Industrial High-Performance Liquid Chromatography Purification of Docosahexaenoic Acid Ethyl Ester and Docosapentaenoic Acid Ethyl Ester from Single-Cell Oil

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ABSTRACT: The use of polyunsaturated fatty acids (PUFA) as medicine or in functional diets requires high purity. An industrial purification method for PUFA from *Schizochytrium* sp. SR21 oil was investigated. This oil contains fewer unwanted components than fish oils. Docosahexaenoic acid and docosapentaenoic acid ethyl esters (DHA-E and DPA-E) were prepared by treatment of this oil with ethanol and 1 N potassium hydroxide in hexane. DHA-E and DPA-E were purified by an industrial high-performance liquid chromatography (HPLC) plant. The separation plant consists of two columns (400 mm i.d., 1,000 mmL) with temperature-controlled water jackets and double-plunger (four heads) injection and eluent pumps. This plant was computer-controlled and equipped with an explosion–prevention system. The packed material was octadecylsilica (reverse-phase ODS), and the eluent was methyl alcohol/water (98:2). DHA-E and DPA-E from single-cell oil were highly purified by this industrial HPLC method in a one-step process. The DHA-E and DPA-E obtained were better than 99% purity.

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KEY WORDS: DHA, docosahexaenoic acid ester, n-6 docosapentaenoic acid ester, DPA, preparative high-performance liquid chromatography, single-cell oil.

Polyunsaturated fatty acids (PUFA), including eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and docosapentaenoic acid (DPA), have attracted our interest for functional diets and as medication (1,2). These applications require high purity of the PUFA.

The main resource for PUFA is fish oil. Purification of DHA and EPA from fish oil is difficult and requires many sophisticated processes, such as fractional extraction with urea (3,4), the silver salt solution method (5), molecular distillation (3), and supercritical fluid chromatography with silver-loaded clay (4).

Recently, large-scale preparative high-performance liquid chromatography (HPLC) has become of interest for application in industrial separations and purifications. In this paper, we describe a preparative (HPLC) method for the purification of PUFA, that consists of stainless-steel columns packed with medium-size particles, which is capable of working at medium to high pressures (1000 to 1500 psi). This equipment is analogous to analytical HPLC except for its size.

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MATERIALS AND METHODS

Single-cell oil. The single-cell oil from *Schizochytrium* sp. SR21 that contains DHA and DPA was developed by the National Institute of Bioscience and Human Technology (Tsukuba, Japan), Nagase Biochemicals, Ltd. (Fukuchiyama, Japan), and Suntory (Osaka, Japan) (6). Cell growth in a fermenter was carried out in a medium that contained glucose, corn steep liquor, $(NH_4)_2SO_4$, and KH_2PO_4 in a half-salt concentration of artificial seawater (6).

Esterification of single-cell oil. The dried cells were extracted by mixing them with glass beads at 10,000 rpm for 5 min in a chloroform/methanol (1/2, vol/vol) solution in a homogenizer. The extracted total lipid was fractionated into polar lipid and neutral lipid by liquid/liquid partitioning in hexane and 90% aqueous methanol. Neutral lipid was dissolved in hexane (1:100, vol/vol) and esterified by 1 N potassium hydroxide/ethanol (oil/ethanol, 1:5, vol/vol) for 30 min at 10–15°C. The hexane solution of the esterified oil was washed with water, and the esterified oil was obtained by fractional distillation of the hexane solution. The yield of esterification was 70%.

Fish oil ester. Fish oil ester (DHA-60E; Nihonkagakushiryo, Hakodate, Japan) was used as starting oil as a control in a comparative study. This esterified fish oil was reasonably pure and was not derived from a direct esterification of crude oil.

Analysis. Analyses of the samples were performed with a gas chromatograph (GC; M-80B, Hitachi, Tokyo, Japan) equipped with a capillary column (TC-1; GL Science, Tokyo, Japan) under temperature programming (100 to 300°C at 10°C/min).

Analyses of the fractionated samples on the industrial preparative HPLC were performed with analytical HPLC (LC6A; Shimadzu, Kyoto, Japan) with a column of i.d. 4.6 mm × 250 mm with 5-µm particle size, 120-Å pore size,

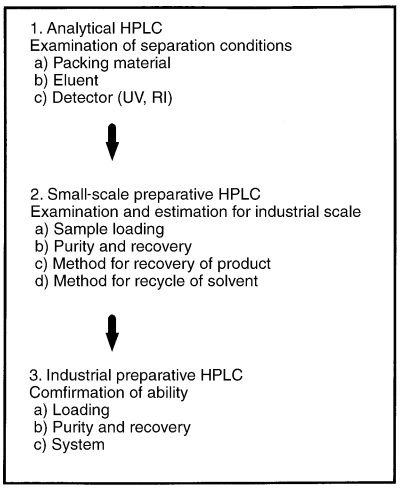
spherical particles, and 17% carbon content (YMC-Pack ODS AM-303; YMC, Kyoto, Japan). The eluent consisted of acetonitrile/water (97.5:2.5, vol/vol). The elution was isocratic, and the flow rate was 1.5 mL/min. Sample detection was conducted at 220 nm wavelength. Samples (2.5 μL) of a 1-g sample in 10 mL methanol solution were injected.

Purification. To determine the purification conditions for industrial-scale preparative HPLC, an analytical HPLC and a small-scale preparative HPLC were used as shown in Scheme 1. First, the packing material, eluent, and detector were examined in the analytical HPLC device. The loading amounts, recovery ratio, purity, and methods of recovery of product and solvent were conducted with the small-scale preparative HPLC. Then, these conditions were confirmed with the industrial-scale preparative HPLC. The best way to achieve a scale-up purification is to use the same packing material and solvent composition. Under these conditions, the scale-up factor depends on the ratio of cross-section of the columns. Scheme 2 summarizes the preparation of esterified DHA and DPA.

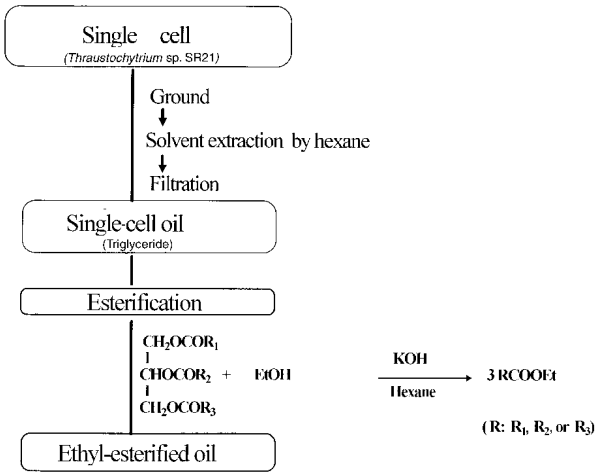
The industrial preparative HPLC separation of esterified DHA and DPA, obtained from both fish oil and single-cell oil, was compared with that on an analytical HPLC.

Small-scale preparative HPLC. A small-scale preparative HPLC was used to study the relationships of the loading conditions (injection volume, concentration), the recovery ratio, and the purity of fractions. The apparatus was an LC8A (Shimadzu) with a column of 6 mm i. d. × 2,000 mm with 50-μm particle size, 120-Å pore size and sphere-shaped gel (YMC-Gel ODS AM 120-S50, YMC). The three different eluents were methanol/water (vol/vol) 100:0, 98:2, and 96:4. The injection sample was prepared at the concentration of 10 vol% in methanol, and the loading amounts were 100, 300, or 500 mg. The elution was isocratic, and the flow rate was 1 mL/min. Sample detection was conducted by ultraviolet light (220 nm) and refractive index (RI) analyzers.

We divided the eluate into 30 fractions from 75 to 105 min retention time at 1-min intervals and analyzed the purity and recovery ratio of each fraction.



SCHEME 1



SCHEME 2

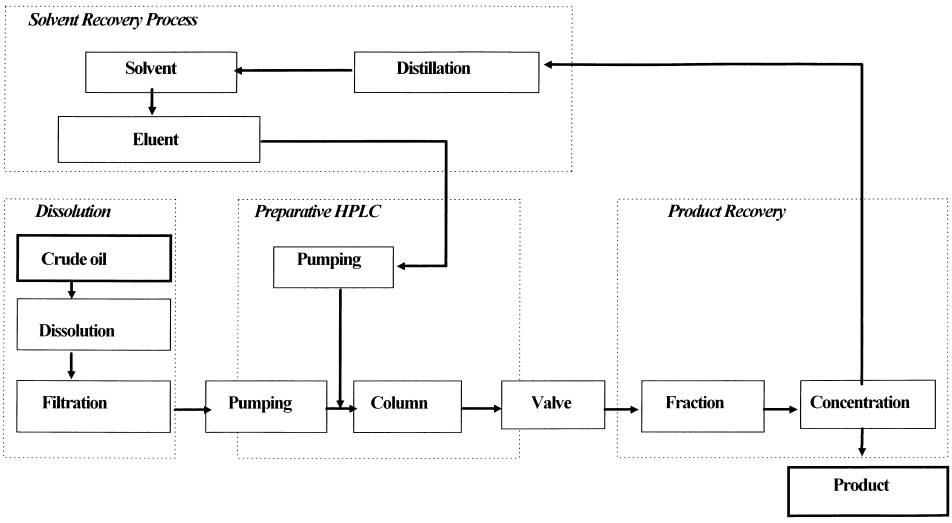
Industrial preparative HPLC. The preparative HPLC, which was controlled by a computer and equipped with an explosion-proof system, was designed and manufactured by YMC. The crucial part of the chromatographic system is the column. The two columns of 400 mm i.d. and 1,000 mm length were provided with temperature-controlled water jackets.

The injection pump was of the double-plunger (four heads) type, and its maximum flow rate was 20 L/min. The eluent pump was also a double-plunger (four heads) type, and its maximum flow rate was 30 L/min. Sample detection was effected at 220 nm with a variable-wavelength absorbance detector (ES-3760, Somakougaku Co., Ltd., Tokyo, Japan). The response was monitored with a personal computer display and a linear recorder. The system also had a dissolution tank, an eluent tank, a fraction tank, a condenser for product oil, and a distillation column for recycling the solvent. Column switching may be employed in this system. A flow diagram for the purification of the ethyl esters of DHA and DPA (DHA-E and DPA-E) appears in Scheme 3.

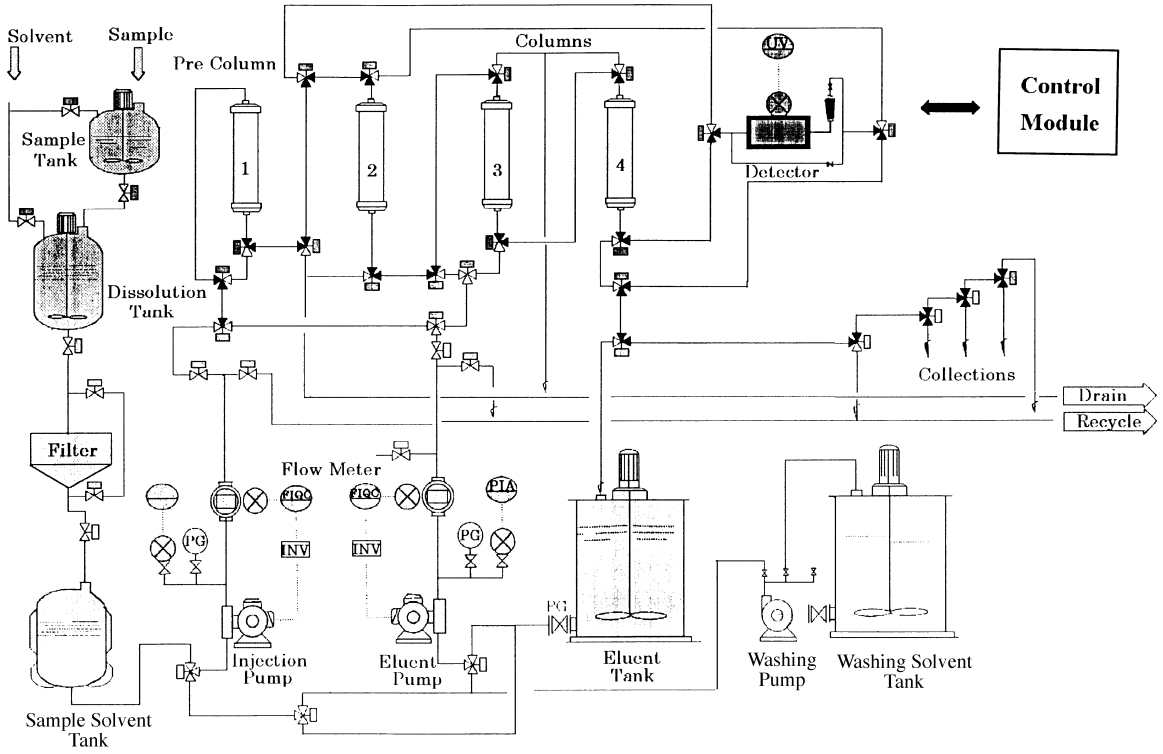
There are four major processes, namely sample dissolution, preparative HPLC, product recovery, and solvent recovery. The flow diagram of industrial preparative HPLC is shown in Scheme 4, and a photograph of the industrial preparative HPLC columns is shown in Figure 1. In Scheme 3, PIA, PG, INV, and FIQC mean, respectively, pressure indicator alarm, pressure gauge, inverter, and float indicator control.

We operated the industrial preparative HPLC under the following conditions, obtained as the result of the small-scale preparative HPLC series. These conditions were changeable according to column size: column, two 400-mm i.d. × 1,000 mm length reverse-phase YMC Gel ODS-AM columns; eluent, methanol/water (98:2, vol/vol); sample concentration, 10 vol% in methanol; loading volume, 6.6 L, total loading amount, 1.33 kg/cycle; flow rate, 4.4 L/min.

The success of producing purified materials of interest



SCHEME 3



SCHEME 4

with preparative HPLC depends on the selection of various factors, such as quality of the packing material, column design, chromatographic conditions, kind of eluent solvent, and loading volume.

RESULTS

High-performance liquid chromatograms of esterified fish oil and single-cell oil along with their separation factors (α) are shown in Figure 5. The separation factor between EPA-E and DHA-E is $\alpha 1$, and the separation factor between DHA-E and unknown component (Peak-X) is $\alpha 2$ in fish oil. The separation factor between DHA-E and DPA-E is $\alpha 3$, and the separation factor between DPA-E and unknown component (Peak-Y) is $\alpha 4$ in single-cell oil. The separation factors of the main components of esterified single-cell oil and esterified fish oil are listed in Table 1. The separation factors of esterified fish oil are roughly 1.1. On the other hand, the separation factors of single-cell oil are roughly 1.3.

FIG. 1. Photograph of columns for industrial preparative high-performance liquid chromatography.

Figure 3 shows an example of small-scale preparative HPLC. The eluate in the range between DHA-E and DPA-E (75–105 min retention time) was collected in 30 fractions, and each fraction was analyzed with regard to its purity and recovery. The fractions with more than 99% purity were collected, and the overall purity and recovery ratio were calculated.

The relationships between loading amounts and recovery ratio (more than 99% purity of esterified DHA and DPA) in three different eluent systems for 99% purity are shown in Figure 4. Therefore, we selected the following conditions for industrial preparative HPLC: The eluent was 98% methanol/2% water; the flow rate was 1 mL/min: the loading was 300 mg per cycle; the concentration of the sample solution was 10% methanol; and the purification of each cycle required 120 min. Suitable fractions with more than 98% purity were collected, and their purity and recovery ratio were measured. The results of DHA-E and DPA-E purification from esterified single-cell oil are listed in Table 2.

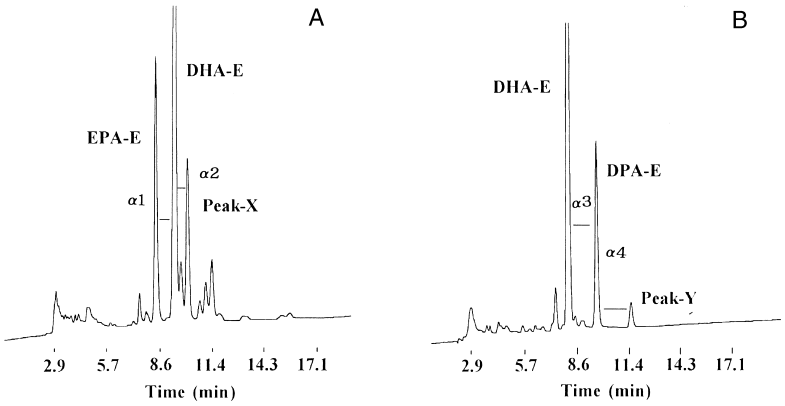


FIG. 2. High-performance liquid chromatograms of esterified fish oil (A) and esterified single-cell oil (B). α1: Separation factor between EPA-E and DHA-E; α2: separation factor between EPA-E and Peak-X in esterified fish oil; α3: separation factor between DHA-E and DPA-E; α4: separation factor between DPA-E and Peak-Y in esterified single-cell oil. Abbreviations: EPA-E, DHA-E, and DPA-E, ethyl esters of eicosapentaenoic acid, docosahexaenoic acid, and docosapentaenoic acid, respectively; Peak-X and Peak-Y, unknown components.

TABLE 1
The Separation Factor (α) of the Main Components of Esterified Single-Cell Oil and Esterified Fish Oil

Peaks		α	Esterified fish oil	α	Esterified single-cell oil
20:5	EPA-E[a]			—	—
		α1	1.189		
22:6	DHA-E				
				α3	1.2912
22:5	DPA-E				
		α2	1.112		
Peak-X				—	—
Peak-Y		—	—	α4	1.278

[a]Abbreviations: EPA-E, DHA-E, and DPA-E, ethyl esters of eicosapentaenoic acid, docosahexaenoic acid, and docosapentaenoic acid, respectively; Peak-X and Peak-Y, unknown peaks in Figure 2.

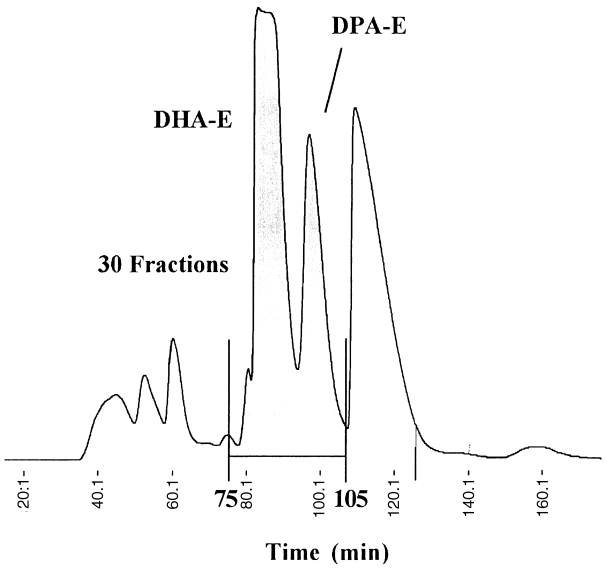


FIG. 3. Chromatogram of esterified single-cell oil from preparative high-performance liquid chromatography. Column: 6 mm i.d. × 2,000 mm length; packing material: YMC Gel ODS-AM S50 (YMC Co., Kyoto, Japan); detector: refractive index.

TABLE 2
The Result of Purification of DHA-E and DPA-E from Esterified Single-Cell Using Industrial Preparative High-Performance Liquid Chromatography

	DHA-E	DPA-E
Purity[a] (%)	99	99
Recovery (%)	23.2	79.6
Productivity (kg/h)	0.10	0.07

[a]Gas chromatographic analysis. Abbreviations: see Table 1.

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The purity of DHA-E obtained was 99%, and that of DPA-E was also 99% as obtained from GC analysis. The recovery ratio of DHA-E was 23.2%, and that of DPA-E was 79.6%. The productivity of DHA-E was 0.10 kg/h and that of DPA-E was 0.07 kg/h.

DISCUSSION

HPLC is known to have speedy and relatively high resolution in the separation of materials. The advantage of preparative HPLC is its increased loading amounts. Good results for purification or separation in preparative HPLC are influenced by many factors besides equipment size. Among these many factors, resolution (R_s) is affected by the separation factor (α) and capacity factor (k') but not much by the theoretical plate number (N).

HPLC runs of fish oil and single-cell oil esters, shown in Figure 2, indicate that there are many components besides the DHA-E peak of esterified fish oil. On the other hand, esterified single-cell oil has fewer components. The separation factors of main components in esterified fish oil are roughly 1.1, and those for single-cell oil are roughly 1.3 (Table 1). This means that single-cell oil is easier to purify.

The relationship between loading amount and recovery ratio for more than 99% purity on a small-scale preparative HPLC, shown in Figure 4, indicates that an increase in loading decreases the recovery ratio. However, the recovery ratio is still maintained at 300 mg loading. For this reason, we applied these conditions to the industrial preparative HPLC.

The results of industrial preparative HPLC were almost the same as those obtained in the small-scale preparative HPLC. With the industrial preparative HPLC, both DHA-E and DPA-E were produced at better than 99.0% purity from esterifed single-cell oil without any other treatment. Single-cell oil seems to be a better resource to produce purified DHA-E and DPA-E when compared with fish oil.

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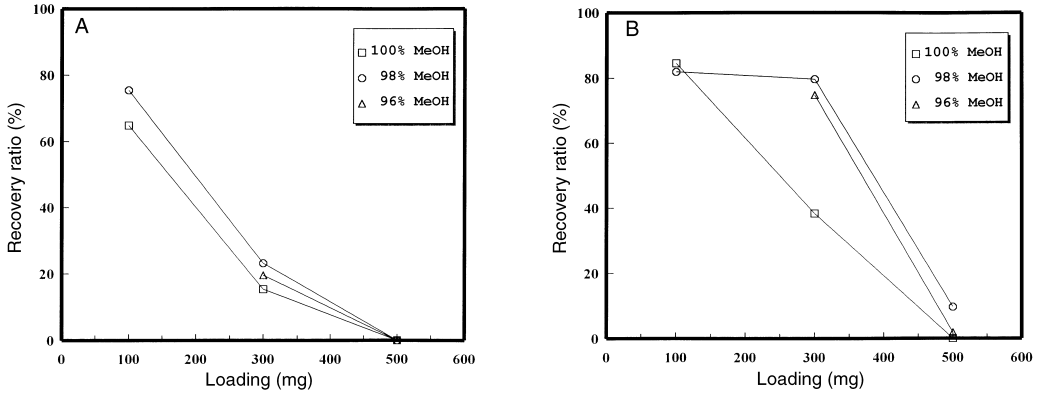

FIG. 4. The relationships between loading amounts and recovery ratios for more than 99% purity. (A) DHA-E; (B) DPA-E. Column: 6 mm i.d. × 2,000 mm length; packing material: YMC Gel ODS-AM S50 (YMC Co.). For abbreviations see Figure 2. For manufacturer see Figure 3.

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