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RESEARCH ARTICLE

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Identification and characterization of supercritical fluid extracts of Rhizoma Chuanxiong by high performance liquid chromatography ion trap mass spectrometry

Abstract The main constituents, senkyunolide A, Z-ligustilide, neocnidilide, 3-butylphthalide, and ligustilide dimers, in supercritical CO_2 fluid extracts of Rhizoma Chuanxiong, a popular Chinese traditional medicine, have been identified and characterized by high performance liquid chromatography tandem mass spectrometry. Separations were carried out on an Agilent (ECLIPSE XDB) C18 analytical column by gradient elution with 0.25% acetic acid and methanol (containing 0.25% acetic acid). An Agilent 1100 series LC/MSD XCT system was operated under positive ESI and auto MS/MS modes for mass spectrometric analysis. Collision-induced dissociation (CID) fragmentations of these phthalides have been investigated and elucidated. Phthalides have primarily undergone two ESI CID pathways: side-chain cleavage with losses of alkenes and ring-opening with eliminations of H_2O followed by losses of CO. Direct neutral loss of CO has not been observed. Sodium adduct ions have demonstrated completely different CID pathways.

Keywords HPLC-MS/MS, Rhizoma Chuanxiong, ligustilide, CID, supercritical fluid extraction

1 Introduction

Rhizoma Chuanxiong is the dried root of the umbelliferae plant *Ligusticum chuanxiong* Hort. It has been widely used as an active ingredient in traditional Chinese medicine (TCM) prescriptions and composite formulae for the treatment of migraine and various cardiovascular diseases, such as angina pectoris and coronary heart disease [1]. Extracts from Rhizoma Chuanxiong have also been used in health food products and in cuisines.

So far, more than 30 compounds have been isolated and identified from Rhizoma Chuanxiong, among which mainly three groups, phenolic acids, alkaloids, and phthalides have been reported to be the biologically active and therapeutically effective components. Examples of the first two are ferulic acid, coniferylferulate, and tetramethylpyrazine (Chuanxiongzine), while the phthalides include *E*- and *Z*-ligustilides, senkyunolide A to Q, neocnidilide, 3-butylphthalide, 3-butylidenephthalide, and phthalide dimers, e.g., riligustilide and levistolide A.

Compared to the traditional steam distillation, supercritical CO_2 fluid extraction (SFE) is more efficient, less time-consuming, operates under low temperature and low oxygen conditions, favorable for the extraction of thermolabile and oxygen-sensitive compounds, organic solvent free, and environment friendly [2, 3].

GC-MS is the most widely-used technique for the analysis of constituents in Rhizoma Chuanxiong [4–6]. However, it was reported that the high temperatures used for sample introduction in GC had undesirable effects on the quantification of those thermolabile phthalides [7]. In recent years, techniques based on high performance liquid chromatography (HPLC) have been frequently applied in the analysis of Rhizoma Chuanxiong, which include HPLC-UV [7], HPLC-NMR [8], and HPLC-MS [9–10]. So far, there is no report on the application of tandem mass spectrometry to this subject.

The two biggest obstacles in the analysis of natural products are their complicated compositions, for example, there are more than 10 phthalides in Rhizoma Chuanxiong, in which some are isobaric isomers, and the lack

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of corresponding standards or reference materials. The technique of choice to alleviate these predicaments is LC-MS/MS. In this study, main constituents in Rhizoma Chuanxiong SFE extract have been identified and characterized. The collision-induced dissociation (CID) fragmentations of the major phthalides have also been investigated and elucidated.

2 Experimental

2.1 Instrumentation and chemicals

An Agilent 1100 series LC/MSD XCT system consisting of a vacuum degasser, binary pump, autosampler, DAD, and dual ESI/APCI ionization source (Agilent, Palo Alto, CA, USA) was used for this research. HA 48 SFE extractor (Nantong, China) was used for the extraction of Rhizoma Chuanxiong samples. HPLC grade methanol (Merck, Darmstadt, Germany) and deionized water produced in this laboratory were used as HPLC mobile phases. All other chemicals were of analytical-reagent grade (Guanghua, China).

Rhizoma Chuanxiong samples were purchased from Dujiangyan, Sichuan, China and were authenticated by professionals at Honsea Sunsine. The voucher specimens of all batches of samples were deposited at Honsea Sunsine.

2.2 Supercritical CO_2 fluid extraction

Rhizoma Chuanxiong raw materials were pulverized to 20–40 meshes. Then, 12 kg of the homogenized powder was placed into an SFE extractor and 10 mL of 95% ethanol in food grade was added as carrier reagent. The temperature was maintained at 40–60°C, pressure 20–35 MPa, and the flow rate of CO_2 was 200–300 kg/h. Extraction took 1.5 to 5 h. Separation temperature was adjusted to 45–60°C and pressure was 8–15 MPa. Ingredients dissolved in CO_2 were released into a separation kettle through a decompress valve. CO_2 was cooled and then pumped back to the storage container for reuse. After dehydration, Rhizoma Chuanxiong volatile oil was stored under a dark and cool environment and shield by nitrogen gas.

2.3 Measurement conditions

Chromatographic analyses were carried out on an Agilent ECLIPSE XDB C18 column (4.6 × 150 mm, 5 μm) with 0.25% acetic acid and methanol (containing 0.25% acetic acid) as mobile phases. Elution gradient was: 0 to 1 min, 70% B; 1 to 21 min, linearly increased from 70 to 85% B; 21 to 23 min, linearly increased from 85 to 100% B; maintain 100% B for 3 min and wash column by 70% B for 5 min before the next run; stop data collection at 26 min. A mass spectrometer was operated under positive ESI mode, auto MS/MS with two precursors and two stages of CID, nebulizer gas 50 psi, drying gas 9 L/min, temperature 350 °C, fragmentation voltage 1.0 V, and monitoring mass range 100–800 Dalton. Rhizoma Chuanxiong volatile oil was diluted 10,000 times with 50% methanol and then stored at 4°C in a refrigerator. A 10 μL sample was injected for analysis.

3 Results and discussion

A typical total ion current (TIC) LC-MS chromatogram of Rhizoma Chuanxiong SFE extract is shown in Fig. 1. Preliminary experiments had shown that no LC-MS chromatogram peak appeared before mobile phase with 50% organic solvent, therefore, elution gradient was started at 70% B to save time for analysis. The predominant three components eluted at 15.8, 19.5, and 20.2 min and the sum of their relative peak areas was greater than 80%.

ESI-MS data of the 10 major constituents in Rhizoma Chuanxiong SFE extract is shown in Table 1, and their corresponding CID MS^n data in Table 2. Eight of these 10 compounds, in which the sum of their relative peak areas are greater than 98%, were identified based on these data and information published in references. The first step was to determine the molecular weight of the target compound from characteristic quasi-molecular ion $[M+H]^+$ and accompanying sodium adduct ions, $[M+Na]^+$, and $[2M+Na]^+$, in Table 1. These three ions would be easily recognizable for a well-separated distinct peak. Otherwise, it would not be clearly determined and would be a judgment call, as components 1, 3, and 9. CID data in Table 2 provided verification for these identifications. In this study, the LC-MS/MS method could not distinguish stereoscopic isomers, senkyunolide H and I, and those phthalide dimers, either naturally occurring riligustilide and levistolide A or those formed when high concentration of extracts were stored for a long period of time and induced by heat or light. MS, MS^2, and

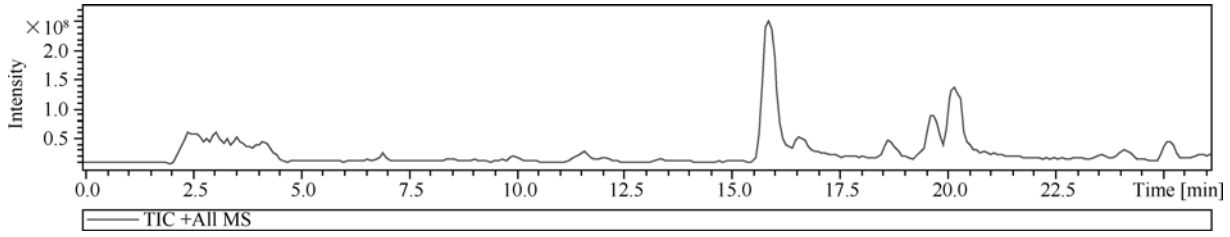


Fig. 1 TIC Chromatogram of *Rhizoma Chuanxiong* Extract

Table 1 LC-MS of *Rhizoma chuanxiong* extract and identifications of its major constituents

Peak	Area (%)	t_R (min)	MS (*m*/*z*)				Identification
			$[M+H]^+$	$[M+Na]^+$	$[2M+Na]^+$	Other ions	
1	~3	4.2	224.8	246.9	470.9	206.9($M+H-H_2O$)	Senkyunolide H/I
2	<0.2	6.9	188.9	211.0	-	228.9($M+Na+H_2O$)	3-Butylenephthalide
3	<0.2	11.6	162.9	184.9	378.8	130.9, 279.0	Not ID
4	45.0	15.8	192.9	214.9	406.9	146.9($M-H_2O-CO+H$), 384.5(M_2+H)	Senkyunolide A
5	6.6	16.5	191.0	213.1	-		3-Butylphthalide
6	4.6	18.6	190.9	212.9	-		E-Ligustilide
7	11.5	19.5	194.9	216.9	411.0	148.9, 177.0($M+H-H_2O$)	Neocnidilide
8	24.5	20.2	190.9	212.9	403.1		E-Ligustilide
9	<0.2	24.1	-	-	-	279.1, 319.1, 437	Not ID
10	~3	25.1	381.0	-	-	190.9($M/2+H$), 398.1($M+H_2O+H$)	Phthalide Dimers

Table 2 Tandem MS of main constituents in *Rhizoma chuanxiong* extract

Peak	Target compound	Target ion	MS^2	MS^3	
				Target ion	Fragment ion.
1	Senkyunolide H/I	207	133，165(C_3H_6)，179($-C_2H_4$)，189($-H_2O$)	189	105，117，161，171
2	3-Butylenephthalide	189	143($-C_4H_8$)，161($-C_2H_4$)，171($-H_2O$)	161	105, 119, 133, 143
4	Senkyunolide A	193	105，137($-C_4H_8$)，147($-H_2O-CO$)，175($-H_2O$)	147	105, 119
5	3-Butylphthalide	191	105, 145, 147, 173	173	105, 117, 145
6	E-Ligustilide	191	147, 173	147	105, 117, 119
7	Neocnidilide	195	149($-H_2O-CO$), 159, 177($-H_2O$)	149	105, 107, 121,
8	E-Ligustilide	191	105, 107, 117, 135($-C_4H_8$), 145($-H_2O-CO$), 149($-C_3H_6$), 155, 163($-C_2H_4$), 173($-H_2O$)	173	105, 117, 131，145, 155
10	Phthalide dimers	381	191	191	105, 145, 155, 163, 173

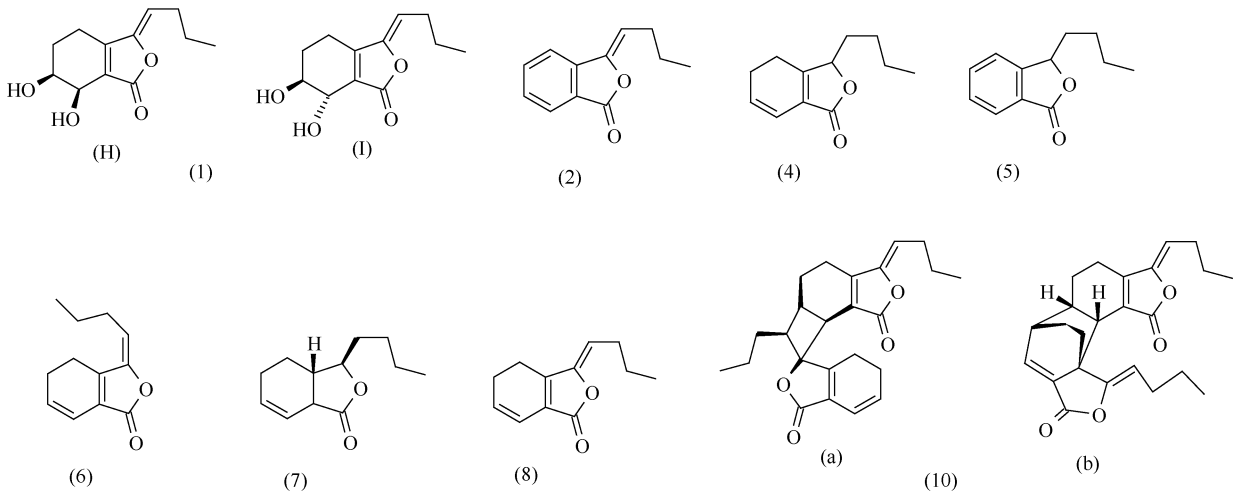
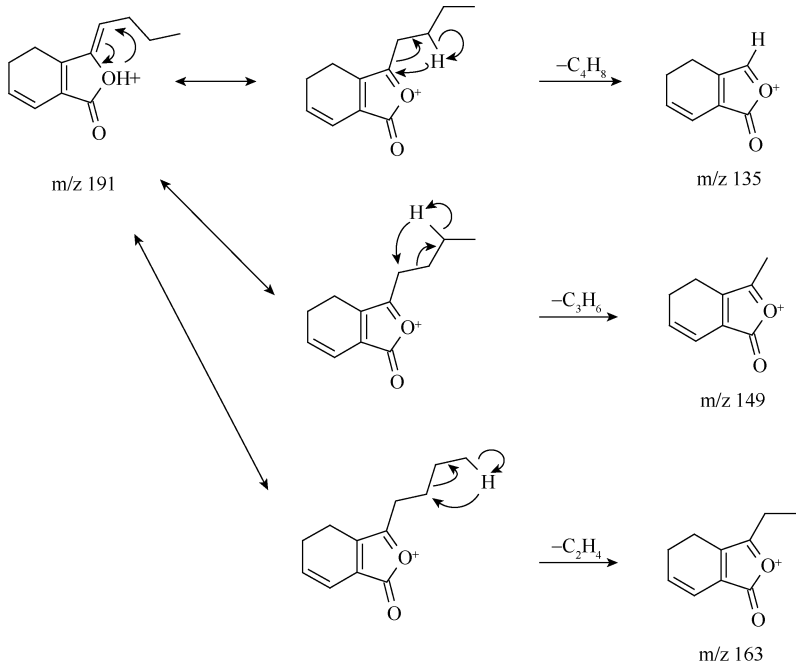


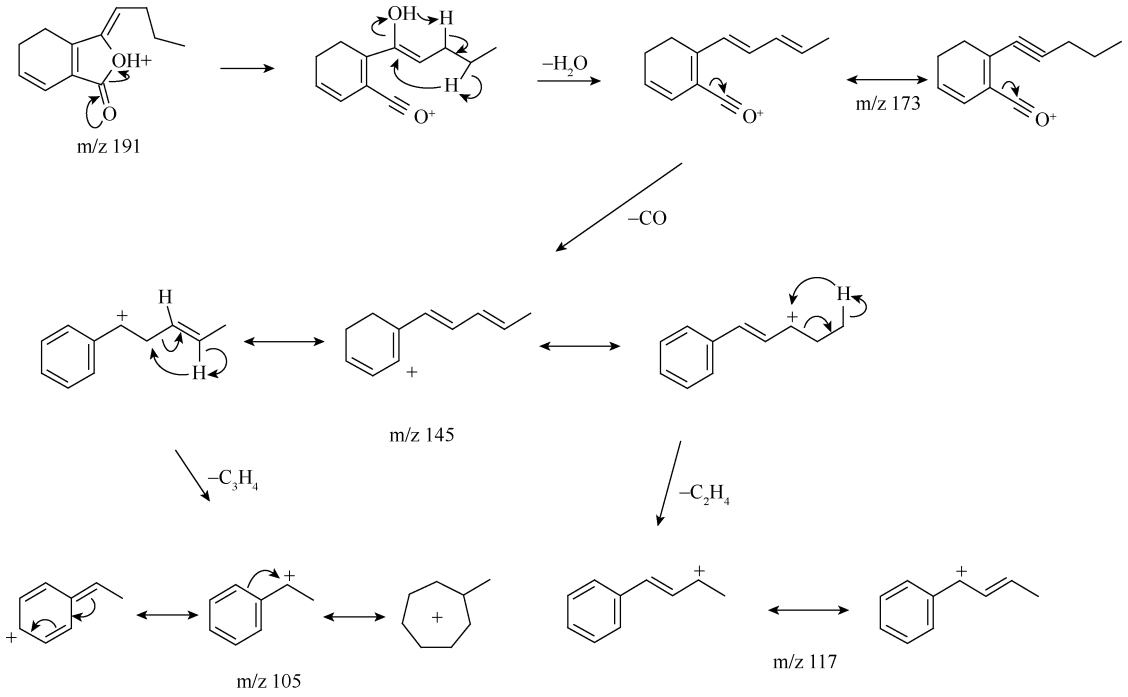
Fig. 2 Structures of main constituents in *Rhizoma chuanxiong* Extract: (1) Senkyunolide H/I, (2) 3-Butylenephthalide, (4) Senkyunolide A, (5) 3-Butylphthalide, (6) E-Ligustilide, (7) Neocnidilide, (8) E-Ligustilide, (10) Pthalide Dimers: (a) Riligustilide and (b) Levistolide A.

MS^3 of these dimers were indistinguishable. The distinction between E-ligustilide, Z-ligustilide, and 3- butylphthalide, which are isobaric isomers, was made based on their concentrations and elution order compared to references. Chemical structures of these identified compounds are shown in Figure 2.

Structural elucidation by LC-MS with soft ionization sources, e.g., ESI and APCI, was based on CID fragmentations. So far, ESI CIDs and their mechanism were not nearly as well-understood as electron impact (EI) in GC-MS. Yi et al



(a) Side-chain cleavage pathways of ligustilide



(b) Ring-opening pathways of ligustilide

Fig. 3 Proposed ESI CID pathways of *Z*-ligustilide

have investigated a group of lactones related to pharmaceuticals and their corresponding hydrolysis products, carboxylic acids and discovered that they underwent different CID fragmentation pathways[11]. They have demonstrated that, under positive ESI mode, lactones had neutral losses of CO but not H_2O and few could loose H_2O and CO together, while acids behaved just the opposite, loosing H_2O, H_2O and CO together, but not CO alone. As shown in Table 2, major phthalides in Rhizoma Chuanxiong SFE extract, such as Z-ligustilide, senkyunolide A, and neocnidilide, have primarily undergone two ESI CID pathways: side-chain cleavage with neutral losses of alkenes and ring-opening with eliminations of H_2O followed by losses of CO. Direct neutral loss of CO was not observed. The ESI CID pathways of

Z-ligustilide are shown in Figure 3. The characteristic ion of *m/z* 105 was common to all fragmentations of phthalides, which could attribute to its high structural stability. A phenomenon that could be explained here was that the ion of *m/z* 155, which was the fragmentation product of quasi-molecular ion of Z-ligustilide, seemingly loses two H_2O in a single step and two steps. A reasonable chemical structure of *m/z* 155 could not be determined. Sodium adduct ions of phthalides have demonstrated completely different CID pathways. While fragmentations of $[2M+Na]^+$ produced only $[M+Na]^+$, CID of $[M+Na]^+$ was hard to control and results were unpredictable. CID of phthalide dimers produced only ion of *m/z* 191, whose CID mass spectra were very similar to those of Z-ligustilide.

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