Chunxia Zhao
Chaohong He

Department of Chemical Engineering, Zhejiang University, Hangzhou, China

Original Paper

Preparative isolation and purification of atractylon and atractylenolide III from the Chinese medicinal plant *Atractylodes macrocephala* by high-speed counter-current chromatography

The medicinal plant *Atractylodes macrocephala* (Baizhu in Chinese) has been widely used in traditional Chinese medicine for energy and stomach complaints, treatment of dyspepsia and anorexia, anti-inflammation, anticancer and for increasing assimilation. A high-speed counter-current chromatography (HSCCC) method was developed for the preparative separation and purification of two main bioactive components, namely, atractylon and atractylenolide III from *A. macrocephala* by using light petroleum (60–90°C)–ethyl acetate–ethanol–water (4:1:4:1 v/v) as the two-phase solvent system in dual-mode elution. Compared with the separation using the normal-mode elution, the dual-mode HSCCC can be achieved with shorter elution time. Atractylenolide III (32.1 mg) at 99.0% purity and 319.6 mg atractylon at 97.8% purity could be obtained from 1000 mg crude sample in a single run. The recoveries of atractylenolide III and atractylon were 95.4 and 92.6%, respectively.

Keywords: Atractylon / Atractylenolide III / *Atractylodes macrocephala* / High-speed counter-current chromatography (HSCCC)

Received: November 30, 2005; revised: March 24, 2006; accepted: March 24, 2006

DOI 10.1002/jssc.200500464

1 Introduction

The rhizome of *Atractylodes macrocephala* (Baizhu in Chinese), as one of the most popular traditional Chinese medicinal herbs, has been widely used for a long time. It is reported as a nutrient for energy and stomach complaints and for treatment of dyspepsia and anorexia in the Pharmacopoeia of People's Republic of China [1]. Atractylon, atractylenolide I, II, III and some other sequensenes are the major bioactive components of *A. macrocephala* [2, 3]. Many studies showed that these compounds had the effects of anti-inflammation, anticancer, and increasing of assimilation [4, 5].

Usually, atractylon is the main bioactive component of *A. macrocephala*. The contents of atractylenolide I, II and III are small, and their contents vary with the production area and cropping season [6]. *A. macrocephala* used in this work is from Xinchang county in Zhejiang Province, which is one of the main producing areas for *A. macrocephala* in China. In this *A. macrocephala*, atractylenolide I and II are too small and can be neglected. So the main bioactive components are atractylon and atractylenolide III. The chemical structures of atractylon and atractylenolide III are shown in Fig. 1.

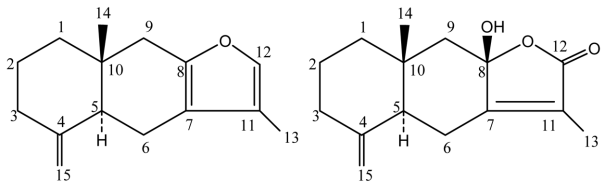

Figure 1. Chemical structures of atractylon and atractylenolide III.

Atractylon is very unstable, and also there are many isomeric compounds existing together in *A. macrocephala* [7, 8]. Thus, the separation of atractylon and atractylenolide III from *A. macrocephala* is quite difficult. The conventional separation method of atractylon and atractylenolide III is the silica gel column chromatography under nitrogen protection, which is time-consuming and has the peril of loss of compounds [9]. Therefore it is of great

Correspondence: Professor Chaohong He, Department of Chemical Engineering, Zhejiang University, Hangzhou 310027, China.
E-mail: chhezju@zju.edu.cn.
Fax: +86-571-87951742.

Abbreviation: HSCCC, high-speed counter-current chromatography

interest to develop a new method to separate atractylon and atractylenolide III from *A. macrocephala*.

The high-speed counter-current chromatography (HSCCC) is a very effective tool for the preparative separation and purification of natural products and Chinese traditional herbs [10–12]. Compared with the traditional liquid–solid column chromatography, HSCCC eliminates irreversible adsorption loss of samples and yields higher recovery and efficiency. So it is widely used in the separation of active components from traditional Chinese medicinal herbs and natural products [13–15].

The present paper deals with the one-step isolation and purification of atractylon and atractylenolide III from *A. macrocephala* by HSCCC.

2 Experimental

2.1 Apparatus

The preparative HSCCC was carried out with a Model TBE-1000A HSCCC (Tauto Biotech, Shanghai, China) equipped with a 1000 mL coil column made of PTFE tubing (1.8 mm) and a 100 mL sample loop. The β-value of the preparative column varied from 0.42 at the internal layer to 0.63 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 the central axis of the centrifuge). The rotation speed of the apparatus can be regulated with a speed controller in the range between 0 and 600 rpm. The HSCCC system is equipped with a Model SD-9002 constant-flow pump, a HX-2050 water bath, a Model 8823B UV monitor operating at 254 nm and a Model N2010 chromatography workstation.

2.2 Reagents and materials

All organic solvents used for the preparation of the crude sample and for the HSCCC separation were of analytical grade (Hangzhou Datong Reagent factory, Hangzhou, China), and the water used was distilled water.

The dried roots of *A. macrocephala* were from Xinchang county in Zhejiang Province, China.

2.3 Preparation of crude sample from *A. macrocephala*

The dried roots of *A. macrocephala* were powdered (about 40 mesh) and extracted with ethyl acetate three times. The extraction time was 2, 2 and 1 h, respectively. The ethyl acetate solutions were combined and evaporated by rotary vaporization at 45°C under reduced pressure. Subsequently, the extract was dissolved in ethanol and was frozen at −4°C for 24 h. The deposit was separated and removed. Then the residue was dissolved with a small amount of ethyl acetate, and eight times the amount of light petroleum (60–90°C) was added to precipitate the residue. After removing the deposit, the crude sample was obtained. It was stored in a refrigerator for the subsequent HSCCC separation.

2.4 Selection of the two-phase solvent systems

Light petroleum (60–90°C)–ethyl acetate–ethanol–water was used as the two-phase solvent system. The composition of the two-phase solvent system was selected according to the partition coefficient (K). The K-values were determined by GC as follows: A suitable amount of crude sample was added to a test tube, to which 2 mL of each phase of the two-phase solvent system was added. After a thorough equilibration, the upper phase and lower phase were separated and analysed by GC. The partition coefficient (K) of the target component in the sample was expressed as the peak area of the solute in the upper phase divided by that in the lower phase.

2.5 Preparation of two-phase solvent system and sample solutions

Light petroleum (60–90°C)–ethyl acetate–ethanol–water (4:1:4:1 v/v) was prepared by adding all the solvents to a separation funnel according to the volume ratios and thoroughly equilibrated by shaking repeatedly. Then the upper phase and the lower phase were separated 30 min prior to use.

The sample solution was prepared by dissolving the crude sample in the solvent mixture of the lower phase and upper phase (1:1 v/v) of the solvent system, because the sample was not easily dissolved in either phase alone.

2.6 HSCCC separation procedure

In each separation, the coiled column was first entirely filled with the upper phase (stationary phase), and then the apparatus was rotated at 450 rpm, while the lower phase (mobile phase) was pumped into the column at a flow rate of 5.0 mL/min. After the mobile phase front emerged and the hydrodynamic equilibrium was established in the column, the retention of the stationary phase can be calculated; then the sample solution of 50 mL, containing the crude extract of 1000 mg was injected through the injection valve. In the dual-mode HSCCC, after about 100 min separation in the head–tail mode, the elution mode was reversed to tail–head, by switching the solvent-changing valve from the head–tail elution mode to the tail–head elution mode, and the upper phase was pumped into the column to obtain tail–head fractions. The effluent of the column was continuously monitored with a UV detector at 254 nm. Fractions were collected every 2 min. Each peak fraction was manually collected according to the chromatogram and evaporated under reduced pressure. The residues were

dissolved in ethanol for subsequent GC analysis. After the separation, the solvents in the column were pushed out.

2.7 GC analysis and identification of fractionated compounds

The crude sample and each HSCCC peak fraction were analysed by GC, and the partition coefficient was also determined by GC. GC analyses were performed with a GC-1690 gas chromatography column (Hangzhou Ke-Xiao) equipped with a FID and SE-54 capillary column (30 m × 0.25 mm; coating thickness 0.50 μm). Oven temperature was programmed to 80°C for 2 min, and then increased to 260°C at a rate of 5°C/min. Injector and detector temperatures were 260 and 240°C, respectively. The carrier gas, nitrogen, was adjusted to 1 mL/min. The crude sample and each peak fraction were diluted 20 times and 0.5 μL of the diluted solution was injected into the GC. The percentages of compounds were calculated by the area normalization method.

The components separated from HSCCC were identified with GC/EIMS, ^{1}H and ^{13}C NMR. GC/EIMS analyses were performed with a Trace 2000 GC equipped with a HP-5 capillary column (30 m × 0.25 mm; coating thickness 0.25 μm) and a Trace 2000 IT mass detector. The injector and transfer line temperatures were 260 and 250°C, respectively. Oven temperature was programmed to 50°C for 2 min, and then increased to 240°C at a rate of 15°C/min. The carrier gas, nitrogen, was adjusted to 1 mL/min. The peak fractions of the target compounds were diluted 10 times and 0.2 μL of the diluted solution was injected. The ionization energy was 70 eV with a scan time of 1 s and a mass range of 30–500 amu. The percentages of compounds were calculated by the area normalization method without considering response factors. ^{1}H and ^{13}C spectra were recorded in $CDCl_3$ on a Bruker AMX 400 spectrometer (Karlsruhe, Germany) using TMS as the internal standard.

3 Results and discussion

3.1 GC analysis of the crude sample

The crude sample obtained from *A. macrocephala* was analysed by GC, and the chromatogram is shown in Fig. 2. The contents of atractylon and atractylenolide III in the crude sample are 34.7 and 3.36%, respectively.

3.2 Optimization of HSCCC conditions

3.2.1 Optimization of the two-phase solvent system

A successful separation by HSCCC depends upon the selection of a suitable two-phase solvent system. A solvent system composed of light petroleum (60–90°C)–ethyl acetate–ethanol–water at different volume ratios was investigated. This two-phase solvent system could be used to isolate and separate a broad range of hydrophobicity by modifying the volume ratio of the four components. Because the polarities of the target compounds in the crude sample were unknown in the beginning, the search started with the two-phase solvent system composed of light petroleum (60–90°C)–ethyl acetate–ethanol–water at a volume ratio of 5:5:5:5 which has a moderate degree of polarity. But the atractylon is mostly distributed in the upper organic phase ($K > 10$) for the solvent system at a volume ratio of 5:5:5:5, which tends to result in a too long elution time and a broad peak. So the volume ratio was changed by reducing the volumes of ethyl acetate and water. The two-phase solvent systems tested in the present study are shown in Table 1(a). The search is directed downwards along the arrow toward more hydrophobic. Light petroleum (60–90°C)–ethyl acetate–ethanol–water at the volume ratio of 6:4:5:5, 6:4:6:4 and 7:3:6:4 were not suitable because the mobile phases also had strong polarity, and atractylon could not be eluted in an acceptable time. The partition coefficients of the target compounds in the other four solvent systems were measured and are shown in Table 1(b). Furthermore, the solvent systems listed in Table 1(b) were tested by HSCCC separation. The results indicated that when light petroleum (60–90°C)–ethyl acetate–ethanol–water (7:3:7:3 or 7:3:8:2 v/v) was used as the solvent system, the elution time was more than 18 or 11 h, respectively. When light petroleum (60–90°C)–ethyl acetate–ethanol–water (9:1:8:2 v/v) was used, atractylenolide III was eluted closer to the solvent front with lower resolution and could not get satisfactory separation. When light petroleum (60–90°C)–ethyl acetate–ethanol–water (4:1:4:1 v/v) was used as the solvent system, good separation results could be obtained and the separation time was acceptable. So the two-phase sol-

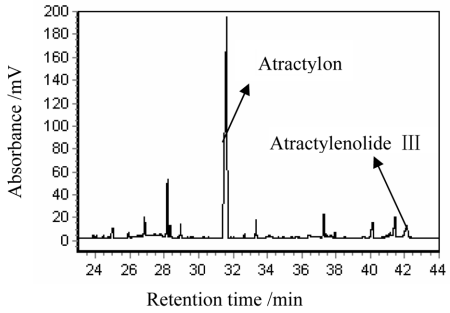

Figure 2. GC chromatogram of the crude sample. Experimental conditions: an SE-54 capillary column (30 m × 0.25 mm; coating thickness 0.50 μm) with a FID; injector temperature 260°C; detector temperature 240°C; oven temperature programmed from 80 (hold 2 min) to 260°C at 5°C/min, then held at 30 min; carrier gas nitrogen, at 1 mL/min; injection of 0.5 μL of the diluted crude sample.

Table 1. Search for a suitable two-phase solvent system

(a) Two-phase solvent systems tested

Solvent system		
Start here →	Light petroleum (60–90°C)–ethyl acetate–ethanol–water (5:5:5:5 v/v)	↓ Hydrophobic
	Light petroleum (60–90°C)–ethyl acetate–ethanol–water (6:4:5:5 v/v)	
	Light petroleum (60–90°C)–ethyl acetate–ethanol–water (6:4:6:4 v/v)	
	Light petroleum (60–90°C)–ethyl acetate–ethanol–water (7:3:6:4 v/v)	
	Light petroleum (60–90°C)–ethyl acetate–ethanol–water (7:3:7:3 v/v)	
	Light petroleum (60–90°C)–ethyl acetate–ethanol–water (7:3:8:2 v/v)	
	Light petroleum (60–90°C)–ethyl acetate–ethanol–water (4:1:4:1 v/v)	

(b) The *K*-values of atractylenolide III and atractylon in light petroleum (60–90°C)–ethyl acetate–ethanol–water

Solvent system v/v	*K*[a)]	
	Atractylenolide III	Atractylon
7:3:7:3	0.42	7.07
7:3:8:2	0.33	4.21
4:1:4:1	0.23	2.11
9:1:8:2	0.17	1.96

[a)] Partition coefficient *K* is expressed as the solute concentration in the upper phase divided by that in the lower phase.

vent system of light petroleum (60–90°C)–ethyl acetate–ethanol–water at a volume ratio of 4:1:4:1 was adopted.

3.2.2 The effect of flow rate and rotation speed on the retention of the stationary phase (S_f)

Retention of the stationary phase (S_f) is one of the most important parameters in HSCCC. It is used for the derivation of column efficiency, peak resolution and solute retention. Successful separation in HSCCC largely depends on the amount of stationary phase retained in the column, that is, the retention of the stationary phase. In general, the higher the retention of the stationary phase, the better the peak resolution.

In this work, the effects of the flow rate and rotation speed on the retention of the stationary phase were investigated by using light petroleum (60–90°C)–ethyl acetate–ethanol–water (4:1:4:1 v/v) as the solvent system. The flow rates were changed from 4 to 12 mL/min under a fixed rotation speed of 450 rpm. The results in Fig. 3 show a good linear relationship between the retention of the stationary phase (S_f) in the HSCCC column and the square root of the flow rate ($F^{0.5}$). Du *et al.* [16], Sutherland [17], and Booth and Sutherland [18] have also shown the linear relationship between S_f and $F^{0.5}$ in the countercurrent chromatography. Considering that a higher flow rate results in a lower S_f and a lower flow rate results in a longer elution time, a flow rate of 5 mL/min was adopted, where S_f and the elution time are both reasonable.

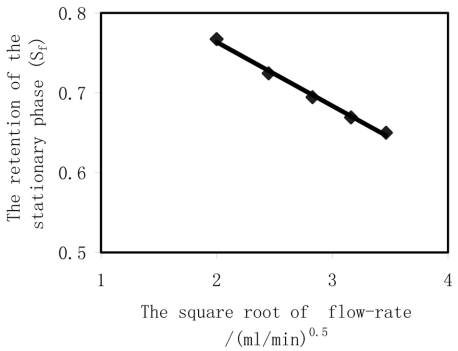

Figure 3. The retention of the stationary phase against the square root of flow rate. HSCCC conditions: solvent system: light petroleum (60–90°C)–ethyl acetate–ethanol–water (4:1:4:1 v/v); flow rate: 4–12 mL/min; rotation speed: 450 rpm; temperature: 25°C.

The effects of changes in rotation speed from 200 to 500 rpm on the retention of the stationary phase were also investigated. The results are summarized in Fig. 4. It is shown in Fig. 4 that with the increase in the rotation speed, S_f increases sharply before 350 rpm, and remains almost constant after 350 rpm. So the rotation speed of 450 rpm was adopted.

3.2.3 The effect of sample loading

Due to the complexity of the crude sample, there are many elution peaks. Usually, introduction of a larger sample loading into the column will reduce peak resolution of the analytes especially with those having small *K* values. The chromatograms of the sample loading of

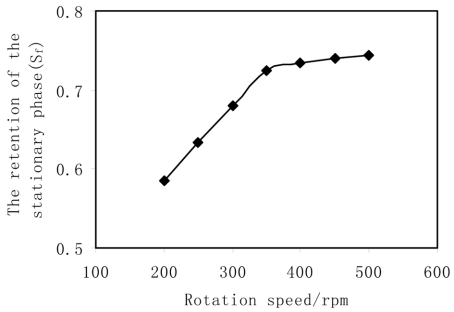

Figure 4. The retention of the stationary phase against the rotation speed. HSCCC conditions: solvent system: light petroleum (60–90°C)–ethyl acetate–ethanol–water (4:1:4:1 v/v); mobile phase flow rate: 5 mL/min; rotation speed: 200–500 rpm; temperature: 25°C.

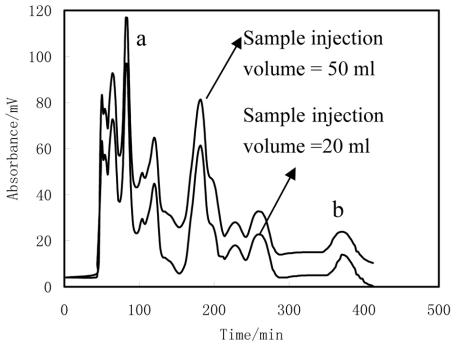

Figure 5. The effect of increased sample loading. (a) Atractylenolide III (retention time: 84 min); (b) atractylon (retention time: 374 min); HSCCC conditions: solvent system: light petroleum (60–90°C)–ethyl acetate–ethanol–water (4:1:4:1 v/v); flow rate: 5 mL/min; rotation speed: 450 rpm; temperature: 25°C.

20 mL containing the crude sample of 400 mg and of 50 mL containing the crude sample of 1000 mg are shown in Fig. 5. Peaks a and b are due to atractylenolide III and atractylon, respectively, and their retention times are 84 and 374 min, respectively. The total elution time was about 7 h. It was found that, with the increase in the sample loading from 20 to 50 mL, the corresponding peaks are similar, and the peak resolution decreases slightly, leading to the decrease of recovery. The recovery of atrctylon decreases from 94.5 to 92.3%, and that of atractylenolide III from 95.7 to 94.6%. Meanwhile, the retention of the stationary phase S_f decreases from 77.0 to 74.5%.

3.2.4 The effect of elution mode

The crude sample used in the study contains solutes with a broad range of hydrophobicity. When light petroleum (60–90°C)–ethyl acetate–ethanol–water (4:1:4:1 v/v) was used, in the normal-mode elution a long time (about 7 h as shown in Fig. 5) was needed for the separation of atractylon which has a higher affinity to the stationary

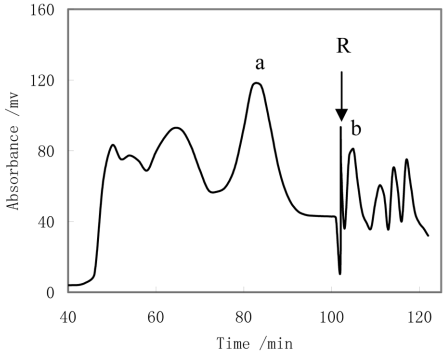

Figure 6. Chromatogram of the crude sample by dual-mode HSCCC. (a) Atractylenolide III (retention time: 84 min); (b) atractylon (retention time: 105 min); HSCCC conditions: solvent system: light petroleum (60–90°C)–ethyl acetate–ethanol–water (4:1:4:1 v/v); flow rate: 5 mL/min; rotation speed: 450 rpm; temperature: 25°C; sample loading: 1000 mg. Phases are reversed at 102 min. Mobile phase: 0–102 min, the lower phase of the solvent system; 102–125 min, upper phase of the solvent system.

phase (K >2). For reducing the elution time, a dual-mode HSCCC in which the initial mobile phase becomes the stationary phase and vice versa [19, 20] was adopted. Compared to the normal-mode HSCCC, the dual-mode HSCCC operation allows faster elution of the solutes with less solvent and elution time, and the peak resolution can also be improved [21–23]. However, there are also some limitations such as disturbing the system equilibrium in the column, leading to leaks in the stationary phase, and also having a strange peak shape due to the reorganization of the phases. But the dual-mode HSCCC offers unique versatility for quick separation of the sample containing solutes with a broad range of hydrophobicity and allows faster elution of the solute with a larger K value.

The dual-mode HSCCC with a two-phase solvent system composed of light petroleum (60–90°C)–ethyl acetate–ethanol–water (4:1:4:1 v/v) was also investigated to separate atractylon and atractylenolide III from *A. macrocephala* (in Fig. 6). The separation started with the organic phase as the stationary phase, and the aqueous mobile phase allowed elution of atractylenolide III (peak a in Fig. 6). After about 100 min separation in the head–tail mode, the elution mode was reversed to tail–head, by switching the solvent-changing valve from the head–tail elution mode to the tail–head elution mode, and the upper phase was pumped into the column. The phase reversal permitted elution of atractylon (peak b in Fig. 6). It can be found in Fig. 6 that the chromatogram before the phase reversal is the same as in Fig. 5, and the dual-mode elution has no other effect on the separation of atractylenolide III. However, the peak b eluted just after the phases was reversed and had a strange peak shape

due to the reorganization of the phases. In the dual-mode HSCCC, 32.1 mg atractylenolide III at 99.0% purity and 319.6 mg atractylon at 97.8% purity could be obtained from 1000 mg of crude sample. The recoveries of atractylenolide III and atractylon were 95.4 and 92.6%, respectively. The recovery and purity in the dual-mode elution had no obvious change compared with those using the normal-mode HSCCC.

The separation in the normal-mode HSCCC would take about 7 h, while in the dual-mode HSCCC it only takes about 2 h. Because the HSCCC apparatus used in the experiments is a large scale preparative model with a 1000 mL column volume, the separation usually needs more than 6 h [24–26]. Even if the K value equals 1, the elution time would be 200 min at a flow rate of 5 mL/min from the basic equations of chromatography $\left(t_R = \frac{V_m}{F} + K\frac{V_S}{F}\right)$, where t_R is the retention time of a solute, V_m is the mobile phase volume, V_S is the stationary phase volume, and F is the flow rate of the mobile phase). So it is very difficult to reduce the elution time any further considering the peak resolution. The use of the dual-mode HSCCC for the preparative purification of atractylon and atractylenolide III is highlighted by comparison with the results of the normal-mode HSCCC; and the elution time can be greatly reduced.

3.3 Structural identification

The chemical structures of the peaks a and b in Figs. 5, 6 were identified according to their GC-MS, ^{1}H and ^{13}C NMR data.

Peak a: EI-MS *m/z*: 248 (M^+), 230, 220, 215, 191, 169, 147 (100), 121, 109. ^{1}H NMR (400 MHz, $CDCl_3$, TMS): 1.03 (3H, s, 14-H), 1.24 (1H, td, J = 12.6 Hz, 1a-H), 1.54 (1H, d, J = 14 Hz, 9-H), 1.60–1.70 (3H, m, 3-H), 1.87 (1H, d, J = 13 Hz, 5-H), 1.97 (1H, t, J = 10 Hz, 3a-H), 2.27 (1H, d, J = 14 Hz, 9-H), 2.37 (2H, d, J = 13 Hz, 3b-H), 2.43 (1H, J = 13 Hz, 6-H), 2.62 (1H, dd, J = 13, 3Hz, 6-H), 4.60 (1H, d, J = 1 Hz, 15-H_a), 4.86 (1H, d, J = 1 Hz, 15-H_b).

Peak b: EI-MS *m/z*: (216, M^+), 173, 145, 108 (100), 79, 41. ^{1}H NMR (400 MHz, $CDCl_3$, TMS): 0.75 (3H, s,14-H), 1.3–1.7 (5H, m), 1.94 (3H, s, 13-H), 2.0–2.4 (6H, m), 4.70 (1H, s, 15-H_a), 4.85 (1H, s, 15H_b), 7.04 (1H, s, 12-H). ^{13}C NMR (400 MHz, $CDCl_3$, TMS): 8.1 (C-13), 17.5 (C-14), 20.9 (C-2), 23.3 (C-6), 36.6 (C-10), 37.3 (C-3), 39.2 (C-1), 41.9 (C-9), 45.7 (C-5), 107.3 (C-15), 116.1 (C-7), 119.5 (C-11), 136.9 (C-12), 149.3 (C-4), 149.8 (C-8).

Compared with the data given in [27], peaks a and b were identified as atractylenolide III and atractylon.

4 Concluding remarks

An efficient HSCCC method for the separation and purification of atractylon and atractylenolide III from *A. macrocephala* was developed by using light petroleum (60–90°C)–ethyl acetate–ethanol–water (4:1:4:1 v/v) as the two-phase solvent system in dual-mode elution. Atractylenolide III (32.1 mg) at 99.0% purity and 319.6 mg atractylon at 97.8% purity could be obtained from 1000 mg crude sample in a single run. The recoveries of atractylenolide III and atractylon were 95.4 and 92.6%, respectively. Compared with the normal-mode elution, the dual-mode elution can greatly reduce the elution time, which is an effective method for quick separation of the sample containing solutes with a broad range of hydrophobicity.

Financial support from the National Natural Science Foundation of the People's Republic of China (project no: 20576113) is gratefully acknowledged.

5 References

[1] China Pharmacopoeia Committee, *Pharmacopoeia of the People's Republic of China*, the first division of 2000 edition, China Chemical Industry Press, Beijing 1999, p. 84.

[2] Endo, K., Toguchi, T., Toguchi, F., Hikino, H., *et al.*, *Chem. Pharm. Bull.* 1979, *12*, 755–760.

[3] Tang, D. F., Hao, Y. H., Liu, Z. Y., Miao, S. L., *et al.*, *Chin. Pharm. Bull.* 1984, *19*, 43.

[4] Wang, C. C., Chen, L. G., Yang, L. L., *Planta. Medica.* 2002, *68*, 204–208.

[5] Wen, H. M., Zhang, A. H., Ge, J. H., Wu, H., *Chin. J. Pharm. Anal.* 2001, *21*, 170–172.

[6] Zhao, Z. F., Shen, J. W., Gong, F., Liang, Y. Z., Qiu, X. M., *Chin. Tradit. Herb. Drugs.* 2004, *35*, 5–8.

[7] Hikino, H., Hikino, Y., Yosioka, I., *Chem. Pharm. Bull.* 1964, *12*, 755–758.

[8] Wen, H. M., Li, W., *Chin. Materia. Medica*. 1997, *22*, 662–663.

[9] Chen, J. M., Yu, M. Q., Shen, Y. Z., Liu, Z. Y., Huang, Z. J., *Acta Bot. Sin.* 1991, *33*, 164–167.

[10] Liu, R. M., Lei, F., Sun, A. L., Kong, L. Y., *J. Chromatogr. A* 2004, *1057*, 89–94.

[11] Wu, S. J., Sun, A. L., Liu, R. M., *J. Chromatogr. A* 2005, *1066*, 243–247.

[12] Liu, R. M., Sun, Q. H., Sun, A. L., Cui, J. C., *J. Chromatogr. A* 2005, *1072*, 195–199.

[13] Li, H. B., Chen, F., *J. Chromatogr. A* 2005, *1074*, 107–110.

[14] Liu, R. M., Chu, X., Sun, A. L., Kong, L. Y., *J. Chromatogr. A* 2005, *1074*, 139–144.

[15] Ma, C. J., Li, G. S., Zhang, D. L., Liu, K., Fan, X., *J. Chromatogr. A* 2005, *1078*, 188–192.

[16] Du, Q., Wu, C., Quian, P., Ito, Y., *J. Chromatogr. A* 1999, *835*, 231–235.

[17] Sutherland, I. A., *J. Chromatogr. A* 2000, *886*, 283–287.

[18] Booth, A. J., Sutherland, I. A., Lye, G. J., *Biotechnol. Bioeng.* 2003, *81*, 640–649.

[19] Slacanin, I., Marston, A., Hostettmann, K., *J. Chromatogr.* 1989, *482*, 234 – 237.

[20] Marson, A., Borel, C., Hostettmann, K., *J. Chromatogr.* 1988, *450*, 91 – 95.

[21] Agnely, M., Thiebaut, D., *J. Chromatogr. A* 1997, *790*, 17 – 30.

[22] Gluck, S. J., Martin, E. J., *J. Liq. Chromatogr*. 1990, *13*, 3559 – 3564.

[23] Menges, R. A., Bertrand, G. L., Armstrong, D. W., *J. Liq. Chromatogr*. 1990, *13*, 3061 – 3065.

[24] Fan, J. P., He, C. H., *J. Liq. Chromatogr. Rel. Technol.* 2006, *29*, 815 – 826.

[25] Aman, R., Carle, R., Conrad, J., Beifuss, U., Schieber, A., *J. Chromatogr. A* 2005, *1074*, 99 – 105.

[26] Peng, J. Y., Fan, G. R., Hong, Z. Y., Chai, Y. F., Wu, Y. T., *J. Chromatogr. A* 2005, *1074*, 111 – 115.

[27] Huang, B. S., Sun, J. S., Chen, Z. L., *Acta Bot. Sin*. 1992, *31*, 614 – 616.

