Separation and Identification of the Phthalic Anhydride Derivatives of Liqusticum *Chuanxiong* Hort by GC–MS, TLC, HPLC–DAD, and HPLC–MS

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Abstract

A simple, sensitive, and rapid method using gas chromatography (GC)–mass spectrometry (MS) is developed for the simultaneous separation and identification of the active ingredients of Liqusticum *Chuanxiong* Hort (*Chuanxiong*). Ten phthalic anhydride derivatives (PADs) are identified in *Chuanxiong* as 3-butylphthalide, 3-butylidenephthalide, 3-butylidene-4-hydroxyphthalide, senkyunolide A, neocnidilide, Z-ligustilide, E-ligustilide, senkyunolide F, senkyunolide-H, and senkyunolide-I. The existence of ferulic acid and vanillin in *Chuanxiong* extract is also demonstrated. Further identification of these compounds is performed by thin-layer chromatography, high-performance liquid chromatography (HPLC), and HPLC–MS analysis. This is the first report of the separation and determination of the PADs in *Chuanxiong* by GC–MS.

Introduction

Liqusticum *Chuanxiong* Hort (*Chuanxiong*), the dried rhizome of *Ligusticum wallichii Franch*, is one of the most frequently occurring drugs in the prescriptions of Chinese traditional medicine, and it has been used to treat headaches, anemia, feelings of cold, and irregularity of menstruation. Its main essential components (ligustilide, other phthalides, ferulic acid, and vanillin) are thought to be the biologically active components of *Chuanxiong* (1,2). Various phthalides derivatives have been isolated from *Chuanxiong* by many workers using silica gel column chromatography (3–8). Some of these phthalides are present in several important medicinal herbs, such as *Cnidium officinale* and Danggui (9). In order to determine the quality of these important herbs and their drug products, it is important to have a rapid, direct, and accurate method for the analysis of these components. Some phthalic anhydride derivatives (PADs) have been analyzed by high-performance liquid chromatography (HPLC) with UV detection (10,11) and gas chromatography (GC)–mass spectrometry (MS) (12–15). Because it is difficult to obtain all the PADs as standards, HPLC with UV detection could not identify most peaks in the *Chuanxiong* extract. In addition to this, the reported GC–MS method only identified 4 or 5 PADs, and the sample preparation was time consuming. In this study, more than 20 volatile compounds were detected by GC–MS in *Chuanxiong*, and ten PADs along with ferulic acid and vanillin were identified. Further identification was performed by thin-layer chromatography (TLC), HPLC with a diode-array detector (DAD), and HPLC–MS analysis.

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Experimental

Instrumentation

GC-MS

The GC-MS system consisted of a GC (HP 6890), a mass selective detector (HP 5973), and an HP ChemStation data analysis system. Separation was carried out on an HP-5 MS capillary column (30-m × 0.25-mm i.d., 0.25-µm film thickness, 5% phenyl methyl siloxane) using the following temperature program. The initial temperature was 80°C, then it increased to 160°C at 25°C/min, then increased to 220°C at 4°C/min, and finally increased to 280°C at 25°C/min. The injector temperature was 250°C, the MS source temperature was 250°C, and the column flow rate of the carrier gas (He) was 1.0 mL/min with a split ratio of 30:1. The full-scan acquisition mode (*m/z* 33–400) was used for detection. Samples of 1 µL were injected manually. Solvent delay was set as 2.0 min.

HPLC

The HP 1100 chromatograph consisted of a quaternary pump, a DAD, and an HP ChemStation data analysis system. A Zorbax SB-C18 ODS column (250- × 4.5-mm i.d., 5-µm particle size) was used for separation. The mobile phase consisted of water and

methanol using the following gradient: 55% methanol for 10 min then increased to 70% methanol within 2.0 min and maintained to the end. The flow rate was adjusted to 0.7 mL/min, and the injected sample volume was 20 µL. The effluent was monitored at 270 nm and scanned between 190 and 500 nm by the DAD.

HPLC–MS

An API3000 liquid chromatograph–MS–MS system (PE SCIEX) was used. The MS was operated in the positive-ion mode with the electrospray ionization mode. A PE Series 200 pump with UV detection set at 270 nm was used for HPLC separation. Chromatography conditions were the same as in the previous HPLC section. The HPLC system was directly connected to the MS without stream splitting. The ion spray was set at 4500 V, the temperature 250°C, the mass range (*m/z*) 100–400, and the scan rate was 2 s per scan.

Chemicals and reagents

HPLC-grade methanol was used for HPLC analysis. Reagent-grade ethanol, hexane, ethyl acetate, and ethyl ether were used for extraction and separation. Normal-phase TLC plates (silica gel 60 F254, 0.25-mm thickness, 10 × 10 cm) were used for TLC. *Ligusticum wallichii* (crude medicinal material) was purchased from Chengdu (Sichuan Province, China).

Extraction of *Chuanxiong*

A 10.0-g amount of crude medicinal material of *Chuanxiong* was crushed into small pieces and extracted with 100 mL ethanol by refluxing on a waterbath at 80°C for 1.0 h, then it was placed in an ultrasonic bath for 10 min. Extraction was repeated three times. The total extracts were combined, and ethanol was removed with a rotary evaporator at 50°C under vacuum. The residue was dissolved with methanol, diluted to appropriate concentration, and filtered through a 0.45-µm filter membrane before analysis.

Preparation of the compounds standards by TLC

An ethanol extract of *Chuanxiong* was dissolved in 50 mL of hot water and extracted three times with 50 mL of ethyl ether and ethyl acetate, respectively. Solvents from all of the fractions were removed with a rotary evaporator to obtain the ethyl ether and ethyl acetate extract. The ethyl ether extract was separated by TLC (normal-phase plates, ethyl ether–hexane, 1:5, v/v), and the nine zones found on the TLC plate could be visualized under UV light at 254 nm. Each zone was scraped from the plate and extracted with methanol. The methanol extract of each fraction was analyzed by GC–MS and HPLC. The ethyl acetate extract was separated by TLC under the same conditions. Only the first two zones (R_F = 0.58 and 0.63) were observed. In this study, ethyl ether extract was used for the preparation of PADs by TLC.

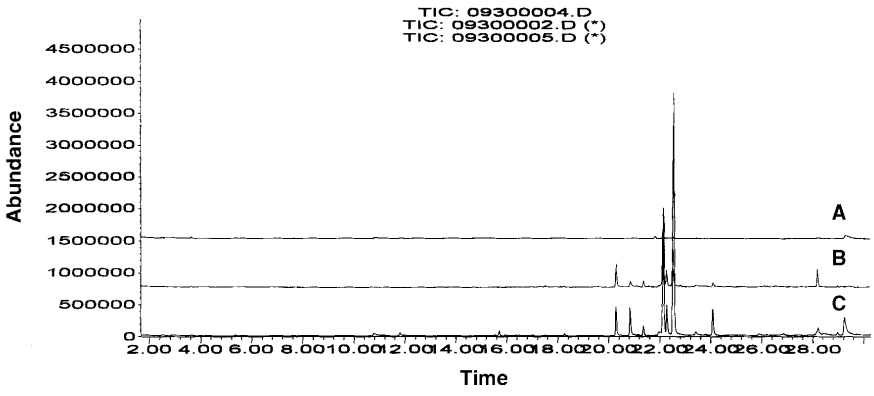

Figure 1. Gas chromatograms of different extracts of *Chuanxiong*: (A) ethyl acetate extract, (B) *n*-hexane extract, and (C) ethanol extract.

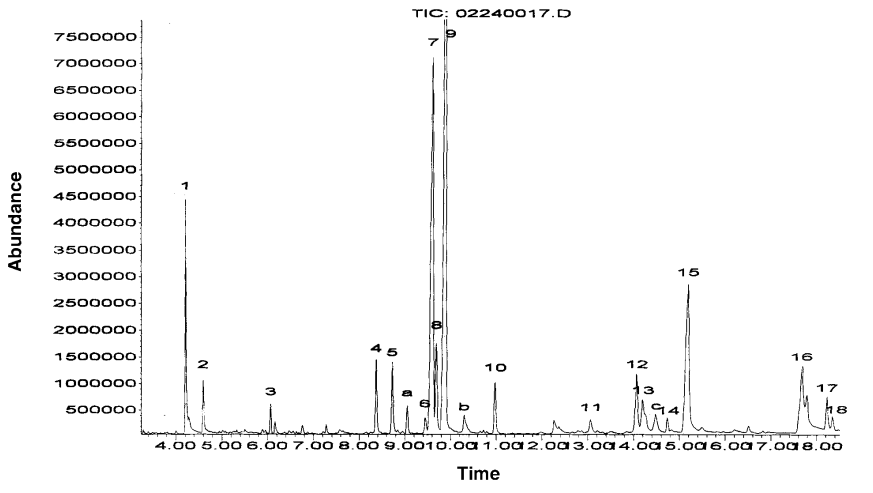

Figure 2. Gas chromatogram of *Chuanxiong* extracts: ferulic acid, 1; vanillin, 2; decahydro-4α-methyl-1-methylene-7-naphthalene, 3; 3-butylphthalide, 4; 3-butylidenephthalide, 5; 3-butylidene-4-hydroxyphthalide, 6; senkyunolide A, 7; neocnidilide, 8; Z-ligustilide, 9; E-ligustilide, 10; senkyunolide F, 11; *n*-hexadecanoic acid, 12; senkyunolide-H, 13; hexadecanoic acid ethyl ester, 14; senkyunolide-I, 15; 9,12-octadecadienoic acid, 16; linoleic acid ethyl ester, 17; ethyl oleate, 18; and unknown, a, b, and c.

Results and Discussion

Optimization of the GC conditions

In order to completely separate the components in *Chuanxiong*, a temperature program was used from 80°C to 280°C at a rate of 4°C/min. The main components started to elute after 20 min, at which time the temperature was increased to 160°C (Figure 1). It was necessary to quickly increase the temperature before the oven temperature reached 160°C. The analysis time was decreased greatly when the temperature program was changed to the following program: the temperature was increased from 80°C to 160°C at 25°C/min, then increased to 220°C at 4°C/min, and finally increased to 280°C at 25°C/min (Figure 2). The other conditions were the same as described in the

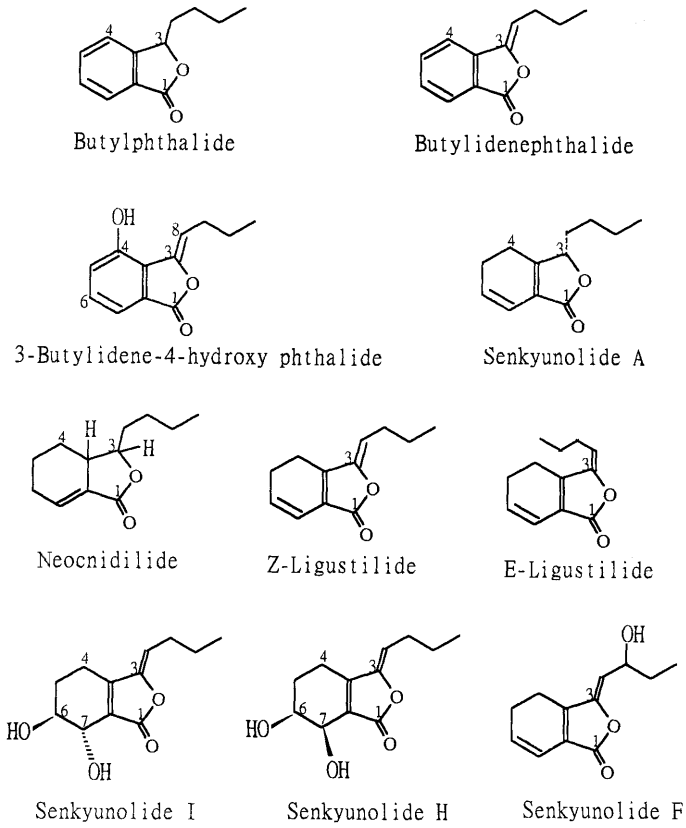

Figure 3. The structures of phthalic anhydride derivatives in *Chuanxiong*.

Experimental section. Under these chromatographic conditions, the separation time decreased greatly and the peaks were completely separated.

Optimization of the extraction of *Chuanxiong*

The crude material of *Chuanxiong* that was extracted with ethanol as described previously was extracted again with hot water by refluxing on a waterbath at 90°C for 1.0 h. Extraction was repeated twice. The total water extracts were combined and concentrated with a rotary evaporator at 60°C under vacuum. The concentrated solution was successively extracted with *n*-hexane and ethyl acetate. Organic solvents from each fraction were removed with a rotary evaporator at 40°C under vacuum in order to obtain *n*-hexane extracts and ethyl acetate extracts. The extracts were dissolved in methanol, diluted to appropriate concentration, and filtered through a 0.45-µm filter membrane for GC–MS analysis.

The separation of the *n*-hexane extract and ethyl acetate extract was performed under the same chromatographic conditions (Figure 1). Only a few of the PADs were detected in the *n*-hexane extracts, and almost no PADs were detected in the ethyl acetate extract. From these results it can be concluded that the PADs were extracted satisfactorily with ethanol.

Separation and identification of PADs in *Chuanxiong* by GC–MS

The ethanol extracts of *Chuanxiong* that were

Table I. Peak Identification in GC–MS

Peak no.	R_t (min)	Mass data*	Compound
1	4.22	150(100),135(80),107(28),77(26)	ferulic acid
2	4.60	152(M^+,100),151(96),123(22),109(22),81(25),53(10)	vanillin
3	6.07	204(M^+,83),189(63),161(68),133(59),105(100),79(75)	decahydro-4α-methyl-1-methylene-7-naphthalene
4	8.38	190(M^+,4),133(100),105(27),77(11)	3-butylphthalide
5	8.73	188(M^+,23),159(100),131(23),103(19),77(19)	3-butylidenephthalide
6	9.44	204(M^+,34),175(100),162(34),147(28),159(73),133(27),103(20),77(20)	3-butylidene-4-hydroxyphthalide
7	9.62	192(M^+,23),163(3),135(5),107(100),79(22)	senkyunolide A
8	9.70	194(M^+,2),137(5),108(100),79(30)	neocnidilide
9	9.90	190(M^+,66),161(100),148(84),133(18),105(51),77(28),55(41)	Z-ligustilide
10	10.98	190(M^+,64),161(100),148(80),133(18),105(34),77(28),55(43)	E-ligustilide
11	13.06	206(M^+,30),188(3),177(100),150(57),135(35)	senkyunolide F
12	14.10	256(M^+,47),213(34),185(19),157(19),129(48),73(100)	*n*-hexadecanoic acid
13	14.20	224(M^+,36),206(3),180(100),151(50),123(16),95(16),77(16),55(26)	senkyunolide-H
14	14.70	284(M^+,11),241(11),157(15),101(58),88(100)	hexadecanoic acid ethyl ester
15	15.19	224(M^+,32),206(3),180(100),151(44),123(12),95(15),77(10),55(25)	senkyunolide-I
16	17.69	280(M^+,24),123(14),109(30),95(63),81(88),67(100),55(58)	9,12-octadecadienoic acid
17	18.23	308(M^+,15),263(20),220(8),178(10),150(16),123(20),95(70),81(92),67(100)	linoleic acid ethyl ester
18	18.35	310(M^+,11),264(46),222(31),180(21),155(16),111(34),88(75),55(100)	ethyl oleate
X1	9.06	150(21),107(10),93(43),79(100)	unknown
X2	10.30	208(100),179(25),151(38),131(67),119(58),91(53)	unknown
X3	14.47	206(9),182(89),139(46),126(100)	unknown

* *m/z*, rel. int. shown in parenthesis.

dissolved in methanol were injected into the GC–MS. The chromatogram is shown in Figure 2. Twenty-one components were separated, and eighteen of them were identified according to the mass spectrum of each component. By comparing the mass spectra data of the sample with literature data, peaks 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 13, and 15 were identified as ferulic acid, vanillin, decahydro-4α-methyl-1-methylene-7-naphthalene, 3-butylphthalide, 3-butylidenephthalide, 3-butylidene-4-hydroxyphthalide, senkyunolide A, neocnidilide, Z-ligustilide, E-ligustilide, senkyunolide F, senkyunolide-H, and senkyunolide-I, respectively. The structures of some phthalides are shown in Figure 3. Peaks 12, 14, 16, 17, and 18 were identified as *n*-hexadecanoic acid, hexadecanoic acid ethyl ester, 9,12-octadecadienoic acid, linoleic acid ethyl ester, and ethyl oleate, respectively. The results are summarized in Table I. The analysis of some of the PADs is described.

Ferulic acid and vanillin

Peaks 1 and 2 were identified as ferulic acid and vanillin, respectively, by comparison of the mass spectra with literature data. Further identification was performed by comparing the retention time and the mass spectra with that of standard ferulic acid and vanillin.

3-Butylphthalide

The mass spectrum of peak 4 showed prominent peaks at *m/z* 190, 133, 105, and 77, which was very similar to the spectrum of 3-butylphthalide (4,16). Based on the mass spectral data, peak 4 was identified as 3-butylphthalide. The main fragment ions were formed at *m/z* 133 ($M^+–C_4H_9$) and 105 ($M^+–C_4H_9–CO$).

3-Butylidenephthalide

The mass spectrum of peak 5 exhibited fragment peaks at *m/z* 188 (M^+, rel. int. 23), 159 (rel. int. 100), 131 (rel. int. 23), 103 (rel. int. 19), and 77 (rel. int. 19). By comparing the mass spectrum with literature data (4), peak 5 was identified as 3-butylidenephthalide.

3-Butylidene-4-hydroxyphthalide

The mass spectrum of peak 6 showed fragment peaks at *m/z* 204 (M^+, rel. int. 34), 175 (rel. int. 100), 162 (rel. int. 34), 147 (rel. int. 28), 159 (rel. int. 73), 133 (rel. int. 27), 103 (rel. int. 20), and 77 (rel. int. 20), which was similar to the mass spectrum of 3-butylidene-4-hydroxyphthalide (5). The main fragment ions were formed as *m/z* 175 ($M^+–C_2H_5$), 162 ($M^+–C_3H_6$), and 147 ($M^+–C_2H_5–CO$).

Senkyunolide A

The mass spectrum of peak 7 was similar to the mass spectrum of senkyunolide A (17), whose mass data were reported as *m/z* 192 (rel. int. 18), 107 (rel. int. 100), and 135 (rel. int. 6). The main fragment ions were formed as *m/z* 192 (M^+), 135 ($M^+–C_4H_9$), 107 ($M^+–C_4H_9–CO$), and 79 ($M^+–C_4H_9–CO×2$). According to the mass spectral data, peak 7 was identified as senkyunolide A.

Neocnidilide

By comparing the mass spectrum of peak 8 with that of neocnidilide, peak 8 was identified as neocnidilide (17), whose mass spectrum was reported as *m/z* 194 (rel. int. 2), 137 (rel. int. 6.4), 108 (rel. int. 100), and 79 (rel. int. 58). The main fragment ions were formed as *m/z* 194 (M^+), 137 ($M^+–C_4H_9$), 108 ($M^+–C_4H_9–CHO$), and 79 ($M^+–C_4H_9–CHO$).

Z-Ligustilide and E-ligustilide

The mass spectra of peak 9 showed fragment ions at *m/z* 190 (M^+, rel. int. 66), 161 (rel. int. 100), 148 (rel. int. 84), 133 (rel. int. 18), 105 (rel. int. 51), 77 (rel. int. 28), and 55 (rel. int. 41). The mass spectra of peak 10 showed fragment ions at *m/z* 190 (M^+, rel. int. 64), 161 (rel. int. 100), 148 (rel. int. 80), 133 (rel. int. 18), 105 (rel. int. 34), 77 (rel. int. 28), and 55 (rel. int. 43). The mass spectra of peaks 9 and 10 were similar because of similar structures. By comparing the mass spectra with literature data (18), peaks 9 and 10 were identified as two isomers of ligustilide. According to the reported GC–MS separation of ligustilide (14), Z-ligustilide eluted faster than E-ligustilide. Based on the mass spectra and the literature data, peaks 9 and 10 were identified as Z-ligustilide and E-ligustilide, respectively.

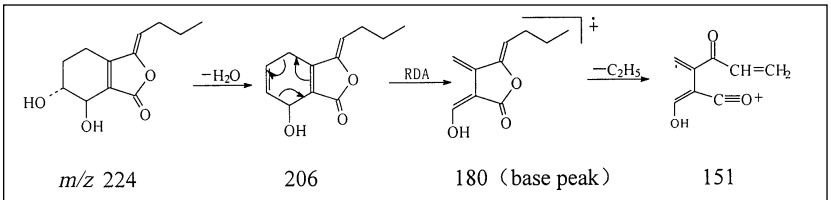

Figure 4. Explanation of the mass splitting path.

Table II. Analytical Results of GC–MS and HPLC–MS for Each Fraction of the TLC Preparation

	GC–MS		HPLC–DAD	
R_f	t_R (min)	MS data	t_R (min)	UV λ_{max} (nm)
0.00	15.16	224,180(100)	3.17	322,290sh
	14.20	224,180(100)		
	9.84	190,161(100)	7.04	280
	4.22	150,135		
0.03	4.30	150,135	3.17	322,290sh
	13.12	206,177(100)	16.03	322
0.12	9.98	190,161(100)	21.07	330,262
	9.44	204,175(100)		
0.17	9.89	192,107(100)	16.99	282
	10.02	190,161(100)	21.07	330,262
0.28	9.78	192,107(100)	16.99	280
0.37	8.66	190,133(100)	21.07	330,262
	10.05	190,161(100)		
0.58	10.06	190,161(100)	21.07	330,262
	9.07	188,159(100)	21.57	320
0.63	9.07	188,159(100)	21.57	320
0.74	18.20	308,263	no peak	

Senkyunolide F

The mass spectrum of peak 11 showed fragments at *m/z* 206 (M^+, rel. int. 30), 188 (rel. int. 3), 177 (rel. int. 100), 150 (rel. int. 57), and 135 (rel. int. 35), which was similar to the mass pattern of senkyunolide F (4) (reported as *m/z* 206 (M^+, rel. int. 18), 188 (rel. int. 9.6), 177 (rel. int. 100), and 150 (rel. int. 62)). According to the mass spectral data, peak 11 was identified as senkyunolide F.

Senkyunolide-H and senkyunolide-I

The mass spectrum of peak 13 showed fragment peaks at *m/z* 224 (M^+, rel. int. 36), 206 (rel. int. 3), 180 (rel. int. 100), 151 (rel. int. 50), 123 (rel. int. 16), 95 (rel. int. 16), 77 (rel. int. 16), and 55 (rel. int. 26), and peak 15 showed fragment peaks at *m/z* 224 (M^+, rel. int. 32), 206 (rel. int. 3), 180 (rel. int. 100), 151 (rel. int. 44), 123 (rel. int. 12), 95 (rel. int. 15), 77 (rel. int. 10), and 55 (rel. int. 25). By comparing with literature data (3,5), it can be observed that the mass spectra of peaks 13 and 15 were similar to the mass spectrum of 6,7-dihydroxyligustilide. Senkyunolide-H and senkyunolide-I are an isomeric pair of dihydroxyphthalides, and senkyunolide-I is the major isomer. It has been reported (5) that the ratio of senkyunolide-I and senkyunolide-H is approximately 5:1 in *Chuanxiong*, which is consistent with the results in this study (the peak area of peak 15 was approximately four times larger than that of peak 13). Based on these facts, peaks 13 and 15 were identified as senkyunolide-H and senkyunolide-I, respectively. It was clear that $[M]^+$ at *m/z* 224 was formed and extensive water loss (formation of the ion at *m/z* 206) took place. The base peak at *m/z* 180 was derived from a Retro-Diels-Alder cleavage of the $[M–H_2O]^+$ ion at *m/z* 206. The main fragment ions were formed as *m/z* 224 (M^+), 206 ($M^+–H_2O$), 180 (RDA rearrangement), and 151 (RDA fragment ion–C_2H_5). The mass splitting path can be explained by the diagram in Figure 4.

Comparing the mass spectra of each peak with literature data along with the elucidation of the mass splitting path enabled ten PADs to be identified in *Chuanxiong* by GC–MS. This demonstrates a powerful tool for the online identification of the PADs directly within the crude extracts.

Separation and identification of the PADs in *Chuanxiong* by TLC, HPLC, and HPLC–MS

The ethyl ether extract of *Chuanxiong* prepared as described in the "Preparation of the compounds standards by TLC" section was repeatedly separated by TLC. Nine zones (including the original zone, $R_F = 0$) were found on the TLC plate. Each zone was scraped from the plate and extracted with methanol. The methanol extract of each fraction was analyzed by GC–MS and HPLC. The analytical results are shown in Table II. Fractions with an R_F of 0.28, 0.63, and 0.74 displayed only one peak in the GC–MS and HPLC chromatograms. Other fractions were mixtures of the compounds with retention times at 3.17, 7.04, 16.03, 16.99, 21.07, and 21.57 min, respectively. In order to further identify these peaks, HPLC–MS was used to determine the molecular weight of each peak. The ethyl ether extract of *Chuanxiong* was analyzed by HPLC–MS. The chromatogram is shown in Figure 5. The retention time and $[M+H]^+$ data are listed in Table III.

According to the retention time and mass spectral data, $[M+H]^+$, and the UV λ_{max} values (Table III), peaks (in the GC–MS chromatogram) with retention times at 4.22, 8.73, 9.62, 10.05, 13.06, and 15.19 min were further identified as ferulic acid, 3-butylidene-phthalide, senkyunolide A, Z-Ligustilide, senkyunolide F, and senkyunolide I.

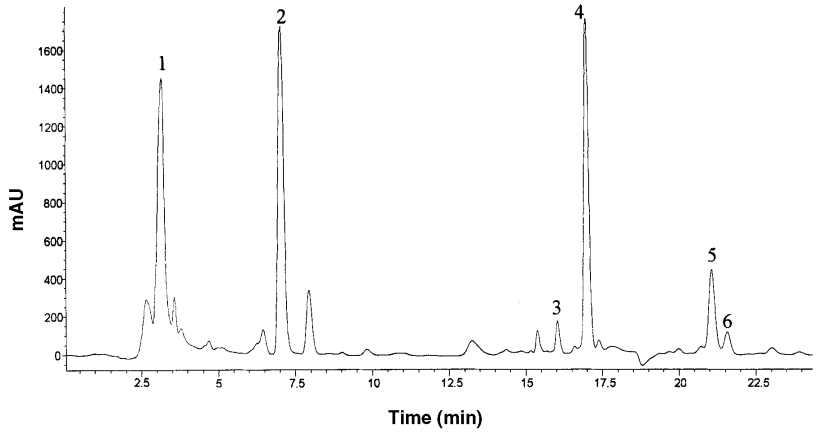

Figure 5. HPLC chromatogram of the ethyl ether extract of *Chuanxiong*: ferulic acid, 1; senkyunolide I, 2; senkyunolide F, 3; senkyunolide A, 4; Z-ligustilide, 5; and butylidene–phthalide, 6.

Table III. Retention Time in the GC Chromatogram, Mass Data, $[M+H]^+$, and UV λ_{max} of the Compounds in *Chuanxiong*

t_R (min) (GC–MS)	MS data (GC–MS)	t_R (min) (LC–MS)	$[M+H]^+$ (LC–MS)	UV λ_{max} (nm) (HPLC–DAD)	Compound
4.22	150,135,107	3.17	195	295sh,322	ferulic acid
8.73	188,159,146	21.57	189	270sh,315	butylidene–phthalide
9.62	192,107,77	16.99	193	277	senkyunolide A
10.10	190,161,148	21.07	191	270,322	ligustilide
13.06	206,177,150	16.03	207	270,296,323	senkyunolide F
15.19	224,180,165	7.04	225	275	senkyunolide H

Conclusion

Ten PADs and seven other compounds were simultaneously determined in *Chuanxiong* by GC–MS without any tedious pretreatment. The identification of some of these compounds was further confirmed by TLC, HPLC, and HPLC–MS. It is a simple, reliable, and quick method for the determination of the PADs in *Chuanxiong* by GC–MS.

Acknowledgments

This research was supported by the Fundamental Research Fund of Tsinghua University. The authors also wish to thank Professor De-lin Liu (China Academy of Traditional Medicine, Beijing, China) for the identification of plant crude material.

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Manuscript accepted December 31, 2001.