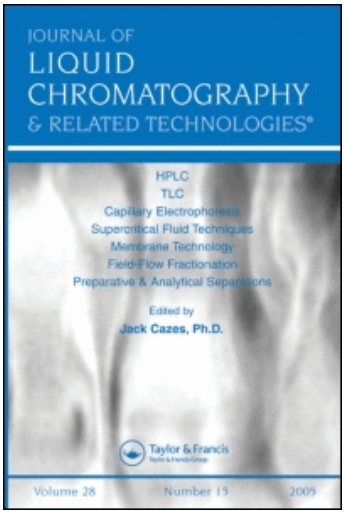

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New Approach for Application of High Speed Countercurrent Chromatography Coupled with Direct Injection of the Powders of a Raw Material without any Preparation, for Isolation and Separation of Four Alkaloids with High Recoveries from *Coptis chinensis* Franch

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New Approach for Application of High Speed Countercurrent Chromatography Coupled with Direct Injection of the Powders of a Raw Material without any Preparation, for Isolation and Separation of Four Alkaloids with High Recoveries from *Coptis chinensis* Franch

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Abstract: Generally, before a successful high speed countercurrent chromatography (HSCCC) separation, crude samples were extracted from medicinal plants by aqueous ethanol or other organic solvents, and then the extracts were cleaned up by liquid–liquid extraction or column chromatography to produce the suitable samples for further purification, which were hostile for our environment and natural resource reserve because more time and solvent were consumed, and there was sample loss with a low recovery. In view of these drawbacks, a new approach of using HSCCC coupled with direct injection of the powders of the raw material without any preparation method, was successfully developed to separate and purify jatrorrhizine, coptisine, palmatine, and berberine from *Coptis chinensis* Franch, with high recoveries of over 92% determined by high performance liquid chromatography and standards compared with approximately 70% recoveries in common HSCCC purification. Our study showed a new approach in the HSCCC research field for combining the extraction and separation process together in the HSCCC column only, to eliminate sample loss and which decreases solvent and length of time.

Keywords: Countercurrent chromatography, Preparative chromatography, Traditional Chinese medicine

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INTRODUCTION

Traditional Chinese herbal medicines have been used to treat human diseases in China for centuries. People are becoming increasingly interested in traditional Chinese medicines because of their high pharmacological activity and low toxicity. In recent years, more and more research, especially in Asia, has become more attractive in this field. Thus, development of an efficient separation technique to purify the active components from natural materials for new product exploitations, pharmacological tests, or quality control is an important research subject.

Countercurrent chromatography (CCC) was first invented by Ito et al. in the early 1970s, which provides an advantage over the conventional separation techniques by not using a solid stationary phase and eliminating the irreversible adsorption of the samples from the support. In the intervening years, the method has been radically improved in terms of resolution, separation time, and sample loading capacity by the development of high speed countercurrent chromatography (HSCCC), which yields a highly efficient separation and has been widely used for isolation and purification of alkaloids,[1,2] flavonoids,[3,4] quinones,[5,6] coumarins,[7] lignans,[8] and other natural products[9] from traditional Chinese medicines (TCMs).

Generally, application of HSCCC to separate chemical constituents from TCMs contained two steps. The first step was the process of preparation of the crude extracts. Crude samples containing the target compounds were extracted from medicinal plants by aqueous ethanol or methanol, or some other organic solvents, and then the extracted samples were cleaned up by liquid–liquid extraction in a separation funnel or conventional column chromatography. Macroporous resin or silica gel, especially, was used as the stationary phase[10–12] for removing the undesired compounds to produce the suitable samples for further purification. The second step was the HSCCC separation process. Although, the first step was beneficial for successful HSCCC purification, more solvents, especially some organic solvents, more time consumption, and more low recoveries of the chemicals were produced because of sample loss during the process, which is hostile to our environment and natural resources reserve. In view of these drawbacks, a new approach to the sample preparation method was required in the HSCCC purification process. Although, many publications including monographs,[13,14] encyclopedia,[15] and review articles,[16] in addition to a great number of research papers on HSCCC in chromatographic journals have been issued during the past 30 years, there have no reports about direct injection of the powders of medicinal herbs into the HSCCC column for chemicals separation as far as we known.

In the present paper, a new approach of using HSCCC coupled with direct injection of the powders of the raw material without any preparation method was successfully developed to separate and isolate jatrorrhizine, coptisine, palmatine and berberine (Figure 1) from *Coptis chinensis* Franch with high recoveries. This new approach in the HSCCC purification field was efficient

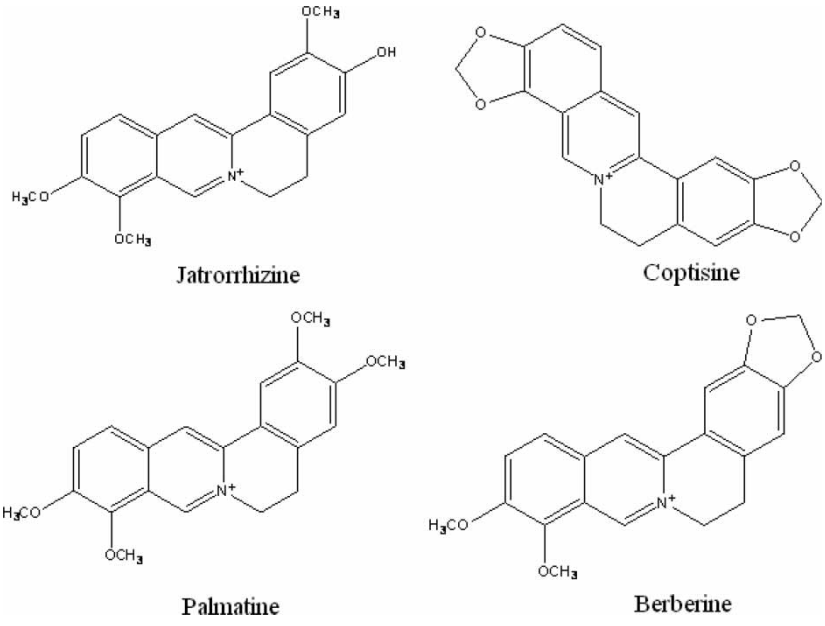

Figure 1. The chemical structures of the compounds.

and simple, with the ability to overcome the drawbacks of more solvent and time consumption and sample loss produced in common HSCCC separation.

EXPERIMENTAL

Chemicals

Ethyl acetate, *n*-hexane, methanol, chloroform, ethanol, KH_2PO_4, SDS, HCl, and acetic acid were analytical grade and purchased from ShenLian Chemical Factory (Shenyang, China). Acetonitrile used for HPLC analysis was HPLC grade (Merck, Germany). Reverse osmosis Milli-Q water (18 MΩ) (Millipore, USA) was used for all solutions. The standards of jatrorrhizine, coptisine, palmatine, and berberine were all purchased from the National Institute of the Control of Pharmaceutical and Biological Products, Ministry of Health, Beijing, China.

HSCCC Separation and Isolation

Preparative HSCCC was carried out with a model TBE–300A high speed countercurrent chromatograph (Shenzhen, Tauto Biotech, China). The apparatus was equipped with a polytetra-fluoroethylene three preparative coil (diameter of tube, 1.6 mm, total volume, 320 mL) and a 20 mL sample

loop. The β value varied from 0.47 at the internal terminal to 0.73 at the external terminal ($\beta = r/R$, $R = 7.5$ cm, where r is the distance from the coil to the holder shaft, and R, the revolution radius or the distance between the hold axis and central axis of the centrifuge). The HSCCC system was equipped with a model *S* constant flow pump, a model 8823B UV detector (Beijing Institute of New Technology Application) operating at 280 nm, and a model N2000 workstation (Zhejiang University, Hangzhou, China). The experimental temperature was adjusted by a HX 1050 constant temperature circulating implement (Beijing Boyikang Lab Implement, Beijing, China).

In HSCCC separation, the coil column was first entirely filled with the upper phases of the solvent system composed of *n*-hexane–ethyl acetate–methanol–1% acetic acid (1:1:1:1, v/v/v/v). Then, the apparatus was rotated at 850 rpm, while the lower phase was pumped into the column at a flow rate of 2.0 mL/min. After the mobile phase front emerged and hydrodynamic equilibrium was established in the column, 15 mL sample solutions containing 500 mg of the powders of *C. chinensis* Franch were directly introduced into the column through the injection valve without any preparation. The effluent of the column was continuously monitored with a UV-Vis detector at 280 nm, and the column temperature was set at 30°C. Peak fractions were collected according to the elution profiles.

HPLC Analysis and Identification of the Obtained Fractions

The HPLC system used throughout this study consisted of one 515 pump (Waters, Milford, MA, USA), a sample injector (Rheodyne, Cotati, CA, USA) with a 20 μL loop, a Waters 2487 UV detector, and a model N2000 workstation (Zhejiang University, Hangzhou, China). The contents of jatrorrhizine, coptisine, palmatine, and berberine in *C. chinensis* Franch, and the peak fractions obtained from HSCCC were all analyzed by HPLC. The column used was a Lichrospher C_{18} (200 × 4.6 mm I.D., 5 μm) (Zhonghuida Science, Dalian, China) with a precolumn equipped with the same stationary phase; the mobile phase used in the present paper was CH_3CN–50 mmol/L KH_2PO_4 (50:50, v/v) (containing 25 mmol/L SDS, pH 3.0). The flow rate was set at 1.0 mL/min, and the effluent was monitored at 345 nm, respectively. Identification of the HSCCC peak fractions was carried out by ESI-MS and the standards.

RESULTS AND DISCUSSION

HPLC Determination of the Contents of the Targets in Plant Materials

The contents of jatrorrhizine, coptisine, palmatine, and berberine were determined in *C. chinensis* Franch, according to the literature,[17] and the

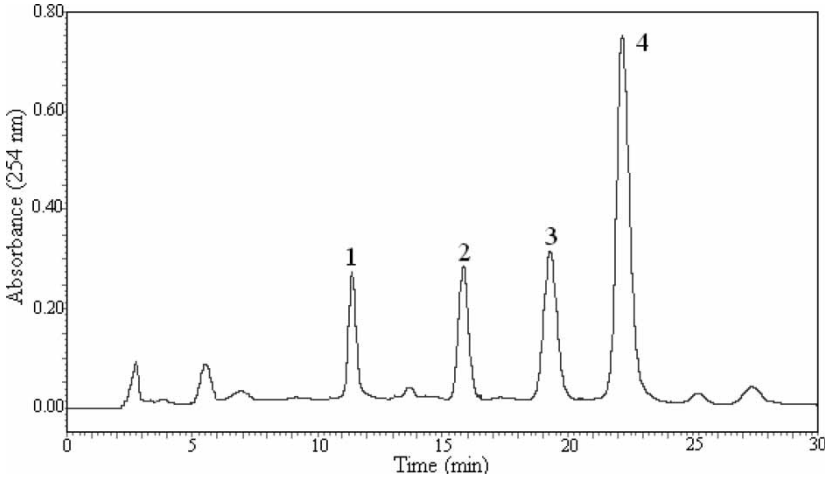

Figure 2. HPLC chromatogram of the extract from *C. chinensis* Franch. Column: Lichrospher C_{18} (200 × 4.6 mm I.D., 5 μm); mobile phase: CH_3CN–50 mmol/L KH_2PO_4 (50: 50, v/v) (containing 25 mmol/L SDS, pH 3.0); flow rate: 1.0 mL/min; detection wavelength: 345 nm. Peaks 1, 2, 3, and 4 correspond to jatrorrhizine, coptisine, palmatine, and berberine, respectively.

chromatogram is shown in Figure 2, in which peaks **1**, **2**, **3**, and **4** correspond to jatrorrhizine, coptisine, palmatine, and berberine, and present the contents of 1.02%, 1.37%, 1.46%, and 6.88% (listed in Table 1), respectively.

Table 1. The contents of the targets in medicinal plant and the amounts, purities and recoveries from HSCCC using the new method

Compounds	Contents in plant materials[a]	Sample size in HSCCC	Amounts from HSCCC	Purity of the obtained fractions[b]	Recovery[c]
Jatrorrhizine	1.02%	500 mg	4.9 mg	95.8%	92.0%
Coptisine	1.37%		6.6 mg	96.4%	92.9%
Palmatine	1.46%		7.0 mg	97.3%	93.3%
Berberine	6.88%		32.1 mg	99.0%	92.4%

[a]The contents of the targets in the medicinal plant determined by HPLC and standards.

[b]The purities of the obtained fractions from HSCCC determined by HPLC and standards.

$$^{c}\text{Recovery (\%)} = \frac{\begin{array}{c}\text{(the amount obtained fraction from HSCCC)} \\ \times \text{ (the purity of the obtained fraction)}\end{array}}{\begin{array}{c}\text{(the content of the target in plant material)} \\ \times \text{ (the sample size in HSCCC)}\end{array}} \times 100\%$$

Optimization Suitable Conditions for HSCCC Separation and Purification

In HSCCC, the selection of the two phase solvent system is most important for the successful separation, and is also the most difficult step; it is estimated that about 90% of the entire work in HSCCC is spent on that. Although, the powders of the raw material, not the crude extract, were used as the HSCCC separation samples, optimization suitable two phase solvents were also critical for success. In the present paper, selection of a suitable solvent system for successful purification of jatrorrhizine, coptisine, palmatine, and berberine from *C. chinensis* Franch, was the most difficult work. However, many studies had been carried out and reported by our scientific workers.[18] Through the reference and our own HSCCC experimental studies, several kinds of solvent systems composed of chloroform–methanol–1%HCl (4:3:2, v/v/v), *n*-hexane–ethyl acetate–ethanol–1% acetic acid (1:1:1:1, 1:2:2:1, v/v/v/v) and *n*-hexane–ethyl acetate–methanol–1% acetic acid (1:1:1:1, 1:0.8:0.8:1, v/v/v/v) were tested, and the results indicated that the solvent system composed of *n*-hexane–ethyl acetate– methanol–1% acetic acid (1:1:1:1, v/v/v/v) was suitable for separation and isolation of the four alkaloids from *C. chinensis* Franch.

At the same time, the influence of the separation temperature, flow rate, and the revolution speed were also investigated. The temperature has significant effect on K values, the retention of stationary phase, and the mutual solvency of the two phases. After testing at 25°C, 30°C, and 35°C, it can be seen that good results can be obtained when the separation temperatures were all controlled at 30°C. The flow rate might also influence the HSCCC separation, and different flow rates (1.0, 1.5, 2.0, and 2.5) of the mobile phases of the selected systems were examined in the present paper. A high flow rate was unfavorable to the retention of the stationary phase, and a low flow rate was satisfactory for our purpose, but the elution time was long and more mobile phase was required. After a series of tests, the flow rates were set at 2.0 mL/min. The revolution speed has a great influence on the retention of the stationary phase, high rotary speed can increase the retention of the stationary phase, and it could not be reserved in the HSCCC column at low revolution speed. In our experiment, the revolution speeds were all set at 850 rpm after trying a lot of experiments.

Under the above optimized separation conditions, the isolation of the target compounds was achieved with good resolution and the retentions of the stationary phase were satisfactory (67%), and the HSCCC separation time was less than 240 min. After the target compounds were eluted out in each separation, in order to save solvents and time, the remaining compounds in the column 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 not to be reused. Figure 3 shows the

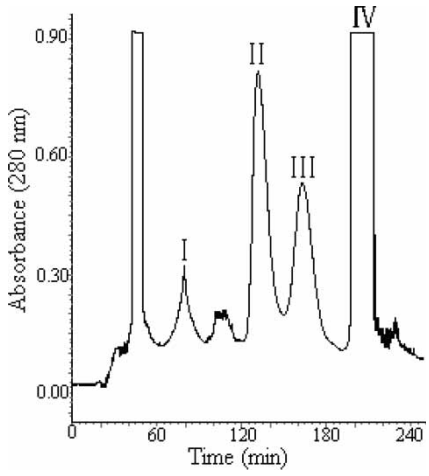

Figure 3. The HSCCC chromatogram of the powders of *C. chinensis* Franch without any preparation. Solvent system: *n*-hexane–ethyl acetate–methanol–1%acetic acid (1:1:1:1, v/v/v/v); flow rate of the mobile phase: 2.0 mL/min; detection wavelength: 280 nm; revolution speed: 850 rpm; column temperature: 30°C; sample size: 500 mg; retention of the stationary phase: 67%. I, II, III, and IV were collected fractions.

preparative HSCCC isolation of 500 mg of the powders of *C. chinensis* Franch using the solvent systems composed of *n*-hexane–ethyl acetate–methanol–1% acetic acid (1:1:1:1, v/v/v/v), and the fractions of I, II, III, and IV were all collected individually according to the elution profiles.

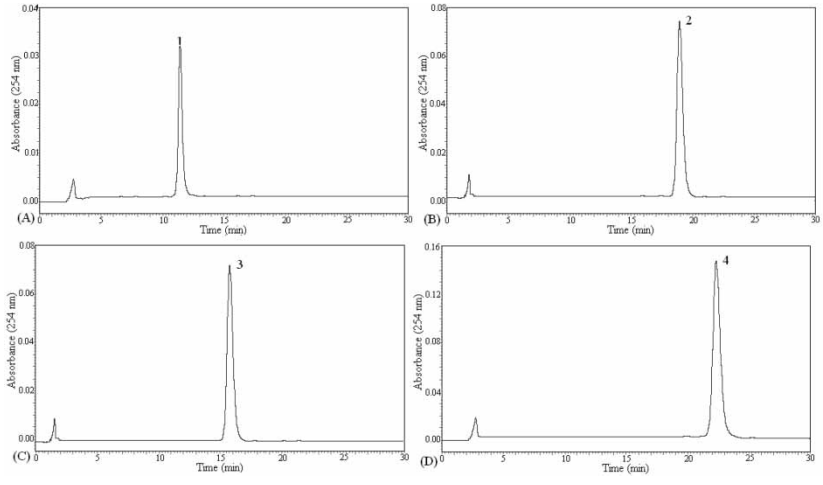

Figure 4. HPLC chromatograms of the obtained fractions from HSCCC. (A) The obtained fraction I from HSCCC; (B) The obtained fraction II from HSCCC; (C) The obtained fraction III from HSCCC; (D) The obtained fraction IV from HSCCC.

Identification, Purity, and Recovery Determination of the Obtained Fractions

The collected fractions were first evaporated to dryness under reduced pressure at 50°C and then analyzed by HPLC, and the chromatograms are shown in Figure 4A, B, C and D, which contained only one peak and correspond to the fractions of I, II, III, and IV, respectively. The collected fractions from HSCCC produced 4.9 mg jatrorrhizine with 95.8% purity, 6.6 mg coptisine with 96.4% purity, 7.0 mg palmatine with 97.3% purity, 32.1 mg berberine with 99.0% purity. Then, the recoveries of the separated compounds were calculated as following formula (1), and the results listed in Table 1 showed high recoveries (from 92.0% to 93.3%) to all the targets in the present separation.

$$\text{Recovery } (\%) = \frac{\begin{array}{c}(\text{the amount of the obtained fraction from HSCCC}) \\ \times (\text{the purity of the obtained fraction})\end{array}}{\begin{array}{c}(\text{the content of the target in plant material}) \\ \times (\text{the sample size in HSCCC})\end{array}} \times 100\% \quad (1)$$

Comparison with Common HSCCC Separation

In general, two steps were required for a successful HSCCC separation. The first step, a process of preparation of the crude extract from plant materials, was critically important for the second step, the HSCCC separation process.

Table 2. The amounts, purities and recoveries of the compounds in the first step from raw material to the crude extract

Compounds	Contents in plant materials	Amounts of plants[a]	Amounts of the crude extracts[b]	Contents of the target compounds[c]	Recovery[d]
Jatrorrhizine	1.02%	1000 g	92.77 g	8.35%	75.9%
Coptisine	1.37%			11.53%	78.1%
Palmatine	1.46%			12.24%	77.8%
Berberine	6.88%			58.44%	78.8%

[a]The powders of the plant material used in the process of preparation the crude extract.
[b]The amounts of the obtained crude extract after solvent extraction and cleaning-up step.
[c]The contents of the target compounds in the crude extracts from raw material

[d]$$\text{Recovery } (\%) = \frac{\begin{array}{c}(\text{the amount of the crude extract}) \\ \times (\text{the content of the target in crude extract})\end{array}}{\begin{array}{c}(\text{the content of the target in medicinal plant}) \\ \times (\text{the amount of the plant used in preparation process})\end{array}} \times 100\% \text{ (in the first step).}$$

However, there was no preparation process before HSCCC isolation and the powders of the medicinal plants were directly introduced into the separation column in our present research, and the outcomes indicated this new approach was very successful to isolate chemical compounds from medicinal plants. In order to compare the two kinds of HSCCC separation methods, the plants of *C. chinensis* Franch were extracted with aqueous ethanol, and then the extracts were cleaned up by conventional column chromatography using macroporous resin as the stationary phase according to the literature,[19] and the contents of jatrorrhizine, coptisine, palmatine, and berberine in the crude extract were determined by HPLC (chromatograms not shown). The amounts and contents of the obtained crude extract from the medicinal plants are listed in Table 2, and the recoveries of the targets in the first step were calculated as the following formula (2). Through the data, we can see the recoveries of all the target compounds in the first step from raw materials to the crude extract after extraction and cleaning up process were ranged from 75.9% to 78.8%.

$$\text{Recovery}(\%) = \frac{\begin{array}{c}(\text{the amount of the crude extract}) \\ \times(\text{the content of the target in crude extract})\end{array}}{\begin{array}{c}(\text{the content of the target in medicinal plant}) \\ \times(\text{the amount of the plant used in preparation process})\end{array}} \times 100\% \quad (2)$$

Subsequently, the second step was carried out according to the optimized HSCCC separation conditions. All the compounds were successfully purified (HSCCC chromato-gram not shown), and the collected fractions were all analyzed and determined by HPLC and the standards. The amounts, purities, and recoveries of jatrorrhizine, coptisine, palmatine, and berberine are listed in Table 3, in which the recoveries of the compounds only in the HSCCC step were calculated as the following formula (3), and their total recoveries from raw material to pure products through the first and the second steps were calculated as the following formula (4).

$$\text{Recovery } (\%) = \frac{\begin{array}{c}(\text{the amount obtained fraction from HSCCC}) \\ \times(\text{the purity of the obtained fraction})\end{array}}{\begin{array}{c}(\text{the content of the target in crude extract}) \\ \times(\text{the samples size in HSCCC})\end{array}} \times 100\% \quad (3)$$

$$\text{Total recovery } (\%) = (\text{the recovery in the first step}) \times (\text{the recovery in the second step}) \quad (4)$$

Table 3. The amounts, purities and recoveries of the compounds in the second step from crude extracts to pure products by HSCCC

Compounds	Sample size in HSCCC	Amounts from HSCCC	Purity of the obtained fractions[a]	Recovery[b]	Total recovery[c]
Jatrorrhizine	400 mg	32.1 mg	95.6%	92.0%	69.8%
Coptisine		44.4 mg	96.4%	92.8%	72.5%
Palmatine		46.2 mg	96.8%	91.3%	71.0%
Berberine		219.2 mg	99.2%	93.0%	73.3%

[a]The purities of the obtained fractions from HSCCC determined by HPLC and standards.

$$[b]\text{Recovery } (\%) = \frac{\begin{array}{c}(\text{the amount of obtained fraction from HSCCC}) \\ \times (\text{the purity of the obtained fraction})\end{array}}{\begin{array}{c}(\text{the content of the target in crude extract}) \\ \times (\text{the sample size in HSCCC})\end{array}}$$

$$\times 100\% \text{ (only in HSCCC separation process).}$$

[c]Total recovery (%) = (the recovery in the first step) × (the recovery in the second step).

The overall data listed in Tables 1, 2, and 3 obviously showed higher recoveries (over 92%) of the targets isolated by HSCCC coupled with direct injection of the powders of the medicinal plant without any preparation method than the two steps purification method (ranged from 69.8% to 73.3% recovery). Although, the amounts of the obtained pure products from the two step isolation were higher than with the one step method, the targets were lost in the process of solvent extraction and liquid–solid or liquid-liquid extraction steps, and there was little loss in our present HSCCC separation protocol. Furthermore, the process of preparation of the crude extract was not required in our present HSCCC isolation. Thus, no extraction solvents and time were needed, which was beneficial for our environment and natural resources reserve. So, we think that the HSCCC coupled with direct injection of the powders of the plant into the separation column could be further developed in future HSCCC research.

CONCLUSION

In the present paper, a new approach of HSCCC coupled with direct injection of the powders of the medicinal plant without any preparation method was developed to separate and isolate jatrorrhizine, coptisine, palmatine, and berberine from *C. chinensis* Franch, with high recoveries of over 92% compared with approximately 70% in common HSCCC separation. Our research also showed that this new approach is beneficial for our environment

and natural resources reserve, and HSCCC is a powerful tool to separate and isolate chemical compounds from medicinal plants.

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