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RESEARCH ARTICLE

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Study of two-dimensional liquid chromatography with high temperature NPLC and room temperature RPLC

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Abstract To overcome the peak band broadening and to increase the peak capacity and separation efficiency of a two-dimensional liquid chromatographic system, a high-temperature normal phase liquid chromatography (HTNPLC) was used as the first dimension (1st-D), and a RPLC was used as the second dimension (2nd-D). The sample was first separated on the 1st-D CN column and the primary eluent stored in the sampling-loop system alternatively (in HTNPLC×RPLC mode) or selectively (in HTNPLC/RPLC mode) and was then transferred to 2nd-D C_{18} column for further separation. The resolution and separation efficiency of the systems were greatly improved. The systems were evaluated by analyzing several polycyclic aromatic hydrocarbons and *Glycyrrhiza uralensis* extract.

Keywords two-dimensional liquid chromatography, HTNPLC /RPLC, sample loops- valve interface, traditional Chinese medicine, *Glycyrrhiza uralensis*

1 Introduction

Direct coupling of NPLC and RPLC without split has been a difficult task since the middle of 1970s due to the high organic solvent content and large volume of primary elution that was injected on the head of the secondary column, causing severe peak dispersion. The peaks insufficiently separated on the primary column still were overlapped on the secondary column. A solute band compression could be used if the elution strength of the mobile phase on the secondary column is stronger than that of the mobile phase in the primary column. In reality, however, such a stratagem will sacrifice the separation efficiency of the secondary column. To solve the problem, the elute from the primary column was diluted by water before injecting onto the secondary column, as reported in literatures, and a split injection has to be adopted. A solute band compression and peak capacity of the secondary column remained in this way, except for the severe sample loss from the 1st-D [1–3].

The drawbacks of the method is that if the diluted elute from the 1st-D was injected directly into the secondary column, the injection volume from the primary onto the secondary column was too large than that of the maximum allowable injection volume of the secondary column. It is not unusual to have distorted peaks because of the high water content of injected solution and the high organic solvent content in the mobile phase of the 2nd-D.

In this study, a high-temperature NPLC was used as the first dimension (1st-D). According to the relation of the retention factors and temperature, an increase of column temperature leads to a decrease in retention [4, 5] and at 225°C the dielectric constant of water was close to that of acetonitrile [6]. Namely, the eluting strength of water was enhanced at elevated temperature and thus, a lower organic solvent content of mobile phase could be used at higher temperature. In this way, low organic solvent content of mobile phase of the primary column could be realized, however the injection volumes of the secondary column were not increased.

In this study, two-dimensional liquid chromatography (HTNPLC×RPLC) systems were constructed by using high-temperature NPLC as the first dimension (1st-D) and a RPLC as the second dimension (2nd-D) with a sample loops-valve interface. The performances of the systems were evaluated by analyzing several polycyclic aromatic hydrocarbons and *Glycyrrhiza uralensis* extract.

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2 Experimental

2.1 Reagents and instruments

Acetonitrile and methanol were purchased from Merck (Merck Company, Darmstadt, Germany). The water used in the experiment was prepared from Wahaha purified water (Wahaha Group, Hangzhou, China). Other chemicals and reagents were analytical grade. Phenanthrene, Carbamazepine, and Phenobarbital were purchased from J & KacrosOrganics (Beijing, China). *o*-Dinitrobenzene was purchased from CMBH & CO (Germany). Benzoic acid (purity 99.15%), Benzaldehyde (purity 98.15%), *o*-Nitrophenol (analytical grade), trichlorophenol (chemical grade), nitrobenzene (chemical grade), and Naphthalene (chromatogramphic grade) were purchased from local companies. *Glycyrrhiza uralensis* was purchased from Meiluo medicine store (Dalian, China).

An Ultra-Plus II liquid chromatograph (MicroTechScientific, USA) was used in the 1st-D with isocratic elution. A binary gradient pump system (Elite P230, Dalian Elite Analytical Instrument) delivered the secondary eluent of the 2D-LC system. Detector 1, CE-1575 UV/VIS Detector (Jasco, Japan), was used for the first dimension detection; detector 2, UV-VIS LC-830 Detector (Soma Optics, Japan), was used for the second dimensional detection.

Column thermostat was laboratory made. Column temperature was controlled by a Model REX-C100 controller (RKC Instrument Inc., Japan) and monitored by Pt-100 (Juchheim, GmbH, Germany).

Chromatogramphic columns used in the experiment were as follows: Normal-phase column 1:200 mm× 0.53 mm i.d., fused silica column packed with 5 μm CN particles (Macherey-Nagel, Germany), and column 2:150 mm×0.53 mm i.d., fused silica column packed with 5 μm CN particles of the same packing, was used in the first dimension in normal-phase mode; Reverse-phase column 3:100 mm×0.53 mm i.d., fused silica column packed with C_{18} (Nuclesil), and column 4:50 mm× 4.6 mm i.d., monolithic column (Merck company, Germany), was used in the second dimension in reversed-phase mode.

2.2 Preparation of samples

One gram of *Glycyrrhiza uralensis* was crushed with a grinder and immersed in 5 mL 80% methanol (volume content) for 1 h and then heated to boiling condition for 3 h. The cooled mixture was then centrifuged (3000 r/min) for 15 min. The supernatant was filtered through a 0.45-μm pore filter and stored in a refrigerator. It was diluted 5 times by methanol/water (50/50, *V*/*V*) before usage.

2.3 HTNPLC×RPLC system

The comprehensive liquid chromatographic (HTNPLC× RPLC) system was constructed by using two loops and a ten-port two-position valve interface, as shown in Fig. 1. Effluent from the first dimension is delivered to the storage loop 1 on the ten-port two-position valve (step 1), while pump 2 deliveries mobile phase through the storage loop 2 entering the 2nd-D RP column and then the detector in the second dimension. The ten-port valve was then switched to step 2, and the analytes retained inside loop 1 were flushed by the mobile phase from pump 2 onto the RP column. Those compounds in loop 1 were subjected to a further separation. Effluent from the 1st-D was transferred to the loop 2 during this period. The fractions from the primary column were transferred alternately to the two loops and then injected onto the 2nd-D column through the ten-port valve switching, realizing an HTNPLC× RPLC system.

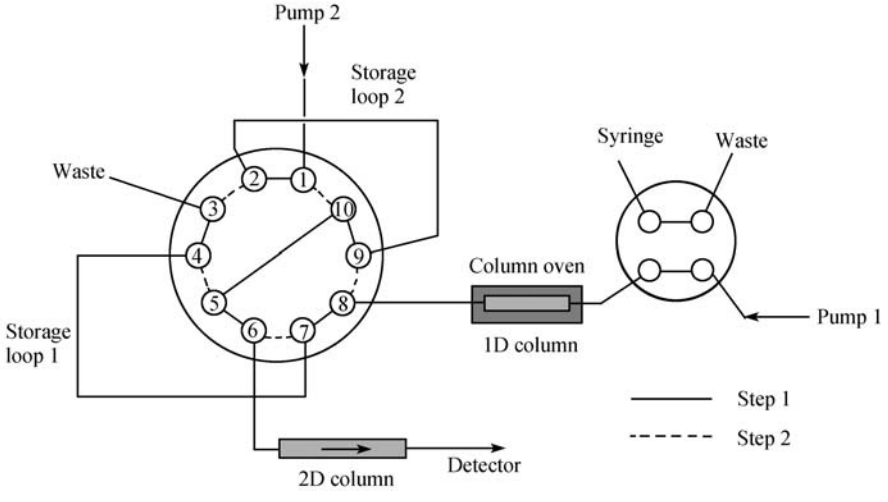

Fig. 1 Schematic diagram of a high-temperature comprehensive two-dimensional liquid chromatograph (HT 2D-LC) system

3 Results and discussion

According to the theory of multidimensional chromatography [7], an orthogonal 2D-LC system can be realized by using two kinds of LC who has difference retention mechanisms. The peak capacities and selectivity of the whole system will reach maximum when true orthogonal 2D-LC is achieved.

3.1 Separation by NPLC as the first dimension

For conventional HPLC, the cold mobile phase is preheated before entering the column in order to reduce temperature gradient within the column. The elute from the 1st-D is then cooled down and passed through the switching valve. However , for the capillary column HPLC, only column and entrance union heating is sufficient because of the very low flow rate of mobile phase, low heat capacities of column and unions, and small size in diameter. Both the radial and axial temperature gradient is very small compared with conventional columns. It was not essential for the cooling of effluent from the primary column because of the low volume and flow rate of mobile phase that reaches room temperature before arriving to detector. Therefore, it is more convenient to manipulate the column temperature in capillary column LC than in conventional column LC.

We investigated the influence of different column temperature on the separation of several polycyclic aromatic hydrocarbons by one-dimensional NPLC (Table 1). The data in Table 1 prove that the higher the column temperature, the stronger the elution strength of the mobile phase. At an elevated temperature, the elution speed of LC was also faster. The influence of temperature on retention of compounds depends on the properties of compounds and the mobile phase composite. The temperature-retention relationship at water-acetonitrile mobile phase is similar to that of water-methanol mobile phase. At elevated column temperature, we found that for every 3°C increase in temperature, there is a corresponding 1% equivalent increase of acetonitrile (volume content) in the mobile phase for polar compounds. While for nonpolar compounds, for every 4°C increase of column temperature, there is a corresponding 1% increase in acetonitrile (volume content) in mobile phase. The experimental results reveal that high-temperature NPLC with low organic phase content in mobile phase has more influence on separation of polar or weak polar compounds. Fig. 2 was the chromatograms of several polycyclic aromatic hydrocarbons by one-dimensional NPLC at normal and at high temperature, respectively. Most of polar and weak polar compounds eluted faster than nonpolar compounds at 80°C with a mobile phase of acetonitrile/water (30/70, *V*/*V*) than that at room temperature with mobile phase of acetonitrile/water (60/40, *V*/*V*), although the elution strength of mobile phases of the former at 80°C was weaker than that of the later at room temperature. In addition, the separation efficiency at 80°C was reasonable. The following NPLC experiments were carried out at 80°C with acetonitrile/water (30/70, *V*/*V*) as mobile phase.

3.2 Evaluation of HTNPLC /μ_RPLC

Two-dimensional liquid chromatographic system using HTNPLC as the first dimension (1st-D) was investigated. When heart-cutting mode was used, a μRPLC was chosen as the second dimension. The selected elutes were cut onto the second dimension (2nd-D) one by one for further separation. The total analysis time is then the sum of each analysis time. Due to the identical inside diameter of the two columns used, the advantages of HTNPLC were fully demonstrated. Fig. 3 showed the chromatograms of 2D separation either by HTNPLC/μRPLC or room temperature NPLC/μRPLC. The former mode utilized a solute band compression on the 2nd-D due to the low organic solvent content of primary elute. While the later mode suffered from high organic solvent content of primary elute, the separation on 2nd-D yield band broadening and distorted peaks, and deterioration of separation efficiency and detection sensitivity (see Table 2, 3). Though the peak broadening could be reduced by operating the 2nd-D at

Table 1 Retention factors of various analytes under different separating column temperatures*

Analyte	Retention factor *k*						
	20°C	30°C	40°C	50°C	60°C	70°C	80°C
Nitrobenzene	0.49	0.48	0.42	0.37	0.33	0.31	0.27
Benzoic acid	0.27	0.26	0.23	0.21	0.20	0.17	0.15
Trichlorophenol	0.75	0.69	0.62	0.55	0.51	0.45	0.39
Benzaldehyde	0.32	0.31	0.26	0.25	0.22	0.21	0.19
Naphthalene	1.15	1.08	0.89	0.78	0.65	0.60	0.52
o-Nitrophenol	0.42	0.38	0.37	0.34	0.30	0.32	0.28
Phenanthrene	2.56	2.25	1.19	1.58	1.33	1.28	1.19
o-Dinitrobenzene	0.77	0.67	0.56	0.51	0.44	0.43	0.36

* Chromatographic conditions: CN column (200 mm×0.53 mm i.d., 5 μm, Macherey-Nagel, Germany); Mobile phase, 50% (by volume) methanol with 0.1% formic acid – 0.1% aqueous solution; Flow rate, 5 μL/min; Detection wavelength, 240–254 nm

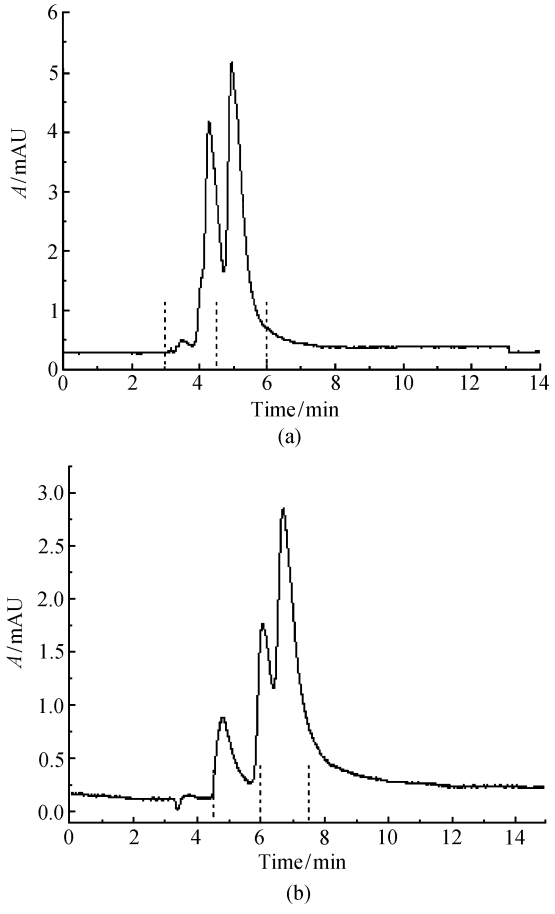

Fig. 2 One dimensional (1-D) normal phase LC (NPLC) separation of standard compounds at room temperature, 22°C (a); and at high temperature, 80°C (b)
conditions: a) CN column (150 mm×0.53 mm i.d., Macherey- Nagel), acetonitrile/water (60/40, *V*/*V*), flow rate, 8 μL/min, column temperature, 22°C, detection wavelength, 254 nm; b) Acetonitrile/water (30/70, *V*/*V*), column temperature, 80°C, the other conditions were the same as (a).

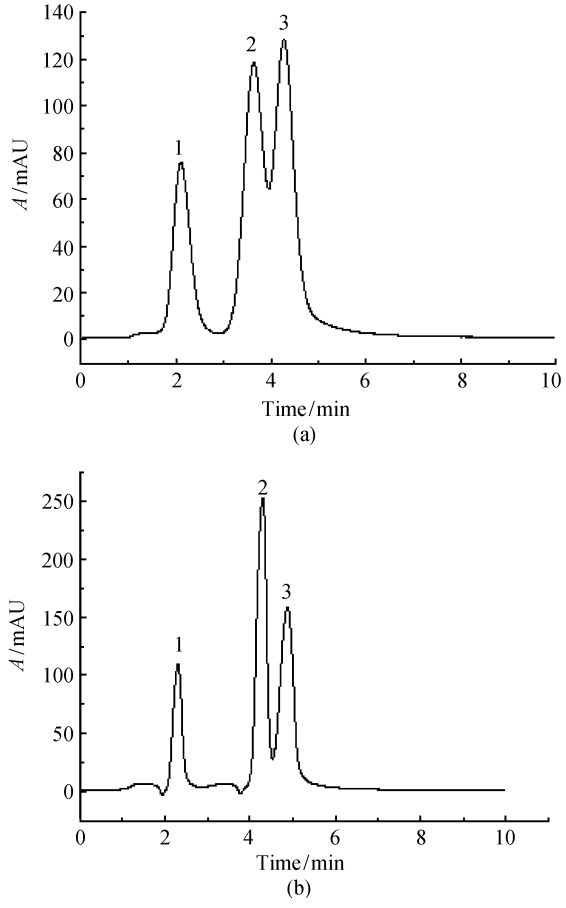

Fig. 3 Chromatograms of standard compounds from 2-D separation at room temperature NPLC/μ_RPLC (a) and at HTNPLC/μ_RPLC (b)
conditions: a) The 1st-D, 150 mm×0.53 mm i.d., 5 μ CN column, acetonitrile/water (60/40, *V*/*V*), flow rate, 8 μL /min; The 2nd-D, 100 mm ×0.53 mm i.d., 5μ C_{18} column (Nuclesil) , mobile phase, water (C)/acetonitrile (D) , linear gradient elution, 60%D to 70%D in 10 min, flow rate, 15 μL/min; Detection wavelength, 254 nm; Column temperature, 22°C; The transferred fractions see Fig. 2(a); b) 1st-D, mobile phase, acetonitrile/water (30/70, *V*/*V*); Column temperature, 80°C; The other conditions were the same as (a) ; The transferred fractions see Fig. 2 (b); Peak identities: 1) Phenobarbital; 2) *o*2Dinitrobenzene; 3) Carbamazepine

higher linear flow rate, the resulting peak width was still larger than that obtained in former mode. We conclude from the above comparative study that the separation efficiency of 2nd-D was not affected by the elution from HTNPLC due to the low organic solvent content of the mobile phase used. All peaks from 2nd-D were sharp and there were not visible peak broadening effect.

3.3 Separation by HTNPLC×RPLC

A monolithic column was used as 2nd-D when the system was configured as HTNPLC×RPLC. The separation speed of the monolithic column was about five times faster than packed column. Fig. 4(a) was the chromatogram of standards separated by NPLC×RPLC at room temperature, and the chromatogram in Fig. 4(b) was obtained by HTNPLC×RPLC system. The separation conditions of

Table 2 Separation results obtained by NPLC/μ_RPLC with NPLC at room temperature.

2nd-D flow rate μL·min^{-1}	Retention time /min	Half-peak width W_h /min	Resolution R_s	Peak area *A* mV·s
	2.10	0.400		1836
15	3.64	0.544	0.69	3368
	4.28	0.551		3863
	1.64	0.337		2009
20	3.19	0.420	0.73	3038
	3.70	0.409		4681

Table 3 Separation results obtained by HTNPLC/μ_RPLC system

2^{nd}-D flow rate μL·min^{-1}	Retention time /min	Half-peak width W_h /min	Resolution R_s	Peak area A/ (mV·s)
	2.31	0.237		1515
15	4.29	0.262	1.12	3709
	4.87	0.348		3830
	1.86	0.177		1654
20	3.70	0.206	1.02	4231
	4.11	0.268		3681

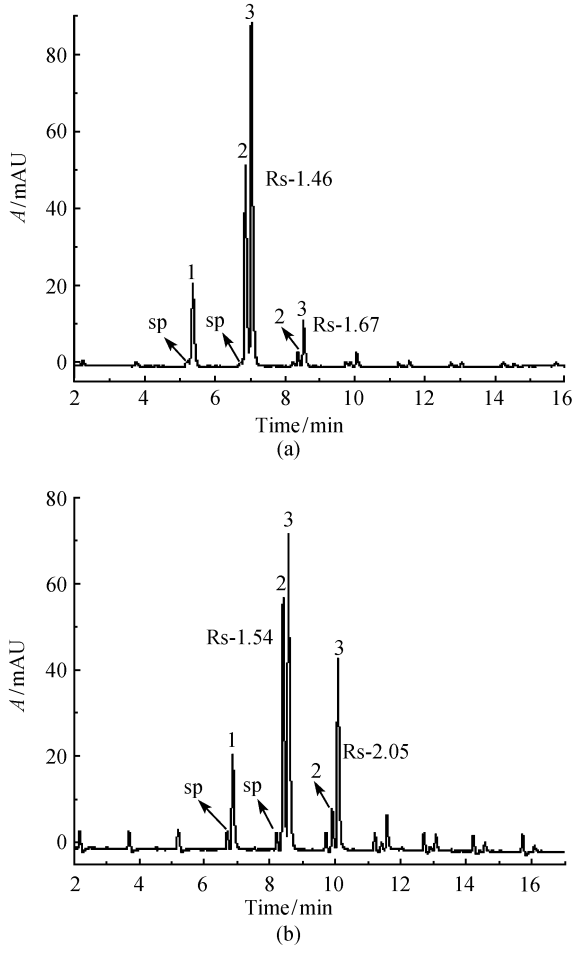

Fig. 4 Chromatograms of 2^{nd} D separation of standard compound (a) in the room temperature NPLC×RPLC system and (b) in the HTNPLC×RPLC system

Conditions: a) 1^{st}-D, CN column (150 mm×0.53 mm i.d., Macherey-Nagel), mobile phase, 40∶60 (by volume)water-acetonitrile, flow rate, 8 μL/min; 2^{nd} -D, RP-18e monolithic column (50 mm×4.6 mm i.d., Merck, Germany), mobile phase, water (C)/acetonitrile (D), linear gradient elution, 60%D to 80%D in 20 min, flow rate, 1 mL/min; Detection wavelength, 254 nm; Column temperature, 22°C; Conditions: b) 1^{st}-D, 70∶30 (by volume) water-acetonitrile, column temperature, 80°C; The other conditions were the same as (a).

2^{nd}-D were same for both modes. Since the flow rate of the 2^{nd}-D was much higher than that of 1^{st}-D, the influence of the high organic solvent content of primary elute on the monolith column should be much less than that on μ_RPLC. Experimental results showed that except peak 1, where the peak width was 0.072 min for HTNPLC×RPLC and 0.079 min for NPLC×RPLC, the peak width of all other peaks (0.06–0.066 min) were the same for both modes. The reason is that the small volume of the elution from 1^{st}-D is only about 1% of the mobile phase flow rate of 2^{nd}-D when monolithic column was used as the 2^{nd}-D column. The separation of system peak from early eluting sample peaks is different between HTNPLC×RPLC and NPLC×RPLC. The organic solvent content of the primary elute could influence the resolution between the system peaks and early eluting peaks of sample. At room temperature NPLC, every system peak brought by switching valve could not be baseline separated with peaks of sample and hence influence the qualitative and quantitative determination of the target peaks from sample. While in HTNPLC, due to peak band compression on the head of the secondary column, the system peaks and components were baseline separated, allowing qualitative and quantitative analysis of the target components. Furthermore, the resolution between peaks was improved by using HTNPLC since the peak band dispersion of the 1^{st}-D was less than that operated at room temperature and the less organic content in mobile phase benefit the band compression on the 2^{nd}-D. The separation capability of the secondary column was preserved completely.

3.4 Separation of real samples by HTNPLC×RPLC

3.4.1 Separation of *Glycyrrhiza uralensis* extract by one dimensional NPLC

Figure 5 was the chromatogram of one dimensional NPLC separation of *Glycyrrhiza uralensis* extract at high temperature. *Glycyrrhiza uralensis* extract could be eluted completely by isocratic elution with water–acetonitrile (70/30) as mobile phase in HTNPLC. However, the major components of *Glycyrrhiza uralensis* extract were not separated because the column efficiency of one-dimensional NPLC was insufficient.

3.4.2 Separation of *Glycyrrhiza uralensis* extract by HTNPLC×RPLC

The performance of the HTNPLC×RPLC system was evaluated by using *Glycyrrhiza uralensis* extract as a test sample since the separation space and peak capacity could be improved by using the comprehensive liquid chromatography. Fig. 6 was the chromatogram of HTNPLC×RPLC separation of *Glycyrrhiza uralensis* extract. The 2^{nd}-D was operated at gradient elution. To avoid baseline drift and disturbance of miscellaneous peaks, the detection wavelength was set to 240 nm. The results of experiment

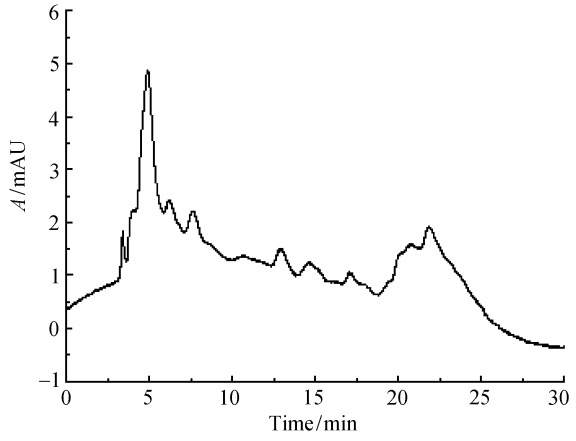

Fig. 5 Chromatogram of one-dimensional NPLC separation of *Glycyrrhiza uralensis* extract at high temperature.
Conditions: 150 mm×0.53 mm i.d., CN column (Macherey-Nagel); Mobile phase, acetonitrile/water (30/70, V/V); Flow rate, 8 μL/min; Detection wavelength, 240 nm

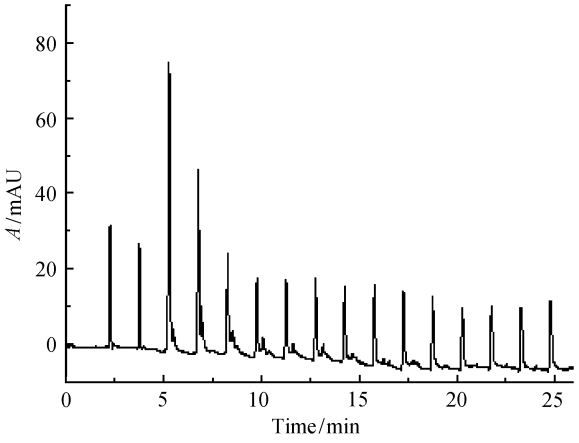

Fig. 6 Chromatogram of comprehensive 2D-LC (HTNPLC× RPLC) separation of *Glycyrrhiza uralensis* extract at high temperature
Conditions: 1st-D, 70:30 (by volume) water–acetonitrile, flow rate, 8 μL/min, column temperature, 80°C; 2nd-D, water (C)-acetonitrile (D), linear gradient elution, 60%D to 85%D in 25 min; Flow rate, 1 mL/min, column temperature, 22°C; Detection wavelength, 240 nm

indicated that this method had an excellent separation capability. Peak band dispersion of the primary fractions on the head of the secondary column was invisible, as indicated from the peaks shape of 2nd-D, owing to the low organic solvent content of primary mobile phase (30%). The total separation time was very short by using the monolith column; the method was suitable for high-speed separation of complex samples.

4 Conclusion

In this study, the organic solvent content of mobile phase could be reduced by using HTNPLC as the first dimension. The peak band dispersion of the primary fractions can be compressed on the head of the 2nd-D column, resulting sharp peaks and enhancement on resolution and peak capacity.

When using stationary phases such as zirconia, polystyrene, or divinybenzene, the organic solvent content of mobile phase could be lower than 10%. The 1st-D can use even subcritical water as mobile phase at 130–200°C. This method could avoid post column dilution and broaden the applications. The method is suitable for separation of polar, weak polar, and non-polar compounds. A parallel RP columns could be used in 2nd-D to achieve high-speed comprehensive separation.

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