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ANALYTICAL-SCALE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF OMEGA-3 FATTY ACID ESTERS DERIVED FROM FISH OILS

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SUMMARY

Fish oil triglycerides were transesterified to their corresponding methyl and ethyl esters. The esters were separated by reversed-phase high-performance liquid chromatography on a 10-cm column of 5-μm octadecylsilyl silica, using a ternary mobile phase of acetonitrile–tetrahydrofuran–water (9:5:11). The separation of principal saturated and unsaturated C_{14}–C_{22} fatty acid esters was accomplished in 60 min. For the polyenoic acid esters, detection limits of *ca.* 26 μg were observed, and baseline resolution of the important critical pair of esters of the ω3 fatty acids, eicosapentaenoic acid (C20:5ω3) and docosahexaenoic acid (C22:6ω3) was achieved. However, to obtain baseline resolution of the esters from minor fatty acid esters, a urea crystallization procedure was required prior to chromatographic analysis. The influence on the reversed-phase chromatographic retention of four different ester moieties on the fatty acids was also studied. The *n*-alkyl esters exhibited an increase in ln k' with increasing chain length and with increasing lipophilic character of the alkyl ester moieties; however, with the aromatic benzyl derivatives there was a decrease in ln k' compared with the alkyl esters.

INTRODUCTION

Interest in ω3 farry acids was initiated by results of epidemological studies performed on Greenland Eskimos. These peoples experienced a low incidence of death from ischemic heart disease (5.3%) compared to Western populations (39.7%)[1]. Further studies[2–8] attribute this low mortality figure and many other health benefits to a diet high in ω3 fatty acids. These acids are long-chain fatty acids which have methylene interrupted double bonds and the last double bond three carbons from the methyl terminus. These particular fatty acids occur naturally at high concentrations in the body oils of cold water fish (*i.e.*, sardine, menhaden, anchovy). The major fatty

[a] The shorthand notation C*n*:*x*ω*y* will be utilized throughout this manuscript. The *n* denotes the carbon chain length, *x* the number of double bonds, and *y* the location of the last double bond in relation to the terminal methyl group of the fatty acid.

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acids of interest are eicosapentaenoic acid (C20:5ω3 or EPA) and docosahexaenoic acid (C22:6ω3 or DHA)[a].

At present, the method of choice for the analysis of complex mixtures of polyenoic fatty acids such as those derived from fish oils, is capillary gas chromatograpy (GC) with pre-chromatographic derivatization and mass spectrometric (MS) detection. However, GC is impractical for the purification of the large amounts of polyenoic fatty acids required for biological and clinical studies. Moreover, the temperatures required in GC may cause degradation of oxidized long-chain polyunsaturated fatty acids which are present as minor components of the mixture.

In the past fifteen years, complex mixtures of these polyunsaturated long-chain fatty acids or their *n*-alkyl esters contained in natural and modified products, such as margarines[9], vegetable oils[10] and evening primrose oils[11] have been successfully separated by reversed-phase high-performance liquid chromatography (RP-HPLC). Under the relatively mild conditions of RP-HPLC, little degradation of polyenoics occur, and methods can be easily scaled-up for semi-preparative or preparative use. Because methylene-interrupted polyunsaturates do not have strongly absorbing chromophores in the UV region, detection by refractive index or far-UV detection (205–214 nm) has been utilized in RP-HPLC separations of free fatty acids and their aliphatic esters. Refractive index detection is less sensitive than UV detection. However, with far-UV detection solvents absorbing UV below 210 nm cannot be used. The RP-HPLC separations reported to date have generally involved derivatization designed to incorporate aromatic chromophores allowing detection by fluorescence[12,13] or UV[14,15] detection. Thus, relatively low levels of fatty acids may be detected in complex sample matrices, such as biological fluids.

Our goal was to develop a RP-HPLC procedure for the analysis of ω3 fatty acid esters which could eventually be adapted to the preparative isolation of quantities of EPA and DHA, suitable for use in nutritional or biochemical studies. In order to accomplish our objective, the factors involved in sample storage, the influence of the ester moiety on retention behavior, and pre-chromatographic procedures were evaluated and the mobile phase for each chromatographic condition was optimized.

EXPERIMENTAL

Materials

Standards of fatty acid methyl and ethyl esters and trimyristin were purchased from Nu-Chek Prep (Elysian, MN, U.S.A.). All solvents used for extraction, transesterification and chromatography were obtained from Fischer Scientific (Springfield, NJ, U.S.A.). Mobile phases were filtered through 0.45-μm Nylon-66 membrane filters (Rainin Instruments, Ridgefield, NJ, U.S.A.), and were degassed by saturation with helium. Butylated hydroxytoluene (BHT) was obtained from Aldrich (Milwaukee, WI, U.S.A.).

Sardine oil was obtained from the commercially available dietary fish oil capsules Promega™ (Parke-Davis Consumer Health Products Group, Morris Plains, N.J., U.S.A.), menhaden from MaxEPA™ (Seven Seas Health Care, Hull, U.K.) and cod liver oil from Dale Alexander™ Cod Liver Oil (Twin Labs., Ronkonkoma, NY, U.S.A.).

Analytical HPLC

The chromatographic system consisted of an M600 pump, equipped with a U6K injector and a R401 differential refractometer, operated at ambient temperature (Waters Division of Millipore, Milford, MA, U.S.A.). The separations were performed on a Whatman ODS-3 RAC II column (100 × 4.6 mm I.D., 5-μm particle size) (Whatman, Clifton, NJ, U.S.A.). A guard column (30 × 4.6 mm I.D.) was dry-packed with Supelcosil LC-18 40-μm material (Supelco, Bellefonte, PA, U.S.A.). The esters were eluted with a mobile phase of acetonitrile–tetrahydrofuran (THF)–water (9:5:11, v/v/v) at a flow-rate of 2.0 ml/min. Data were recorded on an Omniscribe stripchart recorder (Houston Instruments, Austin, TX, U.S.A.).

Stock solutions of ester standards were prepared at concentrations of 100 mg/ml. The diluent was peroxide-free THF containing 0.005% (w/v) BHT. The standards were stored in Reactivials™ (Supelco) blanketed with nitrogen at 4°C. Stored in this manner, the standards were stable for many months. Aliquots of samples or standards were injected with a 100-μl syringe.

Base-catalyzed transesterification

Transesterification of triacylglycerols was accomplished rapidly and quantitatively via reaction with the respective sodium alcoholate–alcohol. The procedure of Christie[16] was used with minor modifications. Typically, the fish oil in one capsule (1 g) was removed with a syringe and immediately dissolved in 20 ml of peroxide-free THF. A 40-ml volume of 0.5 *M* sodium methoxide, sodium ethoxide, sodium *n*-butoxide or sodium benzoxide, freshly prepared by dissolution of freshly cut sodium metal in the corresponding alcohol, was added. After 10 min at 5°C, the reaction was quenched by the addition of 2 ml of glacial acetic acid, followed immediately by the addition of 100 ml of distilled water. The esters were extracted twice with dietyl ether (300 ml) and the ether layer was dried over anhydrous sodium sulfate containing 10% potassium hydrogencarbonate. The resulting slurry was filtered and the remaining solvent was removed under reduced pressure. The residue was dissolved in 1 ml of peroxide-free THF, containing 0.005% (w/v) BHT. Aliquots of solutions were injected into the chromatographic system.

Recovery experiments

In order to confirm the quantitative conversion of the triglycerides to the corresponding esters, 85 mg of the trimyristin was transesterified to methyl myristate in two separate experiments, using the procedure described. An aliquot of the transesterified trimyristin was analyzed chromatographically and quantified by comparison to a methyl myristate standard curve.

Pre-chromatographic procedures

The urea crystallization procedure of Gunstone *et al.*[17] for methyl esters was used with modifications. Following transesterification, the ethyl esters were dissolved in 8 ml of ethanol which contained urea (250 mg urea/ml). The crystallization was initiated at room temperature and then proceeded for 24 h at 4°C. After the solution had thawed, the resulting crystals were washed with urea-saturated ethanol. The ethyl esters were extracted twice from the filtrate with 50 ml of diethyl ether. The remaining solvent was removed by rotary evaporation and the ethyl esters were dissolved in 2 ml

of peroxide-free THF, containing 0.005% (w/v) BHT. Recovery experiments indicated yields after recrystallization were 83% for the EPA ester and 79% for the DHA ester, repectively; yields were in agreement with those reported in the literature[17].

The low-temperature crystallization procedure described by Gunstone *et al.*[17] was applied, unmodified,to both the methyl and ethyl esters.

To minimize degradation of the fish oil esters, methods of storage were evaluated. A sample of fish oil triglycerides was transesterified and the resulting esters were divided into four aliquots: aliquot 1 was dissolved in peroxide-free THF which contained 0.005% BHT (w/v) as an antioxidant, blanketed with nitrogen and stored in a freezer (control sample); aliquot 2 was kept under the same conditions but without BHT; aliquot 3 was not blanketed with nitrogen; aliquot 4 was kept at room temperature in the light.

Column washing procedures

After many weeks of daily operation, the back pressure of the chromatographic system increased but no loss of chromatographic performance was noted. The increase in back pressure was presumably due to the slow precipitation onto the column packing of very-long-chain fatty acids (C_{24} and longer). These acids have a limited solubility in the mobile phase. Therefore, both the analytical and guard columns were washed overnight with degassed, peroxide-free THF at 0.2 ml/min to removed any fatty acid residue. The next day, the column back pressure was normal. After equilibration, the column plate count was checked with the C14:1ω7, C16:1ω7 and C18:1ω9 methyl ester standards.

Physical methods of identification

The ester functionality of benzyl myristate generated by our method was confirmed by IR spectroscopy. The C14:0 benzyl ester, which was collected as it was eluted from the column, was extracted into chloroform. The spectrophotometer employed was a Model FTS-40 Fourier transform IR spectrometer (Digilab, Boston, MA, U.S.A.). Spectral data at 4 cm^{-1} resolution were acquired at 256 scans per file. The spectral window region was 3300–800 $^{-1}$. The sample was analyzed as a thin film, deposited on potassium bromide plates. Peak identification of the C20:5ω3 methyl ester was confirmed by GC–MS by comparison with a standard MS spectrum.

RESULTS AND DISCUSSION

Chromatography

The chromatographic profiles representing saturated, mono-, di- and poly-unsaturated long-chain fatty acid esters derived from marine triacylglycerols, were very complex. Miwa *et al.*[18] were the first to introduce the equivalent chain length (ECL) theory [ECL = (No. of carbon atoms in chain) -2 (No. of bonds)] of retention of long chain fatty acids in chromatographic systems. In general, our data support the ECL,theory; *i.e.* polyunsaturates are eluted before saturates of equal chain length, and shorter-chain before longer-chain fatty acid esters. Compounds with the same ECL are known as critical pairs. In order to obtain the maximum resolution for the critical pairs of long-chain fatty acid esters derived from fish oil, the effect of changing the aqueous content or ratio of organic modifiers (acetonitrile–THF) in the mobile phase was

investigated. Since it has been observed that there is increased selectivity with binary and ternary mobile phases containing THF[9,19] the relationship between ln k' and acetonitrile–THF ratio for the methyl esters of two critical pairs, C20:5ω3 and C22:6ω3 (ECL = 10) and C16:0 and C18:1ω9 (ECL = 16) methyl esters was determined (Fig. 1). The resolution of C20:5ω3 and C22:6ω3 is better than that of the more saturated fatty acid esters with a higher ECL number. Our results are in accordance with those of Baile *et al.*[9] and Tanaka *et al.*[19], who found that the difference in the free energy of interaction ($\Delta G = RT \ln k'$, where R is the universal gas constant and T temperature) between the polyunsaturated critical pair C20:5ω3 and C22:6ω3 is greater than that of the more saturated critical pair C16:0 and C18:1ω9.

Representative chromatograms of fatty acid methyl esters, derived from three fish species most frequently used in dietary supplements, are shown in Fig. 2. In the chromatograms, the number and types of fatty acid methyl esters appear to be the same. However, ratios of the amounts of methyl esters of EPA to DHA differ according to the species of fish. The elution order of the identified fatty acid methyl esters is: C20:5ω3 (ECL = 10)> C14:0 (ECL = 14)> C16:1ω7 (ECL = 14)> C22:6ω3 (ECL = 10)> C18:2ω6 (ECL = 14)> C16:0 (ECL = 16)> C18:1ω9 (ECL = 16). The trend is generally as predicted by the ECL theory, but the C22:6ω3 (ECL = 10) methyl ester is eluted after the C14:0 (ECL = 14) and the C16:1ω7 (ECL = 14) esters. The peaks of the fatty acid methyl esters were identified by comparison of retention times in chromatograms of commercial standards. Peak identification for the C20:5ω3 and C22:6ω3 esters was confirmed by GC–MS.

The reproducibility of retention for the seven identified fatty acid esters was determined by replicate injections of standard compounds. The reproducibility (relative standard deviation) of retention ranged from 0.6 to 4.7% R.S.D. for the methyl esters and from 0.7 to 7.0% R.S.D. for the ethyl esters. The lower limit of detection (LLD), which was defined as the signal-to-noise ratio (S/N) = 2, was 26 μg for the polyenoic acid esters and higher for the saturated and monosaturated acid esters C14:0, C16:0 and C18:1ω9, where the LLD was 42, 86 and 67 μg, respectively.

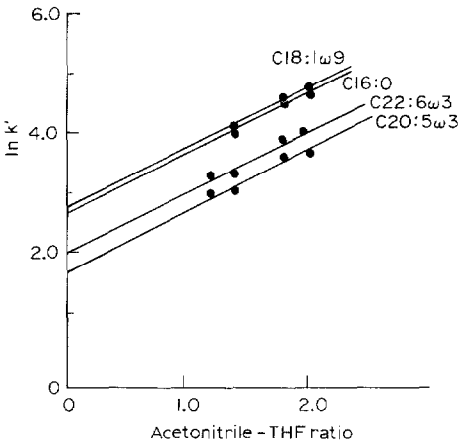

Fig. 1. Mobile phase study: plot of ln k' *versus* acetonitrile–THF ratio. Critical pair 1: C18:1ω9 and C16:0 methyl esters (ECL = 16). Critical pair 2: C22:6ω3 and C20:5ω3 methyl esters (ECL = 10).

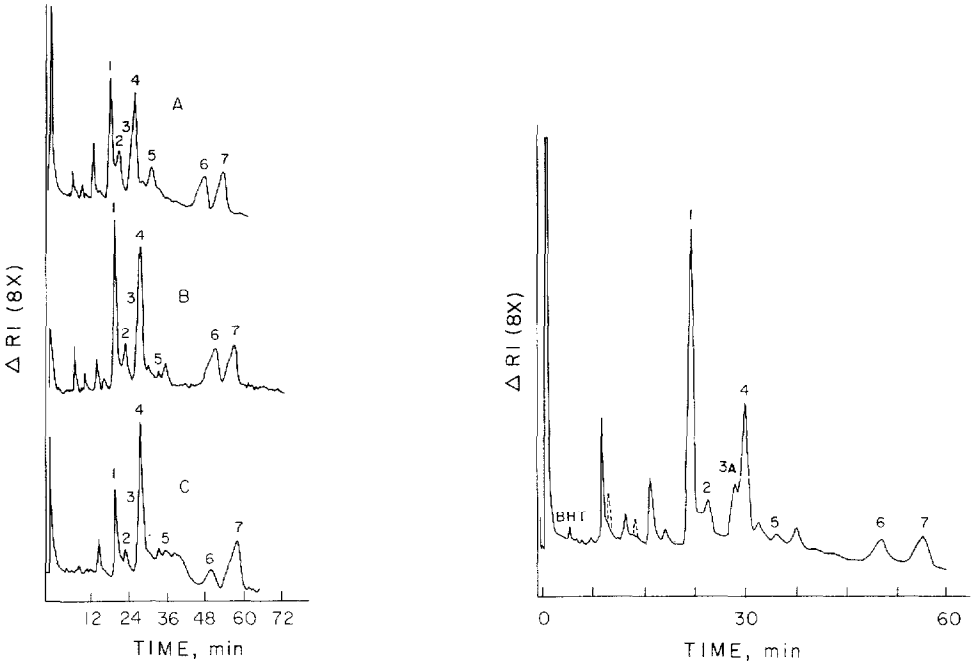

Fig. 2. RP-HPLC of methyl esters, derived from three different fish oil sources: sardine (A), menhaden (B) and cod liver (C). Identified fatty acid methyl esters in order of their elution: (1) C20:5ω3; (2) C14:0; (3) C16:1ω9; (4) C22:6ω3; (5) C18:2ω6; (6) C16:0; (7) C18:1ω9; BHT is eluted right after the void volume; remaining peaks have not been positively identified. Mobile phase: acetonitrile–THF–water (9:5:11) at 2.0 ml/min; stationary phase, Whatman ODS-3 RAC II (100 × 4.6 mm I.D.); detection, refractive index at ambient temperature.

Fig. 3. Degradation study: RP-HPLC of aliquot 2 (no BHT). Analysis day 1, – – –; day 21, ———. For identified methyl esters in order of their elution and chromatographic conditions see Fig. 2.

The linearity of detector response was confirmed for all the fatty acid methyl esters in the range of 100 to 1000 μg.

Storage conditions

Methylene-interrupted polyunsaturated long-chain fatty acids and their esters are susceptible to autoxidation. After 21 days without BHT, a new peak, peak 3A, which was not identified, was present (Fig. 3). In addition two peaks prior to peak 1, which were seen in the chromatogram of the control sample were no longer present (Fig. 3). These peaks were also absent from the aliquots that were not stored under a blanket of nitrogen (aliquot 3) or were kept at room temperature in the light (Aliquot 4). Therefore, throughout our studies, all samples and standards were stored at 4°C in the dark as a solution in peroxide-free THF, containing 0.005% BHT and blanketed with nitrogen. BHT is eluted near the void volume in the chromatogram and does not interfere with the analysis of the long-chain fatty acid esters.

Although the rate of presumed free radical reactions are slowed by the precautions taken in the handling and storage of the fatty acid esters, after six months there was evidence that some of the polyunsaturates were degraded. However, no sign of degradation of EPA and DHA was observed during our work.

Pre-chromatographic procedures

Baseline separation of the critical pairs was achieved when the low-temperature fractionation procedure was used prior to chromatography to diminish the content of the more saturated long-chain fatty acid esters. However, C16:1ω7, which was eluted together with DHA, and C14:0, which was not completely separated from EPA, were not removed by this procedure. The procedure was also applied to decrease substantially the more saturated triglycerides in fish oils, to concentrate triglycerides before transesterification, or to concentrate the fatty acid methyl esters. However in the Promega chromatograms there was no difference in the chromatograms of fatty acid esters before and after low temperature crystallization. Probably, the sardine oil used in these capsules had been concentrated by the low-temperature procedure during processing.

The urea crystallization procedure was successfully utilized to remove a significant amount of the more saturated fatty acid esters. Fig. 4 represents a chromatogram of the sardine oil esters after urea crystallization. This chromatogram clearly shows the prominence of the major omega-3 C20:5ω3 (1) and C22:6ω3 (2) ethyl esters.

Ester moieties

The retention behavior of the methyl, ethyl, *n*-butyl and benzyl esters of six long-chain fatty acids, derived from the sardine oil source, are represented graphically in Fig. 5 as ln *k' versus* the carbon number of the ester moiety. The curves from bottom to top for the following fatty acid esters represent in order of elution: (1) C20:5ω3; (2) C14:0; (3) C22:6ω3; (4) C18:2ω6; (5) C16:0; (6) C18:1ω9. For the aliphatic moieties, retention increases with increasing carbon-chain length[20,21], *i.e.*, retention increases with increasing lipophilicity. However, the aromatic benzyl esters of the fatty acids are less retained than their corresponding methyl esters. This behavior can be attributed to steric hindrance of the more bulky benzyl derivatives than of the *n*-alkyl esters. The polarizability of the benzyl derivatives may also cause a greater affinity for the polar mobile phase. Thus, they are less retained than the shorter, less polarizable *n*-alkyl esters.

Representative chromatograms of the four types of derivatives of the fatty acids in sardine oil are shown in Figs. 2A and 6–8: Fig. 2A, methyl esters; Fig. 6, ethyl esters;

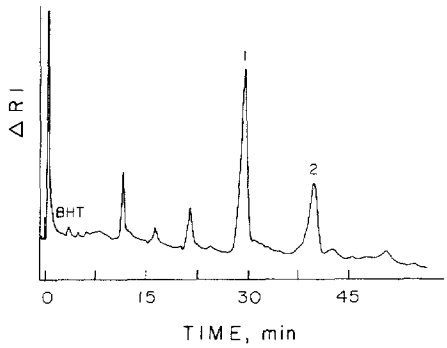

Fig. 4. RP-HPLC of sardine oil long-chain fatty acid ethyl esters after urea crystallization. Identified fatty acid ethyl esters in order of their elution: (1) C20:5ω3; (2) C22:6ω3. For chromatographic conditions see Fig. 2.

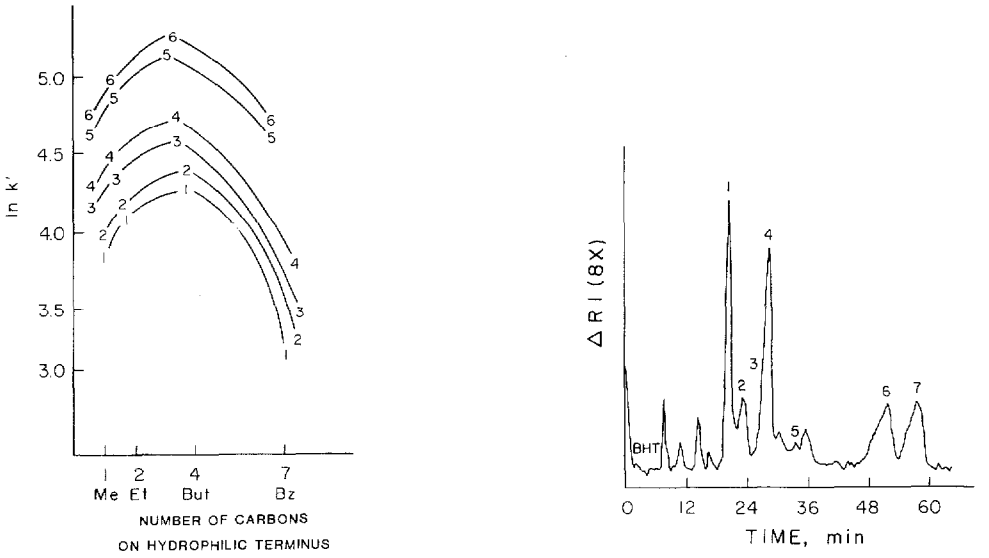

Fig. 5. Plot of ln *k' versus* carbon-chain length of methyl (Me), ethyl (Et), *n*-butyl (Bu) and benzyl (Bz) esters: (1) C20:5ω3; (2) C14:0; (3) C22:6ω3; (4) C18:2ω6; (5) C16:0; (6) C18:1ω9. For chromatographic conditions see Fig. 2.

Fig. 6. RP-HPLC of ethyl esters of long-chain fatty acids, derived from sardine oil. Identified fatty acid ethyl esters in order of their elution: (1) C20:5ω3; (2) C14:0; (3) C16:1ω7; (4) C22:6ω3; (5) C18;2ω6; (6) C16:0; (7) C18:1ω9. For chromatographic condition see Fig. 2.

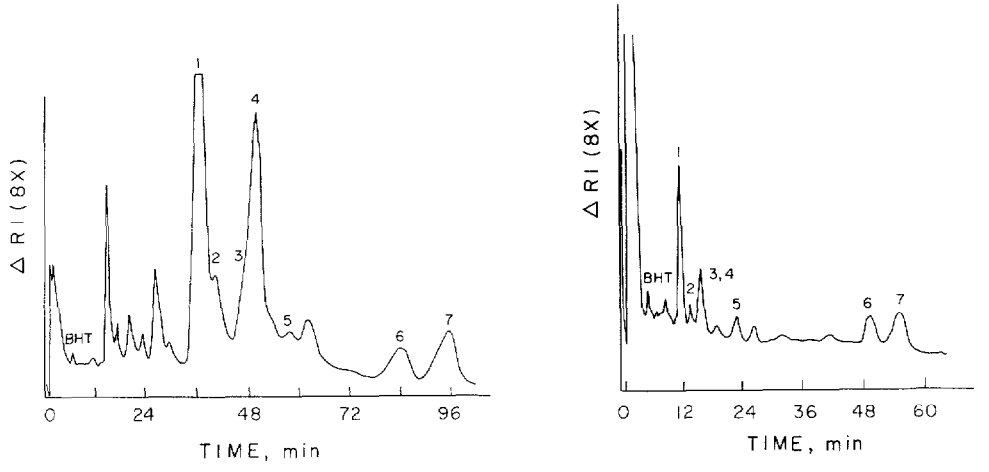

Fig. 7. RP-HPLC of *n*-butyl esters of long-chain fatty acids, derived from sardine oil. Identified fatty acid *n*-butyl esters in order of their elution: (1) C20:5ω3; (2) C14:0; (3) C16:1ω7; (4) C22:6ω3; (5) C18:2ω6; (6) C16-0; (7) C18:1ω9. For chromatographic conditions see Fig. 2.

Fig. 8. RP-HPLC of benzyl esters of long-chain fatty acids, derived from sardine oil. Identified benzyl esters in order of their elution: (1) C20:5ω3; (2) C14:0; (3) C16:1ω7; (4) C22:6ω3; (5) C18:2ω6; (6) C16:0; (7) C18:1ω9. For chromatographic conditions see Fig. 2.

Fig. 7 *n*-butyl esters; and Fig. 8, benzyl esters. The deterioration in peak shape with increased retention may be due to the decreased solubility of the esters in the mobile phase as well as to the band broadening which occurs with increased retention time.

Peak identification

The peak identification for the methyl and ethyl esters was based on comparison with chromatograms of commercially available standard compounds. In addition, the identification of the methyl ester of EPA was confirmed by NMR and GC–MS. The peaks in the *n*-butyl and benzyl ester chromatograms were assigned by analogy to the methyl and ethyl ester profiles, as standards of these compounds are not readily available. The presence of the ester functionality of benzyl myristate was confirmed by IR.

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