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PREPARATIVE-SCALE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF OMEGA-3 POLYUNSATURATED FATTY ACID ESTERS DERIVED FROM FISH OIL

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SUMMARY

Marine triglyceride-derived omega-3 polyunsaturated fatty acid ethyl esters were separated by preparative high-performance liquid chromatography on a 25-μm octadecyl stationary phase using a ternary isocratic mobile phase of acetonitrile–tetrahydrofuran–water (466:233:300, v/v/v). The highest purity first-run fractions obtained were ethyl esters of the major marine polyunsaturates eicosapentaenoic acid (20:5ω3, 97.7%) and docosahexaenoic acid (22:6ω3, 93.7%), and the minor polyunsaturate octadecatetraenoic acid (18:4ω3, 98.1%).

INTRODUCTION

Interest in omega-3 polyunsaturated fatty acids initially was prompted by observations[1,2] that Greenland Eskimos experienced a significantly lower incidence of death from ischaemic heart disease than Western populations[2]. Results of subsequent studies indicated that this alleged or potential health benefit and other physiological effects[3–9] were due to a diet high in marine oils containing esterified omega-3 polyunsaturated fatty acids (PUFAs). The predominant marine triglyceride-derived omega-3 fatty acids are all-*cis*-5,8,11,14,17-eicosapentaenoic acid (20:5ω3, EPA) and all-*cis*-4,7,10,13-16,19-docosahexaenoic acid (22:6ω3, DHA).

Either native winterized or concentrated whole-body fish oils or fish liver oils have been utilized in most studies as dietary sources of EPA and DHA. Also, in many instances clinical subjects have been given relatively large doses of dietary fish oil supplements, for example, ranging from 18 to 50 g per day depending on the study[4–9]. Accordingly, the physiological effects and possible health benefits of administered fish oils generally have been attributed to either EPA or DHA alone, or to a synergistic effect between the two[10,11]. As a result, there has been controversy over the contribution of individual fish oil constituents to particular pharmacological actions and the optimal dosages required for achieving established and/or suspected beneficial effects.

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An obvious need exists, therefore, for amounts of pure EPA and DHA in usable form in order to study these polyunsaturates at all levels of investigation.

Over the past 15 years, long-chain fatty acids and their aromatic and aliphatic esters have been successfully separated by high-performance liquid chromatography (HPLC)[12–15]. Most of these analytical separations have been performed in the reversed-phase (RP) mode with an octadecyl stationary phase. A few reports[16–18] exist on the preparative-scale RP-HPLC of fish oil-derived methyl and ethyl esters of EPA and DHA, the major marine polyunsaturates. This paper describes the separation and purification of ethyl esters of EPA, DHA and the heretofore minor unreported polyunsaturate octadecatetraenoic acid (18:4ω3,OTA) on a preparative scale by modification of an analytical RP-HPLC procedure[19] which we used in preliminary work.

EXPERIMENTAL

Materials

Analytical-reagent grade anhydrous sodium sulfate, urea, potassium hydrogencarbonate, sodium metal and all solvents used for extraction and chromatography were obtained from Fisher Scientific (Springfield, NJ, U.S.A.); butylated hydroxytoluene (BHT) was obtained from Aldrich (Milwaukee, WI, U.S.A.). Promega™, which is commercially available in capsule form from the Parke-Davis Health Care Group (Morris Plains, NJ, U.S.A.), was utilized as a concentrated fish oil source. The concentrations of esterified EPA and DHA were 27.7% and 11.4%, respectively, as determined by gas chromatographic (GC) analysis of methyl esters derived from transesterification of the commercial oil (particular capsules); *ca.* 1% of minor polyunsaturates was also present.

Transesterification

Fish oil triglycerides were transesterified to ethyl esters by a modification of the previously described method of Christie[20]. The fish oil in six capsules (6 g) of Promega was removed with a syringe and immediately dissolved in 120 ml of peroxide-free tetrahydrofuran (THF); 240 ml of freshly prepared 0.5 *M* sodium ethoxide were added. After the reaction had proceeded at 50°C for 10 min, it was quenched by the addition of 12 ml of glacial acetic acid, followed immediately by 600 ml of distilled water. The aqueous phase was extracted twice with diethyl ether (300 ml) and the ether layer was dried over anhydrous sodium sulfate containing 10% solid potassium hydrogen bicarbonate. The resulting slurry was filtered and the solvent removed under reduced pressure to give a quantitative yield of ethyl esters.

Urea crystallization

Concentration of the polyunsatured fatty acid ethyl esters was accomplished using the procedure of Gunstone *et al.*[21] for methyl ester polyunsaturates. Following transesterification, the esters were dissolved in 24 ml of a 250 mg/ml solution of urea in ethanol. The urea crystallization was initiated at room temperature and then proceeded for 24 h at 4°C. On reaching ambient temperature, the resulting solid was washed with urea-saturated ethanol and filtered; the esters then were extracted twice from the filtrate with 50 ml of diethyl ether. The remaining solvent was removed

(rotary evaporator) under reduced pressure followed by addition of 6 ml of peroxide-free THF. Recovery experiments indicated that the yields after crystallization of 93% and 79% for EPA and DHA, respectively, were in agreement with literature values[21].

Optimization of high-performance liquid chromatography

The chromatographic system consisted of an M6000 pump, a U6K injector and an R401 differential refractometer operated at ambient temperature (Waters Division of Millipore, Milford, MA, U.S.A.) and an ST Macrobore® (Separations Technology, Wakefield, RI, U.S.A.) column (35.0 cm × 4.6 mm I.D.) of C_{18} reversed-phase material, 25-μm particle size. A guard column (30 mm × 4.6 mm I.D.) was dry-packed with Supelcosil LC-18 (40-μm) particles (Supelco, Bellefonte, PA, U.S.A.). Mobile phases were prepared by mixing appropriate volumes of acetonitrile, peroxide-free THF and water; all solvents were filtered through 0.45-μm nylon-66 filters (Rainin, Woburn, MA, U.S.A.) and degassed by saturation with helium. The esters were eluted with acetonitrile–THF–water (466:233:300, v/v/v) at a flow rate of 2.0 ml/min. Data were recorded on an Omniscribe strip-chart recorder (Houston Instruments, Austin, TX, U.S.A.). Peak areas were determined by triangulation. Following the transesterification and urea crystallization steps, the concentrated ethyl esters were dissolved in peroxide-free THF containing 0.005% (w/v) 8HT as an antioxidant. Solutions of esters were stored in Reactivials™ (Supelco) at 4°C blanketed with nitrogen. Samples of 10–50 μl containing 10–50 mg total fatty acid ethyl esters were injected with a 100-μl Hamilton (Reno, NV, U.S.A.) syringe.

Analytical high-performance liquid chromatography

Results of methods development experiments were monitored by analytical RP-HPLC of appropriate collected fractions. Prior to chromatography, the esters in each fraction were extracted from the eluent into hexane (2 ml) and the layers were separated. Next, the fractions were brought to dryness under a stream of nitrogen and reconstituted in 200 μl of peroxide-free THF containing 0.005% (w/v) BHT. Aliquots of 50 μl of these concentrates were then analyzed by RP-HPLC. The separations were performed on a Whatman ODS-3 RAC II column (100 mm × 4.6 mm I.D.) (Whatman, Clifton, NJ, U.S.A.); a guard column (30 mm × 4.6 mm I.D.) dry packed with Supelcosil LC-18 (40 μm) was also employed. The esters were eluted with acetonitrile–THF–water (360:200:440, v/v/v) at a flow-rate of 2.0 ml/min. Data were recorded on an Omniscribe strip-chart recorder. This procedure[19] was developed for the quantitative and qualitative analysis of PUFA methyl and ethyl esters derived from fish oil sources.

Preparative high-performance liquid chromatography

A Model ST/800A preparative chromatograph Separations Technology, (Wakefield, RI, U.S.A) fitted with an ST/2000-1 guard column (1 in. × 2 in. I.D.) and an ST/2000B preparative column (25 in. × 2 in. I.D.), each packed with ST Macrobore C_{18} (25 μm particle size), was employed for separation of esters. The system was equipped with a differential refractometer with a flowing reference (as opposed to an air-filled reference) in order to minimize baseline drift. The sensitivity was 8 × at 100 mV full-scale. Samples of 1 or 2 ml (equivalent to 0.8–1.6 g) of total esters were introduced into the system via a 10-ml syringe immediately followed by a 1-ml eluent

flush. The esters were eluted with acetonitrile–THF–water (466:233:300, v/v/v) at a flow-rate of 220 ml/min. Data were recorded with a strip-chart recorder at 12 cm/h. As the esters were eluted, typically 24 fractions of approximately 175 ml each were collected; the fractions were stored in tightly closed containers in a freezer at –60°C until analysed; an aliquot of each fraction was subsequently characterized by GC.

Capillary gas chromatography

Preparative HPLC fraction aliquots were analyzed by GC without further derivatization or purification. The gas chromatograph (Model 8500; Perkin-Elmer, Norwalk, CT, U.S.A.) was operated with an injection inlet temperature of 280°C, a flame ionization detector (splitting ratio 40:1) and a computer-interfaced integrator. The fused-silica capillary stationary phase was Perkin-Elmer BP-1 (12 m × 0.22 mm I.D.) with a 0.25-μm film thickness. The initial oven temperature of 100°C was held for 1 min, then the temperature was increased to 160°C at 32°C/min followed by a 2°C/min gradient to 210°C. The carrier gas linear velocity was 30 cm/s. The ethyl esters were identified by retention times compared with a Menhaden oil-derived ethyl ester standard mixture (Ocean Organics, Wakefield, RI, U.S.A.). Integrated peak areas were used to calculate the percentages of the various esters in each of the 24 fractions.

RESULTS AND DISCUSSION

The experimental conditions of the analytical RP-HPLC method[19] were found to be less than satisfactory for the optimization experiments. The peak shapes were too broad and not well defined; in addition, the analysis time was 2 h or longer. To compensate for the increased carbon load of the optimization packing (*ca.* 18%), as opposed to the carbon load of the analytical packing (*ca.* 10%), the amount of water in the mobile phase was reduced. The optimum mobile phase for use with the semi-preparative stationary phase was found to be acetonitrile–THF–water (466:233:300, v/v/v) at a flow-rate of 2.0 ml/min.

The effect of altering the flow-rate was also investigated in order to optimize the analysis time. Flow-rates of 1.0, 1.8, 2.0, 2.4 and 3.0 ml/min were evaluated. The optimum in terms of resolution and analysis time was found to be 2.0 ml/min. Although the analysis time could be considerably shortened (to 30 min at 3.0 ml/min), the resolution (R_s) of EPA and DHA was not acceptable (R_s = 0.6).

Fig. 1 shows a typical optimization chromatogram. The labeled fractions represent predominantly (A) ethyl octadecatetraenoate (OTA-Et), (B) ethyl eicosapentaenoate (EPA-Et) and (C) ethyl docosahexaenoate (DHA-Et). Fractions corresponding to the separated groups in the chromatogram were collected and subsequently characterized by analytical RP-HPLC[19]. The levels of EPA-Et and DHA-Et in the five fractions indicated in Fig. 1 are summarized in Table I; the fractions containing most EPA-Et and DHA-Et are fractions 3 and 4, respectively. A chromatogram of an analytical-scale separation of the components in fractions 3 and 4 is shown in Fig. 2. The chromatogram of fraction 3 shows that it consists mostly of EPA-Et and that of fraction 4 illustrates that it also contains EPA-Et; however, fraction 4 contains mainly DHA-Et. The relatively high content of DHA-Et in fraction 5 (Table I) is probably due to the tailing of peak C (Fig. 1).

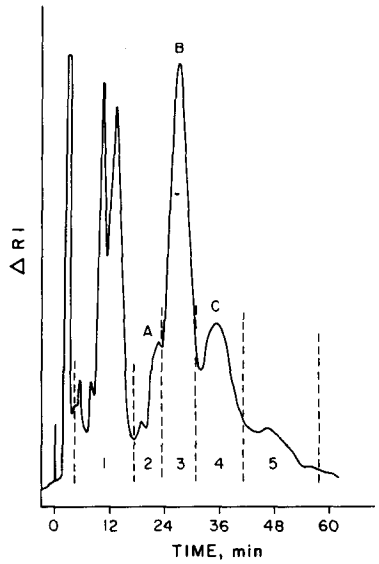

Fig. 1. Typical optimization chromatogram of fish oil-derived fatty acid ethyl esters. Conditions: mobile phase, acetonitrile–THF–water (466:233:300, v/v/v); stationary phase, ST Macrobore C_{18} (35 cm × 4.6 mm I.D., 25 μm); sample load, 35 mg total esters; detection, refractive index (RI) at 16×, ambient temperature with a trapped reference. Major peak components: (A) ethyl octatetraenoate; (B) ethyl eicosapentaenoate; (C) ethyl docosahexaenoate.

Overload experiments were carried out to determine the amount of sample that can be applied to the optimization column without exceeding the column linear capacity (mg of sample per gram of packing). When the column linear capacity is exceeded, overloading occurs[22]. The relationship between the sample load of EPA-Et and the capacity factor (k') is illustrated in Fig. 3. Column overloading occurs at approximately 4.0 mg sample per gram column packing, as is evidenced by the strong deviation in the linear decrease of k' which occurs when this sample load is reached. Hence approximately 36 mg of total fish oil esters may be applied to the optimization column per gram of packing without exceeding its linear capacity.

TABLE I

ANALYTICAL RP-HPLC ANALYSIS OF FRACTIONS FROM OPTIMIZATION EXPERIMENTS

See Fig. 1.

Fraction No.	*EPA-Et (area%)**	*DHA-Et (area%)*
1	0	0
2	1	0
3	93	0
4	2	82
5	0	50

* Values are expressed as area percentage of the sum of the areas of all of the peaks in the chromatogram (Fig. 2), except the solvent peak.

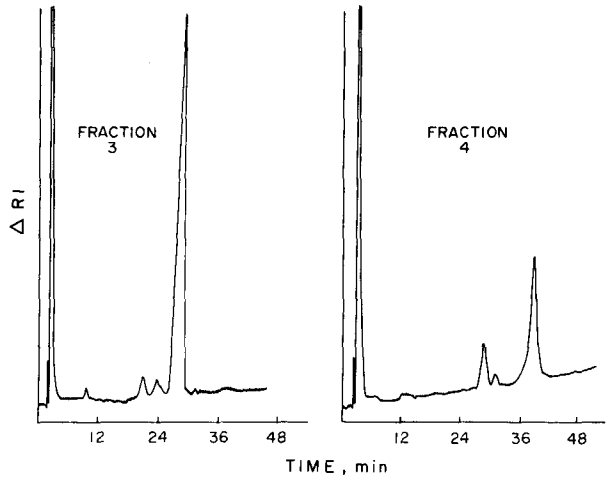

Fig. 2. Analytical RP-HPLC trace of collected fractions 3 and 4 from Fig. 1. Conditions: mobile phase, acetonitrile–THF–water (360:200:400, v/v/v); flow-rate; 2.0 ml/min; stationary phase, Whatman ODS-3 RAC II (100 mm × 4.6 mm I.D.); detection, refractive index at 8 ×, ambient temperature with a trapped reference; chart speed, 5 in./h.

The chromatographic effect of overloading is illustrated in Fig. 4. The chromatogram demonstrated by the broken line corresponds to a sample load of 25 mg of total fatty acid ethyl esters; thus it was obtained without exceeding the linear capacity of the column. On the other hand, the chromatogram (Fig. 4, solid line) obtained under conditions (sample load of a total of 50 mg of ethyl esters) that did exceed the linear capacity of the column showed that the peaks of EPA-Et and DHA-Et are not as well resolved.

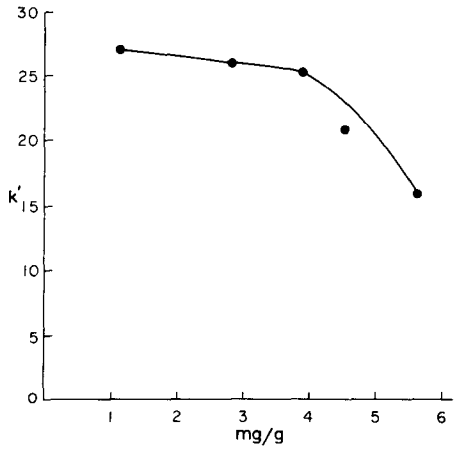

Fig. 3. Plot of k' *versus* sample load: mg/g = mg EPA-Et per gram of column packing. Overloading is defined[22] as the sample load where the solute capacity, k', decreases by 10% from its constant value at smaller sample sizes.

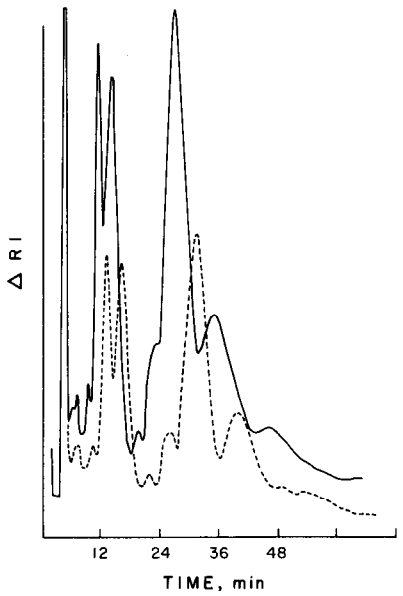

Fig. 4. Chromatographic effects of overloading: broken line, 25-mg fatty acid ethyl ester; solid line, 50-mg fatty acid ethyl ester. Mobile phase, acetonitrile–THF–water (466:233:300, v/v/v); flow-rate, 2.0 ml/min; stationary phase, ST Macrobore (350 mm × 4.6 mm I.D., 25 μm); detection, refractive index at 16×, ambient temperature with a trapped reference.

In the past, separating critical pairs (*i.e.*, fatty acids and esters with the same equivalent carbon length) by liquid chromatographic techniques with reversed-phase analytical, and especially semi-preparative or preparative stationary phases has been challenging. Most separations of fatty acids or their esters on the semi-preparative or preparative scale have required the implementation of argentation chromatography or recycling in order to obtain pure or enriched fractions of components. However, with the recent availability of more versatile semi-preparative and preparative packings of smaller particle sizes coupled with higher carbon loads, increased resolution of closely related compounds can be achieved. In this work, we successfully obtained enriched fractions of EPA-Et and DHA-Et of 93% and 82%, respectively, with a single pass through a Macrobore C_{18} column. Furthermore, results of our optimization studies were directly applicable to preparative-scale HPLC of the marine oil-derived omega-3 polyunsaturated ethyl esters.

Scale-up from the optimization studies gave baseline separation of three omega-3 fatty acid ethyl esters derived from the concentrated fish oil source in 40 min without recycling. A representative chromatogram of the esters with a 1.6-g sample is shown in Fig. 5. The three major peaks in order of elution correspond predominantly to (A) ethyl octadecatetraenoate (18:4ω3), (B) ethyl eicosapentaenoate (20:5ω3) and (C) ethyl docosahexaenoate (22:6ω3). The amounts of omega-3 PUFA ethyl esters in the highest purity (94%) fractions and the percentage composition of the respective esters (18:4ω3, 20:5ω3, 22:6ω3) in all fractions, as determined by capillary GC, are summarized in Table II. The minor omega-3 18:4ω3 ethyl ester eluted first and was found at high concentrations (94%) in fractions 1–4; fraction 3 was the most concen-

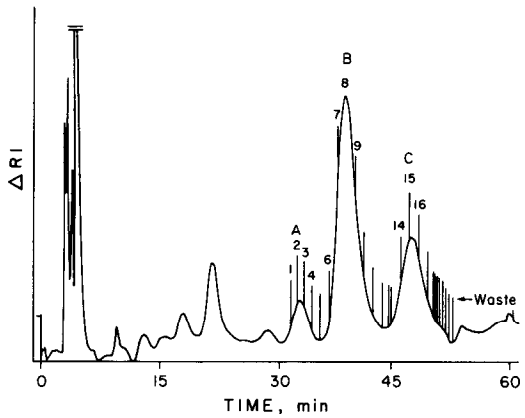

Fig. 5. Preparative HPLC trace of fatty acid ethyl esters derived from a fish oil concentrate. Conditions: sample, 1.6 g total ethyl esters; mobile phase, acetonitrile–THF–water (466:233:300, v/v/v); stationary phase, ST Macrobore C_{18} (25 in. × 2 in. I.D., 25 μm); detection, refractive index, 8 × at 100 mV full-scale, flowing reference at ambient temperature. Peak identification of predominant components: (A) ethyl octadecatetraenoate; (B) ethyl eicosapentaenoate; (C) ethyl docosahexaenoate. The vertical lines indicate fraction collection starting at fraction 1 continuing through fraction 24.

TABLE II

OMEGA-3 FATTY ACID ETHYL ESTER COMPOSITION OF MAJOR PREPARATIVE HPLC FRACTIONS DETERMINED BY GC

Fraction No.	*18:4ω3 (%)**	*20:5ω3 (%)**	*22:6ω3 (%)**	*22:5ω3 (%)**
1	(16)** 94.3			
2	(22) 94.8			
3	(12) 98.1			
4	(5) 95.3			
5	32.5	57.1		
6	1.8	(29)** 95.4		
7	0.2	(113) 97.7		
8		(87) 95.8		
9		(31) 95.9		
10		92.1		
11		86.1		
12		50.2		
13		13.4		
14		3.0		
15			12.5	
16			64.1	
17			86.4	1.5
18			91.1	13.9
19			93.7	1.5
20			84.8	0.5
21			60.7	1.5
22			72.2	3.2
23			70.9	3.8
24			63.6	1.3

* Data are expressed as a percentage of total GC analysis, *i.e.*, area%.
** Amount (mg) in parentheses.

trated at 98.1%. Most of the 20:5ω3 ethyl ester was found in five fractions (6–11), containing the ester at 86% concentration and greater. The highest concentration of 20:5ω3 ester was found in fraction 7 at 97.7%; lower concentrations of this ester occurred in fractions 5, 12, 13 and 14. The other major PUFA, 22:6ω3, ethyl ester was eluted immediately following the 20:5ω3 ethyl ester in fractions 15–24; the highest concentration was found in fraction 19 at 93.7%.

The results of these preliminary preparative HPLC studies, which are comparable[17,18] or superior[16] to previous reports, clearly demonstrate that very pure marine oil-derived polyunsaturates readily can be obtained in sufficient amounts for many laboratory-scale investigations. Optimization of sample load, coupled with repeated preparative runs, will allow the separation of multigram amounts of PUFAs. For example, 99+% pure ethyl eicosapentaenoate and ethyl octadecatetraenoate can be obtained on rechromatography of 94+% first-run eluate by using the present ternary mobile phase (acetonitrile–THF–water), or, preferably ethanol–water[17,18]. Although pure ethyl docosahexaenoate can also be obtained in the same manner, the corresponding free acid (DHA) may be generated in practical, large amounts via a chemical method (iodolactonization) developed by Wright *et al.*[23]. In our studies, the preparation, of ethyl octadecatetraenoate of purity up to 98% (first run), reported for the first time, is significant in view of the paucity of biological data on this minor marine oil-derived omega-3 PUFA.

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