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Liquid chromatographic–electrospray mass spectrometric study of the phthalides of *Angelica sinensis* and chemical changes of *Z*-ligustilide

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Abstract

High-performance liquid chromatography–electrospray ionization–mass spectrometry has been applied to analyze the chemical constituents of Danggui (the rhizome of *Angelica sinensis*) and to study chemical changes of *Z*-ligustilide. Twelve phthalides were unambiguously identified as senkyunolide I (**3**), senkyunolide H (**4**), sedanenolide (**8**), butylphthalide (**9**), *E*-ligustilide (**13**), *Z*-ligustilide (**14**), *Z*-butylidenephthalide (**15**), *Z,Z′*-6.8′,7.3′-diligustilide (**16**), angelicide (**17**), levistolide A (**18**), *Z*-ligustilide dimer E-232 (**19**) and *Z,Z′*-3.3′,8.8′-diligustilide (**20**) in Danggui extract. The existence of 12 other phthalides (**2**, **5**–**7**, **11**, **12**, **22**–**27**), ferulic acid (**1**) and coniferyl ferulate (**10**) in Danggui extract has also been demonstrated. Phthalides **3**, **4**, **16**–**18** and **20** were determined to be the products from chemical change of *Z*-ligustilide. This is the first report of the existence of 16 compounds (**2**–**8**, **10**–**12**, **20**, **22**–**25** and **27)** in Danggui extract.

Keywords: Angelica sinenesis; Danggui; Ligustilide; Phthalides; Coniferyl ferulate

1. Introduction

The rhizome of *Angelica sinensis* (Oliv.) Diels (Umbelliferae), known as Danggui in Chinese, is one of the most important traditional Chinese medicines, used for tonifying the blood and treating female irregular menstruation and amenorrhoea. It is also used for treatment of anemia, hypertension, chronic bronchitis, asthma, rheumatism and cardiovascular diseases [1–5]. Over 70 compounds, such as, phthalides, terpenes, aromatic compounds, etc., have been isolated and identified from Danggui [3–10]. Its main essential component, *Z*-ligustilide (**14**), other phthalides and ferulic acid (**1**) are thought to be the biologically active components of Danggui [3,5,6]. For this study high-performance liquid chromatography–electrospray ionization–mass spectrometry (HPLC–ESP-MS) demonstrated the existence of more than 30 phthalides in Danggui. Eighteen phthalides, along with coniferyl ferulate (**10**), were isolated during this study. Their structures are listed in Fig. 1a,b.

Z-Ligustilide (**14**) is a volatile and unstable liquid compound, which can be changed to other phthalides through oxidation, isomerization, dimerization, etc., [11–17]. *Z*-Ligustilide (**14**) has been also found to exist together with its derivatives in a number of

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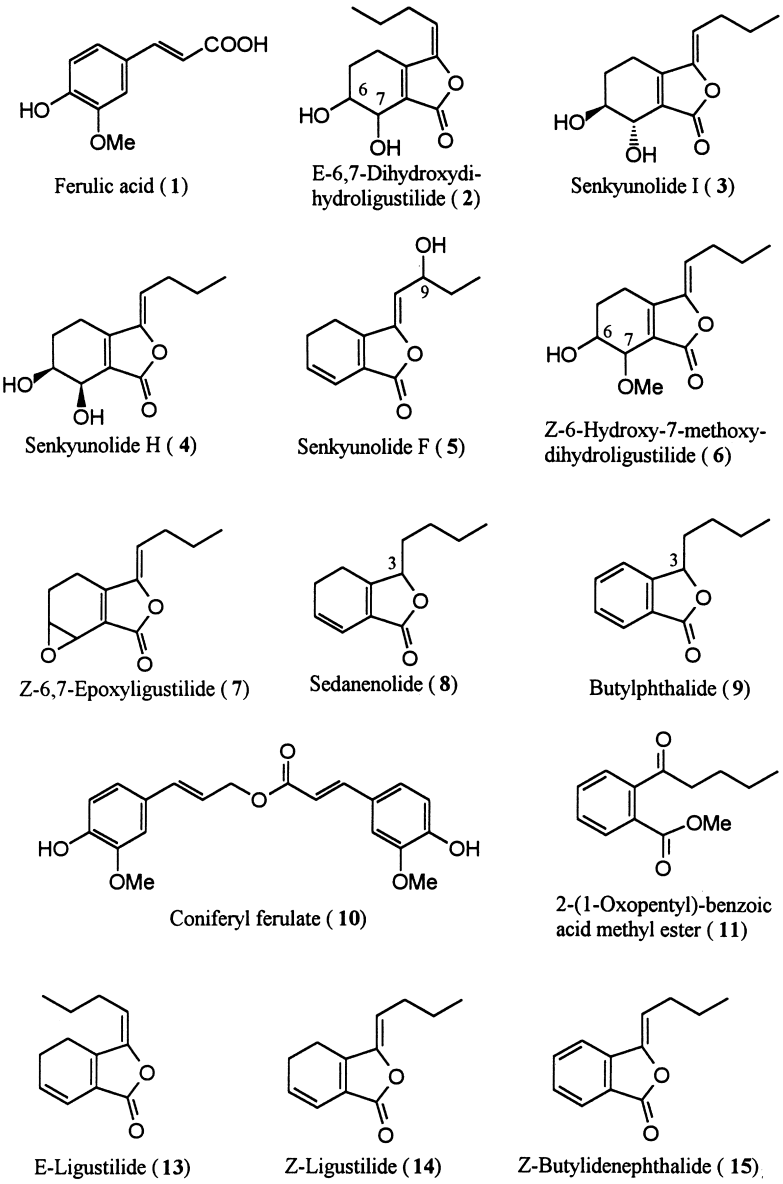

Fig. 1. (a,b) The phthalides and related compounds in Danggui extract.

plants of Unbelliferae [12–25]. These phthalides are also considered to be the biologically active components which are present in several important medicinal herbs, such as the roots of *Ligusticum wallichii* [12], *L. chuangxiong* [18], *L. portere* [21], *Levisticum officinale* (lovage) [23] and *Cnidium officinale* [15,24]. In order to monitor 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 phthalides have been analyzed by gas chromatography (GC)–MS [8–10,19,25] and LC–UV [11,26–

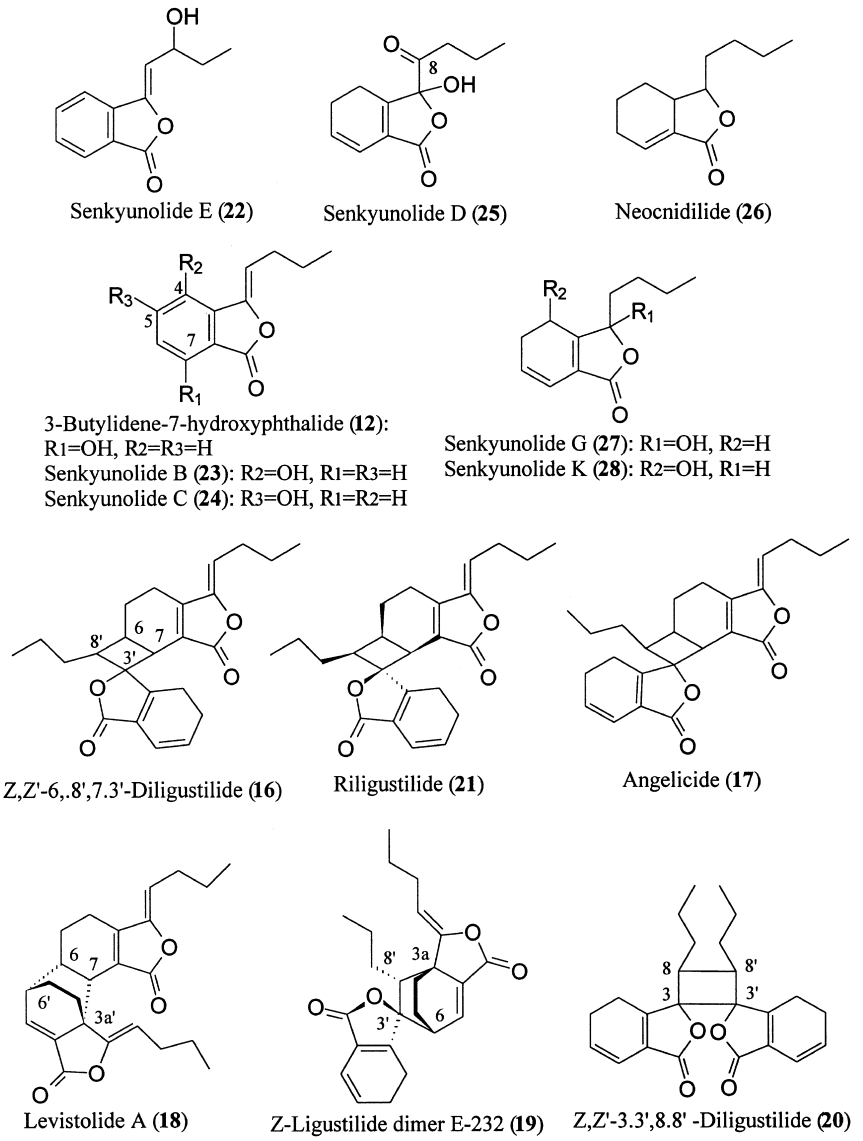

Fig. 1. (*continued*)

31]. Since some phthalides, for example *Z*-ligustilide, decompose rapidly at high temperature, GC is not capable of detecting such unstable compounds [11]. In addition to this, LC–UV could not identify most peaks in Danggui extract. This is the first report to use LC–ESP-MS for identification of the phthalides in Danggui and the main products from chemical changes of **14**.

2. Experimental

2.1. Instrumentation

An HP 1090 Series II LC system (Hewlett-Packard, Palo Alto, CA, USA) with photodiode array detection set at 270 nm was coupled to an HP 5989 B quadrupole mass spectrometer. UV spectra were

obtained by scanning from 200 nm to 500 nm. For chromatography a Waters Symmetry C_{18} column (5 μm, 150×2.1 mm) with a sentry guard column (Symmetry C_{18}, 5 μm, 20×3.9 mm) was used. The mobile phase consisted of (A) water (0.25% HAc) and (B) methanol using a linear gradient of 35–100% B in 40 min, and 100–35% B in 40–42 min. The flow-rate was set at 0.2 ml/min, and the oven temperature was set at 45°C. The ESP-MS spectra were acquired in the positive ion mode, using an electrospray interface Model HP 59987 A. The temperature of the drying gas (N_2) was 350°C, at a gas flow-rate of 40 ml/min, and a nebulizing pressure (N_2) of $5.5 \cdot 10^5$ Pa (80 p.s.i.). The LC system was directly connected to mass spectrometer without stream splitting.

2.2. Solvents and chemicals

HPLC grade water and methanol (VWR, Seattle, WA, USA) were used for HPLC analysis. Reagent grade chloroform, ethanol, hexane and acetone (VWR) were used for extraction and separation. The sorbent for column chromatography was silica gel 60 (>0.063 mm, E. Merck, Darmstadt, Germany). For preparative thin-layer chromatography (TLC) normal-phase TLC plates (silica gel 60 F_{254}, 0.5 mm thickness, 20×20 cm) and reversed-phase TLC plates (RP-18, F_{254}, 0.25 mm thickness, 20·20 cm) (E. Merck) were used.

2.3. Plant material and sample preparation

2.3.1. Extraction of ground Danggui

Danggui was purchased from Asia Natural Products (San Francisco, CA, USA). At room temperature, 0.2 g of ground Danggui was extracted with 10 ml of methanol using sonication for 10 min. The extract was filtered through a 0.45 μm nylon acrodisk 13 filter (Gelman, Ann Arbor, MI, USA). Additionally, a 10-μl volume of the extract was injected onto the analytical column for analysis.

2.3.2. Preparative TLC of Danggui extract

A ca. 500-μl volume of ethanol extract of Danggui (5 g) was separated by preparative TLC (one normal-phase plate, hexane–acetone, 7:3, v/v), and the eight zones founded on the TLC plate could be visualized under UV light 254 nm. Each zone was scraped from the plate, and extracted with methanol. *Z*-Ligustilide (**14**) and *Z*-butylidenephthalide (**15**) are mainly in the first zone (R_F=0.7). The so-called DQSP-B fraction consists of zones 2–8 in 20 ml of methanol, which contains only a small portion of **14**.

2.3.3. Light stressing of Z-ligustilide (*14*)

Oily **14** (10 mg, 95% purity, containing 5% of **15**) was put into a white glass vial, the vial was capped, and held at room temperature for three days with contact to some direct sunlight. In addition to this, 5 mg was dissolved in 5 ml of methanol (sample 1) for LC–ESP-MS analysis.

An additional amount of oily **14** (10 mg) was treated in the same way, but omitting any contact with direct sunlight (sample 2).

2.4. Isolation of standard compounds

Phthalide standards have been isolated from Danggui in the following way: powdered Danggui (3 kg) was extracted with chloroform (3×6 l) and the chloroform extract (120 g) was partitioned between hexane and methanol. The methanol part (50 g) was repeatedly subjected to silica gel column chromatography and preparative TLC to yield 18 phthalides. The solvents for column and normal-phase TLC were hexane–acetone (9:1 to 5:5, v/v, for column; 8:2, 7:3 or 6:4, v/v, for TLC); solvents for reversed-phase TLC were methanol–water (7:3 or 8:2, v/v). The purity of each isolate was checked by both HPLC and TLC, and the identity was confirmed on comparison of NMR data [proton and carbon NMR spectra, taken in $CDCl_3$ solution with Bruker AM-400 NMR instrument, using trimethylsilyl (TMS) as internal standard] with published data [3,5,6,13–18,22,24,32]. The detailed separation procedure will be reported in near future.

The isolated phthalides are **2**, **3**, **4**, **5**, **6**, **7**, **8**, **9**, **11**, **12**, **13**, **14**, **15**, **16**, **17**, **18**, **19**, and **20**.

Sedanenolide (**8**) may also represent its 3-*S* stereoisomer, senkyunolide, which has the same NMR data [24] as that of **8**. Butylidenephthalide (**9**) may also represent its 3-*S* stereoisomer. Z,Z′–6.8′,7.3′-Diligustilide (**16**) represents a mixture of the stereoisomers, and also includes riligustilide (**21**) [6,32]. *Z,Z′*-3.3′,8.8′-Diligustilide (**20**) seems to be a new

compound, a stereoisomer of angelicolide [16,17]. *Z*-6-Hydroxy-7-methoxydihydroligustilide (**6**) is also a new compound.

Ferulic acid (**1**) and coniferyl ferulate (**10**) [15] were also isolated from the extract, and identified in the same manner.

A reference methanol solution (10 ml) contained **1** (2 mg), **2** (1 mg), **3** (1 mg), **4** (1 mg), **5** (1 mg), **6** (1 mg), **7** (1 mg), **8** (0.2 mg), **9** (0.2 mg), **10** (0.4 mg), **11** (2 mg), **12** (2 mg), **13** (0.2 mg), **14** (1 mg), **15** (1.4 mg), **16** (0.5 mg), **17** (1.5 mg), **18** (1 mg), **19** (0.5 mg) and **20** (0.4 mg).

3. Results and discussion

3.1. LC-ESP-MS analysis of phthalide standards

Eighteen phthalides, i.e., **2**–**9**, **11**–**20**, as well as, **1** and **10** were chromatographed in order to determine their retention times (t_R), UV and MS data for comparison with the chromatograms of the plant extract and other samples. Their LC–UV and total ion chromatograms are shown in Fig. 2. Their t_R, $[M+H]^+$, $[M+Na]^+$ and UV λ_{max} values are shown in Table 1.

Table 1 shows that although the isomers of each group have the same mass and similar UV data, they showed differences in t_R. They basically can be distinguished by their different retention times. As a result, it appears possible to enable their identification in Danggui extract.

3.2. LC–ESP–MS of Danggui extract and DQSP-B fraction

The LC–UV and total ion chromatograms of the methanol extract of Danggui are shown in Fig. 3.

The retention time (t_R), $[M+H]^+$, $[M+Na]^+$, UV λ_{max} values and the identification for individual peaks are listed in Table 2. Based on these data, 14 peaks were identified as **1** (P1; P was used to

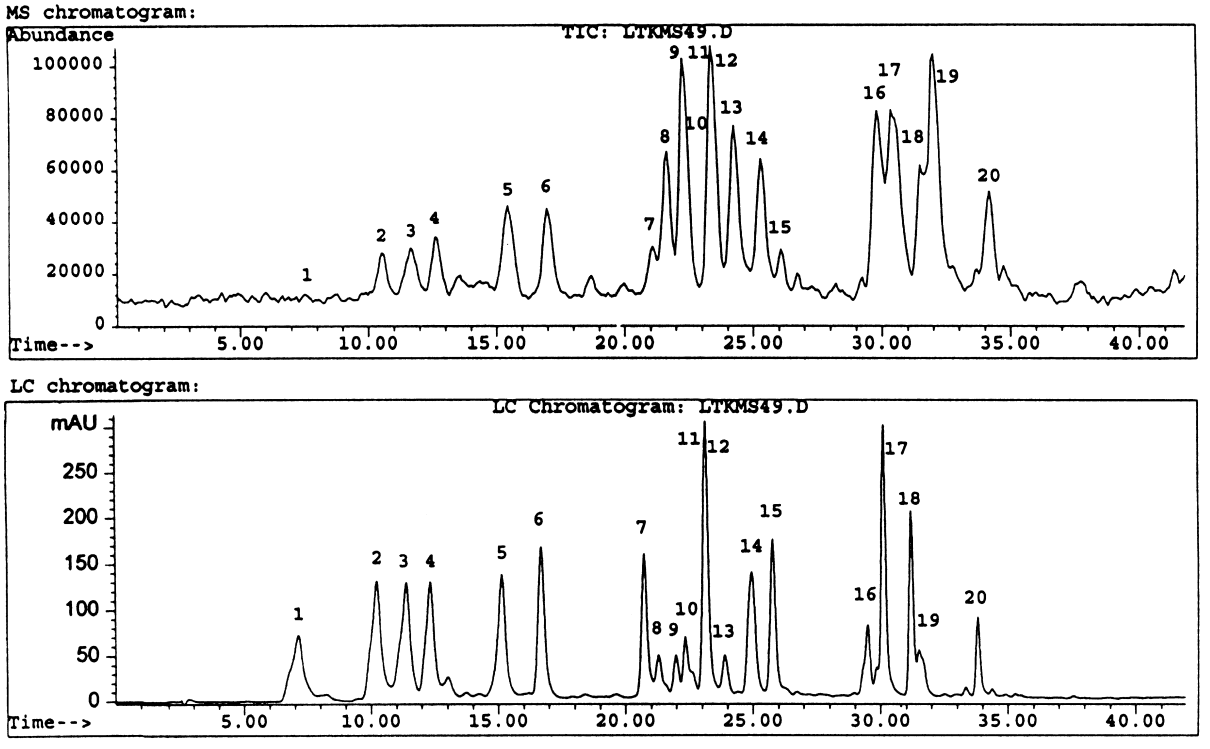

Fig. 2. Simultaneous LC–UV and LC–ESP-MS chromatograms of the Danggui standard compounds. Chromatographic conditions are described in Section 2. The t_R value, MS and UV λ_{max} of each compound are listed in Table 1.

Table 1
Retention time (t_R) value, $[M+H]^+$, $[M+Na]^+$, other MS ions, UV λ_{max} of the standard compounds

Peak No.	Compound	t_R (min)	$[M+H]^+$ (m/z)	$[M+Na]^+$ (m/z)	Other ions (m/z)	λ_{max} (nm)
1	Ferulic acid (**1**)	7.1	195[a]	–	–	295sh, 322
2	*E*-6,7-Dihydroxydihydroligustilide (**2**)	10.2	225	247	207	277
3	Senkyunolide I (**3**)	11.4	225	247	207	277
4	Senkyunolide H (**4**)	12.3	225	247	207	277
5	Senkyunolide F (**5**)	15.2	207	229	189	270, 296, 322
6	*Z*-6-Hydroxy-7-methoxydihydroligustilide (**6**)	16.7	239	261	207	277
7	*Z*-6,7-Epoxyligustilide (**7**)	20.8	207	229	–	277
8	Sedanenolide (**8**)	21.4	193	215	–	277
9	Butylphthalide (**9**)	22.0	191	213	–	270
10	Coniferyl ferulate (**10**)	22.4	–	379[a]	–	272, 298, 320
11	2-(1-Oxypentyl)-benzoic acid methyl ester (**11**)	23.2	221	243	189	230, 276
12	3-Butylidene-7-hydroxyphthalide (**12**)	23.2	205	227	–	285, 335
13	*E*-Ligustilide (**13**)	24.0	191	213	–	270, 322
14	*Z*-Ligustilide (**14**)	25.0	191	213	–	270, 322
15	*Z*-Butylidenephthalide (**15**)	25.9	189	211	–	270sh, 313
16	*Z,Z′*-6.8′,7.3′-Diligustilide (**16**)	29.6	381	403	191	283
17	Angelicide (**17**)	30.2	381	403	191	283
18	Levistolide A (**18**)	31.3	381	403	191	276
19	*Z*-Ligustilide dimer E-232 (**19**)	31.6	381	403	191	279
20	*Z,Z′*-3.3′,8.8′-Diligustilide (**20**)	33.9	381	403	191	296

[a] Ferulic acid and coniferyl ferulated showed only a poor response.

represent peak in the text), **3** (P2), **4** (P3), **8** (P4), **9** (P5), **10** (P6), **13** (P7), **14** (P8), **15** (P9), **16** (P10), **17** (P11), **18** (P-13), **19** (P14) and **20** (P15) based on their t_R, $[M+H]^+$, $[M+Na]^+$, other ions and UV λ_{max} values in the comparison with the data of our isolated standards. Only peak 12 was unidentified.

Because of the instability of *Z*-ligustilide (**14**) and some other phthalides, the sample preparation and HPLC analytical conditions have been strictly controlled to avoid any detectable degradation of the phthalides during the performance. Furthermore, we have analyzed fresh Danggui, which showed the same detectable phthalides. The only difference between the two Danggui samples is the relative ratios among these phthalides. This fact indicated that the phthalides in Table 2 should be the original compounds of Danggui, not artifacts.

The major component (**14**) comprised more than 60% of the total amount of the phthalides. Other phthalides appeared as very minor peaks. In order to detect these minute components, the DQSP-B fraction was subjected to LC–ESP-MS analysis and the t_R, $[M+H]^+$, $[M+Na]^+$, UV λ_{max} values and identification of each of the additional peaks were made. Five of them were identified as stereoisomer of **3** and **4**, **5**, **22**, **7** and **12**, respectively. Peaks 14–17 appear to be unidentified dimers of **14** (chromatograms not shown).

In the same way, analyses of some of other fractions from the column and TLC separation detected the existence of eight other phthalide monomers and at least five other dimers. Using the UV and $[M+H]^+$ or $[M+Na]^+$ data, five monomers could be tentatively identified as senkyunolide B (**23**), senkyunolide C (**24**), senkyunolide D (**25**), neocnidilide (**26**) (or its isomers), senkyunolide G (**27**) [or senkyunolide K (**28**)]. Another three monomers could be identified as **2**, **6** and **11**.

As shown in Tables 1 and 2, although ESP–MS is a rather soft ionization method, it usually showed protonated molecular ions as strong peaks. However, some phthalides still showed some fragments in higher intensity than these of their $[M+H]^+$ and $[M+Na]^+$. So, it is difficult to establish the molecular mass only based on these mass spectra. Therefore, it is better to identify them by direct comparison to the ESP–MS spectra from the standards.

Ferulic acid (**1**) and coniferyl ferulate (**10**) showed

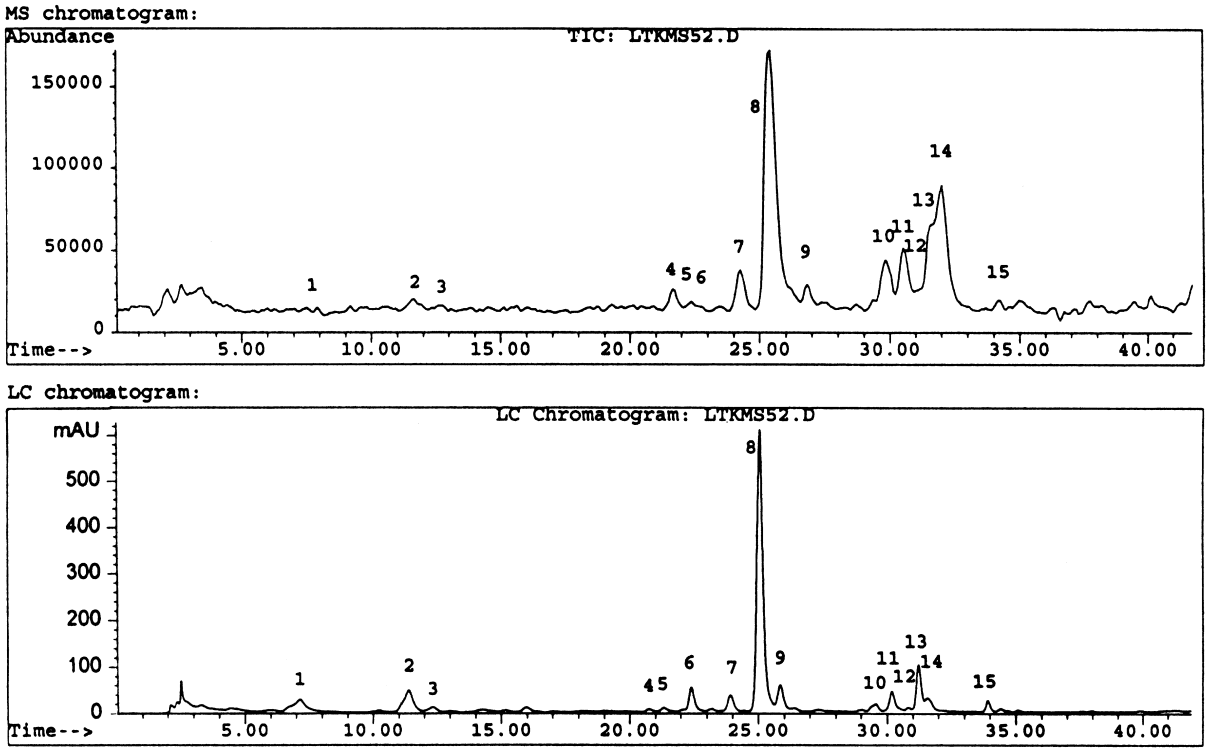

Fig. 3. Simultaneous LC–UV and LC–ESP-MS chromatograms of a Danggui extract. Chromatographic conditions are described in Section 2. Peak assignments are listed in Table 2.

Table 2
Peak assignment for the analysis of the methanol extract of Danggui

Peak No.	t_R (min)	$[M+H]^+$ (m/z)	$[M+Na]^+$ (m/z)	Other ions (m/z)	λ_{max} (nm)	Identification
1	7.1	195	–	–	295sh, 322	Ferulic acid (**1**)
2	11.4	225	247	207	277	Senkyunolide I (**3**)
3	12.3	225	247	207	277	Senkyunolide H (**4**)
4	21.4	193	215	–	277	Sedanenolide (**8**)
5	22.0	191	213	–	270	Butylphthalide (**9**)
6	22.4	–	379	–	272, 298, 320	Coniferyl ferulate (**10**)
7	24.0	191	213	–	270, 322	*E*-Ligustilide (**13**)
8	25.0	191	213	–	270, 322	*Z*-Ligustilide (**14**)
9	25.9	189	211	–	270sh, 313	*Z*-Butylidenephthalide (**15**)
10	29.6	381	403	191	283	*Z,Z′*-6.8′,7.3′-Diligustilide (**16**)
11	30.2	381	403	191	283	Angelicide (**17**)
12	31.1	381	403	191	283	Unknown
13	31.3	381	403	191	276	Levistolide A (**18**)
14	31.6	381	403	191	279	*Z*-Ligustilide dimer E-232 (**19**)
15	33.9	381	403	191	296	*Z,Z′*-3.3′,8.8′-Diligustilide (**20**)

a very low response under this LC–MS condition. It could be suggested that they have different physicochemical properties compared to the phthalides since they do not have phthalide (or partially hydrogenated phthalide) group. They could be identified only based on the retention time and UV data, in comparison with those of their standards.

This study was the one that enabled to determine the existence of compounds **2**–**8**, **10**–**12**, **20**, **22**, **23**, **24**, **25** and **27** (or **28)** in Danggui extract.

It was observed during the study that even when the sample vials were tightly closed to prevent evaporation losses, **14** was gradually lost during storage of Danggui and purified products under normal conditions. Some derivatives were formed. This fact led to the identification of the reaction products of **14**.

Four major produced phthalides of sample 1 were identified as the dimers **16**, **17**, **18**, and **20** based on the direct comparison of their t_R, $[M+H]^+$, $[M+Na]^+$ and UV λ_{max} values with these of the standards. Among the six unidentified peaks, one of them may be a *Z*-ligustilide oxidation compound, the others should be the isomers from *Z*-ligustilide dimer (chromatograms not shown).

In the same way, analysis of *Z*-ligustilide reaction sample 2 found that **3** and **4** were the main products.

The results show that *Z*-ligustilide (**14**) mainly produced its dimers with only a trace amount of its normal oxidation products, for example **3** and **4**, when it is exposed to direct sunlight. This fact suggests that the exposure of **14** (or Danggui, or its product) to direct sunlight seems to be one of the conditions for the dimerization of **14**. Some of the reaction mechanism may be similar to the postulated biogenetic method for the naturally occurring dimers **18** and **21** [13]. However, under normal, non-sun exposed conditions, normal oxidation products of **14**, such as **3** and **4**, have been mainly produced, which might follow the same path as the postulated biosynthetic method for **3**, **4** and **7** [13].

This study showed that LC–ESP-MS was a powerful tool for the rapid and reliable analysis of the constituents of plants, plant extracts and their chemical change products utilizing a relatively small amount of material. The result from LC–ESP-MS provides important information for the further phytochemical studies and quality control of the production process of Danggui. Use of LC–MS for the identification of phthalide isomers still needed comparison with the standards, which was time-consuming. LC–MS–MS can provide more information about the fragmentation of each compound [33,34], thus offering more chance for direct identification. However, combined techniques, such as LC–MS (or LC–MS–MS) and LC–NMR, will be more powerful for on-line identification of the compounds directly within the crude extracts [33,34].

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