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Computation-aided separation of seven components from *Spirodela polyrrhiza* (L.) *via* counter-current chromatography

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ABSTRACT

In this study, seven flavonoids were successfully separated from *Spirodela polyrrhiza* (L.) Schleid. *via* high-speed counter-current chromatography (HSCCC) with elution–extrusion and online recycling elution modes. The first step for successful HSCCC separation is the crucial solvent system selection. In this work, nonrandom two-liquid segment activity coefficient (NRTL-SAC) method, a computational strategy, was used to facilitate the solvent system selection process. According to the NRTL-SAC method, a suitable solvent system can be rapidly predicted and screened by calculating the partition coefficient rather than by performing tedious experimental measurements. Based on the method, two solvent systems of hexane/ethyl acetate/ethanol/water (1:3:1:3 and 1:9:1:9, v/v) were predicted as the most suitable systems for HSCCC separation. To avoid unnecessary waste of the stationary or mobile phase, both the mobile and stationary phases were prepared independently based on the calculated phase compositions by thermodynamic method. Consequently, seven compounds were successfully separated with high purity (>90%). The separated compounds were identified through UV spectra and high-resolution tandem mass spectroscopy. These compounds include orientin (**1**), vitexin (**2**), luteolin 7-O-glucoside (**3**), apigenin 7-O-glucoside (**4**), luteolin 8-C-(2″-O-feruloyl-) glucoside (**5**), apigenin 8-C-(-2″-O-feruloyl-) glucoside (**6**), and luteolin (**7**). This work demonstrates that HSCCC is suitable for separation of natural products, and computational strategy is very helpful for enhancing the efficiency of CCC experiments.

1. Introduction

Spirodela polyrrhiza (L.) Schleid., a type of aquatic plant growing mainly in pools, lakes, and paddy field [1], is a traditional Chinese medicine used to cure urticaria, acute nephritis, and influenza [2]. Flavonoids such as orientin and vitexin are major components of *S. polyrrhiza* [3]. These flavonoids possess several biological activities, such as antioxidant, antibacterial, and anti-inflammatory properties [4–6]. In addition, orientin and vitexin have an adipogenesis inhibitory effect on lipid accumulation [7]. Thus, flavonoids are considered as chemical components that have a major contribution to the pharmacological activity of *S. polyrrhiza*. Investigation into the separation of flavonoids is important to further understand the biological activities of *S. polyrrhiza*.

As a support-free chromatographic technique, HSCCC has been widely utilized for the separation and purification of natural products [8–10]. Compared with conventional solid sorbent-based (such as silica gel and macroporous resin) chromatography, HSCCC possesses distinctive advantages, including easy operation, no irreversible adsorption, and elimination of sample loss [11,12]. For a successful HSCCC separation, the selection of a suitable solvent system is the first crucial step, one that can take 90% of the time for a CCC separation run [13]. Solvent system selection is recognized as a difficult task, particularly for beginners. Solvent system selection is mostly performed by experimentally measuring the partition coefficient (K), a process that is always time consuming and labor intensive. The emergence of predictive methodology provides a novel idea for rapid solvent system selection [14,15]. Given such predictive methods, solvent system selection can be performed by predicting the partition coefficient. In our recent works, nonrandom two-liquid segment activity coefficient (NRTL-SAC) model, a thermodynamic model, was used to develop a predictive method by calculating partition coefficients [16,17]. The NRTL-SAC method is an efficient option for screening a suitable solvent system by calculating the solute partition coefficients in a series of biphasic liquid systems. Based on the calculated results, a suitable solvent system can be rapidly selected for HSCCC separation. The NRTL-SAC method is a promising high-throughput method for rapid solvent system selection, and has been successfully used to

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select a solvent system for HSCCC separation of natural products [18]. Thus, the NRTL-SAC method is selected to facilitate the solvent system selection process in the present work.

In CCC, two-phase solvent systems are conventionally prepared according to the predefined volume ratios [13]. Such preparations cannot independently control the volumes of each phase, which inevitably leads to unnecessary solvent wastage (always the stationary phase). One strategy dealing with this drawback is to prepare the mobile and stationary phases individually (on-demand preparation) [19]. On-demand preparation requires prior knowledge of the phase compositions of the used biphasic systems. In practice, the phase compositions can be calculated by thermodynamic models or measured by experiment (such as gas chromatography) [20,21]. Compared to the conventionally prepared biphasic system, the solvent system prepared separately provided similar solute *K* values and HSCCC chromatogram. By using thermodynamic methods, one can rapidly estimate the physiochemical properties of a specific two-phase solvent system. Previous studies have demonstrated that reliable phase compositions can be calculated by universal quasi-chemical functional-group activity coefficients (UNIFAC) model [22,23]. In this work, the used biphasic systems were prepared independently based on the phase compositions calculated by UNIFAC model.

To the best of our knowledge, few reports on using HSCCC for separating flavonoids from *S. polurrhiza* have been presented in previous works. The ultimate goal of this research is to establish an effective HSCCC method for separating flavonoids from *S. polyrrhiza*. And computational strategy was employed to improve the experimental efficiency of HSCCC separation.

2. Materials and methods

2.1. Reagents and materials

Acetonitrile of HPLC grade (Jiangsu Hanbon Science and Technology Co., Ltd., Huaian, China) was used for UPLC analysis. All organic solvents used for sample preparation and HSCCC separation were of analytical grade (Tianjin Hengxing Chemical Preparation Co., Ltd., Tianjin, China). Deionized water was obtained with a Milli-Q (18.2 MΩ) system (Millipore Bedford, MA, USA).

The whole grass of *S. polyrrhiza* was purchased from local pharmacies (Changsha, China).

2.2. Apparatus

A TBE-300B HSCCC (Tauto Biotechnique Company, Shanghai, China) instrument was used. The apparatus was equipped with three multilayer coil separation columns connected in series (tube diameter = 1.6 mm, total volume = 260 mL) and a 20 mL sample loop. The β-value varied from 0.5 (internal layer) to 0.8 (external layer). The rotation speed of the apparatus could be regulated with a speed controller (0–1000 rpm). The HSCCC system was also equipped with a model S constant-flow pump, a model UV-II detector (254 and 280 nm), a model N2010 chromatography workstation, a temperature controller HX-1050 (Beijing Boyikang Lab Instrument Co. Ltd., Beijing, China), and a BSZ-100 auto parts collector (Shanghai Qingpu-Huxi Instruments Factory, Shanghai, China).

UPLC analysis was performed on a Dionex U3000 Series UPLC system (Dionex, Sunnyvale, CA, USA). The UPLC system consisted of a vacuum degasser, binary pump, autosampler, thermostatted column compartment, and DAD detector.

The ESI-MS (MS/MS) experiments were performed with an IT-TOF mass spectrometer (Shimadzu MS-IT-TOF, Kyoto, Japan) *via* an ESI interface.

2.3. Extraction of crude sample

The dried and pulverized *S. polyrrhiza* (100 g) was extracted with 70% ethanol (1000 mL, 2 × 30 min) in an ultrasonic bath (T = 25 °C). The extract was combined, filtered, and evaporated to dryness *via* rotary evaporation, which yielded 5.7 g of crude extract. The crude extract was then dissolved and suspended in 300 mL water and successively extracted with *n*-hexane (2 × 300 mL), ethyl acetate (2 × 300 mL), and *n*-butanol (2 × 300 mL). The dried ethyl acetate extract (1.97 g) was stored in a refrigerator for subsequent UPLC analysis and HSCCC separation.

2.4. On-demand preparation of two-phase solvent system and sample solution

In current work, on-demand preparation strategy was used to prepare the two-phase solvent systems including hexane/ethyl acetate/ethanol/water (HexEEtWat 1:3:1:3 and 1:9:1:9, v/v). In our previous study (unpublished), UNIFAC model was evaluated for calculating the phase compositions of HexEEtWat solvent systems, and the results is acceptable. Thus, UNIFAC model was used to calculate the phase compositions (liquid–liquid equilibrium data) of each solvent. The accurate volume of hexane, ethyl acetate, ethanol and water with the volume proportion calculated by UNIFAC methods were added into two solvent bottles to constitute the upper phase and lower phase, respectively. Then, the lower and upper phases were separated and degassed *via* sonication for 10 min before use.

The sample solution was prepared by dissolving 500 mg of ethyl acetate extract in 10 mL of pre-equilibrated biphasic system (5 mL upper phase and 5 mL lower phase).

2.5. NRTL-SAC method for solvent system selection

In this work, the NRTL-SAC method was used to select suitable biphasic solvent systems. The entire procedure of the NRTL-SAC method includes three steps. First, the solute *K* values in at least three preselected solvent systems must be determined. Our previous study suggested that hexane/ethyl acetate/methanol/water (HexEMWat 1:9:1:9, 1:1:1:1 and 9:1:9:1) systems should be selected as the initial tested biphasic systems [17]. Second, the measured solute *K* values are used to fit the NRTL-SAC model and identify the solute molecular parameters. Lastly, several *K* values in a large number of solvent systems can be predicted *via* NRTL-SAC method. Based on the calculated *K* values, the best solvent system can be directly selected for separation. A detailed description and procedure of the NRTL-SAC method can be found in our previous reports [17].

2.6. HSCCC separation

In this work, a two-step HSCCC procedure was developed to separate seven components from *S. polyrrhiza*. The elution–extrusion mode of HSCCC was employed in the first step, and then a recycling elution mode was used in the second step. The column was initially filled the stationary phase (upper organic phase) at the beginning of the elution–extrusion HSCCC. Then, the mobile phase was pumped into the column at a flow rate of 2 mL/min with a rotation speed of 900 rpm. The sample solution was injected into the column when a hydrodynamic equilibrium in the column was established. After eluting 400 mL of the mobile phase (lower aqueous phase), the stationary phase (upper organic phase) was pumped into the column to extrude the retained substances under the same instrumental conditions. The effluent was monitored