Characterization of Phthalides in *Ligusticum chuanxiong* by Liquid Chromatographic–Atmospheric Pressure Chemical Ionization–Mass Spectrometry

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Abstract

High-performance liquid chromatography (HPLC) with diode-array detection interfaced to atmospheric pressure chemical ionization (APCI)–mass spectrometry (MS) is applied to analyze phthalides from Chuanxiong (the rhizome of *Ligusticum chuanxiong*). This herb material, containing plenty of phthalide compositions, is selected as the analytical target in this paper for its hematological activity. Some of the phthalides are not stable and are difficult to analyze by gas chromatography–MS. Under optimized LC–MS–MS conditions, six phthalides in the methanol extract of Chuanxiong are unambiguously identified, and characteristic fragments are obtained using homemade reference standards. Ten other phthalides in the extract are confirmed by means of LC–APCI–MS with positive–negative ion mode and collision-induced dissociation in combination with UV spectrophotometry. The results show that LC–MS–MS is a method of choice for fast detection and detailed structural analysis of such mixtures in the crude extract of Chuanxiong.

Introduction

Chuanxiong is the dried rhizome of *Ligusticum chuanxiong* (Umbelliferae), which is frequently used in the prescriptions of traditional Chinese medicine to treat many diseases such as gynecological disorders, chronic bronchitis, or cephalagra (1).

Phthalides have been identified as important constituents of outstanding hematological activity in Chuanxiong. These compounds, which are classified as volatile phthalides, oxidized phthalides, and dimeric phthalide derivatives, are the main components in the methanol fraction of the material (2–4). Some phthalides are biologically active and exhibit promising effects that are used as preventive and therapeutic agents for antiatherosclerosis. The separation and identification of such compounds are therefore of interest in the field of pharmaceutical research.

High-performance liquid chromatography (HPLC) with UV detection is increasingly used to analyze phthalides (5,6). However, the sensitivity and selectivity of UV is insufficient for direct identification of them in complex mixtures. The instability and structural similarity also cause difficulty in their analysis. Some of the volatile phthalides such as Z-ligustilide and sedanenolide are unstable and are easily changed into other phthalides through oxidation, isomerization, dimerization, or rapid decomposition at high temperature because of their active dihydrobenzene structure (7–15). The dimeric phthalides are also thermolabile and retro-Diels-Alder reactions can easily take place even below 100ºC, which can't be detected by gas chromatography (GC)–mass spectrometry (MS) (16). Therefore, application of HPLC coupled to MS is an attractive option to separate and identify such components. Recently, the detection of these compounds by LC–MS was investigated. Long-ze Lin et al. reported the LC–electrospray ionization–MS analysis of phthalides in extract of danggui (17). Sibylle Zschocke et al. used LC–atmospheric pressure chemical ionization (APCI)–MS to analyze dimeric phthalides in extracts of *A. sinensis* and *L. officinale* (16). In order to acquire specific structure information of phthalides, it is necessary to develop a new method to rapidly identify such components in a mixture by using online coupling of HPLC to tandem MS. In this experiment, a gradient separation method was developed using a methanol–water system without any additives, thus a good signal-to-noise (s/n) ratio was obtained in MS analysis. Also, optimized MS conditions were obtained to detect such compounds and specify their structures based on the analyses of six authentic compounds prepared in the lab. This study reports, for the first time, the application of HPLC–APCI–MS–MS for the identification of phthalides in natural products. The estab-

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lished method may be used to accurately monitor the quality of the herb or rapidly analyze the components of the fractions containing various phthalides for bioactivity screening.

Experimental

Chemicals

HPLC-grade methanol was used for HPLC analysis. Water for the HPLC mobile phase was purified on a Milli-Q system (Millipore, Billerica, MA). Reagent-grade petroleum ether, acetate ether, acetone, and ethanol (Lianbang Chemical Co., Shenyang, China) were used for extraction and separation. The sorbent for column chromatography was silica gel 60 (Haiyang Chemical Co., Qingdao, China) and MCI gel CHP-20P (Mitsubishi, Tokyo, Japan). For preparative thin-layer chromatography (TLC), normal-phase TLC plates (silica gel 60 F254) were employed.

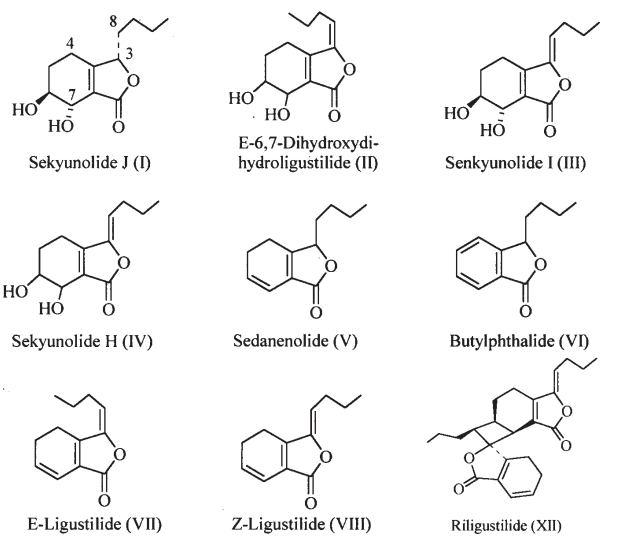

Figure 1. The phthalides analyzed in Chuanxiong extract.

Chromatography

For HPLC separations, a Waters 2690 system (Waters, Milford, MA) equipped with an automatic sample injector was used. All separations were performed with a Hypersil ODS 2 column (Thermo Hypersil-Keystone, Cheshire, U.K.) (5 mm, 4.6 × 250 mm), eluted at 0.8 mL/min with methanol–water (25:75) for 3 min following injection of the sample, then washed with a linear gradient of methanol–water from 25:75 to 100:0 within 47 min. The LC–UV traces were recorded online with a Waters 2690 photodiode-array detector with detection at 280 nm. The LC effluent entered the mass spectrometer without splitting.

MS

MS experiments were performed on a Finnigan MAT TSQ (San Jose, CA) mass spectrometer equipped with an APCI interface.

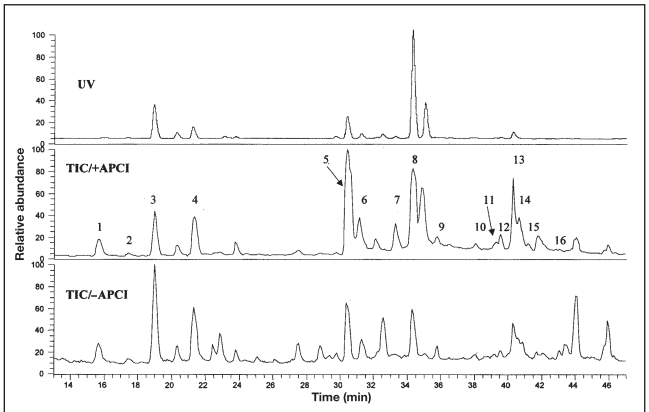

Figure 2. HPLC–UV–APCI–MS analysis of Chuanxiong extract.

Table I. Structure Information from HPLC–UV–APCI–MS Analysis of Chuanxiong Extract

Peak no.	t_R (min)	λ_{max} (nm)	$[M+H]^+$ (*m/z*)	$[M+H+MeOH]^+$ (*m/z*)	Other APCI(+) ions (*m/z*)	$[M-H]^-$ (*m/z*)	Identification
1	15.6	281	227	259	241	225	Senkyunolide J
2	17.4	281	225	257	239	223	E-6,7-Dihydroxydi-hydroligustilide
3	18.9	278	225	257	239	223	Senkyunolide I*
4	20.4	278	225	257	239	223	Senkyunolide H*
5	30.5	281	193	225	–	191	Sedanenolide*
6	31.2	273	191	223	–	–	3-Butylphthalide*
7	33.3	281,328	191	223	–	189	E-Ligustilide
8	34.3	281,328	191	223	–	189	Z-Ligustilide*
9	35.7	281	381	413	191	379	Dimer
10	37.6	283	383	415	191,193	381	Dimer
11	38.1	283	381	413	191	379	Dimer
12	39.3	281	381	413	191	379	Riligustilide*
13	40.7	281	381	413	191	379	Dimer
14	40.9	283	381	413	191	379	Dimer
15	41.5	285	383	415	191,193	381	Dimer
16	42.8	283	381	413	191	379	Dimer

* Standard compound.

The heated capillary temperature was set at 200ºC, vaporizer temperature at 400ºC, auxiliary gas at 10 arbitrary units, and sheath gas at 40 psi. Initially, the mass spectrometer was programmed to perform full scans from *m/z* 100 to 500 to observe molecular ion signals, as well as fragments or adducts in positive–negative ion mode. In MS–MS mode, collision induced dissociation (CID) (collision energy 25 V) of positive molecular ions recorded in the MS spectra were then undertaken to determine the structures of the components, respectively.

Chuanxiong extraction

Chuanxiong was purchased from Sichuan Province, China. At room temperature, 0.5 g Chuanxiong powder was extracted with 10 mL methanol using sonication for 30 min. The methanol extract was filtered through a 0.45-µm syringe filter and injected (10 µL) onto the column for analysis.

Isolation of authentic compounds

Air-dried Chuanxiong (1 kg) was extracted three times with 95% ethanol. The combined extracts were concentrated and suspended into water, followed by partitioning with petroleum ether and ethyl acetate, successively. The petroleum ether extract was chromatographied directly on silica gel (45–70 µm) and eluted using a step gradient of petroleum ether–acetone (90:10, 80:20, 70:30, and finally 60:40). The subfractions were subjected to silica-gel column or normal-phase preparative TLC repeatedly and eluted with the same solvent system as mentioned previously. After repeated chromatography, compounds V, VI, VIII, and XII (Figure 1) were obtained. The ethyl acetate extract was fractionated by MCI gel CHP-20P column chromatography eluted with ethanol–water (40:60, 50:50, and 60:40). Compounds III and IV were obtained. The purity of each component was checked by HPLC. The structure was identified by NMR data (^{1}H NMR, ^{13}C NMR, ^{1}H-^{1}H chemical-shift correlation spectroscopy, nuclear Overhauser-effect spectroscopy, heteronuclear multiple-bond correlation, and heteronuclear multiple quantum correlation spectra taken in Me_2CO solution with a Bruker AM-400 NMR instrument using trimethylsilyl as internal standard) with reported data (8,10,11,18,19). The detailed separation procedure and identification data will be reported later with those of other compounds obtained from Chuanxiong.

Results and Discussion

LC–APCI–MS analysis of standards and Chuanxiong extract

The structures of nine compounds detected in the crude extract of Chuanxiong are listed in Figure 1, and total ion current (TIC) chromatograms are presented in Figure 2.

To achieve a better s/n in MS analysis, a gradient separation method using a methanol–water system without any acid additive was developed. Figure 2 shows the HPLC–UV and TIC chromatograms obtained using the elution system with a good separation of phthalides between 15–45 min and relatively strong MS signals.

In order to demonstrate characteristic MS data for different types of phthalides, six authentic standards of senkyunolide I (III), senkyunolide H (IV), sedanenolide (V), 3-butylphthalide (VI), Z-ligustilide(VIII), and riligustilide (XII), commercial products of which are still not available, were isolated and identified. The standards were first analyzed by LC–MS–MS to determine their retention times, UV, and MS data.

Under the LC–APCI–MS condition, phthalides responded high in positive ion mode, probably because of their lactone structures, which can easily stabilize a proton. Because phthalides possess olefinic bonds and methanol shows high affinity to π-systems, the observed adducts with methanol are common. However, some phthalides also exhibited strong signals in negative ion mode for their active a-H of dihydrobenzene structure or the hydroxyl groups (Table I). Thus, both protonated ions and deprotonated ions obtained in one HPLC run gave more information for determining the molecular mass of a phthalide.

In comparison with the data of six isolated authentic compounds, $[M+H]^+$, $[M-H]^-$, or other ions and UV maximum absorbance values of the analogues were obtained. The values for individual peaks are listed in Table I.

In order to obtain structurally specific data, MS–MS was performed through CID, according to the molecular mass information in MS spectra. The protonated molecular ions were isolated and selectively excited. This mode presents the characterization of compounds not only by their protonated molecular ions, but also by their specific fragmentation. When the collision energy was set at 25 V, CID generally produced a series of product ions for each phthalide.

CID of volatile phthalides

Initial MS–MS work was performed on Z-ligustilide (VIII). It was interesting that this lactone lost 18 Da in the APCI–MS–MS experiment (Figure 3). This type of fragmentation was also observed in the MS–MS spectra of other volatile phthalides, V, VI, and VII. These compounds lost 18 Da from the corresponding protonated molecule that could be attributed to the loss of H_2O, which proved that the proton was conjugated to the oxygen in the five-member cyclic ring of the lactone and that a molecule of H_2O was easily lost after a rearrangement reaction. For butylphthalide, an isomer of ligustilide, the fragmentation of the $[M+H]^+$ ion produced a considerably intense ion at *m/z* 135 that could be attributed to a neutral loss of C_4H_8 (Figure 4). The abundance of

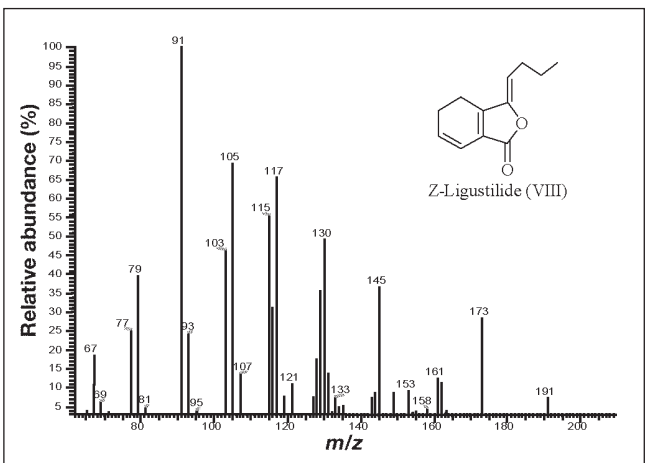

Figure 3. Product ion spectrum of $[M+H]^+$ ion at *m/z* 191 of Z-ligustilide.

the ion at *m/z* 135 was very low in the MS–MS spectrum of ligustilide. The same loss of *m/z* 56 was observed distinctly in the fragmentation of senkyunolide J and sedanenolide (Figure 5). The results revealed that the different substituent with butyl or butenyl at C-3 of a phthalide could be easily distinguished from its product-ion spectrum.

The MS–MS spectrum of the $[M+H]^+$ ion of Z-ligustilide presented ions at *m/z* 173, 162, 145, and 130. In these cases, the fragmentations could be explained assumedly by various radical or neutral losses (or both). It was proposed that these ions were produced as losses of 18 ($-H_2O$), 29 ($-C_2H_5$), 46 ($-H_2O$, $-CO$), and 61 ($-H_2O$, $-CO$, $-CH_3$) Da, respectively. The ions between *m/z* 55 and 120, which were also observed in the MS–MS spectra of the analogues of ligustilide, should be typical characteristic fragments of phthalides. The unknown compound, corresponding to peak 7 in Figure 2, showed the same molecular mass and almost identical MS–MS spectrum with Z-ligustilide. In addition, peaks 7 and 8 presented the same UV maximum absorbance wavelength, hence peak 7 was identified as E-ligustilide (VII) (cis-isomer of Z-ligustilide).

CID of oxidized phthalides

The MS–MS experiment was performed on the $[M+H]^+$ ion of senkyunolide I (III), initially. The product ion at *m/z* 207 corresponded to the loss of water from the protonated molecular ion. With successive or simultaneous loss of water, two ions at *m/z* 189 and 171 were produced. The loss of three molecules of water clearly revealed the dihydroxy substituted structure of senkyunolide I with respect to the loss of water from a lactone observed in the MS–MS experiment of volatile phthalides. The other product ions at *m/z* 165, 161, 147, and 133 showed losses of 60 ($-H_2O$, $-C_3H_6$), 64 ($-2H_2O$, $-CO$), 78 ($-2H_2O$, $-C_3H_6$), and 92 ($-2H_2O$, $-CO$, $-C_2H_4$) Da, respectively (Figure 6). In comparison with senkyunolide I, peak 2 in Figure 2 presented almost identical MS–MS spectrum (Figure 7), with a slight difference in the UV maximum absorbance wavelength. In reference to the report (8) that the cis-trans-isomer coexists commonly in Chuanxiong, peak 2 was tentatively identified as E-6,7-dihydroxydihydroligustilide (the isomer of senkyunolide I and senkyunolide H).

The MS–MS spectrum of the $[M+H]^+$ ion at *m/z* 227 of peak 1 in Figure 2 presented ions at *m/z* 209, 191, 173, 163, 153, 145, and

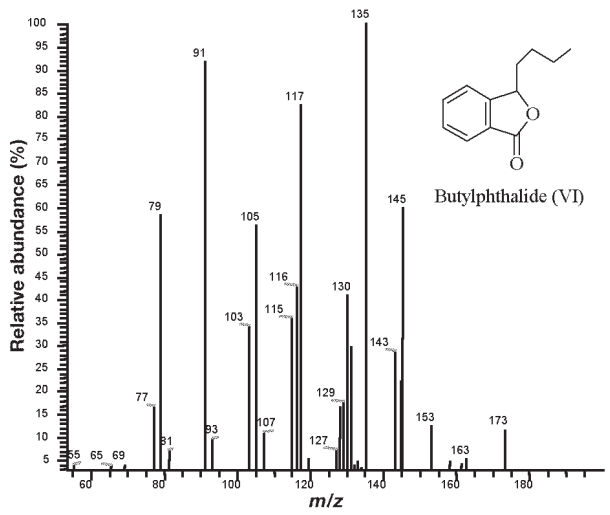

Figure 4. Product ion spectrum of $[M+H]^+$ ion at *m/z* 191 of butylphthalide.

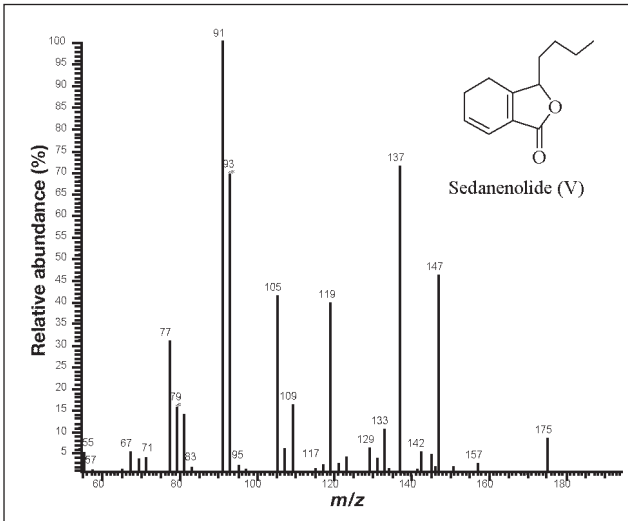

Figure 5. Product ion spectrum of $[M+H]^+$ ion at *m/z* 193 of sedanenolide.

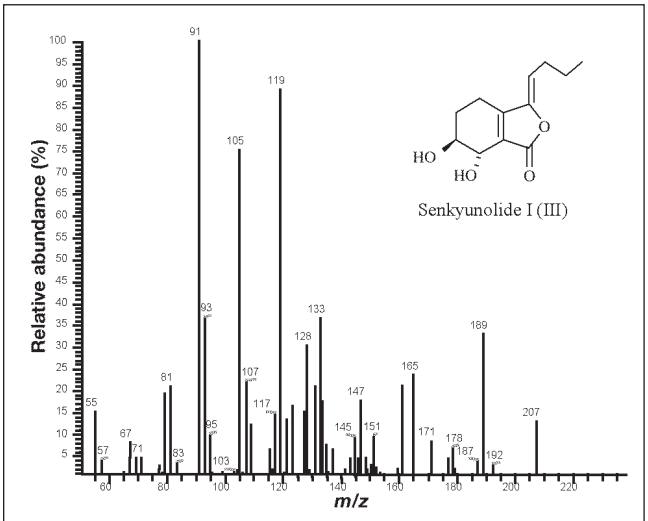

Figure 6. Product ion spectrum of $[M+H]^+$ ion at *m/z* 225 of senkyunolide I.

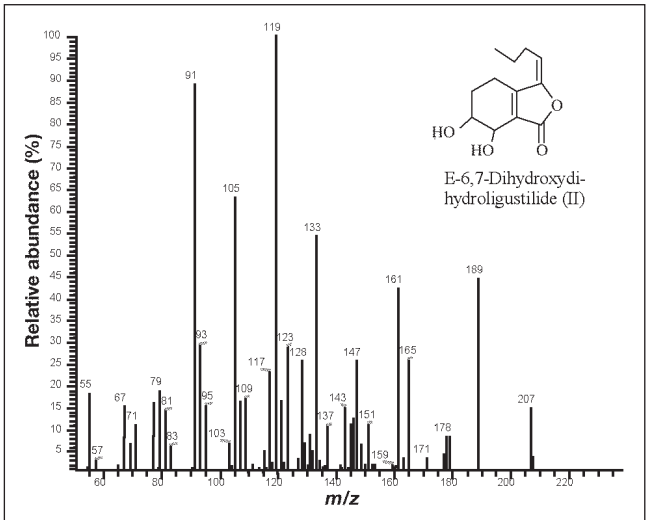

Figure 7. Product ion spectrum of $[M+H]^+$ ion at *m/z* 225 of peak 2.

135 showing characteristic losses of 18 ($–H_2O$), 36 ($–2H_2O$), 54 ($–3H_2O$), 64 ($–2H_2O$, –CO), 74 ($–H_2O$, $–C_4H_8$), 82 ($–3H_2O$, –CO), and 92 ($–2H_2O$, –CO, $–C_2H_4$) Da. In comparison with the product ions of senkyunolide I, it showed that this compound also possessed a dihydroxy structure. The most intense ion at *m/z* 153 suggested the $[M+H]^+$ ion at *m/z* 227 neutral loss of butylene in combination with water ($–H_2O$, $–C_4H_8$). This result indicated that the compound was a 3-butylphthalide derivate. Thus, peak 1 was identified as senkyunolide J (I) (or it's optical isomers).

CID of dimeric phthalides

In MS analysis, dimeric phthalide derivatives showed intense response in both modes under the given conditions. In positive ion mode, riligustilide (XII) showed a protonated ion at *m/z* 381. Like other phthalides, it also formed a stable solvent cluster ion at *m/z* 413 $[M+H+MeOH]^+$. Meanwhile, a fragment ion at *m/z* 191 $[Monomer+H]^+$ was also observed. In negative ion mode, the main peak was the ion at *m/z* 379 $[M-H]^-$. In the product ion spectrum of the protonated molecule ion, the signal corresponding to the protonated monomer ion at *m/z* 191 changed to the base peak (Figure 8). The fragment ion at *m/z* 191 $[Monomer+H]^+$ in MS spectrum was also selected and subject to CID. It was observed that the main product ions were identical with those of the ion at *m/z* 191 $[M+H]^+$ in the fragmentation of ligustilide. The data clearly demonstrated that riligustilide comprised a ligustilide unit. For peak 10, the MS spectrum presented ions at *m/z* 383 $[M+H]^+$, *m/z* 415 $[M+H+MeOH]^+$, and two fragment ions at *m/z* 191 and 193 (Table I). The MS–MS spectrum of the $[M+H]^+$ ion showed that the product ions at *m/z* 191 and 193 became intense (Figure 9). The ion at *m/z* 193 in MS spectrum was also selected and subjected to CID, and it was observed that the main product ions were almost identical with those of sedanenolide. This result indicated the compound had a structure comprised of a sedanenolide and ligustilide unit. Based on similar analysis, Peaks 12–16 were identified as dimeric phthalide derivatives, of which 10 and 15 comprised a sedanenolide unit, and the others were ligustilide dimers. Further structural information of the dimers could not be obtained by tandem MS analysis, but it was rather useful for rapidly detecting such components in complex plant extracts.

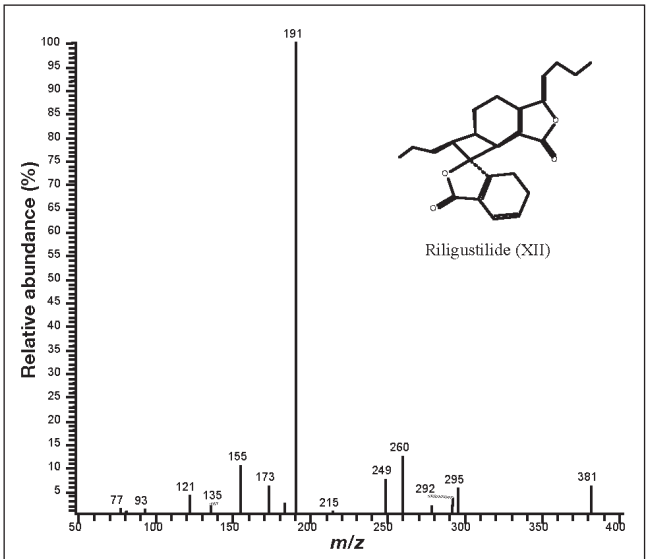

Figure 8. Product ion spectrum of $[M+H]^+$ ion at *m/z* 381 of riligustilide.

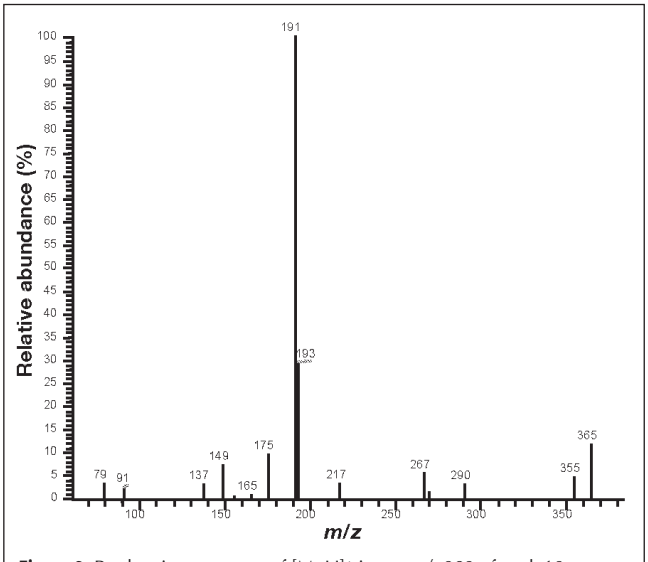

Figure 9. Product ion spectrum of $[M+H]^+$ ion at *m/z* 383 of peak 10.

Conclusion

In this study, both positive and negative ion modes of LC–APCI–MS to analyze phthalides from extract of Chuanxiong were employed. By using optimized instrument parameters, volatile, oxidized, and dimeric phthalides in crude plant extract can be efficiently detected online. MS–MS provides unique structural information to facilitate the identification of phthalides. The potential of LC–APCI–MS–MS in rapidly identifying such compounds in crude plant extract has been demonstrated.

Acknowledgments

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