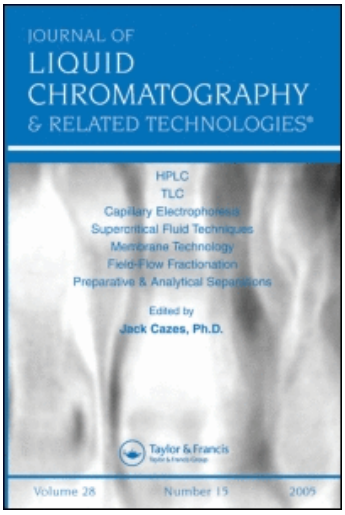

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Separation and Purification of *dl*-Tetrahydropalmatine from *Corydalis Yanhusuo* W. T. Wang by HSCCC with a New Solvent System Screening Method

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Abstract: A fast and simple solvent system screening method was established based on average polarity, which was much easier to find a suitable solvent system for high speed countercurrent chromatography separation. *Dl*-tetrahydropalmatine was separated and purified in one step with *n*-hexane-ethyl acetate-methanol-water (4-6-5-5) on high speed countercurrent chromatography. Acetic acid was successfully used to adjust the pH value of the sample solution to improve recovery of the separation. As a result, 4.37 mg *dl*-tetrahydropalmatine was separated from 25 mg crude extract at the purity of 92.7% with the recovery of 95.1%.

Keywords: Average polarity, *Corydalis yanhusuo*, Countercurrent chromatography, *dl*-tetrahydropalmatine

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INTRODUCTION

Yanhusuo, the dried roots of *Corydalis yanhusuo* W. T. Wang, has been used widely in traditional Chinese medicine (TCM) to promote blood circulation, reinforce vital energy, and alleviate pain such as headache, abdominal pain, menstrual pain, and pain due to injuries.[1,2] The bioactive components in *yanhusuo* are alkaloids. It was demonstrated by pharmacological studies that *dl*-tetrahydropalmatine was the representative active alkaloid for the analgesic activity,[3] sedative tranquilizing, hypnotic, antihypertensive,[4] and anxiolytic[5] activities. The chemical structure of *dl*-tetrahydropalmatine is shown in Figure 1.

High speed countercurrent chromatography (HSCCC) is a continuous liquid–liquid partition chromatography without solid support matrix. The stationary phase is retained in the separation columns by gravity and a centrifugal force field. HSCCC avoids the disadvantages arising from the interactions of samples and the solid support, such as absorption and denaturation of target components. HSCCC is of high recovery and high efficiency. It has been widely used in the separation and purification of natural products to get high purity components.[6–9]

The aim of this work was to develop an efficient method to separate the target compound by HSCCC. The application of HSCCC on the separation of *dl*-tetrahydropalmatine has been cursorily reported by using a two-phase solvent system composed of petroleum ether-ethyl acetate-methanol-water.[10] However, some key parameters such as the boiling point of petroleum ether, the content of the target component in crude extract, and the recovery of *dl*-tetrahydropalmatine were not reported. It was found in our experiments that the retention of the stationary phase composed of petroleum ether was pretty low (36%) and the recovery was 37.36%. Both the low retention of stationary phase and the low recovery indicated that a better solvent system was needed for the separation.

For further study, *n*-hexane was used instead of petroleum ether and that the retention of the stationary phase was 63%. The higher retention of stationary phase indicated the better the peak resolution, and that it was beneficial for the improvement of the purity and recovery of the target compound.

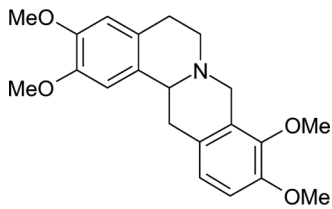

Figure 1. Chemical structure of *dl*-tetrahydropalmatine.

EXPERIMENTAL

Reagents and Materials

Analytical grade reagents used for HSCCC separation *n*-hexane, ethyl acetate, methanol, acetic acid, triethylamine, petroleum ether (30–60°C), butanol, acetonitrile, and ethanol were from Atoz Fine Chemicals Co. Ltd., Tianjin, China. Chromatographic grade methanol (Caledon Laboratories, Canada), acetic acid (Kemiou Chemical, Tianjin, China), and triethylamine (Tedia, USA) were used for high performance liquid chromatography (HPLC) analysis. All aqueous solutions were prepared with pure water produced by Milli-Q system (18MΩ, Millipore, Bedford, MA, USA).

Yanhusuo crude extract was bought from Tianzhe Company, Shanxi, China. The standard sample of *dl*-tetrahydropalmatine (110726–200409) was supplied by the State Food and Drug Administration of China (SFDA).

Apparatus

HSCCC (TBE-300A) is from Tauto Biotech, Shanghai, China, with three preparative coils connected in series (diameter of 2.6 mm, total volume 220 mL) and a 20 mL sample loop. The revolution radius or the distance between the holder axis and central axis of the centrifuge (R) is 5 cm and the β value varied from 0.5 at the internal terminal to 0.8 at the external terminal ($\beta = r/R$, where r is the distance from the coil to the holder shaft). The HSCCC systems are equipped with a Model S constant flow pump, a Model 8823 A UV monitor operating at 280 nm, and a Model 3057 recorder.

Calculation of the Average Polarity (P') of Solvent Systems

A new concept was brought forward named Average Polarity (P'). It was calculated according to the polarity of each solvent (Rohyschneider Snyder, p'_n).[11] The calculated equation was as follows:

$$P' = \frac{p'_1 V_1 + p'_2 V_2 + \ldots + p'_n V_n}{V_1 + V_2 + \ldots + V_n}$$

where P' was the average polarity of the solvent system. p'_n was the polarity of each solvent. V_n was the volume ratio of each solvent that composed of the solvent system.

In the two-phase solvent system n-hexane-ethyl acetate-methanol-water (4-6-5-5, v/v), which was composed of four solvents ($n = 4$), the polarity of *n*-hexane was $p'_1 = 0.1$; the polarity of ethyl acetate was $p'_2 = 4.4$; the polarity of *n*-hexane was $p'_3 = 5.1$, the polarity of *n*-hexane was $p'_4 = 10.2$. The volume ratios were $V_1 = 4$, $V_2 = 6$, $V_3 = 5$, $V_4 = 5$, respectively. Taking these values into the equation, the average polarity of the solvent system was 5.17.

Measurement of Partition Coefficient (*K*)

Partition coefficient was expressed as the absorbency of sample in the upper phase divided by that in the lower phase.

Measurement of *K* value of crude sample was as follows. Solvent systems were prepared and equilibrated. The upper phase of 4 mL and 4 mL of the lower phase were put into a 10 mL test tube. Then, 2.0 mg crude extract was dissolved in the solvent. The test tube was caped and shaken vigorously for 2 min to equilibrate the sample thoroughly. An equal volume of each phase was then analyzed by the UV spectrophotometer at 280 nm to obtain the *K*.

Preparation of Solvent System for HSCCC Separation

A hydrophilic organic/aqueous solvent system was prepared by thoroughly mixing organic solvents and water in a separatory funnel at room temperature. Two phases was separated shortly before use and degassed by sonication for 10 min. The optimized solvent system for dl-tetrahydropalmatine was *n*-hexane-ethyl acetate-methanol-water (4-6-5-5).

Preparation of Sample Solution

The crude sample of *yanhusuo*, 25 mg was dissolved into a mixture of 3 mL upper phase and 3 mL lower phase. Acetic acid, 10 μL was added to the sample solution to adjust the pH value.

HSCCC Operation

The coiled column of HSCCC was filled with the upper phase of the solvent system. Then, the apparatus was rotated at 850 rpm and at a temperature of 30°C, and at the same time the lower phase of solvent system was pumped through the column at a flow-rate of 1.5 mL/min. After the mobile phase emerged in the effluent and hydrodynamic

equilibrium was established in the column, 6 mL of the sample solution containing 25 mg of crude sample of *yanhusuo* was injected through the valve. Retention of the stationary phase was 63%. The effluent was monitored with a UV-Vis detector at 280 nm, and the peak fractions were collected, respectively.

HPLC Analysis

Crude sample and fractions separated by HSCCC were analyzed by HPLC system (10 Avp, Shimadzu, Japan) composed of two pumps, UV detector, oven, system controller, and 20 μL sample loop. The column used was Ultrasphere C_{18} column (250 × 4.6 mm I.D., 5 μm, Agilent, USA). The mobile phases were solvent A (methanol-water-acetic acid-triethylamine = 10-89.1-0.8-0.1) and solvent B (methanol-water-acetic acid-triethylamine = 89.1-10-0.8-0.1) in the gradient mode as follows: 0–10 min: 1–16% B, 10–35 min: 16–40% B, 35–60 min: 40–100% B. The flow rate was 0.9 mL/min. The effluent was monitored at 280 nm. The crude sample and the standard material were dissolved with solvent B.

RESULTS AND DISCUSSION

Analysis of Crude Extract of *Yanhusuo* Containing *dl*-Tetrahydropalmatine by HPLC

Yanhusuo crude extract was analyzed by HPLC with a C_{18} column. Acetic acid was added to the mobile phase to keep it at about pH 3, which made the alkaloids stable. The crude extract was analyzed by HPLC with conditions as shown in HPLC analysis section. Six concentrations of standard material were prepared and analyzed by HPLC to calculate the content of *dl*-tetrahydropalmatine in the crude extract, which was 18.4% (Figure 2).

Fast Solvent System Screening Method Based on Average Polarity (*P'*)

Selection of the two-phase solvent system may be estimated as 90% of the entire work. Conventionally, it searched a suitable solvent system from one solvent system to another. The screening usually started from the system composed of hexane-ethyl acetate-methanol-water, if no ideal K value was found, then shifting to hexane-ethanol-water system, chloroform systems, butanol systems, or modifiers added to the systems. However, it was reported by Ito[13] that over a hundred solvent

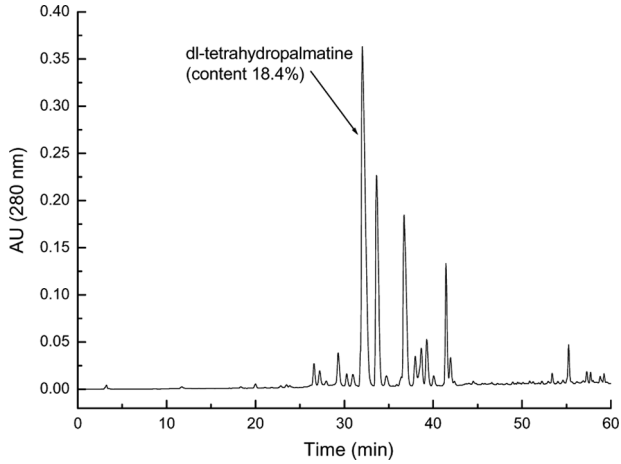

Figure 2. Analysis of crude extract of *yanhusuo* containing *dl*-tetrahydropalmatine by HPLC. The mobile phase was solvent A (methanol-water-acetic acid-triethylamine = 10-89.1-0.8-0.1) and solvent B (methanol-water-acetic acid-triethylamine = 89.1-10-0.8-0.1) in the gradient mode as follows: 0–10 min: 1–16% B, 10–35 min: 16–40% B, 35–60 min: 40–100% B. The flow-rate was 0.9 mL/min. The effluent was monitored at 280 nm.

systems could be used in HSCCC. To select the suitable K from these solvent systems without a systematic method was very time consuming, which gave negative effects on the spread of the HSCCC. It was urgently required to develop a more simple method to screen solvent systems.

Based on the rule of similarity, compounds were easily dissolved by solvents with similar polarities. Polarity was a key parameter in screening of solvent systems for HSCCC separation. A new arrangement method was established based on average polarity of the whole solvent systems, which broke the traditional arrangement of solution systems. Average polarity of the solvent system was calculated with the equation in Calculation of Average Polarity section. Some typical average polarities are listed in Table 1.

It was concluded from our experiment, that separate similar compounds have similar average polarities. For example, two different kinds of solvent systems petroleum ether-EtOAc-MeOH-water = 16-31-19-21 ($P' = 5.15$) and *n*-Hex-EtOAc-MeOH-water = 4-6-5-5 ($P' = 5.16$) have similar P'. Running the two systems on HSCCC with the same operation conditions could obtain similar peak elution sequence, purity, and recovery. The experiment indicated that we could take full advantage of the previous articles for separation and purification of the similar compounds. The searching method of the solvent systems was as follows:

Study the previous articles and find out the solvent systems that have been separated by similar compounds and calculate the P' of them. The

Table 1. Part of solvent systems arranged with increasing average polarity (P')

No.	Solvent system	P'
1	*n*-Hex-EtOAc-MeOH-water = 5-1-5-1	3.38
2	*n*-Hex-EtOAc-MeOH-water = 10-0-5-5	3.88
3	*n*-Hex-EtOAc-MeOH-water = 3-6-2-1	3.93
4	*n*-Hex-EtOAc-MeOH-water = 3-2-2.5-1.5	4.13
5	*n*-Hex-EtOAc-MeOH-water = 5-4-5-4	4.69
6	*n*-Hex-EtOAc-MeOH-water = 6-4-5-5	4.74
7	*n*-Hex-EtOAc-EtOH-water = 1-1-1-1	4.75
8	*n*-Hex-EtOAc-MeOH-water = 1-1-1-1	4.95
9	*n*-Hex-EtOAc-MeOH-water = 4.5-5-4.5-5	5.07
10	petroleum ether-EtOAc-MeOH-water = 16-31-19-21	5.15
11	*n*-Hex-EtOAc-MeOH-water = 4-6-5-5	5.16
12	*n*-Hex-EtOAc-MeOH-water = 4-5-4-5	5.21
13	petroleum ether-EtOAc-MeOH-water = 4-5-4-5	5.23
14	*n*-Hex-EtOAc-MeOH-water = 3.5-5-3.5-5	5.36
15	$CHCl_3$-MeOH-water = 10-7-3	5.37
16	*n*-Hex-EtOAc-MeOH-water = 3-7-5-5	5.38
17	*n*-Hex-EtOAc-MeOH-water = 3-5-3-5	5.54
18	*n*-Hex-EtOAc-MeOH-water = 1-4-2.5-2.5	5.60
19	*n*-Hex-EtOAc-MeOH-water = 1-2-1-2	5.73
20	EtOAc-BuOH-CAN-water = 2-1-2-1	5.75
21	$CHCl_3$-MeOH-AcOH-water = 5-5-0.5-3	5.90
22	*n*-Hex-EtOAc-MeOH-water = 2-5-2-5	5.96
23	*n*-Hex-EtOAc-MeOH-water = 1.5-5-1.5-5	6.22
24	$CHCl_3$-MeOH-AcOH-water = 5-4-0.5-4	6.27
25	EtOAc-EtOH-AcOH-water = 2-1-0.5-1.5	6.28
26	*n*-Hex-EtOAc-MeOH-water = 1-5-1-5	6.52
27	EtOAc-EtOH-AcOH-water = 2-1-0.1-1.9	6.62
28	EtOAc-EtOH-AcOH-water = 5-1-1-4	6.65
29	EtOAc-EtOH-AcOH-water = 2-1-0-2	6.70
30	$CHCl_3$-MeOH-AcOH-water = 2-1-0.2-2	6.71
31	Methyl tert-butyl ether-ACN-water = 2-2-3	6.74
32	EtOAc-EtOH-AcOH-water = 4-1-0.1-3.9	6.92
33	EtOAc-BuOH-ACN-water = 5-1-9-0-8	6.94
34	EtOAc-EtOH-AcOH-water = 5-1-0.5-5	6.98
35	EtOAc-BuOH-ACN-water = 1-1-8-0.2-10	7.76
36	EtOAc-BuOH-ACN-water = 5-2-2-13	7.91

n-Hex: *n*-hexane; EtOAc: ethyl acetate; MeOH: methanol; EtOH: ethanol; AcOH: acetic acid; BuOH: butanol; ACN: acetonitrile; $CHCl_3$: chloroform.

solvent system, methyl tert-butyl ether-acetonitrile-water = 2-2-3[12] ($P' = 6.74$) has been separated with tetrahydropalmatine. The construction of tetrahydropalmatine was similar to *dl*-tetrhydropalmatine.

Average polarity of petroleum ether-ethyl acetate-methanol-water = 16-31-19-21 was 5.15. Therefore, the suitable P' for separation of *dl*-tetrahydropalmatine could lie in between 5.15 or 6.74. As seen in Table 1, there were 5 systems available: petroleum ether-EtOAc-MeOH-water = 16-31-19-21 ($P' = 5.15$), *n*-Hex-EtOAc-MeOH-water = 4-6-5-5 ($P' = 5.16$), $CHCl_3$-MeOH-AcOH-water = 2-1-0.2-2 ($P' = 6.71$), methyl tert-butyl ether-ACN-water = 2-2-3, ($P' = 6.74$), EtOAc-EtOH-AcOH-water = 4-1-0.1-3.9 ($P' = 6.92$).

Two-phase solvent systems containing chloroform and tetrachloromethane were deleterious organic solvents that were dangerous for the environment and health. They were expelled from choices because of being unsuitable for the separation of TCM in industry. Only four solvent were systems left. For further examination the new established method, K values of crude extract of these four systems were measured. The result showed that K values were all in the range of $0.5 \sim 2.0$, which also indicated that the new method was efficient. These systems were performed on HSCCC in the following study.

HSCCC Separation of *dl*-Tetrahydropalmatine from *Yanhusuo* Crude Extract by Adjusting pH of Sample Solution

dl-Tetrahydropalmatine was separated at acceptable purity with the solvent system *n*-hexane-ethyl acetate-methanol-water (4-6-5-5) on HSCCC. But recovery of the purification was low (less than 50%). Further optimization was performed.

The crude extract of *yanhusuo* was dissolved into a mixture of two phases;[13] the upper phase was pH 6.03 and the lower phase was pH 4.45. After adding 25 mg crude extract, the pH value of upper phase was increased to 6.68 and lower phase increased to 6.59. In order to get better performance on HSCCC separation, organic acid and alkali were tried to adjust the pH value of the sample solution (Table 2).

This showed the purity of *dl*-tetrahydropalmatine was 93.2% and the recovery was 40.8% when the crude sample solution was separated on HSCCC without adjusting the pH value of the sample solution (Figure 3a). Then, excessive acetic acid was introduced into the sample solution (upper phase: pH 3.76, lower phase: pH 3.72) to ensure all alkaloids are in salt condition. Only one peak eluted, which indicated that no efficient separation happened (Figure 3b). To ensure all the alkaloids are in a dissociative condition, 20 μL triethylamine was added into a 6 mL sample solution to make the pH value of upper phase 9.25 and lower phase 9.24. Purity of *dl*-tetrahydropalmatine was 88.5% and recovery was 37.4% (Figure 3c). Finally, when 10 μL acetic acid was introduced into the 6 mL sample solution to make the pH value of upper phase

Table 2. The pH of the sample solution in *n*-hexane-ethyl acetate-methanol-water (4-6-5-5) and the yield of dl-tetrahydropalmatine

No.	pH of the sample solution		*K* value of crude extract	Purity of *dl*-THP	Yield of *dl*-THP
	Upper phase	Lower phase			
1	pH 3.76	pH 3.72	0.503	no separation	no separation
2	pH 9.25	pH 9.24	1.492	88.5%	37.4%
3	pH 6.68	pH 6.59	0.990	93.2%	40.8%
4	pH 4.72	pH 4.61	0.592	92.7%	95.1%

dl-THP: *dl*-tetrahydropalmatine.

4.72 and lower phase 4.61 (Figure 3d), 4.37 mg *dl*-tetrahydropalmatine was separated from 25 mg crude extract at the purity of 92.7%, with the recovery of 95.1%.

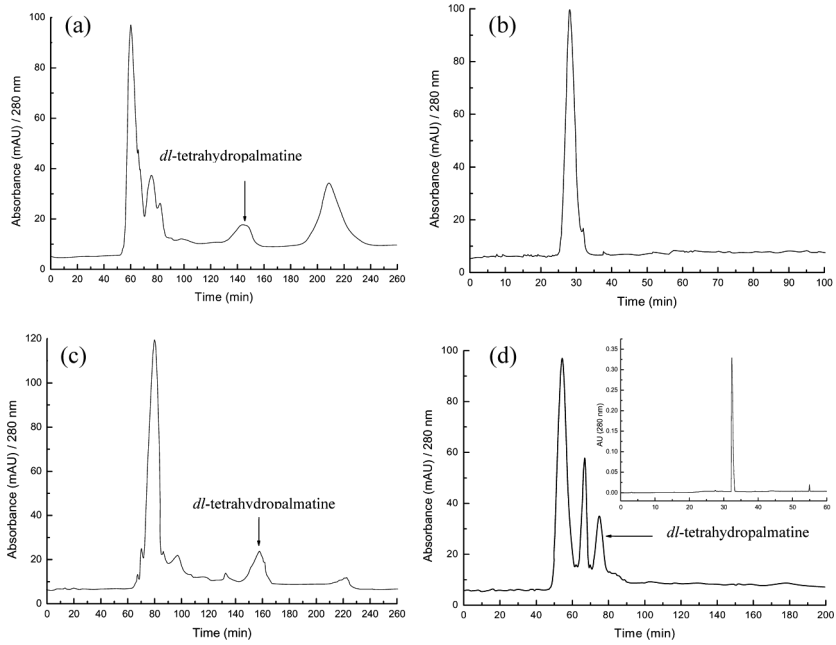

Figure 3. Separation of crude extract of *yanhusuo* by HSCCC. Solvent system of HSCCC: *n*-hexane-ethyl acetate-methanol-water (4:6:5:5); (a) crude sample solution without adjusting pH value, the upper phase was pH 6.68 and the lower phase was pH 6.59; (b) adding 100 μL acetic acid, the upper sample phase was pH 3.76 and the lower phase was pH 3.72; (c) adding 20 μL TEA. The upper phase was pH 9.25 and the lower phase was pH 9.24; (d) adding 10 μL acetic acid. The upper phase was pH 4.72 and the lower phase was pH 4.61.

This indicated that the pH value of the sample solution had considerable effect on separation of alkaloids on HSCCC. Separation of alkaloids with suitable pH value could be improved quite a bit.

CONCLUSIONS

Four solvent systems were obtained from hundreds of solvent systems by using the average polarity method, which was much easier to select a suitable solvent system for HSCCC separation. The result also showed that pH value of sample solution played an important role in purification of alkaloids. *dl*-Tetrahydropalmatine was separated and purified effectively in one step by HSCCC at high purity and high yield, which showed the advantages of this technology.

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