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Optimization suitable conditions for preparative isolation and separation of curculigoside and curculigoside B from *Curculigo orchioides* by high-speed counter-current chromatography

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Abstract

A preparative high-speed counter-current chromatography (HSCCC) was used to isolate and separate curculigoside and curculigoside B from the famous traditional Chinese medicinal herb *Curculigo orchioides*. Some parameters including two-phase solvent system, separation temperature, flow rate of the mobile phase and revolution speed of the apparatus were all investigated, and a successfully isolation and purification was achieved at the following conditions: the two-phase solvent system composed of ethyl acetate–ethanol–water at a volume ratio of 5:1:5 (v/v/v) was selected and the lower phase of the system was used as the mobile phase at the flow rate of 2.0 ml/min, and the isolation temperature and revolution speed were set at 30 °C and 800 rpm. The isolation produced a total of 14.5 mg curculigoside B and 72.8 mg curculigoside with purities of 96.5% and 99.4% determined by high performance liquid chromatography (HPLC) from 300 mg crude extract after cleaning-up by D101 macroporous resin, which was necessary for the excellent purification. The recoveries of the two compounds were 91.6% and 92.5%, respectively, and the chemical structure identification was carried out by UV, MS and NMR. This separation method was more effective than some conventional techniques.

Keywords: *Curculigo orchioides*; Preparative chromatography; Counter-current chromatography; Plant material; Separation; Glycosides

1. Introduction

Generally, preparative isolation and separation of pure products from medicinal plants by some classical methods are tedious, time consuming, requiring multiple chromatographic steps on silica gel, polyamide, macroporous resin column, etc. Thus, an efficient method for the preparative isolation is warranted. High-speed counter-current chromatography (HSCCC), a support free liquid–liquid partition chromatographic technique, eliminates irreversible adsorption of the sample onto solid support, has an excellent sample recovery. The method permits directly introduction of crude samples into the column without more preparation, so it has been successfully applied to isolate and purify a number of natural products [1–5].

Curculigo orchioides (*XianMao* in Chinese), the rhizome of *C. orchioides* Gaertn, family of *Amaryllidaceae*, is a famous traditional Chinese medicine and listed in the Chinese Pharmacopoeia [6]. This plant has been exhibited a widely variety of active effects including anti-osteoporosis, anti-aging effects, anti-inflammatory, enhancement immune function and some actions to the central nervous system [7–9]. The major components of *C. orchioides* are considered to be curculigoside and curculigoside B (shown in Fig. 1).

The aim of the present paper, therefore, was to develop an efficient method to isolate and purify curculigoside and curculigoside B from *C. orchioides* by HSCCC. The best isolation conditions were optimized after investigation the effects of some separation parameters including two-phase solvent system, column temperature, flow rate of the mobile phase and revolution speed of the apparatus. To our best knowledge, this is the first report of using HSCCC to isolate and separate curculigoside and curculigoside B from natural resources.

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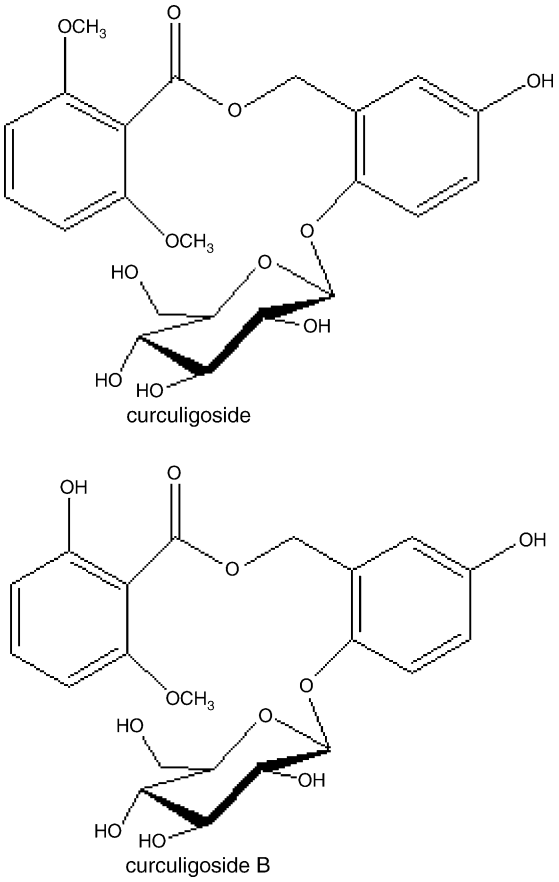

Fig. 1. The chemical structures of curculigoside and curculigoside B.

2. Experimental

2.1. Apparatus

Preparative HSCCC was carried out with a model TBE-300A HSCCC (Shenzhen, Tauto Biotech, China). The apparatus equipped with a polytetrafluoroethylene three preparative coils (diameter of tube, 1.6 mm; total volume, 300 ml) and a 20 ml sample loop. The revolution radius or the distance between the holder axis and central axis of the centrifuge (R) was 5 cm, and the β value varied from 0.5 at the internal terminal to 0.8 at the external terminal ($\beta = r/R$ where r is the distance from the coil to the holder shaft). The HSCCC system was equipped with a model S constant-flow pump, a model UV-II detector operating at 280 nm, and a model N2010 workstation (Zhejiang University, Hangzhou, China). The experimental temperature was adjusted by HX 1050 constant temperature circulating implement (Beijing Boyikang Lab Implement, Beijing, China).

The analytical HPLC system used throughout this study consisted of 515 pump (Waters, USA), 2487 detector (Waters, USA), and a model N2000 workstation (Zhejiang University, Hangzhou, China) and a 20 μl sample loop. D101 macroporous resin was purchased from the Chemical Plant of Nankai University (Tianjin, China).

2.2. Reagents

Ethyl acetate, *n*-hexane, chloroform, *n*-butanol, ethanol and acetic acid were analytical grade and purchased from WuLian Chemical Factory (Shanghai, China), and the standards curculigoside B and curculigoside were purchased from the National Institute for the Control of Pharmaceutical and Biological Products, Ministry of Health, Beijing, China. Acetonitrile and methanol was HPLC grade (Merck, Germany). Reverse osmosis Milli-Q water (18 MΩ) (Millipore, USA) was used for all solutions and dilutions. The *C. orchioides* was purchased from a local drug store (Shanghai, China).

2.3. Preparation of the crude extract

The *C. orchioides* was ground into powder (20–40 mesh), 1000 g of the powder was extracted by 8000 ml 60% aqueous ethanol for two times and 2.0 h for each. United the filter and evaporated to no ethanol under reduced pressure at 60 °C and 400 ml residue was obtained, then the residue was redissolved in water (total volume 800 ml), which was added into a glass column (6.0 cm × 80.0 cm, contained 1500 g D101 macroporous resin). An amount of 6000 ml water was first used to elute the resin until the elution was nearly no color, then 5000 ml 30% aqueous ethanol was used to elute the target compounds, and 10 elution fractions (500 ml for each) were collected and 4 (from 4 to 7 fraction) were united and evaporated to dryness according to HPLC analysis, which was used for further HSCCC isolation and separation.

2.4. Preparation of two-phase solvent system and sample solution

Two-phase solvent system composed of ethyl acetate–ethanol–water (5:1:5, v/v/v) was used in the present study and prepared. The solvent mixture was thoroughly equilibrated in a separated funnel at room temperature and the two phases were separated shortly before use. The sample solution was prepared by dissolving the sample in 15 ml lower phase of the solvent system for isolation and purification.

2.5. HSCCC separation procedure

The coil column was first entirely filled with the upper phase of the solvent system. Then the apparatus was rotated at 800 rpm, while the lower phase was pumped into the column at a flow rate of 2.0 ml/min. After the mobile phase front emerged and hydrodynamic equilibrium was established in the column, about 15 ml sample solution containing 300 mg of the crude extract was injected through the injection value. The effluent of the column was continuously monitored with a UV–vis detector at 280 nm. Peak fractions were collected according to the elution profile. The temperature of the apparatus was set at 30 °C.

2.6. Analysis and identification of HSCCC peak fractions

The crude extract after cleaning-up by macroporous resin and the fractions obtained by HSCCC were all analyzed by HPLC.

The analysis was performed with a reversed-phase Lichrospher C_{18} (150 mm × 6.0 mm I.D., 5 μm) (Hanbang Science, Jiang Su Province, China). The mobile phase composed of CH_3CN–H_2O–HAC (20:80:2, v/v/v) was eluted isocratically at a flow rate of 1.0 ml/min. The column temperature and detection wavelength were set at 25 °C and 280 nm.

3. Results and discussion

3.1. HPLC analysis of the crude extract

In resin column chromatography, water was first used to remove some co-extracted matrix components, which have no or little retention on AB-8 macroporous resin, 30% aqueous ethanol was then used to yield target sample.

The crude extract after resin column chromatography was first analyzed by HPLC. After a lot of experiments, an excellent separation of curculigoside and curculigoside B was achieved under the following conditions: the mobile phase composed of acetonitrile–water–acetic acid (20:80:2, v/v/v) was eluted isocratically at a flow rate of 1.0 ml/min, the column temperature and detection wavelength were set at 25 °C and 280 nm. No complex gradient of mobile phase and no buffer were necessary. The HPLC chromatogram of the crude extract is shown in Fig. 2. Peaks 1 and 2 correspond to curculigoside B and curculigoside, which presented at the contents of 5.09% and 26.08%, respectively.

3.2. Selection suitable two-phase solvent system for HSCCC isolation

The most important step in HSCCC is to optimize suitable conditions for an efficient separation. Various parameters including two-phase solvent system, column temperature, flow rate of the mobile phase and revolution speed are generally considered to be the most important factors. The optimization of suitable HSCCC conditions can be carried out step-by-step or by using an experimental design. In the present study, all selected factors were optimized step-by-step.

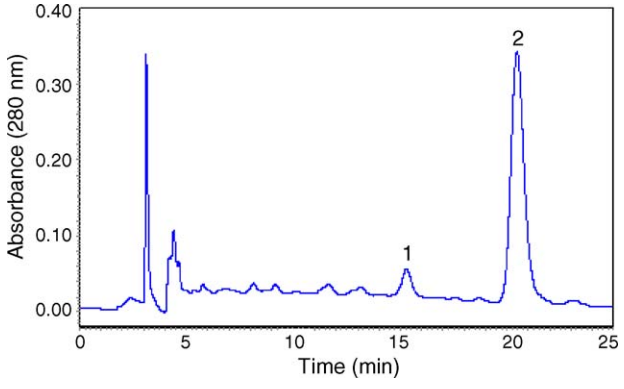

Fig. 2. HPLC chromatogram of crude extract from *C. orchioides* after cleaning-up by D101 macroporous resin. Column: reversed-phase Lichrospher C_{18} (150 mm × 6.0 mm I.D., 5 μm); mobile phase: CH_3CN–H_2O–HAC (20:80:2, v/v/v); flow rate: 1.0 ml/min; UV wavelength: 280 nm; column temperature: 25 °C. Peaks 1 and 2 correspond to curculigoside B and curculigoside, respectively.

Table 1
The partition coefficients (K) of curculigoside and curculigoside B in different solvent systems

Solvent systems	Curculigoside B (K_1)	Separation factor (α)	Curculigoside (K_2)
n-Hexane–ethyl acetate–methanol–water 1:2:2:5 (v/v)[a]	0.034	2.94	0.10
Chloroform–methanol–water 4:3:2 (v/v/v)[b]	3.92	1.59	2.46
Ethyl acetate–*n*-butanol–water 2:1:3 (v/v/v)[a]	6.04	1.71	10.33
Ethyl acetate–*n*-butanol–water 2:3:5 (v/v/v)[a]	7.70	1.69	12.99
Ethyl acetate–*n*-butanol–water 4:1:5 (v/v/v)[a]	3.58	1.55	5.54
Ethyl acetate–ethanol–water 5:1:5 (v/v/v)[a]	1.34	1.81	2.42
n-Butanol–water 1:1 (v/v)[a]	4.43	1.71	7.57
n-Butanol–acetic acid–water 4:1:5 (v/v/v)[a]	3.09	2.04	6.30
Ethyl acetate–methanol–water 4:1:4 (v/v/v)[a]	5.68	2.20	12.48

Experimental protocol: 4 ml of each phase of the equilibrated two-phase solvent system was added to approximately 2 mg of crude sample placed in a 10 ml test tube. The test tube was caped and shaken vigorously for 2 min to equilibrate the sample thoroughly. An equal volume of each phase was then analyzed by HPLC to obtain the partition coefficient (K). The partition coefficient (K) value was expressed as the peak area of the compound in the upper phase divided by the peak area of the compound in the lower phase.

[a] Separation factor, $\alpha = K_2/K_1 (K_2 > K_1)$

[b] Separation factor, $\alpha = K_1/K_2 (K_1 > K_2)$.

In HSCCC, the selection of two-phase solvent system is the most important for successful separation, and is also the most difficult step. In the present paper, nine kinds of two-phase solvent systems were tested and their partition coefficients (K) and separation factors (α) were all examined and listed in Table 1. The results indicated that the solvent system composed of *n*-hexane–ethyl acetate–methanol–water (1:2:2:5, v/v/v) had small K value and the solvent systems composed of chloroform–methanol–water (4:3:2, v/v/v), ethyl acetate–*n*-butanol–water (2:1:3, 2:3:5, 4:1:5, v/v/v), *n*-butanol–water (1:1, v/v), *n*-butanol–acetic acid–water (4:1:5, v/v/v) and ethyl acetate–methanol–water (4:1:4, v/v/v) had large K values, which were not suitable for the isolation and separation. At last, the solvent system composed of ethyl acetate–ethanol–water at a volume ratio of 5:1:5 (v/v/v) was selected for our purification in the present research.

3.3. Selection suitable column temperature for HSCCC isolation

Apart from a suitable two-phase solvent system, other parameters may affect the isolation results in HSCCC, such as column temperature, flow rate of the mobile phase and revolution speed. Among them, column temperature might influence the retention of the stationary phase significantly. High temperature would produce high retention of the stationary phase and low temper-

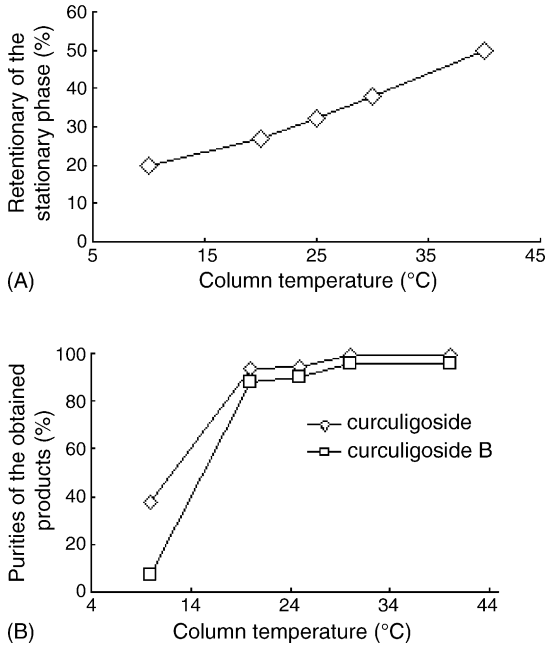

Fig. 3. The influence of the separation temperature to the retention of the stationary phase and the purities of curculigoside and curculigoside B in HSCCC separation. Solvent system: ethyl acetate–ethanol–water (5:1:5, v/v/v); stationary phase: upper phase; mobile phase: lower phase; flow rate: 2.0 ml/min; revolution speed: 800 rpm; sample size: 300 mg; detection wavelength: 280 nm. (A) The relationship between the retention of the stationary phase with the column temperature; (B) the relationship between the purities of the target compounds with the column temperature.

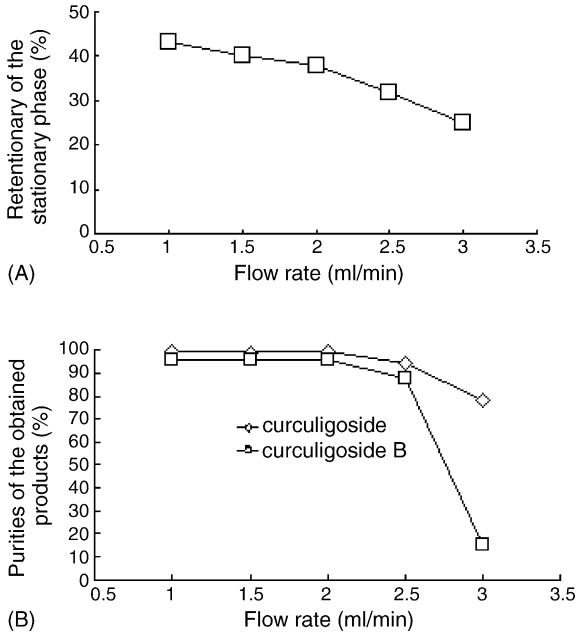

Fig. 4. The influence of the flow rate of the mobile phase to the retention of the stationary phase and the purities of curculigoside and curculigoside B in HSCCC separation. Solvent system: ethyl acetate–ethanol–water (5:1:5, v/v/v); stationary phase: upper phase; mobile phase: lower phase; column temperature: 30 °C; revolution speed: 800 rpm; sample size: 300 mg; detection wavelength: 280 nm. (A) The relationship between the retention of the stationary phase with the flow rate of the mobile phase; (B) the relationship between the purities of the target compounds with the flow rate of the mobile phase.

ature would cause the stationary phase loss, which would affect the purities of the target compounds. In the present paper, five kinds of column temperature (10, 20, 25, 30 and 40 °C) were tested. The highest retention (50%) of the stationary phase of the selected solvent system was obtained at 40 °C and lowest retention (20%) was obtained at 10 °C (Fig. 3A). The purities of curculigoside and curculigoside B decreased when the column temperature was lower than 30 °C (Fig. 3B), and more badly, only one peak was obtained and the purities of the two compounds decreased to 37.4% and 7.2%, respectively at 10 °C (Fig. 6B), which was similar to the crude extract after cleaning-up by D101 macroporous resin without any HSCCC purification. Although the result was satisfactory at 40 °C, a large quantity of air bubble was produced. Furthermore, high temperature would lessen the life of the apparatus. So, the column temperature was set at 30 °C in our isolation mainly considering the above aspects.

3.4. Selection suitable flow rate of the mobile phase for HSCCC isolation

The flow rate might influence the HSCCC separation, too. Different flow rates (1.0, 1.5, 2.0, 2.5 and 3.0 ml/min) of the mobile phase of the selected system were examined in the present paper. Different retentions of the stationary phase (Fig. 4A) and different purities of the compounds were obtained (Fig. 4B). High flow rate was unfavorable to the retention of the stationary phase and the purities of the compounds, and curculigoside and curculigoside B were not separated and combined in one peak (Fig. 6C). On the contrary, low flow rate (1.0 ml/min) was satisfactory to our aim, but the elution time was long and more mobile phase was required. Thus, the flow rate was set at 2.0 ml/min in the present separation according to the overall analysis.

3.5. Selection suitable revolution speed of the apparatus for HSCCC isolation

High revolution speed has the ability to increase the retention of the stationary phase, and it could not be reserved in HSCCC column at low revolution speed. After trying a lot of experiments (700, 800, 900 and 1000 rpm), the curves were draw according to the retention of the stationary phase and the compounds' purities to the revolution speed of the apparatus. Low revolution speed was unfavorable to the retention of the stationary phase (Fig. 5A) and the purities of the compounds (Fig. 5B), and curculigoside and curculigoside B were not separated and combined in one peak (similar to the Fig. 6C and not shown). At last, 800 rpm was selected to separate the target compounds in our paper.

The most suitable isolation conditions, therefore, were selected as follows: the two-phase solvent system was composed of ethyl acetate–ethanol–water (5:1:5, v/v/v), the lower phase was used as the mobile phase at 2.0 ml/min, the column temperature and revolution speed were set at 30 °C and 800 rpm. Under the optimized conditions, three peaks were isolated and two (I and II) were collected in less than 210 min. The HSCCC

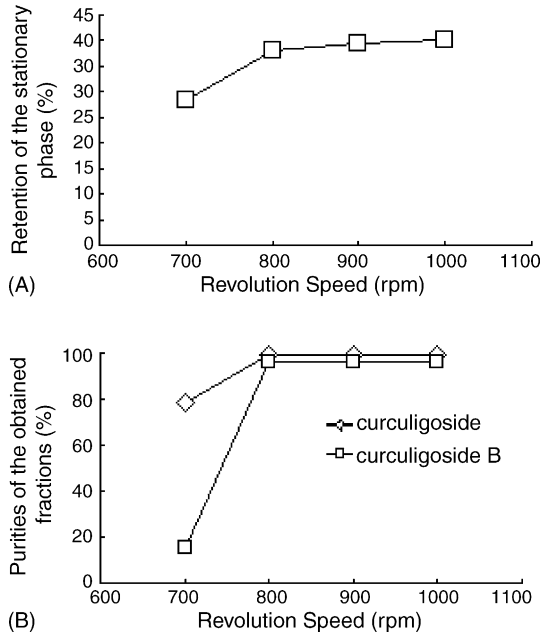

Fig. 5. The influence of the revolution speed to the retention of the stationary phase and the purities of curculigoside and curculigoside B in HSCCC separation. Solvent system: ethyl acetate–ethanol–water (5:1:5, v/v/v); stationary phase: upper phase; mobile phase: lower phase; column temperature: 30 °C; flow rate: 2.0 ml/min; sample size: 300 mg; detection wavelength: 280 nm. (A) The relationship between the retention of the stationary phase with the revolution speed of the apparatus; (B) the relationship between the purities of the target compounds with the revolution speed of the apparatus.

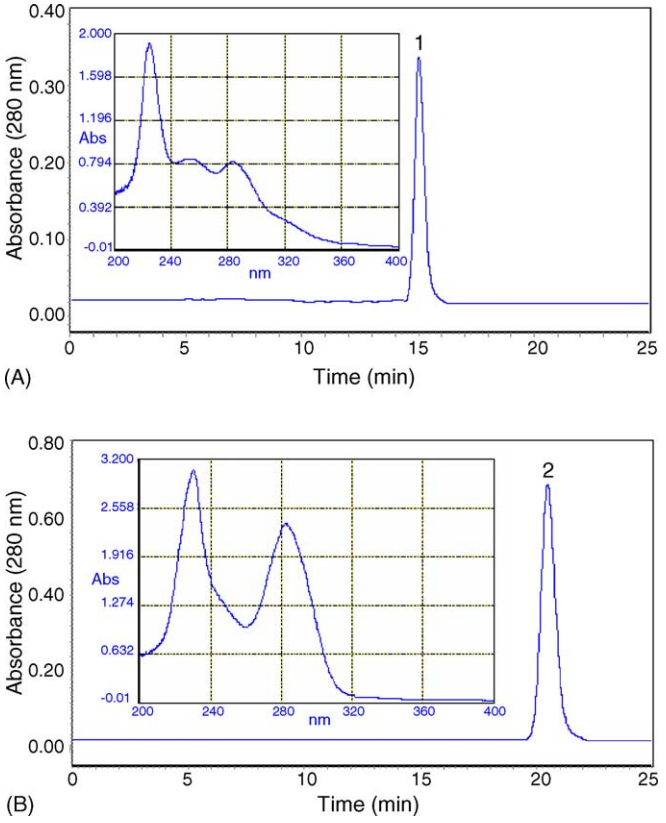

Fig. 7. HPLC chromatograms and the UV spectra of the fractions obtained by HSCCC from *C. orchioides*. Column: reversed-phase Lichrospher C_{18} (150 mm × 6.0 mm I.D., 5 μm); mobile phase: CH_3CN–H_2O–HAC (20:80:2, v/v/v); flow rate: 1.0 ml/min; UV wavelength: 280 nm; column temperature: 25 °C. (A) Fraction I obtained by HSCCC; (B) fraction II obtained by HSCCC; peaks 1 and 2 correspond to curculigoside B and curculigoside.

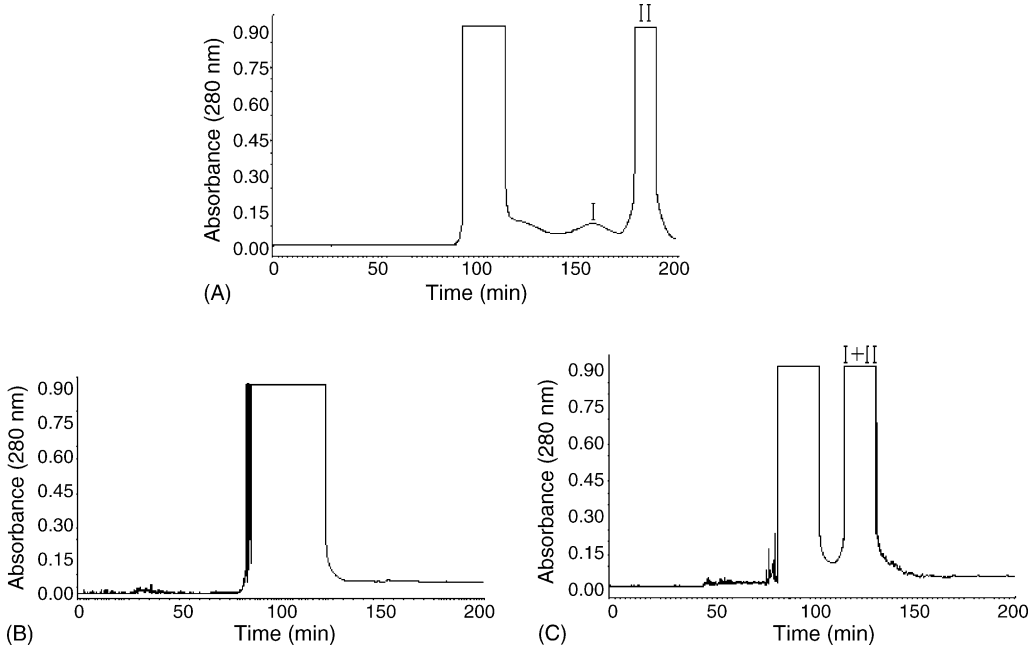

Fig. 6. HSCCC chromatograms of the crude extract from *C. orchioides* after cleaning-up by D101 macroporous resin. Solvent system: ethyl acetate–ethanol–water (5:1:5, v/v/v); stationary phase: upper phase; mobile phase: lower phase; revolution speed: 800 rpm; sample size: 300 mg; detection wavelength: 280 nm. (A) Column temperature: 30 °C; flow rate of the mobile phase: 2.0 ml/min. (B) Column temperature: 30 °C; flow rate of the mobile phase: 3.0 ml/min. (C) Column temperature: 10 °C; flow rate of the mobile phase: 2.0 ml/min.

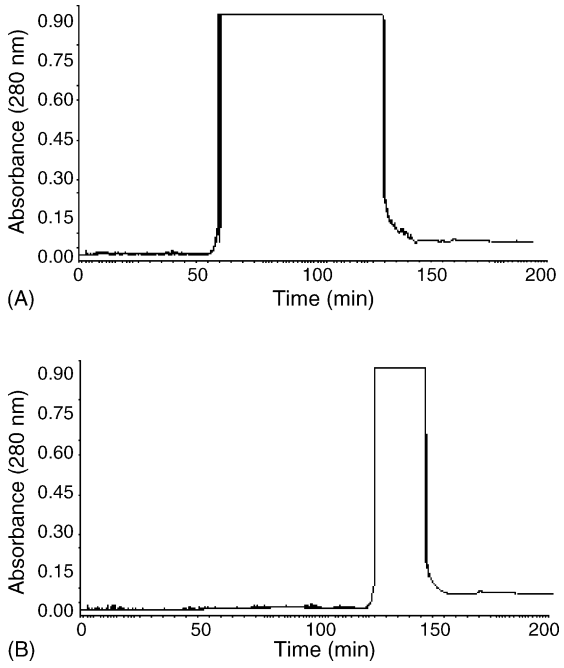

Fig. 8. HSCCC chromatograms of the ethanol extract from *C. orchioides* without D101 macroporous resin cleaning-up step. Solvent system: ethyl acetate–ethanol–water (5:1:5, v/v/v); stationary phase: upper phase; mobile phase: lower phase; revolution speed: 800 rpm; column temperature: 30 °C; flow rate of the mobile phase: 2.0 ml/min; detection wavelength: 280 nm. (A) Sample size: 300 mg; (B) sample size: 80 mg.

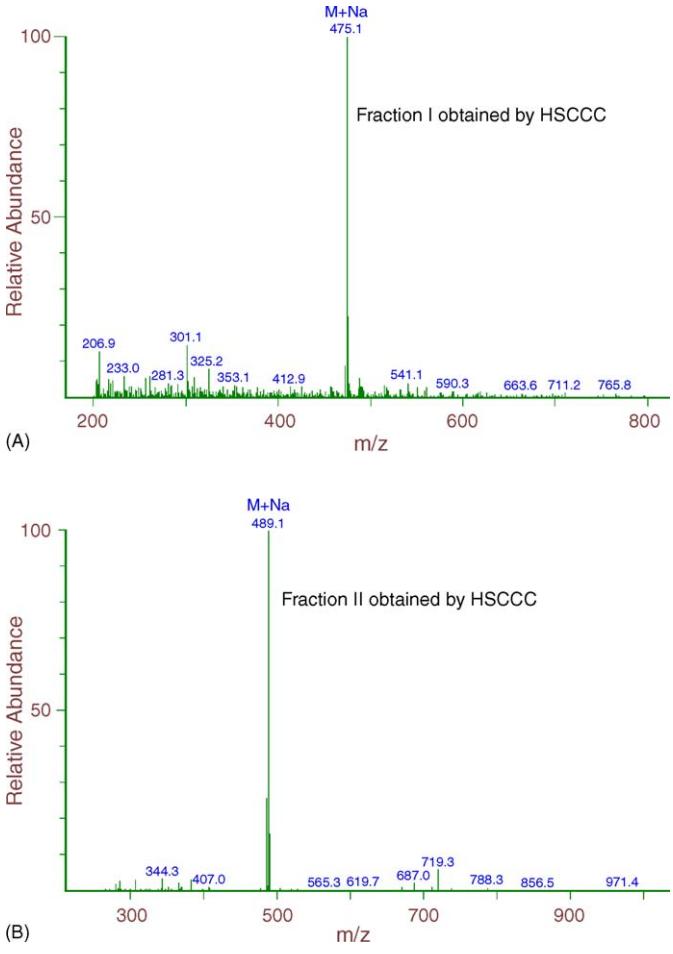

Fig. 9. The MS spectra of the fractions obtained by HSCCC from *C. orchioides*.

chromatogram is shown in Fig. 6A, which was glossy and more beautiful than others. After the two compounds were eluted out, in order to save solvents and time, the remaining compounds in the column were removed by forcing out the stationary phase with pressurized nitrogen gas instead of eluting them with the mobile phase because the stationary phase was not to be reused. The isolation produced a total of 14.5 mg curculigoside B and 72.8 mg curculigoside with purities of 96.5% and 99.4%, respectively as determined by HPLC with external standard method (Fig. 7) from 300 mg crude extract. The recoveries of the two compounds were 91.6% and 92.5%, respectively.

Generally, a cleaning-up step was required after extraction in HSCCC, and suitable samples were obtained and some co-extracted matrix components were eliminated to minimize adverse effects affecting our isolation. In the present paper, the step of D101 resin column chromatography was important for the successful purification. Firstly, we tried to introduce the ethanol extract to HSCCC column without the cleaning-up step, however, the outcome was bad and no pure fraction was obtained under the optimized conditions (Fig. 8A), and even the sample size was decreased to 80 mg (Fig. 8B). Apart from the optimized conditions, a lot of experiments were carried out to isolate the two compounds without resin step, but the results were not satisfactory. Thus, we can say without resin step without excellent purification.

3.6. Chemical structure identification

The chemical structure identification was carried out by UV, MS, and NMR as follows. Fraction I: UV λ_{max}^{MeOH}: 283, 245, 225 nm (Fig. 7A). ESI-MS: 475 $[M+Na]^+$ (Fig. 9A), it showed the molecular was 452, which was in agreement with the molecular formula $C_{21}H_{24}O_{11}$. 1H NMR (500 MHz, DMSO-d_6) δ: 6.97 (1H, d, J = 9.0 Hz, H-3), 6.63 (1H, dd, J = 9.0, 3.0 Hz, H-4), 9.05 (1H, s, 5-OH), 6.86 (1H, d, J = 3.0 Hz, H-6), 5.30 (2H, s, H-7), 10.02 (1H, s, 2′-OH), 6.53 (1H, d, J = 8.3 Hz, H-3′), 7.18 (1H, t, J = 8.3 Hz, H-4′), 6.50 (1H, d, J = 8.0 Hz, H-5′), 3.75 (3H, s, 6′-OCH_3), 4.63 (1H, d, J = 7.0 Hz, H-1″); ^{13}C NMR (125 MHz, DMSO-d_6) δ: 127.7 (C-1), 147.8 (C-2), 117.7 (C-3), 115.2 (C-4), 152.7 (C-5), 114.8 (C-6), 61.3 (C-7), 102.6 (C-1′), 156.2 (C-2′), 111.4 (C-3′), 131.7 (C-4′), 108.8 (C-5′), 157.8 (C-6′), 166.7 (C-7′), 56.3 (–OCH_3–), 103.2 (C-1″), 73.7 (C-2″), 77.4 (C-3″), 70.2 (C-4″), 76.7 (C-5″), 61.7 (C-6″). Comparing with the reported data, the UV, MS and NMR data are in agreement with that of curculigoside B in literature [10]. Fraction II: UV λ_{max}^{MeOH}: 282, 230 nm (Fig. 7B). ESI-MS: 489 $[M+Na]^+$ (Fig. 9B), it showed the molecular was 466, which was in agreement with the molecular formula $C_{22}H_{26}O_{11}$. 1H NMR (500 MHz, DMSO-d_6) δ: 6.98 (1H, d, J = 9.0 Hz, H-3), 6.65 (1H, dd, J = 9.0, 3.0 Hz, H-4), 9.05 (1H, s, 5-OH), 6.80 (1H, d, J = 3.0 Hz, H-6), 5.31 (2H, s, H-7), 3.77 (3H, s, 2′-OCH_3), 6.73 (1H, d, J = 8.5 Hz, H-3′), 7.39 (1H, t, J = 8.5 Hz, H-4′), 6.75 (1H, d, J = 8.5 Hz, H-5′), 3.78 (3H, s, 6′-OCH_3), 4.60 (1H, d, J = 7.5 Hz, H-1″); ^{13}C NMR (125 MHz, DMSO-d_6) δ: 127.7 (C-1), 147.8 (C-2), 117.6 (C-3), 115.2 (C-

4), 152.7 (C-5), 114.8 (C-6), 61.7 (C-7), 104.8 (C-1′), 157.0 (C-2′), 113.1 (C-3′), 131.6 (C-4′), 113.1 (C-5′), 157.1 (C-6′), 166.0 (C-7′), 56.2 (2′-OCH_3), 56.3 (6′-OCH_3), 103.0 (C-1″), 73.7 (C-2″), 77.5 (C-3″), 70.2 (C-4″), 76.8 (C-5″), 61.4 (C-6″). Comparing with the reported data, the UV, MS and NMR data are in agreement with that of curculigoside in literature [10].

4. Conclusion

In the present paper, some parameters including solvent system, column temperature, flow rate and revolution speed were optimized step-by step. Under the selected isolation conditions, two glycosides were successfully separated by HSCCC from the traditional Chinese medicinal plant *C. orchioides*. The results also indicated that HSCCC is a powerful technique to isolate and purify pure materials from natural plants.

Acknowledgement

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