Chemical Constituents in Essential Oil of *Illicium verum* by Supercritical CO_2 Extraction and High-speed Counter-current Chromatography

WANG Dai-jie

WANG Dai-jie[1], GENG Yan-ling[1], LIU Wei[1], WANG Xiao[1*], LIU Jian-hua[1], LIU Jing[1], HUANG Lu-qi[2]

(1. Process Control Research Center of TCM, Shandong Academy of Sciences, Shandong Analysis and Test Center, Jinan 250014, China; 2. Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences, Beijing 100700, China)

Abstract: Supercritical CO_2 extraction was used to extract star anise essential oil from star anise (*Illicium verum* Hook. f.) under the pressure of 25 MPa and temperature of 35 ℃. Three compounds including anisaldehyde, methyl isoeugenol and *trans*-anethole were separated and purified by high-speed counter-current chromatography (HSCCC) with a two-phase solvent system which composed of *n*-hexane-ethyl acetate-methanol-water (1∶0.2∶1∶0.1, volume ratio). The separation yielded a total of 10 mg of anisaldehyde, 7 mg of methyl isoeugenol, and 640 mg of *trans*-anethole from 1.3 g of essential oil in one-step separation with the purity of 98.9 %, 96.8 % and 99.7%, respectively. They are determined by HPLC. The chemical structures of these compounds were identified by EI-MS.

Key words: *Illicium verum* Hook. f.; anisaldehyde; methyl isoeugenol; *trans*-anethole; supercritical CO_2 extraction; high-speed counter-current chromatography

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超临界 CO_2 萃取结合高速逆流色谱分离八角茴香精油中的化学成分

王岱杰[1], 耿岩玲[1], 刘 伟[1], 王 晓[1], 刘建华[1], 刘 静[1], 黄璐琦[2]

(1. 山东省科学院 中药过程控制研究中心; 山东省分析测试中心, 山东 济南 250014;
2. 中国中医科学院 中药研究所, 北京 100700)

摘 要: 建立了超临界 CO_2 提取,高速逆流色谱分离纯化八角茴香中精油的分离方法。首先利用超临界 CO_2 提取八角茴香中的精油,萃取压力为 25 MPa,萃取温度为 35 ℃。1.3 g 粗提物经高速逆流色谱分离,两相溶剂体系为正己烷-乙酸乙酯-甲醇-水(体积比 1∶0.2∶1∶0.1),一次分离出 3 种化合物,经 EI-MS 鉴定为茴香醛 10.3 mg、异丁香酚甲醚 7.1 mg 和反式-茴香脑 636.5 mg,纯度分别为 98.9 %、96.8 % 和 99.7 %。

关键词: 八角茴香; 茴香醛; 异丁香酚甲醚; 反式-茴香脑; 超临界 CO_2 萃取; 高速逆流色谱

Illicium verum Hook. f. (star anise), one of the most popular spices and Chinese traditional medicines, spreads mainly in Guangxi, Guangdong, Guizhou and Yunnan provinces of China. The essential oil from *I. verum* Hook. f. is widely used in food and drink industry. It also has many pharmacologic functions, such

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作者简介: 王岱杰(1981 -),男,山东济南人,博士生,主要从事天然产物化学方面的研究工作
* **通讯作者**: 王 晓,研究员,博士,从事天然产物化学方面的研究; **E-mail**: wxjn1998@126.com。

as ovicidal activity[1], antimicrobial[2-3] and analgesic effect[4]. *Trans*-anethole, a major compound in *I. verum* Hook. f., has been found to have the effect of increasing leukocyte[5]. Anisaldehyde, as an important spice and intermediate, is widely used in fields of food, spices and plating[6].

Supercritical fluid extraction, for its low solvent consumption and mild conditions, has been used to extract edible oil, essential oils and related products from different raw materials[7-8]. High-speed counter-current chromatography (HSCCC) is a support-free liquid-liquid partition chromatographic technique which eliminates irreversible adsorption of the sample onto the solid support. With a large volume of sample injection, multiform relatively pure substances can be obtained at one-step in large amount. It is especially suitable for separation and purification of active components from natural products[9-11]. However, no report has been seen on the use of HSCCC for the isolation and purification of essential oils from *I. verum* Hook. f.. The present paper describes an efficient method for extraction and purification of anisaldehyde, methyl isoeugenol and *trans*-anethole from *I. verum* Hook. f. by SFC and HSCCC.

1 Materials and Methods

1.1 Reagents and materials

Carbon dioxide (99.9 %) was purchased from Deyang Gas Company, Jinan, China. *n*-Hexane, methanol, ethyl acetate were analytical grade (Juye Chemical Factory, Jinan, China). Methanol used for HPLC analysis was of chromatographic grade (Siyou Special Reagent Factory, Tianjin, China). Reverse osmosis Milli-Q water (Millipore, USA) was used for all solutions and dilutions.

I. verum Hook. f. was obtained from a local drug store and identified by Dr Li Jia (College of Pharmacy, Shandong University of Traditional Chinese Medicine, Shandong, China).

1.2 Apparatus

HSCCC was carried out using a Model GS10A-2 commercial instrument (Beijing Emilion Science & Technology Co., Beijing, China), with a multilayer PTFE coil of 1.6 mm i.d. and 110 m in length with a total capacity of 230 mL. The β values of this preparative column range from 0.5 at the internal to 0.8 at the external ($\beta = r/R$, where r is the rotation radius or the distance from the coil to the holder shaft, and R (R = 8 cm) is the revolution radius or the distance between the holder axis and central axis of the centrifuge). The solvent was pumped into the column with a Model NS-1007 constant-flow pump (Beijing Emilion Science & Technology Co., Beijing, China). Continuous monitoring of the effluent was carried out with a Model 8823A-UV detector (Beijing Emilion Science & Technology Co., Beijing, China). A manual sample injection valve with 20 mL loop (Beijing Emilion Science & Technology Co., Beijing, China) was used to introduce the sample into the column. A Model 3057 portable recorder (Yokogawa, Sichuan Instrument Factory, Chongqing, China) was used to draw the chromatogram.

The high-performance liquid chromatography (HPLC) in this study consists of a Waters 996 photodiode array detection (DAD), a Waters 600 Multisolvent Delivery, a Waters 600 system controller, a Waters 600 pump, and a Millennium 32 workstation (Waters, Milford, USA).

The Spe-ed™ supercritical fluid extraction (SFE) system (Applied Separations, Inc., Allentown, PA, USA) was used to extract the crude extract from the material.

1.3 Preparation of sample

Air-dried and ground *I. verum* Hook. f. (400 g) was placed into a 1 L extraction vessel and extracted statically for 1 h followed by further 6.5 h of dynamic CO_2 extraction under the pressure of 25 MPa with a

temperature of 35 ℃. The flow-rate of carbon dioxide supercritical fluid was set at 2 L/min, and the extract in the supercritical fluid was depressed directly into a separation vessel which yielded 53.1 g of crude extract for further isolation and purification by HSCCC.

1.4 Selection of two-phase solvent system

The composition of the two-phase solvent system was selected according to the partition coefficient (K_D) of the target compounds of the samples. The K_D was determined by HPLC as follows: 10 mg of the crude extract was added to a test tube, to which 2 mL of each phase of the two-phase solvent system was added. The test tube was shaken violently for several minutes. Equal volumes of each phase were then analyzed by HPLC to obtain the K_D. The K_D-value was defined as the peak area of compound in the upper phase divided by the peak area of compound in the lower phase[12].

1.5 Preparation of the two-phase solvent system and sample solution

The HSCCC experiments were performed with a two-phase solvent system composed of *n*-hexane-ethyl, acetate, methanol and water(1∶0.2∶1∶0.1, volume ratio, the same in the following). After thoroughly equilibrating the mixtures in a separation funnel at room temperature, two phases were separated shortly before use. The upper organic phase was used as stationary phase, and the lower aqueous phase as mobile phase. The sample solution was prepared by dissolving the crude sample in the mixture solution of organic phase and aqueous phase (1∶1) of the solvent system used for HSCCC separation.

1.6 Separation procedure

HSCCC was performed in the study. Firstly, the multilayer coiled column was entirely filled with the upper phase, and then the lower phase was pumped into the head end of the column inlet at a flow rate of 2.0 mL/min, while the column was rotated at 800 r/min. After hydrodynamic equilibrium was reached as indicated, the sample solution (1.3 g dissolved in 10 mL mixture consisting of equal volumes of each phase of the solvent system) was injected through the sample port. The effluent from the outlet of the column was continuously monitored by UV detector at 254 nm, and the peak fractions were collected according to the chromatogram. The retention of the stationary phase relative to the total column capacity was computed from the volume of the stationary phase collected from the column after the separation was completed.

1.7 HPLC and EI-MS analysis

The crude sample and each purified fraction from the HSCCC were analyzed by HPLC with a Shim-pack VP-ODS column (4.6 mm × 250 mm, 5 μm) and column temperature of 25 ℃. The mobile phase, a solution of methanol and water (80∶20), was set at a flow-rate of 1.0 mL/min. The effluent was monitored by DAD at 254 nm.

The identification of HSCCC peak fractions were performed by mass spectrometry (EI-MS) with an Agilent 5973N mass selective detector (MSD), and Pentium 4 computer with MSD Productivity Chemstation Software.

The mass spectrometer was scanned over the 29–400 u range at scan 1 s, with an ionizing voltage of 70 eV and an ionization current of 150 μA. Components were identified by their mass spectra compared with the mass spectra in commercial mass spectra library (Wiley and NIST 98).

2 Results and Discussion

2.1 Structures of three compounds

The chemical structures of three compounds were showed in Fig. 1.

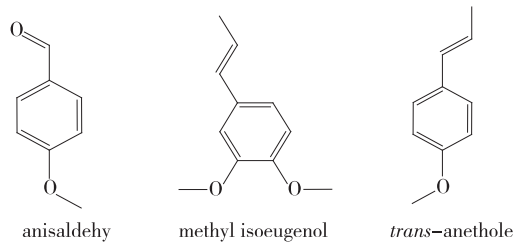


Fig. 1 Chemical structures of three compounds

2.2 Optimization of HSCCC conditions

The selection of the two-phase solvent system is the most important and difficult step. It is estimated that about 90% of the entire work in HSCCC is invested in solvent system selection[13-14]. K_D is the most important parameter in solvent system selection. Successful separation by HSCCC needs a suitable K_D-value. Large K_D-value usually tends to produce excessive sample band broadening, while small K_D-value results in a poor peak resolution[15].

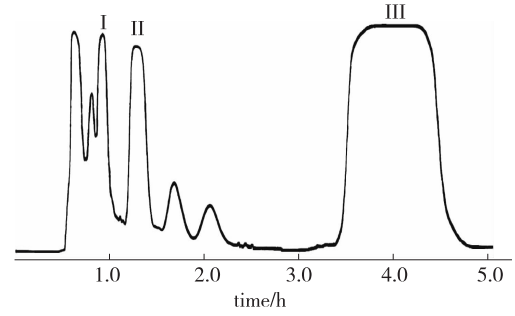
In this work, several two-phase solvent systems were tested and their K_D-values were measured and summarized in Table 1. When *n*-hexane-ethyl, acetate, methanol and water (1∶0.1∶1∶0.1) was used as the two-phase solvent system, compounds 1 and 2 were difficult to separate for the close K_D-value. While the K_D-value of compound 3 was too big with the two-phase solvent system *n*-hexane-ethyl acetate-methanol-water (1∶0.2∶1∶0.2), it and would result in a long separation time. Among these solvents, the ratio of *n*-hexane-ethyl, acetate, methanol and water (1∶0.2∶1∶0.1) was the best for separation. Fig. 2 shows the separation of HSCCC using this solvent system.

Table 1 Partition coefficients (K_D) of three compounds

solvent systems *n*-hexane-ethyl, acetate, methanol and water system	anisaldehyde(K_D1)	methyl isoeugenol(K_D2)	*trans*-anethole(K_D3)
1∶0.1∶1∶0.1	0.45	0.52	1.16
1∶0.2∶1∶0.1	1.42	1.95	6.30
1∶0.2∶1∶0.2	2.32	4.86	24.21

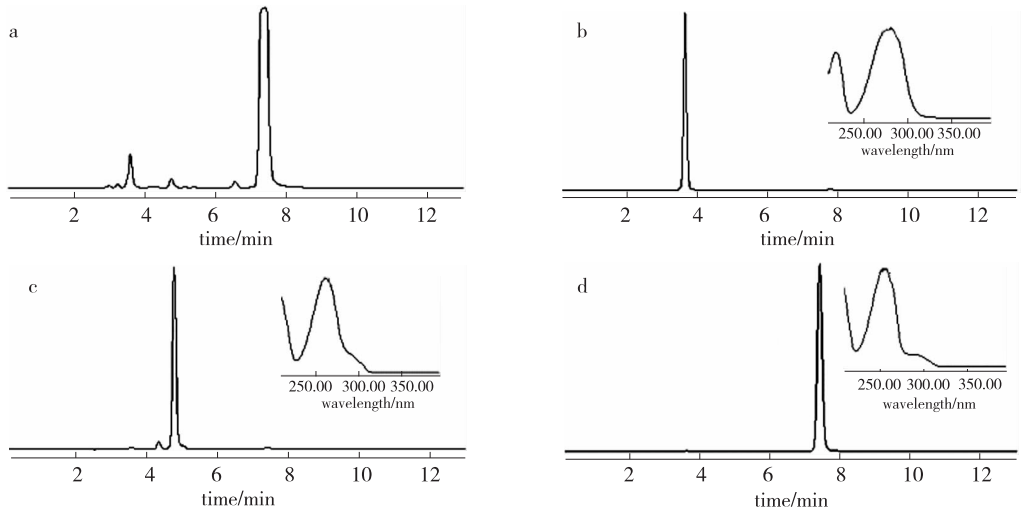
2.3 Separation results by HSCCC

The SFE extracts (1.3 g) from *I. verum* Hook. f. were isolated and purified under the optimum HSCCC conditions. The retention of the stationary phase was 65 %, and the total separation time was about 5 h. The HSCCC fractions were analyzed by HPLC, and their absorbance was measured at 254 nm to draw the elution curve (Fig. 3). Based on the HPLC analysis, three compounds were obtained in one-step separation and yielded 10.3 mg of anisaldehyde (peak I in Fig. 2), 7.1 mg of methyl isoeugenol (peak II in Fig. 2) and 636.5 mg of *trans*-anethole (peak III in Fig. 2). Their purity were 98.9 %, 96.8 % and 99.7 %, respectively (Fig. 3). Their structures were identified by their mass spectra compared with the mass spectra in commercial mass spectra library. Fig. 4 shows their corresponding EI-MS spectrogram.



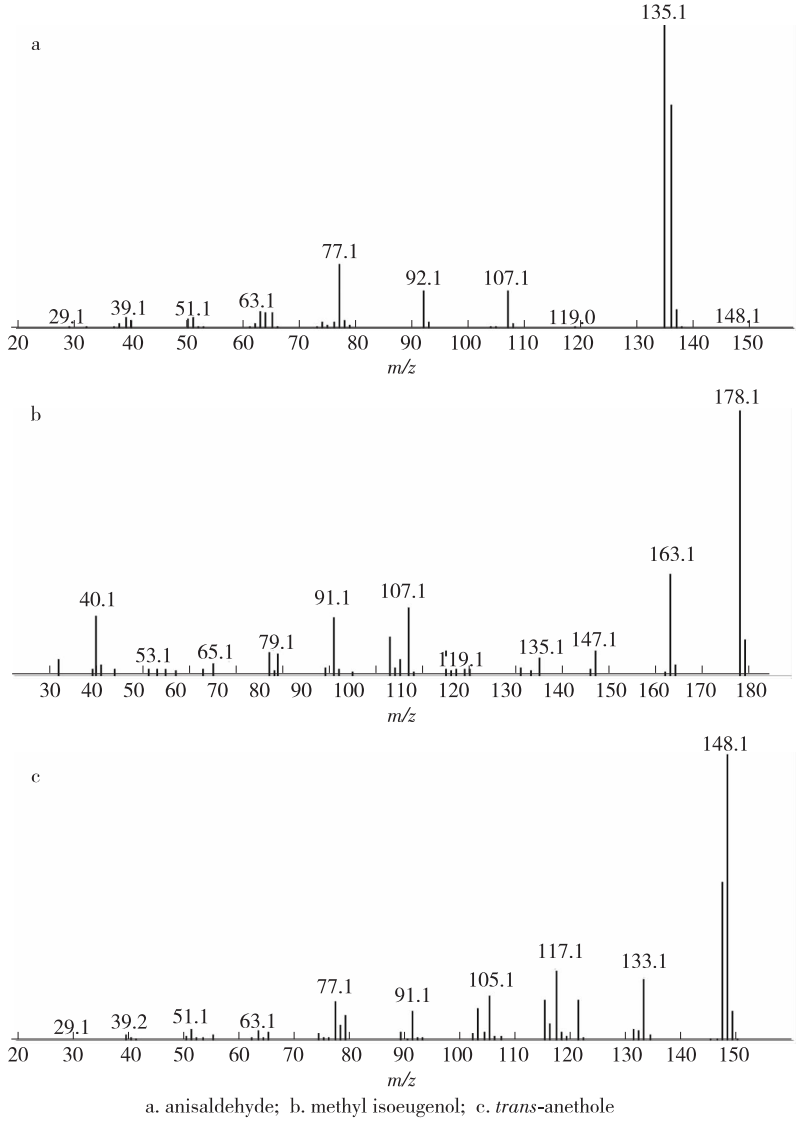
I. anisaldehyde; II. methyl isoeugenol; III. *trans*-anethole

Fig. 2 Chromatogram of the crude extract by HSCCC



a. crude extract; b. anisaldehyde; c. methyl isoeugenol; d. *trans*-anethole

Fig. 3　HPLC analysis and UV spectra of crude extracts and HSCCC fractions from *Illiciumm verum* Hook. f.



a. anisaldehyde; b. methyl isoeugenol; c. *trans*-anethole

Fig. 4　EI-MS spectrograms of the isolated compounds

3 Conclusion

Three compounds including anisaldehyde(10 mg), methyl isoeugenol(7 mg) and *trans*-anethole(640 mg) from *Illicium. verum* Hook. f. were extracted, separated and purified by SFE and HSCCC from 1.3 g of essential oil in one-step separation with the purity of 98.9%, 96.8 % and 99.7 % respectively. Supercritical CO_2 extraction was used under the pressure of 25 MPa and temperature of 35 ℃. HSCCC separation was composed of a two-phase solvent system of *n*-hexane-ethyl acetate-methanol-water(1∶0.2∶1∶0.1). The study demonstrates that SFE and HSCCC are very useful techniques for the extraction, isolation and purification of essential oil from natural products.

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