H_8O$
	227.0689	24.51	–28 (CO)	226.0630	$C_{14}H_{10}O_3$
M3	145.0286	19.01		144.0211	$C_9H_4O_2$
	237.0556	17.30	–18 (H_2O)	236.0473	$C_{15}H_8O_3$
	157.0670	12.41		156.0575	$C_{11}H_8O$
	171.0805	6.67		170.0732	$C_{12}H_{10}O$
	149.0249	6.15		148.0160	$C_8H_4O_3$
	133.0316	3.38		132.0211	$C_8H_4O_2$

Table 1. Continued

	Fragment ions	Ion abundance (%)	The cleavage originated from the fragment ion	MW	Molecule formula
M4	270.0496	100.00	$[M-\cdot CH_3+H]^+$	269.0450	$C_{15}H_9O_5$
	253.0476	58.65	$[M-CH_3OH+H]^+$ 225, 197, 141	252.0423	$C_{15}H_8O_4$
	137.0224	48.46		136.0160	$C_7H_4O_3$
	225.0526	38.23	$[M-CH_3OH-CO+H]^+$ 141	224.0473	$C_{14}H_8O_3$
	286.0782	38.21		285.0763	$C_{16}H_{13}O_5$
	229.0825	9.67		228.0786	$C_{14}H_{12}O_3$
	181.0634	6.68		180.0575	$C_{13}H_8O$
	197.0565	6.64	$[M-CH_3OH-CO*2+H]^+$ 141	196.0524	$C_{13}H_8O_2$
	214.0604	5.25		213.0552	$C_{13}H_9O_3$
	134.0342	3.57		133.0290	$C_8H_5O_2$
	169.0614	0.93		168.0575	$C_{12}H_8O$
	175.0437	0.71		174.0317	$C_{10}H_6O_3$
M5	285.0761	100.00	$[M+H]^+$	284.0685	$C_{16}H_{12}O_5$
	152.0113	74.96		151.0031	$C_7H_3O_4$
	286.0794	40.74		285.0763	$C_{16}H_{13}O_5$
	229.0845	22.76	211, 183	228.0786	$C_{14}H_{12}O_3$
	253.0485	16.45	–16 (CH_4), –16 (O)	252.0423	$C_{15}H_8O_4$
	270.0518	11.54	$[M-\cdot CH_3+H]^+$	269.0450	$C_{15}H_9O_5$
	211.0746	11.10		210.0681	$C_{14}H_{10}O_2$
	183.0816	6.36		182.0732	$C_{13}H_{10}O$
	123.0445	6.04		122.0368	$C_7H_6O_2$
	239.0682	5.21	–28 (CO), –18 (H_2O)	238.0630	$C_{15}H_{10}O_3$
	242.0584	2.56	28 (CO), –15 ($\cdot CH_3$)	241.0501	$C_{14}H_9O_4$
	179.0375	2.46		178.0266	$C_9H_6O_4$
	107.0500	1.67		106.0419	C_7H_6O
M6	269.0794	100	$[M-162+H]^+$	268.0736	$C_{16}H_{12}O_4$
	254.0612	3.11	$[M-162-\cdot CH_3+H]^+$	253.0501	$C_{15}H_9O_4$
	237.0541	2.56	$[M-162-CH_3OH+H]^+$, 209, 181	236.0473	$C_{15}H_8O_3$
	213.0909	1.41		212.0473	$C_{14}H_{12}O_2$
	181.0616	0.25		180.0575	$C_{13}H_8O$
M7	269.0793	100.00	$[M-204+H]^+$	268.0372	$C_{16}H_{12}O_4$
	237.0561	5.11	$[M-204-CH_3OH+H]^+$ 209, 181	236.0473	$C_{15}H_8O_3$
	213.0902	2.42		212.0473	$C_{14}H_{12}O_2$
	253.0532	2.53	$[M-204-CH_4+H]^+$	252.0423	$C_{15}H_8O_4$
	181.0669	0.33		180.0575	$C_{13}H_8O$
M8	285.0739	100.00	$[M+H]^+$	284.0685	$C_{16}H_{12}O_5$
	270.0508	26.65	253, 241, 213, 197	269.0450	$C_{15}H_9O_5$
	137.0226	23.43	$A^{1,3+}$	136.0160	$C_7H_4O_3$
	253.0490	20.29	225, 197, 141	252.0423	$C_{15}H_8O_4$
	225.0530	10.35	–32 (CH_3OH), –28 (CO)	224.0473	$C_{14}H_8O_3$
	181.0644	1.81		180.0575	$C_{13}H_8O$
	197.0610	1.49	–32 (CH_3OH), –28*2 (CO*2)	196.0524	$C_{13}H_8O_2$
M9	286.0444	100.00	$[M-CH_3+H]^+$	285.0399	$C_{15}H_9O_6$
	269.0417	63.82	$[M-CH_3OH+H]^+$, 213, 185, 157	268.0372	$C_{15}H_8O_5$
	152.0095	63.29		151.0031	$C_7H_3O_4$
	153.0170	59.79	$A^{1,3+}$	152.0110	$C_7H_4O_4$
	241.0462	40.98	–32 (CH_3OH), –28 (CO)	240.0423	$C_{14}H_8O_4$
	245.0758	19.53		244.0736	$C_{14}H_{12}O_4$
	185.0589	11.66		184.0524	$C_{12}H_8O_2$
	175.0366	9.94		174.0317	$C_{10}H_6O_3$
	213.0514	7.60	–32 (CH_3OH), –28*2 (CO*2)	212.0473	$C_{13}H_8O_3$
	134.0328	1.88		133.0290	$C_8H_5O_2$

Table 1. Continued

	Fragment ions	Ion abundance (%)	The cleavage originated from the fragment ion	MW	Molecule formula
M10	286.0443	100.00	$[M-CH_3+H]^+$, 257, 229, 153	285.0399	$C_{15}H_9O_6$
	301.0666	65.73	$[M+H]^+$	300.0634	$C_{16}H_{12}O_6$
	153.0173	48.83	$A^{1,3+}$	152.0110	$C_7H_4O_4$
	269.0428	48.66	–32 (CH_3OH)	268.0372	$C_{15}H_8O_5$
	241.0469	29.46	–32 (CH_3OH), –28 (CO)	240.0423	$C_{14}H_8O_4$
	245.0799	8.55		244.0736	$C_{14}H_{12}O_4$
	175.0378	6.80		174.0317	$C_{10}H_6O_3$
	197.0578	2.74		196.0524	$C_{13}H_8O_2$
	171.0429	2.65		170.0368	$C_{11}H_6O_2$
	185.0582	2.23		184.0524	$C_{12}H_8O_2$
	134.0357	1.79		133.0290	$C_8H_5O_2$
M11	285.0754	100.00	$[M-162+H]^+$	284.0685	$C_{16}H_{12}O_5$
	286.0770	22.30		285.0763	$C_{16}H_{13}O_5$
	270.0522	6.48	241, 213, 137	269.0450	$C_{15}H_9O_5$
	253.0519	3.40	M-162, –32 (CH_3OH), 225, 197	252.0423	$C_{15}H_8O_4$
	225.0526	1.63	M-162, –32 (CH_3OH), –28 (CO)	224.0473	$C_{14}H_8O_3$
	269.0458	0.55	M-162, –16 (CH_4)	268.0372	$C_{15}H_8O_5$
	213.0601	0.34	M-162, –16 (CH_4), –28*2 (CO*2)	212.0473	$C_{15}H_8O_3$
	181.0662	0.26		180.0575	$C_{13}H_8O$

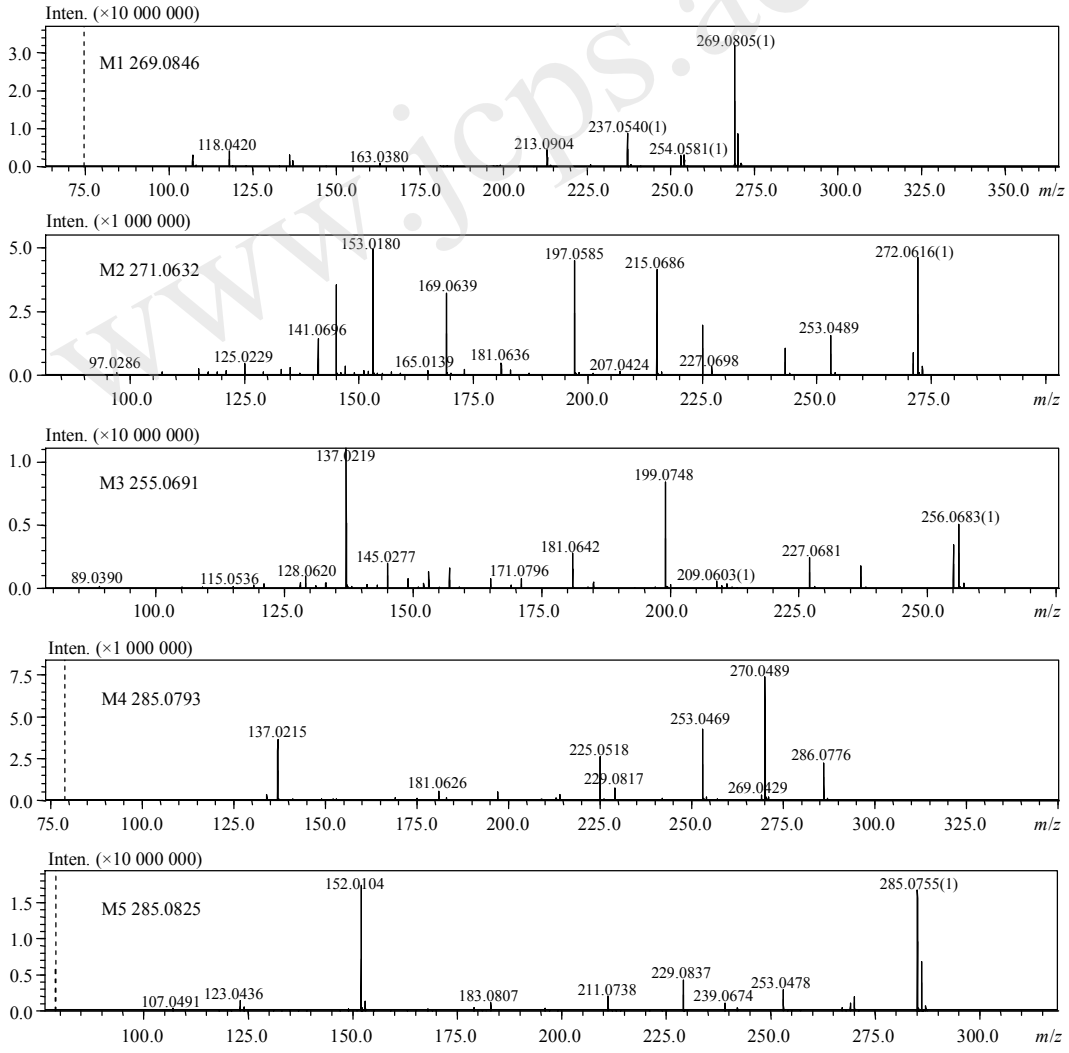

Figure 3. The mass spectra chromatograms of the 11 isoflavones.

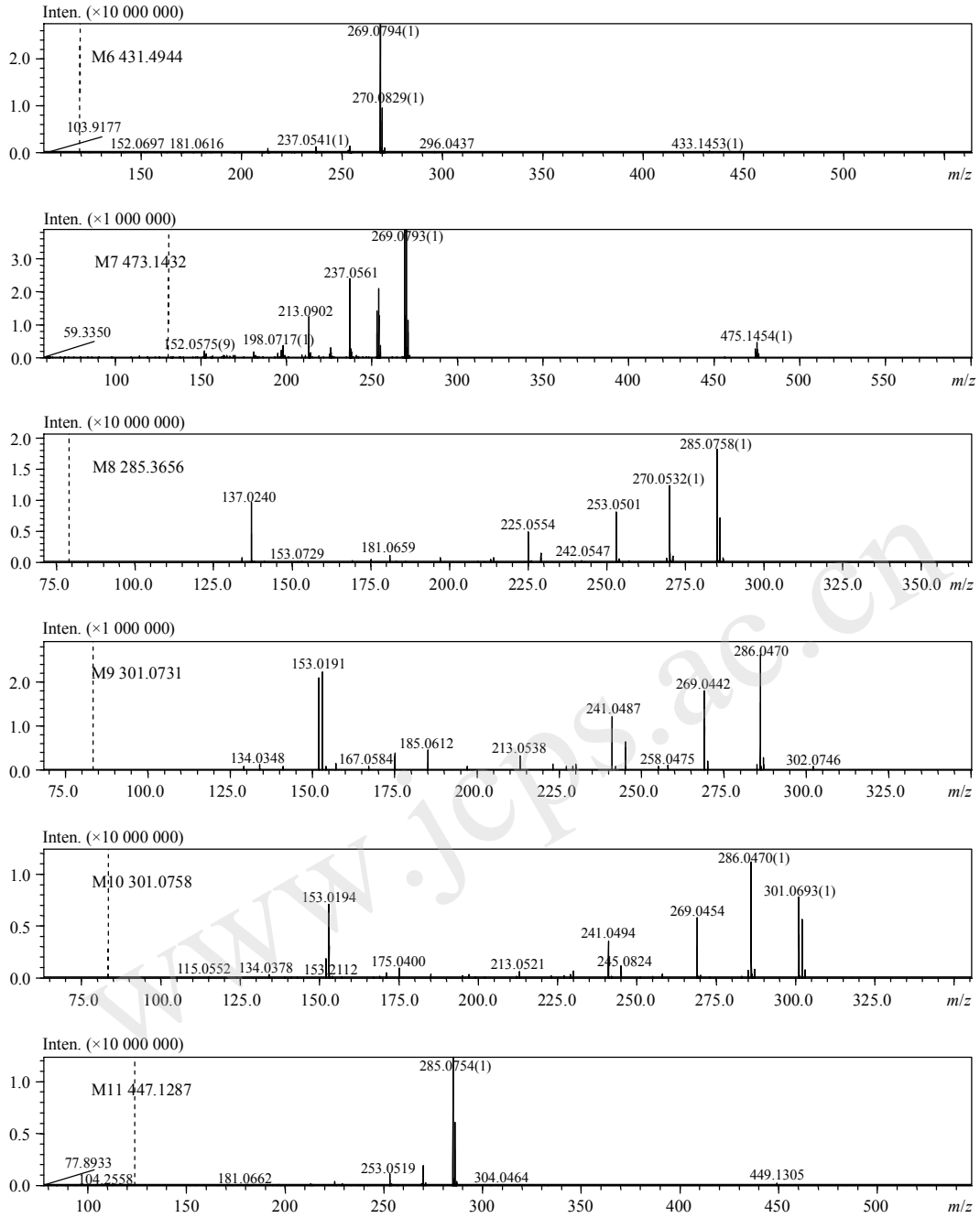

Figure 3. Continued.

3.2.1. Isoflavones and their related isoflavone glycosides: compounds M1, M3, M6, M7

The $[M+H]^+$ ion of **M1** at *m/z* 269 was generated as the base peak. And ions at *m/z* 237 $[M-CH_3OH+H]^+$, *m/z* 253 $[M-CH_4+H]^+$, *m/z* 213 $[M-CO*2+H]^+$ were observed. Ion at *m/z* 163 formed *m/z* 137 $A^{1,3+}$, and $B^{1,3}$ –16 (CH_4) after RDA cracking.

M3 formed *m/z* 137 $A^{1,3+}$ after RDA cracking as the base peak, and other major fragment ions, such as *m/z* 199 $[M-CO*2+H]^+$, *m/z* 181 $[M-CO*2-H_2O+H]^+$, *m/z* 227 $[M-CO+H]^+$, *m/z* 255 $[M+H]^+$ were also observed, and fragment ion *m/z* 145 was generated from B^5-H_2O.

M6 and **M7** are isoflavone glycosides or the isoflavone glycoside acetylated in the glucoside from **M1**. They predominantly yielded ions $[M-162+H]^+$ or $[M-204+H]^+$ as base peak ion, corresponding to the cleavage of glycosidic-bond. The neutral loss of the substituents on **M1**, such as –16 (CH_4), –32 (CH_3OH), –28 (CO), and –56 (CO*2) were apparent (The main fragment ions of **M1**, **M3**, **M6**, **M7** are illustrated in the Fig. 4).

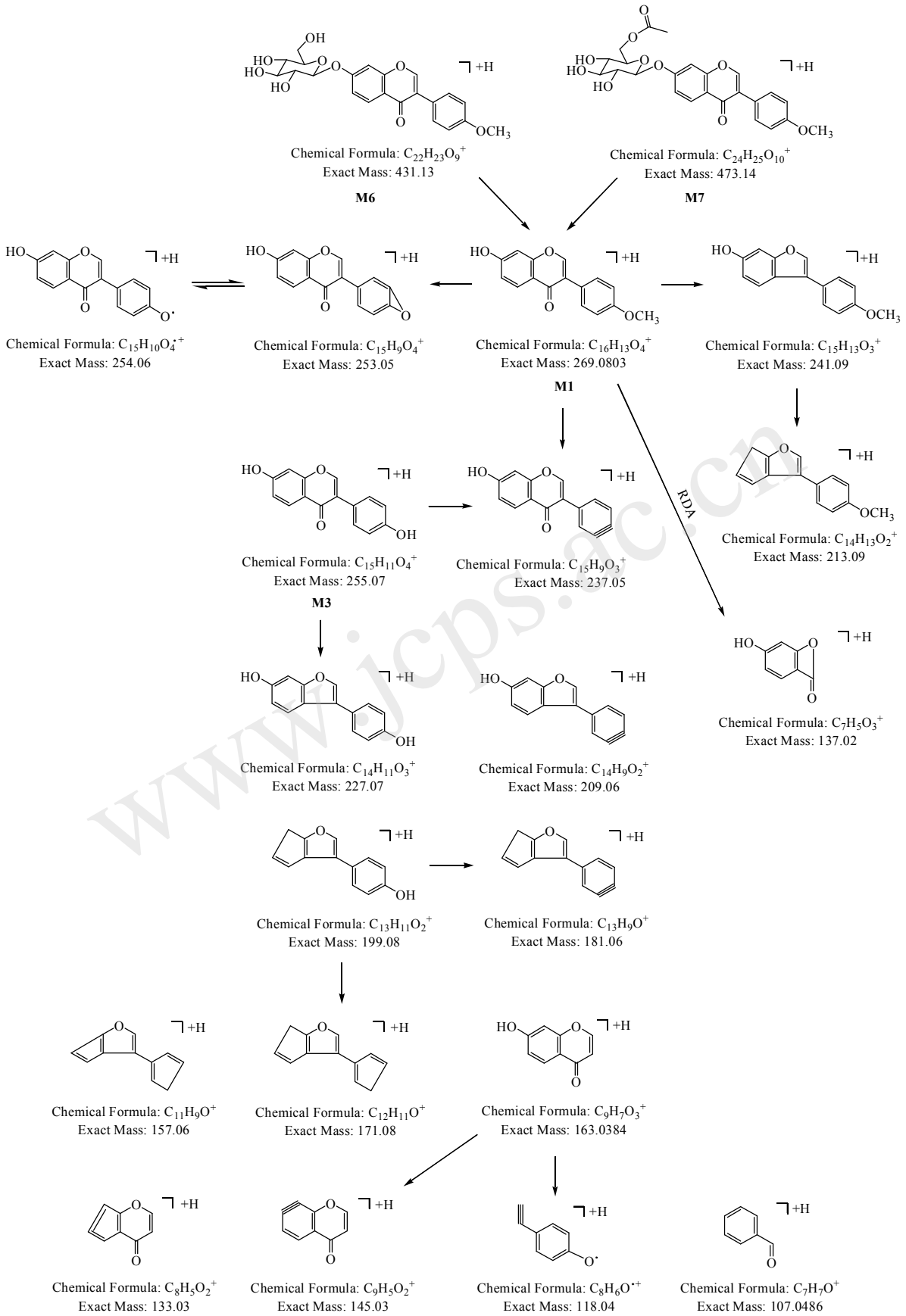

Figure 4. The main proposed fragment ions of **M1**, **M3**, **M6**, **M7**.

3.2.2. One hydroxylation or two hydroxylation with different site in A ring and with the same substituents in B ring: compounds M4, M5

The base peak of **M4** is at *m/z* 270, and other major fragment ions are mainly at *m/z* 253 [M-CH_3OH+H]$^+$, *m/z* 225 [M-CH_3OH-CO+H]$^+$, *m/z* 197 [M-CH_3OH-CO*2+H]$^+$, and *m/z* 181 [M-CH_3OH-CO*2-O+H]$^+$.

The base peak ion of **M5** is at *m/z* 285 [M+H]$^+$, and other major fragment ions include *m/z* 229 [M-CO*2+H]$^+$, *m/z* 253 [M-CH_3OH+H]$^+$, *m/z* 239 [M-CO-H_2O+H]$^+$, *m/z* 211 [M-CO-CH_3OH-H_2O+H]$^+$, and *m/z* 183 [M-CH_3OH-CO*2-H_2O+H]$^+$ (The main fragment ions of **M4**, **M5** are shown in the Fig. 5).

3.2.3. One hydroxylation or two hydroxylation with different site in A ring and with the same substituents in B ring: M8, M9, M10, M11

The base peak of **M8** is at *m/z* 285 [M+H]$^+$, the primary fragment ions are *m/z* 270 [M-·CH_3+H]$^+$, *m/z* 253 [M-CH_3OH+H]$^+$, *m/z* 225 [M-CH_3OH-CO+H]$^+$, and *m/z* 137 $A^{1,3+}$ of RDA cracking in the C-ring.

M9 produced *m/z* 286 [M-·CH_3+H]$^+$ as the base peak, together with the other primary fragment ions at *m/z* 269 [M-CH_3OH+H]$^+$, *m/z* 241 [M-CH_3OH-CO+H]$^+$, and *m/z* 153 $A^{1,3+}$ by RDA cracking in C-ring.

M10 yielded *m/z* 286 [M-·CH_3+H]$^+$ as the base peak. In addition, ions at *m/z* 301 [M+H]$^+$, *m/z* 269 [M-CH_3OH+H]$^+$,

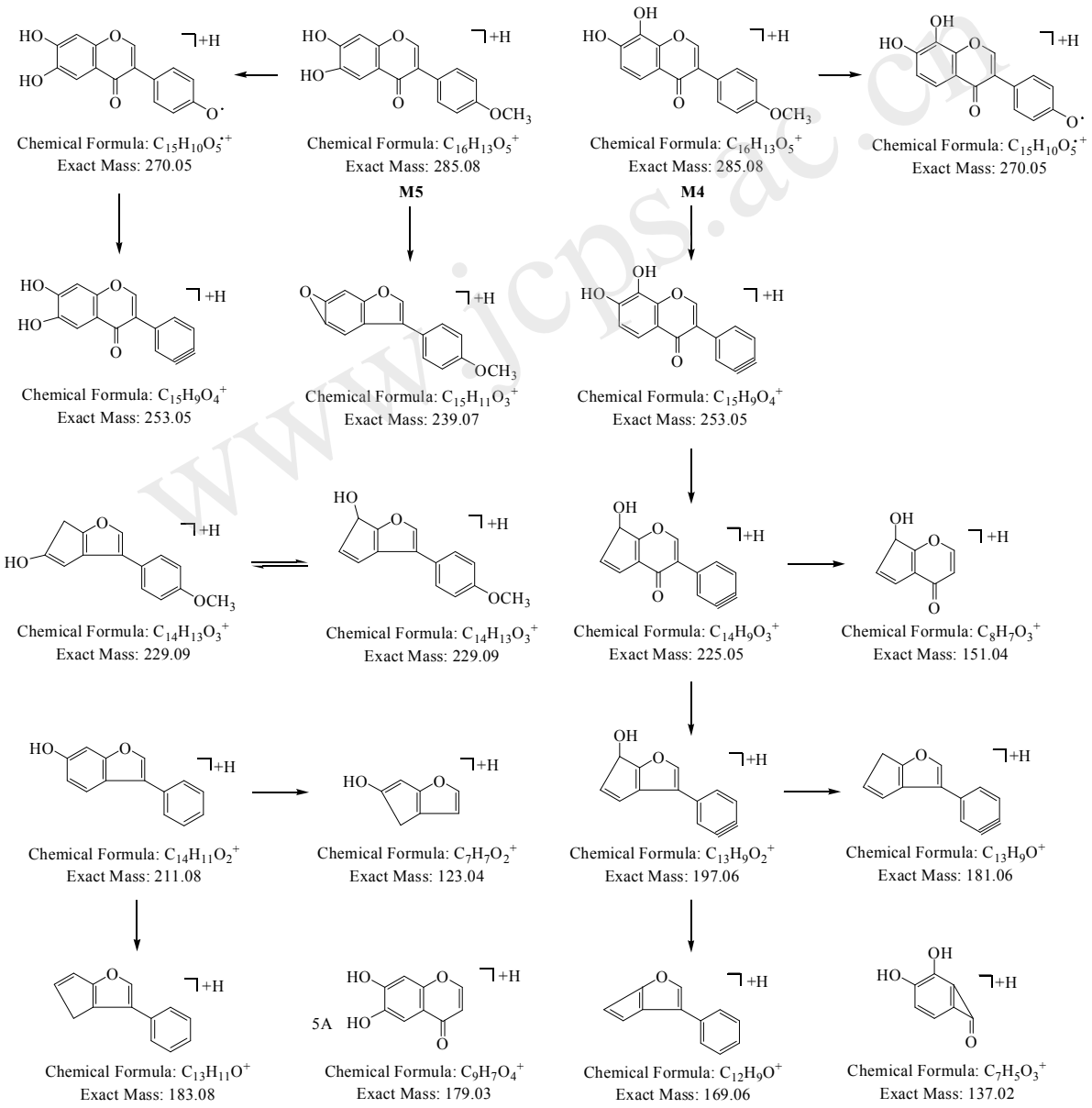

Figure 5. The main proposed fragment ions of **M4**, **M5**.

m/z 241 [M-CH_3OH-CO+H]$^+$, *m/z* 153 $A^{1,3+}$ all were the primary fragment ions.

M11 is the isoflavone glycoside of **M8**, and its mass spectrum showed that *m/z* 285 [M-162+H]$^+$ as the base peak. In addition, the *m/z* 270 [M-162-·CH_3+H]$^+$, *m/z* 253 [M-162-CH_3OH+H]$^+$, and *m/z* 225 [M-162-CH_3OH-CO+H]$^+$ were the primary fragment ions, not exhibiting the fragment ion by RDA cracking or with a low ion abundance (The main fragment ions of **M8**, **9**, **10**, **11** are shown in the Fig. 6).

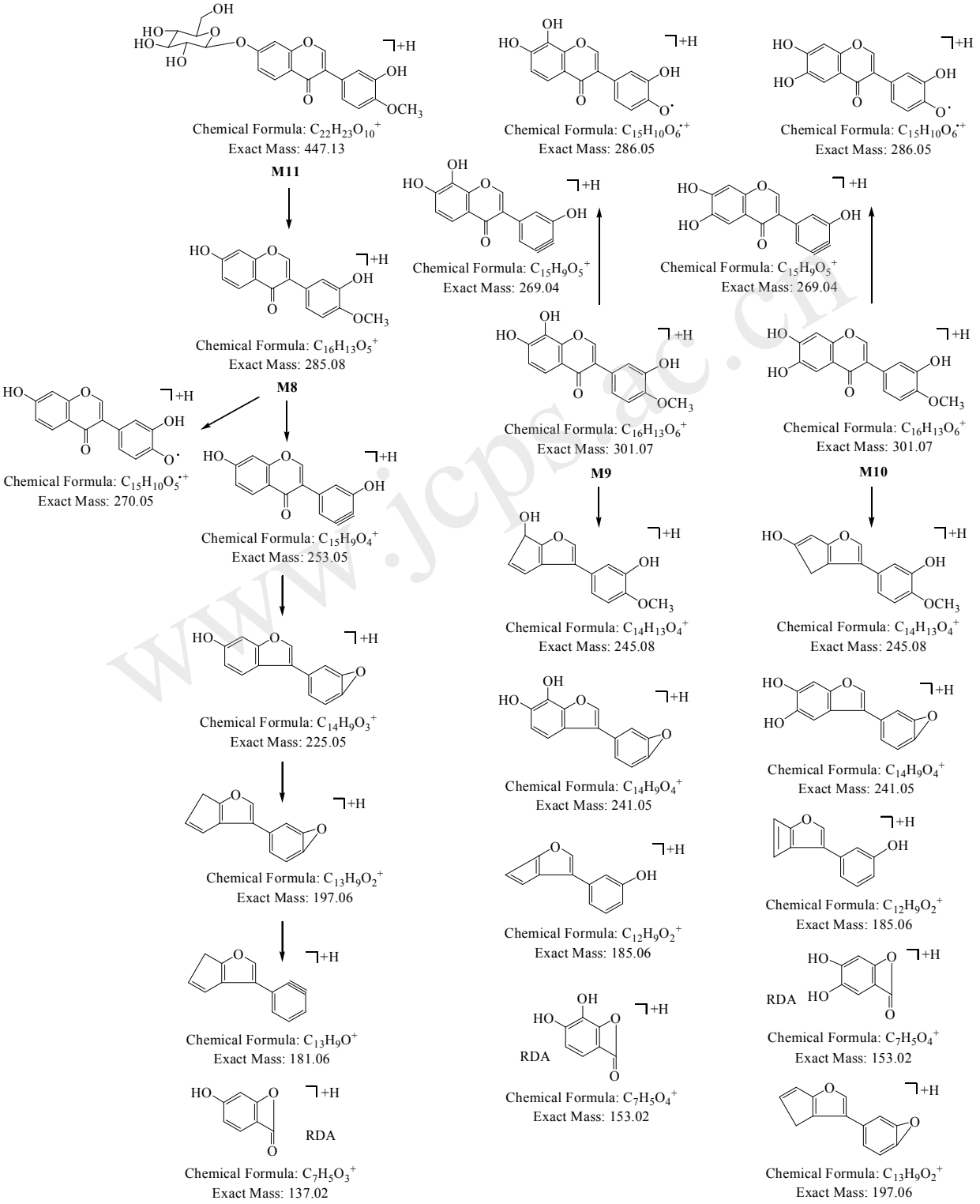

Figure 6. The main proposed fragment ions of **M8**, **M9**, **M10**, **M11**.

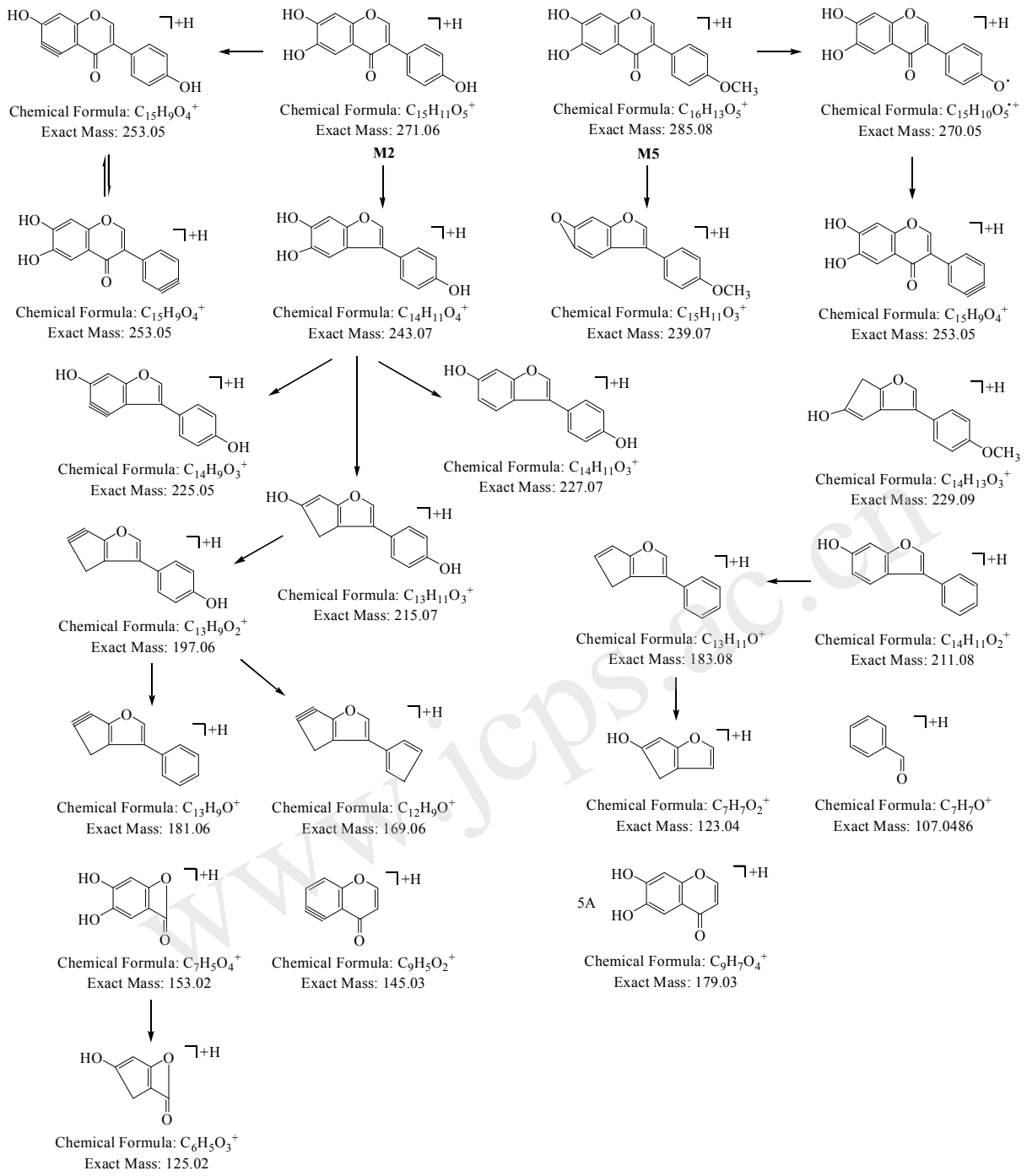

Figure 7. The main proposed fragment ions of **M2**, **M5**.

3.2.4. The different hydroxylation and methylation in B ring with the same substituents in A ring: M2, M5

The base peak ion of **M2** was at *m/z* 197 [M-CO*2-H_2O+H]$^+$, and other major fragment ions were mainly at *m/z* 253 [M-H_2O+H]$^+$, *m/z* 271 [M+H]$^+$, *m/z* 225 [M-CO-H_2O+H]$^+$, *m/z* 215 [M-CO*2+H]$^+$, and *m/z* 153 $A^{1,3+}$ of RDA cracking in the C-ring (The main fragment ions of **M2**, **M5** are shown in the Fig. 7).

4. Conclusions

In the present study, the ESI-IT-TOF-MS^n technique was applied to analyze the fragmentation behavior of isoflavones. The characteristic fragment ions are integral to determining a structure skeleton and substitution patterns for isoflavones. In general, the fragment ions with high ion intensity proved to be the most stable and preferred cleavage.

The neutral loss of 28 u (-CO) from C_4 in the C-ring of isoflavones is the preferred cleavage. The B-ring first underwent the loss of CH_3OH (Δm = 32 u) or H_2O (Δm = 18 u), and fewer cases have a neutral loss of CO (Δm = 28 u) when substituted with hydroxylation. There also occurs a neutral loss of CO, or a loss of H_2O when connected to the adjacent hydroxylation in A-ring.

Based on the fragmentation ions of $A^{1,3+}$ originating from the RDA cracking of isoflavones, we could presume the varying hydroxylate location in A-ring or B-ring (*m/z* 137 $A^{1,3+}$ for one hydroxylation be replaced in A-ring, and *m/z* 153 for A-ring with two replaced hydroxylation). Then the other following fragments in mass spectra are the same as the relating aglycones, with the C_7 glycosidic-bond of isoflavone glycosides in A-ring cleavage in priority. The above fragmentation rules could help to identify the different types of isoflavones.

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ESI-IT-TOF-MS^n方法对11个异黄酮的裂解规律研究

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摘要: 本文对选定的11个异黄酮化合物在ESI-IT-TOF-MS^n的裂解规律进行研究。文章采用ESI-IT-TOF-MS^n仪对样品进行高分辨质谱测定, 正负离子多级切换, 通过对质谱碎片离子进行预测, 并根据结构对其质谱可能的裂解规律进行总结研究。由于在正离子模式下信号较强, 采用正离子模式对11个异黄酮多级碎片离子分析, 发现异黄酮苷的裂解主是优先断裂糖苷键; C环4位羰基易于中性丢失CO (–28), 而C环经过RDA裂解形成的$A^{1,3+}$则能够比较A环与B环上的羟基取代位置; A环上有相邻羟基存在时, 也易中性丢失CO (–28)或H_2O (–18); B环有甲氧基取代时中性丢失CH_4 (–16)、自由基 (CH_3)或CH_3OH (–32)为常见, 但A环与B环有单独羟基时也偶见中性丢失CO (–28)。通过ESI-IT-TOF-MS^n方法, 选定多级正离子模式下对异黄酮的裂解进行了总结, 有利于了解异黄酮在质谱中的裂解行为, 对于异黄酮类化合物相关的结构推测有重要意义。

关键词: 异黄酮; 裂解规律; 高分辨多级质谱技术