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An approach based upon the consecutive separation and the economical two-phase solvent system preparation using UNIFAC mathematical model for increasing the yield of high-speed counter-current chromatography

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ABSTRACT

A HSCCC method combined with the consecutive injection and the economical two-phase solvent system preparation using UNIFAC mathematical model was successfully developed for increasing the yield of target compound. HSCCC was performed with a two-phase solvent system composed of *n*-hexane–ethyl acetate–methanol–water (3:5:3:5, v/v) at a flow rate of 6 mL/min. The components of the upper and lower phases were calculated according to UNIFAC mathematical model to prepare the stationary and mobile phases, separately. Only 300 mL stationary phase was filled into the column, and the crude sample (200 mg, ten times) was consecutive loaded into the column at the given interval time and eluted using the individually prepared mobile phase without reestablishing hydrodynamic equilibrium. After recrystallized, 168 mg tiliroside with the purity of 95.8% was obtained from 2 g crude sample of *Pinus massoniana* L. The structure was identified by ^{1}H NMR and ^{13}C NMR. The present method not only avoided the time consuming of exchanging the new solvent system and reestablishing hydrodynamic equilibrium, but also coped with a large amount of organic solvent waste.

1. Introduction

High-speed counter-current chromatography (HSCCC), a support-free liquid–liquid partition chromatography, is a stable and repeatable method, which has been widely used for the separation and purification of natural products [1–3]. In the past studies, HSCCC with the unique properties, such as high recovery, high efficiency and eliminating irreversible adsorption of sample on the solid stationary phase, has been considered as a powerful tool for the separation and purification of minor compositions and bioactive compounds from the natural resources [4,5].

However, the separation efficiency of targets in HSCCC was significantly restricted by the volume of multilayer coil column [6]. Sometimes, the separation should be repeated several times to enrich minor compounds for the structure identification and bioactivity assays. To cope with this problem, the method of consecutive HSCCC was developed, which avoid time-consuming in exchanging new solvent and the solvent system equilibrium [6–10].

However, the consecutive HSCCC was not significant efficiency for reducing solvent consumption. The volume of stationary organic phase was the capacity of the column in the HSCCC separation. While the volume of mobile aqueous phase based on the flow rate and separation time was much more than that of organic phase. Using the traditional preparative method, the upper and lower phases with the similar volumes were simultaneously obtained before HSCCC separation [2,11]. This meant that a great deal of stationary organic phase was wasted. To solve this problem, UNIFAC mathematical model was introduced to calculate the components of each solvent in the upper and lower phases, respectively [12]. The upper and lower phases can be prepared according to required volumes separately.

In the present study, the consecutive HSCCC combined with the economical two-phase solvent system preparation using UNIFAC mathematical model was established to increase the yield of

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high-speed counter-current chromatography and decrease the organic solvent usages. The consecutive separation of tiliroside (Fig. 1) from *Pinus massoniana* L. is presented as an example, which possess the activities of anti-inflammatory, antioxidant, anticarcinogenic, hepatoprotective and cytochrome P450 inhibitory [13–18].

2. Materials and methods

2.1. Apparatus

The preparative HSCCC instrument employed in this study was a model TBE-300C high-speed countercurrent chromatograph (Shanghai, Tauto Biotech, China) with three polytetrafluoroethylene (PTFE) preparative coils (internal tube diameter: 2.6 mm and total volume: 300 mL). The revolution radius was 5 cm and the β value of the multilayer coil varied from 0.5 at internal to 0.8 at the external terminal. A DC0506 low constant temperature bath (Shanghai, Tauto Biotech, China) was used to maintain the internal HSCCC temperature at 25 °C. The speed of the apparatus could be regulated in a range between 0 and 1000 rpm, where the optimum speed of 800 rpm was used throughout the present study. The solvent was pumped into the column with a model TBP5002 constant-flow pump (Tauto Biotech, Shanghai, China). Continuous monitoring of the effluent was achieved with a model UV2000D monitor (Tauto Biotech, Shanghai, China) at 254 nm, and a manual sample injection valve with a 20 mL loop for the preparative HSCCC was used to introduce the sample into the column. Model V2.0.2B chromatography workstation (Tauto Biotech, Shanghai, China) was used to draw the chromatogram.

The analytical high-performance liquid chromatography (HPLC) equipment included a 230P pump, a Rehodyne 3725i-038 manual sample injector and a UV230II detector. The analysis was carried out with a SinoChrom ODS-BP-C18 column (5 μm, 4.6 mm × 200 mm) from Yilite company, Dalian, China. Evaluation and quantification of data were performed on an EC2006 workstation.

2.2. Chemicals and reagents

Acetonitrile and methanol used for HPLC analysis were of HPLC-grade and purchased from Young Il Metal Co. Ltd (Seoul, South Korea). All organic solvents used for HSCCC were of analytical grade and purchased from Tianjin Chemical Factory (Tianjin, China). Water was distilled and purified using a Milli-Q Water Purification System (Millipore, Bedford, MA, USA).

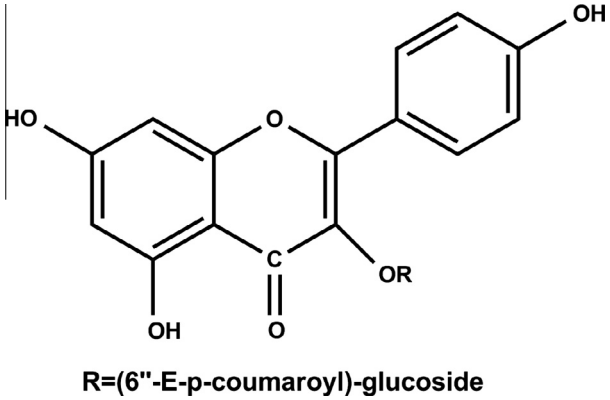

Fig. 1. The chemical structure of tiliroside.

The needles of *Pinus massoniana* L. were collected from Dalian (Liaoning, China) and were verified by Guanmian Shen (Xinjiang Institute of Ecology and Geography, Chinese Academy of Sciences).

2.3. Preparation of crude samples

The dried needles of *Pinus massoniana* L. (2 kg) were refluxed with 6L 70% ethanol (3 h, 3 times). The extracts were combined and evaporated to dryness under reduced pressure. The residue was dissolved in water (1 L), which was extracted by light petroleum (500 mL, 3 times), ethyl acetate (500 mL, 3 times), *n*-butanol (500 mL, 3 times), respectively. The ethyl acetate extract was combined and evaporated to dryness, yielding 30.7 g of crude sample.

2.4. Measurement of partition coefficient (K)

The two-phase solvent systems were selected according to the partition coefficient (*K*) of the target components. Different volume ratio of *n*-hexane–ethyl acetate–methanol–water were prepared and equilibrated in a separatory funnel at room temperature. The *K* values were determined by HPLC analysis as follows: A suitable amount of crude sample (5 mg) was dissolved in 2.0 mL of the mixture containing equal volume of each phase of the two-phase solvent system in a separatory funnel, and the contents were mixed thoroughly. After equilibration was established, the upper phase and the lower phase were analyzed by HPLC separately. The peak area of the upper phase was recorded as A_U and that of the lower phase was recorded as A_L. The *K* value was calculated according to the equation, $K = A_U/A_L$ (Table 1) [3].

2.5. Preparation of two-phase solvent system

In the present study, the two-phase solvent system composed of *n*-hexane–ethyl acetate–methanol–water (3:5:3:5, v/v) was used to separate the target compound. Two methods were used for preparing the above solvent system.

Preparation method I: The solvent mixture was thoroughly equilibrated in a separatory funnel by vigorous shaking and degassing several times. The upper and lower phases were separated shortly before use [19].

Preparation method II [12]: The components of upper and lower phases of the two-phase solvent system were calculated by selection software with UNIFAC mathematical model for solvent system of HSCCC (Tauto Biotech, Shanghai, China). The upper and lower phases were prepared separately. The calculated details, such as the molar concentration, the average density, total volume and viscosity of the solution in each phase, were taken into the Eqs. (1) and (2):

$$M_a = x_i M_i + x_j M_j + x_k M_k + \cdots + x_n M_n \quad (1)$$

$$V_i = \frac{x_i V_t \rho_t M_i}{M_a \rho_i} \quad (2)$$

Table 1
K values of tiliroside in the different two-phase solvent systems composed of *n*-hexane–ethyl acetate–methanol–water.

No.	Solvent system (*v*/*v*)	Compound 1
1	1:1:1:1	0.04
2	4:5:4:5	0.40
3	3:5:3:5	2.60
4	2:5:2:5	16.40
5	1:5:1:5	72.77