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Separation and identification of compounds in *Rhizoma chuanxiong* by comprehensive two-dimensional liquid chromatography coupled to mass spectrometry

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Abstract

A comprehensive two-dimensional liquid chromatographic separation system based on the combination of a CN column and an ODS column is developed for the separation of components in a traditional Chinese medicine (TCM) *Rhizoma chuanxiong*. Two columns are coupled by a two-position, eight-port valve equipped with two storage loops and controlled by a computer. The effluent is detected by both the diode array detector and atmospheric pressure chemical ionization (APCI) mass spectrometer. More than 52 components in the methanol extract of *R. chuanxiong* were resolved and 11 of them were preliminary identified according to their UV and mass spectra.

Keywords: *Rhizoma chuanxiong*; Liquid chromatography, comprehensive two-dimensional; Chinese medicines

1. Introduction

Multi-dimensional chromatography is a powerful separation technique and various multi-dimensional chromatography techniques, such as liquid chromatography (LC) coupled to liquid chromatography [1] and liquid chromatography coupled to capillary zone electrophoresis (CZE) [2], have been developed. As a typical form of multi-dimensional separation system, comprehensive two-dimensional liquid chromatography system has been widely used to characterize and separate biomolecules [1–4], polymers [5], and other complex mixtures [6] due to its high peak capacity, powerful separation and resolution ability since it appeared in 1978 [7]. In ordinary heart cutting two-dimensional system, only a few fractions from the first column are analyzed on the second dimension column, but in comprehensive two-dimensional system all analytes from the first dimension separation are acted upon equality as fractions and analyzed on the second dimension column. The comprehensive two-dimensional system can provide maximum information with minimal amounts of material and allow rigorous quantitative interpretation of the results. Generally, a comprehensive two-dimensional liquid chromatography system combines two or more separation techniques based on different separation mechanisms. Ion exchange chromatography coupled with reversed-phase liquid chromatography [1,4,8], size exclusion chromatography coupled with liquid isoelectric focusing [9], and ion exchange chromatography coupled with size exclusion chromatography [10] etc have been reported to demonstrate that comprehensive two-dimensional liquid chromatography system is a very powerful separation technique for different kinds of samples.

Traditional Chinese medicines (TCMs) are gaining more and more attention in modern pharmaceutical institutes as they provide important resource for drug development. Analysis of TCMs is an important subject in biochemical, pharmaceutical and clinical research. However, these medicinal plants usually contain complex constituents and low content of possible bioactive ingredients. Therefore, efficient methods are required for the separation and

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identification of these ingredients. Different separation techniques including thin-layer chromatography (TLC) [11], gas chromatography (GC) [12], high-performance liquid chromatography (HPLC) [13], biochromatography [14,15] and capillary zone electrophoresis [16] have been developed for this purpose. However, up to now, there are only a few reports on the application of comprehensive two-dimensional system in the separation and identification of TCMs. Zhang et al. [17] applied comprehensive two-dimensional capillary liquid chromatography (LC) with capillary micellar electrokinetic chromatography (MEKC) in the separation of neutral components in TCMs. Recently, comprehensive two-dimensional micro-column chromatography has been developed for separation of components in TCMs [18]. In this paper, a comprehensive two-dimensional liquid chromatography system coupled to mass spectrometry was established to the separation and identification of the components in *Rhizoma chuanxiong*, one of the most commonly used drugs in the prescriptions of TCMs [19]. In the comprehensive two-dimensional system, besides a diode array detector, an atmospheric pressure chemical ionization (APCI) mass spectrometer is also used for the detection of compounds and yields on-line molecular weight information. This also adds a third dimension to this two-dimensional separation system because the overlapped peaks can be identified by mass spectrometer even though they are not resolved by chromatography.

2. Experimental

2.1. Instrumentation

The basic layout of the comprehensive two-dimensional liquid chromatography system draws on the design of Bushey and Jorgenson [10]. Fig. 1 is a schematic representation of the two-comprehensive liquid chromatography system. A pump delivers mobile phase to the injector and into column 1. The outlet of column 1 is attached to a two-position, eight-port valve. Two loops are equipped on the valve. As one loop fills with effluent from column 1, the other loop is being pumped out by another pump and through column 2, and then effluent of column 2 flows through diode array detector and mass spectrometer detector.

The first dimension column was prepared by packing Kromasil-CN (5 μm, 120 Å, Sweden) into a stainless steel column (200 mm × 2.0 mm i.d.), and the mobile phase was adopted by linear gradient elution starting from 100% water to methanol/water (40/60, v/v) in 80 min, then by isocration elution at the composition of methanol/water (60/40, v/v) for another 60 min at the rate of 0.04 ml/min. The mobile phase was delivered by an LC-10ADvp pump (Shimadu, Kyoto, Japan). Kromasil-ODS (5 μm, 120 Å, Sweden) was packed

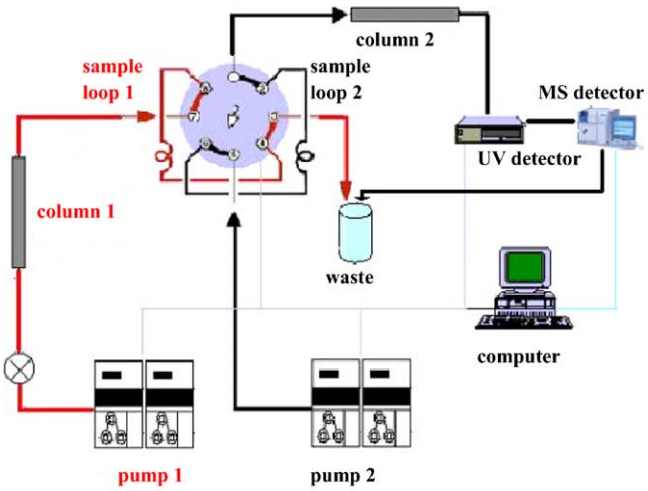


Fig. 1. Schematic representation of a comprehensive two-dimensional liquid chromatography system coupled to mass spectrometry.

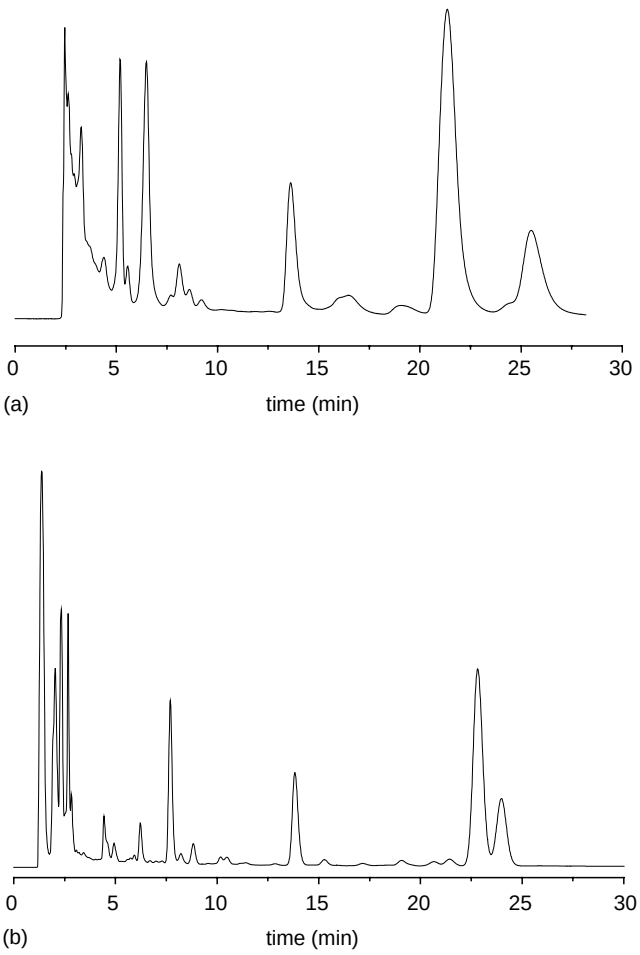


Fig. 2. One-dimensional liquid chromatogram of *Rhizoma chuanxiong*. (a) Experimental conditions: mobile phase, 50% (v/v) methanol in water; column, 150 mm × 4.6 mm i.d. packed with 5 μm Kromasil-CN; flow rate, 0.8 ml/min; injection volume, 10 μl; detection wavelength, 250 nm. (b) Experimental conditions: mobile phase, 50% (v/v) acetonitrile in water; column, 15 mm × 4.6 mm i.d. packed with 5 μm Kromasil ODS. Other conditions are the same as in (a).

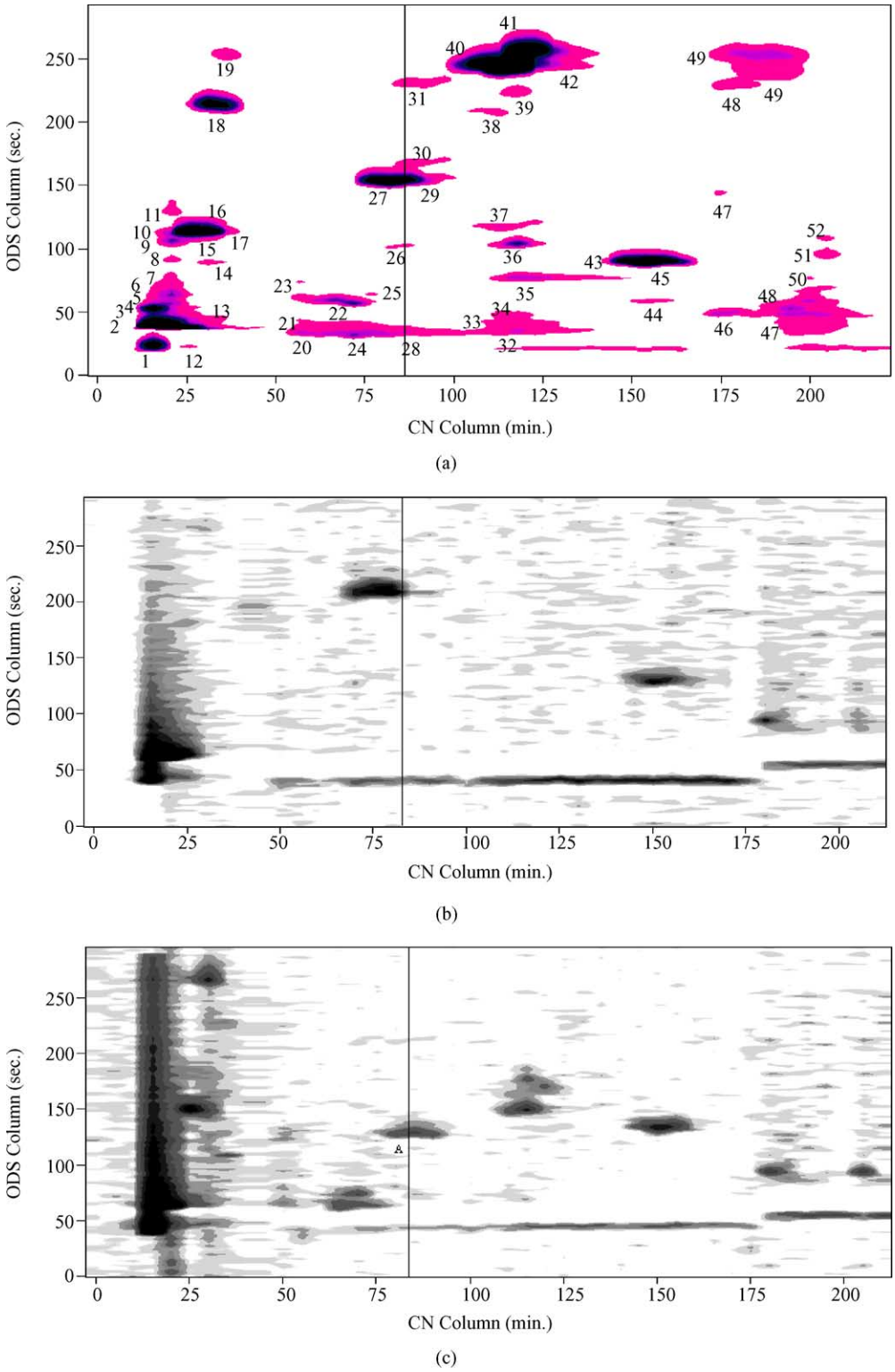


Fig. 3. Two-dimensional chromatogram of *Rhizoma chuanxiong*. Experimental conditions: CN column 200 mm × 2.0 mm i.d. packed in 5 μm Kromasil CN; flow rate, 0.04 ml/min; ODS column 50 mm × 2.0 mm i.d. packed with 5 μm Kromasil ODS; flow rate, 0.7 ml/min; injection volume, 20 μl; detection wavelength, 250 nm. The heights of the two-dimensional pot determined by: (a) the relative UV absorbance; (b) the counts per second using APCI positive ion mode; (c) the counts per second using APCI negative ion mode.

into a column (50 mm × 2.0 mm i.d.) in-house and used as the second dimension column. The mobile phase was adopted with acetonitrile in 0.1% (v/v) acetic acid buffer (25/75, v/v) in the first nine cycles, then changed to acetonitrile/0.1% (v/v) acetic acid buffer (53/47, v/v) for the next twenty-six cycles, and finally changed to acetonitrile/0.1 % (v/v) acetic acid buffer (71/29, v/v) in the rest eight cycles. The second dimension column was supplied with the mobile phase at 0.7 ml/min from another LC-10ATvp pump (Shimadu, Kyoto, Japan). The outlet of the second dimension column was connected to an SPD-M10Avp diode array detector (Shimadzu, Kyoto, Japan) and the outlet of the diode array detector was directly connected to APCI-MS (Shimadzu, Kyoto, Japan). The analytes cut from the second dimensional column was directly detected by mass spectrometry without stream splitting. The APCI probe voltage was set at 1800 V, the nebulizing gas flow was 2.5 L/min, the APCI, CDL and block temperature was set at 400, 250 and 200 °C, respectively. The mass range [*m*/*z*] was from 50 to 1000 and the scan speed was set at 0.5 scan/s. The switching valve was a two-position, eight-port valve (EC8W, Valco Instruments, Houston, TX). Volume of loop on switching valve was determined by multiplying the flow rate of the first column and the run time of the second column.

The two-dimensional chromatographic system was controlled by a computer running a custom program written in-house with visual C++ 6.0 software (Microsoft Corp., Redmond, WA, USA) and the UV data collection were achieved with a data acquisition board (National chromatographic R&A Center, China) and dealt with a program written in house, and APCI-MS data were background subtracted and displayed using the control software LCMS solution (version 2.02, Shimadu, Kyoto, Japan) supplied with the instruments. Two-dimensional data were graphically represented with the aid of software Fortner Transform (version 3.4, Fortner Inc., Savoy, IL, USA).

2.2. Chemical and reagents

Methanol and acetonitrile were chromatographic grade; acetic acid was analytical grade; distilled water used in all experiments was purified by a Milli-Q system (Milford, MA, USA). Ferulic acid was purchased from Sigma (St. Louis, MO, USA), caffeic acid, protocatechuic acid and 3-butylphthalide were ordered from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China) and ligustilide was prepared in our laboratory with purity more than 99%, as determined by HPLC; *R. chuanxiong* was purchased from a local Chinese medicine store.

2.3. Extraction of R. chuanxiong

Three grams of roots of *Ligusticum chuanxiong Hort*. was first crushed with a grinder and immersed in 30 ml methanol for 1 h and then heated to boiling for another 1 h. The methanol extract was filtered through a 0.45 μm membrane and stored at 4 °C in the absence of light for subsequent experiments.

3. Results and discussion

Ferulic acid [20], and ligustilide [11] are known as the main bioactive constitutes of *R. chuanxiong*. The polarity and hydrophobicity of these compounds are significantly different. So, a CN column was selected for the separation of polar compounds whereas an ODS column was selected to separate compounds according to their hydrophobicity. Fig. 2 shows the chromatograms for the methanol extract of *R. chuanxiong* on CN column and ODS column, respectively, and different pattern of chromatograms was observed due to different retention mechanisms on these two columns. In comprehensive two-dimensional liquid chromatography system, the total analysis time is determined by analysis time on the second dimension separation system and the total number of fractions injected into the second dimension, thus

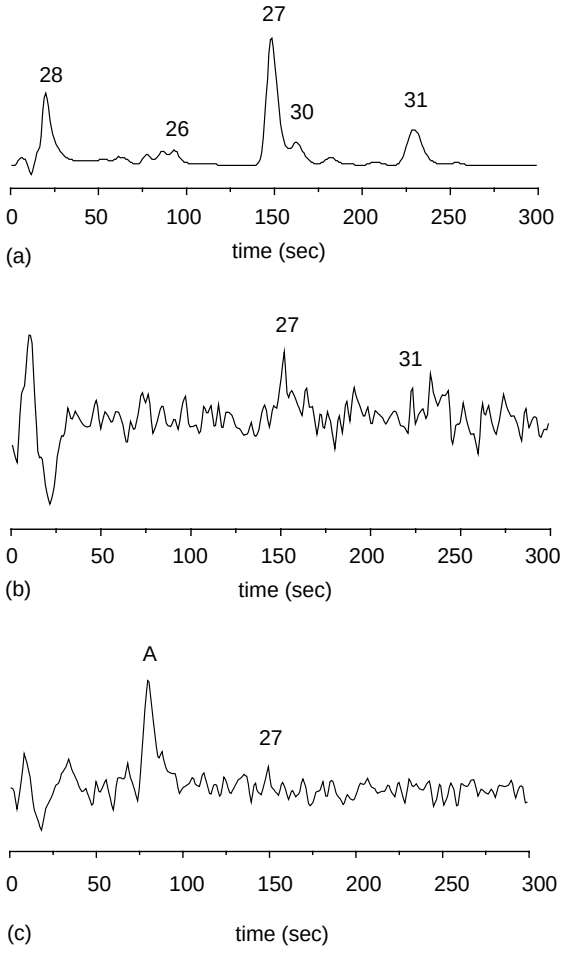


Fig. 4. Chromatograms on the ODS column extracted between 85 and 90 min on CN column. Detection with: (a) UV absorption; (b) APCI positive ion mode; (c) APCI negative ion mode.

Table 1
The molecular structures and mass weights of the identified components in *Rhizoma chuanxiong*

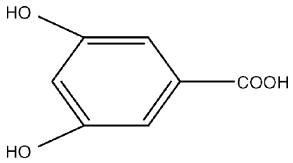
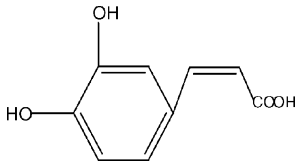
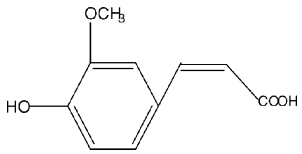
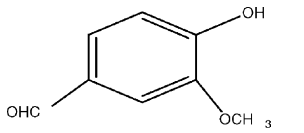
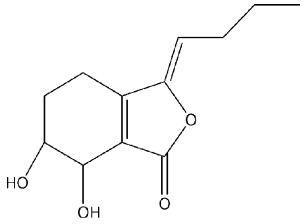
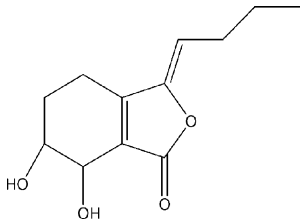
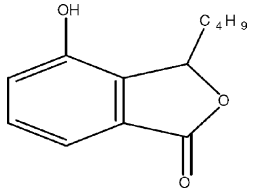
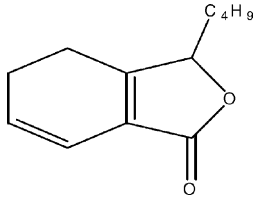
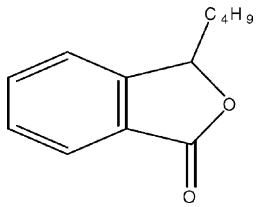
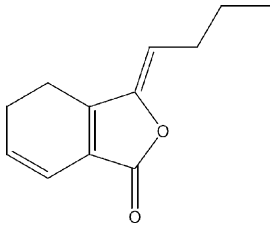
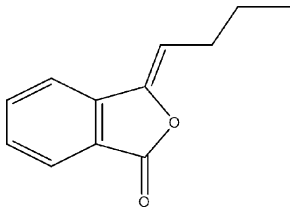
Peak	Compound	Structure	Mass weight
4	Protocatechuic acid		154.12
6	Caffeic acid		180.16
15	Ferulic acid		194.18
16	Vanillin		152.15
18	Senkyunolide I		224.25
19	Senkyunolide H		224.25
A	4-Hydroxy-3-butylphthalide		206.24
27	4,5-Dihydro-3-butylphthalide		192.25
30	3-Butylphthalide		190.24

Table 1 (*Continued*)

Peak	Compound	Structure	Mass weight
41	Ligustilide		190.24
42	3-Butylidenephthalide		188.22

the analysis time of the second dimension should be short enough to allow the analysis of a large number of fractions of the first dimension [21,22]. So, a stationary phase compatible with a high flow rate must be used. Reversed-phase material was readily available and could offer a large body of knowledge about their performance at high flow rates. And according to the experimental results shown in Fig. 2, the ODS column shows better resolution capacity and efficient separations compared with the CN column. So, an ODS column was chosen as the column for the second dimension separation and a CN column for the first dimension separation in this experiment. According to these criteria of a comprehensive two-dimensional system, a high enough flow rate of 0.7 ml/min was used for the second dimension column concerning the limited pressure drop of the pump and the flow rate range for the mass spectrometry in this study. In order to obtain a higher resolution in the first dimension separation, run time of the second dimension was set in 5 min for one cycle. Under these conditions, the mobile phase compositions for the second dimension were optimized with the optimization strategy based on the uniform design and genetic algorithm previously reported [23].

In the comprehensive two-dimensional liquid chromatography system, loop volume is determined by the multiplication of flow rate on the first column and run time on the second column. Thus flow rate of the first dimension separation can be obtained by dividing loop volume with run time on the second column. In this work, the loop volume was set at 0.2 ml, so the flow rate of the first dimension separation was 0.04 ml/min. With these limits, the mobile phase compositions for the first dimension separation were obtained according to the method previously reported [23].

Fig. 3 shows the typical two-dimensional chromatograms for the methanol extract of *R. chuanxiong* on the comprehensive two-dimensional liquid chromatography system described above. The comprehensive two-dimensional chromatogram is the result if each chromatogram on ODS column is stacked side by side and looked upon from a top-down perspective. Retention time of a peak on CN column can be obtained from the *x*-axis, while its retention time on ODS column is depicted on the *y*-axis. The heights of peaks in the two-dimensional pot are determined by the relative UV absorbance in Fig. 3a and the counts per second in Fig. 3b and c. The shapes of peaks in the two-dimensional plot are examined in closer detail by extracting the individual runs on the ODS column in which they appear. For example, single chromatogram of the individual run on the ODS column between 85 and 90 min of it run on the CN column is shown in Fig. 4, where both the UV absorption and total ion intensity data are plotted. Because some components in *R. chuanxiong* cannot be detected with APCI, neither in positive ion nor in negative ion mode, but can be detected with UV detector, so not all the peaks detected in the UV mode can be observed in the ion current data [11]. It can be seen that more than 10 peaks are detected in the UV mode as shown in Fig. 4a, but only the main peaks 26, 27, 28, 30 and 31 are plotted in Fig. 3a. Although most of the peaks can be identified by mass spectrometer with APCI both positive ion mode and negative ion mode, only peaks 27 and 31 can be observed in Fig. 4b and only peak 27 can be observed in Fig. 4c based on the stronger signal of them. Furthermore, a new peak is observed with APCI negative ion mode shown as peak A in Fig. 4c. It is indicated that mass spectrometry could not only be regarded as the third dimension to identify, but determine the components that cannot be detected with UV detector in comprehensive two-dimensional system.

According to the UV detection and the mass spectrometric detection, totally more than 52 components in the methanol extract of *R. chuanxiong* were separated by the comprehensive two-dimensional liquid chromatography system in less than 215 min and 11 of them were identified. Comparing the UV and mass spectra data of the analytes with these reported in literatures [11–13,24–26], peaks 4, 6, 15, 16, 18, 19, A, 27, 30, 41 and 42 were preliminary identified as protocatechuic acid, caffeic acid, ferulic acid, vanillin, senkyunolide I, senkyunolide H,

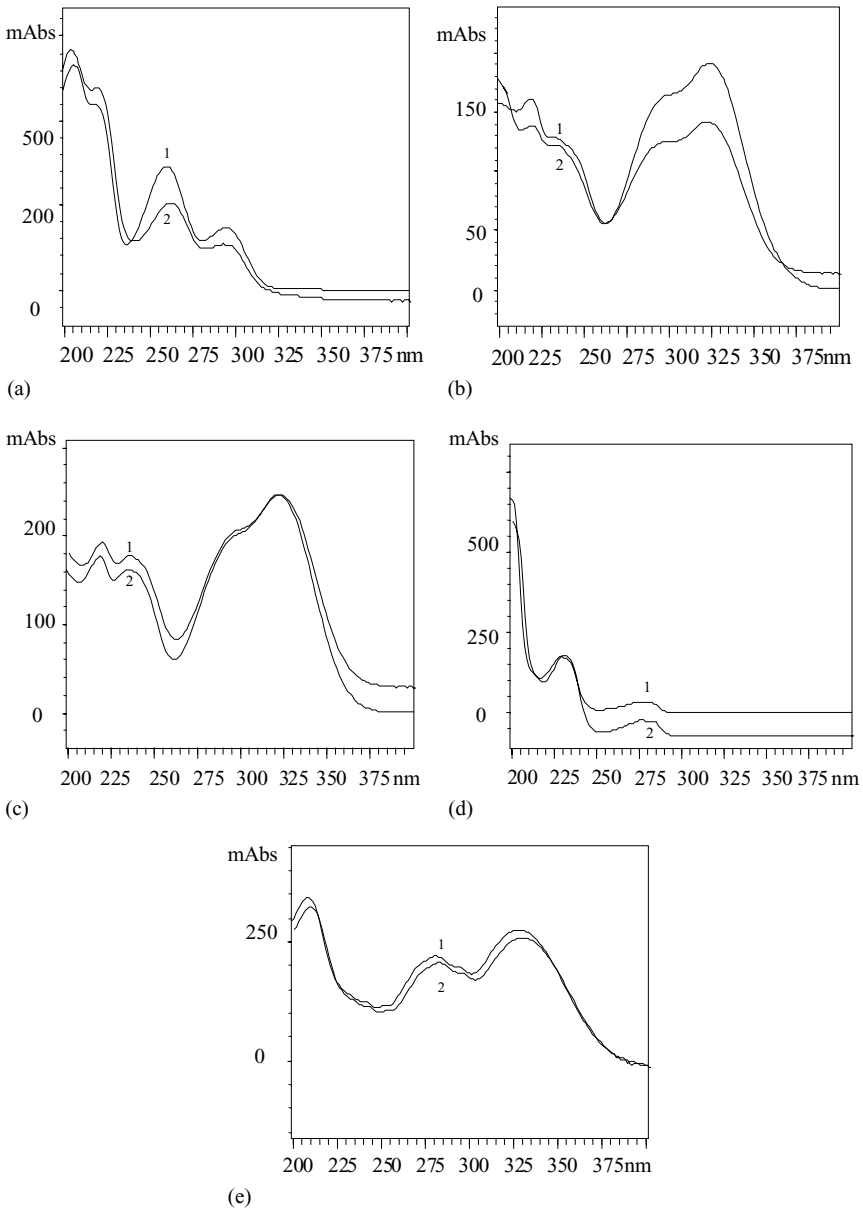


Fig. 5. UV spectra of standards and peaks 4, 6, 15, 30 and 41: (a) peak 4 (1) and protocatechuic acid (2); (b) peak 6 (1) and caffeic acid (2); (c) peak 15 (1) and ferulic acid (2); (d) peak 30 (1) and 3-butylphthalide (2); (e) peak 41 (1) and ligustilide (2).

4-hydroxy-3-butylphthalide, 4, 5-dihydro-3-butylphthalide, 3-butylphthalide, ligustilide and 3-butylidenephthalide, respectively. The molecular structures and molecular weights of these compounds are summarized in Table 1. The analysis of structural information for them obtained from UV and mass spectra is described as follows.

Fig. 5 shows UV spectra of peaks 4, 6, 15, 30, 41 and standard compounds of protocatechuic acid, caffeic acid, ferulic acid, 3-butylphthalide and ligustilide, respectively. The two-dimensional chromatogram for those standards is shown in Fig. 6. Because there was slight fluctuation in the mobile phase composition in every experiment, the retention times of the standards and the peaks have small difference between different injections, the reproducibility of the retention times has been tested by five injections and the relative standard deviation was less than 2.0% in retention times. Comparing with the retention time and UV spectra of the standards and the peaks 4, 6, 15, 30 and 41, their UV spectra are quite similar, but a small difference between standards and corresponding peaks can be observed, which can be explained from the different solvents and amount of analytes that were used to the measure the UV spectra. Identification was further performed by comparing the mass spectra of the peaks with the standards. They were preliminary identified as protocatechuic acid, caffeic acid, ferulic acid, 3-butylphthalide, and ligustilide, respectively.

Fig. 7 shows UV and mass spectra of peaks 16, 18, 19, A, 27 and 42, respectively. The mass spectrum of peak

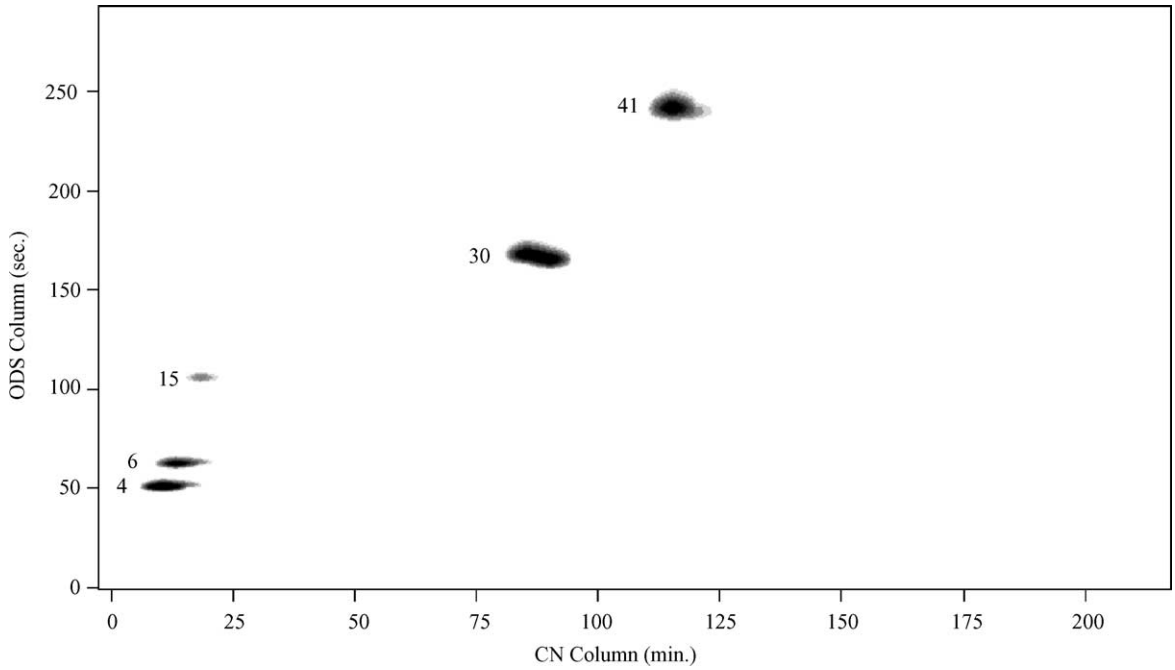


Fig. 6. Two-dimensional chromatogram of the standards. Peaks identification: (4) protocatechuic acid; (6) caffeic acid; (15) ferulic acid; (30) 3-butylphthalide; (41) ligustilide. Experimental conditions: all standards were dissolved in methanol; other conditions are the same as in Fig. 3.

16 shows prominent peak at *m*/*z* 151 (M^-), and its UV spectrum was very similar to vanillin [13], so peak 16 was preliminary identified as vanillin. It can be seen that the mass spectra of peaks 18 and 19 both show a fragment at *m*/*z* 223 (M^-), and their UV spectra with the maximal absorption at about 275 nm are quite similar. Thus it can be expected that the molecular structures of them should be very similar. It was reported that senkyunolide I and senkyunolide H with a significant amount in *R. chuanxiong* have the same UV spectrum with maximal absorption at 275 nm [12] and the amount ratio of senkyunolide I and senkyunolide H is approximately 5–1 in *R. chuanxiong* [13]. With the structural information from UV and mass spectra, peaks 18 and 19 can be preliminary identified as senkyunolide I and senkyunolide H, respectively. The mass spectrum of peak A exhibited fragment peak at *m*/*z* 205 (M^-). By comparing the mass spectrum with literature data [26], peak A was preliminary identified as 4-hydroxy-3-butylphthalide. The mass spectrum of peak 27 was similar to that of 4, 5-dihydro-3-butylphthalide [12,26], whose mass data was reported as *m*/*z* 191 (M^-). According to the mass spectral data, peak 27 was preliminary identified as 4, 5-dihydro-3-butylphthalide. By comparing the mass spectrum of peak 42 with that of 3-butylidenephthalide, peak 42 was preliminary identified as 3-butylidenephthalide [11,12,25], whose mass data showed the fragments at *m*/*z* 187 (M^-).

With further analysis of the result shown in Fig. 6, it can be seen that most of analytes eluted from the CN column are with peak width about 10 min, which means that the peak from the first dimension column is obtained within two fractions to the second dimension column. The time between the dead volume and the last-eluting peak is 120 min. This equals a peak capacity of 12 in the first dimension in the comprehensive two-dimensional system. Returning to Fig. 4a, the peak width is about 20 s; the peak capacity is 14 in the second dimension by dividing the available elution time of 280 s (excluding the dead time) by 20 s. Multiplying 12 by 14 produces a chromatographic peak capacity of 168. The mass spectrometer also has inherent peak capacity, conservatively taken to be 5, because it can identify at least this number of components while scanning a 1000 *m*/*z* range [8]. This makes peak capacity of the entire comprehensive two-dimensional system greater than 840.

Components can be identified based on their retention time in a comprehensive two-dimensional system without detection of mass spectrometry. Of course, this is even more rigorous than a one-dimensional system because of low probability that two components will have the same retention time in two orthogonal modes of separation. However, for the complex samples that are uncharacterized, the addition of mass spectrometry as a detector makes the system a stronger capability of quickly screening for the mass of major components, also it can be regarded as the third dimension to increase the separation capacity.

Although the advantages of comprehensive two-dimensional chromatography coupled with mass spectrometry for separation and identification of TCMs are discussed here, numerous possibilities exist to improve this system.

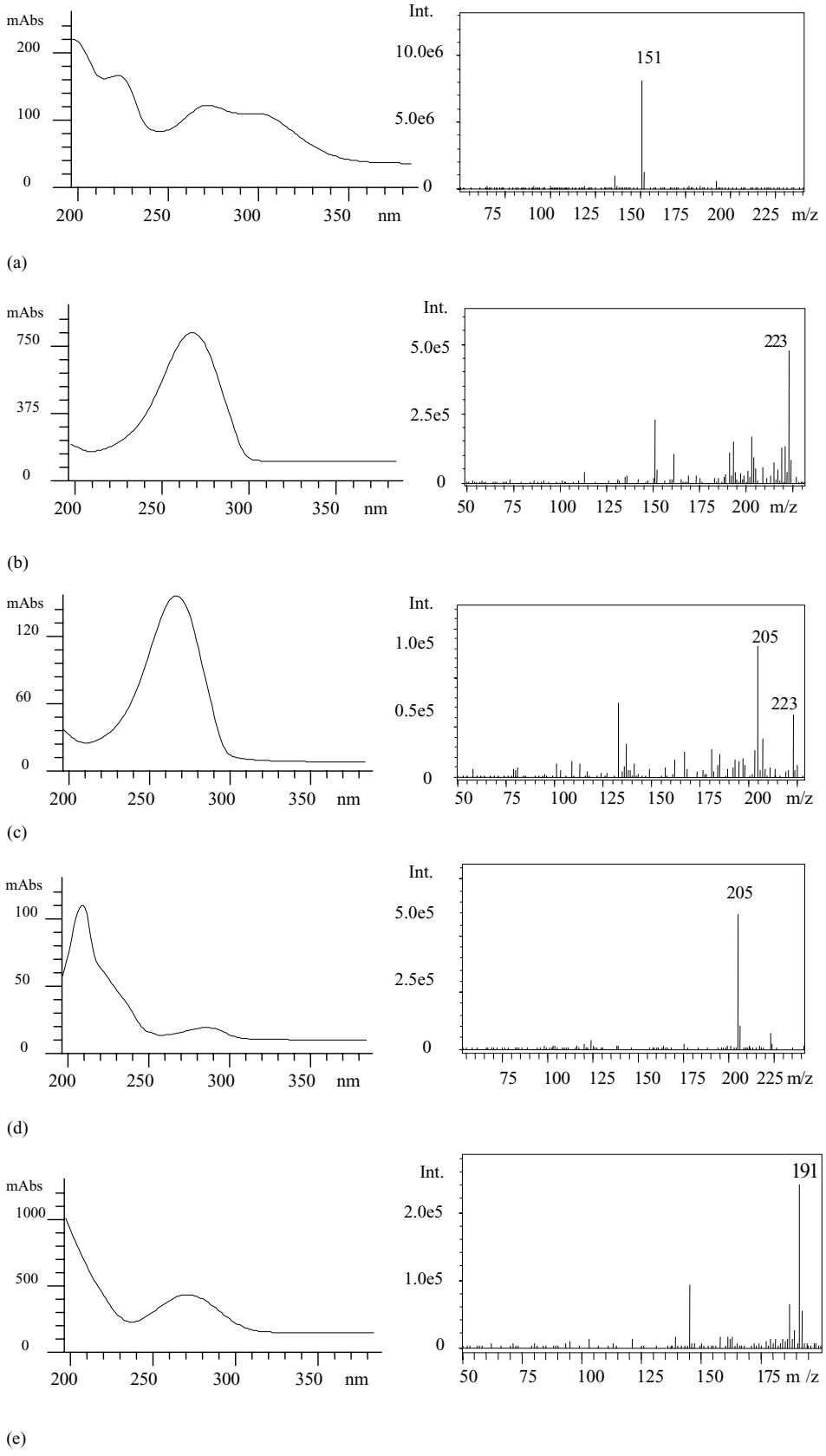


Fig. 7. UV spectra and mass spectra using negative ion mode for detection of peaks 16, 18, 19, A, 27 and 42. Background of all MS spectra was subtracted. (a) Peak 16; (b) peak 18; (c) peak 19; (d) peak A; (e) peak 27; (f) peak 42.

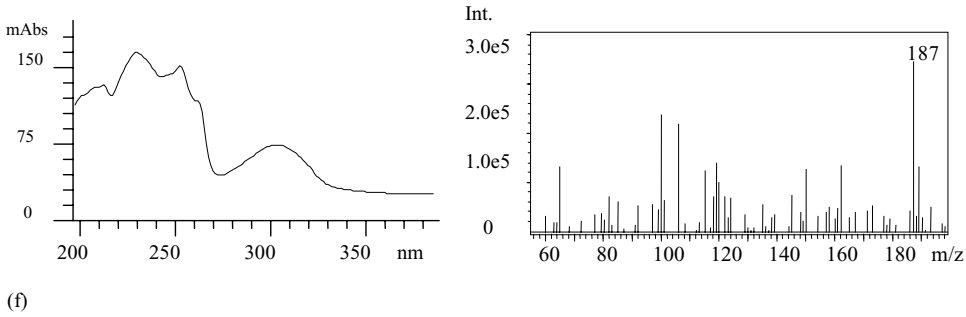


Fig. 7. (*Continued*).

Perhaps, the easiest way is to increase the column length in the first dimension in order to increase efficiency. Unfortunately, this results in an increase in total analysis time. A more efficient way is using a stationary phase with good permeability and high efficiency for the second dimension separation, and also increasing the second dimension separation speed can even improve the separation efficiency by making the peaks sharper and less dilution. With these efficient ways, not only the peak capacity but also the sensitivity and the resolution power of the system would be increased. Small increases in either dimension result in large overall gains in peak capacity, because of the multiplicative effect of combining two separation modes in a comprehensive two-dimensional system. On the other hand, because not all the peaks in the two-dimensional plot have the same heights, the peak cannot be observed if its height is not very high in the two-dimensional chromatogram. So, it will be very necessary to find another way to show all the information of peaks in the two-dimensional plot.

4. Conclusions

A comprehensive two-dimensional liquid chromatographic separation system based on the combination of a CN column and an ODS column is developed for the separation of methanol extract of a traditional Chinese medicine *R. chuanxiong*. Much more components of *R. chuanxiong* can be easily separated and identified with the comprehensive two-dimensional liquid chromatography than traditional one-dimensional liquid chromatography. Moreover, the on-line mass spectrometry further reduced the undetected chances of coeluting peaks and the obtained molecular weight information can be applied for identification of the chromatographic peaks. More than 52 components were separated in less than 215 min and 11 of them were simultaneously identified without tedious pretreatment. The comprehensive two-dimensional liquid chromatography system shows its high peak capacity, sensitivity and powerful resolution potential in separation and identification of complex traditional Chinese medicines.

Acknowledgements

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