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Improved performance of comprehensive two-dimensional HPLC separation of traditional Chinese medicines by using a silica monolithic column and normalization of peak heights

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Abstract

Performance of comprehensive two-dimensional liquid chromatography system is greatly improved than we reported previously by using a silica monolithic column as for the second dimensional separation. Due to the increase of the elution speed on the second dimensional monolithic column, the first dimensional column efficiency and analysis rate can be greatly improved as comparing with conventionally second dimensional column. The developed system was applied to analysis of methanol extraction of two umbelliferae herbs *Ligusticum chuanxiong Hort*. and *Angelica sinensis (Oliv.) Diels* by using CN column as for the first dimensional separation and a silica monolithic ODS column for the second dimensional separation, and the obtained three-dimensional chromatograms were treated by normalization of peak heights with the value of the highest peak or setting a certain value using a software written in-house. It was observed that much more peaks for low-abundant components in TCM extract can clearly be detected here than we reported before, due to the large difference for the amount of components in TCMs' extract. With the above improvements in separation performance and data treatment, totally about 120 components in methanol extraction of *Rhizoma chuanxiong* and 100 in *A. sinensis* were separated with UV detection within 130 min. This result meant that both the number of peaks detected increase twice but the analysis time decease twice if comparing with the previously reported result.

Keywords: Silica monolithic column; Low-abundant components; Traditional Chinese medicines; Data treatment; Liquid chromatography; Comprehensive two-dimensional separation

1. Introduction

Separation of the complex samples requires the analytical methods of extremely high resolving power in order to provide reliable analysis of the sample components. As a powerful tool for the separation of complex mixtures multidimensional separation technique including 2D-LC [1], LC-CE [2,3,10], LC-LC-CE [4] has attracted much attention in recent years.

Two-dimensional system has been developed from heart cutting to comprehensive mode through the past years. As a typical format of multidimensional separation system, comprehensive two-dimensional liquid chromatography system has been widely used to characterize and separate biomolecules [1,5–7], polymers [8], TCMs [9] and other complex mixtures [11] due to its high peak capacity, powerful separation and resolution ability since it appeared in 1978 [12]. A variety of combination modes including ion exchange-reversed phase (IEC-RPLC) [5,7], size exclusion-reversed phase (SEC-RPLC) [4], reversed phase-reversed phase (RPLC-RPLC) [13] and ion exchange-size exclusion (IEC-SEC) [14], etc. have been used in comprehensive two-dimensional liquid chromatography for separation of different kinds of samples.

We have established a comprehensive two-dimensional liquid chromatography separation system for the separation of methanol extraction of a traditional Chinese medicine *Rhizoma chuanxiong* [9]. This system showed its high peak capacity, sensitivity and separation efficiency, but there were

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also numerous problems to be further improved as pointed previously [9]: (1) by using a stationary phase with good permeability and high efficiency for the second dimensional column to increase the second dimensional separation speed and further to improve the first dimensional column efficiency and to decrease sample dilution; (2) by using a software to normalize peak heights of the three-dimensional chromatogram to distinguish low-abundant components. In comprehensive two-dimensional liquid chromatography the analytes on the second dimensional column should be eluted at very high speed to meet the rate of fractionation of the first dimensional separation [15], as practiced in 2D-GC [16]. As the perfusion based and monolithic stationary phase has good permeability, high column efficiency at high linear flow velocities, they are very fitted for fast separation [17,18]. Holland and Jorgenson used perfusive reversed phase column as the second dimensional column to separate tryptic digest of porcine thyroglobulin [19]. Tanaka et al. [15] and Tanaka and co-workers [20], etc. used a monolithic column in two-dimensional liquid chromatography for the analysis of peptides, hydrocarbons and benzene derivatives.

We firstly used a silica monolithic ODS column as for the second dimensional separation to improve the performance of comprehensive two-dimensional liquid chromatography system, and secondly developed a software to treat the obtained two-dimensional chromatograms by normalization of peak heights with setting a certain value to distinguish low-abundant components. The developed method was applied for two-dimensional separation of the components in *R. chuanxiong* and *Angelica sinensis*, the most common used drugs in the prescription of TCMs [21,22]. It was observed that both the number of peaks detected increases twice but the analysis time deceases twice if comparing with the previously reported result.

2. Experimental

2.1. Instrumentation

The basic principle and scheme of 2D-LC/MS system were shown in our previous report [9]. 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. All effluent from the first dimension was injected to the second dimension without splitting. The effluent of column 2 was then split by using a T-joint at a split ratio of approximately 1/4.3, resulting in a flow rate of 0.7 ml/min into diode array detector and mass detector.

The CN column was prepared in-house by packing 5 μm Kromasil-CN (Kromasil, Sweden) into a stainless steel column (150 mm × 4.6 mm, i.d.); SCX column with 150 mm × 4.6 mm, i.d. packed with Hypesil-SCX (5 μm) was pursed from Dalian Elite company (Dalian, China) and the monolithic column was Chromonolith Speed ROD with 50 mm × 4.6 mm, i.d. form the Merck company (Darmstadt, Germany). The 2D-LC/MS system consists of an LC-10ATvp and an LC-10ADvp pump (Shimadu, Kyoto, Japan), a SPD-M10Avp diode array detector (Shimadzu, Kyoto, Japan) and APCI-MS (Shimadzu, Kyoto, Japan). 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 two-dimensional chromatography 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 chromatographic data were collected 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 3.0, Shimadu, Kyoto, Japan) supplied with the instruments. The obtained chromatographic data with normalization of peak heights were also treated by a software program written in-house with visual C++. Three-dimensional projection diagram was displayed using a program written in-house with MATLAB 5.3 software (The MathWorks Inc., Sherborn MA, USA).

2.2. Chromatographic conditions

Mobile phase A_1 was 90% acetonitrile with 0.1% (v/v) acetic acid; B_1 was 10% acetonitrile with 0.1% (v/v) acetic acid; A_2 was 90% methanol with 0.1% (v/v) acetic acid; B_2 was 10% methanol with 0.1% (v/v) acetic acid, respectively.

One-dimensional chromatographic conditions for CN column: the mobile phase was adopted by linear gradient elution starting from 0% A_1 (100% B_1) to 70% A_1 (30% B_1) in 20 min at a rate of 0.8 ml/min. For monolithic column: the mobile phase was adopted by linear gradient elution starting from 0% A_2 (100% B_2) to 100% A_2 (0% B_2) in 5 min at a rate of 3.0 ml/min.

Experimental conditions for two-dimensional chromatography the first column (CN and SCX column): the mobile phase was adopted by linear gradient elution starting from 0% to 70% A_1 in 130 min at a flow rate of 0.133 ml/min. For the second column (monolithic column): the mobile phase was adopted by stepwise linear gradient elution from 0% to 10% A_2 in first 13 cycles; 10% to 30% A_2 in next 17 cycles; 30% to 60% A_2 in another 20 cycles and 60% to 80% A_2 in last 37 cycles at a flow rate of 3.0 ml/min.

2.3. Chemical and reagents

Methanol and acetonitrile were chromatographic grade; naphthalene, fluorene and acetic acid were analytical grade; distilled water used in all experiments was purified by a Milli-Q system (Milford, MA, USA). *Ligusticum chuanxiong Hort*.

and *A. sinensis (Oliv.) Diels* were purchased from a local Chinese medicine store.

2.4. Extraction of Rhizoma chuanxiong and Angelica sinensis

Three grams of *L. 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. *A. sinensis (Oliv.) Diels* was extracted with the same as for *L. chuanxiong Hort*.

3. Results and discussion

3.1. Optimization of the 2D-LC system

TCMs are the complex samples that contain components differing from inorganic to organic, from polar to non-polar, from small molecules to large biomolecules. Different modes of columns should be coupled to obtain a good separation of the sample. We choose CN-column for the separation of polar compounds and ODS-column to separate less polar compounds according to their hydrophobicity. In order to evaluate the complementary separation selectivity between the first and second dimensional columns, the methanol extraction of *R. chuanxiong* was separated on a CN column and monolithic ODS column, respectively. The obtained one-dimensional chromatograms are shown in Fig. 1, and it can be seen that the CN column accomplished the separation process in 20 min but the monolithic one needed only 5 min to give good separation. Also the chromatogram patterns on those two columns are quite different, which means that they provide the different selectivity for separation of the components in *R. chuanxiong*. In comprehensive two-dimensional liquid chromatography the analysis time of the second dimension should be short enough to allow the analysis of a large number of fractions eluted from the first dimensional column, and the separation process on the first dimensional column at a relatively low speed and on the second dimensional column at a relatively high speed. Thus, the monolithic ODS column was selected for the second dimensional separation.

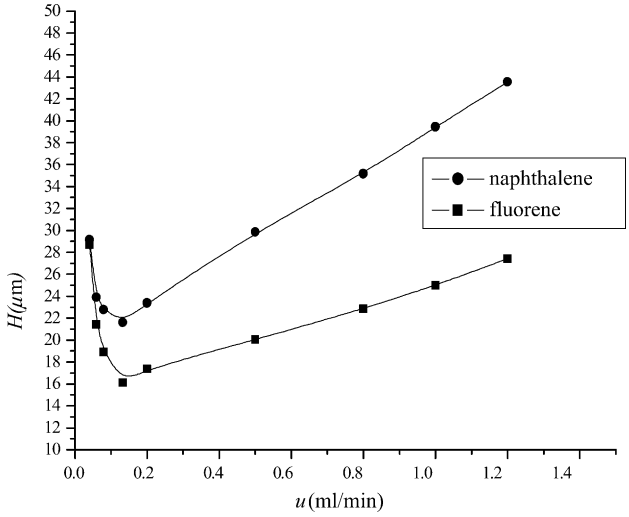

Fig. 2. The van Deemter curve for the CN column we used. Mobile phase: methanol in 0.1% (v/v) acetic acid buffer (50/50, v/v).

The effect of mobile phase velocity on column efficiency for a given column can be expressed by the Van Deemter's equation as follows

$$H = A + \frac{B}{u} + Cu$$

According to this equation, there should be an optimal velocity for a chromatographic column to get the highest column efficiency. The van Deemter curve for solutes of naphthalene and fluorene on the first dimensional CN column were measured, and the obtained results are shown in Fig. 2. It can be seen that the best column efficiency can be achieved at the

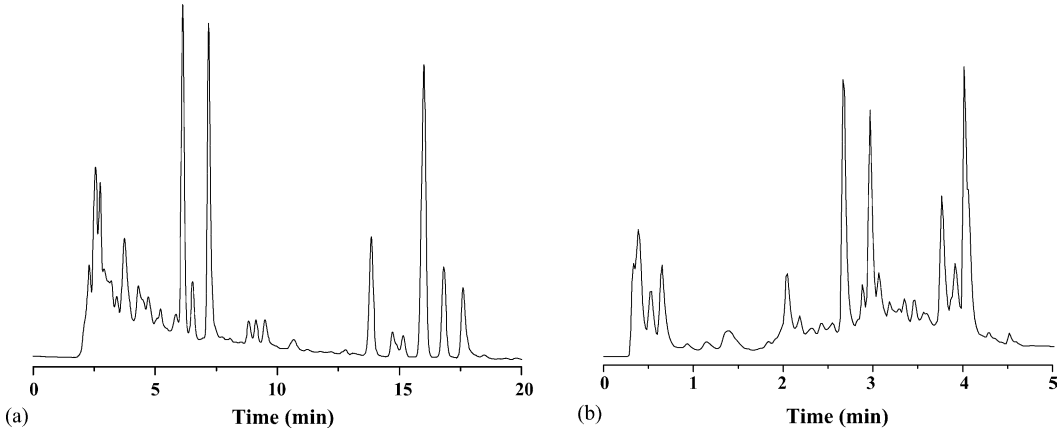

Fig. 1. One-dimensional liquid chromatogram of *Rhizoma chuanxiong*. (a) CN column: gradient elution from 0% to 70% A_1 in 20 min; flow rate, 0.8 ml/min. (b) Silica monolithic ODS column: gradient elution from 0% to 100% A_2 in 5 min; flow rate, 3.0 ml/min. Detection wavelength, 250 nm; injection volume, 5 μl.

flow rate about 0.13 ml/min for both solutes. In our previous work on two-dimensional separation of the components in *R. chuanxiong* [9], the flow rate for the first dimensional CN could only be set at 0.04 ml/min due to the limitation of separation speed on the second dimensional column, thus, the optimal column efficiency on the first dimensional column could not achieved. However, the silica monolithic ODS column has good property for high-speed separation with good column efficiency, thus, the much high velocity of the mobile phase on the first dimensional column can be adopted. In present work, the flow rate on the first dimensional separation was increased to 0.133 ml/min, which is around the optimal velocity for the column efficiency. Sequentially, the column efficiency was increased about twice.

To optimize the chromatography conditions the two-dimensional separation with different second dimensional cycle times was performed, and it was found that the cycle time could not be too long or short. In an extremely short cycle the second dimensional column may not accomplish the analysis process and the sample may coelute in the next cycle. And if the cycle time is too long the organic solvent quantity in the elution would be very large to result in confusing the second dimensional separation. Here, we set 1.5 min for one cycle which is found suitable for our system. We also adopted various modes of gradient elution for separation, and it was found that step linear gradients should be adopted to make the separation of analytes in a single cycle with good efficiency. Different elution speeds were also tested in our two-dimensional separation, and it was observed that too fast elution speed would decrease the resolution and bring the problem of too high pressure of the pump simultaneously resulting in matching difficultly with the mass detector.

3.2. Separation of components in Rhizoma chuanxiong

Usually, TCMs contain the number of components with serious differences in their amounts. For this reason, to express chromatographic peaks of low-abundant components under presence of the high-abundant ones we developed a software program to treat one-dimensional and three-dimensional chromatogram with normalization of peak heights by setting a certain value. The developed software program can be applied to normalize the peak heights with different values according to our requirement. The two-dimensional separation of the components in *R. chuanxiong* under the optimal conditions was performed, and the obtained original three-dimensional projection of chromatogram with normalization by the peak height of the highest peak is shown in Fig. 3(a). The three-dimensional projection of chromatogram is a result if each chromatogram on monolithic 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 monolithic column is depicted on the *y*-axis. The heights of peaks in the two-dimensional pot are determined by the relative UV absorbance. It can be seen that some low-abundant components were blurry in the diagram. Fig. 3(b) is the same three-dimensional chromatogram dealt by our developed software program with normalization by setting one-sixth peak height of the highest peak, and much more peaks were seen clearly than the original chromatogram just as we described before. The 74th cycle separated on the monolithic ODS column was extracted, and the shapes of peaks in the two-dimensional plot are examined in closer detail. The individual run of the 74th cycle is shown on right side of Fig. 3(b), and the five peaks plotted in the extracted cycle can be seen. But in Fig. 3(a), almost no peaks can be seen due to their signals differed greatly from the high-abundant components. As we measured the peak width at baseline on the CN-column was about 2 min at a flow rate of 0.133 ml/min, which indicated that the peak capacity was 65 in 130 min. As we can see in Fig. 3, the average peak width at baseline in the second dimension is about 12 s with peak capacity of 7.5. So the theoretical peak capacity of this 2D-LC system could be estimated as 488, which is about twice of the peak capacity by 2D-LC system previously reported [9]. And the mass spectrometer also has inherent peak capacity, conservatively taken to be 5 [26], so the system's entire capacity could be as high as 2440.

In Fig. 3(b), about 120 components were separated with this system. Table 1 lists the comparison of the separation of the components in *R. chuanxiong* by using 2D-LC previously reported [9] and presently developed. It can be seen that the column efficiency of the first dimensional column can be improved about twice, and analysis time was decreased about twice through increasing the elution speed of the second dimensional column. Also the number of components detected in the methanol extraction of *R. chuanxiong* was increased more than twice by adopting present 2D-LC system with normalization of peak heights by setting a certain value. Thus, it can be concluded that the present 2D-LC system can provide a fast separation ability and more powerful resolving power than that previously reported [9]. By comparing the UV and mass spectra of the analytes with those reported in literatures [9,23–25], five of the components

Table 1
Comparison of analysis results for the methanol extraction of *Rhizoma chuanxiong* by using 2D-LC previously reported [9] and presently developed

2D-LC system	Elution velocity on the first CN column (ml/min)	Plate number on the first CN column ($\times 10^3$)	Analysis time (min)	Number of components detected by UV
2D-LC previously reported	0.04	5.23	215	52
Improved 2D-LC	0.133	9.30	130	120

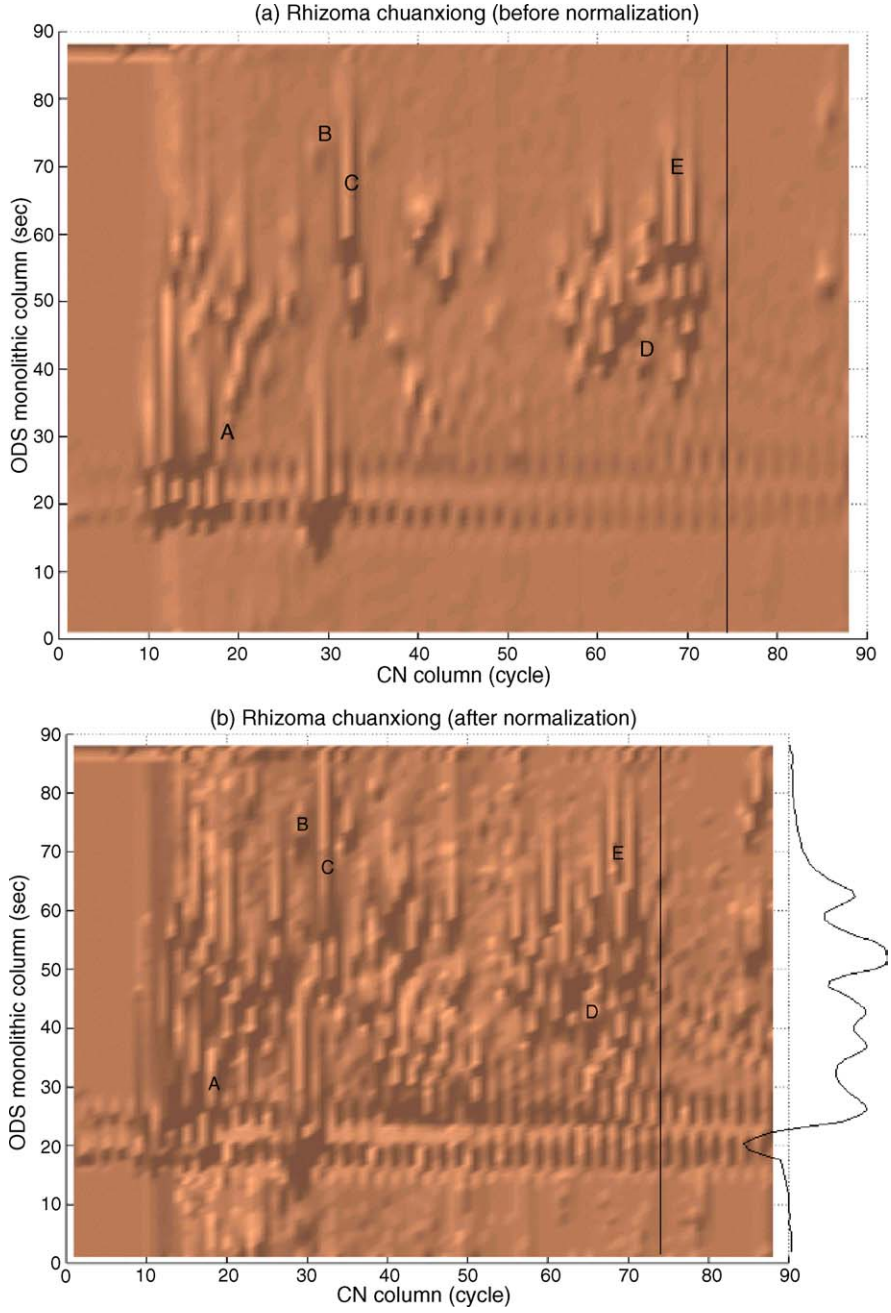

Fig. 3. Three-dimensional projection chromatograms of *Rhizoma chuanxiong* with normalization of peak heights by the value of (a) the highest peak and (b) the one-sixth of the highest peak as well as the extracted chromatogram on the monolithic column of the 74th cycle. Chromatographic conditions for CN column: gradient elution from 0% to 70% A_1 in 130 min; flow rate, 0.133 ml/min. Chromatographic conditions for monolithic ODS column: stepwise linear gradient elution from 0% to 10% A_2 in first 13 cycles; from 10% to 30% A_2 in next 17 cycles; from 30% to 60% A_2 in another 20 cycles and from 60% to 80% A_2 in last 37 cycles; flow rate, 3.0 ml/min. Detection wavelength, 250 nm; injection volume, 20 μl.

can preliminarily be identified as senkyunone, vanillin, ferulic acid, 4,5-dihydro-3-butylphthalide and ligustilide which were marked as A–E in Fig. 3(b).

3.3. Extendibility of the improved 2D-LC system

In comprehensive two-dimensional liquid chromatography, the chromatographic conditions for two columns can be optimized by changing modes of separation, flow rate and mobile phase composition, etc. To demonstrate an extensive utility of this system, we performed the separation of the components in *R. chuanxiong* by using a SCX column as for the first dimensional separation and a silica monolithic ODS column for the second dimensional separation without optimizing any of the chromatographic conditions, and the obtained three-dimensional chromatogram is shown in Fig. 4. It can be seen that also more than 100 components can be well separated after normalization.

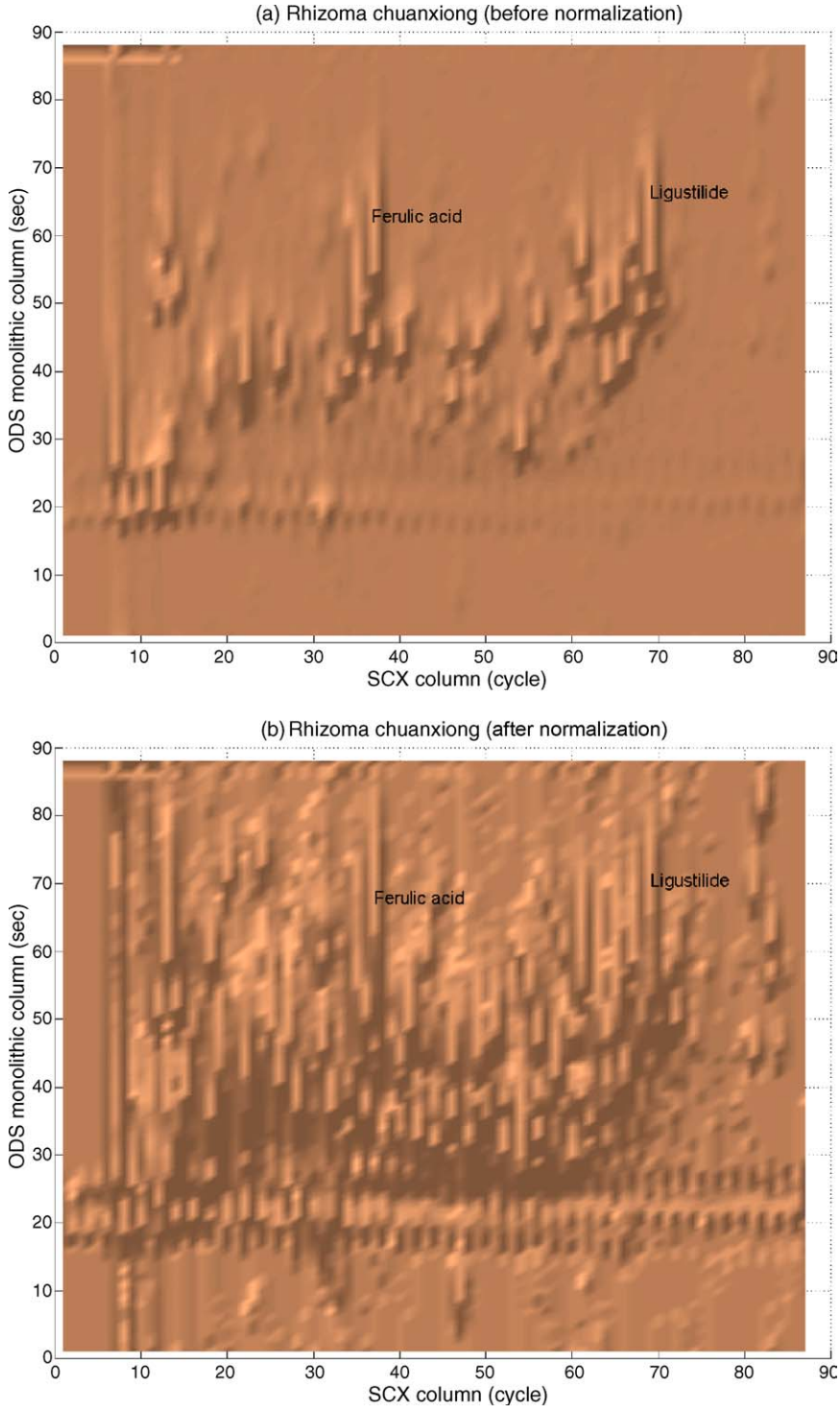

Fig. 4. Three-dimensional projection chromatograms of *Rhizoma chuanxiong* by combination of SCX column with monolithic ODS column with normalization of peak heights by the value of (a) the highest peak and (b) the one-sixth of the highest peak. Conditions are the same as in Fig. 3.

We also attempted this system with almost the same condition with *R. chuanxiong* on another herb, *A. sinensis*, the obtained three-dimensional chromatogram was shown in Fig. 5. It can be seen that also about 100 components were separated. 2D-LC system previously reported [9] has been applied for analysis of number of herbal extracts, and only about 40–50 components could be separated, which means that the separation efficiency of present 2D-LC system was much improved no matter theoretically or practically. By comparing their UV and mass spectra with those reported

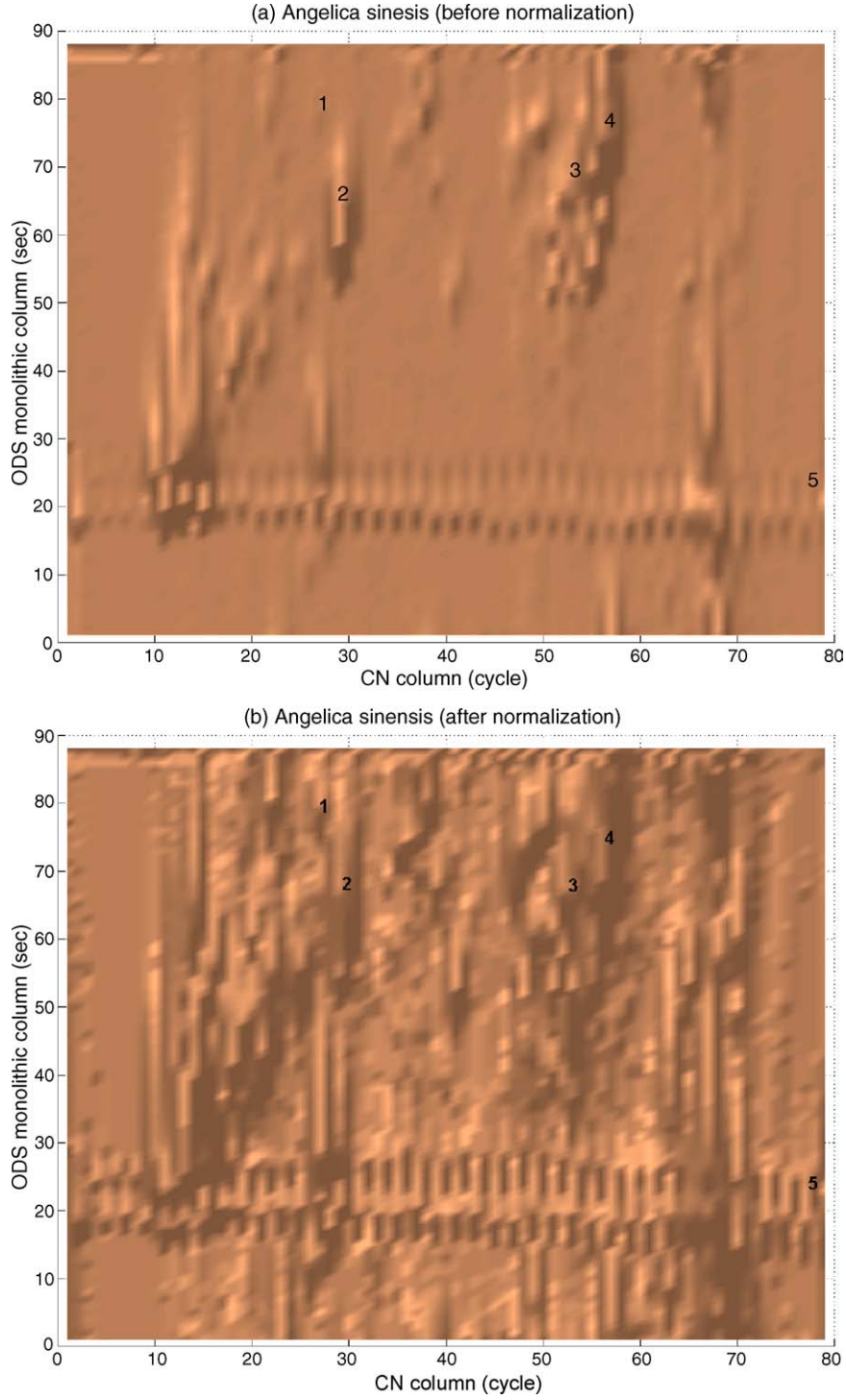

Fig. 5. Three-dimensional projection chromatograms of *Angelica sinensis* by combination of CN column with monolithic ODS column with normalization of peak heights by the value of (a) the highest peak and (b) the one-sixth of the highest peak. Chromatographic conditions for CN column: gradient elution from 0% to 100% A_1 in 130 min; flow rate, 0.133 ml/min. Other conditions are the same as in Fig. 3.

in literatures [9,23,24,27], chromatographic peaks marked as 1–5 in were identified as vanillin, ferulic acid, liqustilide, 3-butylidenephthalide, linoleic acid, respectively. The results for different coupled modes and different TCMs also indicate that the present 2D-LC can provide powerful separation ability for complex samples.

4. Conclusions

By using a silica monolithic column as for the second dimensional separation, we can increase the second dimensional elution speed greatly. About half analysis time was saved and the peak capacity and resolution of this system

were also greatly improved. About 120 components were separated in *R. chuanxiong* and 100 in *A. sinensis* using this system. Through normalization of the peak heights the low-abundant ingredients information can be shown clearly in the three-dimensional plot. This improved comprehensive two-dimensional system affords a powerful tool for the separation of complex mixtures and data displaying in three-dimensional plot.

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