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Application of preparative high-speed counter-current chromatography for separation of chlorogenic acid from *Flos Lonicerae*

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Abstract

Chlorogenic acid, an ester formed between caffeic acid and quinic acid, is a major phenolic compound in the traditional Chinese medicinal herb *Flos Lonicerae*. The separation and purification of chlorogenic acid from the crude extract of *Flos Lonicerae* was achieved by high-speed counter-current chromatography (HSCCC). A high acid, highly polar two-phase solvent system containing *n*-butanol–acetic acid–water (4:1:5) was run on a preparative scale. The upper phase was used as the mobile phase in the head to tail elution mode. A 300-mg quantity of the crude extract containing 5.97% chlorogenic acid was loaded on a 342-ml HSCCC column. Double separations were performed with the same solvent system yielding 16.9 mg chlorogenic acid at 94.8% purity with approximately 90% recovery.

Keywords: Preparative chromatography; Counter-current chromatography; Pharmaceutical analysis; Plant materials; Chlorogenic acid

1. Introduction

Chlorogenic acid (3-*O*-ceffeoyl-D-quinic acid) is an ester formed between caffeic acid and quinic acid, and is one of the major phenolic compounds found in the traditional Chinese medicinal herb *Flos Lonicerae*. It can suppress the *N*-nitrosating reaction and inhibit hepatic glucose 6-phosphatase, which may be a significant factor in the abnormal diabetic state. Moreover, it may serve as antioxidant, antitumor, antimutagenic and anticarcinogenic agents [1–3]. In view of these beneficial effects, an efficient method for the preparative separation and purification of chlorogenic acid from natural sources is warranted. The conventional methods, such as crystallization, column chromatography and high-performance liquid chromatography (HPLC), are tedious and usually require multiple steps [4,5]. High-speed counter-current chromatography (HSCCC) is a useful method for rapid chromatographic purification employing highly efficient fractionation by a hybrid technique of liquid–liquid counter-current distribution and liquid chromatography, in conjunction with the use of centrifugal force. The centrifugal force field generated from both rotational and synchronous planetary motion of coiled columns containing two immiscible liquid phases provides vigorous mixing between stationary and mobile phases, as well as retention of a very large fraction of the stationary phase. HSCCC eliminates the irreversible adsorptive loss of samples onto the solid support matrices used in conventional column chromatography and HPLC. Furthermore, it permits excellent sample recovery and can be employed for preparative-scale separation in a completely straightforward manner. Therefore, HSCCC has recently been used to effectively separate natural products [6–16]. The present paper reported the successful preparative separation and purification of chlorogenic acid from *Flos Lonicerae* by HSCCC.

2. Experimental

2.1. Reagents and materials

Chlorogenic acid was obtained from Wako (Japan). *n*-Butanol, acetone, acetic acid, ethyl acetate, chloroform, methanol, ethanol, calcium hydroxide and sulfuric acid were all of chromatographic grade and obtained from BDH

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(Poole, UK). *Flos Lonicerae* was obtained from Beijing Tong-Ren-Tang drug retail outlet in Hong Kong.

2.2. Apparatus

A Model CCC-1000 high-speed counter-current chromatograph (Pharma-Tech Research, Baltimore, MD, USA) equipped with three preparative coils (342 ml, wound with 1.6 mm i.d. PTFE tubing) was used for the separation and purification of chlorogenic acid. The β value of the preparative column varied from 0.47 at the internal layer to 0.73 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 central axis of the centrifuge). The two-phase solvent system was pumped into the column with a Model Series II HPLC pump (Parma-Tech Research). Continuous monitoring of the effluent was achieved with a Model SPD-10 Avp UV-Vis detector (Shimadzu, Japan), and the chromatogram was drawn with a Model L 120 E flat-bed recorder (Linseis, Germany). A manual injection valve with a 10-ml loop was used to introduce the sample into the column.

Chromatographic analysis was performed on a Waters HPLC system which consisted of two HPLC 510 pumps (Waters, Milford, MA, USA), a sample injector with a 20 μl injection loop (Rheodyne, Cotati, CA, USA), a Waters temperature control module, an RCM-100/column heater, and a Waters 996 photodiode array detection (DAD) system. A reversed-phase Symmetry C_{18} column (150 mm × 3.9 mm i.d., 5 μm, Waters) was used to separate chlorogenic acid. Evaluation and quantification were made on a Millennium chromatography data system (Waters).

2.3. Preparation of crude chlorogenic acid

Flos Lonicerae was dried to constant weight and then ground into powder. Thirty grams of the powder was put into a 500-ml flask, to which 400 ml of deionized water was added. After extracted at 100 °C for 1 h, the mixture was filtrated. The extraction procedure was repeated twice (300 ml of water each time). All the filtrates were combined and concentrated to 80 ml under reduced pressure by rotary evaporation. Then, 20% calcium hydroxide was added until the pH value was adjusted to 10. On separation by centrifugation, the precipitate was mixed with ethanol completely at the volume ratio of 1:2. Subsequently, the pH value of the mixture was adjusted to 3 with 50% sulfuric acid, and separated again by centrifugation. The pH value of the liquid phase was then adjusted to 6. Finally, the solution was evaporated to dryness yielding 1.36 g of crude chlorogenic acid.

2.4. HSCCC separation procedure

2.4.1. HSCCC solvent system

The following six kinds of solvent system at different volume ratios were tested to select a suitable two-phase solvent system based on the partition coefficient (K) of chlorogenic acid: (1) *n*-butanol–acetic acid–water, (2) *n*-butanol–water, (3) *n*-butanol–ethyl acetate–water, (4) ethyl acetate–acetone–water, (5) *n*-butanol–acetone–water, and (6) chloroform–methanol–water. Values of the partition coefficient were determined according to [17]. In brief, 0.1 mg of chlorogenic acid was dissolved in 2 ml of each phase of the pre-equilibrated two-phase solvent system. The solution was shaken vigorously for 10 min to thoroughly equilibrate chlorogenic acid with the two phases. After centrifugation at 4000 × g for 5 min, an aliquot of each phase was pipetted and evaporated to dryness under nitrogen. The residues were dissolved in aliquots of water and analyzed by HPLC. The K value was expressed as the concentration of chlorogenic acid in the upper phase divided by that in the lower phase.

After thorough equilibration of the selected solvent system in a separation funnel by repeatedly vigorously shaking at ambient temperature, the two immiscible phases were separated and degassed by sonication before use. The head to tail elution mode with the lower phase as the stationary phase was adopted in the HSCCC experiments.

2.4.2. HSCCC separation

The multilayer-coiled column was first entirely filled with the lower phase. Then the upper phase was pumped into the head end of the inlet column at a flow-rate of 1.5 ml min^{-1} with an HPLC pump, while the apparatus was rotated at 1000 rpm. When a clear mobile phase was eluted from the tail outlet indicating that a hydrodynamic equilibrium had been established, the sample solution in 10 ml of a mixture of the upper and lower phases (1:1, v/v) was introduced to the column with an injection loop. The effluent from the tail end of the column was continuously monitored with a UV-Vis detector at 325 nm and the chromatogram was recorded. Each peak fraction was collected according to the elution profile and analyzed by HPLC. After the separation was completed, retention of the stationary phase was measured by collecting the column contents by forcing them out of the column with pressurized nitrogen gas.

2.5. HPLC analysis and identification of chlorogenic acid

The crude extract, chlorogenic acid (standard) and each fraction corresponding to various portions of the major peaks in HSCCC were analyzed by HPLC. The analyses were performed on a Waters HPLC system with a C_{18} column at a column temperature of 40 °C. The elution gradient consisted of two solvent components: water (solvent A) and methanol–acetic acid–water (30:6:64, v/v, solvent B). Gradient elution was carried out according to the following programme: solvent B was increased from 10 to 100% in 40 min. The flow-rate was 1.0 ml min^{-1}. The effluent was monitored with DAD, and the absorbance spectra (250–400 nm) were collected continuously during the course of each analytical run. Confirmation of identity was made by comparison of the retention time and spectra

against the pure chlorogenic acid. Routine sample calculations were made by comparison of the peak area with that of the standard.

3. Results and discussion

3.1. Optimization of chromatographic conditions

The crude extract from *Flos Lonicerae* contained several compounds with very similar chemical structures. It was difficult to separate these compounds completely when elution was carried out using a single solvent system. Furthermore, chlorogenic acid tended to appear as a tailing peak due to the residual silanols on the stationary phase. In order to avoid their adsorption on reversed-phase columns, the mobile phases containing various acids were often used [18–20]. In the present study, some organic aqueous-based mobile phases were tested on a reversed-phase C_{18} column, including methanol–water in combination with acetic acid, phosphate buffer, trifluoroacetic acid, phosphoric acid, tetrahydrofuran–phosphoric acid, methanol–acetonitrile–phosphoric acid and methanol–acetonitrile– trifluoroacetic acid. The temperature of the column, which might affect the separation, was also tested. It was found a gradient elution consisting of water (solvent A) and methanol–acetic acid–water (30:6:64, v/v, solvent B) achieved excellent separation at 40 °C. The gradient elution was carried out

Table 1
The *K* (partition coefficient) values of chlorogenic acid in different solvent systems

Solvent system	*K* value
n-Butanol–acetic acid–water (4:1:5)	2.09
n-Butanol–water (1:1)	0.067
n-Butanol–ethyl acetate–water (4:1:5)	0.075
n-Butanol–ethyl acetate–water (3:2:5)	0.037
n-Butanol–ethyl acetate–water (1:1:2)	0.034
n-Butanol–ethyl acetate–water (2:3:5)	0.100
n-Butanol–ethyl acetate–water (1:4:5)	0.060
Chloroform–methanol–water (5:6:4)	0.019
Chloroform–methanol–water (4:3:2)	0.030
n-Butanol–acetone–water (4:1:5)	0.23
n-Butanol–acetone–water (8:3:12)	0.48
n-Butanol–ethanol–water (4:1:4)	0.36
n-Butanol–ethanol–water (8:3:12)	0.41
Ethyl acetate–acetone–water (4:1:5)	0.089
Ethyl acetate–acetone–water (4:2:5)	0.020

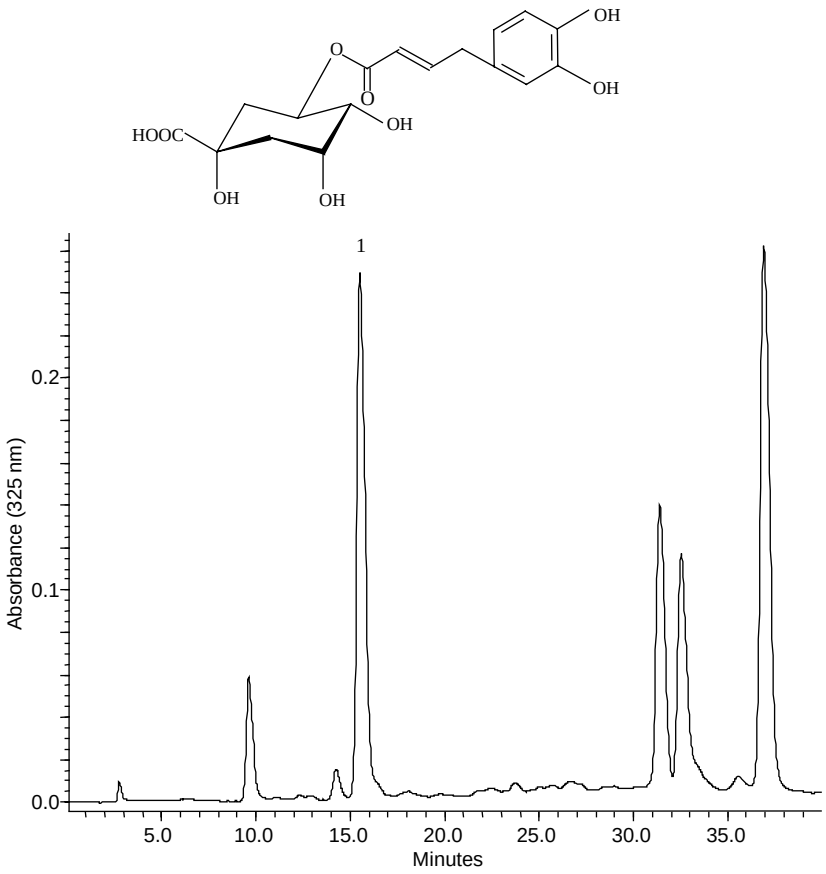

Fig. 1. Chromatogram of the crude sample extracted from *Flos Lonicerae* by HPLC analysis, as well as the chemical structure of chlorogenic acid, (1) chlorogenic acid. Experimental conditions: column: reversed-phase Symmetry C_{18} column (150 mm × 3.9 mm i.d., 5 μm); column temperature: 40°C; Gradient elution: water (solvent A) and methanol–acetic acid–water (30:6:64, v/v, solvent B), solvent B was increased from 10 to 100% in 40 min; flow-rate: 1.0 ml min^{-1}; detection: 325 nm; injection volume, 20 μl.

according to the following programme: solvent B was increased from 10 to 100% in 40 min. UV detection was set at 325 nm, as all the constituents of the extract showed appreciable absorbance at this wavelength. Under these conditions, the crude extract from *Flos Lonicerae* was analyzed (Fig. 1). The content of chlorogenic acid was 5.97%.

3.2. Selection of suitable two-phase solvent system

In using HSCCC, successful separation depends upon the selection of a suitable two-phase solvent system, which requires the following considerations: (1) retention of the stationary phase should be satisfactory; (2) the settling time of the solvent system should be short (i.e. <30 s) [21]; and (3) the partition coefficient (K) of the target compound should fall within a suitable range (i.e. usually between 0.2 and 5) [22]. Small K values usually result in a poor peak resolution, while large K values tend to produce excessive sample band broadening [21]. Chlorogenic acid is not soluble in non-polar solvent, and has little solubility in ethyl acetate, but it has some solubility in methanol and ethanol, and is freely soluble in water. According to these properties of chlorogenic acid, some two-phase solvent systems were tested and their K values are summarized in Table 1. Among them,

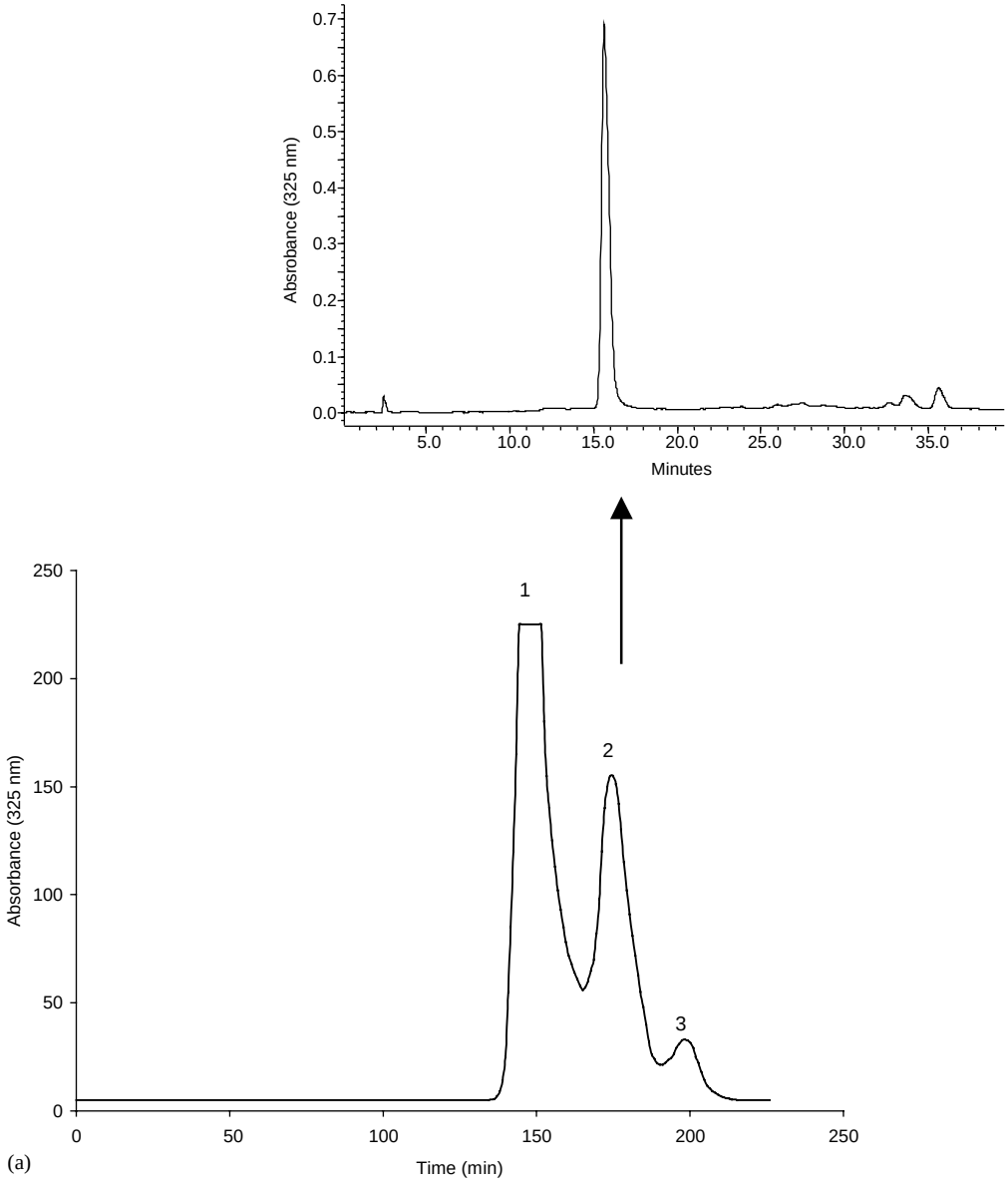

Fig. 2. Chromatograms of the crude sample extracted from *Flos Lonicerae* by HSCCC separation, (2) chlorogenic acid. HSCCC conditions: column: multilayer coil of 1.6 mm i.d. PTFE tube with a total capacity of 342 ml; rotary speed: 1000 rpm; solvent system: *n*-butanol–acetic acid–water (4:1:5, v/v); mobile phase: the upper phase; elution mode: head to tail; flow-rate: 1.5 ml min^{-1}; detection: 325 nm; sample size: 300 mg; injection volume: 10 ml; retention of the stationary phase: 38.0%. HPLC conditions: column: reversed-phase Symmetry C_{18} column (150 mm × 3.9 mm i.d. 5 μm); column temperature: 40 °C; gradient elution: water (solvent A) and methanol–acetic acid–water (30:6:64, v/v, solvent B), solvent B was increased from 10 to 100% in 40 min; flow-rate: 1.0 ml min^{-1}; detection: 325 nm; injection volume: 20 μl. (a) The first separation and (b) the second separation.

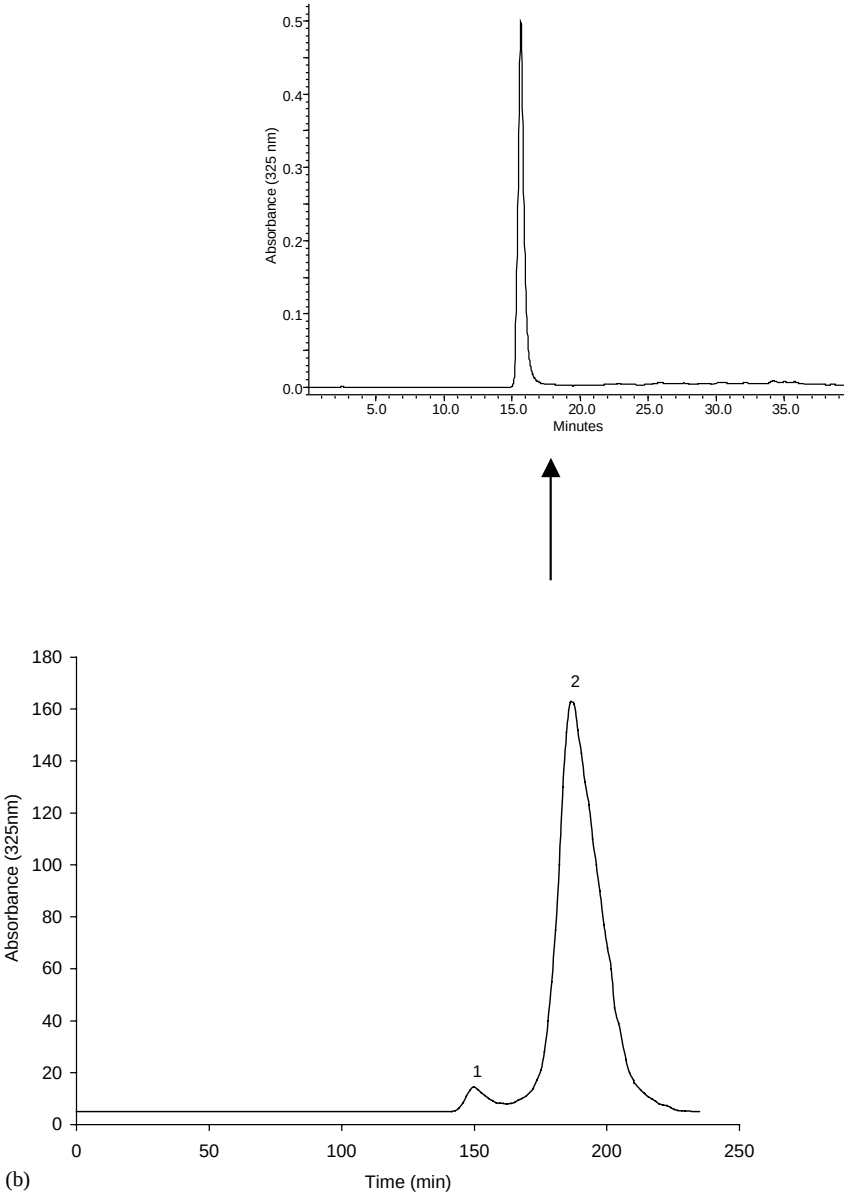

Fig. 2. (*Continued*).

the two-phase solvent systems, including *n*-butanol–water, *n*-butanol–ethyl acetate–water, chloroform–methanol–water and ethyl acetate–acetone–water, had small *K* values. When they were used for the HSCCC separation, chlorogenic acid would be eluted together with other compounds with similar properties near the solvent front resulting in a poor resolution. Thus, these two-phase solvent systems were not suitable for the separation of chlorogenic acid from the crude sample. The other two-phase solvent systems, *n*-butanol–acetic acid–water, *n*-butanol–acetone–water and *n*-butanol–ethanol–water, had suitable *K* values (Table 1). However, when *n*-butanol–ethanol–water was used, the settling time of the solvent system was long (>30 s). When *n*-butanol–acetone–water was used, the retention of the stationary phase was poor (<12%). Thus, these two two-phase solvent systems were neither suitable for the separation of chlorogenic acid from the crude sample. Finally, the two-phase solvent system of *n*-butanol–acetic acid–water at a volume ratio of 4:1:5 was found to be satisfactory for the separation of chlorogenic acid from the crude sample.

3.3. Separation of chlorogenic acid by HSCCC

The design of an HSCCC system is based on the hydrodynamic equilibrium mechanism where the head phase is determined by physical properties of the solvent system. Hydrophobic binary solvent systems with high interfacial tension such as ethyl acetate–water and chloroform–water always distribute the upper phase on the head side and the lower phase on the tail side. Hydrophilic solvent systems

characterized by low interfacial tension and high viscosity, including of *n*-butanol–acetic acid–water (4:1:5, v/v) and 2-butanol–water show the opposite hydrodynamic behaviour which always distribute the lower phase on the head side and the upper phase on the tail side. In the present work, *n*-butanol–acetic acid–water was chosen as the two-phase solvent system. The lower phase was used as the stationary phase while the upper phase was used as the mobile phase in the head to tail elution mode.

A 300-mg quantity of crude extract was separated and purified by HSCCC. The retention of the stationary phase was 38.0%, and the separation time was 230 min in each separation run. Fig. 2a shows preparative HSCCC separation of the crude sample using ethyl *n*-butanol–acetic acid–water (4:1:5, v/v) solvent system for the first run. Based on the HPLC analysis and the elution curve of the preparative HSCCC, peak 2 corresponds to chlorogenic acid. This partially purified fraction was dried, and 23.3 mg of chlorogenic acid was obtained. The purity of chlorogenic acid after the first run separation was 73.3% and there were still impurities in the chlorogenic acid product (Fig. 2a). This product was further dissolved in the both phases and purified again by HSCCC with the same solvent system. Finally, 16.9 mg chlorogenic acid at 94.8% purity was yielded with approximately 90% recovery. Fig. 2b shows preparative HSCCC separation of the crude sample for the second run. The HPLC chromatogram of chlorogenic acid as purified from the preparative HSCCC is also shown in Fig. 2b.

In order to save solvents and time, the slow eluting compounds after chlorogenic acid were removed by forcing out the stationary phase with pressurized nitrogen gas instead of eluting them with the mobile phase because the stationary phase was used only once.

4. Conclusion

With HSCCC we were able to purify the main bioactive compound, chlorogenic acid of the traditional Chinese medicinal herb *Flos Lonicerae* with *n*-butanol–acetic acid–water (4:1:5, v/v) solvent system. From 300 mg of the crude sample extracted from *Flos Lonicerae*, 16.9 mg chlorogenic acid at 94.8% purity was obtained. The purity of chlorogenic acid was increased from 5.97 to 94.8% after a two-step separation. The overall results indicate that HSCCC is a powerful technique in separating and purifying bioactive components from natural products.

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