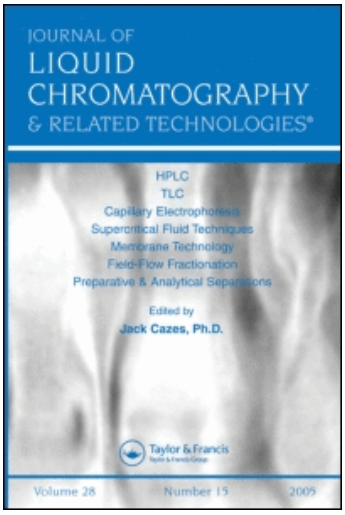

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PURIFICATION OF FISH OIL ETHYL ESTERS BY HIGH-SPEED COUNTERCURRENT CHROMATOGRAPHY USING NON-AQUEOUS SOLVENT SYSTEMS

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ABSTRACT

High-speed countercurrent chromatography (HSCCC) was applied to purification of fish oil ethyl esters (FOEE). The CCC separations were performed using non-aqueous two-phase solvent systems in two steps: The first purification was carried out with a hexane-acetonitrile system where 30 mL of crude FOEE was cleaned in 140 min yielding 23.1 mL of refined FOEE. The product was colorless and free of saturated fatty acid esters. The second and final purification was performed with a hexane-dichloromethane-acetonitrile (5:1:4, v/v/v) where 1 g of the refined FOEE was resolved into three fractions, ethyl hexadecenoate, ethyl octadecenoate and the third composed of

56.4% of ethyl docosahexaenoate and 39.3% of ethyl eicosapentenoate. The separation was completed in 145 min. However, polyunsaturated fatty acids are not resolved with this solvent system due to their low partition coefficients.

INTRODUCTION

Since the last decade, high-speed countercurrent chromatography (HSCCC) has been extensively applied to separation and purification of natural products mostly using organic/aqueous two-phase solvent systems.[1,2]

This paper describes purification of a gram quantity of fish oil ethyl esters (FOEE) which contains two major components, ethyl docosahexaenoate (EDHA) and ethyl eicosapentenoate (EEPA). It is claimed that these esters possess the medicinal property of preventing cardiovascular diseases and exhibit higher antioxidant activities than the free acid form.[3,4] Using two different non-aqueous two-phase solvent systems, these major components were separated as a mixture from minor components.

Recently, an analytical-scale separation of polyunsaturated fatty acids by HSCCC has been reported using organic-aqueous two-phase solvent systems. Application of the method to a gram quantity, however, produced a mixture of 4 components.[5] On the other hand, the HSCCC separation of polyunsaturated fatty acid ethyl esters has not been reported.

EXPERIMENTAL

Apparatus

HSCCC experiments were performed using a coil planet centrifuge equipped with a multilayer coil separation column that was designed and fabricated at the Beijing Institute of New Technology Application, Beijing, P. R. China. The multilayer coil was prepared by winding a 1.6 mm ID PTFE (polytetrafluoroethylene) tube coaxially onto the column holder hub. The total column capacity measured 230 mL.

The HSCCC centrifuge was rotated at 800 rpm with an 8 cm revolution radius. The system was equipped with an FMI pump (Zhejiang Instrument Factory, Hangzhou, P. R. China), a fraction collector and a sample injection valve.

Reagents

Hexane, acetonitrile and dichloromethane were of an analytical grade and purchased from Shanghai Chemical Factory, Shanghai, China. A standard sample mixture for fatty acid ethyl esters was a gift from the Second Oceanography Institute, the Ocean Bureau of P.R. China, Hangzhou, China.

Materials

The fish oil ethyl esters (FOEE), a yellow oily liquid, was purchased from Puto Medicine factory, Zhousan, Zhejiang, P. R. China. This material was used for HSCCC refinement.

HSCCC Refinement of Crude FOEE

The HSCCC experiment was performed with a non-aqueous two-phase solvent system composed of hexane-acetonitrile. The solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature and the two phases were separated shortly before use. In each refinement the multilayer coil column was first entirely filled with the upper stationary phase. Then the lower mobile phase containing 30 mL of crude FOEE was pumped into the inlet of the column at a flow rate of 3.0 mL/min, while the apparatus was rotated at 800 rpm. After 220 mL of mobile phase was pumped and relatively polar impurities were eluted from the column, the HSCCC centrifuge was stopped and the column contents were collected by pressure with nitrogen. The contents were evaporated to dryness to yield the refined FOEE which was further subjected to separation as described below.

Separation of the Refined FOEE

Final purification was performed with a non-aqueous solvent system composed of hexane-dichloromethane-acetonitrile (5:1:4, v/v/v). The solvent mixture was equilibrated and separated as described earlier. In each separation, the multilayer coil was first entirely filled with the upper stationary phase, followed by injection of sample solution (1 mL of the refined FOEE in 10 mL of the mobile phase) through the injection valve. Then the mobile phase was pumped into the inlet of the column at a flow rate of 2.0 mL/min, while the apparatus was rotated at 800 rpm. The effluent from the outlet of the column was collected into test tubes using a fraction collector. Each fraction was subjected to GC analysis for fatty acid ethyl esters.

GC Analysis of Fatty Acid Ethyl Esters

A Shimadzu GC-7AG gas chromatography equipped with a flame ionization detector (FID) was used for GC analysis of fatty acid ethyl esters. GC separations were performed on a Bpl capillary column (50 m X 0.22 mm ID) (SEG Company). The temperature was programmed from 150°C to 280°C at the rate of 3°C/min increment, while the temperature at the injector and the detector was kept constant at 280°C. The carrier gas was nitrogen.

RESULTS AND DISCUSSION

Refinement of Crude FOEE

GC analysis of the FOEE showed that it contained 1.1% of ethyl myristate, 1.7% of ethyl hexadecenoate, 1.8% of ethyl palmitate, 5.5% of ethyl octadecenoate, 0.9% of ethyl octadecanoate, 28.8% of ethyl eicosapentenoate (EEPA) and 41.0% of ethyl docosahexaenoate (EDHA) (Fig. 1 A). Using HSCCC 30 mL of the FOEE was refined with the non-aqueous solvent system of hexane-acetonitrile to produce 23 mL of colorless refined FOEE which contained 51.6% of EDHA, 36.0% of EEPA, 6.7% of ethyl octadecenoate and 2.0% of ethyl hexadecenoate (Fig. 1 B).

We found that refined FOEE was colorless and free of saturated fatty acid esters. It appears that unsaturated fatty acid esters are more hydrophobic and therefore have higher partition coefficients than the saturated fatty acid esters.

Separation of Refined FOEE

Table 1 shows the separation of 1 g of the refined FOEE by HSCCC with the hexane-dichloromethane-acetonitrile (5:1:4, v/v/v) system. Three components I, II and III were obtained. Component III (corresponding to peak C16:1 in GC) and component II (corresponding to peak C18:1 in GC) were ethyl hexadecenoate and ethyl octadecenoate, respectively (see Fig. 1 A and B).

Component I (corresponding to peak C20:5 and C22:6 in GC in Fig. 1 A and C) was a mixture of EDHA and EEPA. Fractions containing component I was collected and dried in vacuum to yield 910 mg of mixture containing 56.4% of EDHA and 39.3% of EEPA (Fig. 1 C).

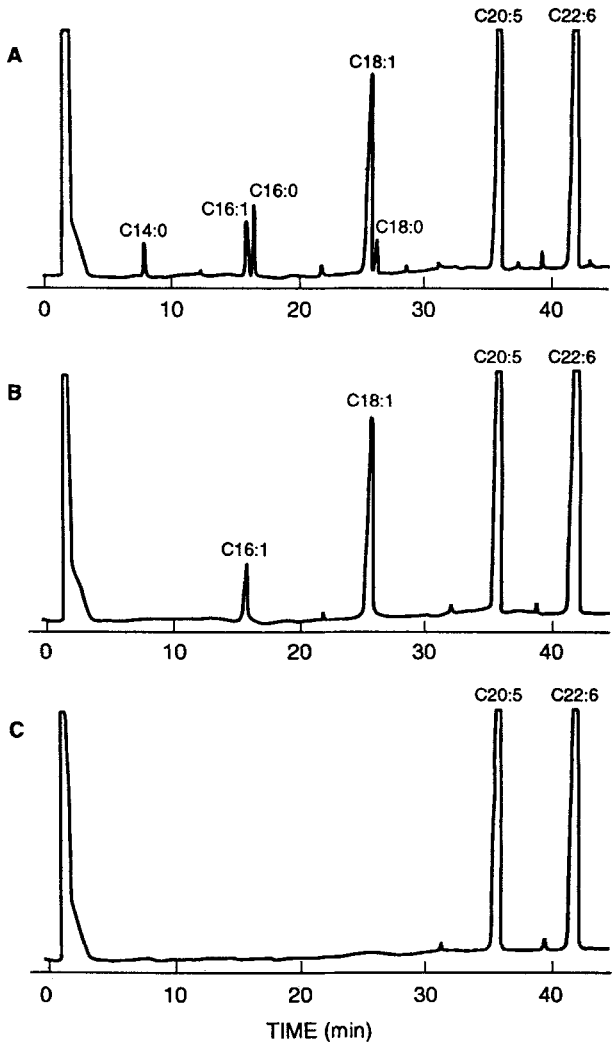

Figure 1. GC analysis of three samples. A: Fish oil ethyl ester (FOEE); B: the refined FOEE obtained from HSCCC refinement of crude FOEE; C: component I obtained from HSCCC separation of the refined FOEE. C14:0 (ethyl myristate), C16:1 (ethyl hexadecenoate), C18:1 (ethyl octadecenoate), C18:0 (ethyl octadecanoate), C20:5 (ethyl eicosapentenoate) (EEPA), C22:6 (ethyl docosahexaenoate) (EDHA).

Table 1

GC Analysis of HSCCC Fractions Obtained from the Refined FOEE

Fraction No.[1]	Retention Time (min)	Detection[2]	Component[3]
1	35	-	
3	40	-	
5	45	-	
7	50	+	I
9	55	++	I
11	60	++++	I
13	65	+++++	I
15	70	++++++	I
17	75	+++++	I
19	80	++++	I
21	85	+++	I
23	90	++	I
25	95	+	I
27	100	-	
29	105	+	II
31	110	+	II
33	115	+	II
35	120	+	II
37	125	-	
39	130	+	III
41	135	+	III
43	140	+	III
45	145	-	

[1] Fraction volume: 5 mL/min.

[2] GC analysis: 0.5 μL of each fraction was injected. -: no fatty acid ester. +: small amounts of fatty acid esters. ++ to ++++++: larger amounts of fatty acid esters.

[3] Component I: a mixture of ethyl docosahexaenoate (EDHA) and ethyl eicosapentenoate (EEPA); Component II: ethyl octadecenoate; Component III: ethyl hexadecenoate.

The overall results of the present studies indicate that HSCCC can be used for separation and purification of fatty acid ethyl esters with non-aqueous solvent systems. The method may play an important role in the field of oil chemistry in future.

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