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Pressurized liquid extraction coupled with countercurrent chromatography for systematic isolation of chemical constituents by preprogrammed automatic control

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ABSTRACT

Pressurized liquid extraction (PLE) coupled with high-speed countercurrent chromatography (HSCCC) *via* an automated procedure was firstly developed to extract and isolate ginsenosides from *Panax quinquefolium*. The experiments were designed under the guidance of mathematical model. The partition coefficient (*K*) values of the target compounds and resolutions of peak profiles were employed as the research indicators, and exponential function and binomial formulas were used to optimizing the solvent systems and flow rates of the mobile phases in a three-stage separation. In the first stage, ethyl acetate, *n*-butanol, and water were simultaneously pumped into the solvent separator at the flow rates 11.0, 10.0, and 23.0 mL/min, respectively. The upper phase of the solvent system in the solvent separator was used as both the PLE solvent and the HSCCC stationary phase, followed by elution with the lower phase of the corresponding solvent system to separate the common ginsenosides. In the second and third stages, rare ginsenosides were first separated by elution with ethyl acetate, *n*-butanol, methanol, and water (flow rates: 20.0, 3.0, 5.0, and 11.0 mL/min, respectively), then with *n*-heptane, *n*-butanol, methanol, and water (flow rates: 17.5, 6.0, 5.0, and 22.5 mL/min, respectively). Nine target compounds, with purities exceeding 95.0%, and three non-target compounds, with purities above 84.48%, were successfully separated at the semipreparative scale in 450 min. The separation results prove that the PLE/HSCCC parameters calculated *via* mathematical model and formulas were accurately and scientifically. This research has opened up great prospects for industrial automation application.

1. Introduction

At present, in order to purify natural products, extraction, concentration and separation processes are performed independently. To expedite these time-consuming processes, integrated techniques [1], as well as automated extraction and separation processes [2] have been pursued for the extraction and isolation of natural products.

In this work, pressurized liquid extraction (PLE) and high-speed countercurrent chromatography (HSCCC) were employed for automated extraction and separation. PLE has several advantages over traditional solvent extraction methods, including short extraction times, adjustable extraction temperature (room temperature to 300 °C), on-line extract purification, high reproducibility, and low extraction discrimination [3,4]. PLE has been used for extractions in the food and pharmaceutical fields [5,6]. HSCCC is an advantageous separation technique because in this technique the analytes do not interact with a solid stationary phase, which prevents irreversible adsorption; this technique is particularly suitable for the preparative isolation of natural products in the chemical industry [7,8].

However, HSCCC has some drawbacks as well. First is the requirement that the compounds must be distributed between two immiscible phases [9]. However, the polarity range of the compounds separated by HSCCC technique is very narrow, and hence, most of the reports have focused on several key components

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in a specific fraction only [10–12]. Nowadays, many laboratories are focusing on the separation and evaluation of natural products obtained from plants for high-throughput screening [13–16]. However, such compounds have a wide range of polarity. Thus, it becomes imperative that a rapid multistage single-step separation method be developed that can process a large number of compounds with varying polarity.

Second, the screening method used in the current HSCCC solvent systems are usually prepare 4–10 candidate solvent systems, and the optimal solvent system has to be selected by determining the *K* values of these systems [17,18]. However, this screening method has a great deal of randomness and blindness, and the solvent system so obtained may not be the best solvent system. During our research, we found that the *K* values of target compounds have a nonlinear relationship with the solvent composition in two-phase solvent systems. Therefore, it is necessary to apply a mathematical method to calculate the relationship between the solvent composition of the two-phase solvent system and the *K* value of the target compound by using the obtained *K* values; the optimal solvent ratios of the two-phase solvent system can then be calculated, and the solvent system obtained so can be identified as the optimum solvent system in our work.

Third, the use of organic solvents such as *n*-heptane, ethyl acetate, methanol, and in particular chloroform and methylene chloride are highly toxic, and their excessive exposure can lead to serious diseases [19]. Therefore, it is very important to develop a suitable instrumental setup for industrial separation and to prevent their coming into contact with human population.

Fourth, in conventional extraction and separation processes, parameters such as pump flow rates, PLE temperature, extraction time, rotation speed of HSCCC columns, and flow rates of HSCCC mobile phases are manually controlled, which involves a great deal of human resources. In order to save the resources and automate the process, it is necessary to develop a software control system for the complete process control of the entire PLE/HSCCC system.

Herein, a novel, rapid PLE-coupled HSCCC method (Fig. 1), which involves an automated three-stage extraction and online separation process, was developed that can process compounds, of varying polarity, in plant samples. *Panax quinquefolium* has a broad range of common ginsenosides, including Rb_1, Rb_2, Rc, Rd, Re, and Rg_1, and was employed as the target test herb in this study because of its medicinal properties [20]. Previous work has shown that the pharmacological activities of the rare ginsenosides, Rg_3, Rk_1, Rg_5, Rh_4, Rk_3, Rs_4, and Rs_5, are higher than those of common ginsenosides [21,22]. It is important to note that common ginsenosides in *P. quinquefolium* can be converted into rare ginsenosides by high temperature and pressure extractions. To simultaneously obtain common and rare ginsenosides, appropriate temperatures for the three-stage PLE of *P. quinquefolium* were determined. The extract was subsequently separated on-line using HSCCC.

In the first stage of the PLE/HSCCC process, the ethyl acetate:*n*-butanol:water ratio was optimized using a binomial formula, and the separation was then carried out with the calculated solvent mixture. More than three chromatographic peaks were detected following the separation. In the second and third stages, the raw *P. quinquefolium* plant material was extracted at higher temperature ($T > 100\,^{\circ}C$) to hydrolyze common ginsenosides into rare ginsenosides. The rare ginsenosides were then extracted and eluted with solvent composions of ethyl acetate, *n*-butanol, methanol, and water in stage 2, and solvent composions of *n*-heptane, *n*-butanol, methanol, and water in stage 3. The optimal solvent ratios were calculated with an exponential function model using MATLAB software, and six to seven peaks were detected following stages 2 and 3.

Using this online extraction and isolation process, 12 ginsenosides – Rb_1, Re, Rg_1, F_4, Rh_4, 20S-Rg_3, Rk_1, Rs_4, Rs_5, 20S-Rg_3, Rg_5, and 20R-Rs_4 – were separated and purified from *P. quinquefolium* in 450 min. The process was highly automated: the pump flow rates, flow rate of the mobile phase pump, extraction temperature, and adjustable "T" splitters, which connected the PLE and HSCCC, were controlled by software programs designed in lab. The PLE temperature [23], *K* values of the target compounds [24], flow rate of the mobile phase, and retention of the stationary phase [25] were essential experimental variables and were optimized using computer programs. This automated method can efficiently extract and isolate natural products from plants and has great potential for industrial applications.

2. Experimental

2.1. Correlation of PLE solvent ratio with target compound yields

A two-phase solvent system was used for the PLE-coupled HSCCC process. In the first PLE stage, extractions were performed at 60 °C; in this stage, the upper phase of the solvent was composed of ethyl acetate, *n*-butanol, and water. The correlation analysis of the three solvents was then conducted (the *n*-butanol:water ratio was maintained constant, *i.e.*, 1:5, because the amount of water dissolved in *n*-butanol was constant regardless of how much water was added in the solvent system).

In the second PLE stage, the upper phase of the solvent consisted of ethyl acetate, *n*-butanol, methanol, and water, and extractions were performed at 110 °C. In the third stage, the upper phase was composed of *n*-heptane, *n*-butanol, methanol, and water, and extractions were carried out at 130 °C. For these extractions, the ratios of ethyl acetate:*n*-butanol and *n*-heptane:*n*-butanol were critical, because ginsenosides dissolve readily in *n*-butanol, but are poorly soluble in ethyl acetate and *n*-heptane. The ratios of *n*-butanol to methanol and water were maintained constant. First, the optimal ethyl acetate:*n*-butanol and *n*-heptane:*n*-butanol ratios were determined based on the yield of Rb_1. For the second stage, the ethyl acetate:*n*-butanol ratio was determined based on the yield of 20S-Rg_3. For the third stage, the optimal *n*-heptane:*n*-butanol ratio was determined based on the yield of Rs_4. The proportion of methanol:*n*-butanol was determined based on the *K* values obtained in the subsequent experiments.

2.2. Partition coefficients

In this study, ginsenosides of varying polarities from *P. quinquefolium* were separated by a three-stage PLE/HSCCC process. A mathematical model was used to determine the *K* values of the target compounds. In the first stage, a solvent system composed of ethyl acetate, *n*-butanol, and water was used to separate the hydrophilic ginsenosides. The upper phase of the solvent system was used as the PLE solvent for extracting common polar ginsenosides. Following the extraction, 3 mL of the PLE solution was added to a test tube, and then 3 mL of the lower phase was added to it. The mixture was vigorously shaken for 10 min, and then separated by centrifugation for 3 min. In the next step, 0.5 mL aliquot of each phase was separately placed into a test tube, diluted with 1 mL of methanol, and analyzed by HPLC. The *K* value was expressed as the peak area of the target compound in the upper phase divided by that in the lower phase (*K*, for polar ginsenosides). A nonlinear correlation was used for analyzing the *K* value of the target compounds and the volume ratio of ethyl acetate/*n*-butanol; then, the optimal volume ratio was calculated.

In the second stage, the moderately polar ginsenosides were extracted, and in the third stage, the low polar ginsenosides were obtained. The experiments for the second and third stages were performed as follows. The upper phases of the solvent systems were