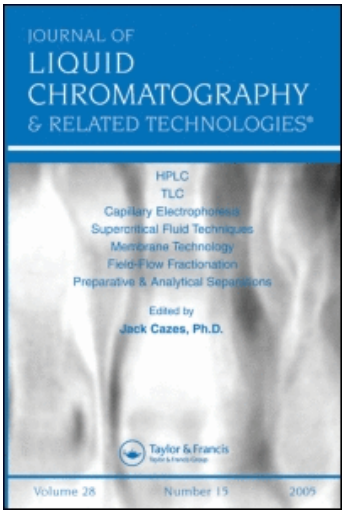

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Preparative Isolation and Purification of Alkaloids from *Corydalis yanhusuo* W. T. Wang by High Speed Counter-Current Chromatography

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Abstract: Four alkaloids including dehydrocorydaline, palmatine, coptisine, and columbamine were successfully isolated from the total alkaloids of *Corydalis yanhusuo* W. T. Wang using a stepwise elution by high speed counter-current chromatography (HSCCC). A pair of two-phase solvent systems consisting of carbon tetrachloride–trichloromethane–methanol–0.2 mol/L hydrochloric acid (1 : 7 : 3 : 4, v/v/v/v) and trichloromethane–methanol–0.2 mol/L hydrochloric acid (7 : 3 : 4, v/v/v) was successively used, which yielded four relatively pure alkaloids from 200 mg of the total alkaloids in a single run. The purities of dehydrocorydaline, palmatine, coptisine, and columbamine were 99.1, 97.1, 99.7, and 94.9%, respectively. The structures were identified by ^{1}H NMR and ^{13}C NMR.

Keywords: Counter-current chromatography, C*orydalis yanhusuo* W. T. Wang, Dehydrocorydaline, Palmatine, Coptisine, Columbamine

INTRODUCTION

Corydalis yanhusuo W. T. Wang (*C. turtschaninovii Bess. f. yanhusuo* Y. H. Chou et C. C. Hsu), a perennial herb up to 30 cm tall, is one of the medicinally

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important species of *Corydalis* and it is well known as a traditional Chinese herbal medicine.[1] The dried and pulverized tubers of *Corydalis yanhusuo*, also called *Rhizoma Corydalis* or yan-hu-suo, are used as traditional Chinese medicine in the treatment of gastric and duodenal ulcer, cardiac arrhythmia disease, rheumatism, and dysmenorrhea.[2] Also, it has been used to promote blood circulation, reinforce vital energy, and alleviate pain such as headache, chest pain, hypochondriac pain, epigastric pain, abdominal pain, backache, arthralgia, or trauma.[3]

Corydalis yanhusuo is traditionally and mainly cultivated in Zhejiang province of China as an annual crop using tubers, and its tubers are partially exported to other countries. The tuber contains several tertiary and quaternary alkaloids that form the main bioactive components. Some of the important quaternary alkaloids are dehydrocorydaline (DHC), palmatine, coptisine, columbamine, and so on.[1] They exhibit various kinds of bioactivity.[4–6] For instance, studies show that DHC not only inhibits antibody-mediated allergic reactions but also influences cell-mediated allergic reactions.[7]

Previous studies show that the alkaloid constituents isolated from the extract of *Corydalis yanhusuo* were almost purified by classical methods such as silica gel chromatography or alumina chromatography,[8,9] which usually need several steps, resulting in time- and solvent-consuming. HSCCC has been widely used as a convenient tool to isolate alkaloids from natural products.[10–12]

Our paper introduces a method of preparative HSCCC separation of four quaternary alkaloids (Figure 1) from the crude extract of *Corydalis yanhusuo*, using a pair of two-phase solvent systems with their combined use in stepwise elution.

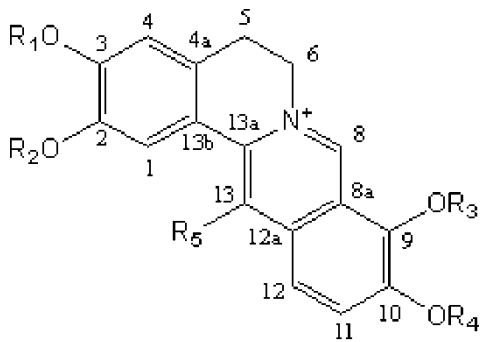

Figure 1. Chemical structures of the four alkaloids (a) Dehydrocorydaline: $R_1 = R_2 = R_3 = R_4 = R_5 = CH_3$; (b) Palmatine: $R_1 = R_2 = R_3 = R_4 = CH_3$, $R_5 = H$; (c) Coptisine: $R_1 + R_2 = -CH_2-$, $R_3 + R_4 = -CH_2-$, $R_5 = H$; (d) Columbamine: $R_1 = R_3 = R_4 = CH_3$, $R_2 = R_5 = H$.

EXPERIMENTAL

Reagents and Materials

All organic solvents used for HSCCC were of analytical grade and purchased from Hangzhou HuiPu Chemical Factory (Hangzhou, China). Acetonitrile used for HPLC analysis was of chromatographic grade. Potassium dihydrogen phosphate and sodium dodecyl sulphonate (SDS) were of chemical grade. The dried tubers of *Corydalis yanhusuo* were cultivated and collected from Dongyang County (Zhejiang, China).

Apparatus

A Model TBE-300A high speed counter-current chromatograph (Shanghai Tauto Biotechnique, Shanghai, China) equipped with three preparative multilayer coils (260 mL, wound with 1.6 mm I.D. PTFE tubing) was used. The β values of this column range from 0.46 to 0.73 ($\beta = r/R$, $R = 6.0$ cm, where r is the distance from the coil to the holder shaft, and R, the revolution radius or the distance between the holder shaft and central axis of the centrifuge). The revolution speed of the apparatus can be regulated with a speed controller in the range between 0 rpm and 1000 rpm. The columns of HSCCC were installed in a vessel that was retained at 20°C by a Model HX-1050 constant-temperature controller (Beijing Boyikang Lab Instrument Co. Ltd., Beijing, China). The solvent was pumped into the column with a Model NS-1007 constant-flow pump (Beijing Shengyitong Technique Co. Ltd., Beijing, China). Continuous monitoring of the effluent was achieved with a Model UV-II detector Monitor (Shanghai Institute of Biochemistry of Academy of Science, Shanghai, China) at 254 nm. A manual sample injection valve with a 20 mL loop (Shanghai Tauto Biotechnique, Shanghai, China) was used to introduce the sample into the column. Sepu3000 workstation (Hangzhou PuHui Technology, Hangzhou, China) was employed to record the chromatogram. Eluate was collected with a Model BSZ-100 fraction collector (Shanghai Huxi Tech, Shanghai, China), 10 mL for each fraction.

The high performance liquid chromatograph (HPLC) used was a CLASS-VP Ver.6.1 system (Shimadzu, Japan) comprised of a Shimadzu SPD10Avp UV detector, a Shimadzu LC-10ATvp Multisolvent Delivery System, a Shimadzu SCL-10Avp controller, a Shimadzu LC pump, and a CLASS-VP Ver.6.1 workstation.

Extraction of Total Alkaloids

The dried tubers of *Corydalis yanhusuo* (15 g) were milled into powder (about 50 mesh) by using a model FZ-102 pulverizer (Tianjin Taisite Instrument

Co. Ltd., Tianjin, China). The powder was soaked in 10 mL of 10% ammonia solution for 12 h and then extracted with chloroform at room temperature three times (1 d, 1 d, 1 d). Each time, the extraction mixture was filtered. Finally, the extracts were combined and evaporated to dryness under reduced pressure, which yielded 236 mg of total alkaloids that were directly subjected to HSCCC.

Preparation of Two-phase Solvent System and Sample Solution

For the present study, we selected a pair of two-phase solvent systems composed of carbon tetrachloride–trichloromethane–methanol-0.2 mol/L hydrochloric acid (1 : 7 : 3 : 4, v/v/v/v) and trichloromethane–methanol–0.2 mol/L hydrochloric acid (7 : 3 : 4, v/v/v). The solvent mixture was thoroughly equilibrated in a separation funnel at the same temperature as in the vessel of HSCCC and the two phases were separated shortly before use.

The sample solution was prepared by dissolving the crude sample in the mixture solution of lower phase and upper phase (1 : 1, v/v) of solvent system used for HSCCC separation.

Separation Procedure

HSCCC was performed as follows. The multilayer-coiled column was first entirely filled with the upper phase as a stationary phase. The lower organic mobile phase was then pumped into the head end of the column inlet at a flow-rate of 2.0 mL/min, while the apparatus was run at a revolution speed of 810 rpm. After hydrodynamic equilibrium was reached, as indicated by a clear mobile phase eluting at the tail outlet, the sample solution (200 mg dissolved in 10 mL mixture solution of lower phase and upper phase (1 : 1, v/v) of the solvent system) was injected through the sample port. The effluent from the tail end of the column was continuously monitored with a UV detector at 254 nm. Each peak fraction was manually collected according to the elution profile and determined 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 air.

HPLC Analysis and Identification of HSCCC Peak Fractions

The total alkaloids extracted from *Corydalis yanhusuo* and each peak fraction from HSCCC was analyzed by HPLC. The analyses were performed with a Shim-Pack CLC-ODS C_{18} column (250 mm × 4.6 mm I.D.). The mobile phase composed of acetonitrile–0.03 mol/L potassium dihydrogen phosphate (32 : 68, v/v, added 1.670 g of SDS to 1000 mL mobile phase) was eluted at

a flow-rate of 0.6 mL/min, and the effluent was monitored by a Shimadzu SPD10Avp UV detector at 345 nm.

Identification of HSCCC peak fractions was carried out by ^{1}H NMR and ^{13}C NMR spectra. NMR spectra were recorded on a Bruker Avance 400 MHz spectrometer with TMS (tetramethylsilane) as internal standard.

RESULTS AND DISCUSSION

Selection of the Method for Extracting Total Alkaloids

Attention was paid to selecting the method for extracting the total alkaloids from natural products, as there were several schemes to choose from. The following two schemes had been tried for extracting total alkaloids from *Corydalis yanghusuo*:

Scheme 1: The dried tubers of *Corydalis yanhusuo* (15 g) were milled into powder (about 50 mesh) and extracted with 75% ethanol at room temperature three times (1 d, 1 d, 1 d). The extracts were combined and evaporated to dryness under reduced pressure, which yielded 610 mg extract. The ethanol extract was dissolved in 15 mL 2% hydrochloric acid and filtered. The filtrate was extracted with chloroform twice and ammonia added to adjust pH = 9, and then extracted with chloroform several times until the chloroform layer showed weak action to the Dragendorff reagent. The chloroform extract was evaporated to dryness under reduced pressure, which yielded 185 mg of total alkaloids.

For Scheme 2: see EXPERIMENTAL: Extraction of Total Alkaloids.

HSCCC separation of the total alkaloids obtained from both two schemes showed that scheme 2 was more ideal for isolation of the four target compounds, while in HSCCC separation of the total alkaloids from scheme 1, except for dehydrocorydaline, the other three alkaloids couldn't be well isolated.

Selection of Two-phase Solvent System for HSCCC

Successful separation of the target compounds using HSCCC depends on the selection of the suitable solvent systems which should be focused on the following points: (1) no decomposition or denaturation of the sample; (2) sufficient sample solubility; (3) suitable partition coefficient (K) values of the target compound (i.e. usually between 0.2 and 5); (4) satisfactory retention of the stationary phase; and (5) the settling time of the solvent system should be short (i.e. <30 s).[13] A series of experiments were performed to determine the optimal two-phase solvent system for the HSCCC separation. The following systems at different volume ratios were tested: *n*-butanol–ethyl acetate–water (4 : 1 : 5), (1 : 1 : 2), and (2 : 3 : 5);

hexane–ethyl acetate–methanol–water (5 : 5 : 5 : 5), (3 : 7 : 5 : 5), and (4 : 6 : 4 : 6); hexane–ethanol-0.2 mol/L hydrochloric acid (6 : 5 : 5), (6 : 4 : 6), and (6 : 3 : 7); carbon tetrachloride–methanol–water (10 : 10 : 1) and (10 : 9 : 2); trichloromethane–methanol-water (2 : 1 : 1) and (4 : 3 : 2); trichloromethane–methanol–0.2 mol/L hydrochloric acid (7 : 3 : 4, v/v/v) (solvent system 1) and carbon tetrachloride–trichloromethane–methanol–0.2 mol/L hydrochloric acid (1 : 7 : 3 : 4, v/v/v/v) (solvent system 2). Among those solvent systems, 1 could give approving separation of palmatine, coptisine, and columbamine, but failed to separate dehydrocorydaline and Peak I (Figure 2A). On the other hand, solvent system 2 gave a satisfying separation of dehydrocorydaline and Peak I, but coptisine required much more than 8 h to be eluted out and columbamine would still be retained in stationary phase after 11 h (Figure 2B). Therefore, the combined use of the above solvent systems would lead to a successful separation and purification of four alkaloid compounds in a single run. Our experiment clearly confirmed this conclusion. Figure 3 shows the result obtained from 200 mg of the total alkaloids of *Corydalis yanhusuo* by HSCCC. After 150 min of elution using the lower phase of solvent system 2, the mobile phase was changed to the lower phase of solvent system 1 to elute the retained two peaks. This elution mode yielded four relatively pure peak fractions each at 99.1, 97.1, 99.7, and 94.9% purity, respectively, as determined by HPLC (Figure 4). The retention of the stationary phase was 57.7%, and the separation time was 8 h for a separation run.

In addition to the isocratic elution usually employed, HSCCC enables stepwise and gradient elution. However, the successful application requires a particular choice of the solvent systems as follows: the key element producing the gradient (such as acid, neutral salt, etc.) should be partitioned almost entirely into the mobile phase, and at the same time should not significantly alter the volume ratio of the two solvent phase after the two phases reestablish the hydrodynamic equilibrium in the column.[14] This method facilitates us to choose the second mobile phase with suitable K values for the target compounds with much greater partition coefficient, so that the peak resolution is further increased in a short period time.

HPLC Analysis of The Crude Sample and of HSCCC Peak Fractions

The total alkaloids of *Corydalis yanhusuo* was analyzed by HPLC. The result indicates that under UV 345 nm it contained several major quaternary alkaloids including dehydrocorydaline (49.6%), palmatine (9.9%), coptisine (23.4%), and columbamine (8.0%) and some unknown compounds (see Figure 4A).

After HSCCC separation, the fractions containing dehydrocorydaline, palmatine, coptisine, and columbamine were collected, respectively.

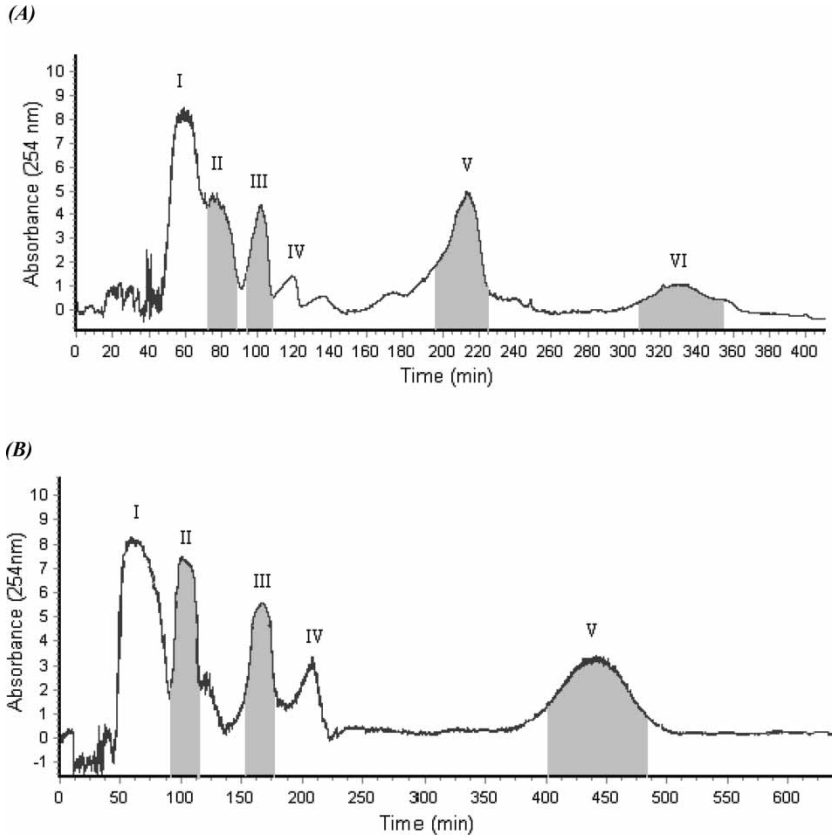

Figure 2. Chromatogram of the total alkaloids from *Corydalis yanhusuo* W. T. Wang by HSCCC. Peak I: unknown compounds; Peak II dehydrocorydaline; Peak III: palmatine; Peak IV: unknown compounds; Peak V: coptisine; Peak VI: columbamine. Solvent system: trichloromethane–methanol–0.2 mol/L hydrochloric acid (7 : 3 : 4, v/v/v) (A) and carbon tetrachloride–trichloromethane–methanol–0.2 mol/L hydrochloric acid (1 : 7 : 3 : 4, v/v/v/v) (B); stationary phase: upper aqueous phase; mobile phase: lower organic phase; flow-rate: 2.0 mL/min; revolution speed: 810 rpm; sample: 200 mg dissolved in 10 mL mixture solution of lower phase and upper phase (1 : 1, v/v) of the solvent system; retention of the stationary phase: 61.6% (A) and 53.8% (B).

The analysis of these fractions indicated that the Peak II fraction contained dehydrocorydaline, which weighed 27.1 mg at over 99.1% purity, the Peak III fraction contained palmatine which weighed 11.5 mg at over 97.1% purity, the Peak V fraction contained coptisine, which weighed 20.0 mg at over 99.7% purity, and the Peak VI fraction contained columbamine, which weighed 3.0 mg at over 94.9% purity, as determined by HPLC (Figure 4B–4E).

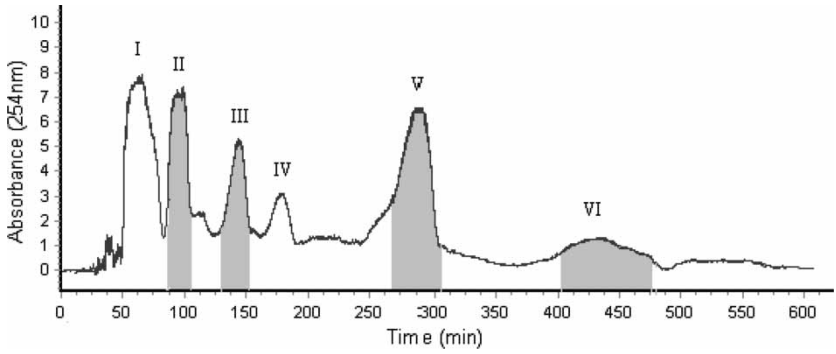

Figure 3. Chromatogram of the total alkaloids from *Corydalis yanhusuo* W. T. Wang by HSCCC. Peak I: unknown compounds; Peak II dehydrocorydaline; Peak III: palmatine; Peak IV: unknown compounds; Peak V: coptisine; Peak VI: columbamine. Solvent system: carbon tetrachloride–trichloro–methane–methanol–0.2 mol/L hydrochloric acid (1 : 7 : 3 : 4, v/v/v/v) (0–150 min) and trichloromethane–methanol–0.2 mol/L hydrochloric acid (7 : 3 : 4, v/v/v) (150–600 min); stationary phase: upper aqueous phase; mobile phase: lower organic phase; flow-rate: 2.0 mL/min; revolution speed: 810 rpm; sample: 200 mg dissolved in 10 mL mixture solution of lower phase and upper phase (1 : 1, v/v) of the solvent system; retention of the stationary phase: 57.7%.

Structural Identification

The structural identification of the four components was carried out by ^{1}H NMR and ^{13}C NMR spectra.

Peak II: ^{1}H NMR ($CDCl_3$) δ ppm: 10.68 (1H, s, H-8), 7.92 (1H, d, $J = 9.10$, H-12), 7.87 (1H, d, $J = 9.10$, H-11), 7.16 (1H, s, H-1), 6.93 (1H, s, H-4), 5.30 (2H, br. s, H-6), 3.25 (2H, br. s, H-5), 3.94 (3H, s, OCH_3-2), 4.35 (3H, s, OCH_3-3), 4.08 (3H, s, OCH_3-9), 4.00 (3H, s, OCH_3-10), 2.97 (3H, s, CH_3-13). ^{13}C NMR ($CDCl_3$) δ ppm: 151.3, 150.5, 147.7, and 146.3 (C-2, C-3, C-9, and C-10), 146.5 (C-8), 128.5 (C-13), 125.4 (C-12), 119.7 (C-11), 136.3, 133.7, 132.2, 121.7, and 119.2 (C-4a, C-8a, C-12a, C-13a, C-13b), 113.9 (C-4), 110.7 (C-1), 63.2 (C-6), 28.2 (C-5), 57.1 (3-OCH_3), 56.9 (OCH_3-9), 56.5 (OCH_3-10), 56.2 (OCH_3-2), 17.9 (CH_3-N). After comparing the data with spectral information from the literature,[9] peak II was confirmed as dehydrocorydaline.

Peak III: ^{1}H NMR (DMSO-d_6) δ ppm: 9.89 (1H, s, H-8), 9.08 (1H, s, H-13), 8.19 (1H, d, $J = 9.10$, H-12), 8.02 (1H, d, $J = 9.10$, H-11), 7.71 (1H, s, H-1), 7.08 (1H, s, H-4), 4.94 (2H, t, $J = 6.00$, H-6), 3.21 (2H, t, $J = 6.00$, H-5), 3.85 (3H, s, OCH_3-2), 4.08 (3H, s, OCH_3-3), 4.05 (3H, s, OCH_3-9), 3.92 (3H, s, OCH_3-10). ^{13}C NMR (DMSO-d_6) δ ppm: 151.9, 150.6, 149.1, and 144.0 (C-2, C-3, C-9, C-10), 145.8 (C-8), 120.4 (C-12), 119.3 (C-11), 123.8 (C-13), 138.1, 133.5, 129.0, 127.2, and 121.7 (C-4a,

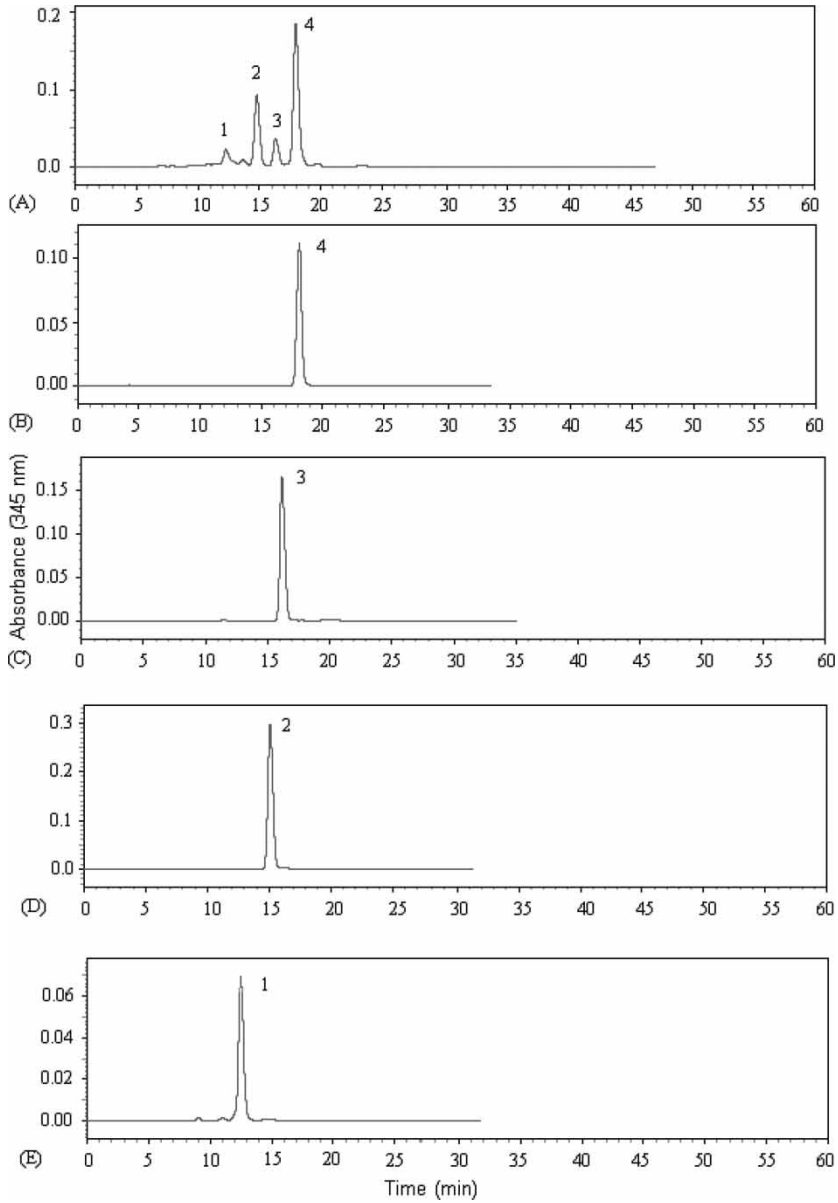

Figure 4. Results of HPLC analyses of the total alkaloids of *Corydalis yanhusuo* and its HSCCC fractions. Column: Shim-Pack CLC-ODS C_{18} column (250 mm × 6 mm I.D.); mobile phase: acetonitrile–0.03 mol/L potassium dihydrogen phosphate (32:68, v/v, 1000 mL mobile phase was added with 1.670 g SDS); flow-rate: 0.6 mL/min; UV detector: 345 nm. (A) The total alkaloids; (B) HSCCC fraction from Peak II (Figure 3); (C) HSCCC fraction from Peak III (Figure 3); (D) HSCCC fraction from Peak V (Figure 3); (E) HSCCC fraction from Peak VI (Figure 3).

C-8a, C-12a, C-13a, C-13b), 111.7 (C-4), 109.2 (C-1), 62.3 (C-6), 26.4 (C-5), 57.4 (OCH_3-3), 56.6 (OCH_3-9), 56.2 (OCH_3-10), 55.8 (OCH_3-2). After comparing the data with spectral information from the literature, [15] peak III was confirmed as palmatine.

Peak V: ^{1}H NMR (DMSO-d_6) δ ppm: 9.96 (1H, s, H-8), 8.98 (1H, s, H-13), 8.04 (1H, d, $J = 8.58$, H-12), 7.83 (1H, d, $J = 8.70$, H-11), 7.80 (1H, s, H-1), 7.09 (1H, s, H-4), 6.54 (2H, s, OCH_2O-9), 6.18 (2H, s, OCH_2O-2), 4.89 (2H, t, $J = 6.10$, H-6), 3.20 (2H, t, $J = 6.10$, H-5). ^{13}C NMR (DMSO-d_6) δ ppm: 150.2, 148.1, 147.5, and 144.3 (C-2, C-3, C-9, C-10), 145.0 (C-8), 121.5 (C-13), 120.9 (C-12), 112.1 (C-11), 137.2, 132.8, 131.0, 122.2, and 121.4 (C-4a, C-8a, C-12a, C-13a, C-13b), 108.8 (C-4), 105.7 (C-1), 102.5 (OCH_2O-2), 104.9 (OCH_2O-9), 55.5 (C-6), 26.7 (C-5). After comparing the data with spectral information of reference compound, peak V was confirmed as coptisine.

Peak VI: ^{1}H NMR (DMSO-d_6) δ ppm: 9.90 (1H, s, H-8), 9.51 (1H, s, H-13), 8.22 (1H, d, $J = 9.17$, H-12), 8.09 (1H, d, $J = 9.12$, H-11), 7.60 (1H, s, H-1), 6.91 (1H, s, H-4), 4.96 (2H, t, $J = 6.10$, H-6), 3.22 (2H, t, $J = 6.10$, H-5), 4.10 (3H, s, OCH_3-3), 3.92 (3H, s, OCH_3-9), 4.12 (3H, s, OCH_3-10). ^{13}C NMR (DMSO-d_6) δ ppm: 150.9, 150.2, 148.4 and 143.9 (C-2, C-3, C-9, C-10), 145.5 (C-8), 119.6 (C-13), 129.2 (C-12), 127.0 (C-11), 138.7, 133.7, 123.9,121.7,117.2 (C-4a, C-8a, C-12a, C-13a, C-13b), 115.1 (C-4), 109.7 (C-1), 55.8 (C-6), 26.2 (C-5), 57.2 (OCH_3-3), 56.6 (OCH_3-9), 62.1 (10-OCH_3). Compared with the reported data,[16] the spectra data of peak VI was in agreement with those of columbamine.

The result of our studies described above, clearly demonstrated that when using stepwise elution, HSCCC is successful in the preparative separation of dehydrocorydaline, palmatine, coptisine, and columbamine from total alkaloids of *Corydalis yanhusuo.*

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