Journal of Chromatography A, 1218 (2011) 3124–3128

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Short communication

Elution–extrusion counter-current chromatography separation of five bioactive compounds from *Dendrobium chrysototxum* Lindl

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ARTICLE INFO

Article history:
Received 14 January 2011
Received in revised form 7 March 2011
Accepted 8 March 2011
Available online 16 March 2011

Keywords:
Elution–extrusion counter-current chromatography
Dendrobium chrysototxum Lindl

ABSTRACT

The elution–extrusion counter-current chromatography (EECCC) method was firstly developed by Berthod in 2003 and has been used in natural products separation in recent years. The advantages of this method have been well documented such as reducing the separation time and solvent consumption. In the EECCC method, the time point of the extrusion step is very important during the whole separation process as it directly affects the resolutions, separation time and solvent consumption. However, how to choose a suitable time point to perform the extrusion step without decreasing the resolution has not been studied yet. In the present study, a strategy for systematically calculating the time point for extrusion was developed in theory and five bioactive compounds from the extract of *Dendrobium chrysototxum* Lindl. were separated and compared using normal CCC and EECCC method. Our results demonstrated that the accurate time point to perform the extrusion could be calculated and reduced both separation time and solvent consumption without losing separation performance. Using this EECCC method, five bioactive compounds were separated and purified with high purity. The separation time and solvent consumption were decreased from 200 min to 100 min and 5–2.5 L during the separation process while the resolutions were still acceptable. Finally, 63 mg, 48 mg, 97 mg, 162 mg and 43 mg of hydroxyl phenanthrenes and bibenzyls with the purity of 98.7%, 98.0%, 98.2%, 99.0% and 98.7%, respectively were isolated from 1.2 g crude extract of *D. chrysototxum* Lindl. initially purified by column chromatography in one step separation. The purities of compounds were determined by HPLC. Their structures were identified by electrospray ionization-mass spectrometry (ESI-MS) and NMR.

1. Introduction

Counter-current chromatography (CCC) is a separation technique that uses a support-free liquid stationary phase [1]. The elution–extrusion counter-current chromatography (EECCC) method which takes advantage of the liquid nature of the stationary phase by combining normal elution and extrusion in a single run was developed by Berthod in 2003 [2] and widely used in natural products separation recent years [3]. After an initial elution stage, extrusion of the stationary phase is achieved by switching the supply of flowing liquid from the mobile phase to the originally stationary phase, while maintaining the centrifugal force through continued rotation. The advantages of this method have been well documented such as reducing the separation time and solvent consumption. However, few researches focus on the accurate time point when the extrusion step should be started during the separation process. In the EECCC method, the time point of the extrusion step is very important during the whole separation process as it directly affects the resolutions, separation time and solvent consumption.

In the present study, a strategy for calculating the time point was developed theoretically based on previous researches [4]. The accurate time point made it possible to reduce both separation time and solvent consumption without losing separation performance. In order to explore the relationship of time point to perform the extrusion step and separation time and resolution, the extract of *Dendrobium chrysototxum* Lindl were chosen to separate their bioactive compounds.

The stem of the oriental herb *D. chrysototxum* (also known as Shihu) has been used in traditional China due to its gastric acid

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doi:10.1016/j.chroma.2011.03.015

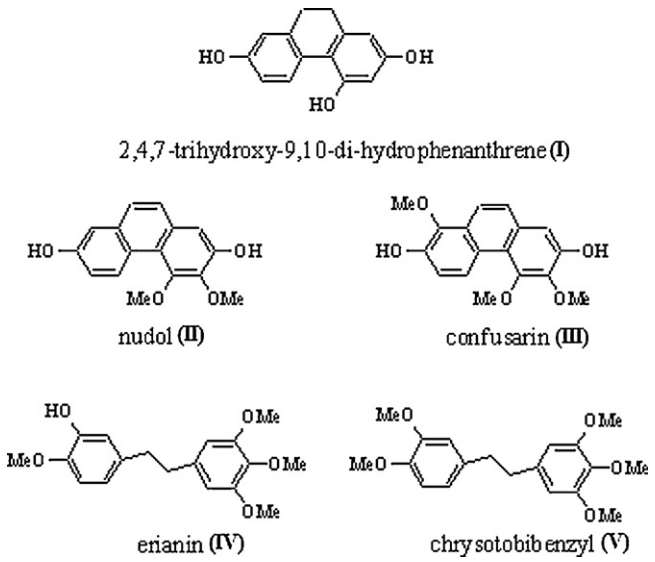

Fig. 1. The structures of the five compounds.

stimulation, metabolism enhancement and anti-aging properties [5]. Erianin, one of the major bioactive constituents of *D. chrysotoxum* Lindl. had been demonstrated to have metabolic inhibition [6], anti-tumor [7], anti-proliferative [8] and anti-angiogenesis [9] activities. Some compounds with similar structures were isolated from *D. chrysototxum* Lindl. In order to further study the biological activities of these compounds, a large quantity of compounds is in great need (Fig. 1).

2. Experimental

2.1. Apparatus

The Spectrum (Dynamic Extraction, Slough, UK) has four coils on two bobbins all integrated in one machine—an analytical and preparative column on each bobbin. The analytical columns use stainless steel tubing of 0.8 mm diameter with column volumes for coils 1 and 2 being 18.5 and 18.0 ml, respectively. The preparative columns use a 4 mm polyfluoro alkoxy (PFA) tubing with volumes for coils 3 and 4 being 460.5 and 452.0 ml, respectively [10]. HX-2050 constant temperature regulator (Beijing Boyikang Lab Implement, Beijing,China) was used to control the separation temperature. The CCC system was equipped with ÄKTA BASIC system (Amersham Pharmacia Biotechnique Group, Uppsala, Sweden), which contained a P-900 pump, an UV-900 detector and a UNICORN work station.

2.2. Reagents and materials

All the organic solvents used for CCC were of analytical grade and purchased from Chengdu Changzheng Chemical Factory, Sichuan, China. Reverse osmosis Milli-Q water (18 MX) (Millipore, Bedford, MA, USA) was used for all the solutions and dilutions. Methanol used for HPLC analysis was of chromatographic grade and purchased from Fisher Chemical (Loughborough, UK). The dried *D. chrysototxum* Lindl were purchased from Yunan province in China.

2.3. Preparation of the crude extract of D. chrysototxum Lindl

The dried *D. chrysototxum* Lindl was ground to powder (2 kg) which was extracted with 95% ethanol (5 L) at room temperature for 5 days. The extraction procedure was repeated three times. The ethanol extract was combined and evaporated under reduced pressure by rotary vaporization to yield a dark brown residue (115.2 g). The residue was dispersed in the distilled water and the suspension was extracted with ethyl acetate (EtOAc) to afford an EtOAc extract (25.9 g). The EtOAc extract was applied to the silica gel column and eluted with petroleum ether–EtOAc (10:1, 5:1, 2:1, 1:1, v/v) and EtOAc–methanol (5:1, 0:1, v/v) successively to obtain seven fractions (fraction A–G). Fraction C (4.2 g) was used to make the crude extract. It was stored in a refrigerator (4 °C) for the CCC separation.

Table 1
The K_D values of target compounds in different solvent systems.

Solvent system	I	II	III	IV	V
HEMWat (1:1:1:1, v/v)	0.151	0.774	0.809	1.417	2.828
HEMWat (1:2:1:2, v/v)	2.643	12.680	10.906	22.290	>50
HEMWat (2:1:2:1, v/v)	0.044	0.044	0.049	0.136	0.166
HEMFa (1:1:1:1, v/v)	0.082	0.508	0.607	0.829	1.452
HEMFa (2:3:2:3, v/v)	0.609	2.214	4.078	6.139	9.319

2.4. Selection and preparation of two-phase solvent system and sample solution

The two-phase solvent system was selected according to the K_D of each target component. The K_D values were measured by HPLC as follows: Add a small amount (5 mg) of crude extract to the two mutually equilibrate solvent phase (2 ml each) in a test tube. After shaking vigorously for 10 min, deliver 20 μL of each phase into a separate test tube, dilute each into 1 ml with methanol and analyze by HPLC. The K_D value was expressed as the ratio of the peak area of target compound in the upper phase divided by that in the lower phase.

Each solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature. The two phases were separated and degassed by supersonic bath for 20 min before use. The sample solution was prepared by dissolving 1.2 g crude extract in a solvent mixture consisting of equal volumes of both upper and lower phases (10 ml of each phase).

2.5. CCC procedure

In each separation, the coiled column was first entirely filled with the upper phase (stationary phase) then the Spectrum was rotated at 1200 rpm, while the lower phase (mobile phase) was pumped into the column in the "head-to-tail" elution mode at a flow rate of 25 ml/min. After the mobile phase front emerged and hydrodynamic equilibrium was established in the column, about 20 ml of the sample solution containing 1.2 g was injected into the head of the column through the injection valve. The effluent of the column was continuously monitored with a UV detector at 280 nm. Peak fractions were collected according to the elution profile. After the separation, the solvents in the column were pushed out with nitrogen.

2.6. HPLC analysis and identification of CCC peak fractions

The crude extract and peak fractions obtained by EECCC were analyzed by HPLC with a phodediode array detector (Waters, Milford, MA, USA). The chromatographic column was an Atlantis C18 (150 mm × 4.6 mm I.D. 5 μm) (Waters), the mobile phase was methanol–water (60:40, v/v). The flow rate was 1 ml/min, and the effluent was monitored at 200–400 nm. Their structures were identified by ^{1}H and ^{13}C-NMR spectroscopy using a Varian spectrometer (Varian, Palo Alto, CA, USA) model Gemini 400 at 400 MHz. The mass measurement of the compounds was measured by Q-TOF Premier mass spectrometer (Micromass, Manchester, UK).

3. Results and discussion

3.1. Selection of a suitable two-phase solvent system for CCC separation

In CCC separation, a suitable solvent system was critical for success of separation and preparation, which should provide ideal K_D as well as good solubility of the sample. In this work, several solvent systems were tested and the K_D values were measured and listed in Table 1. According to the results, the solvent systems composed of *n*-Heptane–EtOAc–Methanol–Water (HEMWat, 1:2:1:2, v/v) and HEMWat (2:1:2:1, v/v) were unsuitable, for the K_D values they provided were too large or too small and compounds mainly partitioned in only one phase. The HEMWat systems did not discriminate correctly Compounds II and III likely due to a partial ionization of Compound III that made it elute faster. Adding formic acid suppressed the Compound III ionization giving a nice repartition of K_D for the five compounds. As a result, the solvent system of *n*-Heptane–EtOAc–Methanol-0.1% formic acid solution (pH 2) (HEMFa, 2:3:2:3, v/v) was most suitable, for it could provide suitable K_D as well as a good solubility of sample. This acid buffered HEMWat system might hydrolyze faster that the unbuffered systems.

3.2. Theoretical calculation of the time point of extrusion and the separation procedure

The EECCC method was developed by Berthod. His research indicated that two solutes might be separated well before leaving the column. And in this condition, the extrusion step could be employed to save separation time and solvent consumption during the separation process. This method takes advantage of the liquid nature of the stationary phase by combining normal elution and extrusion in a single run. After an initial elution stage, extrusion of the stationary phase is achieved by switching the supply of flowing liquid from the mobile phase to the originally stationary phase, while maintaining the centrifugal force through continued rotation. According to previous research [2], the method of calculating the R_s between solutes inside the column was given as follows:

$$R_s = \frac{1}{2}\sqrt{Nv}\left(\sqrt{\frac{1}{V_{R1}}} - \sqrt{\frac{1}{V_{R2}}}\right) \quad (1)$$

The resolution was proportional to the square root of the column plate number N and also with the square root of the eluted mobile phase volume, v. The last term of Eq. (1) depended on the retention volume of the solutes (V_{R1} and V_{R2}). Introducing the retention time t_R into Eq. (1), using V_R/u (u is the flow rate). And it becomes:

$$R_s = \frac{1}{2}\sqrt{Nt}\left(\sqrt{\frac{1}{t_{R1}}} - \frac{1}{t_{R2}}\right) \quad (2)$$

The time of certain resolution of two solutes was reached, t, could be obtained by:

$$t = \frac{\left((2R_s)/\left(\sqrt{1/t_{R1}} - \sqrt{1/t_{R2}}\right)\right)^2}{N} \quad (3)$$

in which t represents the time when two solutes are separated with the R_s. According to Eq. (3) we can calculate when the solutes are completely separated inside the column and the separated solutes can be recovered by an extrusion step very fast.

In this work, the crude extract was separated with the solvent system of HEMFa (2:3:2:3, v/v) by the normal CCC method at first (shown in Fig. 2A). Every target compound could be obtained in a single run, but the whole separation process would consume more than 3 h (200 min) and nearly 5 L solvent. To calculate the time point of extrusion, every parameter needed for the EECCC method was collected and listed in Table 2.

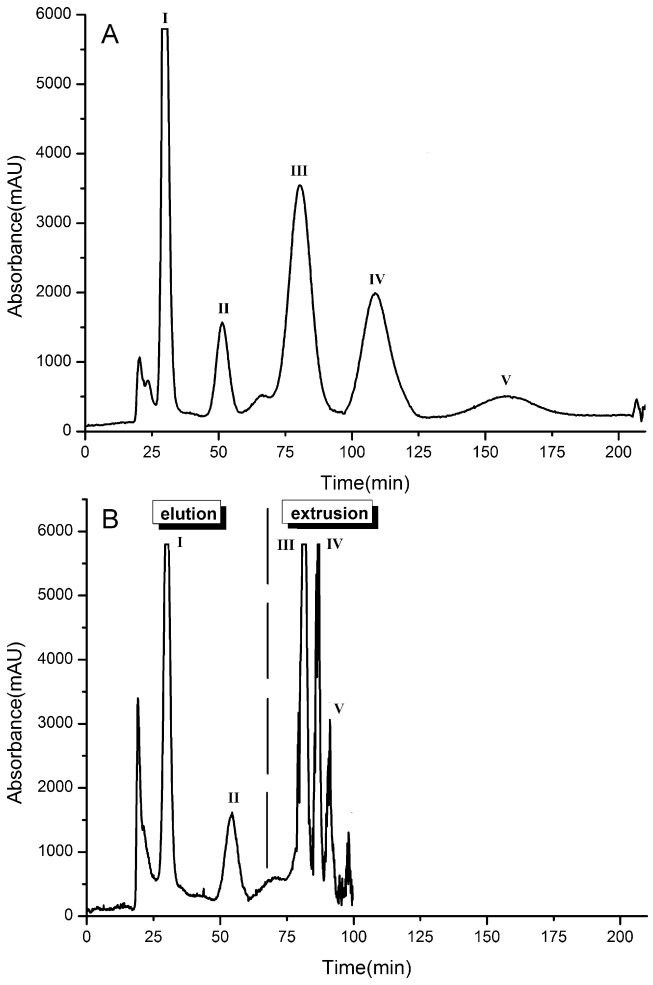

Fig. 2. Chromatograms of the crude extract separation by Spectrum. Solvent system: HEMFa (2:3:2:3, v/v); coil volume: 912.5 ml; rotation speed: 1200 rpm; stationary phase: the upper phase; mobile phase: the lower phase; flow rate: 25 ml/min; detection wavelength: 280 nm; sample loading: 1.2 g; retention of the stationary phase: 44%. (A) The normal elution CCC method. (B) The EECCC method with extrusion at 65 min.

In preparative CCC, it is very hard to make every two solutes baseline separated ($R_s \geq 1.5$). In fact, it is not necessary for preparative separation. For two neighboring peak 1 and peak 2, $W_1 \approx W_2 = 4\sigma$. If $R_s = 1.1$, there will be a cover area, but the bare area of the peak is 97.2% ($t_R \pm 2.2\sigma$). If the $R_s = 1.5$, two peaks are separated completely, and the bare area of the peak reach 99.7%

Table 2
The experimental results obtained from the normal CCC method.

Fraction	K_D	t_R (min)[a]	W (min)	N[b]	R_S[c]
I	0.609	29.77	6.77	309	2.2
II	2.214	51.37	12.30	279	1.9
III	4.078	80.33	18.08	315	1.3
IV	6.139	108.80	25.09	300	1.6
V	9.319	158.48	35.62	316	

[a] Solvent system: HEMFa (2/3/2/3, v/v); mobile phase: lower phase; retention of the stationary phase: Sf = 0.44; volume of mobile phase: V_M = 511.0 ml; volume of stationary phase: V_S = 401.5 ml; volume of column: V_T = 912.5 ml.

[b] Experimental efficiency in plate number estimated using $16(t_R/W)^2$ measured on chromatography for normal elution CCC method.

[c] R_s was calculated using $2(t_{R2} - t_{R1})/(W_1 + W_2)$.

Table 3
The time t needed to reach certain R_s.

Fraction	K_D	t (min)			
		R_s = 1.0	R_s = 1.1	R_s = 1.2	R_s = 1.5
I	0.609	6.87	8.32	9.90	15.46
II	2.214	16.84	20.38	24.25	37.90
III	4.078	53.36	64.57	76.84	120.06
IV	6.139	48.71	58.94	70.14	109.60
V	9.319				

($t_R \pm 3\sigma$). So when the R_s changes from 1.0 to 1.5, the recovery of compounds increases only 2.5% and R_s = 1.1 is enough for the preparation and most compounds could still be obtained. Four resolution factors (R_s = 1.0, 1.1, 1.2, 1.5) were set in this study and the time points to start the extrusion step with these R_s were calculated by Eq. (3), respectively. The results were listed in Table 3.

According to the calculated results shown in Table 3, when the extrusion step began at 65 min, all the compounds could be eluted out from the column within 100 min. The R_s in this EECCC separation were 2.2, 1.9, 1.1 and 1.2 in sequence. Finally, 63 mg, 48 mg, 97 mg, 162 mg and 43 mg of compounds could be isolated in 100 min with only 2.5 L solvent (see Fig. 2B). Compared with the normal CCC method, about 100 min and 2.5 L solvent were saved by this EECCC method.

3.3. Results of HPLC analysis

The crude extract and EECCC peak fractions were analyzed by HPLC and the results were shown in Fig. 3. The purities of peaks I–V from EECCC separation were 98.7%, 98.0%, 98.2%, 99.0% and 98.7%, respectively.

3.4. Structural identification

All the compounds obtained were identified by their spectrum data (ESI-MS, ^{1}H-NMR, ^{13}C-NMR). The structural elucidations of compounds were as follows:

3.4.1. 2,4,7-Trihydroxy-9,10-di-hydrophenanthrene (I)

ESI–MS (m/z): [M+H]$^+$: 305. ^{1}H-NMR (400 MHz, CDCl3) δ ppm: 2.62 (4H, s, H-9, 10), 6.23 (1H, d, J = 2.4 Hz, H-3), 6.39 (1H, d, J = 2.4 Hz, H-1), 6.59 (1H, dd, J = 9.5, 2.7 Hz, H-6), 6.60 (1H, d, J = 2.7 Hz, H-8), 8.11(1H, d, J = 9.5, H-5). ^{13}C-NMR (400 MHz, acetone-d$_6$) δ ppm: 108.2(C-1), 157.3(C-2), 102.8(C-3), 156.4(C-4), 130.1(C-5), 113.7(C-6), 156.2(C-7), 115.2(C-8), 31.2(C-9), 31.8(C-10), 141.8(C-10a), 115.4(C-4a), 126.6(C-4b), 140.1(C-8a).

3.4.2. Nudol (II)

ESI–MS (m/z): [M+H]$^+$: 271. ^{1}H-NMR (400 MHz, CDCl3) δ ppm: 3.98(3H, s, OMe-4), 4.11(3H, s, OMe-5), 7.170(1H, dd, J = 9.2, 2.4 Hz, H-6), 7.172(1H,s, H-1), 7.20(1H, d, J = 2.4 Hz, H-8), 7.50(1H, d, J = 8.8 Hz, H-10), 7.53(1H, J = 8.8 Hz, H-9), 9.36(1H,d, J = 9.2 Hz, H-4). ^{13}C-NMR (400 MHz, CDCl3) δ ppm: 109.8(C-1), 148.0(C-2), 140.7(C-3), 147.1(C-4), 121.1(C-5), 116.3(C-6), 153.9(C-7), 120.6(C-8), 128.2(C-9), 129.0(C-10), 131.0(C-10a), 117.0(C-4a), 118.0(C-4b), 134.3(C-8a), 61.2(–OCH$_3$), 62.7(–OCH$_3$).

3.4.3. Confusarin (III)

ESI–MS (m/z): [M+H]$^+$: 301. ^{1}H-NMR (400 MHz, CDCl3) δ ppm: 3.97(6H, s, OMe-1 or OMe-6), 4.11(3H, s, OMe-5), 5.79(1H, s, OH-7), 5.99(1H, s, OH-2), 7.19(1H, s, H-8), 7.29(1H, d, J = 9.2 Hz, H-3), 7.60(1H, d, J = 9.2 Hz, H-9), 7.85(1H, d, J = 9.2 Hz, H-10), 9.19(1H, d, J = 9.2 Hz, H-4). ^{13}C-NMR (400 MHz, CDCl3) δ ppm: 140.40(C-1), 144.92(C-2), 115.56(C-3), 123.38(C-4), 150.16(C-5), 140.40(C-6), 147.12(C-7), 107.61(C-8), 126.83(C-9), 118.83(C-10), 124.27(C-4a), 118.45(C-4b), 128.74(C-8a), 125.73(C-10a), 61.42(–OCH$_3$), 60.76(–OCH$_3$), 59.20(–OCH$_3$).

3.4.4. Erianin (IV)

ESI–MS (m/z): [M+H]$^+$: 319. ^{1}H-NMR (400 MHz, CDCl3) δ ppm: 2.82(4H, s, H-α, α′), 3.83(9H, s, OMe-3′, 4′, 5′), 3.87(3H, s, OMe-5), 6.38(2H, s, H-2′, 6′), 6.64(1H, dd, J = 8.4, 2.0 Hz, H-4), 6.76(1H, d, J = 8.4 Hz, H-3), 6.81(1H, d, J = 2.0 Hz, H-6). ^{13}C-NMR (400 MHz, CDCl3) δ ppm: 37.32(C-α), 38.38(C-α′), 136.47(C-1), 110.70(C-2), 145.62(C-3), 144.94(C-4), 114.75(C-5), 119.84(C-6), 135.14(C-1′), 105.67(C-2′, 6′), 153.14(C-3′, 5′), 137.38(C-4′), 56.10(–OCH$_3$), 56.14(–OCH$_3$), 60.77(–OCH$_3$).

3.4.5. Chrysotobibenzyl (V)

ESI–MS (m/z): [M+H]$^+$: 333. ^{1}H-NMR (400 MHz, CDCl3) δ ppm: 2.85(4H, s, H-α, α′), 3.82(9H, s, OMe-3, 4, 5), 3.84(3H, s, OMe-3′), 3.86(3H, s, OMe-4′), 6.37(2H, s, H-2, 6), 6.66(1H, d, J = 1.85 Hz, H-2′), 6.72(1H, dd, J = 8.4, 1.85 Hz, H-6′), 6.80(1H, d, J = 1.85 Hz, H-5′). ^{13}C-NMR (400 MHz, CDCl3) δ ppm: 37.58(C-α), 38.57(C-α′), 134.29(C-1), 111.32(C-2), 148.97(C-3), 147.37(C-4), 112.03(C-5), 120.38(C-6), 134.29(C-1′), 105.57(C-2′, 6′), 153.49(C-3′, 5′), 137.49(C-4′), 55.84(–OCH$_3$), 55.98(–OCH$_3$), 60.86(–OCH$_3$), 60.80(–OCH$_3$).

On the basis of spectroscopic data analysis (NMR and MS) and comparison with reports in the literature, compounds I–V were identified to be 2,4,7-Trihydroxy-9,10-di-hydrophenanthrene (I) [11], Nudol (II) [12], Confusarin (III) [13], Erianin (IV) [13] and Chrysotobibenzyl (V) [14], respectively.

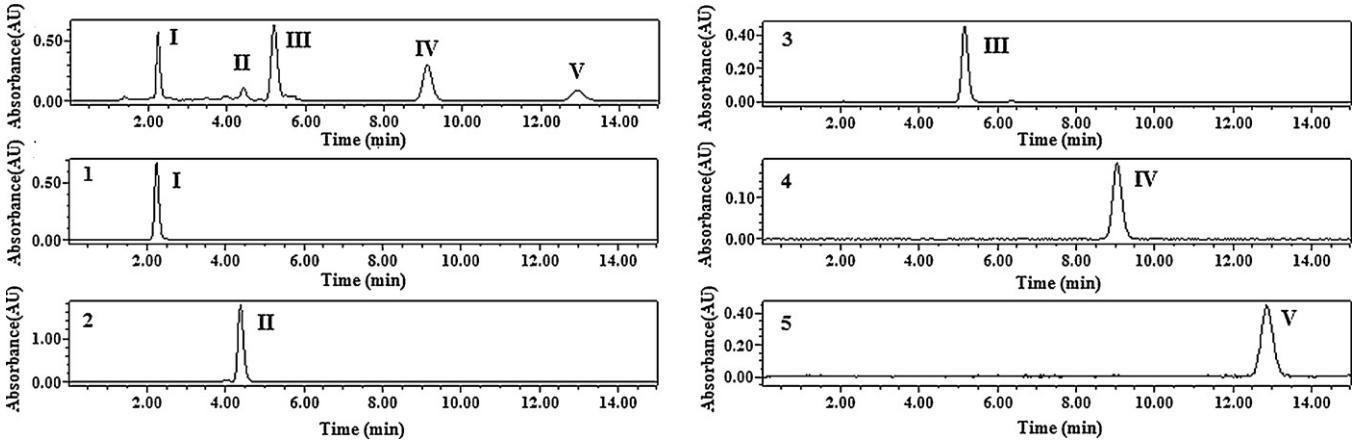

Fig. 3. Chromatograms of crude extract and the EECCC peak fractions by HPLC analysis. Column: reversed-phase column Atlantis C18 (150 mm × 4.6 mm I.D. 5 μm) (Waters); mobile phase: methanol–water (60:40, v/v); flow rate: 1 ml/min; detection wavelength: 280 nm.

4. Conclusions

In this study, we successfully calculated the time point to start extrusion step theoretically in the EECCC method. An efficient EECCC method was developed for the separation and purification of five major compounds from *D. chrysototxum* Lindl. based on our results. Finally, 63 mg (I), 48 mg (II), 97 mg (III), 162 mg (IV) and 43 mg (V) of hydroxyl phenanthrenes and bibenzyls with the purity of 98.7%, 99.0%, 98.2%, 98.0% and 98.7%, respectively, were obtained from 1.2 g crude extract during a single separation process. Compared with normal elution CCC method, the EECCC method with the extrusion at 65 min reduced the separation time and solvent consumption from 200 min to 100 min and 5–2.5 L during the separation process while the resolutions were still acceptable. Our research showed a great potential of EECCC on preparative separation.

Acknowledgement

The work was supported by National Key Programs of China during the 11th Five-Year Plan Period (2009ZX09501-015), the National Natural Science Foundation of China (81071251) and the Open-Study Funds of State Key Laboratory Breeding Base of Systematic Research, Development and Utilization of Chinese Medicine, Chengdu University of Traditional Chinese Medicine.

References

[1] Y. Ito, J. Chromatogr. A 1065 (2005) 145.
[2] A. Berthod, M.J.R. Angel, S.C. Broch, Anal. Chem. 75 (2003) 5886.
[3] Y.B. Lu, W.Y. Ma, R.L. Hu, A. Berthod, Y.J. Pan, J. Chromatogr. A 1216 (2009) 4140.
[4] A. Berthod, J. Chromatogr. A 1126 (2006) 347.
[5] Chinese Pharmacopoeia Commission, Chinese Pharmacopoeia, Chemical Industry Press, Beijing, 2005.
[6] Y.Q. Gong, Y. Fan, L. Liu, D.Z. Wu, Z.L. Chang, Z.T. Wang, In Vivo 18 (2004) 223.
[7] M. Cushman, D. Nagarathnam, D. Gopal, A.K. Chakraborti, C.M. Lin, E. Hamel, J. Med. Chem. 34 (1991) 2579.
[8] G.X. Ma, G.J. Xu, L.S. Xu, J. China Pharm. Univ. 25 (1994) 188.
[9] Y.Q. Gong, Y. Fan, D.Z. Wu, H. Yang, Z.B. Hu, Z.T. Wang, Eur. J. Cancer 40 (2004) 1554.
[10] H.Y. Ye, S. Ignatova, H.D. Luo, Y.F. Li, A.H. Peng, L.J. Chen, I. Sutherland, J. Chromatogr. A 1213 (2008) 145.
[11] H. Yang, Z.T. Wang, L.S. Xu, Z.B. Hu, J. China Pharm. Univ. 33 (2002) 367.
[12] R.S. Li, X. Yang, P. He, N. Gan, J. Chin. Med. Mater. 32 (2009) 220.
[13] G.X. Ma, G.J. Xu, L.S. Xu, Z.T. Wang, T. Kickuchi, Acta Pharm. Sin. 29 (1994) 763.
[14] H. Yang, Y.Q. Gong, Z.T. Wang, L.S. Xu, Z.B. Hu, G. Xu, Chin. Tradit. Herbal Drugs 32 (2001) 973.