**Improvement of interface in comprehensive two-dimensional liquid chromatography and its application in the research of proteomics**

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Abstract: A two-dimensional liquid chromatography (2D-LC) system was constructed with improved trap column interface. Weak anion exchange (WAX) LC was used as the first dimension separation mode and reversed-phase (RP) LC as the second dimension. Two 35 mm long columns, column 1 and column 2, were used as trap columns to retain the fraction from the first dimension and forward-flushing was employed to pre-separate the components when the trap column was connected to the second dimension. The interface greatly increases the efficiency of the second dimension column without losing the separation speed. Rat serum sample was separated on this system to evaluate the performance of the constructed system. The viscous fingering (VF) phenomenon was generated due to the difference in the velocities of the mobile phases of the two dimensions. The separation efficiency was theoretically increased by 70% when the 35 mm trap columns were used in the forward-flush mode.

Key words: comprehensive two-dimensional liquid chromatography, improved trap column interface, rat serum, proteomics

CLC number O658 Document code A Article IC 1000-871 201002-0163-05
In recent years, the comprehensive two-dimensional liquid chromatography (2D-LC) has gained much attention for the separation of complex samples in various fields of research. Several 2D-LC systems have been developed through the past over a decade, 3–5. Column switching between the two dimensions is a delicate operation to design and implement for its functions of connections, valve storage and enrichment, 6.

There are several kinds of interfaces, 7,8, have been used in online 2D-LC system like sample loop parallel column and trap column interface. Sample loop interface is widely used in 2D-LC system. However, small loop volume limits the flow rate and column dimension of the first dimension. The use of parallel column in the second dimension columns can carry out several second dimension separations simultaneously, 9. Although this improves the overall speed of the 2D-LC analysis, it requires more elaborate instrumentation and causes difficulty in the data analysis, 10,11.

The trap column interface, 12, refers to packing suitable adsorbent in the sample loop to collect the fractions eluted from the first dimension column and storing them for periodic re-injection into the second dimension column. While one column traps the components at its head, the other column back-flushes to induce the components to the second dimension separation, 6,13.

Except for the various combinations of the two dimensions, most systems emphasized the separation speed of the second dimension, 14,15. Several kinds of the fast separation fast gradient elution or/and short column, were used in the second dimension in the online 2D-LC system, 16–19. However, the second dimension could achieve high separation speed but lose efficiency when a shorter column was used. In this paper, a longer trap column was employed to construct an interface that allowed increased separation efficiency in the second dimension without affecting the valve switching frequency. The components could be pre-separated through interface before entering to the second dimension. The rat serum sample was used to evaluate the performance of the 2D-LC system.

1 Experiment

1.1 Chemicals and reagents

All of the water used in the experiment was prepared using a Sartorius Arium 611 System. Sartorius, Germany. Trifluoroacetic acid, 99% was obtained from J & K Chemical, Beijing, China. Acetic acid, sodium acetate and NaCl of analytical grade were purchased from Sinopharm Chemical Reagent Co. Ltd., Shanghai, China. Acetonitrile, HPLC gradient grade, was purchased from Xingke Shanghai Xingke Biochemistry Co. Ltd., Shanghai, China. Rat serum sample was kindly donated by Laboratory of Systems Biology, Institutes of Biomedical Sciences, Fudan University. Rat blood was centrifuged at 5000 r/min for 10 min the upper layer was taken as the sample.

1.2 Instrumentations

For the first dimension separation, a standard Elite 230 Series chromatographic instrument controlled by EC2000 Chemstation software version v1.5.5, Elite HPLC Ltd., Dalian, China was used. This instrument was equipped with Model 7725 injection valve, Rheodyne USA, with a home made 300 μL sample loop and a binary pumping system. A weak anion exchange, WAX, column, AX 250 mm × 4.6 mm 5 μm BioBasic was used as the first dimension. A reversed-phase, RP column, ODS-BP 50 mm × 4.6 mm 5 μm, SinoChrom was chosen as the second dimension. Independent binary pump system identical to first dimension was used. Two RP columns, ODS-BP 35 mm × 4.6 mm 5 μm, SinoChrom were connected by 10-port valve Model EV750-102 Rheodyne USA through 0.01” i.d. PEEK tubing to serve as trap column. The UV detector was set at the end of the second dimension column.

2 Results and discussion

2.1 Principal of the interface

In order to increase the speed of the second dimensional separation, fast separation of second
dimension is often used in the 2D-LC systems. Although it increases the valve switching the frequency it also causes column efficiency loss. In our work to acquire the enhanced column efficiency and maintain the separation speed longer trap columns were employed and were always flushed forward whatever in the loading status or elution status. The trap columns had two functions one was to trap the elution the other was to pre-separate the components retained on the column. The principles of improved trap column interface are shown in Fig. 1.

![Diagram of 2D-LC system](image)

**Fig. 1 Principles of the interface in 2D-LC system**

Column 1 and 2 served as the trap columns and had the function of pre-separation. At position 1 column 1 was connected to the first dimension. The fractions from the previous dimension were captured by column 1 and retain at the head of the column. Solvent of the first dimension was drained to the waste directly. When changed to position 2 the column 1 was connected to the second dimension directly. Increased organic solvent gradient from the pump system eluted the column 1 and second dimension column in series. Component enriched at the head of column 1 in position 1 got retention at column 1 and the second dimension column and were eluted by the organic solvent gradient gradually. At position 2 column 1 performed the function of pre-separation. Simultaneously column 2 plays the role of trap column. When the separation of the second dimension completed the valve was switched to the position 1 and column 2 was eluted by the pump system and the circulation process started.

The pre-separation was carried out through the longer trap column before components arrived the second dimension. The switching frequency of the valve can increased the separation efficiency of the second dimension.

### 2.2 Chromatographic conditions

Before the 2D-LC analysis the rat serum was investigated by the one dimensional mode. The WAX column and the RP column were used and conditioned to get the optimized chromatographic parameters. The chromatograms of the one dimensional separation are shown in Fig. 2. In Fig. 2a only a few peaks are recognized in the ion exchange chromatography IEX separation mode which did not give a powerful resolution. The same situation exists in the RP separation in Fig. 2b. However the one dimensional separation was restricted by its lower capacity.

![Chromatograms](image)

**Fig. 2 One dimensional separations of a rat serum sample a. IEX separation b. RP separation**

Conditions a. BioBasic AX column 250 mm × 4.6 mm i.d. 5 μm mobile phases A [20 mmol/L Tris-HCl pH 7.5] and B [20 mmol/L Tris-HCl 3 mol/L NaCl] flow rate 0.2 mL/min gradient elution [0 – 60 min] 0% B – 60% B detection wavelength 280 nm b. SinoChrom ODS-BP column 250 mm × 4.6 mm i.d. 5 μm mobile phases A [water containing 0.1% TFA] and B [acetonitrile containing 0.1% TFA] flow rate 1 mL/min gradient elution [0 – 8 min] 30% B – 40% B [8 – 40 min] 40% B – 60% B detection wavelength 280 nm.
2.3 2D-LC separation of rat serum sample

When the 35 mm column was used as the trap column to perform the pre-separation function, the separation power of the second dimension, which was 50 mm long, would theoretically increase by 70%. Because of the volume of the mixer and the trap column which generated a delay time, the time of mobile phase of the initial gradient reaching the separation column was postponed by 2.5 min. As a result, the valve was switched when the gradient had passed 2.5 min and data acquisition started at the same time. The modulation time of the second dimension was 5 min. The chromatograms of rat serum samples separated on this 2D-LC system are shown in Fig. 3.

![2D-LC chromatograms of aqueous extract of rat serum samples](image)

Conditions: first dimension BioBasic AX column 250 mm × 4.6 mm 5 μm mobile phases A 20 mmol/L Tris-HCl pH 7.5 and B 3 mol/L NaCl 20 mmol/L Tris-HCl flow rate 0.2 mL/min gradient elution 0 – 60 min 0% B – 60% B. Second dimension SinoChrom ODS-BP column 50 mm × 4.6 mm 5 μm mobile phases A water containing 0.1% TFA and B acetonitrile containing 0.1% TFA flow rate 1 mL/min gradient elution 0 – 2.5 min 30% B – 60% B 2.5 – 5 min 0% B detection wavelength 280 nm.

A total of 16 fractions of the first dimension were introduced to the second dimension. The negative peak between 0.8 min and 1.6 min attributed to the UV absorbance of the mobile phase captured in the trap column. When comparing with the traditional trap column interface, the elution from 0 – 0.8 min was additional separation power provided by the forward-flushed longer trap column.

According to Shalliker’s report, the viscous fingering VF phenomenon was generated from the velocities difference of the mobile phases of the two dimensions in the region they contacted directly. When the viscosity of injection plug was bigger than that of the mobile phase VF generated at the rear of the injection plug. On the contrary, VF would be generated at the head of the injection plug. Since the mobile phase in the first dimension was pure buffer whose viscosity was bigger than that of mobile phase of the second dimension so the VF generated at around 1.6 min. Mass transfer in direct contact regions was very slow. The intact region between the mobile phases of two dimensions was just like the moving reaction boundary. So the desalting of the fraction and re-equilibrium of the column can be avoided. Fast gradient elution of the second dimension can be carried out directly.

3 Conclusion

A WAX-RP 2D-LC system was constructed with improved trap column interface. This interface greatly increases the efficiency of the second dimension column without affecting the separation speed. When 35 mm trap columns were used comparing to a 50 mm second dimensional column the separation efficiency was theoretically increased by 70%. Primary separation of the rat serum sample was used to analysis and evaluate the utility of the system. The VF phenomenon was generated attributing to viscosity of the mobile phases of the two dimensions. This interface would be an alternative selection to the traditional trap column interface in 2D-LC system for the separation of complex mixture in proteomics. Based on the improved interface the WAX-RP 2D-LC system also could be applied in the other areas such as the research of traditional Chinese medicines.

References

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