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Application of step-wise gradient high-performance counter-current chromatography for rapid preparative separation and purification of diterpene components from *Pseudolarix kaempferi* Gordon

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ABSTRACT

In general, simultaneously separation and purification of components with a broad polarity range from traditional Chinese medicine (TCM) is a challenge by an ordinary high-speed counter-current chromatography (HSCCC) method. In this paper, we describes a rapid and efficient separation method of combining three-step gradient elution and two-step flow-rate gradient elution using high-performance counter-current chromatography (HPCCC) to separate 8 diterpene compounds simultaneously within 80 min in a single run from the alcohol extract of *Pseudolarix kaempferi* Gordon. This separation process produced 166 mg pseudolaric acid B O-β-D-glucopyranoside (PABGly), 152 mg pseudolaric acid C (PAC), 8 mg deacetylpseudolaric acid A (deacetylPAA), 5 mg pseudolaric acid A O-β-D-glucopyranoside (PAAGly), 484 mg pseudolaric acid B (PAB), 33 mg pseudolaric acid B methyl ester (PAB methyl ester), 10 mg pseudolaric acid A (PAA) and 18 mg pseudolaric acid H (PAH) from 1.0 g crude sample with purities of 98.6%, 99.6%, 92.3%, 92.2%, 99.2%, 99.4%, 98.3%, 91.0%, respectively. Our study indicates that the suitable combination of step-wise gradient elution and flow-rate gradient elution using HPCCC is an effective strategy to separate complex components from natural products.

1. Introduction

HPCCC is now accepted as an advanced preparative technique, and widely used for separation and purification of various natural products [1–6]. In recent years, several CCC methods which could shorten separation time have been developed, such as elution-extrusion counter-current chromatography (EECCC), step-wise gradient elution, and flow-rate gradient elution. In the EECCC method, when the solutes have been fully separated inside the column before eluting out of the column, it is possible to use the liquid stationary phase to extrude the column contents in order to shorten separation time. As an efficient CCC technique, the EECCC method was developed by Berthod et al. and it has already been applied in the separation of natural products [7–10]. In traditional liquid chromatography, gradient elution is widely employed to improve the resolution efficiency and reduce separation time. Step-wise gradient elution with HPCCC is a useful method when one or more compounds need to be obtained from complex sample [11]. Flow-rate gradient elution is also a conventional method to separate the compounds with different polarity by a step-wise increase of the mobile phase flow-rate. It had been applied in the separation of compounds from plants by CCC [12–15]. However, there are few reports on the combination of step-wise gradient elution and flow-rate gradient elution using HPCCC for the separation and purification of components from natural products.

The root bark of *Pseudolarix kaempferi* Gordon, which is known as "Tu-Jin-Pi" in traditional Chinese medicine, has been employed for the treatment of fungal infections [16]. Pseudolaric acid B (PAB), which is a major constituent in *P. kaempferi* Gordon, was found to be effective on anti-cancer activity [17]. Due to the unique structural scaffold and the significant anti-angiogenic activity, PAB has been regarded as an anti-cancer drug lead and the pathway of PAB has also been demonstrated by other researchers [18–20]. For developing anti-cancer drugs with high activity and low toxicity, more compounds with similar structure to PAB need to be obtained. So

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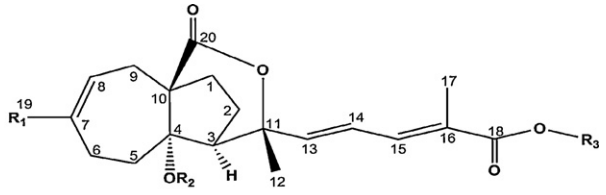
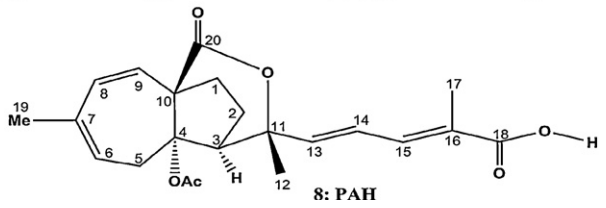


	R_1 (19 23)	R_2 (21 22)	R_3
1: PABGly	COOMe	$COCH_3$	Glc
2: PAC	COOMe	H	H
3: deacetylPAA	Me	H	H
4: PAAGly	Me	$COCH_3$	Glc
5: PAB	COOMe	$COCH_3$	H
6: PAB methyl ester	COOMe	$COCH_3$	CH_3
7: PAA	Me	$COCH_3$	H



8: PAH

Fig. 1. The structures of diterpene compounds **1**–**8** isolated from *P. kaempferi* Gordon. 1 = pseudolaric acid B O-β-D-glucopyranoside (PABGly); 2 = pseudolaric acid C (PAC); 3 = deacetylpseudolaric acid A (deacetylPAA); 4 = pseudolaric acid A O-β-D-glucopyranoside (PAAGly); 5 = pseudolaric acid B (PAB); 6 = pseudolaric acid B methyl ester (PAB methyl ester); 7 = pseudolaric acid A (PAA); 8 = pseudolaric acid H (PAH).

it is significantly important to find a fast and effective method to separate components from *P. kaempferi* Gordon.

Although Han et al. had separated four diterpenoids from *P. kaempferi* Gordon with solvent systems composed of *n*-hexane-ethyl acetate-methanol-water (HEMWat) at two ratios (5:5:5:5 and 1:9:4:6, v/v/v/v) by HSCCC, the whole process for the two-stage separation took about 8 h [21]. In the present study, three different CCC elution modes, such as EECCC, step-wise gradient elution, and flow-rate gradient elution were tested, and finally a method of combining three-step gradient elution and flow-rate gradient elution was established. After parameter optimization, the separation method was put into effect by HPCCC and 8 diterpene compounds were obtained simultaneously within 80 min. Their structures have been elucidated by means of spectroscopic methods including MS, ^{1}H NMR and ^{13}C NMR and compared with the published data (see Fig. 1).

2. Experimental

2.1. Apparatus

HPCCC was performed on a HPCCC centrifuge (Dynamic Extraction, Slough, UK). The apparatus has four columns 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 columns 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 columns 3 and 4 being 460.5 and 452.0 ml, respectively. The revolution radius or the distance between the column axis and central axis of the centrifuge (R) for all of these columns is 11 cm with a β value varying from 0.52 at the internal terminal to 0.86 at the external terminal. The rotational speed is adjustable from 200 to 1400 rpm. A HX-2050 constant temperature regulator (Beijing Boyikang Lab Implement, Beijing, China) was used to control the separation temperature. The HPCCC system was equipped with AKTA Basic system (Amersham Pharmacia Biotechnique Group, Uppsala, Sweden), which contained a P-900 pump, an UV-900 detector and an UNICORN work station.

HPLC analysis was performed on Waters 2695 coupled with 2996 photodiode array detector (Waters, Milford, MA, USA). The MS analyses were performed with a Q-TOF Premier Mass Spectrometer (Waters Micromass, Milford, MA, USA) coupled with an ESI source. The nuclear magnetic resonance (NMR) spectrometer used here was a Mercury Plus 400 NMR system (Varian, Palo Alto, CA, USA).

2.2. Reagents and materials

Analytical grade *n*-hexane, ethyl acetate and methanol for HPCCC separation were purchased from Changzheng chemical factory (Chengdu, China). Methanol used for HPLC was chromatographic grade and purchased from Fisher Chemical (Loughborough, UK); water was produced by Milli-Q system (18 MΩ) (Millipore, Bedford, MA, USA).

The root barks of *P. kaempferi* Gordon were obtained from Chengdu TCM Market in China and identified by Dr. Yanfang Li (Department of Pharmaceutical Engineering, College of Chemical Engineering, Sichuan University). A voucher specimen (TJP-201011) was deposited in the State Key Laboratory of Biotherapy, Sichuan University, Chengdu, China.

2.3. Preparation of two-phase solvent systems and sample solution for HPCCC separation

The two-phase solvent system was composed of HEMWat at various volume ratios. The solvent mixture was equilibrated in a separated funnel at room temperature, and the two phases were separated shortly before use.

The root barks of *P. kaempferi* Gordon (5 kg) were powered and extracted with 95% ethanol (25 L) three times. After recovering solvent under vacuum at 40 °C, a total of 300 g crude sample for HPCCC separation was obtained. The crude sample was dissolved in a solvent mixture consisting of equal volumes of both upper and lower phases before every separation process.

2.4. Measurement of partition coefficient (K_D)

Measurement of K_D values of target compounds from crude sample was as follows: crude sample (1 mg) was weighed into a 10 ml glass tube and added 1 ml of each phase of a pre-equilibrated two-phase solvent system. The glass tube was then shaken vigorously for 5 min to thoroughly equilibrate the sample between the two phases. After settling, 500 μl of each phase was transferred to two separate test tubes and evaporated to dryness. The residues were diluted with 1 ml methanol and analyzed by HPLC. The K_D value was expressed as the peak area of target compounds in the upper phase (stationary phase) divided by that in the lower phase (mobile phase).

2.5. HPCCC separation procedure

2.5.1. Analytical HPCCC separation procedure

The analytical separation was carried out on analytical column 2 (18 ml) and contained 3 steps. In each separation run, the multiple layer coiled column was first entirely filled with the lower phase (stationary phase) of HEMWat (1:1:1:1) in the tail to head mode. Then the upper phase (mobile phase) of HEMWat (1:1:1:1) was pumped into the column at a flow rate of 0.5 ml/min while the rotor was rotated at 1250 rpm. When hydrodynamic equilibrium was established, 1 ml of the sample solution (20 mg/ml) was injected into the column through the sample valve. In the first step, the mobile phase of HEMWat (1:1:1:1) was used and the flow rate

Table 1
The partition coefficients (K_D) of target compounds for *n*-hexane–ethyl acetate–methanol–water (HEMWat) solvent system (normal phase).

HEMWat solvent system	Partition coefficient (K_D)							
	PABGly (**1**)	PAC (**2**)	deacetylPAA (**3**)	PAAGly (**4**)	PAB (**5**)	PAB methyl ester (**6**)	PAA (**7**)	PAH (**8**)
2:1:2:1	>100	>100	43.48	>100	6.67	1.92	1.30	1.04
1:1:1:1	99.11	1.85	1.37	56.87	1.01	0.43	0.32	0.22
2:3:2:3	34.48	1.08	0.66	20.83	0.34	0.15	0.12	0.08
1:3.5:1:3.5	1.20	0.63	0.34	0.98	0.15	0.07	0.06	0.04

was 0.5 ml/min. While the upper phase (mobile phase) of HEMWat (2:3:2:3) and HEMWat (1:3.5:1:3.5) were used in the second and third step, the flow rate of mobile phase was set at 1 ml/min. The effluent was continuously monitored with a UV-900 detector at 262 nm. Peak fractions were manually collected according to the chromatogram. Each fraction was evaporated under reduced pressure and dissolved in methanol for HPLC analysis.

2.5.2. Preparative HPCCC separation procedure

The preparative separation was carried out on two preparative columns (912.5 ml), which were connected in series for the scale-up and the process was similar to the analytical separation procedure except that the sample loading and flow rate were linearly scaled up. In the preparative separation process, the initial flow rate of mobile phase was 25 ml/min in the first step and then increased to 50 ml/min in the second and third step. 50 ml sample solution containing 1.0 g of the crude sample was injected into the column via the sample valve (50 times the sample loading in the analytical separation).

2.6. HPLC analysis of crude sample and fractions from HPCCC separation

The crude extract and fractions separated by HPCCC were analyzed by HPLC coupled with a photodiode array detector. The column used in this study was Sunfire C_{18} column (150 mm × 4.6 mm I.D., 5 μm, Waters). The mobile phase was methanol and 0.1% acetic acid water solution in the gradient model as follows: 0–18 min, 50–75% methanol; 18–20 min, 75–95% methanol. The flow rate was set at 1 ml/min, and the temperature was 30 °C. The detection wavelength was 200–400 nm. The crude extract and fractions were dissolved in methanol.

2.7. MS analysis of compounds

The ESI-MS experiments were performed on ESI-Q-TOF Premier instrument in positive ion mode. After evaporated under reduced pressure, the fractions were dissolved in methanol and injected at a rate of 50 μl/min. Capillary voltage was set at 2.6 kV; sampling cone voltage was set at 40.0 V; source temperature was 90 °C; Desolvation gas flow was set at 450 l/h and collision energy was set at 10.0 eV. The mass spectrometer was scanned from *m*/*z* 200 to 1000 in full scan mode.

3. Results and discussion

3.1. Evaluation of the distribution coefficients (K_D)

An appropriate solvent system providing a suitable range of the distribution coefficients (K_D) for target compounds is the key to a successful HPCCC separation. HEMWat is a classic two-phase solvent system because it could provide a broad polarity range [22]. In this experiment, the K_D values of target compounds in different ratios of HEMWat were determined by HPLC and listed in Table 1.

The large differences of K_D values in different HEMWat solvent systems showed that the target compounds covered a wide polarity range, indicating that it was impossible to separate them with a conventional HPCCC method. Thus, the present study aimed to explore a fast and efficient separation method to separate all target compounds with HPCCC in a single run.

3.2. Exploration of the separation method and analytical separation of target compounds on Mini-DE HPCCC

The EECCC method was proposed firstly. According to Ref. [23], the time point to start extrusion step could be calculated theoretically. Here, we selected HEMWat (1:1:1:1) as a relatively suitable solvent system to calculate the theoretical time of extrusion and the results showed that due to the large K_D values of PABGly (1) and PAAGly (4), if the two compounds were separated with satisfactory resolution by extrusion, the extrusion step would be started at 240 min (if R_s = 1.0). So it is too long for a HPCCC separation process. Considering the variations of K_D values of the target compounds in the above solvent systems, a three-step gradient elution was designed: first, to separate PAH (8), PAA (7), and PAB methyl ester (6) with HEMWat (1:1:1:1); then to isolate PAB (5), deacetylPAA (3) and PAC (2) with HEMWat (2:3:2:3); last, to separate PAAGly (4) and PABGly (1) with HEMWat (1:3.5:1:3.5).

Since the K_D values of compounds 6–8 ranging from 0.22 to 0.43 were very close in HEMWat (1:1:1:1), their peaks were partly overlapped. Therefore, flow-rate of the mobile phase was reduced to 0.5 ml/min at the first step of the three-step gradient elution to obtain better resolutions and satisfactory stationary phase retention.

As mentioned above, a successful separation of all the components within 80 min in a single run was achieved by combining three-step gradient elution and two-step flow-rate gradient elution on analytical Mini-DE HPCCC (see Fig. 2). Three solvent systems of HEMWat (1:1:1:1), HEMWat (2:3:2:3) and HEMWat (1:3.5:1:3.5)

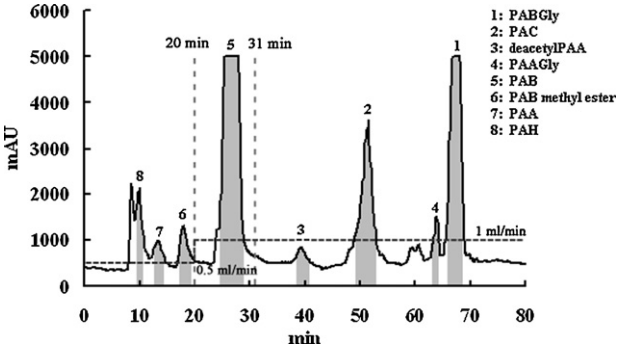


Fig. 2. Separation chromatogram of the crude sample by Mini-DE HPCCC. Coil volume: 18 ml; two-phase solvent system: HEMWat (1:1:1:1, 2:3:2:3, 1:3.5:1:3.5, v/v/v/v) in three-step gradient elution; stationary phase: the lower phase of HEMWat (1:1:1:1); mobile phase: the upper phase of HEMWat (1:1:1:1) from 0 to 20 min, the upper phase of HEMWat (2:3:2:3) from 20 to 31 min, the upper phase of HEMWat (1:3.5:1:3.5) from 31 to 80 min (the vertical dashed lines represent the time points of replacing mobile phase); flow rate: 0.5 ml/min from 0 to 20 min and 1 ml/min from 21 to 80 min (the horizontal dashed line shows the change of flow rate in the whole process); sample loading: 20 mg; rotation speed: 1250 rpm; temperature: 25 °C; detection wavelength: 262 nm; retention of the stationary phase: 67%.

were selected for the separation. The solvent system of HEMWat (1:1:1:1) was used firstly and the separation was started at a slow flow rate of 0.5 ml/min to separate PAH (8), PAA (7), and PAB methyl ester (6). After PAB methyl ester (6) was eluted out, the mobile phase was switched to the upper phase of HEMWat (2:3:2:3) and the flow rate was increased to 1.0 ml/min simultaneously to elute PAB (5). At last, the upper phase of HEMWat (1:3.5:1:3.5) was pumped into the coil after PAB (5) was eluted out and the flow rate of 1.0 ml/min was kept to elute deacetylPAA (3), PAC (2), PAAGly (4) and PABGly (1).

3.3. Preparative separation of target compounds on Midi-DE HPCCC

The theory of linear scale-up of HPCCC was demonstrated by Sutherland et al. [24,25]. In this study, the separation conditions were scaled up to preparative Midi-DE HPCCC as shown in Fig. 3.

Fractions were collected as described in *section 2.5* and evaporated under vacuum. Finally, 166 mg PABGly (1), 152 mg PAC (2), 8 mg deacetylPAA (3), 5 mg PAAGly (4), 484 mg PAB (5), 33 mg PAB methyl ester (6), 10 mg PAA (7) and 18 mg PAH (8) were obtained from 1.0 g crude sample.

In this process, 8 compounds were separated simultaneously within 80 min by HPCCC in a single operation. Compared to previous study [21], the number and throughput of purified compounds were increased greatly, and the separation time was shortened

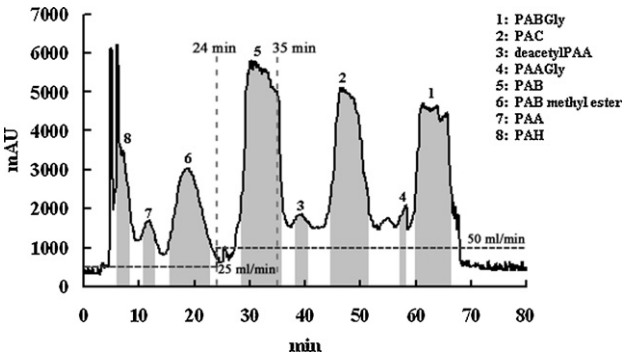


Fig. 3. Separation chromatogram of the crude sample by Midi-DE HPCCC. Coil volume: 912.5 ml; two-phase solvent system: HEMWat (1:1:1:1, 2:3:2:3, 1:3.5:1:3.5, v/v/v/v) in three-step gradient elution; stationary phase: the lower phase of HEMWat (1:1:1:1); mobile phase: the upper phase of HEMWat (1:1:1:1) from 0 to 24 min, the upper phase of HEMWat (2:3:2:3) from 24 to 35 min, the upper phase of HEMWat (1:3.5:1:3.5) from 35 to 80 min (the vertical dashed lines represent the time points of replacing mobile phase); flow rate: 25 ml/min from 0 to 24 min and 50 ml/min from 25 to 80 min (the horizontal dashed line shows the change of flow rate in the whole process); sample loading: 1.0 g; rotation speed: 1250 rpm; temperature: 25 °C; detection wavelength: 262 nm; retention of the stationary phase: 82%.

remarkably. Two key factors contribute to the improvement of separation performance: (1) the column volume is an important factor of separation performance. Comparing the TBE-300A HSCCC with column volume of 300 ml in Ref. [21], the Midi-DE HPCCC

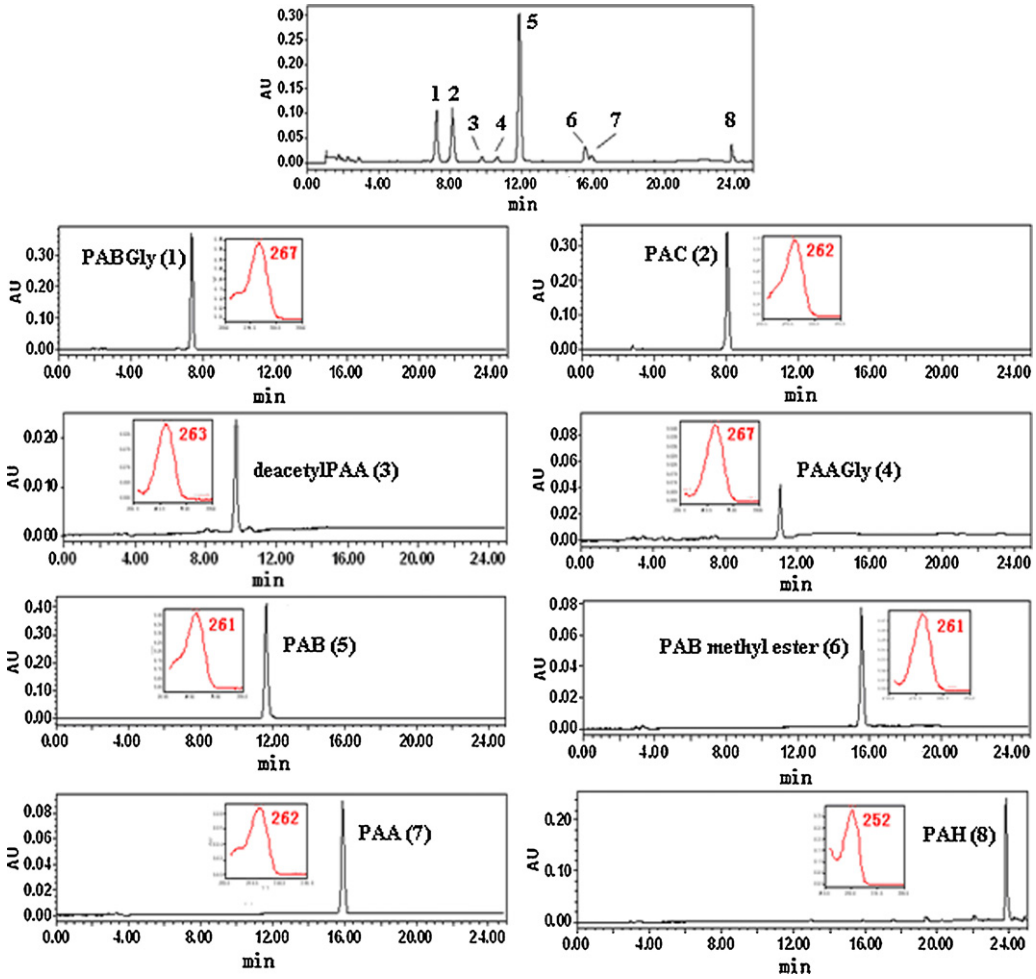


Fig. 4. Chromatograms of the crude sample and peak fractions **1**–**8** by HPLC analysis. Column: reversed-phase Sunfire C_{18} column (150 mm × 4.6 mm I.D., 5 μm, Waters); mobile phase: methanol–0.1% acetic acid water (methanol: 0–18 min, 50–75%; 18–20 min, 75–95%); flow rate: 1 ml/min; temperature: 30 °C; detection wavelength: 200–400 nm.

instrument used here has a column volume of 912.5 ml, which is 3.04 × (912.5/300). Hence, the enlarged column volume can effectively improve the sample loading; (2) Midi-DE HPCCC can provide a rotation speed of 1250 rpm, gaving a high "g" value of 192 × g. Hence, the mobile phase flow-rate in this instrument could be as high as 50 ml/min with a satisfactory stationary phase retention, resulting in a short separation time of 80 min. Thus, our results have demonstrated that the HPCCC instrument has the great advantages of high throughput and higher separation efficiency [26–29].

3.4. HPLC analysis of crude sample and HPCCC fractions

The purities of HPCCC fractions were determined by HPLC. As shown in Fig. 4, the purity of compounds **1**–**8** were 98.6%, 99.6%, 92.3%, 92.2%, 99.2%, 99.4%, 98.3%, 91.0%, respectively.

3.5. Structure identification

The structure identification of compounds **1**–**8** was performed with MS, ^{1}H NMR and ^{13}C NMR. Comparing with the published data, compounds **1**–**8** were identified as PABGly (1) [21], PAC (2) [30], deacetylPAA (3) [31], PAAGly (4) [21], PAB (5) [30], PAB methyl ester (6) [30], PAA (7) [32] and PAH (8) [33], respectively.

4. Conclusions

In this study, we applied three-step gradient elution and two-step flow-rate gradient elution together on HPCCC, for rapid preparative separation and purification of 8 diterpene compounds from *P. kaempferi* Gordon. The results of our study indicate that the combination of step-wise gradient elution and flow-rate gradient elution is a powerful method for the separation of the components with a broad polarity range. The HPCCC technology by varying solvent system and flow-rate of the mobile phase has a great potential on natural products separation.

Conflict of interest

The authors have declared no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2012.01.040.

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