Journal of Chromatography B, 878 (2010) 1929–1933

Contents lists available at ScienceDirect

Journal of Chromatography B

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

Predictable and linear scale-up of four phenolic alkaloids separation from the roots of *Menispermum dauricum* using high-performance counter-current chromatography

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ARTICLE INFO

Article history:
Received 16 August 2009
Accepted 2 May 2010
Available online 1 June 2010

Keywords:
HPCCC
Menispermum dauricum
Phenolic alkaloids
Linear scale-up

ABSTRACT

This paper describes how distribution ratios were used for prediction of peak elution in analytical high-performance counter-current chromatography (HPCCC) to explore the method for separation and purification of bioactive compounds from the roots of *Menispermum dauricum*. Then important parameters related to HPCCC separations including solvent systems, sample concentration, sample loading volume and flow rate were optimized on an analytical Mini-DE HPCCC and finally linearly scaled up to a preparative Midi-DE HPCCC with nearly the same resolutions and separation time. Four phenolic alkaloids were for the first time obtained by HPCCC separation with a two-phase solvent system composed of petroleum ether–ethyl acetate–ethanol–water (1:2:1:2, v/v). This process produced 131.3 mg daurisolin, 197.1 mg dauricine, 32.4 mg daurinoline and 14.7 mg dauricicoline with the purity of 97.6%, 96.4%, 97.2% and 98.3%, respectively from 500 mg crude extract of the roots of *M. dauricum* in a one-step separation. The purities of compounds were determined by high-performance liquid chromatography (HPLC). Their structures were identified by electrospray ionization mass spectrometer (ESI-MS) and nuclear magnetic resonance (NMR).

1. Introduction

The roots of *Menispermum dauricum*, known as Bei-Dou-Gen in Chinese, are widely used for the treatment of a number of immune disorders such as inflammation, allergy and arrhythmia in traditional Chinese medicine [1–3]. The main active constituents of the herb belong to the alkaloid family, including daurisolin, dauricine, daurinoline and dauricicoline (see Fig. 1 for their chemical structures). Dauricine, a calcium channel blocker, is the main toxin that makes *M. dauricum* poisonous [4], has been studied by many scientists for its high pharmacological activities [5–7]. However, little pharmacological research has been done on its structure analogues such as daurisolin, daurinoline and dauricicoline. In order to acquire structural derivation of dauricine with lower toxicity and study potential bioactivity of its structure analogues *in vitro* and *in vivo*, a rapid and reproducible method to obtain large quantity of these components needed to be developed.

High-speed counter-current chromatography (HSCCC), first invented by Ito [8], is a continuous liquid–liquid partition chromatography with no solid support matrix. Compared with traditional separation methods, such as silica gel column chromatography, thin-layer chromatography (TLC), HSCCC has a series of advantages including high sample loading capacity, high sample recovery, low solvent consuming and no irreversible absorption. It is especially suitable for separation and purification of active components from natural products [9–14]. According to our literature research, up to now, there is no report on the use of HSCCC for the separation and purification of daurisolin, dauricine, daurinoline and dauricicoline from the roots of *M. dauricum*.

It is difficult for an ordinary HSCCC instrument to perform linear scale-up process from analytical to preparative HSCCC [15], because the preparative HSCCC can neither provide identical "*g*" value producing nearly the same stationary phase retention nor stand the high pressure brought by the same mobile phase linear flow velocity as analytical HSCCC to keep retention time identical. Thus, the difficulty lies in matching machines and using high-performance instruments with "*g*" fields as high as 250 × *g*, which allow much higher flow and throughput for a given stationary phase retention. The Midi-HPCCC (preparative centrifuge) employed in this experiment (for structural description see Section 2.1) can provide the same "*g*" value (191 × *g*) as Mini-HPCCC (analytical centrifuge). At the same time, HPCCC can stand high flow rate up to 100 ml/min compared to 2–3 ml/min of an ordinary preparative HSCCC. Thus, Mini-HPCCC matched with Midi-HPCCC can be excellent combination for linear scale-up process. In the present study, distribution

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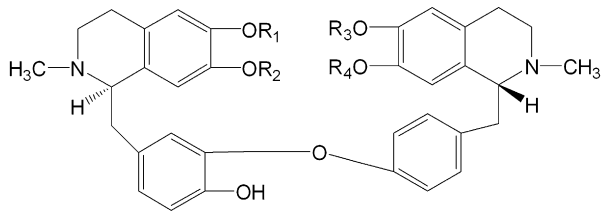
1570-0232/$ – see front matter © 2010 Published by Elsevier B.V.
doi:10.1016/j.jchromb.2010.05.002



		R_1	R_2	R_3	R_4
Compound I	daurisoline	Me	H	Me	Me
Compound II	dauricine	Me	Me	Me	Me
Compound III	daurinoline	Me	Me	H	Me
Compound IV	dauricicoline	H	Me	Me	H

Fig. 1. Structures of phenolic alkaloids from the roots of *Menispermum dauricum*.

ratio was initially used to predict peak elution and guided analytical HPCCC separation and critical separation parameters were then optimized at the analytical scale and finally transferred directly to Midi-HPCCC for the linear scale-up separation and purification of daurisolin, dauricine, daurinoline and dauricicoline from the roots of *M. dauricum*.

2. Experimental

2.1. Apparatus

The analytical HPCCC instrument employed in the present study was an analytical Mini-DE centrifuge (Dynamic Extractions, Slough, UK). The coil of 0.8 mm bore PTFE tubing had a capacity of 18 ml and the extra coil volume was 3.0 ml. The instrument had a planet radius (R) of 50 mm and β-value ranged 0.68–0.79 ($\beta = r/R$). It was run at a speed of 1850 rpm. The preparative HPCCC instrument employed for scale-up was the Midi-DE centrifuge (Dynamic Extractions, Slough, UK) equipped with two experimental bobbins. One bobbin contained an analytical coil of 20.1 ml and a preparative coil of 455.7 ml and the other one included an analytical coil of 19.3 ml and a preparative coil of 459.8 ml. The diameters of analytical and preparative coil were 0.8 mm and 4 mm, respectively. In the present study, two preparative coils were connected in series for the scale-up. It was run at a speed of 1250 rpm.

Both analytical and preparative HPCCC systems were equipped with ÄKTA BASIC system (Amersham Pharmacia Biotechnique Group, Sweden) containing a P-900 pump, an UV-900 detector and a UNICORN work station. The HPLC equipment was an Alliance 2695 system coupled with 2996 photodiode array detector (Waters, Milford, MA, USA). The MS analysis was performed with a quadrupole time of flight (Q-TOF) premier system including an ESI source (Micromass, Simonsway, Manchester, UK). The nuclear magnetic resonance (NMR) spectrometer was a Mercury Plus 400 NMR system (Varian, USA).

2.2. Reagents and materials

All solvents for HPCCC separation were of analytical grade and purchased from Changzheng Chemical Factory, Sichuan, China. Methanol used for HPLC was of chromatographic grade and purchased from Fisher Chemical (Loughborough, UK). Water was purified by Milli-Q system (18 MΩ) (Millipore, Bedford, MA, USA).

The dried roots of *M. dauricum* were purchased from Wukuaishi Herbs Market (Chengdu, China).

2.3. Preparation of crude sample

The dried roots of *M. dauricum* (3 kg) were pulverized and extracted three times with 95% ethanol (3:1, v/w). Ethanol extract was evaporated to dryness under vacuum and the residue was extracted with 800 ml 5% NaOH solution for 2 h under stirring. Then the solution was filtered off, adjusted to pH 4 with 2 M HCl and lay for 12 h. The acid solution with the precipitate was extracted with 400 ml EtOAc (repeated five times). The EtOAc solution was evaporated to obtain 57 g of crude sample.

2.4. Measurement of partition coefficient (K)

The composition of the two-phase solvent system was selected according to the partition coefficient (K) and peak resolution of the target compounds. According to the literature [16], the measurement of K values was performed as follows: 3 mg crude sample was weighed into a test tube to which 1 ml of each phase of the pre-equilibrated two-phase solvent system was added. The test tube was then shaken vigorously for 3 min to thoroughly equilibrate the sample between the two-phase. Then, an aliquot of each phase (500 μl) was delivered into a test tube separately and evaporated to dryness. The residues were diluted with methanol to 1 ml and analyzed by HPLC. The K value was defined as the peak area of target compound in the upper phase (stationary phase) divided by the peak area of compound in the lower phase (mobile phase).

2.5. Preparation of the two-phase solvent systems and sample solution

In the present study, the two-phase solvent system composed of light petroleum (b.p. 60–90 °C)–ethyl acetate–ethanol–water was used for HPCCC separation. Each component of the solvent system was added to a separated funnel at various volume ratios and thoroughly equilibrated at room temperature. The two phases were separated shortly before use.

Since neither upper nor lower phase alone can dissolve EtOAc crude of *M. dauricum*, a solvent mixture consisting of equal volumes of two phases was considered to dissolve the crude sample at suitable concentration. And it showed better solubility.

2.6. HPCCC separation procedure

In the separation process, the column was firstly filled with upper phase (stationary phase) of the solvent system. Then, the apparatus was rotated at a suitable speed. At the same time, the lower phase (the mobile phase) was pumped into the column. After hydrodynamic equilibrium was established, the prepared sample solution was injected into the column through an injection valve and the temperature was held at 25 °C. The effluent from the tail end of the column was monitored with an UV detector at 254 nm. The peak fractions were collected and evaporated under reduced pressure. The residues were dissolved by methanol for purity analysis using HPLC.

2.7. HPLC analysis and identification of HPCCC fractions

The crude extract and fractions separated by HPCCC were analyzed by HPLC. The HPLC chromatogram of the crude extract is shown in Fig. 2. The column used was an Xterra C_{18} column (150 mm × 4.6 mm, I.D. 5 μm, Waters, Milford, MA). Methanol–0.1% formic acid aqueous system was used as the mobile phase in gradient mode as follows: methanol: 0–12 min, 60–72%. The flow rate was set at 1 ml/min and the temperature was set at 40 °C. The

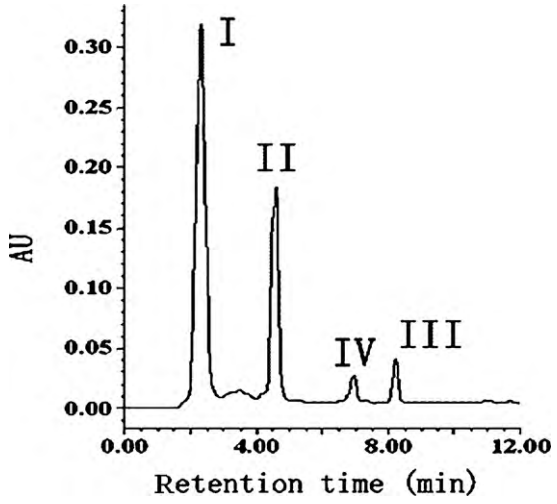


Fig. 2. The HPLC chromatogram of the *M. dauricum* crude sample. HPLC conditions: column: Xterra C_{18} column (150 mm × 4.6 mm, I.D. 5 μm); mobile phase: methanol–0.1% formic acid (methanol: 0–12 min, 60–72%); flow rate: 1.0 ml/min; temperature: 40 °C; detection wavelength: 254 nm.

effluents were monitored at 254 nm by a diode array detector. Identification of the HPCCC fractions was performed by ESI-MS, ^{1}H NMR and ^{13}C NMR.

3. Results and discussion

3.1. Selection of solvent system for HPCCC and peak elution time prediction

It is essential to select a suitable solvent system for HPCCC separation. The ideal *K* values of target compounds should be in a proper range. Generally, small *K* values usually result in poor peak resolution, while large *K* values tend to cause excessive band broadening. For this crude, preliminary studies showed that the most commonly used HEMWat (Hexane–ethyl acetate–methanol–water) system provided similar *K* values of the four target compounds due to the similarity in structure (data not shown). Then, a two-phase solvent system composed of petroleum ether–ethyl acetate–ethanol–water (PEEW) at various volume ratios (1:1:1:1, 1:2:1:2, 1:3:1:3 and 1:4:1:4, v/v) were further tested and their *K* values were measured. The *K* values of compounds in different ratios of PEEW determined by HPLC were listed in Table 1. As shown in Table 1, this solvent system has a better selectivity with respect to HEMWat system. When PEEW (1:1:1:1, v/v) was selected as a solvent system, the *K* values were too small and would result in poor peak resolution. It can be seen that the *K* values of the PEEW (1:4:1:4, v/v) and PEEW (1:3:1:3, v/v) were too large and would lead to a long separation time and broad peaks. PEEW (1:2:1:2, v/v) was finally chosen for CCC separation as it gave a reasonable range of *K* values and a better resolution of target compounds.

Table 1
K values of target compounds in different ratios of light petroleum (b.p. 60–90 °C)–ethyl acetate–ethanol–water solvent system.

Solvent system (v/v)	Daurisolin	Dauricine	Daurinoline	Dauricicoline
1:1:1:1	0.09	0.15	0.37	0.46
1:2:1:2	0.31	0.83	1.46	1.93
1:3:1:3	0.95	1.57	2.61	3.98
1:4:1:4	1.84	2.65	3.74	6.12

Table 2
Comparison of the peak prediction and experimental time of bioactive compounds in the root of *Menispermum dauricum* on analytical HPCCC.

Compound	Mini-HPCCC			
	C1	C2	C3	C4
K value	0.31	0.83	1.46	1.93
Predictive time (min) *Sf* = 60%	12.3	18.9	26.8	32.7
Experimental time (min) *Sf* = 56.3%	10	18.2	24.3	27

According to the *K* values of solvent system PEEW (1:2:1:2, v/v) in Table 1 and the stationary phase retention (*Sf*) of 60% data gained from prior experience with this solvent system, the peak elution time could be predicted as shown in Table 2. Compared with analytical HPCCC running (see Fig. 3, 1st injection), the elution time had a little difference due to reasons as different *Sf* values, sample loading and tested temperature which results *K* value discrepancy. However, the predictive time can provide general information for the separation.

3.2. Sample concentration optimization on Mini-DE

The results of increasing sample concentration at a stable sample loading volume of 0.5 ml are shown in Fig. 3. It was under the following running conditions: PEEW phase system of 1:2:1:2, reversed-phase mode, rotation speed of 1850 rpm, flow rate of 1.0 ml/min and temperature 25 °C. As the sample concentration was increased to 5 mg/ml, 10 mg/ml and 20 mg/ml, resolution of target compounds and stationary phase retention showed marginal

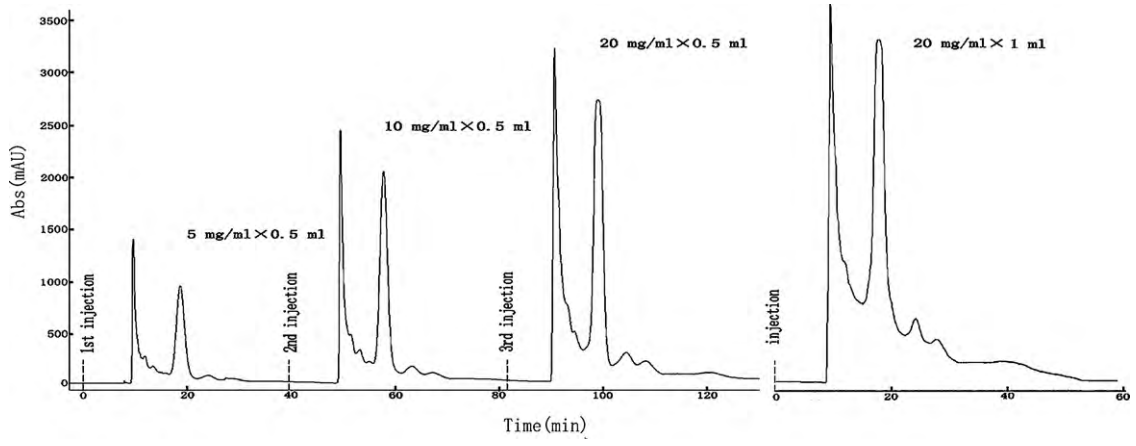


Fig. 3. Sample concentration and loading volume optimization on Mini-DE. Solvent system: PEEW (1:2:1:2, v/v), stationary phase: upper phase; flow rate: 1 ml/min; revolution speed: 1850 rpm; temperature: 25 °C; detection wavelength: 254 nm; *Sf* = 56.3% and 50.3%.

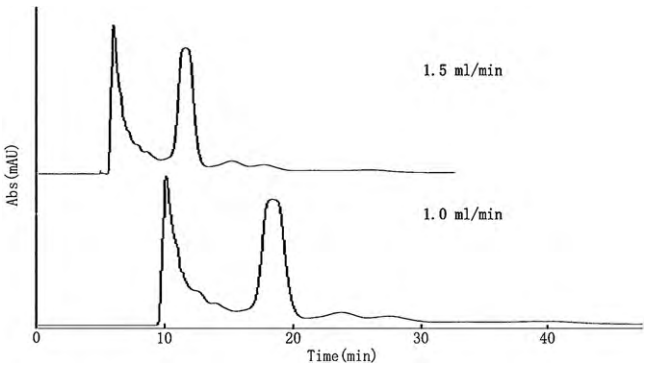


Fig. 4. Flow rate optimization on Mini-DE: solvent system: PEEW (1:2:1:2, v/v), stationary phase: upper phase; sample loading: 20 mg/ml × 0.5 ml; revolution speed: 1850 rpm; temperature: 25 °C; detection wavelength: 254 nm.

difference. When increasing sample concentration to a higher value, the sample precipitated. Therefore, 20 mg/ml was chosen as the maximum and optimum concentration for this crude in the sample solvent.

3.3. Sample loading volume optimization on Mini-DE

The result of increasing sample loading volume at a stable sample concentration of 20 mg/ml is shown in Fig. 3. It ran in the same operating conditions as given in Section 3.2 except that the sample concentration was held at 20 mg/ml and the sample loading volume increased from 0.5 ml to 1 ml. When the sample loading volume was increased to 1 ml, there was considerable loss of stationary phase and it resulted in poor peak resolution. For this reason, 0.5 ml was chosen as the optimum sample loading.

3.4. Flow rate optimization on Mini-DE

The result of increasing flow rate at a stable sample concentration of 20 mg/ml and sample loading volume of 0.5 ml is shown in Fig. 4. As the flow rate increased, the separation time was reduced while the peak resolution would be poor due to the loss of stationary phase. When the flow rate reached to 1.5 ml/min, the stationary phase lost significantly, resulting in the reduction of peak resolution of target compounds. Thus, the flow rate was optimized to 1 ml/min.

3.5. Scaling up linearly from Mini-DE to Midi-DE

When systematic optimization of parameters on Mini-DE HPCCC was finished, the optimized parameters were transferred to the Midi-DE HPCCC for scale-up. The volume ratio between the Mini-DE HPCCC (18 ml) and Midi-DE HPCCC (912.5 ml) centrifuges is approximately 1–50. The flow rate used on the Midi-centrifuge was therefore chosen to be 50 ml/min based on the scale-up principle described by Wood et al. [17], with all other parameters kept the same. The sample loading volume was also adjusted proportionally to 25 ml (0.5 ml × 50). For rotation speed, in order to keep the same "*g*" value (191 × *g* at 1850 rpm) as Mini-DE HPCCC, the rotation speed of Midi-DE HPCCC was set to be 1250 rpm (192 × *g*). The result indicated that these scale-up settings had good stationary phase retention with nearly the same retention time and peak resolution for all target compounds, see Fig. 5.

3.6. HPLC analysis of HPCCC fractions

Fractions collected were analyzed by HPLC and the purity of compounds I, II, III and IV was 97.6%, 96.4%, 97.2%, and 98.3%, respectively. All compounds were separated and purified in one-step separation from crude extract of the roots of *M. dauricum*.

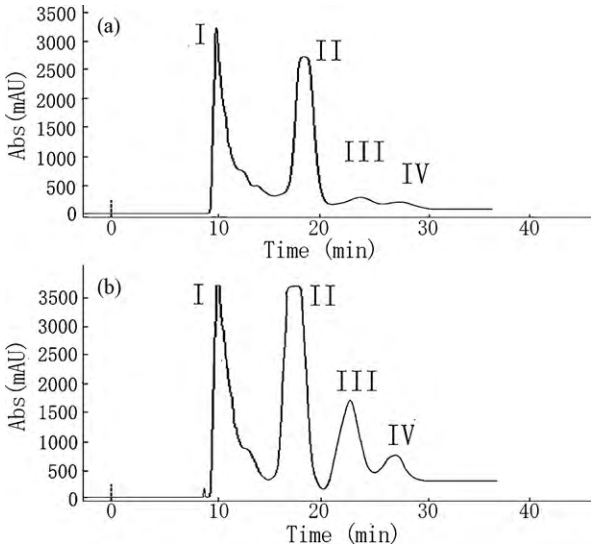


Fig. 5. (a) The chromatogram of Mini-DE HPCCC: solvent system: PEEW (1:2:1:2, v/v), stationary phase: upper phase; sample loading: 20 mg/ml × 0.5 ml; flow rate: 1 ml/min; revolution speed: 1850 rpm; temperature: 25 °C; detection wavelength: 254 nm; *Sf* = 56.3%. (b) The chromatogram of Midi-DE HPCCC: solvent system: PEEW (1:2:1:2, v/v), stationary phase: upper phase; sample loading: 20 mg/ml × 25 ml; flow rate: 50 ml/min; revolution speed: 1250 rpm; temperature: 25 °C; detection wavelength: 254 nm; *Sf* = 56.8%.

3.7. Structure identification of HPCCC fractions

The chemical structures of the peaks were identified according to their MS, ^{1}H and ^{13}C NMR data.

Peak I: ESI-MS (*m/z*): $[M+1]^+$ 611, Molecular formula: $C_{37}H_{42}N_2O_6$. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 2.44 (3H, s), 2.51 (3H, s), 3.61 (3H, s), 3.75 (3H, s), 3.81 (3H, s), 5.01 (2H, s), 6.11–7.14 (11H, m). Comparing the above data with [18], the obtained product was identified as daurisolin.

Peak II: ESI-MS (*m/z*): $[M+1]^+$ 625, Molecular formula: $C_{38}H_{44}N_2O_6$. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 2.45 (3H, s), 2.51 (3H, s), 3.42 (1H, m), 3.59 (6H, s), 1.81, 1.84, 2.01, 2.16, 2.21, 2.31 (each 3H, s), 2.34 (H, q, J = 7.0 Hz), 4.72 (H, d, J = 2.6 Hz), 3.77 (3H, s), 3.80 (3H, s), 6.01–7.05 (11H, m). Comparing the above data with [19], the obtained product was identified as dauricine.

Peak III: ESI-MS (*m/z*): $[M+1]^+$ 611, Molecular formula: $C_{37}H_{42}N_2O_6$. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 2.54 (3H, s), 2.62 (3H, s), 3.59 (3H, s), 3.84 (3H, s), 2.64–3.50 (12H, m), 3.86 (1H, s), 5.93 (1H, s), 6.55 (1H, d, J = 1.8 Hz), 6.82 (2H, d, J = 8.5 Hz), 6.93 (1H, d, J = 8.2 Hz), 7.00 (2H, d, J = 8.5 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 11.13, 24.44, 24.77, 39.97, 41.02, 41.93, 42.43, 45.98, 46.19, 55.47, 55.66, 64.47, 64.72, 110.53, 111.05, 114.45, 117.55, 119.98, 125.30, 125,83, 126.47, 127.61, 128.48, 131.11, 132.01, 134.91, 143.35, 144.12, 145.76, 146.12, 147.25, 154.99. Comparing the above data with [20], the obtained product was identified as daurinoline.

Peak IV: ESI-MS (*m/z*): $[M+1]^+$ 597, Molecular formula: $C_{36}H_{40}N_2O_6$. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 2.43 (3H, s), 2.52 (3H, s), 2.50–3.41 (12H), 3.62 (1H, m), 3.73 (1H, m), 5.96 (1H, s), 6.47 (1H, s), 6.55 (1H, d, J = 1.2 Hz), 6.77 (1H, dd, J = 8.4, 1.2 Hz), 6.83 (2H, d, J = 8.4 Hz), 7.00 (2H, d, J = 8.4 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 7.0, 24.5, 25.1, 40.5, 40.6, 42.1, 42.4, 46.1, 55.6, 55.8, 64.5, 64.6, 110.3, 110.5, 114.1, 114.5, 115.6, 117.5, 117.8, 120.1, 125.2, 125.8, 126.1, 127.8, 129.6, 130.9, 131.0, 131.9, 134.5, 143.2, 143.4, 144.1,

144.5, 145.1, 145.6, 155.2. Comparing the above data with [21], the obtained product was identified as dauricicoline.

4. Conclusions

This work presents a successful application of the linear scale-up separation from analytical HPCCC instrument to the preparative one demonstrating that the HPCCC centrifuge with the high "*g*" value operating at the high flow rate is a reliable method for linear scale-up separation with a large amount of sample injection. In this separation procedure, four phenolic alkaloids including daurisolin (131.3 mg, 97.6%), dauricine (197.1 mg, 96.4%), daurinoline (32.4 mg, 97.2%) and dauricicoline (14.7 mg, 98.3%) were obtained from 500 mg of crude extract of the roots of *M. dauricum* in 30 min.

Acknowledgements

This project was supported by the National Major Program of China during the 11th Five-Year Plan Period (2009ZX09501-015).

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