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Journal of Chromatography A, 1139 (2007) 254–262
JOURNAL OF CHROMATOGRAPHY A
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Preparative isolation and purification of chemical constituents from the root of *Adenophora tetraphlla* by high-speed counter-current chromatography with evaporative light scattering detection

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Received 29 July 2006; received in revised form 6 November 2006; accepted 10 November 2006
Available online 5 December 2006

Abstract

Preparative high-speed counter-current chromatography (HSCCC), as a continuous liquid–liquid partition chromatography with no solid support matrix, combined with evaporative light scattering detection (ELSD) was employed for systematic separation and purification of non-chromophoric chemical components from Chinese medicinal herb *Adenophora tetraphlla* (*Thunb.*), *Fisch*. Nine compounds, including α-spinasterol, β-sitosterol, nonacosan-10-ol, 24-methylene cycloartanol, lupenone, 3-*O*-palmitoyl-β-sitosterol, 3-*O*-β-D-glucose-β-sitosterol, eicosanoic acid and an unknown compound, were obtained. The compounds were all above 95% determined by high-performance liquid chromatography (HPLC)–ELSD, and their structures were identified by ^{1}H NMR and chemical ionization mass spectroscopy (CI-MS). The results demonstrate that HSCCC coupled with ELSD is a feasible and efficient technique for systematic isolation of non-chromophoric components from traditional medicinal herbs.

Keywords: High-speed counter-current chromatography; Evaporative light scattering detection; *Adenophora tetraphlla* (*Thunb.*), *Fisch*; Systematic isolation and purification

1. Introduction

High-speed counter-current chromatography (HSCCC) is a kind of support-free all-liquid partition chromatography that was first invented by Ito [1]. As no solid stationary phase is used, it eliminates irreversible adsorption of sample onto the solid support used in the conventional chromatographic column. What is more, it has a large amount of sample injection; multiform relative pure substances can be obtained at one time in large amount. So it is especially suitable for separation and purification of active components from natural products. As an advanced separation technique, HSCCC has been widely used for separation of active components from traditional Chinese herbs and other natural products in recent years. Successful application of HSCCC has been reported for the purification of alkaloids [2–4], quinones [5–7], flavonoids [8,9], coumarins [10,11], other natural products [12–14] and so on. For these chromophoric constituents, UV detector has become the major detection instrument of HSCCC to monitor the column effluent [15]. But its application to HSCCC is limited by its inherent shortcomings. It cannot be used as the detector for separation of non-chromophoric components and makes the application of HSCCC restricted to some degree. So the study on the application of other kind of detector for HSCCC separation is of great interest.

It is well recognized that evaporative light scattering detection (ELSD) can outperform traditional detection methods when analyzing samples having no or end ultraviolet absorption by high-performance liquid chromatography (HPLC). Analytical HSCCC coupled with ELSD had been reported previously [16,17]. Preparative HSCCC combined with ELSD was also reported [18]. A split valve was used to connect the preparative HSCCC and ELSD system. But the split parameters and the ELSD condition were not given, and the purity of the obtained compounds was also determined with a UV detector at 204 nm. The two-phase solvent system used also contained $CHCl_3$ which is deleterious to humans and environment. In the present paper, diverse methods of preparative HSCCC coupled with ELSD

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doi:10.1016/j.chroma.2006.11.056

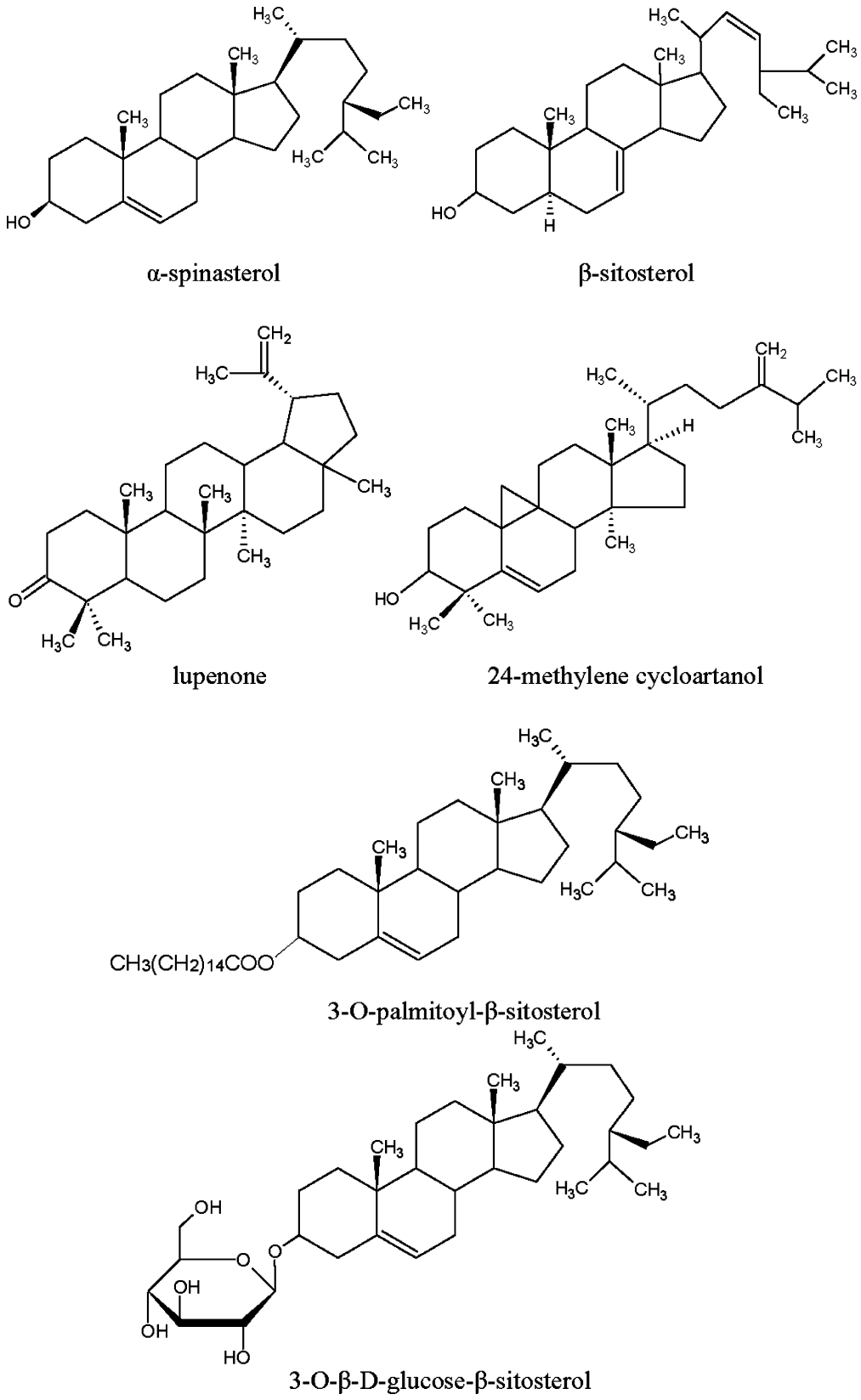

Fig. 1. Chemical structures of compounds from *A. tetraphlla* (*Thunb.*), *Fisch.*

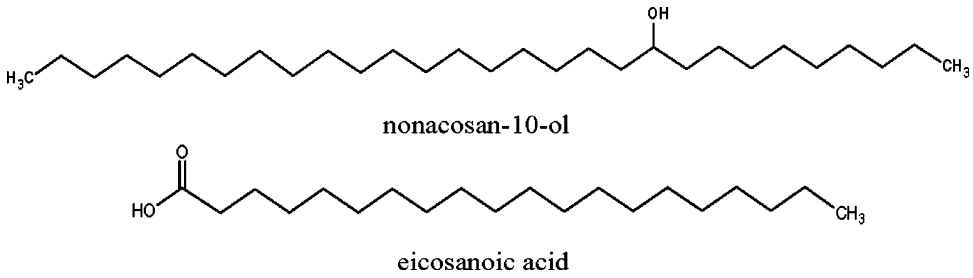

Fig. 1. (*Continued*).

were established for systematic separation and purification of chemical components from different fractions of Chinese medicinal herb *Adenophora tetraphlla* (*Thunb*.), *Fisch*, which is listed in China Pharmacopoeia as an antiinflammatory and antitussive drug used in the treatment of lung disease [19]. Nine compounds with non-chromophoric absorption were obtained. The chemical structures of the compounds are given in Fig. 1.

2. Experimental

2.1. Apparatus

The HSCCC instrument employed in the present study was a TBE-300 high-speed counter-current chromatography (Tauto Biotechnique, Shanghai, China) with three multilayer coil separation columns connected in series (I.D. of the tubing = 1.5 mm, total volume = 300 ml) and a 20 ml sample loop. The revolution radius was 5 cm, and the values β ($\beta = r/R$, where r is the rotation radius or the distance from the coil to the holder shaft, and R is the revolution radius or the distance between the holder axis and central axis of the centrifuge) of the multilayer coil varied from 0.5 at internal terminal to 0.8 at the external terminal. The revolution speed of the apparatus can be regulated with a speed controller in the range between 0 and 1000 rpm. The system was also equipped with one S-1007 constant flow pump (Shenyitong Tech & Exploitation, Beijing, China), an Alltech 2000ES ELSD (Alltech, USA). The connection of the system is shown in Fig. 2. A tee junction valve was used to connect the outlet of HSCCC column with the inlet of the ELSD system and pipeline of fraction collector. The splitflow of the effluent was adjusted by adjusting the valve, and half of the effluent from the outlet of the column was monitored by ELSD. The data were collected with the model N2000 chromatography workstation (Zhejiang University, Hangzhou, China). The HPLC equipment used was Agilent 1100 series system and Agilent HPLC workstation (Agilent, USA). Nuclear magnetic resonance (NMR) spectrometer used here was Bruker AM-500 MHz (Bruker, Switzerland). Chemical ionization mass spectroscopy (CI-MS) used was Shimadzu GC-MS 2010QP (Shimadzu, Japan).

2.2. Reagents

All solvents used for HSCCC were of analytical grade (Hanbon Sci & Tech, Jiangsu, China). Methanol and acetonitrile used for HPLC were of chromatographic grade (Hanbon Sci & Tech); D-101 macroporous resin (Chemical Plant of Nankai University, Tianjin, China) was used for sample purification and the water used was distilled water.

The dried roots of *A. tetraphlla* (*Thunb*.), *Fisch* were purchased from a local drug store and identified by Professor Mingjian Qing, Department of Medicinal Plant, China Pharmaceutical University.

2.3. Preparation of crude extract

Eight hundred grams dried roots of *A. tetraphlla* (*Thunb*.), *Fisch* was powdered and extracted by 8 l 90% ethanol, and the extract was evaporated to form a syrup. The syrup was then

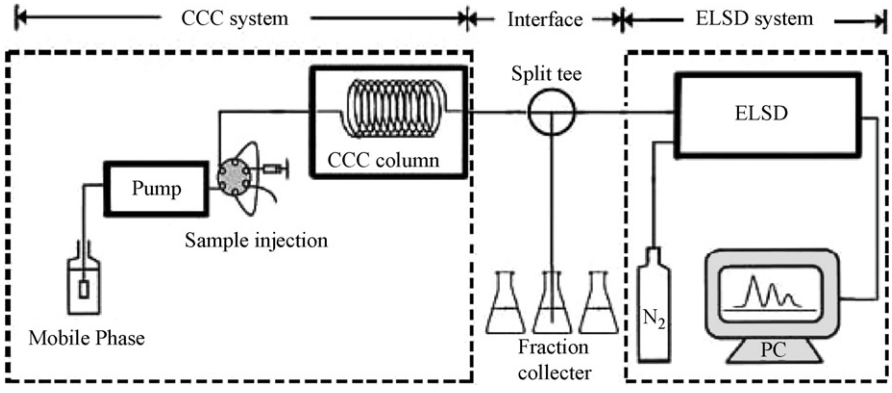

Fig. 2. Roadmap of extraction and separation. Solvent system 1: *n*-hexane–ethyl acetate–acetonnitrile 5:1:5 (v/v/v); solvent system 2: *n*-hexane–ethyl acetate–acetonnitrile 5:1:4 (v/v/v); solvent system 3: *n*-hexane–ethyl acetate–acetonnitrile 5:2:5 (v/v/v); solvent system 4: light petroleum (b.p. 60–90 °C)–ethyl acetate–ethanol–water 6:4:5:5 (v/v/v/v).

dissolved in 1.5 l water by sonication and partitioned with ether and *n*-butanol of equal volume three times successively. Both ether and *n*-butanol solution were vacuum evaporated at 65 °C. About 20 g residue of ether and 18 g residue of *n*-butanol were obtained, respectively. In order to enrich the target components and remove impurities, the residue of *n*-butanol was loaded on D-101 macroporous resin column (35 cm × 3.4 cm, the volume of column was 170 ml) and eluted with 1.7 l distilled water and 1.5 l 75% ethanol, respectively. The 75% ethanol effluent was collected and evaporated at 65 °C in vacuum and about 810 mg residues were obtained. All the residues were stored in a refrigerator (5 °C) for further use.

2.4. Preparation of two-phase solvent system and sample solution

In the present study, the two-phase solvent system composed of *n*-hexane–ethyl acetate–acetonnitrile 5:1:5 (v/v/v), 5:1:4 (v/v/v), 5:2:5 (v/v/v) and light petroleum (b.p. 60–90 °C)–ethyl acetate–ethanol–water 6:4:5:5 (v/v/v/v) were used for HSCCC separation. Each component of the solvent system was added to a separatory funnel and thoroughly equilibrated at room temperature for 12 h. The upper phase and lower phase were separated and degassed by sonication for 30 min shortly before use.

For HSCCC separation of ether extracts, the sample solution was prepared by dissolving 360 mg of ether extract in 3 ml of the upper phase and 3 ml of the lower phase of *n*-hexane–ethyl acetate–acetonnitrile 5:1:5 (v/v/v), and seriously filtering with micropore membrane filters ($\Phi = 0.45$ μm). The sample solution for HSCCC separation of refined *n*-butanol fraction was prepared by dissolving 400 mg of refined *n*-buthanol fraction in 5 ml of the lower phase of light petroleum (b.p. 60–90 °C)–ethyl acetate–ethanol–water 6:4:5:5 (v/v/v/v) and seriously filtering with micropore membrane filters ($\Phi = 0.45$ μm).

2.5. HSCCC separation

The HSCCC system was connected according to Fig. 2. In each separation process, the coiled column was first entirely

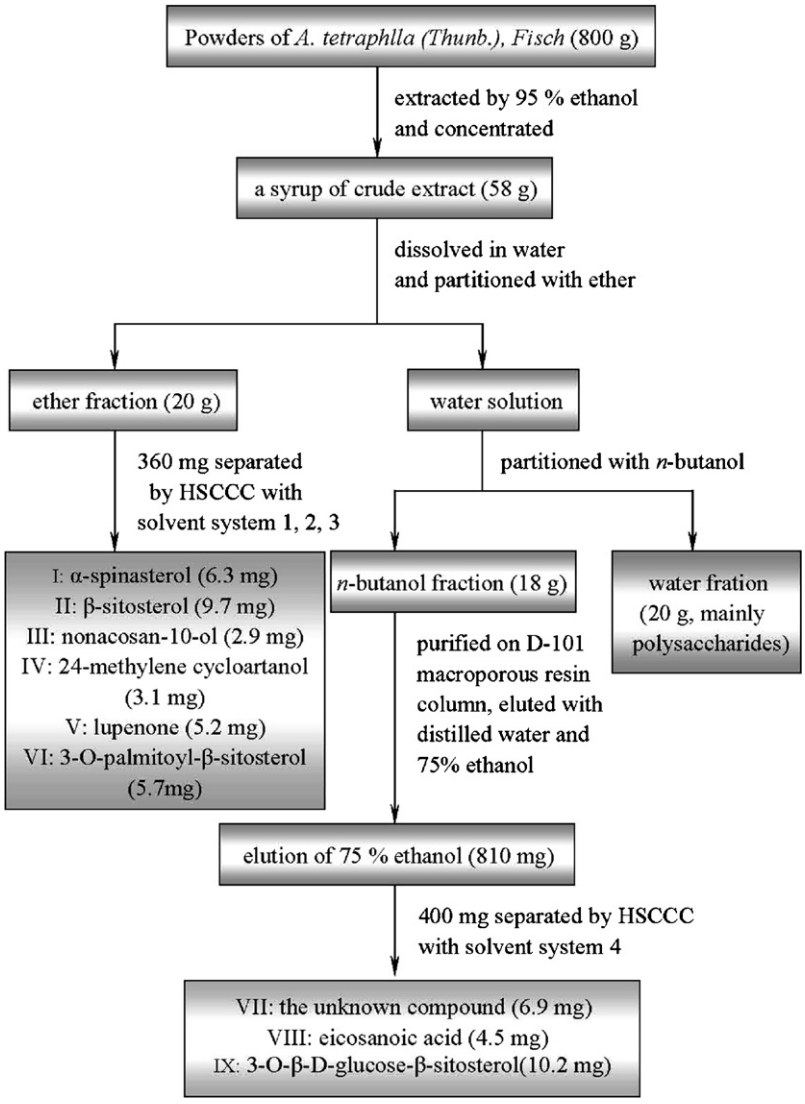

Fig. 3. Schematic diagram of the HSCCC–ELSD system and design of T-split.

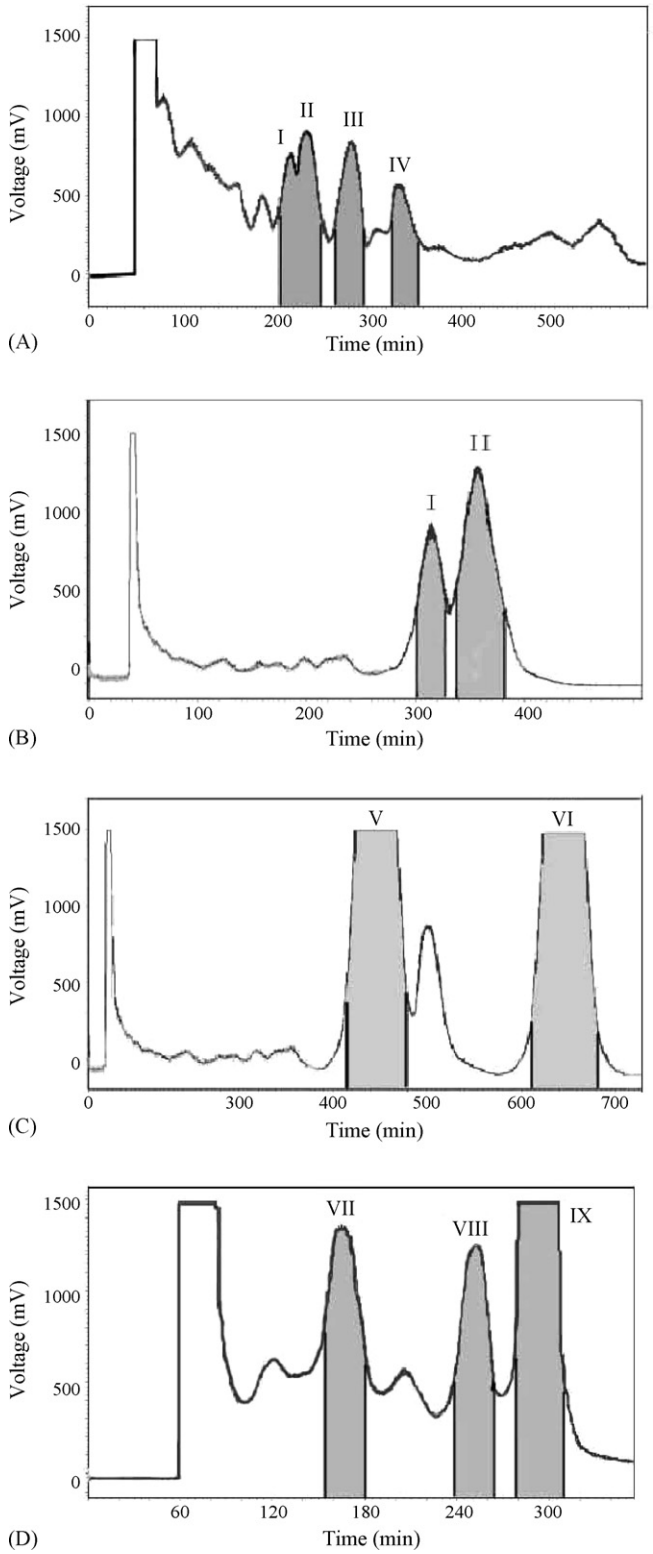

Fig. 4. HSCCC chromatograms of two fractions of ether (A–C), *n*-butanol (D) from *A. tetraphlla* (*Thunb.*), *Fisch*. Conditions of (A) two-phase solvent system: *n*-hexane–ethyl acetate–acetonnitrile 5:1:5 (v/v/v); stationary phase: upper organic phase; mobile phase: lower phase; flow rate: 1.5 ml/min; revolution speed: 800 rpm; sample size: 360 mg of ether extracts dissolved in 3 ml of the upper phase and 3 ml of lower phase; retention of the stationary phase: 59%. Conditions of (B) two-phase solvent system: *n*-hexane–ethyl acetate–acetonnitrile 5:1:4 (v/v/v), mobile phase: lower aqueous; flow rate: 2.0 ml/min; revolution speed: 800 rpm; retention of the stationary phase: 69%. Conditions of (C) two-phase solvent system: *n*-hexane–ethyl acetate–acetonnitrile 5:2:5 (v/v/v), mobile phase: lower aqueous; flow rate: 2.0 ml/min; revolution speed: 800 rpm; retention of the stationary phase: 66%. Conditions of (D) two-phase solvent system: light petroleum (b.p. 60–90 °C)–ethyl acetate–ethanol–water 6:4:5:5 (v/v/v/v); mobile phase: the lower phase; flow rate: 1.5 ml/min; revolution speed: 800 rpm; sample size: 400 mg of refined *n*-buthanol extract in 5 ml of the lower phase, retention of the stationary phase: 61%. ELSD condition: tube temperature was 110 °C, gas flow was 1.5 l/min. All the samples for HSCCC were filtered with micropore membrane filters ($\Phi = 0.45\ \mu m$) strictly, and separation temperature was all under room temperature (22.0–25.5 °C).

filled with the upper organic stationary. Then the apparatus was rotated at 800 rpm, while the lower aqueous mobile phase was pumped into the column at a flow rate of 1.5 ml/min for preliminary separation and 2.0 ml/min for further separation. After the mobile phase front emerged and the system established a steady state hydrodynamic equilibrium, the sample solution was injected into the separation column through the injection valve. The split-flow of the effluent was adjusted by adjusting the switch valve, and half of the effluent from the outlet of the column was monitored by ELSD. The tube temperature of ELSD was 110 °C, and gas flow rate was set at 1.5 l/min in the above course of detection. The whole process of separation was carried out under room temperature (22.0–25.5 °C). Each peak fraction was manually collected according to the chromatogram and concentrated under reduced pressure. The residuals were dissolved in methanol for subsequent HPLC analysis.

2.6. HPLC analysis and identification of HSCCC peak fractions

The HPLC analysis of every fraction of *A. tetraphlla* (*Thunb.*), *Fisch* crude extract and HSCCC peak fraction was performed with a Shim-Pack CLC-ODS column, (150 mm × 4.6 mm I.D., 5 μm) at room temperature. For analysis of the fraction of ether, the mobile phase was acetonnitrile–water (98:2, v/v) and the flow rate was set at 1.0 ml/min constantly. When the refined *n*-buthanol fraction was analyzed, the mobile phase was methanol and water in gradient mode as follows: 5:95–100:0 in 60 min, and the flow rate was kept at 1.0 ml/min constantly. Tube temperature of ELSD was 110 °C, and gas flow was set at 1.5 l/min in the above analysis same as HSCCC separation process.

3. Results and discussion

In the present study, D-101 macroporous resin was used to purify crude extract of *n*-butanol fraction. Water was first used to remove plentiful hydrosoluble chemicals such as pigments and polysaccharides, which had no or little retention on D-101 macroporous resin. Secondly, 75% ethanol was used to elute most target compounds, which was prepared for further HSCCC isolation and purification. At last, 95% ethanol was used to activate the resin for another use. Consequently, 810 mg refined *n*-butanol fraction was obtained from 800 g *A. tetraphlla* (*Thunb.*), *Fisch*, using this purification process. A road map

of the whole work is given in Fig. 3, and the HSCCC chromatograms are shown in Fig. 4.

3.1. HPLC analysis and identification of HSCCC peak fractions

The ether fraction, refined *n*-butanol fraction and each peak fraction of HSCCC was analyzed by HPLC. On the basis of the research of refs. [20,21], good HPLC conditions were investigated in this research. Isocratic and gradient elution were employed to obtain optimum resolution equally. According to the adopted methods, the purities of the eight compounds were all above 95%, and their HPLC chromatograms and corresponding purity were shown in Fig. 5. Identification of the HSCCC peak fractions was based on retention time together with ^{1}H NMR and CI-MS.

3.2. Selection of two-phase solvent system and other conditions of HSCCC

Successful separation by HSCCC largely depends upon the selection of suitable two-phase solvent system. As for the constituents mainly composed of sterols, their separation is not an easy task. In this experiment, several kinds of solvent systems were tested. The ideal *K*-values of target compounds should be in a proper range. In general, small *K*-values usually result in poor peak resolution, while large *K*-values tend to produce excessive sample band broadening.

For separation of sterols, which were the main constituents of ether fraction, *n*-heptane–ethyl acetate–acetonnitrile was never employed before [22]. Because the polarity of target compounds is too low, perfect *K*-value cannot be gained in the solvent systems containing water. Considering that *n*-hexane

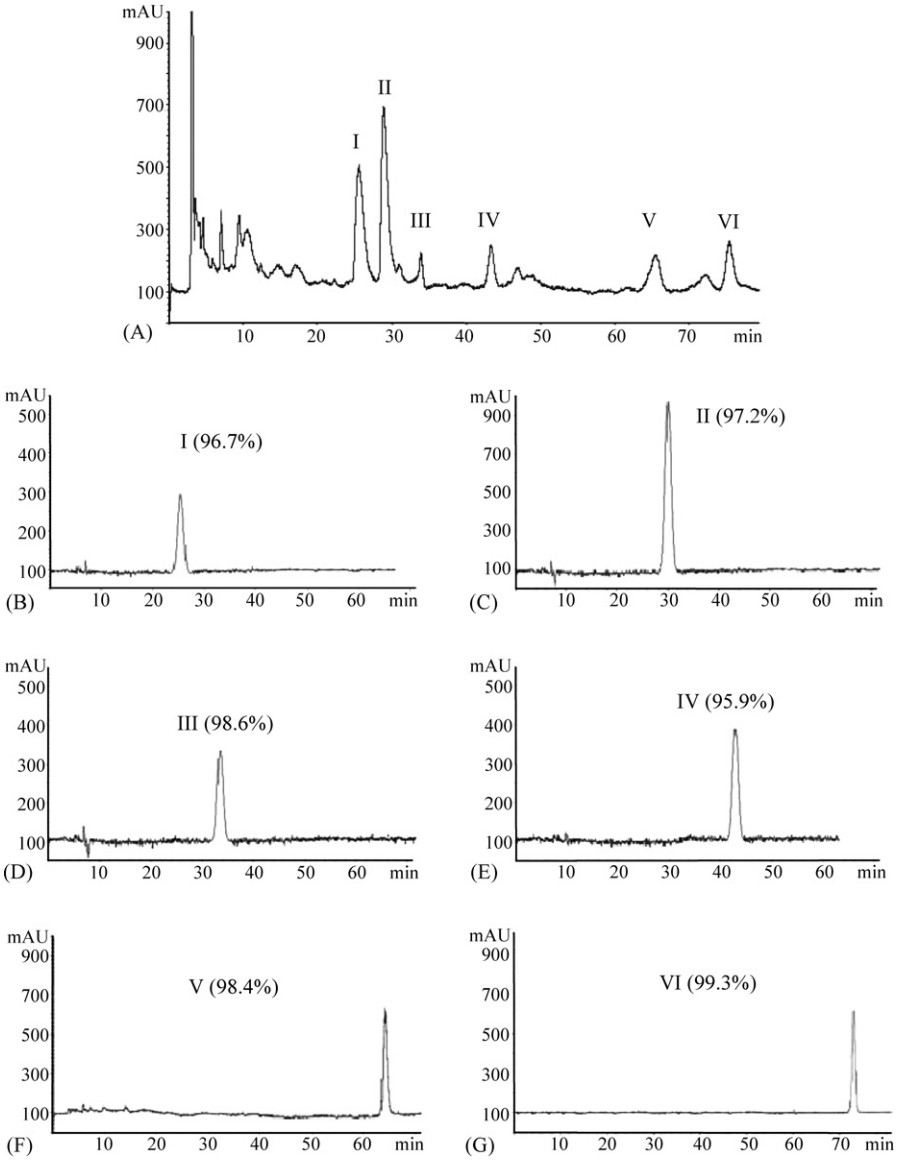

Fig. 5. HPLC chromatograms of two fractions of ether (A) and *n*-butanol (H) from *A. tetraphlla* (*Thunb.*), *Fisch* and HSCCC peak fractions of them (B–G, H–K). Column: Shimadzu VP-ODS (150 mm × 4.6 mm I.D., 5 μm); mobile phase (A–G): acetonnitrile–water (98:2, v/v); flow rate: 1.0 ml/min; mobile phase (H–K): methanol–water (methanol: 5–100% in 60 min); flow rate: 1.0 ml/min; ELSD tube temperature: 110 °C, gas flow rate: 1.5 l/min.

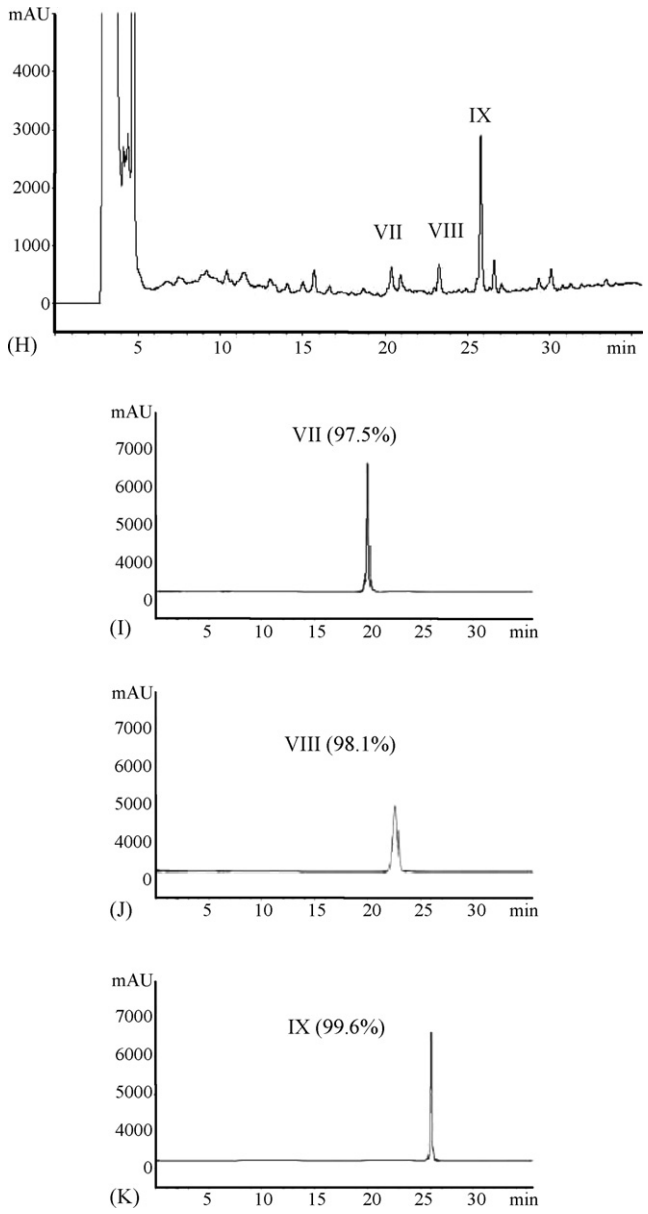

Fig. 5. (*Continued*).

was more inexpensive, it was advisable to substitute *n*-heptane with *n*-hexane from the point of view of mass production. So *n*-hexane–ethyl acetate–acetonnitrile was chosen as the two-phase solvent system for HSCCC separation of ether fraction. The *K*-values of the target constituents in several solvent systems are shown in Table 1. From Table 1, it can be seen that for separation of ether fraction, when *n*-hexane–ethyl acetate–acetonnitrile (5:1:4, v/v/v) was used as the solvent system, compounds I and II could be separated well, but the *K*-values of compounds III, IV, V and VI were too high. When *n*-hexane–ethyl acetate–acetonnitrile (5:1:5, v/v/v) was used as the solvent system, the *K*-values of compounds I and II were too close, and that of V and VI too high. But in this system, compounds III and IV could be separated. When *n*-hexane–ethyl acetate–acetonnitrile (5:2:5, v/v/v) was used as the solvent system, compounds V and VI could be separated well. So the ether fraction was first separated with *n*-hexane–ethyl acetate–acetonnitrile (5:1:5, v/v/v). The HSCCC chromatogram is shown in Fig. 4(A). During this separation process, four peaks (I, II, III and IV in Fig. 4) were collected. Peaks III and IV were approved to be nonacosan-10-ol and 24-methylene cycloartanol. Peaks I and II were too close and the purities of I and II were not satisfactory, so peaks I and II were collected together and further separated with *n*-hexane–ethyl acetate–acetonnitrile (5:1:4, v/v/v). The HSCCC chromatogram is shown in Fig. 4(B). Peak I was identified as α-spinasterol and peak II as β-sitosterol. The compounds with large *K*-values were still maintained in the separation column. So the residual solution in the separation column was blown out with compressed nitrogen gas after the four major peaks were obtained. Then the residual solution was concentrated to dryness and separated with *n*-hexane–ethyl acetate–acetonnitrile (5:2:5, v/v/v) as the solvent system. The HSCCC chromatogram is shown in Fig. 4(C). At last, another two compounds (peaks V and VI) were obtained and identified as lupenone and 3-*O*-palmitoyl-β-sitosterol.

Enlightened by Ito's strategy of solvent system selection [23], light petroleum (b.p. 60–90 °C)–ethyl acetate–ethanol–water 5:5:5:5 (v/v/v/v) was tried for separation of the refined *n*-buthanol fraction at first. But it was given up quickly for poor retention of the stationary phase and resolution. So light petroleum (b.p. 60–90 °C)–ethyl acetate–ethanol–water 6:4:5:5 (v/v/v/v) was used as the two-phase solvent system. The upper organic phase was used as the stationary phase and the lower aqueous phase as the mobile phase. Three compounds including an unknown compound, eicosanoic acid and 3-*O*-β-D-glucose-β-sitosterol (peaks VIII, IX and X in Fig. 4), were acquired from refined *n*-buthanol fraction. The HSCCC chromatogram is shown in Fig. 4(D).

3.3. The structural identification

The structural identification of peak fractions was performed with CI-MS and ^{1}H NMR, with TMS as internal standard. Data of each compound were given as follows.

Data of HSCCC peak I (6.3 mg): CI-MS (*m*/*z*): 413($[M+H]^+$); ^{1}H NMR (500 MHz, $CDCl_3$): 5.16 (1H, s), 5.13(1H, dd), 5.02 (1H, dd), 3.58 (1H, m), 1.03 (3H, d, J = 6.6 Hz), 0.87 (3H, t, J = 8.9 Hz), 0.82 (6H, d, J = 8.0 Hz), 0.79 (3H, s), 0.55 (3H, s). The results were very similar to those in ref. [24], peak I corresponded to α-spinasterol.

Data of HSCCC peak II (9.7 mg): CI-MS (*m*/*z*): 415 ($[M+H]^+$); ^{1}H NMR (500 MHz, $CDCl_3$): 5.40 (1H, br d), 3.58(1H, m), 0.69–1.01 ($CH_3 \times 6$). The results were very similar to those in ref. [25], peak II corresponded to β-sitosterol.

Data of HSCCC peak III (2.9 mg): CI-MS (*m*/*z*): 425 ($[M+H]^+$); ^{1}H NMR (500 MHz, $CDCl_3$): 3.63 (1H, m), 1.44–1.24 (m, 23H), 0.89 (6H, t, J = 7.1 Hz, $CH_3 \times 2$). The results were very similar to those in ref. [26], peak III corresponded to nonacosan-10-ol.

Data of HSCCC peak IV (3.1 mg): CI-MS (*m*/*z*): 441 ($[M+H]^+$); ^{1}H NMR (500 MHz, $CDCl_3$): 4.72, 4.67 (2H, d, J = 2.7 Hz), 3.21 (1H, m), 1.35 (6H, d, J = 7.0 Hz), 1.07, 1.06, 0.99, 0.91, 0.87 (s, $CH_3 \times 5$), 0.96 (3H, d, J = 5.1 Hz), 0.57 (1H, d, J = 4.5 Hz), 0.34 (1H, d, J = 4.5 Hz). The results were very sim-

Table 1
The *K*-values of target components measured in different solvent systems

Solvent system		*K*-value			
		n-Hexane–ethyl acetate–acetonnitrile (5:1:4, v/v/v)	*n*-Hexane–ethyl acetate–acetonnitrile (5:1:5, v/v/v)	*n*-Hexane–ethyl acetate–acetonnitrile (5:2:5, v/v/v)	Petroleum ether–ethyl acetate–ethanol–water (6:4:5:5, v/v/v/v)
	I	2.11	1.07	–	–
	II	2.49	1.12	–	–
Ether	III	>5	1.41	–	–
fraction	IV	–	1.63	–	–
	V	–	>5	2.93	–
	VI	–	–	4.27	–
Refined *n*-butanol fraction	VII	–	–	–	0.85
	VIII	–	–	–	1.22
	IX	–	–	–	1.45

Experimental protocol: 4 ml of each phase of the equilibrated two-phase solvent system was added to approximately 8 mg of crude sample placed in a 10 ml test tube. 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 HPLC to obtain the partition coefficient (*K*). The partition coefficient (*K*) value was expressed as the peak area of the compound in the upper phase divided by the peak area of the compound in the lower phase.

ilar to those in ref. [27], peak IV corresponded to 24-methylene cycloartanol.

Data of HSCCC peak V (5.2 mg): CI-MS (*m/z*): 425 ($[M+H]^+$); ^{1}H NMR (500 MHz, $CDCl_3$): 4.57, 4.68 (1H each, m), 2.42 (2H, m), 0.80–1.68 ($CH_3 \times 6$, s). The results were very similar to those in ref. [25], peak V corresponded to lupenone.

Data of HSCCC peak VI (5.7 mg): CI-MS (*m/z*): 653 ($[M+H]^+$), 397 ($[M+H\text{-}162]^+$); ^{1}H NMR (500 MHz, CD_3OD): 5.56 (1H, br), 4.60 (1H, br), 2.30 (2H, t, $J = 6.5$ Hz), 1.25–1.83 (55H, m), 0.67–1.14 ($CH_3 \times 7$). The results were very similar to those in ref. [28], peak VI corresponded to 3-*O*-palmitoyl-β-sitosterol.

Data of HSCCC peak VII (6.9 mg) showed the CI-MS quasi-molecular ion ($[M+H]^+$) peak (*m/z*: 283), its further structural identification will be continued.

Data of HSCCC peak VIII (4.5 mg): CI-MS (*m/z*): 313 ($[M+H]^+$); ^{1}H NMR (500 MHz, $CDCl_3$): 0.86 (3H, t, CH_3), 1.27 (32H, m, $CH_2 \times 16$), 1.64 (2H, q), 2.35 (2H, t), which matched with the reported the data given in ref. [29], peak VIII corresponded to eicosanoic acid.

Data of HSCCC peak IX (10.2 mg): CI-MS (*m/z*): 577 ($[M+H]^+$), 415 ($[M+H\text{-}162]^+$). Compared with NMR data of peak II, there is a group of glucose protons at δ 3.8–5.2 ppm in addition. The results were very similar to those in ref. [26], peak IX corresponded to 3-*O*-β-D-glucose-β-sitosterol.

4. Conclusions

Some of the chemical constituents from the different fractions of *A. tetraphlla* (*Thunb.*), *Fisch*, one of the most popular Chinese medicinal herbs, were isolated and purified systematically by HSCCC. The results of the research provided a successful method for isolation of chemical compounds having no or end ultraviolet absorption with the help of ELSD on line. The introduction of online ELSD with HSCCC has dramatically extended the field of this technique by overcoming the drawbacks of UV detector. The effluent from the outlet of HSCCC was splitted into two parts: one was collected, while the other was introduced directly into ELSD. The present study indicates that this new strategy, which integrates all the advantages of HSCCC and ELSD, is a very powerful technique possessing general utility in the preparative separation and purification of all kinds of bioactive components from traditional Chinese medicine (TCM), leaving alone their chromophoric property.

Acknowledgement

This research work was supported by the National Scientific Foundation of China for Outstanding Young Scientists (No. 30525032).

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