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Rational approach to solvent system selection for liquid–liquid extraction–assisted sample pretreatment in counter–current chromatography

Jiajia Wang[a], Dongyu Gu[b,*], Miao Wang[c], Xinfeng Guo[c], Haoquan Li[c], Yue Dong[c], Hong Guo[a], Yi Wang[c,*], Mengqi Fan[a], Yi Yang[a,*]

[a] School of Light Industry and Chemical Engineering, Dalian Polytechnic University, Dalian 116034, China
[b] School of Marine Science and Environment Engineering, Dalian Ocean University, Dalian 116023, China
[c] School of Biological Engineering, Dalian Polytechnic University, Dalian 116034, China

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ABSTRACT

A rational liquid–liquid extraction approach was established to pre–treat samples for high–speed counter–current chromatography (HSCCC). *n*-Hexane–ethyl acetate–methanol–water (4:5:4:5, v/v) and (1:5:1:5, v/v) were selected as solvent systems for liquid–liquid extraction by systematically screening *K* of target compounds to remove low- and high-polarity impurities in the sample, respectively. After liquid–liquid extraction was performed, 1.4 g of crude sample II was obtained from 18.5 g of crude sample I which was extracted from the flowers of *Robinia pseudoacacia* L., and then separated with HSCCC by using a solvent system composed of *n*–hexane–ethyl acetate–methanol–water (1:2:1:2, v/v). As a result, 31 mg of robinin and 37 mg of kaempferol 7–*O*–α–L–rhamnopyranoside were isolated from 200 mg of crude sample II in a single run of HSCCC. A scale–up separation was also performed, and 160 mg of robinin with 95% purity and 188 mg of kaempferol 7–*O*–α–L–rhamnopyranoside with 97% purity were produced from 1.2 g of crude sample II.

1. Introduction

High–speed counter–current chromatography (HSCCC), a liquid–liquid partition technology, has been widely applied to separate chemical compositions [1–4]. Before HSCCC separation is performed, the crude samples extracted from natural resources are refined with pretreatment methods, such as macroporous resin chromatography [5,6], Sephadex LH–20 column [7], silica gel column [8], and liquid–liquid extraction [9], to increase the yield and purity of target compounds. Among these methods, liquid–liquid extraction is the most simplest and commonly used method to remove the impurities [10–14]. In traditional liquid–liquid extraction, solvent systems are mainly composed of single water–immiscible solvent and water, such as *n*–hexane–water, ethyl acetate–water, chloroform–water and *n*–butanol–water. But the extraction with those solvent systems may induce loss of target compounds or incomplete removal of impurities. However, the partial loss of minor components in extraction may render them undetectable for further separation.

In our previous study, an ionizable sample pretreatment method based on acid–base extraction was established for pH–zone–refining CCC. *K* values were considered to select two solvent systems which were used to remove impurities whose acidity was higher or lower than that of target compounds. This strategy had been successfully employed to prepare alkaloids [15]. Based on the above results, two–phase solvent systems for sample pretreatment in regular HSCCC separation can be selected by evaluating the *K* values of target compounds. *K* value between 0.5 and 2.5 was suitable for HSCCC separation [16]. A solvent system with small low *K* values was inappropriate for HSCCC separation, but was applicable to remove low–polarity impurities. Simultaneously, a solvent system with high *K* can be used to remove high–polarity impurities.

Robinia pseudoacacia L. (RPL), a traditional Chinese medicine, is a widely distributed species with interesting biological activities, including anticancer potential [17], improving capillary elasticity, reducing blood lipids [18], as well as strong antioxidant potential [19] and immune enhancement [20]. The major chemical ingredients of PRL were tannins, flavonoids, sterol and choline [21,22].

In the present study, a rational solvent system selection for the pretreatment of samples for traditional HSCCC was established. Two active flavonoids, namely, robinin and kaempferol 7-*O*-α-L-rhamnopyr-

* Corresponding authors.
E-mail addresses: gudongyu@dlou.edu.cn (D. Gu), wangyi@dlpu.edu.cn (Y. Wang), yangyi105@mails.ucas.ac.cn (Y. Yang).

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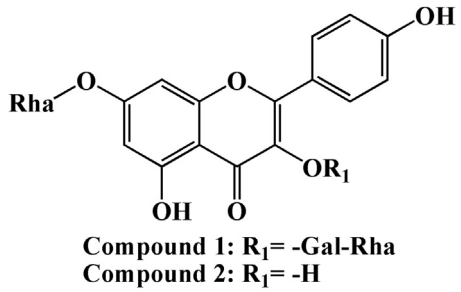

Fig. 1. The structures of robinin and kaempferol 7–*O*–α–L–rhamnopyranoside.

anoside (Fig. 1), from RPL flowers were separated and presented as an example.

2. Materials and methods

2.1. Apparatus

The HSCCC separation system included a model TBE–300C high–-speed countercurrent chromatograph (Tauto Biotech, Shanghai, China) with three polytetrafluoroethylene (PTFE) coils (internal tube diameter: 1.9 mm), a model TBP5002 constant–flow pump (Tauto Biotech, Shanghai, China), a model UV2000D monitor at 254 nm (Sanotac Scientific Instruments CO., LTD, Shanghai, China) and a model V2.0.2B workstation (Tauto Biotech, Shanghai, China). The total volume was 300 mL.

HPLC equipment (Yilite company, Dalian, China) included a UV230II detector, two 230P pumps and a Rehodyne model 3725i-038 sample injector. The analysis was carried out with a SinoChrom ODS-BP-C18 column (5 μm, 4.6 mm × 200 mm). The data were processed on EC2006 workstation (Yilite company, Dalian, China).

2.2. Chemicals and reagents

HPLC–grade methanol was purchased from Young Metal Co. Ltd. (Seoul, South Korea). All analytical grade solvents were purchased from Beijing Chemical Works (Beijing, China).

The flowers of *Robinia pseudoacacia* L. (RPL) were collected from Dalian, China and were authorized by Guanmian Shen (Xinjiang Institute of Ecology and Geography, Chinese Academy of Sciences).

2.3. Preparation of crude sample I

The dried flowers of RPL (221 g) were refluxed with light petroleum (b.p. 30–60 °C) (2.5 L, 3 times) and ethanol (2.5 L, 3 times) for 3 h. 18.5 g of ethanol extracts (crude sample I) was obtained.

2.4. Measurement of partition coefficient (K)

The two solvent systems were prepared by mixing different volume ratio of *n*–hexane–ethyl acetate–methanol–water. After equilibration was reached, a suitable amount of crude sample was dissolved in the mixture of upper and lower phase. The contents were mixed thoroughly in a separatory funnel. The *K* value can be calculated based on the equation, $K = A_U/A_L$ (Table 1), where A_U and A_L were the peak area of the upper phase and the lower phase, respectively [23].

2.5. Pretreatment of crude sample I

The crude sample I (18.5 g) was dissolved in the upper phase (1.0 L) of *n*–hexane–ethyl acetate–methanol–water (4:5:4:5, v/v) and extracted twice by 1.0 L of the lower phase. The sample of lower phase was combined and concentrated to dryness and then dissolved in the lower phase (1.0 L) of *n*–hexane–ethyl acetate–methanol–water (1:5:1:5, v/v) and extracted twice by 1.0 L of the upper phase. The upper phase was combined and evaporated to dryness. 1.4 g of crude sample II was obtained and used for HSCCC separation.

Table 1
K values of robinin and kaempferol 7-*O*-α-L-rhamnopyranoside in the different two-phase solvent systems composed of *n*-hexane-ethyl acetate-methanol-water.

No.	Solvent system (*v/v*)	Compound 1	Compound 2	Separation factor (α)
1	1:1:1:1	–	0.02	–
2	4:5:4:5	0.04	0.09	2.25
3	3:5:3:5	0.57	0.86	1.51
4	1:2:1:2	1.02	1.56	1.53
5	2:5:2:5	3.33	3.86	1.16
6	1:5:1:5	9.95	11.23	1.13

2.6. HSCCC separation procedure

The upper phase was pumped into the coiled column as stationary phase. After the rotational speed reached 800 rpm, the sample solution was loaded into the column. The lower phase was pumped into the head end of the separation column as mobile phase at a flow rate of 2.0 mL/min. The effluent was detected at 254 nm.

2.7. HPLC analysis and identification of isolated fractions

The crude samples and isolated compounds were analyzed by a SinoChrom ODS–BP–C18 column (4.6 mm i.d. × 250 mm, 5 μm). The gradient elution of methanol (A) and 0.2% formic acid (B) was carried out from 15% to 100% A in 50 min at a flow rate of 1.0 mL/min. The detection wavelength of effluent was 254 nm.

3. Results and discussion

3.1. HPLC analysis

Several solvent systems, such as methanol–water, and methanol–-formic acid with gradient elution, are employed to separate crude samples. Good separation can be achieved when methanol (A) and 0.2% formic acid (B) were utilized as the mobile phase and the gradient was from 15% to 100% A in 50 min at a flow rate of 1.0 mL/min. Formic acid was used to alleviate the peak tailing of compositions [8]. Crude samples and purified fractions were analyzed under the optimum condition (Fig. 2).

3.2. Selection of HSCCC solvent system

K values of compounds 1 and 2 were systematically examined using different two–phase solvent systems composed of n–hexane–ethyl acetate–methanol–water (Table 1). Solvent system selection was initiated with two–phase solvent system 1 composed of hexane–ethyl acetate–methanol–water at a volume ratio of 1:1:1:1 because the polarity of the target compound was unknown [1]. The target compounds exhibited low *K* values ($K < 0.1$) in solvent systems 1 and 2 and were mostly distributed in the lower aqueous phase. In solvent systems 5 and 6, the target compounds were mostly found in the upper organic phase (*K* was approximately 10). Solvent systems 3 and 4 provided suitable *K* ranging from 0.5 to 2.5 [24]. Solvent system 4 with a larger separation factor ($\alpha = K_2/K_1$) was selected for HSCCC seperation.

3.3. Two–phase solvent system selection for the sample pretreatment

The selection of two–phase solvent systems is an important step in sample pretreatment. The two–phase solvent system was also selected according to *K* value of target compound. A low *K* contributed to the