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Preparative separation of four major alkaloids from medicinal plant of *Tripterygium Wilfordii Hook F* using high-speed counter-current chromatography

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Abstract

A high-speed counter-current chromatography (HSCCC) method was developed for preparative isolation and purification of alkaloids from *Tripterygium Wilfordii Hook F*. Preparative HSCCC with a two-phase solvent system composed of petroleum ether–ethyl acetate–ethanol–water (6:4:5:8, v/v/v/v). The organic phase was used as the stationary phase of HSCCC, and the aqueous phase as the mobile phase. Seven hundred milligrams total alkaloids yielded 210 mg of wilfortrine, 90 mg of wilfordine, 220 mg of wilforgine and 100 mg of wilforine, with the purity of 90.3%, 92%, 99.5% and 93.5% in one step separation, respectively.

Keywords: *Tripterygium Wilfordii Hook F*; Preparative separation; Alkaloid; HSCCC

1. Introduction

Tripterygium Wilfordii Hook F (TWHF) is a traditional Chinese herb grown in the south of China and used for various immune and inflammatory diseases in China [1]. The extracts of the roots of TWHF by ethanol, ethyl acetate and other solvents have potent immunosuppressive, anti-cancer and anti-inflammatory properties. The diverse extracts are used widely in China for the treatment of a number of autoimmune disorders such as rheumatoid arthritis, systemic lupus erythematosus, and skin diseases [2–6].

Pharmacological properties of TWHF were studied extensively over the last years, and the results obtained could explain the traditional use of the plant and confirm its efficacy. The main active constituents of the herb have been reported to be diterpenes, triterpenes and alkaloid compounds [7,8]. Wilfortrine, wilfordine, wilforgine and wilforine (the chemical structures shown in Fig. 1) were originally separated from TWHF [9]. Wilfortrine and wilforine have been reported to possess immunosuppresive effects, wilforine is effective in treatment of rheumatoid arthritis [10]. Wilfortrine can inhibit leukemia cell growth in mice [11,12], and show anti-HIV activity [13].

So far, wilfortrine, wilfordine, wilforgine and wilforine have been purified from TWHF by several steps, including chromatography and crystallization. However, those conventional methods may encounter various problems. For example, the compound of interest is often strongly adsorbed onto the solid support of conventional silica gel column chromatography, which result in low recoveries. Existing HPLC methods are not suitable for large-scale isolation of wilfortrine, wilfordine, wilforgine and wilforine. Further studies on pharmacological and clinical effects of wilfortrine, wilfordine, wilforgine and wilforine necessitate the development of an efficient preparative separation method of these compounds. Such a method will also facilitate quality control and improvement of the quality of existing TWHF products.

High-speed counter-current chromatography (HSCCC), first invented by Ito [14], is a support-free liquid–liquid partition chromatographic technique, and eliminates irreversible adsorption of the sample onto the solid support [15]. With a large amount of sample injection, multiform relative pure substances can be obtained at one step in large amount. It is especially suitable for separation and purification of active components

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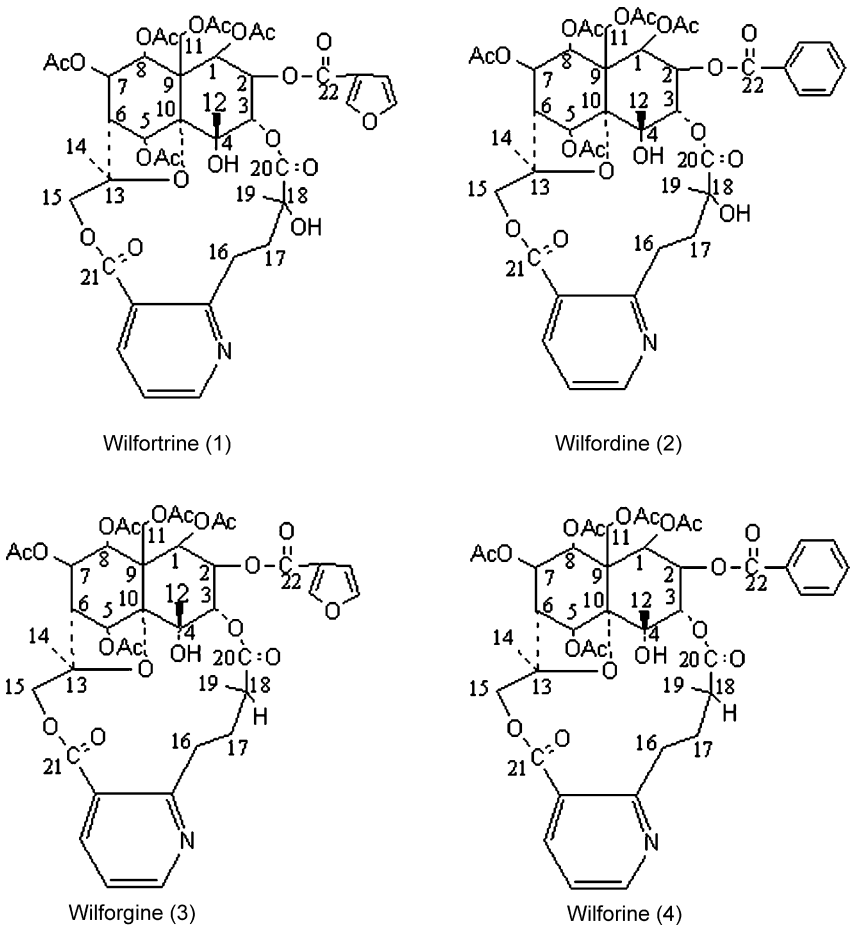

Fig. 1. Structure of alkaloids **1**–**4** isolated from *Tripterygium Wilfordii Hook F*.

from natural products [16–22]. However, no report has been published on the use of HSCCC for the isolation and purification of wilfortrine, wilfordine, wilforgine and wilforine from TWHF.

The present paper describes the successful preparative separation and purification of wilfortrine (component **1**), wilfordine (component **2**), wilforgine (component **3**) and wilforine (component **4**) from the partially purified total alkaloids from TWHF by HSCCC.

2. Experimental

2.1. Reagents and materials

Acetonitrile (MeCN) was HPLC grade. Deionized water was prepared by a Milli-Q water purification system from Millipore (Molsheim, France). The other regents were of analytical grade. The root bark of TWHF was obtained from DND Pharmaceutical Co. Ltd. (XinChang, Zhejiang province, China). Wilfortrine, wilfordine, wilforgine and wilforine standards (purity >95%, determined by HPLC) were obtained in our lab by column chromatography, identity of the isolated compounds were confirmed by spectral (MS, ^{1}H NMR and ^{13}C NMR) methods.

2.2. Apparatus

HSCCC was performed using a Model TBE-1000A HSCCC system manufactured by Tauto Biotech Co. Ltd., Shanghai, China, equipped with a 1000 ml coil column made of polytetrafluoroethylene tubing (3.0 mm). The β-value of the preparative column varied from 0.59 at the internal layer to 0.75 at the external layer ($\beta = r/R$, where r is the distance from the coil to the holder shaft, and R is the revolution radius or the distance between the holder axis and the central axis of centrifuge). The revolution speed of the apparatus could be adjusted in a range between 0 and 600 rpm. The solvent was pumped into the column by a Model SD-9002 constant-flow pump (Beijing Shengyitong Technology Development Co. Ltd.), and continuously delivered by 254 nm absorption with a Model 8823B UV detector (Beijing Institute of New Technology Application), the data was displayed and analyzed simultaneously on a Model N2010 workstation (Zhejiang University, Hangzhou, China). The experimental temperature was adjusted by HX-2050 constant temperature circulating implement (Beijing Boyikang Lab Implement, Beijing, China). A manual injection valve with an 80 ml loop was used to introduce the sample into the column.

The HPLC equipment used was an Agilent 1100 system, consisting of a quaternary pump (G1311A), a column ther-

mostat (G1316A), a degasser unit (G1379A), an autosampler (G1313A), and an Agilent LC-MSD Trap SL mass spectrometer with ESI interface (Agilent Technologies, Germany), a diode array detector and data were processed using with LC-MSD Trap Software 4.2 (Bruker). The nuclear magnetic resonance (NMR) spectrometer used here was a Bruker AMX 500 spectrometer (Karlsruhe, Germany) using TMS as internal standard.

2.3. Measurement of partition coefficient and separation factor

The two-phase solvent system was selected according to the partition coefficient (K) of each target component. The K was defined as the peak area of component in the upper phase divided by the peak area of component in the lower phase. The K values were determined by HPLC. In brief, suitable amount of crude extract was dissolved in a 20 ml test tube to which 5 ml each pre-equilibrated two-phase solvent system was added. The test tube was stoppered and shaken vigorously for several minutes to thoroughly equilibrate the sample with two phases. Then, equal volumes (1 ml) of the upper and lower phases were evaporated to dry separately under a gentle stream of nitrogen. The residues were diluted with MeCN 55% to 1 ml and analyzed by HPLC to determine K of each component.

2.4. Preparation of sample and sample solution

The powder of dried root bark of TWHF was leached with 90% ethanol for 10 h (ratio volume to weight, 3:1, V/W), and then the solution was concentrated, the residual solution was extracted with chloroform to obtain crude extract. The crude extract (200 g) was then thoroughly mixed with 600 ml of 10% ammonium hydroxide for 3 h under constant stirring, and then extracted with 300 ml ether (repeated eight times), the ether extract was then extracted with 2% hydrochloric acid four times (each for 100 ml), the acid extract was pooled and decolorized thrice by active carbon, treated with ammonia until alkaline to litmus, an hour later the precipitated crude alkaloid fraction was filtered off, washed with distilled water, and dried. The dried crude fraction was dissolved in sufficient methanol to form a heavy syrup and then filtered off, washed with a minimum of cold methanol and dried to yield 1.8 g crude alkaloid powder, which was directly used for HSCCC separation. The filtrate was evaporated to dryness and set aside for future study.

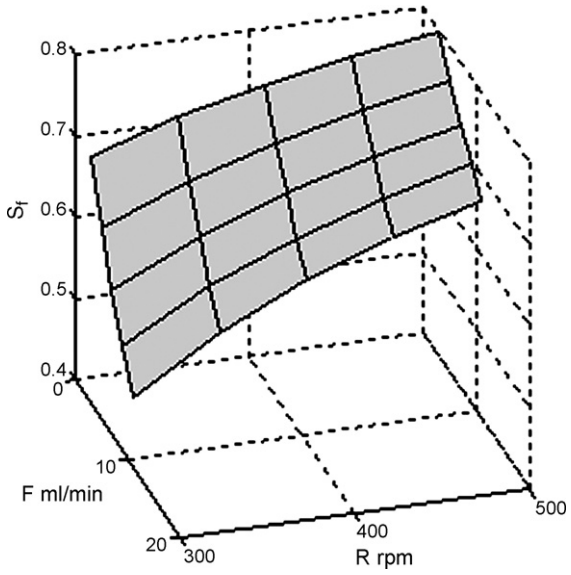

Fig. 2. Influence of the flow rate of mobile phase and revolution speed on the retention of stationary phase (R: revolution speed, rpm; F: flow rate of mobile phase, ml/min; S_f: retention of stationary phase, %).

For the present study, several two-phase solvent systems with suitable K values were selected. Each solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature and the two phases separated shortly before use. The upper phase was used as the stationary phase, while the lower phase was used as the mobile phase in the head-to-tail elution mode.

The sample solutions were prepared by dissolving the sample in the mobile phase of the solvent system used for separation at suitable concentrations according to the preparative purpose.

2.5. HSCCC separation procedure

The selection of suitable solvent system is the first and most important step in performing preparative HSCCC. In the present studies, a suitable solvent system with suitable K values for the separation of target compounds was selected, the solvent systems composed of petroleum ether–ethyl acetate–ethanol–water (6:6:6:6, 6:4:5:8, 6:5:5:8, 6:6:5:8, 6:7:5:8, v/v/v/v) were studied.

The preparative separation was performed with the optimal solvent system composed of petroleum ether–ethyl acetate–ethanol–water (6:4:5:8, v/v/v/v). The multilayer coil column was first entirely filled with the upper phase (stationary phase). The lower phase (mobile phase) was then pumped into the head end of the inlet column at a flow rate of 5.0 ml/min,

Table 1
The partition coefficients (K) of the target components in different ratio of volume in petroleum ether–ethyl acetate–ethanol–water solvent system (component **1**, wilfortrine; component **2**, wilfordine; component **3**, wilforgine; component **4**, wilforine)

Petroleum ether–ethyl acetate–ethanol–water ratio of volume	Component **1**	Component **2**	Component **3**	Component **4**
6:6:6:6	0.22	0.30	0.39	0.78
6:4:5:8	0.424	0.642	0.817	1.20
6:5:5:8	0.833	1.19	1.50	2.09
6:6:5:8	1.53	2.15	2.68	3.71
6:7:5:8	2.23	3.08	3.77	5.34

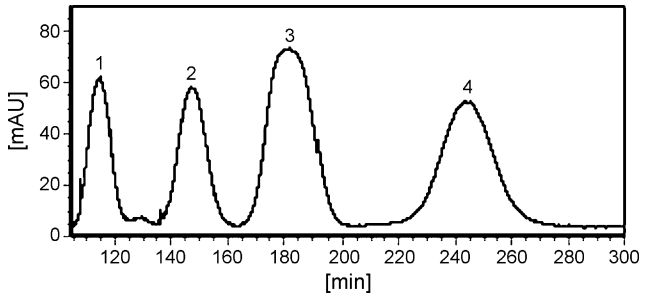

Fig. 3. HSCCC separation chromatogram of 700 mg crude sample. Solvent system: petroleum ether–ethyl acetate–ethanol–water (6:4:5:8, v/v/v/v); stationary phase: upper phase; flow-rate of the mobile phase: 5 ml/min; revolution: 500 rpm; retention of stationary phase: 72%; UV wavelength: 254 nm; column temperature: 25 °C (peak 1: wilfortrine; peak 2: wilfordine; peak 3: wilforgine; peak 4: wilforine).

while the apparatus was rotated at 500 rpm. After reaching hydrodynamic equilibrium, as indicated by a clear mobile phase eluting at the tail outlet, the sample solution (700 mg of the crude alkaloid in 30 ml of the upper phase of the solvent system) was injected into the column through the injection valve. The effluent from the tail end of the column was continuously monitored with a UV detector at 254 nm as stated earlier and the chromatogram was recorded. Eluate was collected with test tube at 3 min intervals per tube. After running, the solvents in the column were pushed out and the retention of stationary phase was measured. Final purity of wilfortrine, wilforgine, wilfordine and wilforine was valued by HPLC.

2.6. HPLC analysis and identification of HSCCC peak fraction

The crude alkaloid powder and each peak fraction obtained by HSCCC were analyzed by HPLC. The column used was a reversed-phase C-18 (Zorbax SB 250 mm × 4.6 mm i.d., 5 μm

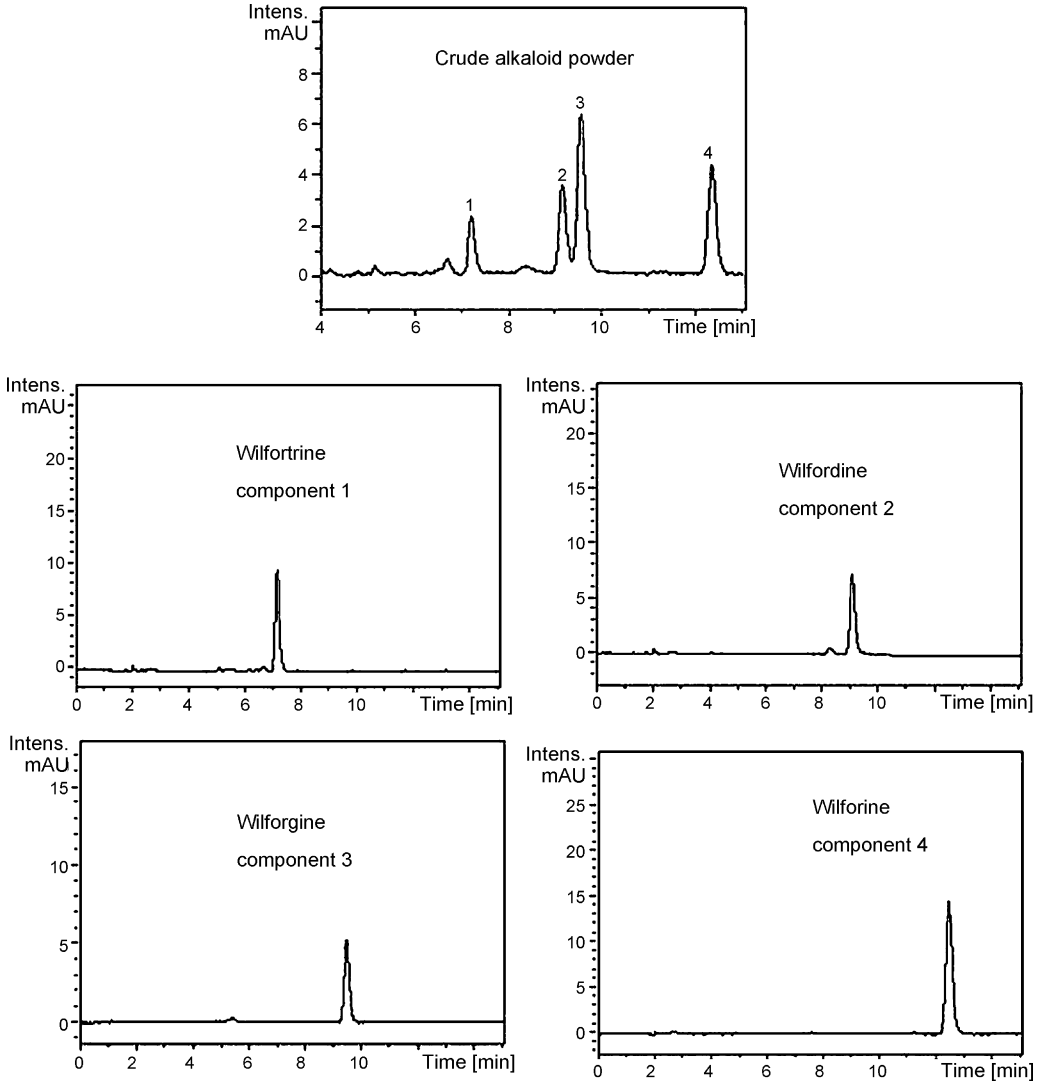

Fig. 4. HPLC analysis of crude alkaloid powder and the components (**1**–**4**) obtained from HSCCC separation. Experimental conditions: Agilent Zorbax SB-C18 reserved-phase column (5 μm, 250 mm × 4.6 mm, i.d.); mobile phase: acetonitrile–water (60:40, v/v); flow-rate: 0.8 ml/min, with the UV detector: 230 nm; column temperature: 35 °C.

particle size, Agilent, U.S.A.). The mobile phase was composed of acetonitrile-water (60:40, v/v), which was isocratically eluted at a flow-rate of 0.8 ml/min, with the detector set at 230 nm.

The purity of wilfortrine, wilforgine, wilfordine and wilforine was compared with standards by HPLC.

3. Results and discussion

3.1. Optimization of suitable HSCCC solvent system

Various parameters including two-phase solvent system, revolution speed, flow rate and column temperature of the mobile phase are generally considered to be the most important factors. The most important step in HSCCC is to optimize suitable conditions for an efficient separation. The optimization of suitable HSCCC conditions can be carried out step-by-step or by using an experimental design.

A successful separation of the target compounds using HSCCC requires a careful search for a suitable two-phase solvent system to provide an ideal range of partition coefficients (K) for the applied materials. In order to select a suitable system, previous articles on HSCCC should be carefully consulted, and some rules need to be considered [23,24]. The target compound should be soluble and stable in the solvent system; the settling time of the solvent system should be short (<30 s); the partition of the target compounds between two phases should be appropriate ($0.5 \leq K \leq 1$); the retention of the stationary phase should be satisfactory (>50%) [25]. In order to achieve efficient separation of target compounds from the crude alkaloid, four solvent systems at different volume ratios were tested. Their K values were measured and summarized (Table 1).

The two-phase solvent system containing *n*-hexane–ethyl acetate–methanol–water (1:1:1:1, v/v/v/v) was a versatile system [26–28], but this system (in our paper we used petroleum ether and ethanol to replace *n*-hexane and methanol, respectively) was not suitable for separation of target alkaloids according to the rules mentioned in the literature [23], because the K values of the system are very close between components **1**, **2** and **3**.

So other four two-phase solvent systems (petroleum ether–ethyl acetate–ethanol–water) were tested by changing the volume ratio of the solvent to obtain the optimum composition that gave an ideal range of partition coefficient (K) values and an appropriate settling time of the two-phase solvent system. It can be observed that the K values of the target compounds increased along with the increasing of the ratio of ethyl acetate. According to the rules of selecting solvent system, the range of $0.5 \leq K \leq 1.0$ was the best K values when the lower aqueous phase was mobile phase [23].

Finally, when petroleum ether–ethyl acetate–ethanol–water (6:4:5:8, v/v/v/v) was used, good separation results could be obtained and the separation time was acceptable.

3.2. Selection suitable revolution speed, column temperature and flow rate of the mobile phase for HSCCC isolation

Apart from a suitable two-phase solvent system, other parameters may affect the isolation results in HSCCC, such as revolution speed, flow rate of the mobile phase and column temperature.

Although high temperature would produce high retention of the stationary phase and low temperature would cause the sta-

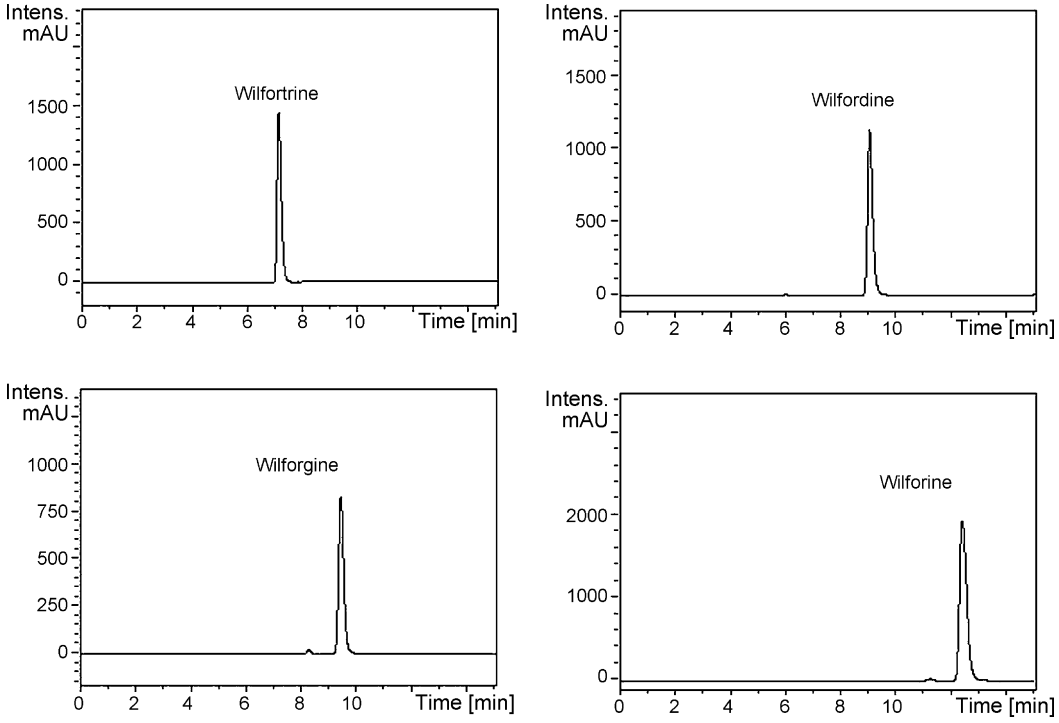

Fig. 5. HPLC chromatograms of standard wilfortrine, wilfordine, wilforgine and wilforine.

tionary phase loss, air bubble increased along with the increasing of temperature and high temperature would lessen the life of the apparatus. The column temperature was set at 25 °C in our experiments.

Different flow rates (3, 5, 7, 9 and 11 ml) of the mobile phase and different revolution speed (300, 350, 400, 450 and 500 rpm) of the selected system were examined in the present paper. The variation of the retention of the stationary phase (S_f: %) with the revolution speed of the apparatus (R: rpm) and the flow rate (F: ml/min) was shown in Fig. 2. It was clear that high flow rate was unfavorable to the retention of the stationary phase. On the contrary, low flow rate (3 ml/min) was satisfactory to our aim, but the elution time was long and more mobile phase was required. Then the flow rate was set at 5 ml/min in the present separation according to the overall analysis and 500 rpm was selected to separate the target compounds in our paper.

3.3. HSCCC separation

The crude alkaloid sample was separated by HSCCC using a single solvent system composed of petroleum ether–ethyl acetate–ethanol–water (6:4:5:8, v/v/v/v) (HSCCC chromatogram shown in Fig. 3). The solvent system possessed a good retention character at the operation condition, i.e., 72% of retention rate of the stationary phase after the separation. The analytical results monitored by HPLC, all fractions with the same value of R_f were combined to evaporated to dryness under reduced pressure, and then yielded component **1** (210 mg) upon recrystallization from MeOH and component **2** (90 mg), **3** (220 mg), **4** (100 mg) upon recrystallization from MeOH. Component **1** elution time 107–124 min. Component **2** elution time 140–158 min. Component **3** elution time 166–198 min. Component **4** with elution time 224–272 min. Each component was determined by HPLC (see Fig. 4), the purified wilfortrine (component **1**), wilfordine (component **2**), wilforgine (component **3**) and wilforine (component **3**) showed apparent single peaks in the analytical HPLC chromatograms, and the final purity was determined by HPLC with the standard samples (HPLC chromatograms of each standard sample shown in Fig. 5). Seven hundred milligrams total alkaloids yielded 210 mg of wilfortrine, 90 mg of wilfordine, 220 mg of wilforgine and 100 mg of wilforine, with the purity of 90.3%, 92%, 99.5% and 93.5% in one step separation, respectively

4. Conclusion

The results of our study clearly demonstrate that HSCCC can provide highly efficient preparative separation of wilfortrine, wilfordine, wilforgine and wilforine from TWHF. In combination with a suitable extraction and cleanup procedure prior to HSCCC separation, pure reference compounds are obtained on a preparative scale. HSCCC offers several advantages compared to preparative HPLC. HSCCC uses no solid stationary phase and gentle operation conditions, artifact formation is minimized, moreover, the separation column consists of an inert system of Teflon tubing and the separations are run at ambient temperature. The sample load capacity in HSCCC is superior to that in preparative HPLC, isolation of several hundred milligrams of pure compounds can be obtained during a working day.

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