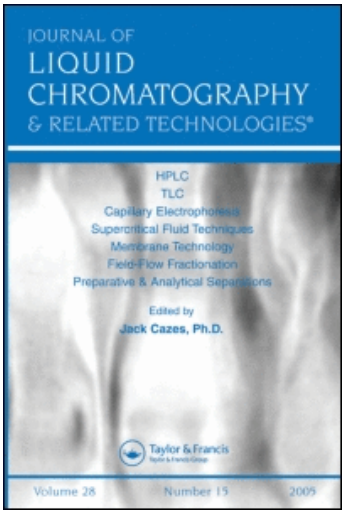

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Preparative Separation and Purification of β-Caryophyllene from Leaf Oil of Vitex negundo L. var. heterophylla (Franch.) Rehd. by High Speed Countercurrent Chromatography

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Abstract: High speed countercurrent chromatography (HSCCC) coupled with an evaporative light scatter detector (ELSD) was successfully applied to preparative isolation and purification of β-caryophyllene from essential oil of Vetix negundo L. var. heterophylla (Franch.) Rehd. Using either n-hexane-chloroform-acetonitrile (6:2:5) or *n*-hexane-dichloromethane-acetonitrile (10:3:7) as the two-phase solvent system, β-caryophyllene was separated in one run from the crude essential oil by HSCCC. However, the application of *n*-hexane-dichloromethane-acetonitrile (10:3:7) resulted in a higher purity and recovery of β-caryophyllene. About 85 mg of β-caryophyllene representing 97.3% by GC, was yielded from 600 mg crude essential oil when eluted with the lower phase at a flow rate of 1.5 mL.min^{-1}.

Keywords: β-Caryophyllene, Essential oil, HSCCC, Vetix negundo L. var. heterophylla (Franch.) Rehd

INTRODUCTION

Vetix negundo L. var. heterophylla (Franch.) Rehd., family Verbenaceae, is a perennial deciduous shrub or a small multi-stemmed tree,

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growing wildly over most mountain or hill areas of China. It has gray-green, finely dissected foliage, flexible stems, and panicles of lavender-blue flowers that bloom in summer. The leaves, flowers, and stems bear strong herbous, cool, and refreshing odors. The essential oils of the leaves are known in Chinese traditional medicine for the treatment of common coughs, asthma, and chronic bronchitis. Constituents of the leaf oil mainly included monoterpenoids, sesquiterpenoids, and diterpenoids, among which β-caryophyllene was present in high amounts.[1,2] The chemical structures of β-caryophyllene and three other sesquiterpenes from the leaf oil of Vetix negundo L. var. heterophylla (Franch.) Rehd. are shown in Figure 1. β-Caryophyllene is a flavor compound. It is an important clinical drug for bronchitis and often used in the synthesis of β-caryophyllene alcohol,[3] a flavor and also a novel drug for asthma, excellent for a strong and permanent effect and low toxicity.[4]

HSCCC is a support free liquid–liquid partition chromatography technique. It eliminates irreversible adsorption of samples on solid phase and offers various advantages including high purity of fractions, high sample recovery, high loading capacity, and is easy to scale up. In recent years, HSCCC has been widely applied for separation of active components from traditional Chinese herbs and other natural products.[5–11] Here, β-caryophyllene, a common functional sesquiterpene hydrocarbon, also found existing in essential oils of Vitex negundo L. var cannabifolia (Sieb et Zucc) Hand-Mazz, clove, lavender, copaiba, and folium Artemisiae argyi,[12] was successfully purified from leaf oil of Vetix negundo L. var. heterophylla (Franch.) Rehd by HSCCC. In contrast to our previous practice of vacuum distillation in combination with repeated silica gel column chromatography for β-caryophyllene separation,[13] the HSCCC proved rather efficient. To our knowledge, such reports using HSCCC to separate sesquiterpene hydrocarbons from essential oils are not yet found.

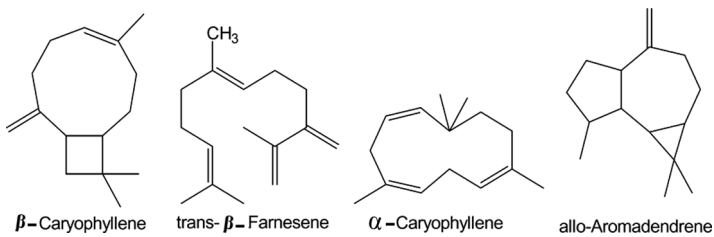

Figure 1. Chemical structures of β-caryophyllene and three other sequiterpenes from leaf oil of Vetix negundo L. var. heterophylla (Franch.) Rehd.

EXPERIMENTAL

Apparatus

A model TBE 300A high speed countercurrent chromatography (Shanghai Tauto Biotech, Shanghai, China) with three ploytetrafluoroethylene coils (tube diameter 2.6 mm, total volume 300 mL) and a 20 mL manual injection sample loop was employed in the preparative separation. The revolution radius (R) was 5 cm, and the β values of the multilayer coil varied from 0.5 at the internal terminal to 0.8 at the external terminal ($\beta = r/R$, where r is the distance between the coil and the holder shaft). The rotary speed of the apparatus can be regulated at $700 \sim 1000$ rpm with a speed controller. The solvents were pumped into the column by an ÄKTA prime system (Amersham Pharmacia Biotechnique Group, Sweden). The column temperature was controlled by a HX 1050 water circulating constant temperature implement (Beijing Tianyou Science Development Co. Ltd., Beijing, China). The effluent was monitored by a Model 2000 evaporative light scattering detector (ELSD) (Alltech Science Co. Ltd., USA) with the flow rate of air set at $1.5\,L \cdot min^{-1}$, impactor on, drift tube temperature at 25°C and gain at 1. The ELSD chromatogram was recorded by a N2010 workstation (Zhejiang University, Hangzhou, China).

An Agilent 6890N/5973i gas chromatograph and mass spectrometer (GC-MS) and an Agilent 6890 gas chromatograph coupled with a flame ionization detector (GC-FID) (Agilent Technologies, USA) were used for analysis.

Materials and Reagents

Fresh leaves at the flowering stage of mid July were collected from the mountain area, northwest in Beijing suburb. They were room naturally dried and then sealed in boxes.

β-Caryophyllene (98.2%) was previously prepared from the leaf oil of Vetix negundo L. var. heterophylla by vacuum distillation combined with silica gel column chromatography. The organic solvents, methanol, acetonitrile, ethyl acetate, *n*-hexane, chloroform, and dichloromethane, were all of analytical grade and purchased from Beijing Chemical Reagent Company, Beijing, China.

Preparation of Essential Oil by Steam Distillation

Batches of 1 kg of pulverized leaves were steam distilled for 2 h. The installation consisted of a cooking autoclave, a 1000 mL round bottom

extraction flask, a glass jacketed condenser, and a 200 mL graduated cylinder glass receiver. The steam was produced by the cooking autoclave and then transferred to the extraction flask with glass tubules. The upper layer of the condensate was collected and dehydrated over anhydrous Na_2SO_4 and the pale yellow oil was obtained in a yield of 0.25%. The oil was stored in a refrigerator ($-20^{\circ}C$) for GC-MS analysis and HSCCC separation.

Selection of Two-Phase Solvent System

The composition of the two-phase solvent system was selected according to the partition coefficient (K) of β-caryophyllene. The K values were determined as follows: a small amount of β-caryophyllene was dropped into a 10 mL test tube to which 2.0 mL of each phase of the equilibrated two-phase solvent system was added. The tube was shaken vigorously for 2 min to equilibrate the sample thoroughly with the two phases. Then equal volumes of sample solutions from the two phases were taken and analyzed separately by GC. The peak areas of β-caryophyllene in the upper phase and the lower phase were recorded as A_1 and A_2, respectively. The K value was calculated by the equation $K = A_1/A_2$.

Preparation of Two Phase Solvent Systems and Sample Solutions

The solvent systems, *n*-hexane-chloroform-acetonitrile (6:2:5, v/v) and *n*-hexane-dichloromethane-acetonitrile (10:3:7, v/v) were used in HSCCC separation. At the temperature similar to that of HSCCC separation, the solvents were mixed according to the volume ratios selected and then thoroughly equilibrated by shaking repeatedly in a separation funnel. Prior to use, the upper phase and the lower phase were separated and degassed by sonication for 25 min.

The sample solution near 20 mL was prepared by the dissolution of 600 mg essential oil into the lower phase of the solvent system.

HSCCC Separation

The upper phase was used as stationary phase and the lower phase was used as mobile phase. The multilayer coiled column was first entirely filled with the stationary phase at a flow rate of $30\,mL \cdot min^{-1}$. Then, the mobile phase was pumped through the column at a flow rate of $1.5\,mL \cdot min^{-1}$ with the HSCCC apparatus rotating at a speed of 850 rpm. After hydrodynamic equilibrium was reached, the sample solution

was injected into the separation column. The column temperature was controlled at 22°C. For the sake of continuous ELSD detection, as well as for fraction collecting, a splitter was installed at the outlet of the column, and the ratio of the effluent to the ELSD versus that to the receiver was kept at 1:20. According to the chromatograms recorded, the fractions were manually collected and then concentrated by N_2 blowing. The residue liquids were analyzed by GC.

GC-MS and GC Analysis

HP-5, 30 m × 0.25 mm × 0.25 μm capillary column (Agilent Technologies, USA) was used in GC-MS analysis. The carrier gas was helium in $1\,mL \cdot min^{-1}$. The initial oven temperature was at 44°C, holding for 2 min; then raised to 280°C at $8°C\,min^{-1}$, holding for 2 min. The sample of 1.0 μL was injected at 280°C in split mode (20:1). The mass detector was operated at 150°C in electron impact mode at 70 eV. The ion source temperature was at 230°C. The transfer line temperature was at 250°C. The chromatograms were recorded by monitoring the total ion current in 40–450 mass range.

The HP-5, 30 m × 0.32 mm × 0.25 μm capillary column (Agilent Technologies, USA) was used in GC analysis. The carrier gas was nitrogen in $1\,mL \cdot min^{-1}$. The initial oven temperature was at 45°C, holding for 2 min; then raised to 280°C at $8°C\,min^{-1}$ holding for 2 min. The sample of 0.5 μL was injected at 280°C in split mode (20:1).

RESULTS AND DISCUSSION

Analysis of the Essential Oil

The total ion current chromatogram of the essential oil in GC-MS was shown in Figure 2, which could be largely divided into three retention regions that corresponded to skeletal types of monoterpenoids (0 ~ 15.50 min), sesquiterpenoids (15.50 ~ 23.60 min), and diterpenoids (23.60 ~ 30.00 min) respectively. Some lipid derived or aromatic compounds were also found from the essential oil, but they were minor.[1] In Figure 2, based on NIST02 mass spectra library as well as retention indices (RI), the peaks of some sesquiterpenes including β-caryophyllene, β-farnesene, α-caryophyllene, allo-aromadendrene, γ-muurolene, and α-muurolene were marked in the order of numbers. β-Caryophyllene was the most abundant in the oil, representing 22.6% of the total peak areas.

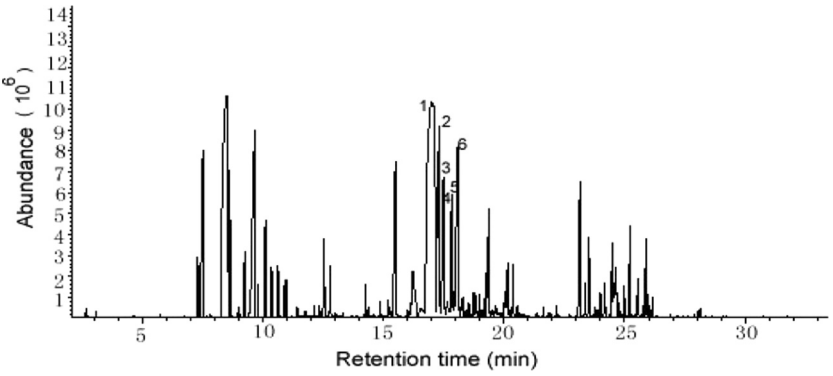

Figure 2. Total ion current chromatogram of the leaf oil of Vetix negundo L. var. heterophylla (Franch.) Rehd. on a HP–5 30 m × 0.25 mm × 0.25 μm capillary column in GC-MS. Peak 1 ~ 6 were β-caryophyllene (RI 1420), β-farnesene (RI 1452), α-caryophyllene (RI 1462), allo-aromadendrene (RI 1468), γ-muurolene (RI 1473) and α-muurolene (RI 1492) in turn (RI, retention index).

Optimization of HSCCC Separation

The separation by HSCCC depends mainly upon the selection of a suitable two-phase solvent system, which should provide an ideal range of partition coefficient (*K*) for the target compounds. Several two-phase solvent systems tested together with their *K* values measured are presented in Table 1.

The n-Hexane-ethyl acetate-methanol-water system was tried first since it has been applied successfully for the separation of compounds with various polarities.[5] However, it turned out that β-caryophyllene was much inclined to partition in the upper phase of this solvent system. For example, the *K* value was up to 10 when *n*-hexane-ethyl acetate-methanol-water (32:8:20:1) was tested. The addition of *n*-hexane could somewhat improve the partition. And following this way, the best case obtained was *n*-hexane-ethyl acetate-methanol-water (144:16:80:1) with the *K* value of 1.3. Yet, retention of solid phase for this solvent system proved rather low (approximately 30%), which can seldom achieve a good HSCCC separation.

In contrast to most target compounds reported in HSCCC separation,[6–11] β-caryophyllene is a volatile sesquiterpene, which can often be properly analyzed by GC not HPLC. However, for the present measurement of *K* value in aqueous two-phase solvent system by GC, the removal of water was necessitated since water in samples harms the GC capillary column. Besides, the recovery of volatile targets by evaporation of HSCCC fractions would be complicated if water was contained

Table 1. The partition coefficients (K) of β-caryophyllene in several two-phase solvent systems

Solvent system (v/v)	K value
n-hexane-ethyl acetate-methanol-water (32:8:20:1)	10
n-hexane-ethyl acetate-methanol-water (144:16:80:1)	1.30
n-hexane-chloroform-acetonitrile (6:1:4)	5.68
n-hexane-chloroform-acetonitrile (5:1:5)	4.47
n-hexane-chloroform-acetonitrile (5:2:5)	1.41
n-hexane-chloroform-acetonitrile (6:2:5)	1.84
n-hexane-ethyl acetate-acetonitrile (5:1:4)	5.61
n-hexane-ethyl acetate-acetonitrile (5:1:5)	3.55
n-hexane-ethyl acetate-acetonitrile (5:2:3)	1.86
n-hexane-dichloromethane-acetonitrile (10:1:9)	7.27
n-hexane-dichloromethane-acetonitrile (10:2:8)	4.61
n-hexane-dichloromethane-acetonitrile (10:3:7)	2.56

in the two-phase solvent systems. Therefore, the *n*-hexane-ethyl acetate-methanol-water system was abandoned and non-aqueous two-phase solvent systems were taken into account in the following.

n-Hexane (or *n*-heptane)-acetonitrile and *n*-hexane (or *n*-heptane)-methanol are the usual non-aqueous two-phase solvent systems to be chosen.[14–17] Considering that sesquiterpene hydrocarbons are not so soluble in polar protonic solvents like methanol, the partition of *β*-caryophyllene in *n*-hexane-acetonitrile was carefully studied with halogen containing hydrocarbons and ethyl acetate as modifiers.

In Table 1, for all *n*-hexane-acetonitrile systems tested, the partition of *β*-caryophyllene in the upper phase was more than that in the lower phase. As we know, the suitable experiential K values are generally considered as $0.5 \sim 2$. In comparison, *n*-hexane-chloroform-acetonitrile (5:2:5), *n*-hexane-chloroform- acetonitrile (6:2:5), *n*-hexane-dichloromethane-acetonitrile (10:3:7), and *n*-hexane-ethyl acetate-acetonitrile (5:2:3) revealed K values approximate to 2, indicating their potential of HSCCC separation. But in the further selection, the solvent systems of *n*-hexane-chloroform-acetonitrile (5:2:5) and *n*-hexane-ethyl acetate-acetonitrile (5:2:3) had to be discarded. The fact was, that for *n*-hexane-chloroform-acetonitrile (5:2:5), the volume ratio of the two phases was about 1:4, which led to the wastage of solvents; while for *n*-hexane-ethyl acetate-acetonitrile (5:2:3), not only the retention of solid phase was rather low (27%) but also the drop of solid phase present all the time during HSCCC separation. Finally, the solvent systems of *n*-hexane-chloroform-acetonitrile (6:2:5) and *n*-hexane-dichloromethane-acetonitrile (10:3:7) were chosen and employed in the separation of *β*-caryophyllene from the essential oil by HSCCC.

From HSCCC chromatograms in Figure 3, it could be seen that whether by *n*-hexane-chloroform-acetonitrile (6:2:5) or *n*-hexane-dichloromethane- acetonitrile (10:3:7), *β*-caryophyllene was one step separated from the crude essential oil; and owing to the *K* value of *β*-caryophyllene, was larger in *n*-hexane-dichloromethane-acetonitrile (10:3:7) than in *n*-hexane-chloroform-acetonitrile (6:2:5) and a base-line separation was achieved though the retention time was a bit longer. Otherwise, GC analysis of *β*-caryophyllene fraction from HSCCC (Figure 4) also substantiated that the separation by *n*-hexane-dichloromethane-acetonitrile (10:3:7) was better than by *n*-hexane-chloroform-acetonitrile (6:2:5). In Figure 4b, the percentage of peak areas of *β*-caryophyllene from HSCCC by *n*-hexane-dichloromethane-acetonitrile (10:3:7) amounted to 97.3% of the total; whereas by

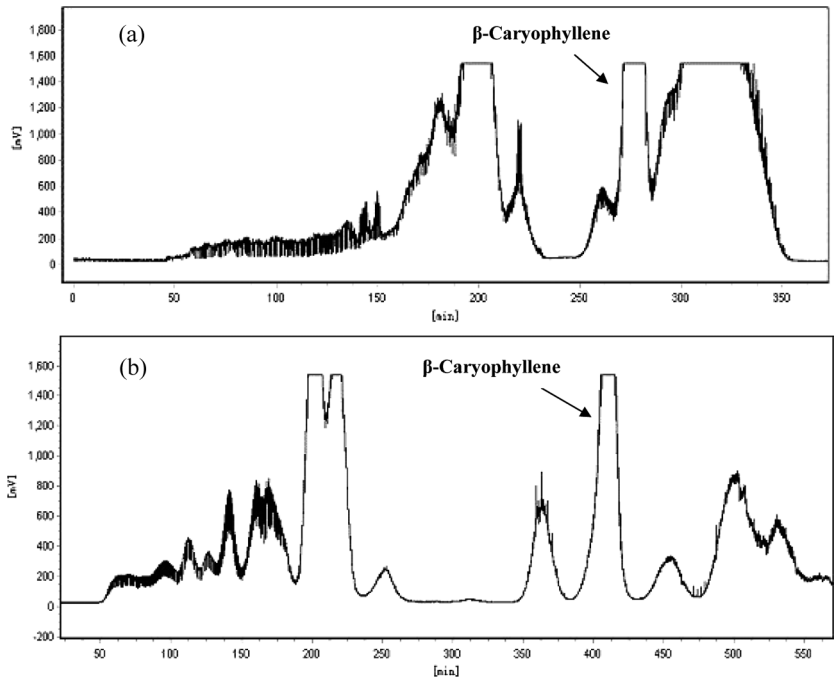

Figure 3. HSCCC chromatograms of the crude essential oil from leaves of Vetix negundo L. var. heterophylla (Franch.) Rehd. with the detection of ELSD, the effluent split ratio (20:1), the flow rate of air 1.5 $L \cdot min^{-1}$, impactor on, drift tube temperature 25°C and gain at 1. The two-phase solvent system in HSCCC (a) *n*-hexane-chloroform-acetonitrile (6:2:5), (b) *n*-hexane-dichloromethane-acetonitrile (10:3:7); the lower phase as mobile phase, flow rate, 1.5 $ml \cdot min^{-1}$; revolution speed, 850 rpm; separation temperature 22°C; sample size, 600 mg of crude oil diluted to near 20 mL with the lower phase.

n-hexane-chloroform-acetonitrile (6:2:5) (Figure 4a), that of β-caryophyllene accounted for 95.3% of the total and even appreciable amount of α-caryophyllene (the peak following β-caryophyllene in Figure 4a) was detected. What was more, the recovery of β-caryophyllene from 600 mg crude oil in HSCCC by *n*-hexane-dichloromethane-acetonitrile (10:3:7) and *n*-hexane-chloroform-acetonitrile (6:2:5) was 85.4 mg and 71.7 mg, respectively. In fact, the smaller amount of product by *n*-hexane-chloroform-acetonitrile (6:2:5) resulted from only part of β-caryophyllene effluent being collected due to the presence of peak overlapping.

Above all, the influences of flow rate of mobile phase and concentrations of sample solutions injected on HSCCC separation were investigated, respectively, using *n*-hexane-dichloromethane-acetonitrile (10:3:7) as the two-phase solvent system. When the flow rate of mobile phase was increased from $1.5\,mL \cdot min^{-1}$ to $2\,mL \cdot min^{-1}$, β-caryophyllene fraction was eluted out earlier ($287 \sim 303$ min). However, GC analysis revealed that the content of β-caryophyllene in this β-caryophyllene fraction was reduced to 95.0%. This proved that the concentration of the sample solution could only be increased in a limited amount, or else

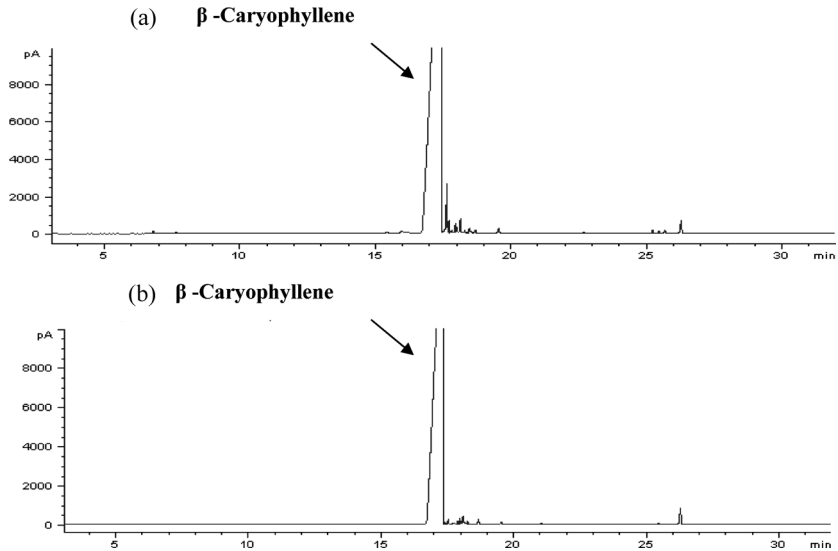

Figure 4. GC chromatograms of the fractions from HSCCC separation on a HP-5 30 m × 0.32 mm × 0.25 μm capillary column; (a) the HSCCC fraction in Figure 3a by *n*-hexane-chloroform-acetonitrile (6:2:5); (b) the HSCCC fraction in Figure 3b by *n*-hexane-dichloromethane-acetonitrile (10:3:7).

the drop of solid phase was observed from HSCCC fractions and consequently affected the separation.

In conclusion, the optimal HSCCC conditions found for one step purification of β-caryophyllene from leaf oil of Vetix negundo L. var. heterophylla were as follows: *n*-hexane-dichloromethane-acetonitrile (10:3:7) as the two-phase solvent system, the lower phase as the mobile phase, the flow rate at 1.5 mL $\cdot$ min^{-1} and sample loading of 600 mg crude oil diluted to 20 mL by the mobile phase.

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