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# A comprehensive two-dimensional normal-phase × reversed-phase liquid chromatography based on the modification of mobile phases

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ABSTRACT

A comprehensive orthogonal two-dimensional liquid chromatography (2D-LC) based on the modification of mobile phases was developed with a sample loop–valve interface. To improve the compatibility of mobile phases and analysis speed, some special solvents were chosen as the mobile phases, and the column temperature was elevated to decrease the viscosity of mobile phases of reversed-phase liquid chromatography (RPLC). Based on this principle, the first dimension was normal-phase liquid chromatography (NPLC) with a $SiO_2$ column, and the second dimension was reversed-phase liquid chromatography containing two tandem C18 columns. 1,4-Dioxane was used in the NPLC mobile phase, and isopropyl alcohol was employed in the RPLC mobile phase. Moreover, the elevated column temperature enabled the reduction of the backpressure and using tandem C18 columns to improve the resolving power in RPLC. The new comprehensive 2D-LC system and applied strategy offered a novel idea for construction of 2D-LC system. A traditional Chinese medicine, Zhengtian pill, was used as the test sample to evaluate the constructed 2D-LC system. 876 peaks were detected, and the peak capacity reached 1740.



## 1. Introduction

Liquid chromatography has been considered as a powerful technique for the analysis of complex samples. Compared with one-dimensional HPLC, two-dimensional approach is intensively investigated to increase resolving power and peak capacity [1], which can be used to analyze various samples, such as polymers, oligomers [2,3], environmental compounds [4], proteomes [5–8], traditional Chinese medicines [9–11], and pharmaceutical compounds [12,13].

In general, 2D HPLC system was combined with two LC modes with different separation mechanisms, such as size exclusion or ion-exchange coupled to reversed-phase (RP) [7,8,14,15], hydrophilic interaction chromatography (HILIC) coupled to RP [16–19], and normal-phase (NP) coupled to RP [10,11,20,21]. In the available 2D HPLC systems, there still exist some problems, for instance, the mobile phase exchange [22], especially between the normal-phase and the reversed-phase. Of all the LC × LC approaches, the combination of NP and RP modes is probably the most orthogonal in nature, and also one of the most difficult to achieve for mobile phase immiscibility [23–25]. In most cases, normal-phase techniques use organic solvents, such as hexane or ethyl acetate, as mobile phases, which are difficult to combine with modes using predominantly aqueous mobile phases [14].

Several strategies were proposed in the establishment of comprehensive NPLC × RPLC. Wise and co-workers [26] used a trap column as an interface and online solvent evaporation by $N_2$ to achieve solvent exchange. However, the application of the method was restricted to the complex instrumentation and time of solvent evaporation. Murphy et al. [27] reported the method of using a water/ACN gradient to separate alcohol ethoxylates in both NPLC and RPLC system. Dugo et al. [12,20] used a microbore silica column with *n*-hexane/ethyl acetate as mobile phase in the first dimension and a monolithic type C18 column with water/ACN in the second dimension to enable the solvent compatibility of NPLC × RPLC. Guan and co-workers [10,11] developed a vacuum-evaporation loop-type valve interface in the orthogonal 2D-LC system. A vacuum pump was used to evaporate solvents in the loop of the interface under vacuum conditions, and the analytes in the loop were dissolved by the mobile phase of the second dimension and injected to the secondary column. Sandra and co-workers [28] used parallel columns in the second dimension. The features were that the separation space in the second dimension was enlarged and the

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risk of losing resolution was reduced for the increasing of fractions transferred from the first to the second dimension. Furthermore, Jandera et al. [17] developed a RP × NP system for the separation of ethylene oxide–propylene oxide (EO–PO) (co)oligomers. A C18 microbore column was employed in the first dimension with an acetonitrile–water mobile phase and the second one was HILIC with an aminopropyl silica column (APS). Also, it was proved that HILIC was highly orthogonal to RPLC based on the retention behaviors [16,18,19]. But in practice, it is difficult to online hyphenate the HILIC and RPLC columns because of the incompatibility of the mobile phases [19].

Zhengtian pill is a prepared Chinese medicine for treating headache, processed with 15 traditional Chinese medicines [29]. Its components are very complex. Thus, few reports [29–33] were published about its separation. He and Xiong [31] and Wang et al. [33] determined the content of paeoniflorin from extracts of Zhengtian pills and capsules by RPLC, respectively. Furthermore, Zheng and Ge [29] improved thin-layer chromatography (TLC) identification of *Uncaria rhynchophylla* from Zhengtian pills by optimizing extraction methods and coloration. The above reports are restricted to the determination of certain components. Our group [32] reported the separation of Zhengtian pills by 2D-LC. 733 peaks were detected. Also, that proves the complexity of Zhengtian pill extracts.

In this paper, a simple orthogonal 2D-LC system (NPLC × RPLC) based on the adjustment of mobile phases was developed by using a sample loop–valve interface. Theoretically the combination of NP and RP modes is probably the most orthogonal. Zhengtian pill, a complicated and prepared Chinese medicine, was used to evaluate the establish 2D-LC system. Several solvents were chosen as mobile phases on the basis of physical and chemical properties. Ultimately, the compatibility of mobile phases was solved by employing 1,4-dioxane in NPLC and isopropyl alcohol in RPLC. Also a higher column temperature in the second dimension was used to decrease the viscosity of mobile phases and improve their miscibility. Moreover, two tandem C18 columns were applied in the RPLC to improve the resolving power. Zhengtian pill, was used as the test sample to evaluate the constructed 2D-LC system.

## 2. Experimental

### 2.1. Chemicals and reagents

*n*-Hexane, 1,4-dioxane, ethanol and isopropanol were purchased from Kermel Chemical Reagent Co., Ltd. (Tianjin, China), and methanol was from Fisher Scientific (Waltham, USA). Zhengtian pill was supplied by Sanjiu Medical & Pharmaceutical Co. Ltd. (Shenzhen, China). The water used in the experiment was prepared by a Milli-Q system (Millipore, ELPaso, TX, USA). All solvents were HPLC grade.

### 2.2. Sample preparation

Ten grams of Zhengtian pills were crushed with a grinder, packed in a filter paper and placed in a 250 mL Soxhlet extractor. Then the samples were extracted by 100 mL ethanol for 15 h and evaporated under vacuum at 50 °C to a final volume of 10 mL. The ethanol extract was filtered through a 0.45 μm filter and stored at 4 °C for subsequent experiments.

### 2.3. Chromatographic instrumentation

NPLC × RPLC was performed on the Elite P230 HPLC System (Dalian Elite Analytical Instruments Co., Ltd., Dalian, China). The first dimension separation was carried out by a P230 isocratic system (Dalian Elite Analytical Instruments Co., Ltd., Dalian, China). The isocratic system included a high-pressure pump (P230) and a UV/vis detector (UV 230+). A P230 binary gradient system was used for the second dimension separations, containing two high-pressure pumps (P230), a UV/vis detector (UV 230+), a column oven (ZW), and a self-made mobile phase heat and cool apparatus. Other tools included two six-port valves (7725i) and a 2-position 10-port switching valve (Rheodyne, Rohnert Park, USA) with a 200 μL sample loop. Instrument control and data acquisition were performed by a chromatography data-processing workstation, and the data were processed by a software (EC2000, Elite, Dalian, China).

### 2.4. Chromatographic conditions

The first dimension separation was performed on a Hypersil $SiO_2$ column, 50 mm × 4.6 mm I.D., 5 μm $d_P$ (Elite). The column was operated in isocratic mode with *n*-hexane/1,4-dioxane (99.5:0.5) at a flow rate of 100 μL/min.

Two tandem Kromasil C18 columns, 250 mm × 4.6 mm I.D., 5 μm $d_P$ (Elite) were used in the second dimension. The mobile phase in the second dimension was isopropanol/water (2:98) (phase A) and methanol (phase B) at a flow rate of 1.0 mL/min with the linear gradient 10%B at 0 min, and 90%B at 30–40 min, 10%B at 45–60 min for the equilibrium of columns. The first dimension column temperature was ambient, and the secondary ones were thermostated at 60 °C. The injected sample volume was 20 μL in the NP mode, while the injection volume of RP mode was 200 μL with the sample–loop interface. The detection wavelength was 240 nm.

## 3. Results and discussion

### 3.1. NPLC × RPLC system

The system configuration was shown in Fig. 1. A 10-port 2-position valve and a 200 μL sample loop were used as the interface. In the first dimension, solvents were delivered by pump 1. The effluent from the first dimension was transferred from the column to the sample loop, and the analytes were stored in the sample loop (position 1). Meanwhile, the RP columns in the second dimension were in line with the pump2, column oven, and detector 2. After 2 min of the sample collection, the valve was switched to position 2, following pump 1 stopped and then the analytes in the loop were delivered to the second dimension by pump 2. The further separation was achieved by the RP columns based on different retention mechanism. The valve was switched back to position 1 after 60 min, the sample loop was ready for the next collection of analytes, and also the RP columns were ready for next separation. After 20 switches, the 2D-LC analysis data were obtained and processed by EC2000.

### 3.2. Choice of a NP column

Of all the LC × LC approaches, the combination of NP and RP modes is probably the most orthogonal in nature. NPLC is suitable for the group separation, and RPLC possesses high resolution. Considering the complexity of a traditional Chinese medicine, its group separation was carried out in NPLC followed by a high-resolution analysis in RPLC. Using a silica column in NP may lead to the best orthogonality, so a silica column is applied in NPLC. In the first dimension, we had tried gradient elution; however, the system lacked good repeatability. Therefore, isocratic elution was chosen in NPLC. Isocratic elution was optimized (data not listed). Furthermore, to obtain more information about the effect of columns on group separation in NPLC, a cyano column was investigated to the analysis of Zhengtian pill extracts. The results showed that analytes were strongly retained in a cyano column. This contributed to the non-polar substances in the sample. The first peak was eluted almost at 30 min, and the total analysis time was more than 70 min.

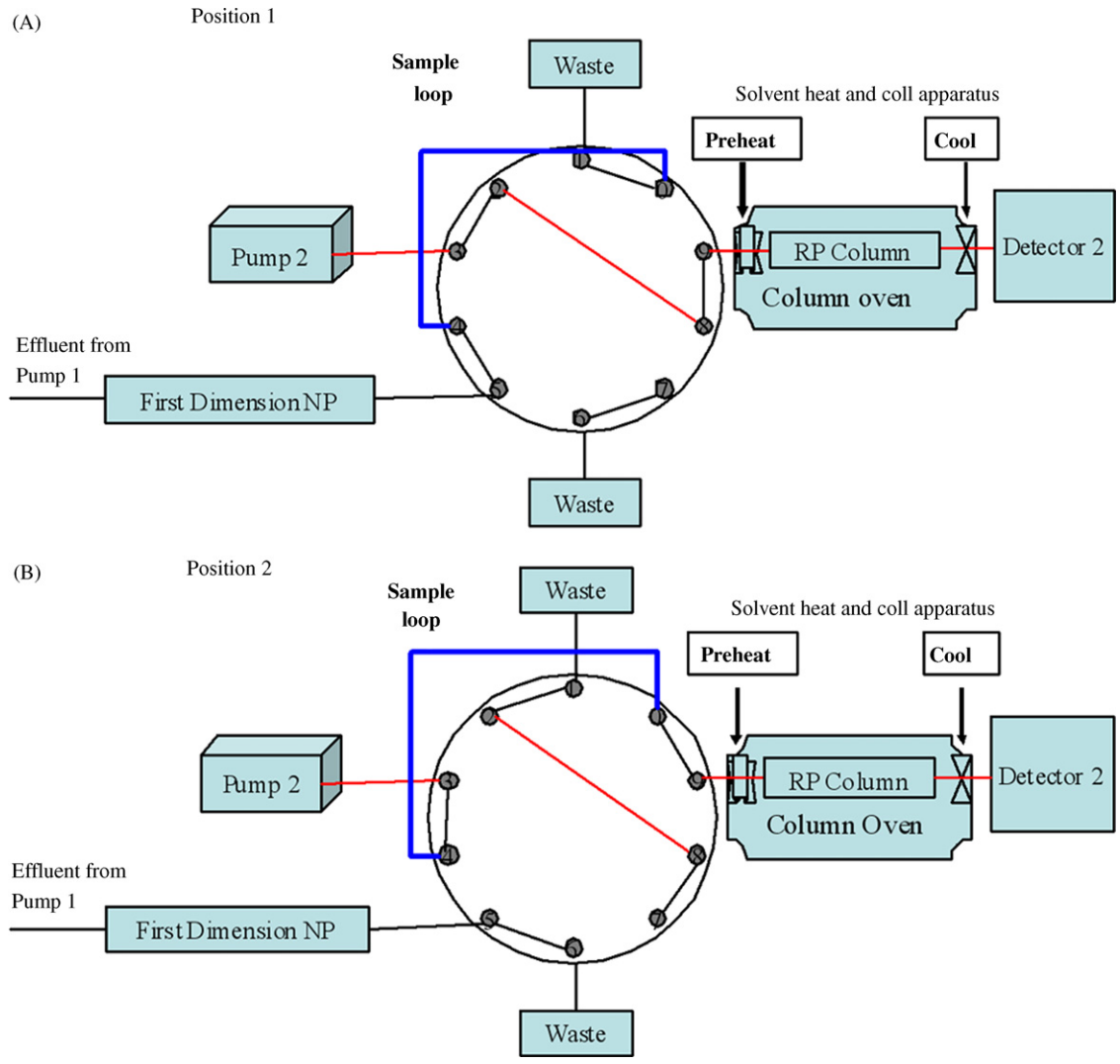


**Fig. 1.** Schematic diagram of the interface of two-dimensional LC.

However, as the column was changed to a silica column, the first peak was eluted less than 10 min, and the components were separated in a short period of time. Therefore, a silica column was chosen in NP.

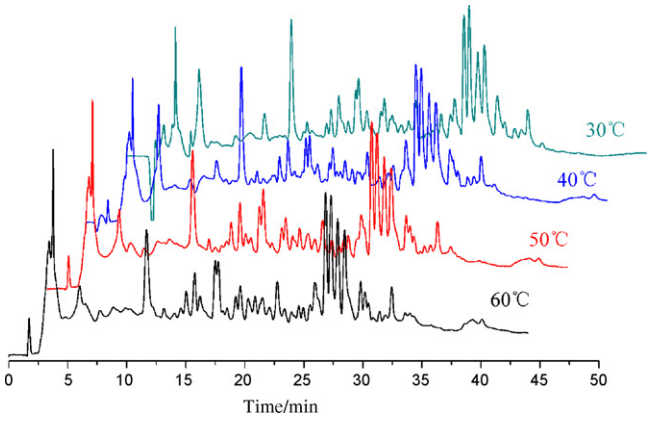


**Fig. 2.** Chromatogram of Zhengtian pills at different temperatures in RP. Separation conditions: column, Kromasil C18, 250 mm × 4.6 mm I.D., 5 μm $d_P$. Mobile phase: isopropanol/water (2:98) (phase A) and methanol (phase B), gradient: 10%B at 0 min, 90%B at 30–40 min, and 10%B at 45–60 min for equilibrium of the column. Flow rate: 1.0 mL/min. Injected sample volume: 20 μL. Detection wavelength: 240 nm.

### 3.3. Choice of mobile phases

In 2D-LC, the compatibility of mobile phases is a Gordian knot. To solve the problem, we collected and analyzed the physical and chemical parameters of a large number of solvents. And the separation was undertaken via comparison of different solvents. 1,4-Dioxane was selected as a solvent modifier in NPLC. 1,4-Dioxane is a typical water-soluble non-polar solvent with a moderate viscosity. 1,4-Dioxane possesses a special structure, which could form intermolecular hydrogen bond between dioxane and RPLC mobile phases. In NPLC, hexane, alone or with small quantities of more polar solvents, is the most frequently used mobile phase. In the present work, several solvents [34] (ethyl acetate, isopropyl alcohol and 1,4-dioxane) mixed with hexane were investigated to test the compatibility of mobile phases in NPLC and RPLC. The results showed that a mixture of 1,4-dioxane and hexane as mobile phases was more efficient due to the special structure of 1,4-dioxane. The concentration of 1,4-dioxane has effects not only on the separation results but also compatibility of 2D-LC. Subsequently, different proportions (0.5%, 1%, 2%, 5%, 10%) of 1, 4-dioxane were investigated in the analysis of Zhengtian pills. Better results were achieved using 0.5% (v/v) of 1,4-dioxane, allowing a good separation and peak capacity of Zhengtian pills. Therefore, we chose *n*-hexane/1,4-dioxane (99.5:0.5) as the NP mobile phase. Although the use of isopropyl alcohol (IPA) is limited in HPLC for its high vis-

cosity, it is a good solvent for RP and NP since it is miscible with both types of solvents. Furthermore, the viscosity can be decreased at a higher column temperature, so isopropyl alcohol was applied in the second dimension.

### 3.4. Effect of column temperature in reversed-phase chromatography

In general, the total analysis time of the 2D-LC is limited by that of the second dimension. Therefore, it is necessary to improve the speed of the second dimension. Elevating the column temperature is one of the most effective approaches. The elevation of temperature leads to the decrease of viscosity of mobile phases and back pressure in RPLC, which allows a longer column and higher eluent linear velocity through the column [35]. Also, high column temperatures can improve the compatibility of the two-dimensional (NP and RP) mobile phases. Furthermore, a change of temperature can be used to control sample retention.

Since Zhengtian pills consist of 15 traditional Chinese medicines, their extracts are very complex. The effect of column temperature in RPLC was investigated by using an ethanol extract of Zhengtian pills as a test sample, as shown in Fig. 2. As a result, high temperatures facilitate reduction of analysis time and gradient cycle time. When the column temperature increased from 30 °C to 60 °C, the reduced analysis time was more than 5 min. Taking into account 20 times of the switch, the total analysis time could be shortened by more than 1 h.

Moreover, numbers of peaks at different temperatures were achieved by EC2000. When the column temperature was lower than 60 °C, the peak numbers increased dramatically. However, the trend of increased peaks turned slow when the column temperatures were higher than 60 °C. Considering composition of Zhengtian pills, the elevation of the column temperature favored the elution of the substances from a RP column. However, if a conventional RP column was used at a temperature higher than 60 °C for a long time, the column life would be shortened. Thereby, 60 °C was selected as the optimal RPLC column temperature.

### 3.5. Effect of column length on RP analysis

In NPLC × RPLC, the resolving power of RPLC plays an important part in the achievement of high peak capacity. There are many methods to improve the resolving power of RPLC, but using a longer column is undoubtedly much easier than others to realize. Furthermore, as described above, high temperature enables the decrease of viscosity and back pressure in RPLC, which allows using a longer column to obtain higher resolution. Herein, we want to search for an appropriate column length for the separation of Zhengtian pills under an acceptable back pressure, as well as separation time in the conventional HPLC instruments. Therefore, 4 different column lengths (150 mm, 200 mm, 250 mm and 500 mm including two 250 mm columns in series) were examined at 60 °C in RPLC analysis. An ethanol extract of Zhengtian

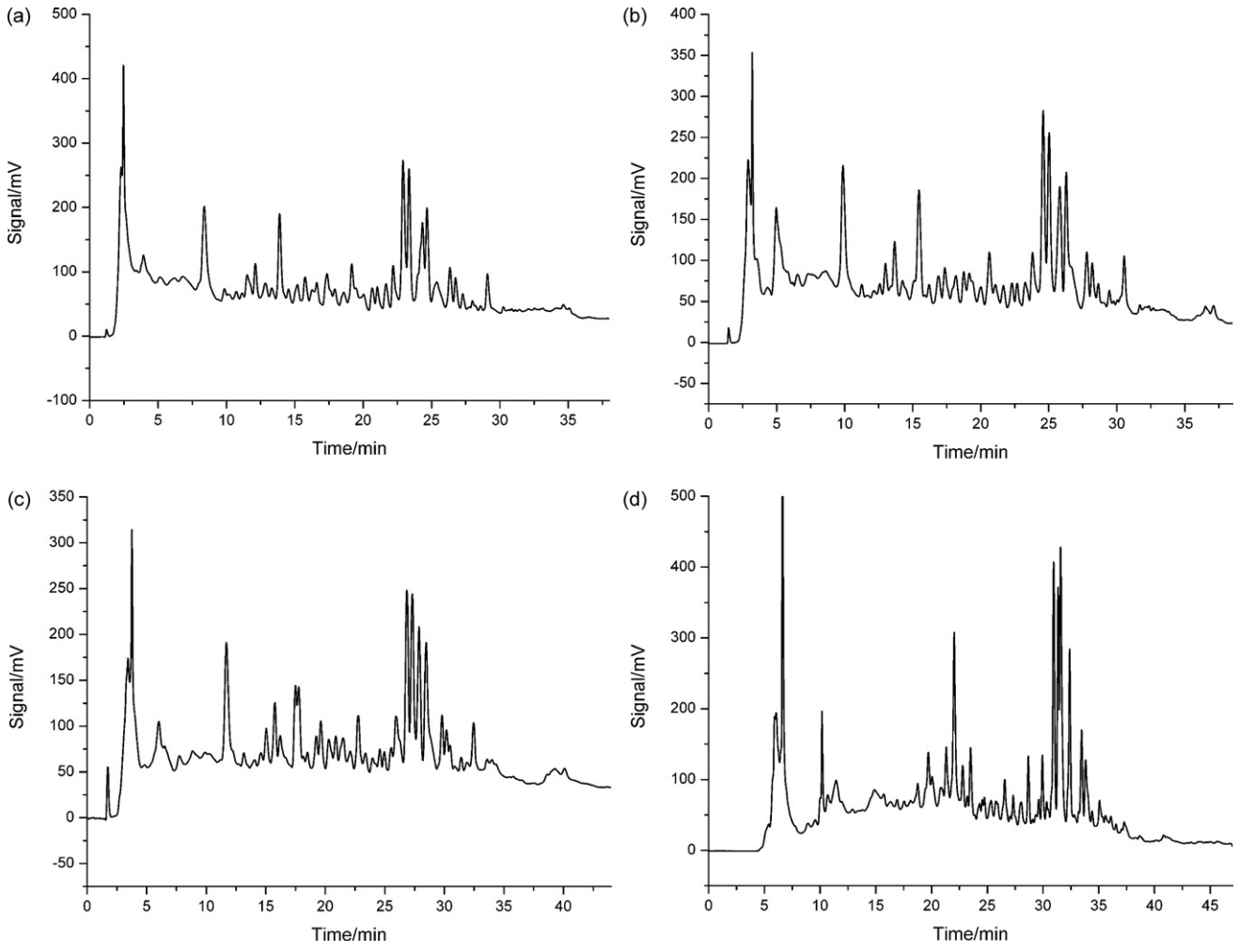


**Fig. 3.** Chromatograms of different-length RP columns separation of Zhengtian pills. (a) 150 mm; (b) 200 mm; (c) 250 mm; (d) 500 mm. Separation conditions: column, Kromasil C18, 4.6 mm I.D., 5 μm $d_P$. Column temperature: 60 °C. Other conditions see Fig. 2.

pills was used as the test sample (chromatograms shown in Fig. 3).

The detected peak numbers increased significantly with the raise of column length. As two tandem 250 mm columns were used in RPLC, 78 peaks were achieved, which was far more than that of a single 250 mm column (64 peaks). Furthermore, as the tandem columns were used in RPLC at 60 °C, the back pressure of the system was 17–19 MPa which was close to the back pressure of a conventional 250 mm column in RPLC analysis. Thereby, the tandem columns could be used for the separation of complex samples.

### 3.6. Application of the 2D-LC based on the modification of mobile phases

A traditional Chinese medicine always contains hundreds or thousand components, so it would be a laborious and even impossible mission to separate these complex samples by means of simple one-dimensional HPLC. In theory, assuming that the peaks of a chromatogram distribute uniformly and the resolution achieves 1.5, the separation of 500 peaks would require the theoretical plate number of 2 million in a conventional one-dimensional HPLC. This far exceeds the separation ability of the conventional one-dimensional HPLC at present. In view of this, the separation of complex samples requires the use of two-dimensional or even multi-dimensional liquid chromatography to provide sufficient peak capacity and high selectivity.

In our previous report [32], a NPLC × RPLC system based on the adjustment of mobile phases were established with a 250 mm Kromasil C18 column in the second dimension to test the ethanol Zhengtian pill extract. It was found that 733 peaks were detected and the peak capacity reached 1470. Therefore, the NPLC × RPLC system showed a good resolving power for analysis of Zhengtian pills. In order to obtain more peaks and better peak capacity, a tandem-column NPLC × RPLC system were built, in which two 250 mm Kromasil C18 columns were used in series in the second dimension. Also, the ethanol Zhengtian pill extract was employed again to evaluate the tandem-column NPLC × RPLC system. The separation of Zhengtian pills on the tandem-column 2D-LC was shown in Fig. 4. As a result, 876 peaks were detected, and the total peak capacity reached 1740.

Comparing with Fig. 2 and Fig. 3, the distribution of peaks in Fig. 4 is much more uniform, and the phenomena of the peak overlap are improved greatly. In conclusion, the tandem-column 2D-LC system is capable of separating complex samples, and can provide higher resolving power.

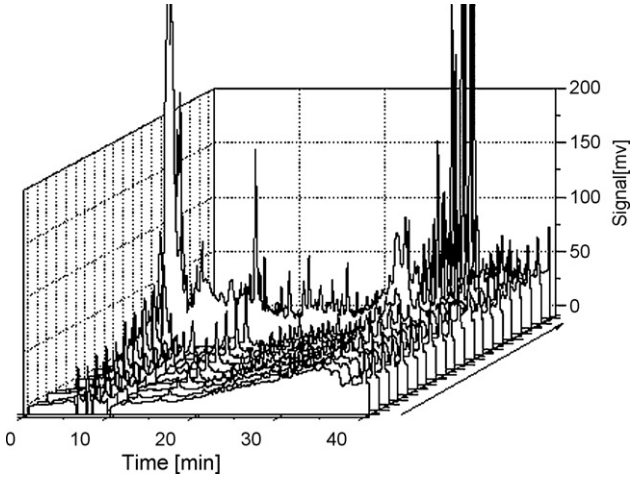


**Fig. 4.** Chromatogram of tandem-column NPLC × RPLC separation of Zhengtian pills. Conditions: NPLC, column, Hypersil $SiO_2$, 50 mm × 4.6 mm I.D., 5 μm $d_P$; mobile phase, *n*-hexane/1,4-dioxane (99.5:0.5); flow rate, 100 μL/min; injected sample volume 20 μL; column temperature, ambient; UV at 240 nm. RPLC, tandem columns, Kromasil C18, 250 mm × 4.6 mm I.D., 5 μm $d_P$; other conditions see Fig. 3.

## 4. Conclusions

The compatibility of mobile phases is one of the most difficult problems in orthogonal two-dimensional liquid chromatography (2D NPLC × RPLC), and the speed of the second dimension is the chief limitation of the total speed of a 2D-LC. To solve the problems, new strategies were proposed, which were an application of special solvents as mobile phases and elevation of column temperature in RPLC. As a result, 1,4-dioxane was selected as the modifier of hexane in NPLC, and isopropyl alcohol was used in the RPLC. Based on the adjustment of mobile phases, an orthogonal comprehensive two-dimensional NPLC × RPLC system was established. Furthermore, the elevation of the column temperature decreased the viscosity of solvents which enabled a lower back pressure and a longer column in the RPLC to obtain higher resolving power. Two tandem columns were used to the RPLC. A traditional Chinese medicine, Zhengtian pill, was used as a test sample to evaluate the constructed 2D-LC. As a result, 876 peaks were detected, and the total peak capacity reached 1740. In conclusion, the system was easy to operate and might be applied for routine analysis of complex samples, such as, traditional Chinese medicine extracts and samples. Meanwhile, the proposed strategies could be used for reference in the construction of other 2D-LC.

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