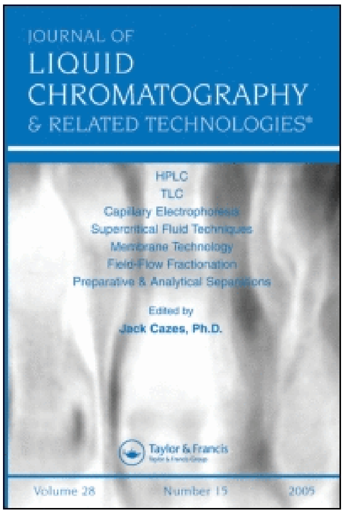

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DETERMINATION OF THE ACTIVE INGREDIENTS IN *CHUANXIONG* BY HPLC, HPLC-MS, AND EI-MS

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ABSTRACT

The main active ingredients, ferulic acid, vanillin, and three senkyunolides in Chinese traditional herb medicine, *Chuanxiong,* were separated and identified by HPLC, HPLC-MS, and EI-MS. Tetramethylpyrazine (TMP), which was reported as the major active ingredient in *Chuanxiong*, could not be detected by HPLC in the present paper.

Although TMP is the major active ingredient of *Chuanxiong*, its content was below 1.2×10^{-7} g/g, which is less than the lower detection limit by HPLC.

INTRODUCTION

Chuanxiong (Japanese name ‘Senkyu’) is the dried rhizome of *Ligusticum wallichii Franch*, and is one of the most important crude drugs among traditional

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Chinese medicines, and has been used to treat headaches, feeling of coldness, and menstrual disorders.[1,2] Tetramethylpyrazine (TMP) has already been identified as one of the active principles of *Chuanxiong* and has been in clinical use for ischemic cerebrovascular disease in China.

Because TMP had the same pharmacological roles as *Chuanxiong* and was first isolated from *Chuanxiong* herb, it was named as *Chuanxiongzine* by researchers.[3,4] Several methods have been reported for the determination of TMP, i.e., high-performance liquid chromatography (HPLC)[5-7] and gas chromatography (GC).[8]

In the present paper, TMP could not be detected by HPLC because its content was found to be below 1.2×10^{-7} g/g, although TMP appears to be the major active ingredients of *Chuanxiong*. Phenolic compounds, ferulic acid and vanillin, are also two active ingredients of *Chuanxiong*. There are reports about the determination of ferulic acid and vanillin by capillary electrophophoresis[9] and HPLC.[5,10] Senkyunolides are a kind of phthalides which are also important active ingredients of *Chuanxiong*. Several phthalides have been isolated and identified by column chromatography[11-13] and several volatile phthalides have also been analyzed by GC-MS.[14]

In the present paper, ferulic acid, vanillin, and three senkyunolides were first simultaneously separated and identified by HPLC. Ferulic acid and vanillin were identified by comparing the retention time and UV spectrum with that of the standard preparations. Senkyunolides were isolated and identified by HPLC-UV, HPLC-MS, and EI-MS.

EXPERIMENTAL

Chemicals and Reagents

Ferulic acid, vanillin, and TMP were of analytical grade and were purchased from the Corp. of Chinese Biological Institute. *Ligusticum wallichii* (crude medicinal material) was obtained from Chengdu, Si Chuan Province. Methanol was of HPLC grade. Other chemicals were of analytical grade.

Apparatus and Conditions

HPLC

The chromatographic system (HP series 1100) consisted of a quaternary pump, a degasser, a diode array detector (DAD), and a HP ChemStation Data

Analysis. Separation was achieved on a Zorbax SB-C18 (250×4.5 mm, I.D., 5 μm). The mobile phase consisted of methanol-water-acetic acid (45:55:0.5, v/v/v) and the flow rate was 0.6 mL/min. The mobile phase was filtered before use by a Millipore vacuum filter system equipped with a 0.45 μm filter.

Because the maximal absorption of TMP was 290nm, the detector was set at 290 nm. The column temperature was 30°C. The injected volume of samples was 20 μL.

HPLC-MS

HPLC-MS system was HP series 1050 and HPLC conditions were the same as described above. MS conditions: Tuning Parameters, APCI; Corona, 3.36 kV; HV Lens, 0.34 kV; Cone, 14 V; Source temp, 150°C; APCI probe temp, 550°C; ion energy, 2.0 V; Scan duration, 1.00 s; slonization mode, AP^+.

EI-MS

England VG20-253. MS source temperature, 230°C; injector temperature, 250°C; electron impact ionization energy, 70 eV.

Sample Preparation

200.0 g crude medicinal material of *Chuanxiong* was crushed into small pieces and immersed in water for one night and was extracted with 250 mL water by refluxing on a water bath at 80°C for 1.5 hr. Extraction was repeated three times. The extract was concentrated to about 200 mL with a rotary evaporator at 40°C under vacuum.

Preparation of the Benzene Extract, Ethyl Ether Extract, and Ethyl Acetate Extract

100 mL water extract obtained above, was successively extracted with benzene, ethyl ether, and ethyl acetate four times each, respectively. Solvents from each fraction were removed with a rotary evaporator at 30°C under vacuum, to obtain benzene, ethyl ether, and ethyl acetate, and the extracted solids were dissolved in methanol, diluted to appropriate concentration, and were filtered through a 0.45 μm filter membrane for HPLC analysis.

Preparation of Total Alkaloids

100 mL concentrated water extract was further extracted with ethyl ether three times and the pooled ethyl ether extract was concentrated to 60 mL in a 40°C water bath; then the concentrated ethyl ether extract was further extracted three times with 1.0 mol/L sulphuric acid. The acid-water extract was adjusted to pH 10 with saturated Na_2CO_3 and extracted three times with chloroform. The organic solvent was removed and the residue was dissolved in 10mL methanol. The methanol solution was filtered through a 0.45 μm filter membrane before HPLC and LC-MS analysis.

RESULTS AND DISCUSSIONS

Determination of TMP

Analysis of Benzene Extract, Ethyl Ether Extract, and Ethyl Acetate Extract

The extracts of phenyl-ethyl ether, ethyl acetate, and water residues obtained as described above from 100g crude *Chuanxiong* material, were 0.099, 0.31, 0.114, and 14.693g, with the extraction rates of 0.10%, 0.31%, 0.11%, and 14.69%, respectively. From HPLC chromatograms of benzene extract, ethyl ether extract, and ethyl acetate extract (Fig.1), we can see that there were three similar peaks with retention time at 14.5, 16.9, 19.9 min in benzene extract and ethyl ether extract. There were no interested peaks except for the polar impurities in water residue (figure omitted).

After successive extractions with phenyl ethyl ether and ethyl acetate, the main ingredients in *Chuanxiong* were completely extracted. In each extract, there was no TMP detected which has the retention time at 13.2 min. The chromatogram of water extract (Fig.2) from *Chuanxiong* without treatment with solvents, has a similar HPLC profile as in Fig.1a, which suggested that peaks 3,4,5 had significant amounts in *Chuanxiong*.

Analysis of Total Alkaloids

In order to detect TMP, we prepared total alkaloids to enhance the concentration of TMP. The extraction rate of alkaloids was 0.43%. From the HPLC chromatograms of alkaloids (Fig.3), we can see that there were the same three main peaks as Fig.1 and TMP was not detected. Adding some amounts of standard TMP into the alkaloids extract (Fig.3 upper window), the TMP peak was identified before peak 3 with the present HPLC conditions. The detection limit of TMP was 0.03 g/mL (signal-to-noise ratio as 3, S/N=3). The sample concentration was 250 mg dry material/mL, and the content of TMP in dry *Chuanxiong* was lower than 1.2×10^{-7} g/g. Although TMP was found to be the main active ingredient in *Chuanxiong*, its content was too low for detection by HPLC.

While, in all the extracts of benzene, ethyl ether, and ethyl acetate, five peaks were obtained with retention times of 8.7, 9.3 14.5, 16.9, 19.9 min, the peak size showed a significant concentration, which suggested that these five peaks may be the main ingredients in *Chuanxiong*. We identified these compounds using HPLC, LC-MS, and EI-MS.

Identification of Vanillin and Ferulic Acid (FA)

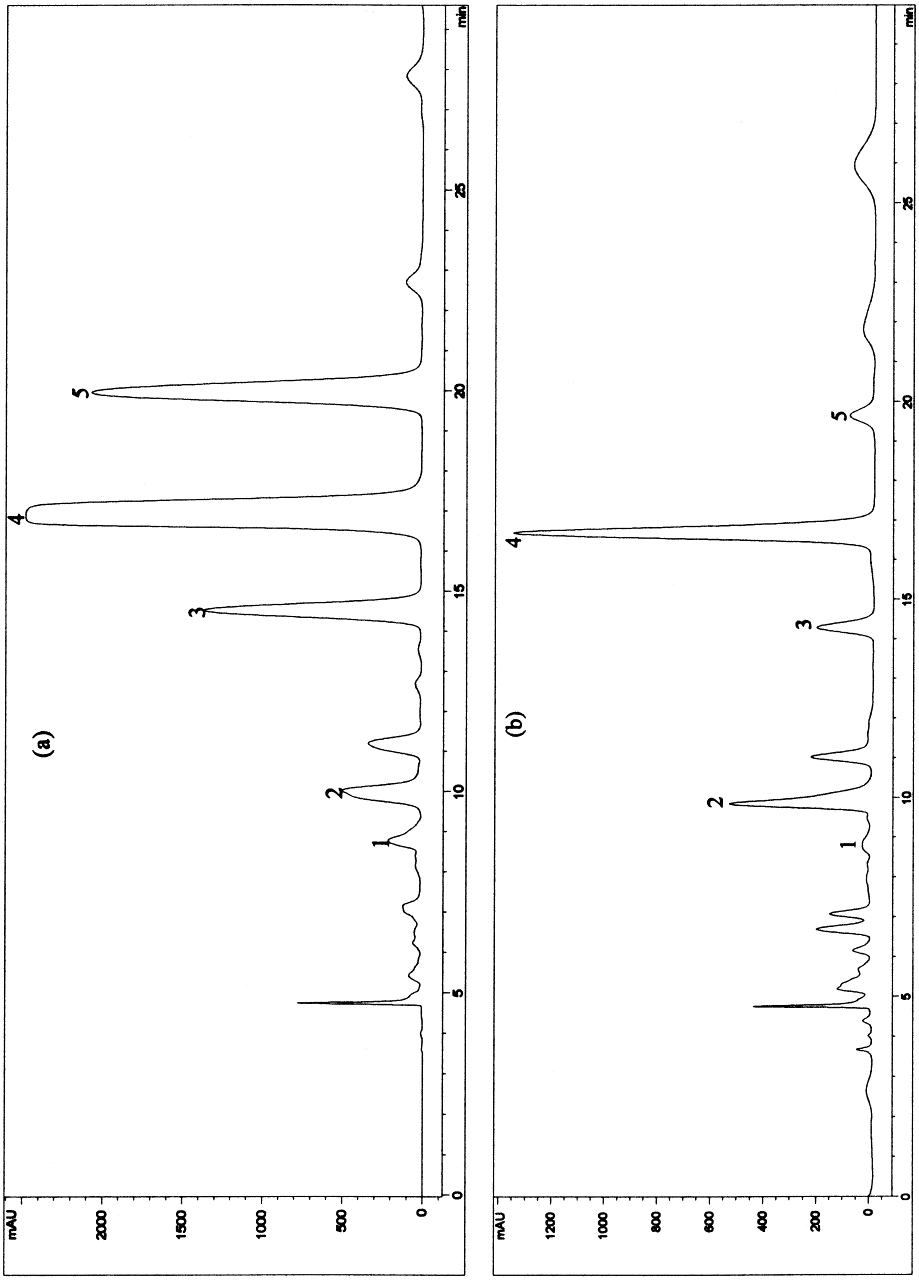
In the chromatogram of benzene extract of *Chuanxiong* (Fig.1a), there were peaks 1, 2, 3, 4, and 5 with high amounts. By comparing with the retention time and the UV spectra of the standards of FA and vanillin, the peaks with retention time at 8.7 min and 9.3 min were identified as vanillin and FA, respectively. The UV spectra of peaks 1 and 2 were very similar to that of vanillin and FA (Fig.4~ Fig.5).

Identification of Senkyunolides

From the UV spectra (Fig.6) of peaks 3, 4, and 5 collected at the apex of each chromatographic peak by DAD detector in Fig.1, we found that their UV spectra were quite similar with the maximal absorption at about 275 nm. This demonstrated that maybe they had very similar molecular structures. It is reported, that the Senkyunolide-H (cis-6,7-Dihydroxyligustilide) and Senkyunolide-I (trans-6,7-Dihydroxyligustilide) with a significant amount in *Chuanxiong* have the same UV spectra with maximal absorption at 274 nm.[11] We surmised that peaks 3, 4, and 5 were separately corresponding to Senkyunolide-I, Senkyunolide-H, and isomer of Senkyunolide-I. Further identification was made by LC-MS and EI-MS.

LC-MS Analysis

The total ion chromatogram of benzene extract of *Chuanxiong* was the same as Fig.1a (Figure omitted). The MS chromatograms of peaks 3, 4, and 5 are shown in Fig.7. It is clear that $[M+H]^+$ at m/z 225 is formed and extensive water


(a)
mAU
2000
1500
1000
500
0
0
5
10
15
20
25
min
1
2
3
4
5
(b)
mAU
1200
1000
800
600
400
200
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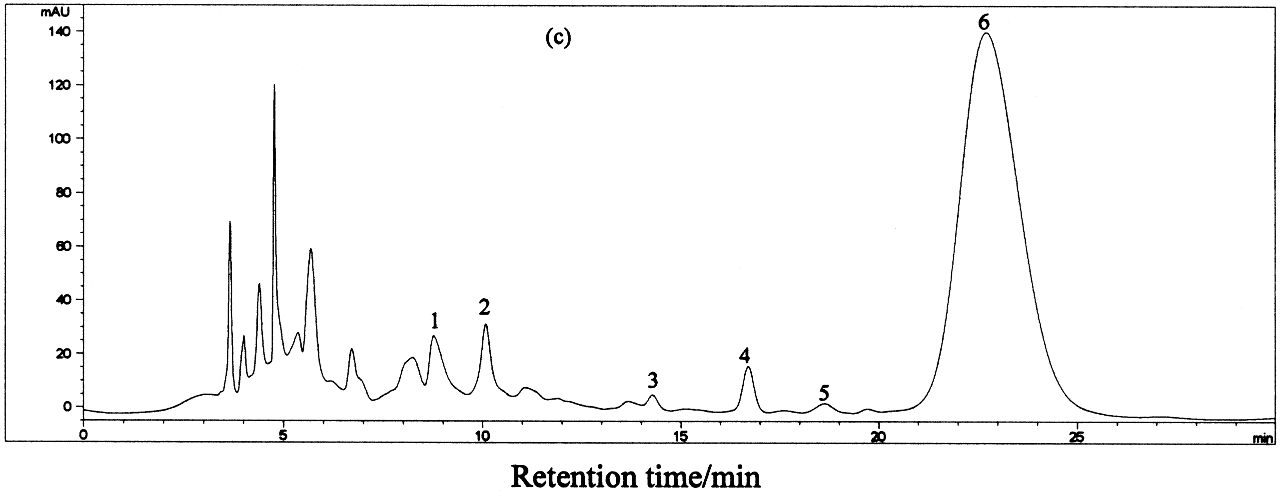


Figure 1. Chromatograms of benzene extract, ethyl ether extract, and ethyl acetate extract from *Chuanxiong*. (a) benzene extract, (b) ethyl ether extract, (c) ethyl acetate extract.

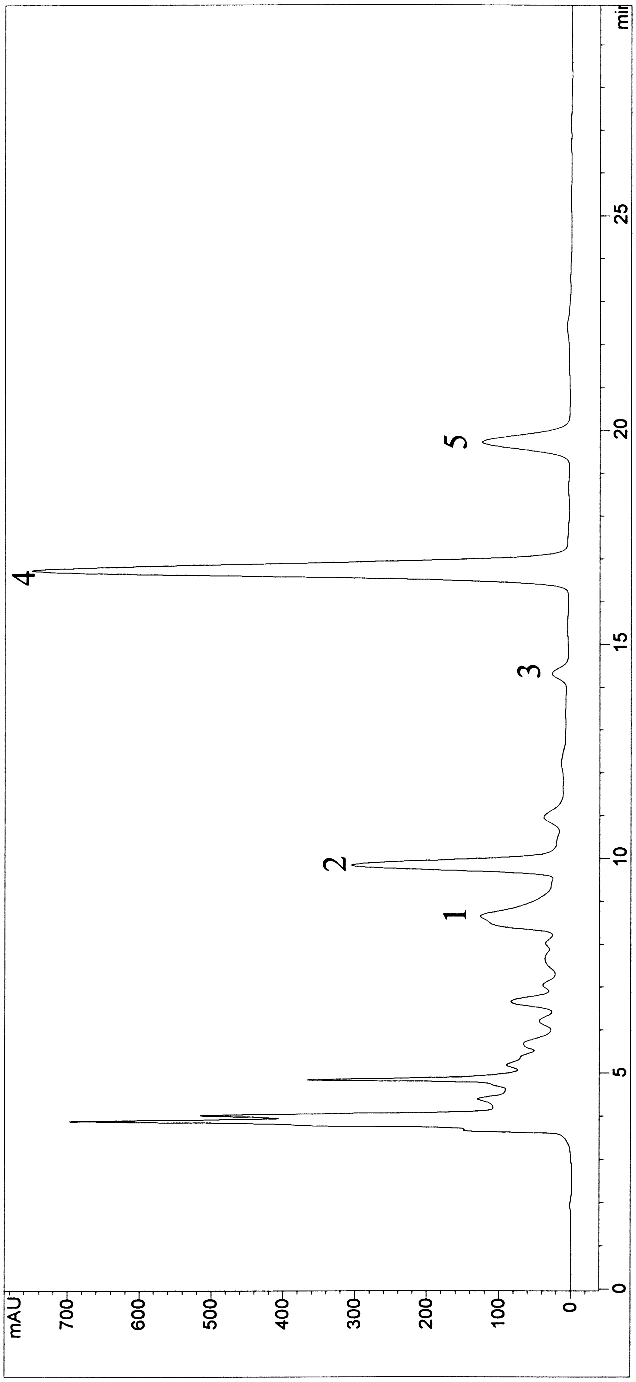


Figure 2. Chromatograms of water extract from *Chuanxiong*.

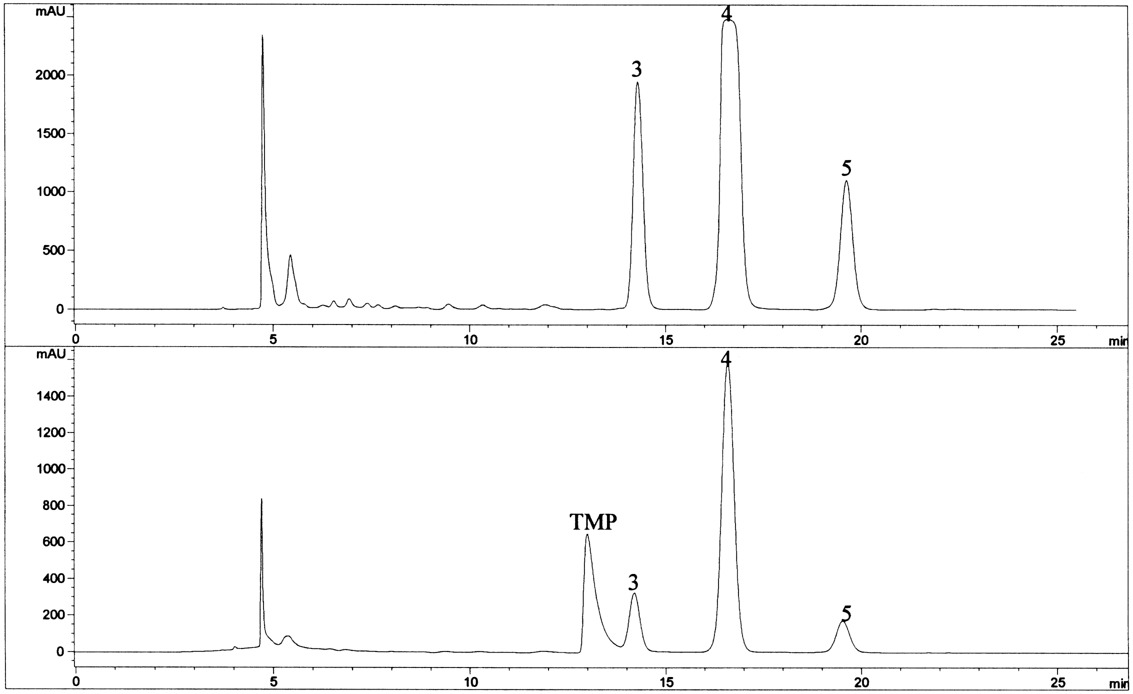


Figure 3. The chromatograms of alkaloids and alkaloids added TMP. Upper window: total alkaloids, Lower window: alkaloids added TMP

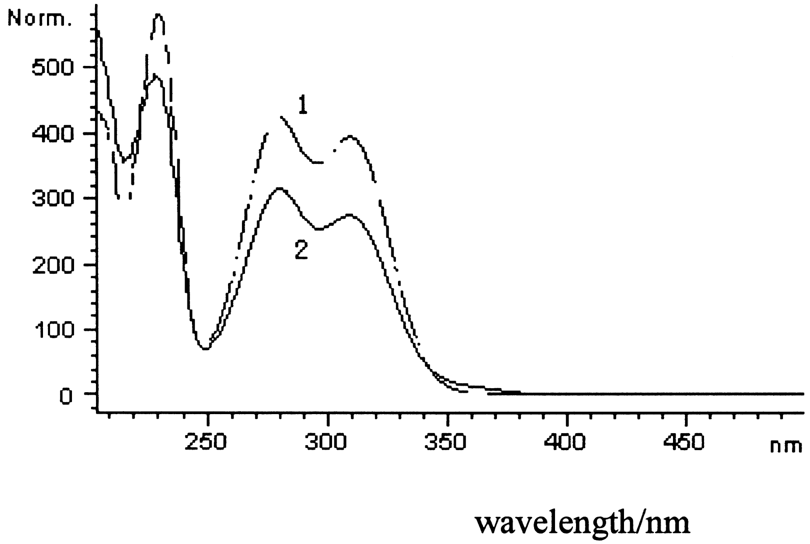


Figure 4. UV spectra of standard vanillin and peak 1. 1- vanillin, 2- peak1

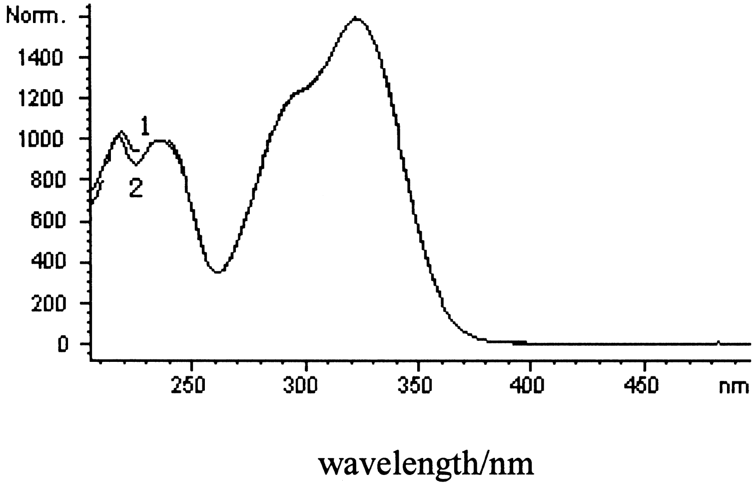


Figure 5. UV spectra of standard FA and peak 2. 1- FA, 2- peak 2.

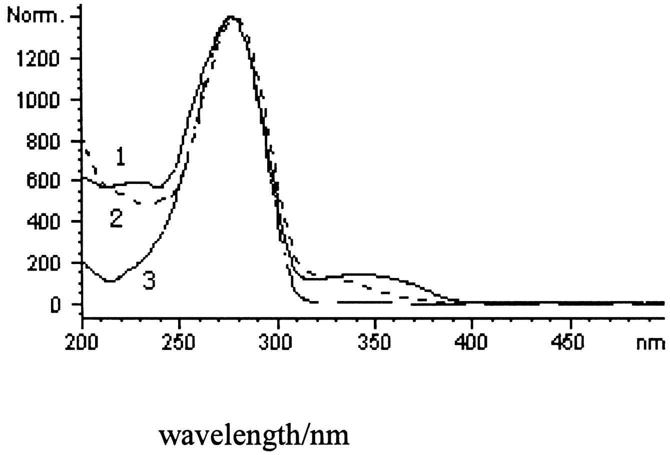


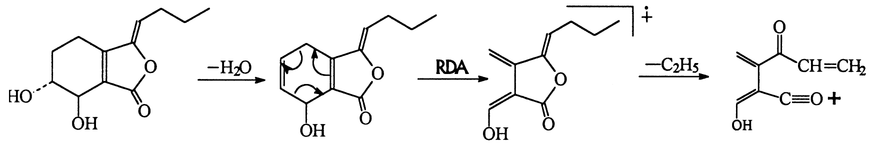
Figure 6. UV spectra of peak 3, 4, and 5. 1- peak 3, 2- peak 4, 3- peak 5.

loss (formation of the ion at m/z 207) takes place. The m/z 207 is characterized as $[M+H-H_2O]^+$.

The molecular mass of Senkyunolide-H, Senkyunolide-I, and isomer of Senkyunolide-I was all 224. They were prone to take off one H_2O molecule to form the $[M+H-H_2O]^+$ ion exactly at m/z 207. These facts were identical with the LC-MS results.

EI-MS Analysis

We manually prepared the compounds of peak 4 in Fig.1 using semi-preparation HPLC column and analyzed the compound by EI-MS. The MS spectrum (Fig.8) showed a base peak at m/z 180, which was derived from a Retro-Diels-Alder cleavage of the $[M-H_2O]^+$ ion at m/z 206. The main fragment ions in Fig.8 were as follows: m/z 224(M^+, 33), 206(M^+- H_2O, 2), 180(RDA rearrangement, 100), 151(RDA fragment ion-C_2H_5, 54), 138(RDA fragment ion-C_3H_6, 18). The mass splitting path was explained as following:



m/z 224 206 180°C base peak 151

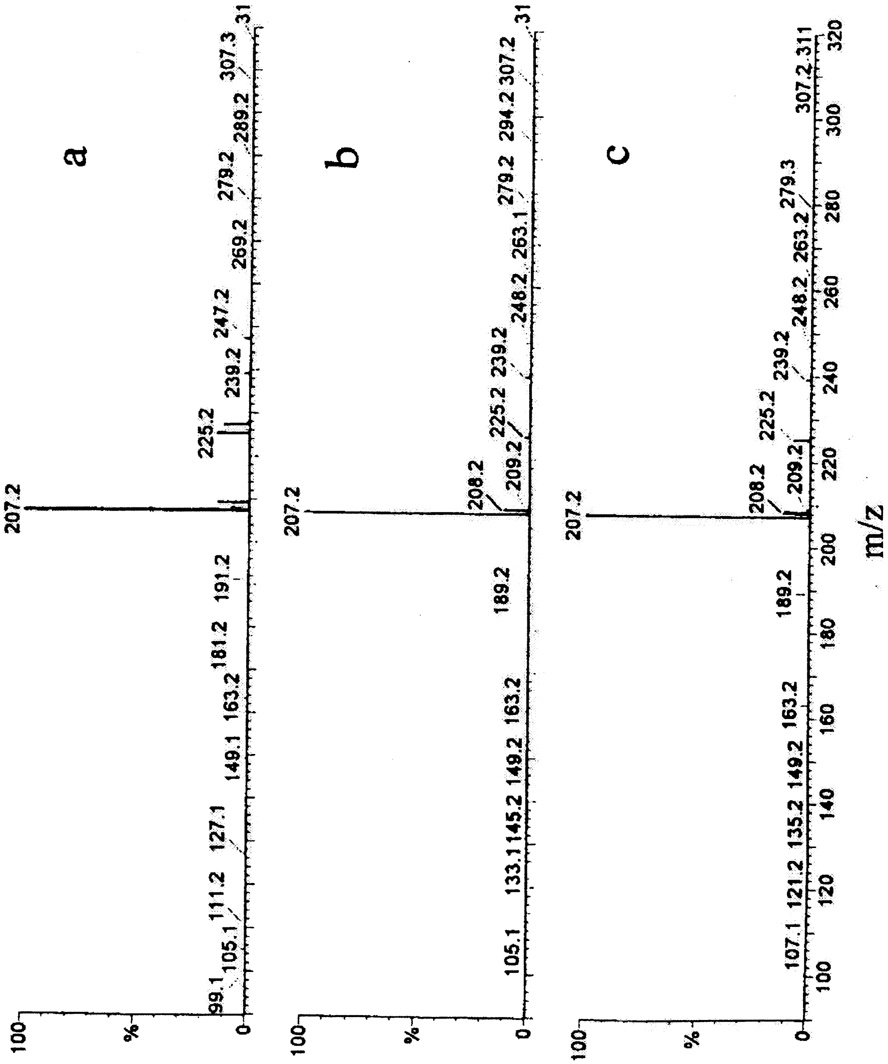


Figure 7. MS spectra of peaks 3, 4, and 5 in benzene extracts chromatogram. a: peak 3, b: peak 4, c: peak 5.

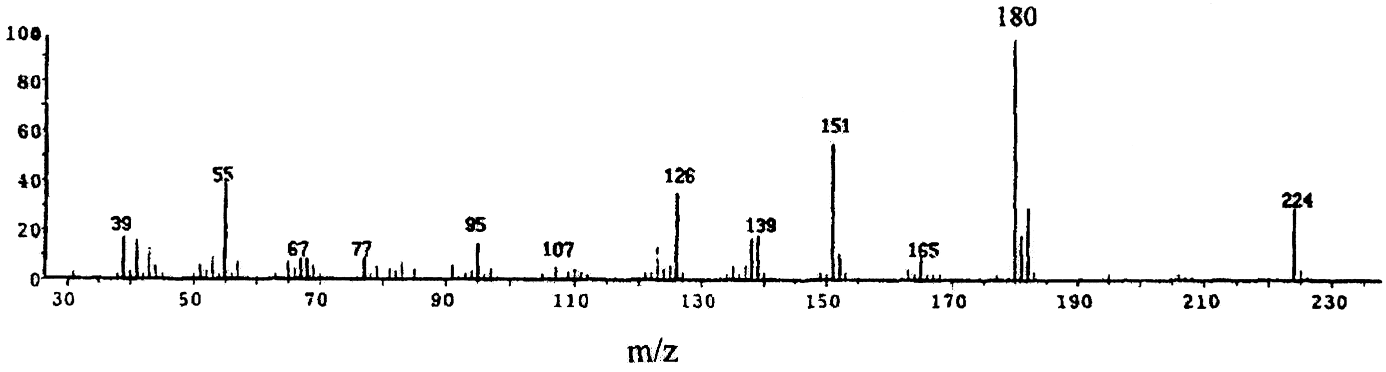


Figure 8. Mass spectrum of compound collected at peak 4.

The EI-MS pattern of Senkyunolide-H separated from *Chuanxiong* was reported as 224 (M^+, 26), 206 (3), 180 (100), 165 (17), 151 (41), 138 (15), 123 (12), 95 (20), 55 (38).[12] It was very similar to our results, not only in fragment ions but also in relative intensity.

From UV, HPLC-MS, and EI-MS, peaks 4 can be identified as Senkyunolide-H and peak 3 and 5 were identified as Senkyunolide-I and isomer of Senkyunolide-I, respectively. Further identification of senkyunolides will be performed after the pure senkyunolides are isolated and purified from *Chuanxiong*.

Quantification of FA and Vanillin in *Chuanxiong*

The contents of FA and vanillin in *Chuanxiong* were quantified from the corresponding peak area, using the external standard method to calculate. The water extract of *Chuanxiong*, diluted with methanol, was filtered through a 0.45 μm filter membrane before being injected into the HPLC. The chromatogram of the extract was shown in Fig.2. The contents of FA and vanillin in *Chuanxiong* material were calculated to be 0.05% and 0.047%, respectively. Chromatographic precision, expressed as relative standard deviation (RSD), was calculated by injecting five replicates of the extract. RSD was varied between 0.1-0.3%. The detection limits (S/N=3) for FA and vanillin were 1.5 and 5.0 g/dm^3, respectively.

Because no standard senkyunolides were available, the quantitative analysis of the senkyunolides was not performed.

CONCLUSIONS

FA and vanillin in *Chuanxiong* were identified and quantified by HPLC. Senkyunolide-H, Senkyunolide-I, and isomer of Senkyunolide-I, presented in *Chuanxiong*, were identified by HPLC, HPLC-MS, and EI-MS.

TMP reported as the major active ingredient in *Chuanxiong* could not be detected by HPLC in the present paper.

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