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Enrichment of eicosapentaenoic acid and docosahexaenoic acid esters from esterified fish oil by programmed extraction–elution with supercritical carbon dioxide

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ABSTRACT

Methyl esters of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in esterified fish oil were extracted by supercritical fluid extraction with carbon dioxide and directly introduced into a silica gel column coated with silver nitrate. Supercritical fluid chromatography with carbon dioxide was then performed by changing stepwise the pressure of the column outlet. The EPA and DHA methyl esters thus separated were fractionated by reducing the pressure of column effluent to atmospheric. In this way, EPA and DHA methyl esters were enriched from 12% to 93% and from 13% to 82%, respectively.

INTRODUCTION

Recent studies have revealed that polyunsaturated fatty acids can prevent diseases such as arteriosclerosis and myocardial infarction by lowering the concentration of lipids and cholesterol in blood. This stimulated considerable interest in the enrichment of these fatty acids, especially, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). It is known that fish oil is a rich source of such fatty acids. These polyunsaturated fatty acids exist in the form of triglycerides.

A number of methods have been developed to isolate EPA and DHA from esterified fish oil, including molecular distillation[1], liquid chromatography (LC)[2–5], supercritical fluid extraction (SFE) with carbon dioxide[6,7] and supercritical fluid chromatography (SFC) with carbon dioxide[8]. SFE with carbon dioxide permits operation at moderate temperatures and treatment of samples without exposure to oxygen; this advantage prevents compounds with many C = C double bonds from being oxidized by oxygen and from being degraded by heat. Further, SFE allows fractionation by simply reducing the pressure of carbon dioxide to atmospheric; this results in precipitation of solutes because of a significant decrease in their solubility. However, a simple SFE system does not have sufficient selectivity to obtain highly purified EPA and DHA. Therefore, SFE in combination with other techniques was examined previously, *e.g.*, SFE–reflux column[6,9] and SFE–urea adduction[10].

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In LC, it is known that a silica gel column coated with silver nitrate is very suitable for separation of alkenes with *cis* configurations from *n*-alkanes, because *cis*-alkenes form silver chelates that are adsorbed on the stationary phase more strongly than *n*-alkanes. The use of this technique for the concentration of esters of EPA and DHA has been reported[11,12]. If the compounds behave in the same way in supercritical carbon dioxide mobile phase, then SFC using a silver nitrate-coated silica gel column can enrich EPA and DHA esters efficiently. Such a separation system will have the advantages of both SFE and LC.

EXPERIMENTAL

Materials

Fish oil was derived from sardines (Hokkaido, Japan). Hydrogen chloride–methanol solution (Reagent 10) was obtained from Tokyo Kasei (Tokyo, Japan) and silver nitrate from Wako (Osaka, Japan). Silica gel (10–20 μm, 90 Å) was purchased from Nomura Kagaku (Aichi, Japan). Standard-grade carbon dioxide was obtained from Toyoko Kagaku (Kanagawa, Japan) and was used as the extraction medium and mobile phase. The fish oil was esterified with the hydrogen chloride–methanol solution in the usual way[13].

The separation column was prepared as follows. A 1-g amount of silver nitrate

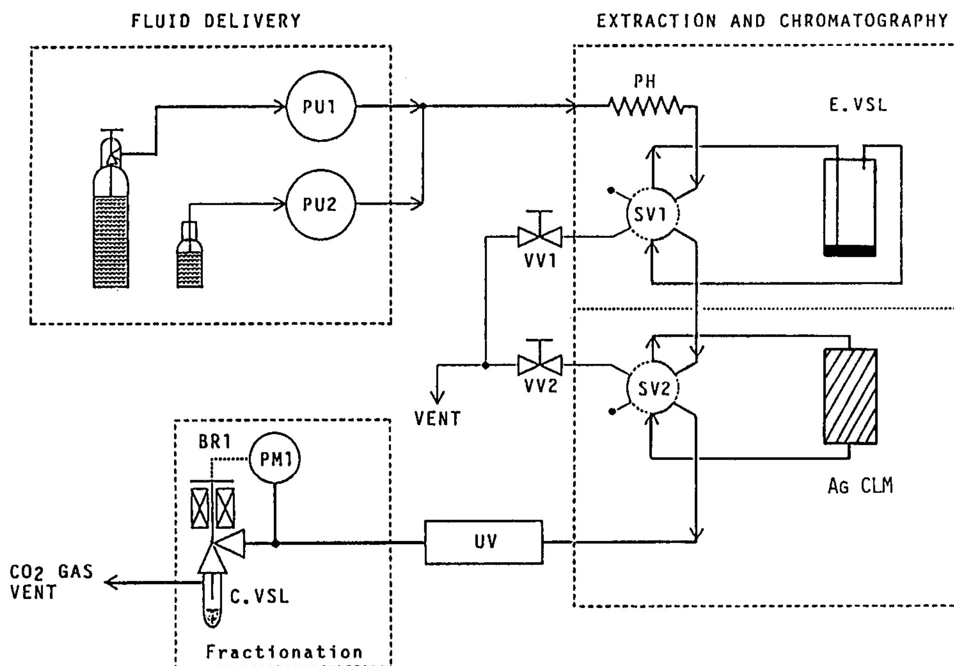

Fig. 1. Schematic diagram of the system. Components: PU1 = CO_2 pump; PU2 = modifier pump; PH = preheating coil; SV1 and SV2 = six-way switching valves; E.VSL = extraction vessel; Ag CLM = separation column; UV = photodiode-array UV detector; PM1 = pressure transducer; BR1 = back-pressure regulator; C.VSL = collection vessel.

dissolved in 50 ml of acetonitrile was added to 10 g of silica gel and the mixture was evaporated to dryness at 40°C. A 6-g amount of the residue obtained was packed into a stainless-steel tube (125 mm × 10 mm I.D.).

Apparatus

A Jasco (Tokyo, Japan) Super-200 System 3 (Fig. 1) was used. It consisted of three sections: fluid delivery, extraction and chromatography, and fractionation. The fluid-delivery section included two pumps, which delivered liquid carbon dioxide and a modifier solvent separately. In the extraction and chromatography section, programmed extraction–elution was performed with supercritical carbon dioxide modified with ethanol. The column effluent was monitored with a Jasco Multi-330 photodiode-array multi-wavelength UV detector. The fractionation section included a back-pressure regulator, which kept the pressure of the extraction vessel and the column at a desired value[14]. The column effluent flowing through the regulator reduced its pressure to atmospheric and thereby the solubility of the solutes in the effluent was reduced virtually to zero. In this way, the solutes were deposited and collected in the collection vessel.

An HP 5890A capillary gas chromatograph (Hewlett-Packard, Avondale, PA, U.S.A) was used for the determination of the EPA and DHA methyl ester contents in each step. Each fraction was dissolved in *n*-hexane or dichloromethane at a concentration of 10 or 20 mg/ml and a 1-μl portion was injected into the column.

SFE and SFC

A 0.5-ml volume (445 mg) of the esterified fish oil was placed in the extraction vessel (E.VSL), which was then connected to the separation column (SEP.CLM) by a switching valve (SV1) and SFE was performed for 20 min at 8 MPa and 40°C. The flow-rate of liquid carbon dioxide was 9 g/min. Thus, the extract from the fish oil was directly introduced into the separation column.

When the extraction was completed, the extraction vessel was bypassed by the switching valve (SV1), then SFC was performed by a programmed extraction–elution method. The pressure of the back-pressure regulator (BR1) was changed stepwise to 8, 12 and 20 MPa and ethanol was delivered as a modifier[15,16]. In this way, the column effluent was fractionated into five portions: fraction 1 (8 MPa, 0–110 min), fraction 2 (8 MPa, 110–180 min), fraction 3 (12 MPa, 180–250 min), fraction 4 (20 MPa, 250–310 min) and fraction 5 (20 MPa, 310–370 min). In the last fractionation, the flow-rate of liquid carbon dioxide was changed from 9 to 5 g/min and at the same time the modifier was added to the carbon dioxide at a flow-rate of 0.1 ml/min.

RESULTS AND DISCUSSION

The extraction vessel was used in order to introduce only constituents of the esterified fish oil that are soluble in supercritical carbon dioxide into the separation column. This was successful in the fractionation of EPA and DHA methyl esters. However, direct injection of the esterified fish oil was unsuccesful in this fractionation, because constituents of the esterified fish oil insoluble in supercritical carbon dioxide precipitated and covered the stationary phase, resulting in a decrease in the selectivity of the column.

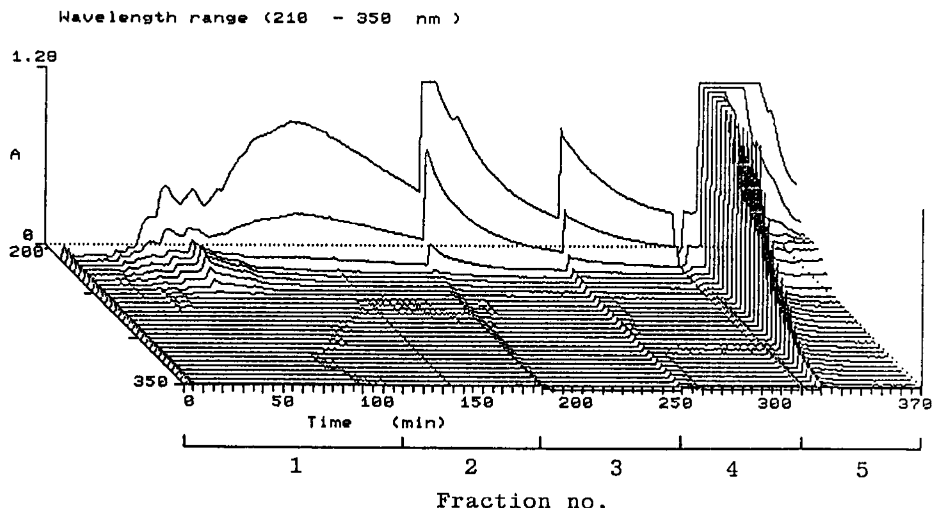

Fig. 2. Three-dimensional chromatogram obtained from SFC of esterified fish oil. The numbers under the time axis correspond to fraction numbers.

A three-dimensional chromatogram obtained from SFC of the esterified fish oil is shown in Fig. 2. Fractionation was achieved by means of real-time monitoring of the column effluent.

The gas–liquid chromatogram of the esterified fish oil is shown in Fig. 3. Peaks of fatty acid methyl esters from C_{14} to C_{22} appear. The peak areas of EPA and DHA methyl esters relative to those of all the other peaks except the solvent peak were 12% and 13%, respectively.

Each fraction obtained was of oil. The gas–liquid chromatograms of fractions 1–5 are shown in Figs. 4–8. Each chromatogram indicates the EPA and DHA methyl ester contents of the corresponding fraction. Fraction 1 (8MPa, 0–110 min) included mainly C_{16} and C_{18} fatty acid methyl esters. However, EPA and DHA methyl esters were not detected by gas–liquid chromatographic (GLC) analysis. Fraction 2 (8MPa, 110–180 min) included 57% of EPA methyl ester and 39% of other fatty acid methyl esters from C_{16} to C_{18}. Fraction 3 (12 MPa, 180–250 min) included 93% of EPA methyl ester. However, DHA methyl ester was not detected by GLC. Fraction 4 (20 MPa, 250–310 min) included 46% of EPA methyl ester and 18% of DHA methyl ester. Fraction 5 (20 MPa, 310–370 min), which was obtained with addition of the modifier (ethanol), included 82% of DHA methyl ester. However, EPA methyl ester was not detected by GLC. Because the solvent power of supercritical carbon dioxide increases with increase in its pressure or with addition of a modifier, these GLC results indicate that adsorption of fatty acid methyl esters on the stationary phase increases with increase in the number of C=C double bonds.

The amount of each fraction, the EPA and DHA methyl ester contents of the fraction and the recoveries of these esters are given in Table I. SFE of 0.5 ml (445 mg) of the esterified fish oil yielded 352 mg of oil. The contents of both EPA and DHA

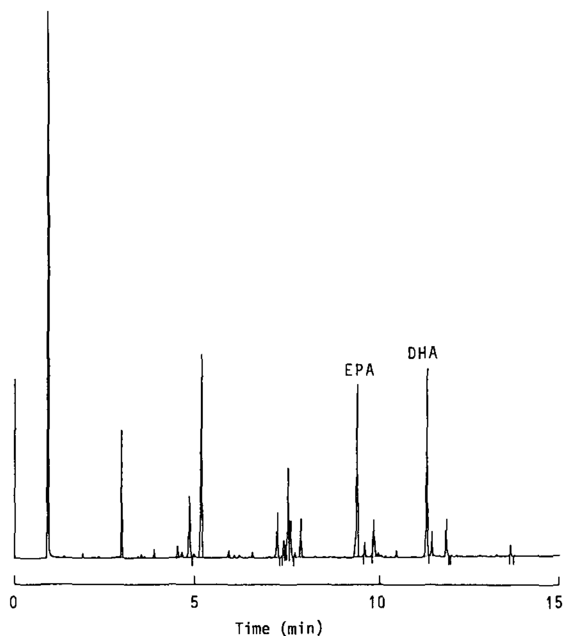

Fig. 3. GLC of esterified fish oil. Conditions: column, HP-1 (cross-linked methylsilicone, 20 m × 0.2 mm I.D.; Hewlett-Packard); detector, flame ionization; column temperature, initial 200°C, held for 5 min, increased at 10°C/min to 300°C; injection volume, 1 μl (splitting ratio = 1:100); injection temperature, 300°C; carrier gas, helium at 180 kPa.

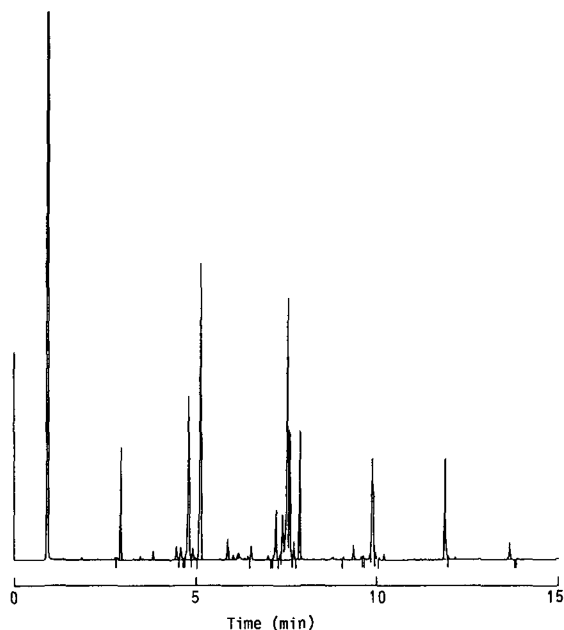

Fig. 4. GLC of fraction 1. Conditions as in Fig. 3.

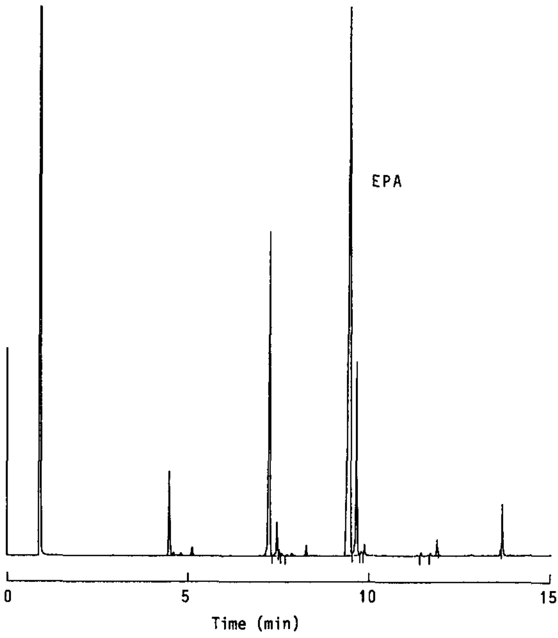

Fig. 5. GLC of fraction 2. Conditions as in Fig. 3.

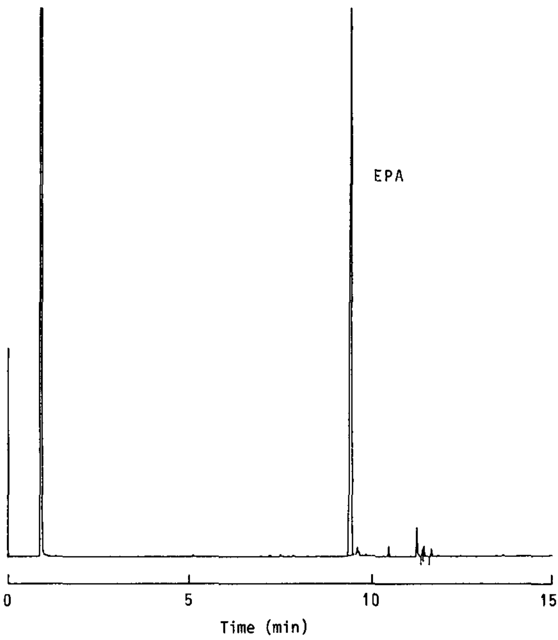

Fig. 6. GLC of fraction 3. Conditions as in Fig. 3.

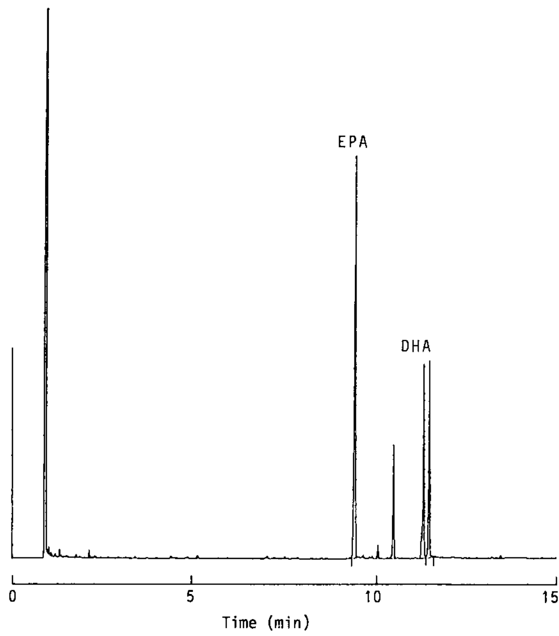

Fig. 7. GLC of fraction 4. Conditions as in Fig. 3.

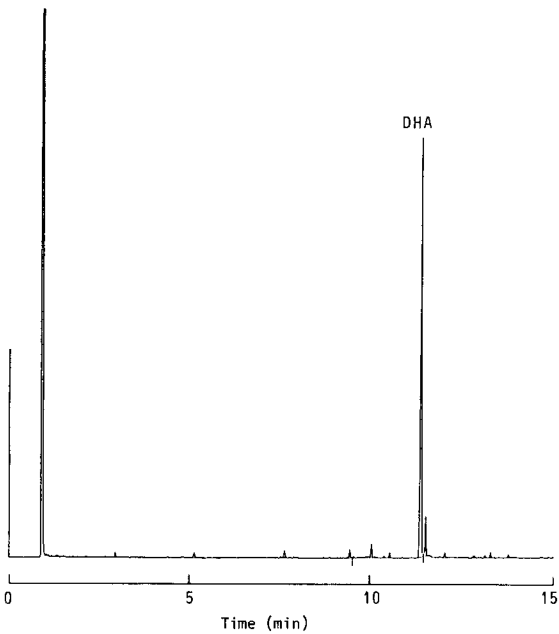

Fig. 8. GLC of fraction 5. Conditions as in Fig. 3.

TABLE I

EPA AND DHA METHYL ESTER CONTENTS IN EACH STEP AND THEIR RECOVERIES

Starting material or fraction	*EPA content (recovery, %[a])*	*DHA content (recovery, %[a])*	*Amount (mg)*
Fish oil	12% (53.4 mg, 100%)	13% (57.9 mg, 100%)	445
SFE fraction	13% (45.8 mg, 86%)	13% (45.8 mg, 79%)	352
SFC fraction No.:			
1	–	–	88.9
2	57% (7.5 mg, 14%)	–	13.1
3	93% (11.3 mg, 21%)	–	12.2
4	46% (1.2 mg, 2%)	18% (0.5 mg, 1%)	2.6
5	–	82% (39.2 mg, 68%)	47.8
Total	12% (20.0 mg, 37%)	24% (39.7 mg, 69%)	164.6

[a] Recovery (%) = $\frac{\text{amount of EPA (DHA) ester in each fraction}}{\text{amount of EPA (DHA) ester in the esterified fish oil}} \cdot 100.$

methyl esters in this oil were shown to be 13% by GLC. With reference to the amount of EPA methyl ester included in the esterified fish oil (53.4 mg, 12% of 445 mg), the total amount of EPA methyl ester obtained by SFC (fractions 2, 3 and 4) was 20 mg; the recovery was 37%. This low recovery may be due to the EPA methyl ester being sprayed out of the regulator outlet nozzle and taken by gaseous carbon dioxide to the vent line in collection. This recovery can be increased by adding a small amount of ethanol to supercritical carbon dioxide containing EPA methyl ester just upstream of the regulator. With this arrangement the compound can be collected as an ethanolic solution. The recovery of DHA methyl ester was then calculated to be 69%.

Although the separation column was loaded with esterified fish oil weighing nearly 6% of the weight of the stationary phase, 11.3 mg of highly purified EPA methyl ester and 39.2 mg of highly purified DHA methyl ester were obtained by using a 125 mm × 10 mm I.D. column. This result indicates that the stationary phase has a good selectivity for polyunsaturated fatty acid methyl esters, and that the separation column has a high loading capacity in spite of its small dimensions.

In conclusion, the programmed extraction–elution method provided the isolation of highly purified EPA and DHA methyl esters; preconcentration of these esters with urea was not needed before SFC. In this method, solute–fluid separation was performed by simply reducing the pressure of carbon dioxide to atmospheric; fractionation in LC need to be followed by removal of the solvents used for elution, such as methanol, acetonitrile and water.

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