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Preparative separation of quaternary ammonium alkaloids from Caulis Mahoniae by conventional and pH-zone-refining counter-current chromatography

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Abstract

In this work, the preparative separation of quaternary ammonium alkaloids from Caulis Mahoniae by pH-zone-refining counter-current chromatography (PZRCCC) was compared to a conventional high-speed counter-current chromatography

(HSCCC). A flow-rate changing strategy was performed in conventional CCC separation with two-phase solvent systems composed of chloroform-methanol-0.5mM HCl water solution (4:1.5:2, *v/v*). Compared to conventional CCC separation, 3.0 g crude alkaloid extracts was carried out by PZRCCC with solvent system of chloroform-methanol-water (4:3:3, *v/v*). The retainer acid and eluter base were optimized by changing the concentration ratios, 60 mM HCl in upper aqueous phase and 7.5 mM TEA in lower phase. From 3.0 g of the crude alkaloid extracts, stepharanine (53.7 mg, 97.4%), columbamine (28.1 mg, 96.5%), jatrorrhizine (150.6 mg, 99.0%), palmatine (169.8 mg, 99.0%) and berberine (157.2 mg, 99.5%) were obtained. The compounds were identified by ESI-MS and ^{1}H-NMR data. The results indicated that PZRCCC is an excellent separation mode for separating quaternary ammonium alkaloids compared with conventional CCC.

1 Introduction

Caulis Mahoniae, the dried stem of *Mahonia bealei* (Fort) Carr. or *Mahonia fortune* (Lindl.) Fedde, is widely distributed in southeast of China. As a famous folk medicine, Caulis Mahoniae is widely used for treating jaundice hepatitis, jaundice, skin ulcer, etc [1, 2]. Caulis Mahoniae is also known for its effects of anti-cancer, anti-viral, anti-inflammatory, anti-arrhythmia, anti-bacterial, and hypoglycemic. Major effective compounds in Caulis Mahoniae are alkaloids which belong to quaternary ammonium [3-6].

A number of traditional chromatography techniques are used for separating alkaloids of quaternary ammonium. (e.g. pre-HPLC, alkaline silica gel and sephadex LH-20). Although traditional methods are widely used, the disadvantages of them could not be neglected, such as large solvent consumption, irreversible sample

adsorption and require complex multiple steps. It is necessary to establish an efficient method to separate alkaloids of quaternary ammonium from Caulis Mahoniae.

High-speed counter-current chromatography (HSCCC) is a liquid-liquid partition chromatography which can eliminate the irreversible adsorption without solid packing support. Due to the advantages of HSCCC, such as large sample injection, high recovery rate, simple sample preparation, and excellent repeatability [7], HSCCC has been developed to separate and isolate various samples [8-16]. pH-zone-refining counter-current chromatography (PZRCCC), derived from HSCCC and initially invented by Ito, can separate ionic compounds including alkaloids and organic acids. The chromatography peaks are rectangular with highly concentrated sample according to their hydrophobicities and pK_a values. Compared to conventional CCC, PZRCCC is approximate 10-fold increasein sample loading, enrichment of minor impurities, and high concentration of peaks.

In this paper, two CCC methods including conventional CCC and PZRCCC are comparative employed for preparative separation alkaloids of quaternary ammonium from Caulis Mahoniae. Fig. 1 shows the chemical structures of quaternary ammonium alkaloids.

2 Experimental

2.1 Reagents and materials

Petroleum ether, chloroform, methanol, alcohol, triethylamine (TEA), and hydrochloric acid (HCl) were analytical grade purchased from Siyou Chemical Reagent Factory. Acetonitrile, phosphoric acid and TEA used for HPLC were of chromatographic grade (Tedia Company, Inc., Fairfield, USA). Ultrapure water (18.2 MΩ) was purified by osmosis Milli-Q water system (Millipore, Bedford, MA, USA). Caulis Mahoniae was purchased from Jinan Jian-lian TCM store and the specie was

identified by Professor Jia Li (Shandong University of Traditional Chinese Medicine, Jinan, China). The voucher specimen (No. MD201509) was stored in Shandong Analysis and Test Center, Jinan, Shandong, China.

2.2 Apparatus

HSCCC separation was performed by TBE-300A (Shanghai Tauto Biotechnique, Shanghai, China) equipped with three multilayer coil separation PTFE columns connected in series (total volume 300 mL, I.D. of the tube diameter 2.6 mm) with a 20 mL sample loop. The rotation speed was adjusted from 0 to 1000 rpm by speed controller. During the separation process, the temperature of separation columns were maintained at 25°C using a model HX series constant temperature circulator instrument (Changcheng Company, Zhengzhou, China). TBP-5002 constant-flow pump (Shanghai Tauto Biotechnique, Shanghai China) was used to pump the two solvent systems. The chromatogram data of alkaloid extracts was collected using Model 3057 portable recorder (Yokogawa, Sichuan Instrument Factory, Sichuan, China). Spectra were generated from 8823A-UV detector (Beijing Emilion Technology, Beijing, China) at the wavelength of 254 nm.

HPLC was performed on Waters e2695 equipment (Waters, Milford, MA, USA) including a Waters 2998 diode assay detector (DAD) system, an automatic sample injection, a Waters 2695 quaternary-solvent delivery system, and a millennium 32 workstation.

2.3 Preparation of crude extracts from Caulis Mahoniae

The dried rhizomes of Caulis Mahoniae (4 kg, 40-60 mesh) were extracted under reflux (70°C) with 2 L of 95% ethanol twice for two hours. After extraction, filtrated extracts under decompression, then combined together and concentrated them by rotary evaporation at 50°C.

The pH value of the liquid concentration was adjusted to 3 with 2% HCl. After that, extracted the acid solution with petroleum ether for five times. The extraction solution was slowly alkalized with 10% ammonia until the pH value was 10. After rotary evaporation, 10.2 g of yellow precipitate was obtained and stored in refrigerator at 5°C.

2.4 Preparation of solvent systems and sample solutions

In conventional CCC, flow-rate changing strategy was used to separate total alkaloids in Caulis Mahoniae. The two-phase solvent system used for conventional CCC was chloroform-methanol-0.5mM HCl water (4:1.5:2, volume ratio, the same as follows). After equilibrated in a separating funnel, the solvent system that used for the following experiment was separated into two phases. 250 mg of crude alkaloid extracts were dissolved in 5 mL stationary phase and 5 mL mobile phase.

In PZRCCC separation, a two phase solvent system consisted of chloroform-methanol-water (4:3:3, *v/v*) was used. After equilibrated in a separating funnel, the solution was divided into two phases for the following experiment. The upper phase was acidified with 60 mM of HCl (stationary phase) and lower organic phase was alkalified with 7.5 mM TEA (mobile phase). 3.0 g of crude alkaloid extracts were dissolved in lower phase without alkalization and acidified upper phase.

2.5 Separation procedure

2.5.1 Conventional CCC separation

The multilayer-coiled columns were first filled with upper phase at 20.0 mL/min. Then the HSCCC equipment was rotated at 800 rpm in a clockwise mode while the lower mobile phase was pumped into the apparatus at 2.0 mL/min. After hydrodynamic equilibrium established, sample solution with 250 mg total alkaloids from Caulis Mahoniae was injected via the sample loop. The effluent was monitored

continuously with the UV detector at 254 nm. When elution time reached to 200 minutes, the mobile phase was changed into 10.0 mL/min until 310 min. The stationary phase retention was defined as the ratio of stationary phase divided by the total volume in column after separation.

2.5.2 PZRCCC separation

The PZRCCC was first filled with upper phase, and sample solution with 3.0 g total alkaloids from Caulis Mahoniae was injected. The lower mobile phase was pumped into the CCC column at 2.0 mL/min while rotated at 800 rpm in a clockwise mode. The effluent was monitored at 254 nm and records were collected by portable recorder. pH values of all fractions were tested at room temperature. The stationary phase retention in PZRCCC was defined the same as conventional CCC.

2.6 Analysis and identification of separation products

Analysis used for HPLC was Waters Symmetry® C_{18} column (4.6 mm×250 mm, i.d., 5 μm) at 25°C. The mobile phase was acetonitrile-1% TEA (adjust the pH value to 3 with phosphoric acid) solution (25:75, *v/v*) and the flow-rate was 1.0 mL/min. And the detector wavelength was 265 nm.

Agilent 5973N mass selective detector was used to detect the molecular weight of pure compounds with ESI interface. The NMR spectrum was tested by Varian-600 spectrometer with TMS as internal standard and DMSO as the solvent (Varian, Palo Alto, CA, USA).

3. Results and discussion

3.1 Conventional CCC separation

According to the alkaloid properties and the previous test on HSCCC separation, the solvent systems composed petroleum ether-ethyl acetate-methanol-water (1:1:1:1.1, *v/v*), ethyl acetate-*n*-butanol-0.5mM HCl water solution (4:1:5, *v/v*), and

chloroform-methanol-0.5mM HCl water solution (4:1.5:2, *v/v*) were designed to get an efficient separation of the alkaloid compounds. The K_D values of solvent systems of HSCCC are presented in Table 1. Table 1 shows that the K_D values were too small and unsuitable to separate alkaloids from Caulis Mahoniae when using petroleum ether-ethyl acetate-methanol-water (1:1:1:1, *v/v*) and ethyl acetate-n-butanol-0.5mM HCl solution (4:1:5, *v/v*) as the solvent system. In view of alkaloids were soluble in chloroform, the solvent system composed of chloroform-methanol-0.5mM HCl water solution were chosen for the separation. When chloroform-methanol-0.5mM HCl water solution (4:1.5:2, *v/v*) was used, compounds A, D and E afforded a suitable K_D value for separating but the elution time of compounds B and C were too long. In consideration of increasing the flow-rate had little effect on the stationary phase retention in chloroform series solvent systems, a flow-rate changing strategy was used in compounds B and C separation.

It was then assayed the HSCCC separation with chloroform-methanol-0.5mM HCl water solution (4:1.5:2, *v/v*). The lower phase of chloroform-methanol-0.5mM HCl water solution (4:1.5:2, *v/v*) were used as mobile phase in flow-rate changing strategy (0-200 min, 2.0 mL/min; 200-240 min, 10.0 mL/min), a good separation result could be obtained. After that, five compounds were successfully isolated from 250 mg crude alkaloid extracts of Caulis Mahoniae (shown in Fig. 3) including stepharanine (compound A, 13.2 mg), columbamine (compound B, 6.6 mg), jatrorrhizine (compound C, 17.3 mg), palmatine (compound D, 13.4 mg), and berberine (compound E, 14.7 mg) with the purities of 97.5%, 96.0%, 98.2%, 99.0%, and 99.5% as determined by HPLC, and the compound recoveries were 98%, 97%, 98%, 93%, and 97%, respectively. The productivities of the organic solvent were also calculated. In conventional CCC, the productivities per hour of the five compounds (compound A,

B, C, D, E) were 2.6, 1.3, 3.4, 2.6, and 2.9 mg/h, respectively, while 9.4, 4.7, 12.4, 9.6, and 10.5 mg/L (per liter of the solvent), respectively.

3.2 PZRCCC Separation

Through preliminary experiment, the solvent system composed of chloroform-methanol-water (4:3:3, *v/v*) was selected with different concentration of retainer acid and eluter base in PZRCCC separation. When 1.0 g crude alkaloid extracts were separated with 30 mM HCl as retainer acid (upper phase) and 10 mM TEA as eluter base (lower phase), compound A was successfully separated with a purity over 98% (Fig. 4I) while compounds B, C and D, E were not fully separated. When 60 Mm HCl and 10 mM TEA was used, the peak resolution was improved with elution time longer. As shown in (Fig. 4II), 1.0 g crude alkaloid extracts were completely separated. Then, the sample amount increased to 3.0 g, compound A was separated successfully while compounds B, C and D, E partly separated. When the stationary phase was acidified with 60 mM HCl and the mobie phase was alkalified with 7.5 mM TEA, 3.0 g crude alkaloid extracts were successful separation as shown in (Fig. 4III). Ultimately, five compounds were isolated and were identified as stepharanine (compound A, 53.7 mg), columbamine (compound B, 28.1 mg), jatrorrhizine (compound C, 150.6 mg), palmatine (compound D, 169.8 mg), and berberine (compound E, 157.2 mg) with the purities of 97.4%, 96.5%, 99.0%, 98.7%, and 98.5%, respectively, while they were determined by HPLC. Recoveries of five compounds were 33%, 34%, 71%, 98%, and 86%, respectively. In PZRCCC, the productivities per hour of the five compounds (compound A, B, C, D, E) were 7.4, 3.9, 20.6, 23.3, and 21.5 mg/h, respectively. And the productivities per liter of the organic solvent of the five compounds were 44.8, 23.4, 125.5, 141.5, and 131.0 mg/L, respectively.

3.4 Identification of the isolated compounds

Compound A (peak A in Fig. 3, peak A in Fig. 4III): light yellow crystal in chloroform methanol, bismuth potassium iodide reaction was positive. UV (λ_{max}^{MeOH}): 227, 282, 348 nm. Positive ESI-MS (*m/z*): 324.1 $[M+H]^+$. ^{1}H-NMR (125 MHz, DMSO-d_6) δ_{ppm}: 9.70 (1H, s, H-8), 8.73 (1H, s, H-13), 7.89 (1H, d, J = 9.0 Hz, H-12), 7.85 (1H, d, J = 9.0 Hz, H-11), 7.54 (1H, s, H-1), 7.03 (1H, s, H-4), 4.89 (2H, t, J = 6.0 Hz, H-6), 3.17 (2H, t, J = 6.0 Hz, H-5), 4.04 (3H, s, 9-OCH_3), 3.88 (3H, s, 3-OCH_3). Compared to literature [27], this compound was identified as stepharanine.

Compound B (peak B in Fig. 3, peak B in Fig. 4III): light yellow spiculas in chloroform methanol, bismuth potassium iodide reaction was positive. UV (λ_{max}^{MeOH}): 263, 345 nm. Positive ESI-MS (*m/z*): 338.4 $[M+H]^+$. ^{1}H-NMR (125 MHz, DMSO-d_6) δ_{ppm}: 9.87 (1H, s, H-8), 8.80 (1H, s, H-13), 8.21 (1H, d, J = 8.4 Hz, H-11), 8.03 (1H, d, J = 8.4 Hz, H-12), 7.58 (1H, s, H-1), 7.10 (1H, s, H-4), 4.96 (2H, t, J = 5.4 Hz, H-6), 4.10 (3H, s, 10-OCH_3), 4.08 (3H, s, 9-OCH_3), 3.91 (3H, s, 3-OCH_3), 3.20 (2H, t, J = 5.4 Hz, H-5). Compared to literature [28], this compound was identified as columbamine.

Compound C (peak C in Fig. 3, peak C in Fig. 4III): light red spiculas in chloroform methanol, bismuth potassium iodide reaction was positive. UV (λ_{max}^{MeOH}): 265, 345 nm. Positive ESI-MS (*m/z*): 338.4 $[M+H]^+$. ^{1}H-NMR (125 MHz, DMSO-d_6) δ_{ppm}: 9.85 (1H, s, H-8), 9.01 (1H, s, H-13), 8.11 (1H, d, J = 8.4 Hz, H-12), 8.00 (1H, d, J = 8.4 Hz, H-11), 7.29 (1H, s, H-1), 6.96 (1H, s, H-4), 4.91 (2H, t, J = 5.4 Hz, H-6), 4.08 (3H, s, 10-OCH_3), 4.06 (3H, s, 9-OCH_3), 3.95 (9H, s, 2-OCH_3), 3.15 (2H, t, J = 5.4 Hz, H-5). Compared to literature [29], this compound was identified as jatrorrhizine.

Compound D (peak D in Fig. 3, peak D in Fig. 4III): Yellow needle crystal in chloroform methanol, bismuth potassium iodide reaction was positive. UV (λ_{max}^{MeOH}):

273, 346 nm. Positive ESI-MS (*m/z*): 352.4 $[M+H]^+$. ^{1}H-NMR (125 MHz, DMSO-d_6) δ_{ppm}: 7.72 (1H, s, H-1), 7.09 (1H, s, H-4), 9.89 (1H, s, H-8), 8.22 (1H, d, *J* = 8.4 Hz, H-11), 8.02 (1H, d, *J* = 8.4 Hz, H-12), 9.04 (1H, s, H-13), 4.95 (2H, t, *J* = 5.4 Hz, H-6), 4.12 (3H, s, 10-OCH_3), 4.07 (3H, s, 9-OCH_3), 3.96 (3H, s, 2-OCH_3), 3.87 (3H, s, 3-OCH_3), 3.24 (2H, t, *J* = 5.4 Hz, H-5). Compared to literature [30], this compound was identified as palmatine.

Compound E (peak E in Fig. 3, peak E in Fig. 4III): Yellow needle crystal in chloroform methanol, bismuth potassium iodide reaction was positive. UV (λ_{max}^{MeOH}): 263, 346 nm. Positive ESI-MS (*m/z*): 336.4 $[M+H]^+$. ^{1}H-NMR (125 MHz, DMSO-d_6) δ_{ppm}: 9.91 (1H, s, H-8), 8.95 (1H, s, H-13), 8.20 (1H, d, *J* = 8.4 Hz, H-11), 8.01 (1H, d, *J* = 8.4 Hz, H-12), 7.78 (1H, s, H-1), 7.07(1H, s, H-4), 6.16 (2H, s, 2, 3-OCH_2O), 4.93 (2H, t, *J* = 5.4 Hz, H-6), 4.08 (3H, s, 10-OCH_3), 4.06 (3H, s, 9-OCH_3), 3.22 (2H, t, *J* = 5.4 Hz, H-5). Compared to literature [31], this compound was identified as berberine.

4. Conclusions

In this paper, two separation models conventional HSCCC and PZRCCC were successful used to preparative separation quaternary ammonium alkaloids from Caulis Mahoniae.. Five compounds were obtained in one-step separation with two CCC separation models and were identified as stepharanine, columbamine, jatrorrhizine, palmatine and berberine with the purities over 96%. The results demonstrated that in order to save elution time, flow-rate changing strategy may be employed in conventional HSCCC separation. Due to quaternary ammonium alkaloids are high polarity, reverse elution PZRCCC can be used for the low organic phase as mobile phase. And the concentration of the eluate (mobile phase) and retainer (stationary phase) are optimized by alkali and acid. Compared with the conventional CCC, PZRCCC is an efficient and rapid method for separation alkaloids of quaternary

ammonium because of the high concentration of fractions and large sample loading capacity.

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322 **Captions to the figures**

323 **Fig. 1** Chemical structures of compounds separated from Caulis Mahoniae.

324

325 **Fig. 2** The HPLC chromatogram of crude alkaloids from Caulis Mahoniae.
326 Experimental conditions: column, Waters Symmetry® C_{18} column (4.6 mm×250 mm,
327 i.d., 5 μm); mobile phase, acetonitrile-1% TEA (adjust the pH value to 3 with
328 phosphoric acid) solution (25:75, *v/v*); detection, 265 nm; flow-rate, 1.0 mL/min.

329

330 **Fig. 3** Conventional CCC separation and HPLC analysis to the HSCCC peak fractions.
331 Conditions: stationary phase, the upper phase of chloroform-methanol-0.5mM HCl
332 water solution (4:1.5:2, *v/v*); 0-200 min, flow-rate 2.0 mL/min, 200-240 min,
333 flow-rate 10.0 mL/min; sample size, 250 mg; detection, 254 nm; stationary phase
334 retention, 66.7% (flow-rate 2.0 mL/min), 52.8% (flow-rate 10.0 mL/min); revolution
335 speed, 800 rpm. HPLC conditions are as in Fig. 2.

336

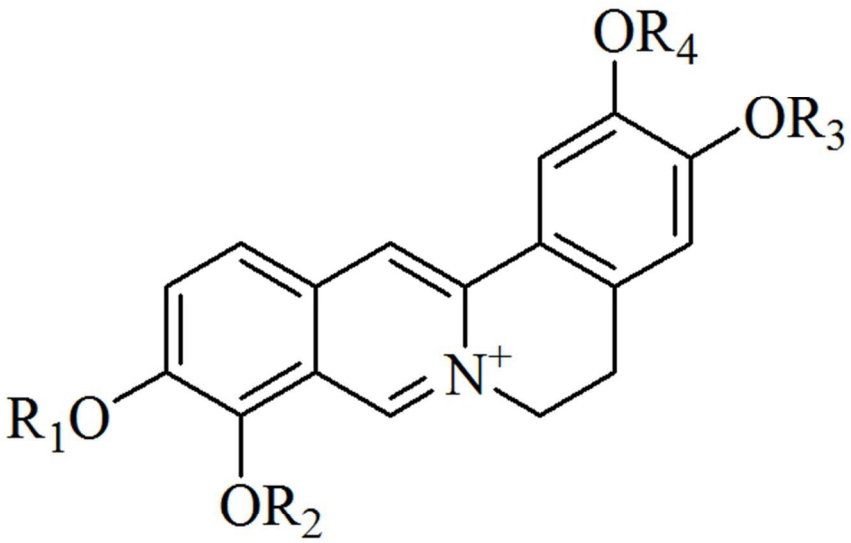
337 **Fig. 4** PZRCCC separation and HPLC analysis to the HSCCC peak fractions.
338 Conditions: stationary phase, the upper phase of chloroform-methanol-water (4:3:3,
339 *v/v*); detection, 254 nm; flow-rate, 2.0 mL/min; revolution speed, 800 rpm. I: 30 mM
340 HCl in upper stationary phase and 10 mM TEA in lower phase, sample loading 1.0 g,
341 stationary phase retention 40%; II: 60 mM HCl in upper stationary phase and 10 mM
342 TEA in lower phase, sample loading 1.0 g, stationary phase retention 41.7%; III: 60
343 mM HCl in upper stationary phase and 10 mM TEA in lower phase, sample loading
344 3.0 g, stationary phase retention 36.9%; IV: 60 mM HCl in upper phase and 7.5 mM
345 TEA in lower phase, sample loading 3.0 g, retention of stationary phase 36.4%.

346

347 **Table 1** The K_D-values of target compounds in different solvent systems

Solvent system	K_D-values of compounds A-E				
	A	B	C	D	E
petroleum ether-ethyl acetate-methanol-water 1:1:1:1.1	0.24	0.32	0.22	0.19	0.33
ethyl acetate-*n*-butanol-0.5mM HCl water solution 4:1:5	0.07	0.12	0.16	0.11	0.29
chloroform-methanol-0.5mM HCl water solution 4:1.5:2	1.27	4.75	5.50	0.40	0.57

348



	R 1	R 2	R 3	R 4
stepharanine	H	CH_3	CH_3	H
columbamine	CH_3	CH_3	CH_3	H
jatrorrhizine	CH_3	CH_3	CH_3	H
palmatine	CH_3	CH_3	CH_3	CH_3
berberine	CH_3	CH_3	— CH_2 —	

Fig. 1 Chemical structures of compounds separated from Caulis Mahoniae.

59x61mm (300 x 300 DPI)

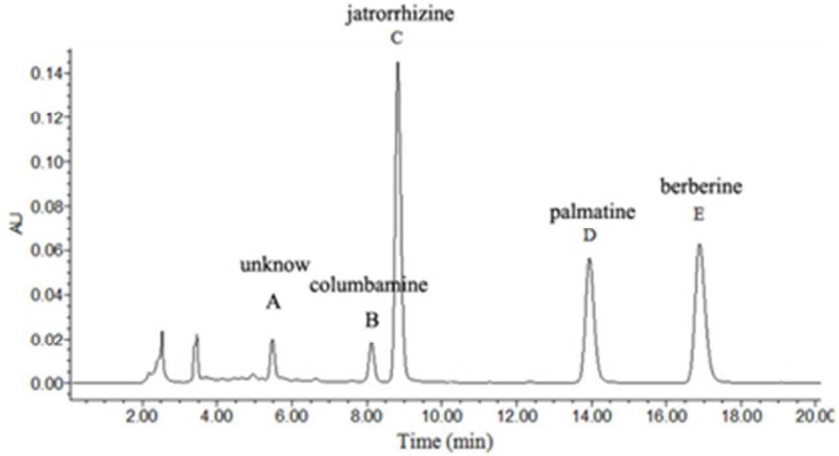


Fig. 2 The HPLC chromatogram of crude alkaloids from Caulis Mahoniae. Experimental conditions: column, Waters Symmetry® C18 column (4.6×250 mm, i.d., 5μm); mobile phase, acetonitrile-1% TEA (adjust the pH value to 3 with phosphoric acid) solution (25:75, v/v); detection, 265 nm; flow-rate, 1.0 mL/min.

36x19mm (300 x 300 DPI)

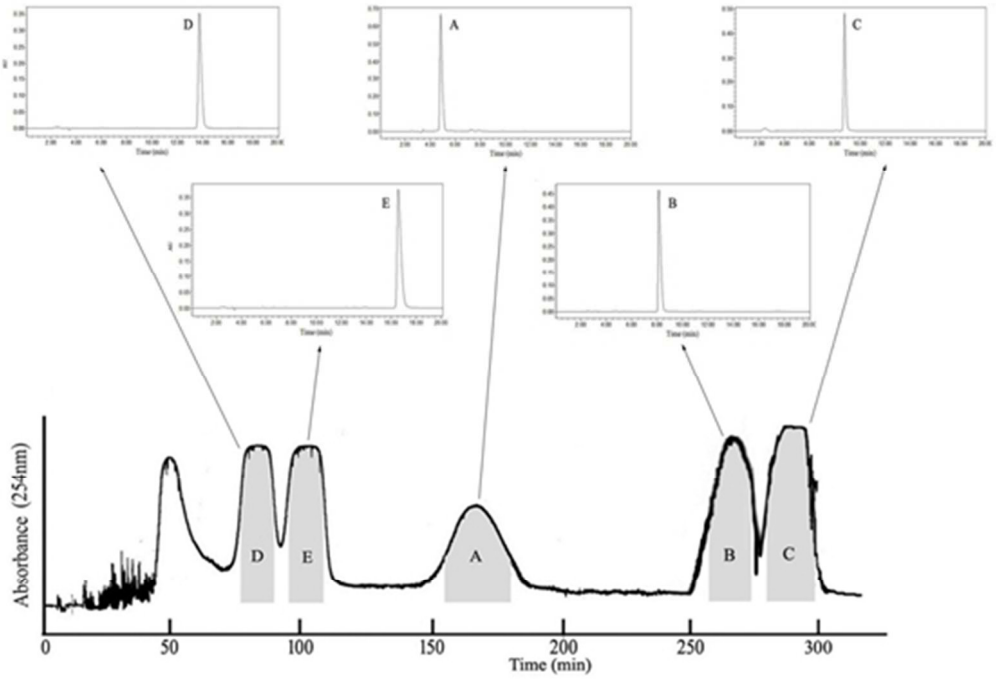


Fig. 3 Conventional HSCCC separation and HPLC analysis to the HSCCC peak fractions. Conditions: stationary phase, the upper phase of chloroform-methanol-0.5mM HCl water solution (4:1.5:2, v/v); 0-200 min, flow-rate 2.0 mL/min, 200-240 min, flow-rate 10.0 mL/min; sample size, 250 mg; detection, 254 nm; stationary phase retention, 66.7%; revolution speed, 800 rpm. HPLC conditions are as in Fig. 2.

46x33mm (300 x 300 DPI)

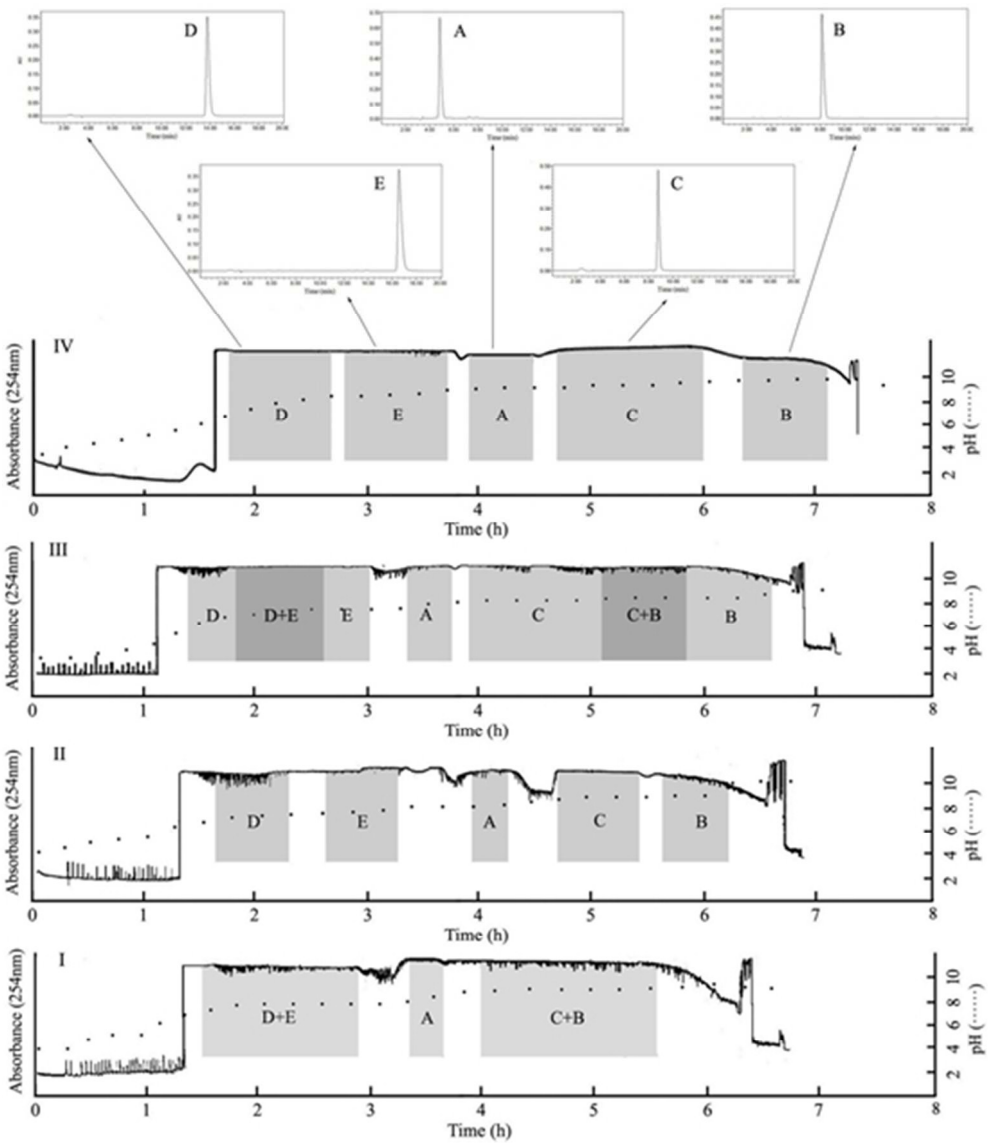
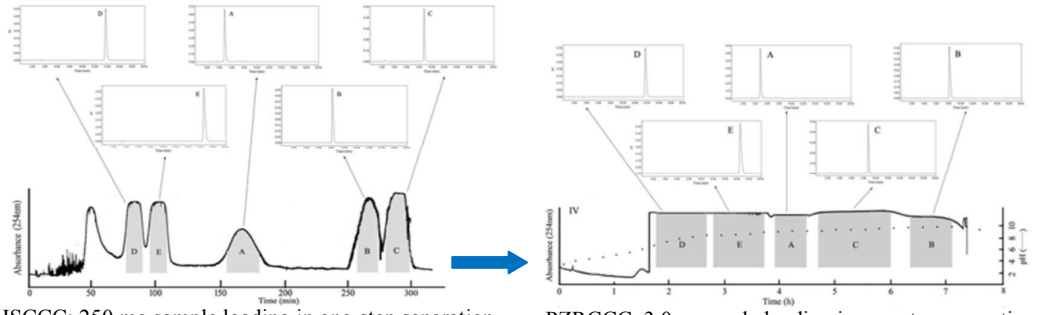


Fig. 4 PZRCCC separation and HPLC analysis to the HSCCC peak fractions. Conditions: stationary phase, the upper phase of chloroform-methanol-water (4:3:3, v/v); detection, 254 nm; flow-rate, 2.0 mL/min; revolution speed, 800 rpm. I: 30 mM HCl in upper stationary phase and 10 mM TEA in lower phase, sample loading 1.0 g, stationary phase retention 40%; II: 60 mM HCl in upper stationary phase and 10 mM TEA in lower phase, sample loading 1.0 g, stationary phase retention 41.7%; III: 60 mM HCl in upper stationary phase and 10 mM TEA in lower phase, sample loading 3.0 g, stationary phase retention 36.9%; IV: 60 mM HCl in upper phase and 7.5 mM TEA in lower phase, sample loading 3.0 g, retention of stationary phase 36.4%.

47x53mm (300 x 300 DPI)

Graphical Abstracts



HSCCC: 250 mg sample loading in one-step separation

PZRCCC: 3.0 g sample loading in one-step separation

Compared with the conventional HSCCC, PZRCCC is an efficient and rapid method for separation alkaloids of quaternary ammonium because of the high concentration of fractions and large sample loading capacity