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Preparative isolation and purification of five steroid saponins from *Dioscorea zingiberensis* C.H.Wright by high-speed counter-current chromatography coupled with evaporative light scattering detector

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Abstract

A high-speed counter current chromatography (HSCCC) method was successfully applied to separate and purify steroid saponins from the traditional Chinese medicine *Dioscorea zingiberensis* C.H.Wright for the first time. Ethyl cetate-n-butanol-methanol-water (4:1:2:4, v/v) was used as the two-phase solvent system, and evaporative light scattering detector (ELSD) was used as the detector in this method. The method separated in a single run the following five steroid saponins: 26-O-β-D-glucopyranosyl-(25R)-furost- 5-en-3β,22ζ,26-triol-3- O-[β-D-glucopyranosyl-(1→3)-β-D-glucopyranol- (1→4)-α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside (**Compound A**); 26-O-β-D-glucopyranosyl-(25R)-furost-5-en-3β,22ζ,26-triol-3-O-[β-D-glucopyranosyl (1→3)-α-L-rhamnopyranosyl(1→2)]-β-D-glucopyranoside (**Compound B**); 26-O-β-D-glucopyranosyl-(25R)-furost-5-en-3β, 22ζ, 26-triol- 3-O- [α-L-rhamnopyranosyl (1→4)]-β-D-glucopyranoside (**Compound C**); 26- O-β-D- glucopyranosyl-(25R)- furost-5, 20(22)-diene-3β, 26-diol-3-O-{ α-L-rhamnopyranosyl- (1→4)-[β-D-glucopyranosyl-(1→3)-β-D-glucopyranosyl-(1→2)]}-β-D-glucopyranoside (**Compound D**); and 26-O-β-D-glucopyranosyl-(25R)-furost-5, 20(22)-diene-3β, 26-diol-3 -O-[β-D-glucopyranosyl-(1→4)-α-L-rhamnopyranosyl(1→2)]-β-D-glucopyranoside (**Compound E**). Their structural identification of the five steroid saponins was performed by means of ESI-MS, and ¹³C NMR.

Keywords

Dioscorea zingiberensis C.H.Wright; ELSD; HSCCC; steroid saponins; ethyl acetate-n-butanol-methanol-water (4:1:2:4, v/v) was used as the two-phase solvent system

INTRODUCTION

Dioscorea zingiberensis C.H.Wright, named “huangjiang”, is a Chinese medicinal plant widely distributed in many districts of China [1-3]. Its rhizomes contain some steroid saponins that have been used as a Traditional Chinese Medicine (TCM) for many years, and they are applied as a folk treatment for cough, anthrax, rheumatism, tumefaction, sprain as

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well as cardiac disease [4-8]. Because of their diverse bioactivities, separation and purification of steroid saponins have been attempted by some researchers for recent years. However, due to their similar structures and relatively higher polarity, separation and purification using the conventional chromatographic methods became difficult, leading to unsatisfactory separation results [9]. The conventional analytical methods such as column chromatography and preparative thin-layer chromatography (prep-TLC) require repeated steps which is tedious, time-consuming, and vulnerable to contamination [10-11]. Furthermore, the overall yield of the target compounds, especially for the trace compositions is reduced due to irreversible adsorption onto the solid support during separation. Therefore, development of a highly efficient and green separation method is justified [12-13].

Among of modern separation techniques, high-speed counter-current chromatography (HSCCC) [14-15], is a unique liquid-liquid partition technique, and it has been widely used for separation and purification of active components from natural products [16-18]. The method has various advantages over conventional chromatography, such as a larger sample loading capacity, relatively shorter isolation time, wider range of applicable two-phase solvent systems, higher purity and total sample recovery [19-21]. Consequently, HSCCC is considered to be an important alternative for the conventional chromatographic technique especially for the separation of natural products. Although separations of various kinds of steroids by HSCCC have been reported, to our knowledge there is no report on isolating steroid saponins. This paper describes for the first time an efficient method for separation and purification of five steroid saponins from *Dioscorea zingiberensis* C.H.Wright by HSCCC.

EXPERIMENTAL

Apparatus

The present studies were carried out with a TBE-300A preparative HSCCC instrument purchased from Tauto Biotech Co., Shanghai, China. The apparatus is equipped with a set of three-multilayer coil separation columns and a 20 mL sample loop. The coiled columns were made of polytetrafluoroethylene (PTFE) tubing of 1.5 mm I.D. with a total capacity of 300 mL. The β values ranged from 0.5 at the internal layer to 0.8 at the external layer. ($\beta = r/R$, where r is the rotation radius or the distance from the coil to the holder shaft, and R is the revolution radius or the distances between the holder axis and central axis of the centrifuge). The revolution speed of the apparatus was regulated from 0 to 1000 rpm with a speed controller, while 800 rpm was used throughout this study. The solvent was pumped into the column with a model TBP5002 constant flow pump (Tauto Biotech Co. Ltd, Shanghai, China), and the effluent was continuously monitored with an Alltech 800 evaporative light scattering detector. The N2000 chromatography workstation (Zhejiang University, Hangzhou, China) was used to record the chromatogram. The high-performance liquid chromatography (HPLC) analysis was performed using a Waters Alliance 2695 equipment (Waters, Milford, MA, USA) with a vacuum degasser, a low pressure quaternary pump, an autosampler, and an Alltech 2000 evaporative light scattering detector. The analysis of steroid saponins was carried out with a Welchrom C₁₈ column (250×4.6 mm, 5 μ m), and evaluation and quantification were made on an Empower Workstation. Nuclear magnetic resonance (NMR) spectrometer was Bruker Avance 500 MHz (Bruker BioSpin, Rheinstetten, Germany), while ESI-MS was performed with a Thermo LTQ- XL ion trap mass spectrometer (Thermo Scientific, USA).

Reagents and Materials

All organic solvents used for HSCCC were of analytical grade, and purchased from Tianjin Chemical Factory (Tianjin, China). Acetonitrile used for analytic HPLC was of an HPLC

grade, and double-distilled water was obtained with a Milli-Q plus system (Millipore, Madrid, Spain).

The rhizomes of *Dioscorea zingiberensis* C.H.Wright were provided by Yangtze River Pharmaceutical Industry Co., Ltd. (Jiangsu, China).

Preparation of Total Steroid Saponins

Dried raw material of *Dioscorea zingiberensis* C.H.Wright (3.5 Kg) was powdered and extracted with 70% ethanol for three times. The ethanol solution was combined and evaporated to dryness under reduced pressure by a rotary evaporator, and the residue was redissolved in water, and subjected to centrifugation. The supernatant was separated on a D-101 macroporous resin column, by eluting with 60% ethanol. The effluent was concentrated under reduced pressure. The syrup thus obtained was dissolved in 1 L of water again, and extracted with an equal volume of n-butanol six times successively. The n-butanol solution was concentrated to obtain 140 g of residue. In order to enrich the target components and remove impurities, the residue was separated on silica gel column (100-160 mesh) by eluting with dichloromethane-methanol-water (70:30:10). The effluent was collected according to the R_f value between 0.2-0.5 on the Thin Layer Chromatography (TLC) with a solvent composed of dichloromethane-methanol-water (65:35:10), and then the collecting effluent was evaporated at 100°C under reduced pressure. Finally, about 20 g of residue was obtained for subsequent HSCCC isolation and separation.

Selection of Two-phase Solvent System

The two-phase solvent system was selected according to the partition coefficient (K) of each target component. Several different kinds of solvent systems were prepared each at various volume ratios, and equilibrated in a separation funnel at room temperature. The K values were determined by HPLC analysis as follows: a suitable amount of total steroid saponins (10 mg) was added to 4.0 mL of the pre-equilibrated two-phase solvent system consisting of equal volumes of each phase in a 10 mL test tube. The capped test tube was shaken vigorously to thoroughly equilibrate the sample between the two phases. After the equilibration was established, 1.5 mL of each phase was transferred to a separate test tube and evaporated. The residue of each phase was dissolved in 2 mL of methanol and analyzed by HPLC. The K values were calculated according to the equation: $K = A_U/A_L$, while A_U and A_L were the peak area of each compound in the upper phase and the lower phase, respectively.

Preparation of Two-phase Solvent System and Sample Solution

Ethyl acetate-n-butanol-methanol-water (4:1:2:4, v/v) was finally chosen as the two-phase solvent system. The solvent system was mixed and shaken vigorously to thoroughly equilibrate in a separatory funnel at room temperature and left over night. The two phases were then separated and degassed by sonication for 30 min before use. An amount of 100 mg of total steroid saponins dissolved in 10 mL of the lower phase was used as the sample solution for HSCCC separation.

HSCCC Separation Procedure

In this study, the head-tail elution mode was adopted. The three-multilayer coil separation column was first completely filled with the upper stationary phase at a flow rate of 25 mL min⁻¹. Then the apparatus was rotated at 850 rpm, while the lower mobile phase was pumped into the head inlet of the column at a flow rate of 2.0 mL min⁻¹. When a mobile phase emerged at the tail outlet which indicated a steady state hydrodynamic equilibrium was established, the sample solution was injected through the injection valve. By applying a

flow splitter, a small fraction of the effluent from the outlet of the column was monitored with an Alltech 800 ELSD detector (the drift tube temperature was 55°C, and the gas flow rate was 3.0 L min⁻¹), while the rest of the effluent was manually collected according to the chromatogram. Throughout the experiment, the separation temperature was controlled at 25°C. After peak fractions A-D were collected, the run was terminated and the fraction E which still retained in the column was collected by emptying the column contents by nitrogen gas.. Five fractions were concentrated under reduced pressure and their residuals were ready for subsequent analysis.

HPLC Analysis of HSCCC Peak Fractions

Purity of each peak fraction from HSCCC separation determined by HPLC was performed on a Welchrom C₁₈ column (250×4.6 mm, 5 μm) at 25°C. The mobile phase was acetonitrile-water (30:70, v/v) with isocratic elution, the flow rate was 1.0 mL min⁻¹, and an Alltech 2000 evaporative light scattering detector (the drift tube temperature was 104°C, and the gas flow rate was 2.8 L min⁻¹) was used to monitor the effluent online. The purity of HSCCC fractions was calculated in this method with area normalization.

Identification of HSCCC Peak Fractions

Identifying the structure of each peak fraction was performed by means of ESI-MS, ¹³C NMR. The spectrogram of ESI-MS was obtained by Thermo LTQ- XL ion trap mass spectrometer, and the spectrogram of NMR by Bruker Avance 500.

Results and Discussion

HSCCC Separation and HPLC Analysis—Fig. 3 shows the separation of crude extract of *Dioscorea zingiberensis* C.H.Wright by HSCCC with a two-phase solvent system composed of ethyl acetate-n-butanol-methanol-water (4:1:2:4, v/v) at a flow rate of 2 mL/min. Four peaks (A-D) were eluted, and one component (E) still retained in the column was recovered from the column contents. From 100 mg of the crude extract, five target steroid saponins were separated in less than 3.5 h with the total elution volume of 400 mL in one separation. This one-step work produced 20.1 mg of compound A (collected during 100-120 min), 25 mg of compound B (collected during 130-148 min), 17 mg of compound C (collected during 160-180 min), 12.3 mg of compound D (collected during 190-205 min), and 11.2 mg of compound E (collected from the column contents). The retention rate of the stationary phase was 42% after the separation was finished. The purities of these five target compounds were 95.8%, 97.2% and 96.4%, 96.1% and 95.2%, respectively. The HPLC analytical chromatogram was displayed in Fig. 2.

Identifying the Structures of the Compounds—The structural identification of five compounds was confirmed as the steroid saponins A-E shown in Fig 1 by means of modern spectroscopic techniques, including ESI-MS in Fig. 4, and ¹³C NMR.

Data of compound A: ESI-MS(m/z): 1249 ([M+Na]⁺), molecular formula: C₅₇H₉₄O₂₈; ¹³C NMR (125 MHz, pyridine-d₅): 37.4(C-1), 30.5(C-2), 78.6(C-3), 38.8(C-4), 141.3(C-5), 122.5(C-6), 32.4(C-7), 30.9(C-8), 49.9(C-9), 37.5(C-10), 21.2(C-11), 40.8(C-12), 41.5(C-13), 56.8(C-14), 32.1(C-15), 81.2(C-16), 63.8(C-17), 16.7(C-18), 20.0(C-19), 40.9(C-20), 16.6(C-21), 110.7(C-22), 36.8(C-23), 28.5(C-24), 34.7(C-25), 75.6(C-26), 17.8(C-27), 100.9(C-28), 77.2(C-29), 77.0(C-30), 82.1(C-31), 78.4(C-32), 62.9(C-33), 102.0(C-34), 72.4(C-35), 72.1(C-36), 74.2(C-37), 69.6(C-38), 18.2(C-39), 105.7(C-40), 88.1(C-41), 74.6(C-42), 69.2(C-43), 78.9(C-44), 61.1(C-45), 105.2(C-46), 75.4(C-47), 78.3(C-48), 71.1(C-49), 62.3(C-50), 78.5(C-51), 105(C-52), 75.1(C-53), 78.2(C-54), 71.7(C-55), 78.4(C-56), 62.4(C-57). Compared the above data with Ref [20], compound A was identified as 26-O-β-D-glucopyranosyl-(25R)-furost-5-en-3β,22ξ,26-triol-3-O-[β-D-

glucopyranosyl-(1→3)-β-D-glucopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside. Data of compound B: ESI-MS(*m/z*): 1087 ([M+Na]⁺), molecular formula: C₅₁H₈₄O₂₃; ¹³C-NMR (125 MHz, pyridine-*d*₅): 37.2(C-1), 31.5(C-2), 71.7(C-3), 42.5(C-4), 140.2(C-5), 121.9(C-6), 32.1(C-7), 31.7(C-8), 49.5(C-9), 36.1(C-10), 20.5(C-11), 39.4(C-12), 41.9(C-13), 56.1(C-14), 31.2(C-15), 81.0(C-16), 62.5(C-17), 16.4(C-18), 19.1(C-19), 41.4(C-20), 14.8(C-21), 108.9(C-22), 31.3(C-23), 28.4(C-24), 30.1(C-25), 66.9(C-26), 18.4(C-27), 100.1(C-28), 77.3(C-29), 88.7(C-30), 69.9(C-31), 78.0(C-32), 62.1(C-33), 101.1(C-34), 72.0(C-35), 72.2(C-36), 73.5(C-37), 69.8(C-38), 18.8(C-39), 103.1(C-40), 74.0(C-41), 77.5(C-42), 71.9(C-43), 78.3(C-44), 60.3(C-45), 100.2(C-46), 78.4(C-47), 91.3(C-48), 69.2(C-49), 62.6(C-50), 61.7(C-51). Compared the above data with Ref [21–22], compound B was identified as 26-O-β-D-glucopyranosyl-(25R)-furost-5-en-3β, 22ζ, 26-triol-3-O-[β-D-glucopyranosyl(1→3)-α-L-rhamnopyranosyl(1→2)]-β-D-glucopyranoside. Data of compound C: ESI-MS(*m/z*): 925 ([M+Na]⁺), molecular formula: C₄₅H₇₄O₁₈; ¹³C-NMR (125 MHz, pyridine-*d*₅): 37.8(C-1), 30.2(C-2), 78.6(C-3), 39.9(C-4), 141.4(C-5), 121.5(C-6), 32.4(C-7), 31.2(C-8), 50.7(C-9), 37.2(C-10), 21.2(C-11), 40.9(C-12), 40.5(C-13), 56.7(C-14), 32.7(C-15), 81.4(C-16), 64.2(C-17), 16.8(C-18), 19.9(C-19), 41.5(C-20), 16.2(C-21), 110.7(C-22), 37.1(C-23), 28.4(C-24), 34.1(C-25), 75.2(C-26), 17.8(C-27), 102.3(C-28), 73.1(C-29), 73.5(C-30), 72.2(C-31), 74.6(C-32), 69.2(C-33), 105.2(C-34), 75.7(C-35), 78.5(C-36), 71.2(C-37), 78.3(C-38), 63.5(C-39), 100.3(C-40), 78.7(C-41), 78.9(C-42), 62.5(C-43), 79.1(C-44), 72.6(C-45). Compared above the data with Ref [25], compound C was identified as 26-O-β-D-glucopyranosyl-(25R)-furost-5-en-3β, 22ζ, 26-triol-3-O-[α-L-rhamnopyranosyl(1→4)]-β-D-glucopyranoside.

Data of compound D: ESI-MS(*m/z*): 1231.67 ([M+Na]⁺), molecular formula: C₅₇H₉₂O₂₇; ¹³C-NMR (125MHz, pyridine-*d*₅): 37.0(C-1), 31.4(C-2), 71.6(C-3), 42.5(C-4), 140.5(C-5), 121.0(C-6), 32.0(C-7), 31.2(C-8), 50.3(C-9), 36.4(C-10), 20.7(C-11), 39.7(C-12), 40.0(C-13), 56.6(C-14), 31.6(C-15), 80.8(C-16), 62.3(C-17), 16.5(C-18), 19.7(C-19), 41.6(C-20), 14.3(C-21), 109.3(C-22), 31.6(C-23), 28.6(C-24), 30.1(C-25), 66.8(C-26), 17.2(C-27), 100.3(C-28), 81.5(C-29), 76.2(C-30), 78.6(C-31), 77.4(C-32), 61.6(C-33), 104.3(C-34), 73.5(C-35), 88.2(C-36), 69.7(C-37), 77.6(C-38), 61.8(C-39), 101.7(C-40), 72.8(C-41), 72.7(C-42), 74.1(C-43), 69.4(C-44), 18.9(C-45), 106.3(C-46), 75.7(C-47), 78.6(C-48), 71.9(C-49), 78.7(C-50), 62.6(C-51), 105.2(C-52), 75.4(C-53), 78.3(C-54), 71.8(C-55), 78.4(C-56), 63.3(C-57). Compared the above data with Ref [24], compound D was identified as 26-O-β-D-glucopyranosyl-(25R)-furost-5, 20(22)-diene-3β, 26-diol-3-O-[α-L-rhamnopyranosyl-(1→4)]-β-D-glucopyranosyl-(1→3)-β-D-glucopyranosyl-(1→2)]-β-D-glucopyranoside.

Data of compound E: ESI-MS(*m/z*): 1069.33([M+Na]⁺), molecular formula: C₅₁H₈₂O₂₂; ¹³C-NMR (125 MHz, pyridine-*d*₅): 37.5(C-1), 30.1(C-2), 78.0(C-3), 38.9(C-4), 140.7(C-5), 121.8(C-6), 32.4(C-7), 31.4(C-8), 50.3(C-9), 37.1(C-10), 21.2(C-11), 39.6(C-12), 43.4(C-13), 54.9(C-14), 34.5(C-15), 84.4(C-16), 64.5(C-17), 14.1(C-18), 19.4(C-19), 104.9(C-20), 11.8(C-21), 152.5(C-22), 23.7(C-23), 31.4(C-24), 33.5(C-25), 74.9(C-26), 17.3(C-27), 17.3(C-28), 99.9(C-29), 77.2(C-30), 77.7(C-31), 82.1(C-32), 76.2(C-33), 61.5(C-34), 101.8(C-35), 72.5(C-36), 72.7(C-37), 74.1(C-38), 69.5(C-39), 18.7(C-40), 104.9(C-41), 78.6(C-42), 71.7(C-43), 78.5(C-44), 62.8(C-45), 105.2(C-46), 75.0(C-47), 78.6(C-48), 71.2(C-49), 78.5(C-50), 61.9(C-51). Compared the above data with Ref [27], fraction III was identified as 26-O-β-D-glucopyranosyl-(25R)-furost-5, 20(22)-diene-3β, 26-diol-3-O-[β-D-glucopyranosyl-(1→4)-α-L-rhamnopyranosyl(1→2)]-β-D-glucopyranoside.

Optimization of the HSCCC Conditions—In the HSCCC separation, the first step is to select a two-phase solvent system which provides a satisfactory partition coefficient (*K*) to

the target compounds. This task is most critical and may account for almost 90% of the entire work [28]. The suitable K values for HSCCC are in a proper range of 0.5-2 to get an efficient separation and a suitable run time. A smaller K value elutes the solute closer to the solvent front with lower resolution, while a larger K value tends to give better resolution but broader, more dilute peaks due to a longer elution time. The steroid saponins from the rhizomes of *Dioscorea zingiberensis* C.H. Wright has high carbohydrate contents, which makes these saponins comparatively polar to provide a satisfactory solubility in hydrophilic solvents. In order to obtain suitable partition coefficients of the target compounds, the two-phase solvent system should provide moderate hydrophobicity. Therefore, the following five solvent systems have been examined: (1) chloroform-methanol-water, (2) chloroform-n-butanol-methanol-water, (3) n-hexane-ethyl acetate-methanol-water, (4) ethyl acetate-n-butanol-water, (5) ethyl acetate-n-butanol-methanol-water. K values of target compounds in these solvent systems are listed in Tables 1 and 2. These tables indicate that the K values of the solvent systems of chloroform-methanol-water and chloroform-n-butanol-methanol-water were too large to have satisfactory separation, while the solvent system of ethyl acetate-n-butanol-methanol-water (6:0.5:2:4, 4:1:2:4, and 4:2:2:4, v/v) gave acceptable K values. Since the HSCCC technology uses liquid as the stationary phase in separation column, successful separation largely depends on the amount of the stationary phase retained in the column. In other words, the higher the retention of the stationary phase, the better separation is expected. The retention of stationary phase of ethyl acetate-n-butanol-methanol-water (4:1:2:4 and 4:2:2:4, v/v) is better, but the K values of target compounds D and E in the latter are too large which would lead to broad and dilute peaks as mentioned before. Considering all these aspects discussed above, ethyl acetate-n-butanol-methanol-water with the volume ratio of 4:1:2:4 was selected as the two-phase solvent system for HSCCC.

Apart from optimizing the conditions of the solvent system, other influencing factors including revolution speed, the flow rate of mobile phase and the temperature were also investigated. The results indicated that the experimental conditions at a flow rate of 2 mL min⁻¹ and resolution speed of 850 rpm in 25°C produced the best separation.

Although the UV detector is widely used in HPLC, it has not ever been adopted in HSCCC for separation of saponins in wavelength of 200-210 nm [29]. Since these steroid saponins have few chromophores in their structures, they give weak UV absorption. In order to obtain satisfactory sensitivity and stable baseline, evaporative light scattering detection (ELSD) was used in the present study.

CONCLUSION

Five steroid saponins were successfully isolated and purified from crude extract of *Dioscorea zingiberensis* C.H. Wright for the first time by high-speed counter-current chromatography with the two-phase solvent system composed of ethyl acetate-n-butanol-methanol-water (4:1:2:4, v/v) in one-step operation. The effluent was monitored with evaporative light scattering detector in this research work. The method provides various advantages over the conventional preparative chromatographic methods such as high partition efficiency, total sample recovery, high reproducibility and almost no need for pretreatment of the crude sample.

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Fig. 1.
Chemical structures of compounds from *Dioscorea zingiberensis* C.H.Wright.
(A): Compound A; (B): Compound B; (C): Compound C; (D): Compound D; (E):
Compound E

Fig. 2.

HPLC chromatograms of total saponins and HSCCC peak fractions.

Conditions: column: Welch C₁₈ column (250×4.6 mm, 5 μm); mobile phase: acetonitrile-water (30:70, v/v); flow rate: 1 mL min⁻¹; Alltech 2000 ELSD condition: drift tube temperature: 104°C; gas flow rate 2.8 L min⁻¹; column temperature: 25°C; injection volume: 10 μL. (a): Total steroid saponins; (b): Compound A; (c): Compound B; (d): Compound C; (e): Compound D; (f): Compound E

Fig. 3. HSCCC chromatogram of total steroid saponins. Solvent system: ethyl acetate-n-butanol-methanol-water (4:1:2:4, v/v); stationary phase: upper organic phase; mobile phase: lower aqueous phase; flow-rate: 2.0 mL min⁻¹; revolution speed: 850 rpm; sample: 100 mg dissolved in 10 mL lower phase; retention of the stationary phase: 42%; Alltech 800 ELSD condition: drift tube temperature: 55°C; gas flow rate 3.0 L min⁻¹.

Fig. 4.
ESI-MS chromatogram of five compounds.
(a): Compound A; (b): Compound B; (c): Compound C; (d): Compound D; (e): Compound E

Table 1

The K values of the target fractions A–E in various two-phase solvent systems used in HSCCC.

No.	Solvent systems	Ratio(v/v)	K				
			A	B	C	D	E
1	Chloroform-methanol-water	4:3:2	22.2	132.89	25.45	35.1	37.2
2	Chloroform-n-butanol-methanol-water	4:0.2:3:2	136.4	27.5	14.2	33.1	35.9
3	n-hexane-ethyl acetate-methanol-water	1:1:1:1	-	-	-	-	-
4	Ethyl acetate-n-butanol-water	4:1:5	0.16	0.02	0.08	0.32	0.41
5	Ethyl acetate-n-butanol-methanol-water	4:1:0.5:6	0.21	0.04	0.14	0.41	0.53

Table 2

The K values of the target fractions A–E in ethyl acetate-n-butanol-methanol-water with different ratio

No.	Ratio(v/v)	retention of stationary phase	K				
			A	B	C	D	E
1	8:0.5:2:4	28%	0.28	0.10	0.19	0.55	0.72
2	7:0.5:2:4	30%	0.32	0.14	0.24	0.84	1.03
3	6:0.5:2:4	32%	0.40	0.21	0.54	1.03	1.52
4	4:1:2:4	42%	0.49	0.98	1.23	1.58	1.97
5	4:2:2:4	45%	0.53	1.15	1.74	1.98	2.35