Analysis of Homoisoflavonoids in *Ophiopogon japonicus* by HPLC-DAD-ESI-MS^n

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The homoisoflavonoids in *Ophiopogon japonicus* (Thunb.) Ker-Gawler were analyzed by high-performance liquid chromatography-diode array detection-electrospray ion trap tandem mass spectrometry (HPLC-DAD-ESI-MS^n). Homoisoflavonoids gave prominent $[M - H]^-$ ions by electrospray ionization monitored in the negative ion mode. They could be classified into two types depending on the fragmentation behavior of their $[M - H]^-$ ions in the ion trap mass analyzer. The $[M - H]^-$ ions of homoisoflavonoids with a saturated $C_{2\text{-}3}$ bond underwent $C_{3\text{-}9}$ bond cleavage to lose the B-ring, which was followed by the loss of a molecule of CO. The $[M - H]^-$ ions of homoisoflavonoids with a $C_{2\text{-}3}$ double bond usually eliminated a CO molecule first, and then underwent the cleavage of $C_{3\text{-}9}$ or $C_{9\text{-}1'}$ bonds. For homoisoflavonoids with a C-6 formyl group, however, the neutral loss of CO was the first fragmentation step; the presence of a methoxyl group at C-8 could lead to the cleavage of C-ring. No retro Diels-Alder (RDA) fragmentation characteristic for normal flavonoids was observed. The above fragmentation rules were reported for the first time, and were implemented for the analysis of homoisoflavonoids in *O. japonicus*. The $CHCl_3$-MeOH extract was separated on a Zorbax Extend-C_{18} column, eluting with a acetonitrile-0.3% acetic acid gradient. A total of 18 homoisoflavonoids, including seven new minor constituents, were identified or tentatively characterized based on the UV spectra and tandem mass spectra of the HPLC peaks. (J Am Soc Mass Spectrom 2005, 16, 234–243) © 2004 American Society for Mass Spectrometry

Flavonoids are a class of natural products with a C_6-C_3-C_6 skeleton widely distributed in the plant kingdom. They have been reported to be the major bioactive constituents of medicinal herbs because of their antioxidant, anti-inflammatory, and anti-carcinogenic activities [1, 2]. The development of simple and rapid methods for the analysis of flavonoids is therefore of great significance in the quality control of herbal medicine. The plant extract, however, is a very complex mixture, and contains a variety of secondary metabolites. A potent and currently widely used technique to analyze plant extracts is fingerprinting, especially by high-performance liquid chromatography-diode array detection (HPLC-DAD) [3]. An informative and convincing fingerprint should have most of the diagnostic peaks elucidated. Unfortunately, such routine techniques as HPLC-DAD could only provide very limited structural information like UV spectrum; standard compounds, which are commercially unavailable in most cases, are usually necessary for the characterization of individual constituents. As a result, the isolation and purification from crude plant extracts of adequate amounts (at least 5–10 mg) of pure compounds (>90% purity) for nuclear magnetic resonance (NMR) identification was needed before they could serve as reference compounds. The whole process is tedious, laborious, and expensive. Moreover, some constituents are only present in raw plant materials in very low amounts, and their enrichment and purification are extremely difficult. High-performance liquid chromatography-mass spectrometry (HPLC-MS) combines the efficient separation capabilities of HPLC and the great power in structural characterization of MS, and provides a new powerful approach to identify the constituents in plant extracts rapidly and accurately [4]. In addition, mass spectrometer is a very sensitive detector. In many cases, minor constituents in plant extracts that are difficult to obtain by conventional phytochemical means could be detected, especially when selected ion monitoring (SIM) technique was used. Thus, HPLC-MS could also be used to discover new constituents from medicinal herbs.

Presently, HPLC-MS has been used for the analysis of a variety of natural products. Flavonoids, especially

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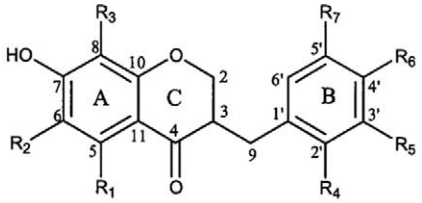
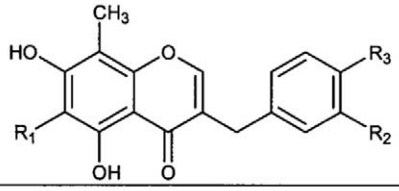


No.	Trivial name	R_1	R_2	R_3	R_4	R_5	R_6	R_7	MW
1	Methylophiopogonanone A	OH	CH_3	CH_3	H	-O-CH_2-O-		H	342
2	Methylophiopogonanone B	OH	CH_3	CH_3	H	H	OCH_3	H	328
3	6-formyl-isoophiopogonanone A	OH	CHO	CH_3	H	-O-CH_2-O-		H	356
6	Ophiopogonanone A	OH	CH_3	H	H	-O-CH_2-O-		H	328
7	Ophiopogonanone E	OH	CH_3	OCH_3	OH	H	OCH_3	H	360
8	Ophiopogonanone F	OCH_3	CH_3	OCH_3	OH	H	OCH_3	H	374
9		OH	CHO	CH_3	H	H	OCH_3	H	342
10		OH	CH_3	CH_3	H	OCH_3	OH	OCH_3	374



No.	Trivial name	R_1	R_2	R_3	MW
4	Methylophiopogonone A	CH_3	-O-CH_2-O-		340
5	6-aldehydo-isoophiopogonone A	CHO	-O-CH_2-O-		354
11	6-aldehydo-isoophiopogonone B	CHO	H	OCH_3	340

Figure 1. Chemical structures of homoisoflavonoids identified from *Ophiopogon japonicus*.

flavonols, flavones, and their glycosides, are the most frequently reported [5–12]. Electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) sources are usually used as the interface between HPLC and a mass spectrometer.

Homoisoflavonoids are a type of special flavonoids, with their B- and C-rings connected by an additional CH_2 group. These compounds are rare in plants and few reports can be found on their HPLC-MS analysis. *Ophiopogon* species (Liliaceae) are abundant in homoisoflavonoids. Up to now, at least 30 homoisoflavonoids have been isolated from these plants [13–19] (Figure 1). Recently, we isolated five pure homoisoflavonoids from *O. japonicus*, a traditional Chinese medicine (Mai-Dong) frequently used as a tonic drug in the clinic. These compounds have been reported to be important active constituents for its therapeutic effects [15]. In this study, we investigated the fragmentation behaviors of homoisoflavonoids in an electrospray ion trap mass spectrometer, and analyzed the homoisoflavonoids in *O. japonicus* by high-performance liquid chromatography-diode array detection-electrospray ionization-tandem mass spectrometry (HPLC-DAD-ESI-MS^n).

Experimental

Standards and Reagents

Pure homoisoflavonoids **1**–**5** were isolated from the tubers of *Ophiopogon japonicus* (Thunb.) Ker-Gawler by the authors. Their structures were unambiguously identified by NMR techniques, and their purities were above 98% as determined by HPLC.

HPLC grade acetonitrile (Fisher, Loughborough, UK) and ultra-pure water were used for all analyses. The chloroform and methanol for plant extraction and acetic acid used in mobile phase was of AR grade,

purchased from Beijing Chemical Corporation (Beijing, China).

Plant Materials and Sample Preparation

The tubers of *O. japonicus* were purchased from a drug store in Beijing. The materials were pulverized and dried to constant weight before use. An aliquot of 1 g of the sample was extracted with 50 ml of chloroform-methanol (1:1) in an ultrasonic water bath for 30 min and then filtered. The filtrate was evaporated to dryness and the residue was dissolved in 1 ml of acetonitrile. The solution was filtered through a 0.45-μm micropore membrane prior to use. A volume of 10 μl was injected into the HPLC instrument for analysis.

HPLC Conditions

The analyses were performed on an Agilent series 1100 HPLC instrument (Agilent, Waldbronn, Germany) equipped with a quaternary pump, a diode-array detector, an autosampler, and a column compartment. The sample was separated on a Zorbax Extend-C_{18} column (5 μm, φ 4.6 $\times$ 250 mm, Agilent). The mobile phase consisted of acetonitrile (A) and water containing 0.3% acetic acid (B). A gradient program was used as follows: 40% A in the first 10 min, linearly gradient to 65% A over 25 min, then hold for 10 min. The mobile phase flow rate was 1.0 ml/min; the detector was monitored at 285 nm; column temperature was set at 25 °C.

Mass Spectrometry

For HPLC-MS analysis, a Finnigan LCQ Advantage ion trap mass spectrometer (ThermoFinnigan, San Jose, CA) was connected to the Agilent 1100 HPLC instrument via an ESI interface. The LC effluent was introduced into the ESI source in a post-column splitting ratio of 2:1. Ultrahigh pure helium (He) was used as the collision gas and high purity nitrogen (N_2) as the nebulizing gas. To tune the mass spectrometer, a 0.1 mg/ml solution of methylophiopogonanone A (**1**) in acetonitrile (MeCN) was pumped into a 0.5 ml/min MeCN-0.3% HOAc (1:1, vol/vol) LC eluate at a steady rate of 2.5 μl/min by using a TEE union. The combined flow was allowed to enter the ESI source for ionization. The mass spectrometer parameters were adjusted to obtain maximum response of the $[M - H]^-$ ion at *m/z* 341. The optimized parameters in the negative ion mode were as follows: ion spray voltage, 4.5 kV; sheath gas (N_2), 45 arbitrary units; auxiliary gas (N_2), 10 units; capillary temperature, 320 °C; capillary voltage, −15 V. For full scan MS analysis, the spectra were recorded in the range of *m/z* 120–800. A data-dependent program was used in the liquid chromatography-tandem mass spectrometry analysis so that the two most abundant ions in each scan were selected and subjected to MS/MS and MS^3 analyses. The collision-induced dissociation (CID) energy was adjusted to 45%. The isolation width of precursor ions was 3.0 mass units.

Results and Discussion

Tandem Mass Spectrometry of Pure Standards

APCI and ESI sources were tried for the ionization of homoisoflavonoids both in positive and negative ion modes. Either source gave $[M - H]^-$ ions as the base peak in the negative mode, while $[M+H]^+$ ions with remarkably lower intensity were obtained in the positive mode. APCI and ESI produced very similar ions. Therefore, ESI in the negative ion mode was selected as the ion source for follow-up analyses.

Pure Compounds **1**–**5** (0.1 mg/ml in MeCN) were respectively injected into the ESI source by continuous infusion. The $[M - H]^-$ ions were selected for CID fragmentation to produce MS/MS spectra. The prominent MS/MS ions were then selected for further MS^n analysis (n = 3 up to 5). The collision energy ranged from 30 to 50%.

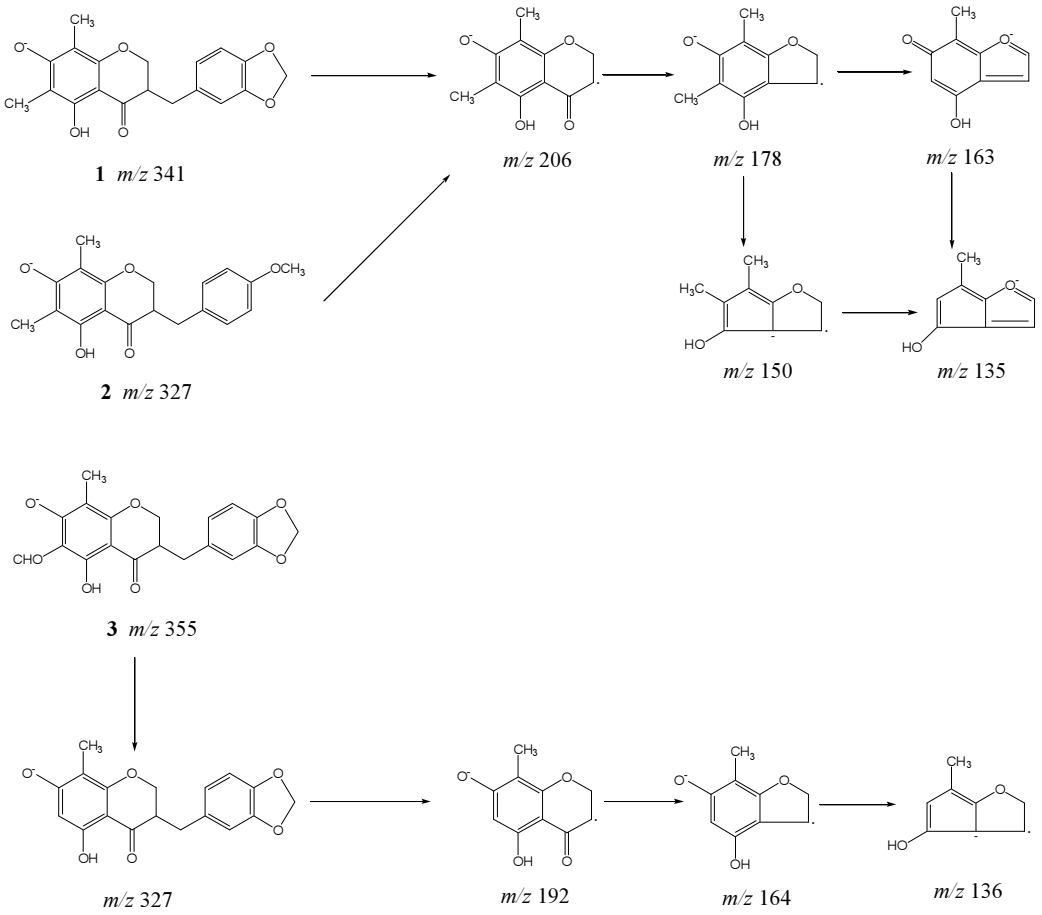
Unexpectedly, the homoisoflavonoid $[M - H]^-$ ions underwent new and unique fragmentations, which were completely different from the retro Diels-Alder (RDA) cleavage°characteristic°for°flavones°and°flavonols°[20].

The five analyzed homoisoflavonoids could be clearly classified into two groups according to their fragmentation behavior. Interestingly, the classification was well correlated with the saturation status of their $C_{2\text{-}3}$ bond.

For Compounds **1**, **2**, and **3**, which contain saturated $C_{2\text{-}3}$ bonds, the predominant fragmentation of their $[M - H]^-$ ions was the cleavage of $C_{3\text{-}9}$ bond to lose the B-ring, followed by the neutral loss of a molecule of CO (Scheme **1**). This class of homoisoflavonoids would be cited as Type I in this paper for convenience. For Compounds **4** and **5**, where the C-2 and C-3 are connected by a double bond (Type II), however, the $[M - H]^-$ ions appeared to be more stable. They produced a major product ion by losing a CO molecule first, and then underwent the cleavage of $C_{3\text{-}9}$ or $C_{9\text{-}1'}$ bonds to lose the B-ring (Scheme **2**). For any homoisoflavonoid with a formyl group at C-6, the fragmentation was triggered by the initial loss of a molecule of CO.

Fragmentation of Standards 1, 2, and 3

When applied to an collision energy of 50%, the $[M - H]^-$ ion at *m/z* 341 of methylophiopogonanone A (**1**) produced a prominent ion at *m/z* 206, which should result from the cleavage of $C_{3\text{-}9}$ bond. Similar ions could be found in the electron impact (EI) mass spectra of homoisoflavonoids. The major difference was that the B-ring fragments were usually base peaks in the EI spectra, whilst they could not be observed in the ESI spectra. The *m/z* 206 ion was further subjected to MS^3 analysis to produce a signal at *m/z* 178, which should be attributed to the loss of the carbonyl group (CO, Δm =



Scheme 1. Proposed MS fragmentation pathway for the $[M - H]^-$ ions of Compounds **1**, **2**, and **3**.

28) at C-4. The *m/z* 178 ion then underwent successive losses of CH_3 ($\Delta m = 15$) or CO to generate ions at *m/z* 163,°150,°and°135°(Figure°2).°The°above°product°ions allowed us to propose a fragmentation pathway for Compound **1**, as depicted in Scheme **1**.

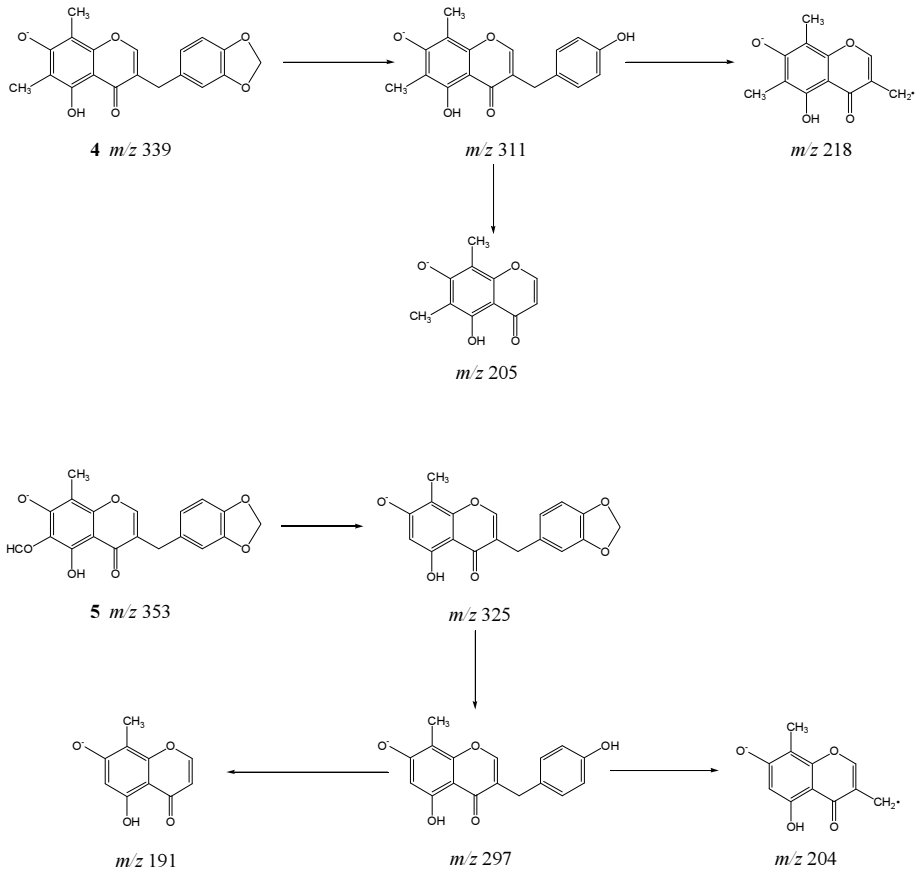
The fragmentation behaviors of **1** could also be applied to Compounds **2** and **3**. The $[M - H]^-$ ion of Compound **2** at *m/z* 327 gave a prominent product ion at *m/z* 206, which further yielded ions at *m/z* 178, 163, 150, and 135 in the MS^3 spectra by the same pathway as Compound **1**.

The fragmentation of Compound **3** was somewhat different from **1** and **2**. The MS/MS spectrum of its $[M - H]^-$ ion at *m/z* 355 gave a base peak at *m/z* 327, due to the neutral loss of a CO molecule. We assumed this fragmentation should involve the loss of the formyl group at C-6, since free carbonyl group is easy to lose by α-cleavage. The obtained product ion at *m/z* 327 then undertook similar fragmentations as quasi-molecular ions of **1** and **2** (Scheme **1**).

Fragmentation of Standards 4 and 5

The $[M - H]^-$ ion (*m/z* 339) of methylophiopogonone A (**4**) fragmented into *m/z* 311 at a 50% energy collision. There could be two possible ways to form this ion. One was to lose the carbonyl group (CO) at C-4, and the other involved the conversion of methylenedioxyl group at B-ring into a hydroxyl group (Scheme **2**). The first pathway was normal for the loss of 28 mass units, and was described above for Type I homoisoflavonoids. Unfortunately, no supporting product ions could be observed in the MS^3 spectrum of Compound **4**. Thus, $C_{2\text{-}3}$ double bond seemed to inhibit the cleavage of C-4 carbonyl group. The second fragmentation pathway had rarely been reported before, but the MS^3 ions at *m/z* 205 and *m/z* 218 strongly suggested the presence of a carbonyl group in their parent *m/z* 311 ion. Very similar fragmentations had been reported recently for aporphine° alkaloids° with° a° methylenedioxyl° group° [21]. With its extended π-conjugation, the ion at *m/z* 311 was relatively stable and fragmented only upon 50% energy collision to form the ions at *m/z* 205 and 218, presumably due to the cleavages of $C_{3\text{-}9}$ and $C_{9\text{-}1'}$ bonds, respectively.

Also with a formyl group at C-6, the $[M - H]^-$ ion at *m/z* 353 of **5** first underwent the loss of CO like Compound **3**. The obtained *m/z* 325 ion then produced



Scheme 2. Proposed MS fragmentation pathway for the $[M - H]^-$ ions of Compounds **4** and **5**.

ions at *m/z* 297, 204, and 191 in the same pathways as Compound **4**.

HPLC-DAD-ESI-MS^n Analysis of the Plant Extract

Figure°3°shows°the°HPLC-UV°and°TIC°profiles°of°the extract of *Ophiopogon japonicus*.

A variety of solvents were tried for the extraction of homoisoflavonoids from *Ophiopogon* tubers. Pure methanol (MeOH) could extract homoisoflavonoids effectively, but suffered from the concomitant extraction of large amounts of saponins and saccharides, which remarkably affected the HPLC-MS chromatogram. Chloroform ($CHCl_3$) and acetonitrile extracts gave good HPLC profiles, while the more polar homoisoflavonoids could not be readily extracted. Finally, a mixture of MeOH-$CHCl_3$ (1:1, vol/vol) was used. The homoisoflavonoids, including the minor ones, could be effectively extracted, and most HPLC peaks were baseline resolved in a 45-min run.

Acetic acid was added in the mobile phase to improve the ionization of homoisoflavonoids, though they did not alter their chromatographic retention significantly°[22].

The UV detector was monitored at 285 nm to make sure all homoisoflavonoids gave good responses.

The HPLC peaks were preliminary identified as homoisoflavonoids according to their on-line UV spectra. Because of different π-conjugation extensions, Type I homoisoflavonoids show a maximum absorption band at 294–298 nm, while Type II at 262–266 nm. When a C-6 formyl group is present, however, the band shifts to 274–276 nm. The structures were further elucidated based on their tandem mass spectra. Fragmentation rules described above for standards **1–5** were extensively used for the elucidation.

At least 25 homoisoflavonoids were detected from the extract of *O. japonicus*, and 18 of them were characterized°(Table°1).°Compounds°**1–5** were°identified°by comparing their HPLC retention times and UV spectra with the standards. In the HPLC-MS analysis, they gave MS and MS^n spectra almost identical with those obtained by continuous infusion.

Identification of Type I Homoisoflavonoids

In addition to **1–3**, eleven $C_{2\text{-}3}$ saturated homoisoflavonoids (Type I) were plausibly identified. Five of them were known constituents previously reported from *Ophiopogon* species. The other six compounds were reported for the first time and were tentatively characterized on the basis of their tandem mass spectra.

The peak at 11.23 min (no. 4) gave a $[M - H]^-$ ion at

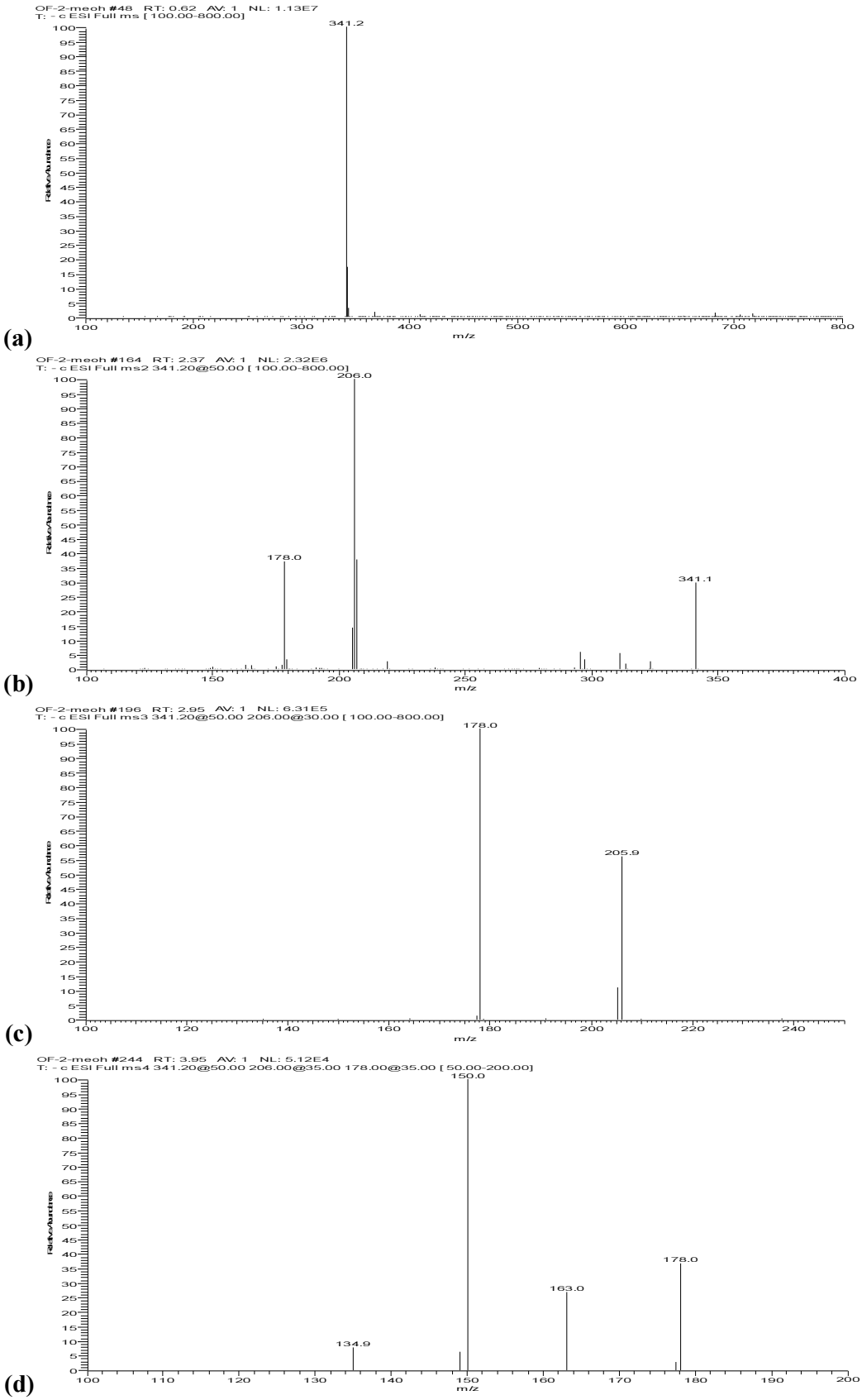


Figure 2. Full scan MS and MS^n spectra for Compound **1**. (**a**) full scan MS spectrum; (**b**) MS^2 spectrum of the $[M - H]^-$ ion at *m/z* 341; (**c**) MS^3 spectrum of the *m/z* 206 ion (341 > 206); (**d**) MS^4 spectrum of the *m/z* 178 ion (341 > 206 > 178).

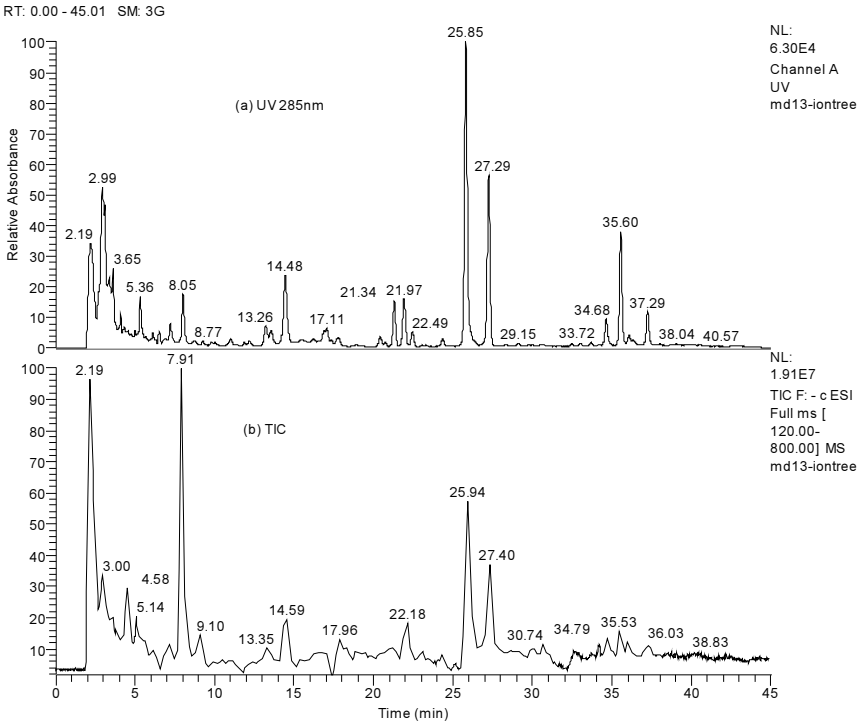


Figure 3. HPLC-DAD-ESI-MS^n analysis of the $CHCl_3$-MeOH (1:1) extract of *Ophiopogon japonicus*. (**a**) HPLC-UV chromatogram monitored at 285 nm. (**b**) LC-negative ion ESI-MS total ion current (TIC) profile.

m/z 373. Its MS/MS and MS^3 spectra yielded prominent ions at *m/z* 207 and *m/z* 179, respectively. These ions should result from $C_{3\text{-}9}$ bond cleavage, followed by the neutral loss of CO, which were characteristic for Type I homoisoflavonoids. The above fragments were consistent with the known 5,7,4′-trihydroxy-3′,5′-dimethoxy-6,8-dimethyl homoisoflavanone (**10**), and allowed the identification of Peak 4.

The pseudo-molecular ion (*m/z* 343) of Peak 6 (t_R = 13.35 min) yielded very similar MS/MS and MS^3 spectra as Peak 4, with *m/z* 207 and *m/z* 179 ions as the base peak, respectively. However, ions at *m/z* 192 and 163 resulting from the neutral loss of 15 mass units were also observed as abundant fragments in the MS^3 spectrum of Peak 6, suggesting the presence of a methoxyl group in A-ring [8, 11]. Although a Type I homoisoflavonoid (5,7,4′-trihydroxy-3′-methoxy-6,8-dimethyl homoisoflavanone, without a methoxyl group at A-ring) with molecular weight of 344 Da had been isolated from *O. japonicus*, Peak 6 was tentatively identified as the new 5,4′-dihydroxy-7,3′-dimethoxy-8-methyl homoisoflavanone.

Peak 9 (t_R = 21.22 min) and Peak 14 (t_R = 27.42 min, **2**) both gave a $[M - H]^-$ ion at *m/z* 327. This pair of isomers could be distinguished by their MS/MS spectra, which yielded *m/z* 192 and 206 ions as the base peak, respectively. They suggested different substitution patterns at the A-ring. By examining known homoisoflavonoids, Peak 9 was characterized as ophiopogonanone A (**6**).

Peak 1 (t_R = 7.23 min) gave a $[M - H]^-$ ion at *m/z* 329. Its MS/MS spectrum gave a base peak at *m/z* 207, consistent with it being a Type I homoisoflavonoid. In the MS^3 spectrum, an abundant demethylated product at *m/z* 192 (75% relative intensity, Δm = 15) was observed besides the expected *m/z* 179 ion (Δm = 28), suggesting the presence of a methoxyl group in A-ring. The neutral loss of a methyl radical was common for methoxylated flavonoids [8, 11]. Thus, Peak 1 was plausibly identified as 5,3′,4′-trihydroxy-7-methoxy-8-methyl homoisoflavanone.

Peak 8 (t_R = 17.69 min) gave a $[M - H]^-$ ion at *m/z* 355. Its MS/MS spectrum yielded prominent ions at *m/z* 340 and 205, and excluded the possibility of the known ophiopogonanone C (5,7-dihydroxy-3′,4′-methylenedioxy-8-aldehydo-6-methyl homoisoflavanone, MW = 356). These two ions should result from the loss of a methyl radical and the cleavage of $C_{3\text{-}9}$ bond, respectively. Thus, Peak 8 was tentatively identified as 5-hydroxy-7-methoxy-3′,4′-methylenedioxy-6,8-dimethyl homoisoflavanone.

Peak 10 (t_R = 22.35 min) gave a $[M - H]^-$ ion at *m/z* 313. No homoisoflavonoid with molecular weight of 314 Da had been reported from *Ophiopogon* species, hence it should be a new constituent. The MS/MS spectrum gave ions at *m/z* 192 and 164. Based on the general fragmentation rules of Type I homoisoflavonoids, Peak 10 was preliminarily characterized as 5,7-dihydroxy-4′-methoxy-6-methyl homoisoflavanone.

Three peaks in the HPLC chromatogram gave a quasi-molecular ion at *m/z* 341, including Compound

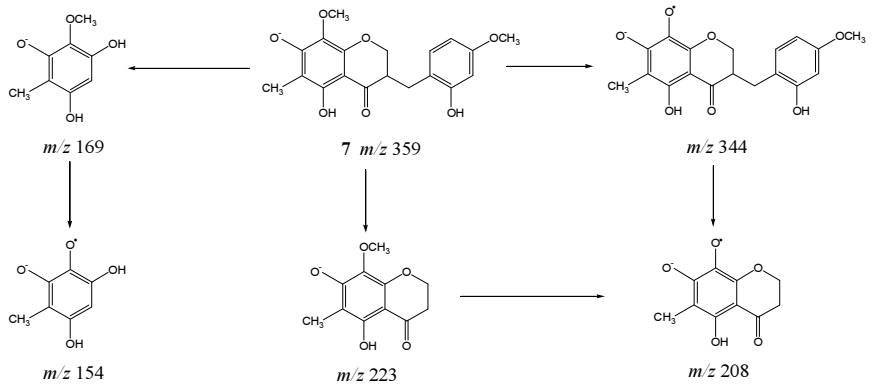
Table 1. Characterization of homoisoflavonoids by HPLC-DAD-tandem mass spectrometry from *Ophiopogon japonicus*

Peak no.	Retention time (t_R, min)	UV λ_{max} (nm)	$[M - H]^-$ *m/z*	HPLC-ESI-MS[a] *m/z* (% base peak)	Identification
1	7.23	296	329	MS^2[329]: 207(100) MS^3[207]: 192(75), 179(100), 165(25), 163(40)	5,3′,4′-trihydroxy-7-methoxy-8-methyl homoisoflavanone
2	8.18	296	373	MS^2[373]: 358(20), 355(30), 222(5), 183(100), 168(35), 153(20) MS^3[183]: 168(100)	5,2′-dihydroxy-7,8,4′-trimethoxy-6-methyl homoisoflavanone
3	9.92	–	339	MS^2[339]: 324(100), 311(5) MS^3[324]: 296(30), 295(100), 237(5)	5-hydroxy-7,4′-dimethoxy-6, 8-dimethyl homoisoflavone
4	11.23	–	373	MS^2[373]: 207(100) MS^3[207]: 179(100)	5,7,4′-trihydroxy-3′,5′-dimethoxy-6,8-dimethyl homoisoflavanone **(10)**
5	12.20	–	341	MS^2[341]: 326(50), 191(100) MS^3[326]: 191(100)	5-hydroxy-3′,4′-dimethoxy-6,8-dimethyl homoisoflavanone
6	13.35	296	343	MS^2[343]: 207(100) MS^3[207]: 192(45), 179(100) 163(15)	5,4′-dihydroxy-7,3′-dimethoxy-8-methyl homoisoflavanone
7	14.37	296	359	MS^2[359]: 344(100), 223(15), 208(12), 169(85), 154(43) MS^3[344]: 326(20), 208(100), 154(15) MS^3[169]: 154(100)	Ophiopogonanone E (**7**)
8	17.69	296	355	MS^2[355]: 340(40), 205(100) MS^3[340]: 205(100) MS^3[205]: 190(35) 177(100), 161(60)	5-hydroxy-7-methoxy-3′,4′-methylenedioxy-6,8-dimethyl homoisoflavanone
9	21.22	294	327	MS^2[327]: 205(20), 192(100), MS^3[192]: 164(40)	Ophiopogonanone A **(6)**
10	22.35	294	313	MS^2[313]: 192(100), 164(15)	5,7-dihydroxy-4′-methoxy-6-methyl homoisoflavanone
11	24.23	–	339	MS^2[339]: 311(100), 219(25)	Methylophiopogonone A (4)
12	25.67	296	341	MS^2[341]: 206(100), 178(40) MS^3[206]: 178(100) MS^3[178]: 150(100)	Methylophiopogonanone A **(1)**
13	25.71	–	373	MS^2[373]: 358(30), 237(100), 222(20), 183(55), 168(20), 153(5) MS^3[237]: 222(100)	Ophiopogonanone F **(8)**
14	27.42	296	327	MS^2[327]: 206(100), 178(15) MS^3[206]: 178(100) MS^3[178]: 150(100)	Methylophiopogonanone B **(2)**
15	34.79	276	353	MS^2[353]: 325(100) MS^3[325]: 297(80), 204 (20)	6-aldehydo-isoophiopogonone A **(5)**
16	35.53	274	355	MS^2[355]: 337(30), 327(100), 307(65), 193(45) MS^3[327]: 192(100), 164(30) MS^3[307]: 279(100)	6-formyl-isoophiopogonanone A **(3)**
17	36.03	–	339	MS^2[339]: 324(8), 311(100), 296(5) MS^3[324]: 296(100) MS^3[311]: 296(100)	6-aldehydo-isoophiopogonone B **(11)**
18	37.23	274	341	MS^2[341]: 323(50), 313(100), 220(50), 193(45) MS^3[313]: 192(100), 164(10) MS^3[220]: 192(100), 178(10)	5,7-dihydroxy-4′-methoxy-6-aldehydo-8-methyl homoisoflavonanone **(9)**

–UV absorption maximum unavailable because of low amounts.

1. Peak 5 (t_R = 12.20 min) gave prominent MS/MS ions at *m/z* 326 and 191, suggesting the presence of a methoxyl group. Its structure was plausibly established as 5-hydroxy-3′,4′-dimethoxy-6,8-dimethyl homoisoflavanone. The third peak appeared at 37.23 min (no. 18). Its MS/MS spectrum gave a base peak at *m/z* 313 (Δm = 28). The preferential loss of CO was characteristic for homoisoflavonoids with a formyl group. The MS^2 and MS^3 spectra were consistent with the known 5,7-dihydroxy-4′-methoxy-6-aldehydo-8-methyl-homoisoflavanone (**9**), and allowed the partial characterization of Peak 18.

Peak 7 (t_R=14.37 min) represented a main constituent of *O. japonicus*. It gave a $[M - H]^-$ ion at *m/z* 359. A moderate intensity ion at *m/z* 223 was observed in the MS/MS spectrum, attributed to $C_{3\text{-}9}$ bond fragmenta-



Scheme 3. Proposed MS fragmentation pathway for the $[M - H]^-$ ions of Compounds **7**.

tion. The base peak at *m/z* 344 suggested the presence of a methoxyl group. Thus, Peak 7 was identified as the known ophiopogonanone E (**7**). The *m/z* 344 ion was further subjected to MS^3 analysis, and a prominent ion at *m/z* 208 resulting from $C_{3\text{-}9}$ bond cleavage was produced, while no signal at *m/z* 223 was observed. Hence, the *m/z* 344 ion should be attributed to the loss of CH_3 at A-ring. Another abundant ion at *m/z* 169 in the MS/MS spectrum could not be explained by previously known fragmentation pathways. We assumed it should result from the simultaneous cleavage of C_2—O and $C_{4\text{-}11}$ bonds. This ion could further fragment into *m/z* 154 by CID. The fragmentation pathway for Compound **7** is given in Scheme **3**.

The new fragmentation behavior for Compound **7** was also observed in Peak 2 (t_R = 8.18 min) and Peak 13 (t_R = 25.71 min). They represented a pair of isomers, both giving a $[M - H]^-$ ion at *m/z* 373. Their MS/MS spectra gave ions at *m/z* 358, 237, and 222, generated by the loss of a methyl radical and $C_{3\text{-}9}$ bond cleavage. They were 14 units greater than the corresponding ions at *m/z* 344, 223, and 208 observed in Compound **7**, suggesting the presence of an additional methoxyl group in the A-ring, presumably at C-5 or C-7. The above fragments were consistent with the known ophiopogonanone F (**8**), containing a C-5 methoxyl group. Coincidentally, Compounds **7** and **8** both had a C-8 methoxyl group. This structural characteristic might be the reason for the new fragmentation pathway. Indeed, an abundant ion at *m/z* 183 was observed in the MS/MS spectra for both Peak 2 and Peak 13, which corresponded to the *m/z* 169 ion in Compound **7**. The next work was to differentiate the two isomers. Their MS/MS spectra were extremely similar, except that an ion at *m/z* 237, due to the direct $C_{3\text{-}9}$ bond cleavage from $[M - H]^-$ ions, was observed as the base peak in the spectrum for Peak 13, while it was not observed in Peak 2. We assumed it depended on whether or not the $[M - H]^-$ ion was easy to lose a methyl radical. When a methoxyl group was present at C-7, the $[M - H]^-$ ion favored to eliminate a methyl group to form a more extended and stable π-conjugation structure. All further product ions would derive from the resulting *m/z* 358 ion. On the other hand, the loss of a methyl radical at C-5 was not likely to produce a more stable structure. Therefore, Peak 13 (with the *m/z* 237 ion) was identified as the known ophiopogonanone F (**8**), and Peak 2 was tentatively characterized as a C-7 methoxylated isomer, 5,2′-dihydroxy-7,8,4′-trimethoxy-6-methyl homoisoflavanone.

Identification of Type II Homoisoflavonoids

Relatively fewer Type II homoisoflavonoids were detected from *O. japonicus*. Besides standards **4** and **5**, only one known compound was identified. It appeared at 36.03 min in the HPLC chromatogram (Peak 17) and gave a $[M - H]^-$ ion at *m/z* 339. In addition to the ion at *m/z* 311 attributed to the initial loss of CO, an ion at *m/z* 324 also was observed in the MS/MS spectrum, indicating the presence of a methoxyl group. Based upon the above evidences, Peak 17 was identified as the known 6-aldehydo-isoophiopogonone B (**11**). In its MS/MS spectrum, the *m/z* 311 ion was much more abundant than *m/z* 324, since the elimination of formyl group was easier than methyl radical. No $C_{3\text{-}9}$ or $C_{9\text{-}1'}$ bond fragmentation characteristic for Type II homoisoflavonoids was observed in the HPLC-MS analysis because of the low amounts of precursor ions and their relatively stable structures. Another peak at t_R 9.92 min (Peak 3) gave almost identical MS and MS/MS spectra to Compound **11**, except that the *m/z* 324 ion was more abundant than *m/z* 311. This result excluded the presence of a formyl group in the molecule, and the *m/z* 311 ion should result from the cleavage of C-4 carbonyl group. Peak 3 was thus tentatively characterized as 5-hydroxy-7,4′-dimethoxy-6,8-dimethyl homoisoflavone, which was reported from *Ophiopogon* species for the first time. This structure was consistent with our assumption that C-7 methoxyl group could trigger the loss of 15 mass units, as observed for Peak 2.

Conclusions

In this paper, the fragmentation behavior of homoisoflavonoids in electrospray ion trap mass spectrometer was studied. The observed fragmentation pathway was very different from those of flavones and flavonols. The major fragmentation of $C_{2\text{-}3}$ saturated homoisoflavonoids (Type I) was $C_{3\text{-}9}$ bond cleavage, which was followed by the loss of carbonyl group (CO). Homoisoflavonoids with a $C_{2\text{-}3}$ double bond (Type II) usually eliminated a CO molecule first, and then underwent the cleavages of $C_{3\text{-}9}$ or $C_{9\text{-}1'}$ bonds. The pathway for CO elimination in Type II homoisoflavonoids was still unclear, and needs further investigation with compounds devoid of a methylenedioxyl group. When a C-6 formyl group was present, the homoisoflavonoids readily lost one molecule of CO to form a base peak. The unique MS fragmentation of homoisoflavonoids should be due to the additional CH_2 group (C-9), which divided the structure into two isolated conjugation parts. Like other flavonoids, methoxylated homoisoflavonoids could lead to the loss of 15 mass units. This fragmentation appeared to take place more easily for C-7 or C-8 methoxyl groups than that at C-4′. As a result, demethylated ions were low or could not be observed in compounds with only a C-4′ methoxyl group, such as Compounds **2** and **9**, and Peak 10. In addition, the presence of a C-8 methoxyl group appeared to trigger the cleavage of C-ring, which might be characteristic for this substitution. However, this fragmentation should be further confirmed with standard compounds.

Based on the above MS fragmentation rules, 18 homoisoflavonoids were identified or tentatively characterized from *Ophiopogon japonicus* in one LC-MS^n run. Seven of them were reported from *Ophiopogon* species for the first time. Isomers and close analoges could be distinguished from each other by comparing their MS/MS and MS^3 spectra. This research set a good example for the rapid identification of bioactive constituents in plant extracts and their products.

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