Eicosapentaenoic Acid Geometrical Isomer Artifacts in Heated Fish Oil Esters

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Gas liquid chromatography (GLC) on polar capillary columns showed that the ester of eicosapentaenoic acid (EPA) in distilled fish oil methyl and ethyl esters sometimes is accompanied by several artifacts. The same EPA artifacts did not arise from saponification and/or esterification but were formed in a significantly high yield during prolonged heating of the authentic acid. Physicochemical studies, isolation and partial degradation showed that these artifacts are mainly geometrical (*cis-trans*) isomers of the natural *cis* ethylenic bonds of eicosapentaenoic acid. Several C_{20} mono- and diethylenic geometrical isomers were prepared and the GLC equivalent chain length values determined. On the basis of measured and calculated equivalent chain length values on two different GLC phases, some of the EPA artifacts were identified as 20:5-Δ*trans*-5,*cis*-8,*cis*-11,*cis*-14,*cis*-17; 20:5-Δ*cis*-5,*trans*-8,*cis*-11,*cis*-14,*cis*-17; and 20:5-Δ*cis*-5,*cis*-8,*cis*-11,*cis*-14,*trans*-17. No evidence was found for any positional isomers.

The omega-3 (or n-3) polyunsaturated fatty acids (PUFA), especially eicosapentaenoic (EPA or 20:5n-3) and docosahexaenoic (DHA or 22:6n-3), present in fish and fish oils have an imputed major positive role in human health and disease (1-8). It has been shown that n-3 PUFA in fish oils have an inhibitory effect on platelet aggregation, and this reduces the risk of thrombosis (2,3,7,9), which is a major cause of strokes and heart attacks. Furthermore, the n-3 PUFA in fish oils are very effective in lowering serum triglycerides and, in some cases, also lower blood pressure (7,9,10). As a consequence of popular awareness of these possible beneficial effects, increased consumption of omega-3 fatty acids in the form of either dietary fish or fish oil capsules is a recognized change in the nutritional habits of many individuals. In either form, the fish lipid fatty acids are subjected to a certain degree of heat treatment before consumption.

Many chemical reactions including hydrolysis, thermal oxidation, polymerization and isomerization occur during heating of unsaturated fats and fatty acids (11). These reactions lead to a number of undesirable unnatural components, including various geometrical isomers of naturally existing unsaturated fatty acids, cyclic fatty acids, and dimers and higher polymers of triglycerides and fatty acids. The vegetable oil α-linolenic acid (18:3n-3), the most common member of the omega-3 family, undergoes mainly geometrical isomerization during heat treatment and deodorization of vegetable oils (12,13). The more highly unsaturated nature of EPA and DHA suggested that these fatty acids could easily undergo similar structural modifications during oil processing and refining, or any other thermal treatment, and even during the cooking of fish.

In our laboratory, small amounts of EPA and DHA artifacts were observed in distilled omega-3 PUFA concentrates (14), and also in methyl esters of the fatty acids from the contents of a number of retail fish oil capsules (15). This paper describes the characterization of some of the major EPA artifacts, specifically the geometrical isomers.

EXPERIMENTAL PROCEDURES

Materials. Menhaden oil was a gift from the Zapata-Haynie Corporation (Reedville, VA). An omega-3 PUFA concentrate enriched with EPA (30%) and DHA (17%), in the fatty acid ethyl ester form, was prepared from menhaden oil via urea fractionation followed by short-path distillation (14,16,17). EPA, 90% pure, and arachidonic acid, 99% pure, (20:4-Δ5*c*,8*c*,11*c*,14*c*), were purchased from the Sigma Chemical Company (St. Louis, MO). The authentic fatty acids 20:1-Δ8*c*, 20:1-Δ11*c*,20:2Δ11*c*,14, 20:3-Δ11*c*,14*c*,17*c*, and 20:3-Δ8*c*,11*c*,14*c*, were purchased from Nu-Chek-Prep Inc. (Elysian, MN). Meadowfoam (*Limnanthes douglasii*) oil was obtained from the Oregon Meadowfoam Growers Association (Salem, OR). The saturated fatty acid methyl esters (C_3 to C_{15}) and the diesters of dicarboxylic acids used for the identification of the oxidative ozonolysis products were purchased from either Nu-Chek-Prep or the Polyscience Corp. (Niles, IL).

A number of uncommon C_{20} monoethylenic and diethylenic fatty acids required for gas liquid chromatography (GLC) equivalent chain length (ECL) calculations were generated from authentic standards by subjecting them to partial hydrazine reduction, fractionation of the products by silver nitrate thin-layer chromatography ($AgNO_3$-TLC), and subsequent *cis-trans* isomerization of the required products by *p*-toluenesulfinic acid. The details of the procedures have been described elsewhere (12,18-22). The 20:1-Δ14 and a mixture of 20:1-Δ11*c* and 20:1-Δ17*c* were obtained by partial hydrazine reduction and $AgNO_3$-TLC of 20:3-Δ11*c*,14*c*, 17*c*. The same procedure also gave a mixture of 20:2-Δ11*c*,14*c* and 20:2-Δ14*c*,17*c*. A similar procedure applied to 20:3-Δ8*c*,11*c*,14*c* furnished the monoethylenic esters 20:1-Δ8*c*, 20:1-Δ11*c*, 20:1-Δ14*c* and the diethylenic esters 20:2-Δ8*c*,11*c*, and 20:2-Δ11*c*,14*c*. The diene 20:2-Δ5*c*,8*c*, mixed with 20:2-Δ8*c*,11*c*, was obtained from 20:4-Δ5*c*,8*c*,11*c*,14*c*. A mixture of benzene:chloroform (95:5 v/v) was used as the developing solvent for the $AgNO_3$-TLC (see below). Each of the *cis* isomers or pair of isomers was converted to *cis-trans* mixtures according to the method of Snyder and Scholfied (22).

Pure 20:1-Δ5*c* was isolated from meadowfoam oil. The oil (~50 mg) was transmethylated with 10% BF_3-

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MeOH (1 ml), and the methyl esters were fractionated by $AgNO_3$-TLC (developed in benzene:chloroform, 95:5 v/v). The monoethylenic band afforded 20:1-Δ5*c*.

Heat Treatment of EPA. The EPA concentrate (ethyl ester) or authentic EPA (free acid) were heated at 220 ± 5°C (silicone oil bath) for five hr in a 10 ml centrifuge tube flushed with nitrogen and fitted with a leak-tight, Teflon-lined, screw cap.

Isolation of EPA artifacts (EPAA) by silver ion column chromatography. The heated EPA ethyl ester concentrate (0.5 g) was fractionated on 40 g silica gel impregnated with silver nitrate (25% $AgNO_3$, 100/140 mesh, ADSORBOSIL-CABN, Applied Science Laboratories Inc., State College, PA). Hexane containing increasing amounts of a mixture of freshly distilled diethyl ether (23) and methyl ethyl ketone (85:15 v/v) was used as the eluting solvent (24). Saturates, monoenes, dienes and most of the trienes were eluted with HE 18 (hexane containing 18% v/v of diethyl ether and methyl ethyl ketone mixture). With solvent HE 20, enriched isomers (fraction EPAA) eluted just ahead of EPA and were recovered mixed with EPA (Fig. 1c), small amounts of C_{18} tetraenes, and C_{18}, C_{21}, and C_{22} pentaenes (not shown).

Characterization of EPA artifacts. The EPAA artifact fraction, EPAA (25 mg) was saponified in 5 ml of ethanolic KOH under N_2. After dilution with H_2O (10 ml) and acidification (6N HCL, 10 ml) the free fatty acids were extracted with hexane (3 × 10 ml). A small portion of the fatty acids (~1 mg) was methylated with 10% BF_3-MeOH (1 ml) and analyzed by GLC. The GLC showed that the composition of the artifacts was unaffected by the saponification and reesterification.

The EPAA fatty acids (20 mg) were partially reduced (hydrogenated) to a mixture of tetraenoic, trienoic, dienoic, monoenoic and saturated fatty acids, by 95% hydrazine in 96% ethanol (40 ml) for three hours as described by Ratnayake (18). The mixture of reduced fatty acids was methylated as above and the monoethylenic fatty acid methyl ester fraction was isolated via the bromomercuric adduct fractionation technique of Sebedio and Ackman (25). The C_{20} monoethylenic fatty acids were separated from other monoethylenics by reversed phase-high performance liquid chromatography (HPLC).

The isolated 20:1 methyl ester fraction was ozonized in 10% BF_3-MeOH as described by Ackman (26). The ozonolysis products, monomethyl- and dimethyl esters, were analyzed by GLC and identified by comparing their retention time with authentic standards.

Hydrogenation of fatty acid methyl esters. A solution of fatty acid methyl esters (5 mg) in methanol (30 ml) and platinum oxide catalyst (1 mg) was agitated for one hr at room temperature while maintaining a continuous flow of hydrogen over the reaction mixture. The solution was filtered, and the filtrate was concentrated (~2 ml) in a rotary evaporator. The hydrogenated esters were extracted with hexane (3 × 5 ml), washed with water (3 ml), and then concentrated under a slow stream of nitrogen.

Gas liquid chromatography. Analytical gas-liquid chromatography (GLC) was carried out on the following fused silica capillary columns from Supelco, Inc. (Bellefonte, PA): SUPELCOWAX-10, 30 m × 0.32 mm i.d., film thickness 0.25 μm and SP-2340, 60 m × 0.25 mm i.d., film thickness 0.20 μm. The SUPELCOWAX-10 column was installed in a Perkin Elmer model 8420 capillary gas chromatograph coupled to a Perkin Elmer GP-100 graphic printer. The other column was used with a Perkin Elmer 990 instrument coupled to a Perkin Elmer LCI-100 computing integrator. Helium carrier gas was used at a pressure of 12 psig with the former column, and of 26 psig with the latter. The SUPELCOWAX-10 column was operated isothermally at 195°C, except for ozonolysis products, for which the temperature was programmed at a rate of 15°C/min from 60°C to 240°C, and then held at that temperature for 30 min. The SP-2340 column was operated isothermally at 200°C. In all cases, a flame ionization detector was used. The injector and detector temperatures were 250°C and 270°C, respectively.

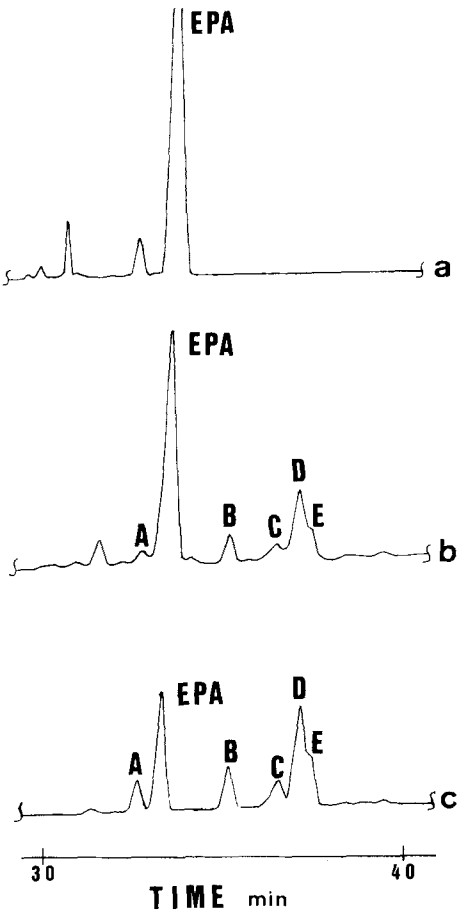

FIG. 1. The GLC C_{20} region of menhaden omega-3 PUFA concentrate (ethyl ester): — (a) before and (b) after heat treatment at 220°C, and (c) the 20:5 region of the artifact concentrate (EPAA) isolated by $AgNO_3$ column chromatography. Peaks A-E refer to artifacts formed after heat treatment. Analysis on a SUPELCOWAX-10 fused-silica capillary column operated isothermally at 195°C.

High performance liquid chromatography. The reverse-phase HPLC was performed on a Waters μ-BONDAPAK C_{18} column (3.9 mm × 30 cm, 10 μm, Waters Associates, Milford, MA). An isocratic mixture of methanol:water (92:8 v/v) at a flow rate of 1.0 ml min^{-1} was used as the eluent. The chromatographic

system was comprised of a Waters 6000 A solvent system, a Waters R401 differential refractive index detector, and a 1 mv pen recorder (Fisher Recordall Series 5000).

Argentation thin layer chromatography ($AgNO_3$-TLC). Preparative $AgNO_3$-TLC of the methyl esters of eicosapentaenoic acid was carried out on precoated Adsorbosil Plus-1 silica gel TLC plates (0.25 mm thick coating, Alltech Ass. Inc., Deerfield, IL) impregnated with $AgNO_3$ by immersion in a 10% (w/v) solution of $AgNO_3$ in acetonitrile, dried horizontally, and activated at 115°C for one hr. After development with a suitable solvent system, the plates were sprayed with an ethanolic solution of 2′,7′-dichlorofluorescein (0.2% w/v), and viewed under ultraviolet (UV) light to locate the separated components. The separated bands were scraped off the plate, extracted with hexane: chloroform (1:1 v/v, 50 ml) and concentrated for GLC analysis.

Spectroscopy. The UV and IR spectra were recorded on Unicam spectrophotometers SP 800 and SP 1000, respectively (Pye Unicam Ltd., Cambridge, U.K.), and the proton magnetic resonance (PMR) spectra on a Nicolet 360 NB instrument (General Electric Co., Fremont, CA).

Gas chromatography/mass spectrometry. Electron impact mass spectra were obtained on a model 700 Finnigan MAT Ion Trap Detector (ITD) System (Finnigan MAT, San Jose, CA), interfaced to a Perkin-Elmer model 990 gas chromatograph (27). The chromatography was executed on a DB-Wax fused silica capillary column (30 m × 0.25 mm ID; J and W Scientific Inc., Folsom, CA). The column was operated at 170°C and 10 psig helium.

All studies were conducted with version 3.0 of the ITD software supplied by the Finnigan Corp. The ITD was operated in the full scan mode and scanned with a 1-s cycle time.

Results. Figures 1a and 1b, respectively, show the 20:5 region of the GLC chromatogram of omega-3 PUFA ethyl ester concentrate before and after heat treatment at 220°C. Heated omega-3 PUFA concentrate contained several unknown peaks in the vicinity of EPA, designated A, B, C, D and E. These EPA artifacts (EPAA) moved with EPA on silicic acid TLC and were converted to 20:0 after hydrogenation, suggesting that they are positional and/or geometrical isomers of EPA. For further characterization the EPAA were isolated by silver nitrate column chromatography. EPAA eluted just ahead of EPA, GLC analysis showed

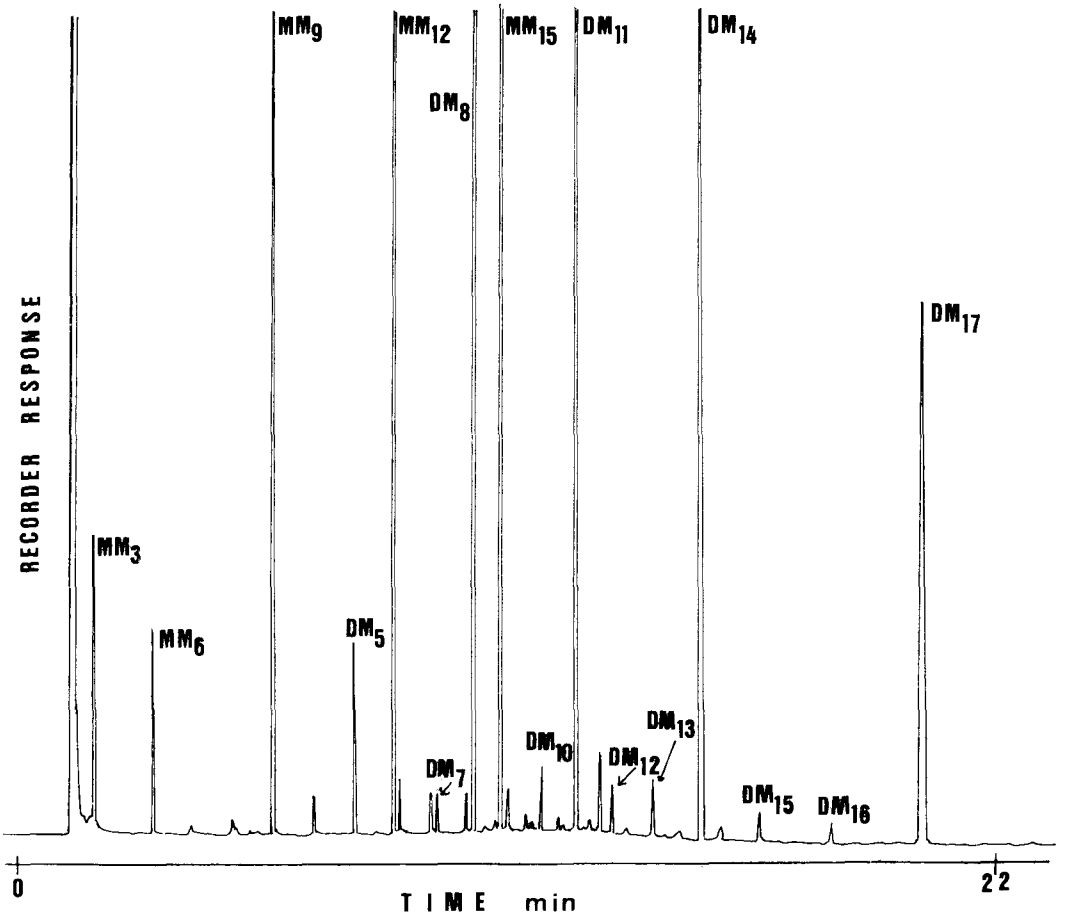

FIG. 2. GLC profile of the ozonolysis products of the pure C_{20} monoethylenic fraction isolated from the partial hydrazine reduction product of menhaden oil EPA artifacts (EPAA). Analysis on a SUPELCOWAX-10 capillary column temperature programmed for 60–240°C at the rate of 15°C/min and held at the final temperature for 30 min. Notations such as MM_{12} and DM_8, etc., represent C_{12} monoester and C_8 diester, respectively.

that the EPAA fraction was made up of 47% of artifacts, 21% of EPA (Fig. 1c), and 32% of C_{18}, C_{21} and C_{22} pentaenoates (not shown in Fig. 1c).

The above EPAA fraction was partially reduced with hydrazine, which is known to reduce double bonds without affecting either the geometry or the position of the remaining double bonds (28). Conditions were chosen to optimize monoethylenic ester formation. The monoethylenic esters were isolated by preparative TLC after conversion into methoxy-bromomercuric adducts. This technique separates the esters according to the degree of unsaturation irrespective of the geometry or the position of the double bonds, and was preferred to direct $AgNO_3$-TLC (21, 25). The isolated monoethylenic fraction was comprised predominantly of C_{20} chain length with relatively small amounts of monoethylenics of C_{18}, C_{21} and C_{22} chain lengths. These C_{18}, C_{21} and C_{22} chain lengths were readily removed by reverse-phase HPLC. Oxidative ozonolysis of the purified C_{20} monoethylenic esters furnished major mono- and diester fragments attributable to unsaturation at Δ5, Δ8, Δ11, Δ14, and Δ17 only (Fig. 2). The mono- and diester fragments were identified by comparing the GLC retention time with authentic standards.

Although several minor peaks representing C_7, C_{10}, C_{12}, C_{13}, C_{15}, and C_{16} diesters were present, they each amounted to less than 2% of the principal diesters, and can be dismissed as either secondary oxidation products (29) or fragments from minor positional isomers. Obviously, no significant positional isomerization of bonds in the original EPA had occurred as a result of heat treatment.

When chromatographed on SUPELCOWAX-10, the HPLC-pure C_{20} monoethylenic fraction showed peaks for *trans*-Δ14 and *trans*-Δ17 esters, in addition to the original *cis* isomers (Fig. 3). No peaks for *trans* isomers of the Δ5, 8 and 11 positions were visible, but it should be noted that *trans*-Δ11 and *trans*-Δ8 overlapped with *cis*-Δ11 on the SUPELCOWAX-10 column employed, and *trans*-Δ5 separates poorly from *cis*-Δ11 (Table 1). SP-2340 gave excellent separation between *cis* and *trans* isomers for a given double bond position (Fig. 4). However, overlap occurred between a *cis* monoene and the *trans* monoene isomer with the double bond position six carbon units further away from the carboxyl end; for example, *cis*-Δ5 with *trans*-Δ11, *cis*-Δ8 with *trans*-Δ14 and *cis*-Δ11 with *trans*-Δ17. Evidence for the presence of *trans*-Δ5 and/or *trans*-Δ8 was found in the leading edge of the *cis*-Δ5/*trans*-Δ11 peak (Fig. 4, Table 1).

More artifacts for spectroscopic investigation were readily prepared by heat treatment of authentic eicosapentaenoic acid at 220°C for five hr. The gas-chromatogram of the product from this treatment, after conversion into methyl esters, is shown in Figure 4a. The absence of the shoulder peak E and the sharpness of the artifact peaks when compared with the (menhaden) ethyl ester artifacts (Fig. 1b and c) are noteworthy. However, when the ethyl esters were methylated the chromatogram (not shown) resembled that shown in Figure 6a; the artifact peaks became sharper and the peaks D and E merged into one. This showed that the dissimilarities between Fig. 5a and Fig. 1b and c are due to differences in chromatographic behavior between methyl and ethyl esters, rather than arising from different artifact compositions.

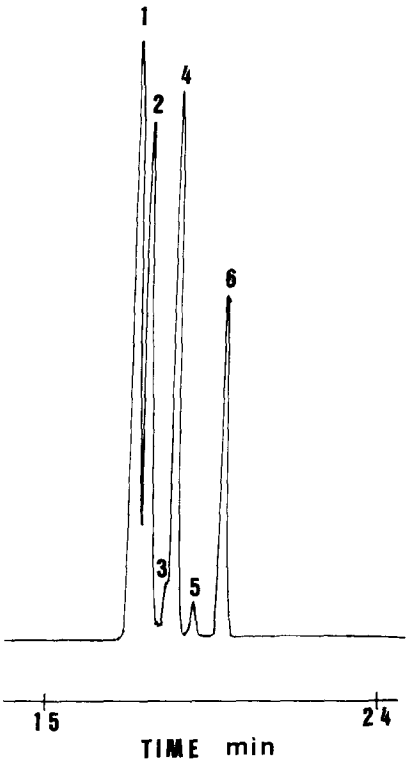

FIG. 3. GLC profile of the C_{20} monothylenic fraction isolated from the partial hydrazine reduction product of menhaden oil EPA artifacts (EPAA; Fig. 1) after purification by reversed phase HPLC. Analysis on a SUPELCOWAX-10 capillary column operated isothermally at 195°C. Identifications: 1 = 20:1-Δ5*c*+20:1-Δ8*c*; 2 = 20:1-Δ11*c*+20:1-Δ8*t*+20:1-Δ5*t*; 3 = 20:1-Δ14*t*; 4 = 20:1-Δ14*c*; 5 = 20:1-Δ17*t* and 6 = 20:1-Δ17*c*.

The artifacts formed by heat-treatment of authentic EPA were separated from the unreacted EPA by $AgNO_3$-TLC after conversion into methyl esters; a broad band moving slightly above that of unchanged EPA methyl ester contained the EPA artifacts (Fig. 5b). This mixture of artifacts derived from authentic EPA is referred to as EPAI. The high resolution (360 Mz) proton magnetic resonance of EPAI showed signals at δ $^{CDCl_3}_{TMS}$, 5.37 (-C$\underline{H}$=C$\underline{H}$-), 3.66 (-$CO_2C\underline{H}_3$), 2.76 (-CH=CHC$\underline{H}_2$CH=CH), 2.33 (-$CH_2C\underline{H}_2CO_2CH_3$), 2.10 ($CH_3C\underline{H}_2CH$=CH- and CH=CHC$\underline{H}_2CH_2$-), 1.70 (-$C\underline{H}_2CH_2CO_2CH_3$), a complex set of signals at 1.2–1.6 (?), and 0.8–1.0 ($C\underline{H}_3CH_2$- + ?). No signals were found in the ppm 4.5–5.0 region where vinyl hydrogen (CH_2=CH-) usually appears, and hence the absence of terminal ethylenic bonds in the artifacts was confirmed. The UV spectrum of EPAI did not show any absorptions characteristic of double bonds conjugated with carbonyls, and hence the presence of double bonds at the Δ2 position was also eliminated. A UV absorption maximum at 233 nm (conjugated dienes) and maxima at 263 nm and 274 nm (conjugated trienes) were observed, but they represented only 0.9% and 0.4%, respectively of conjugated dienes and conjugated trienes

TABLE 1

Fractional Chain Length Values[a] and Diethylenic Interaction Corrections[b] for Ethylenic Bond Positions Related to EPA

Monoethylenic isomers

	SP-2340				SUPELCOWAX-10			
	ECL		FCL		ECL		FCL	
Δ[c]	*Cis*	*Trans*	*Cis*	*Trans*	*Cis*	*Trans*	*Cis*	*Trans*
5	20.47	20.38	0.47	0.38	20.15	20.25	0.15	0.25
8	20.57	20.40	0.57	0.40	20.15	20.20	0.15	0.20
11	20.62	20.44	0.62	0.44	20.21	20.22	0.21	0.22
14	20.78	20.55	0.78	0.55	20.36	20.33	0.36	0.33
17	20.99	20.65	0.99	0.65	20.59	20.44	0.59	0.44

Diethylenic Isomers

Isomer[d]	ECL	Diethylenic Interaction Correction	ECL	Diethylenic Interaction Correction
5*c*,8*c*	21.07	0.03	20.40	0.10
5*t*,8*c*	21.01	0.06	20.61	0.21
5*c*,8*t*	20.97	0.10	20.53	0.18
5*t*,8*t*	20.86	0.08	20.62	0.17
8*c*,11*c*	21.27	0.08	20.48	0.12
8*t*,11*c*	21.21	0.19	20.65	0.24
8*c*,11*t*	21.17	0.16	20.62	0.25
8*t*,11*t*	20.97	0.13	20.62	0.20
11*c*,14*c*	21.55	0.15	20.70	0.13
11*t*,14*c*	21.42	0.20	20.83	0.25
11*c*,14*t*	21.32	0.15	20.74	0.20
11*t*,14*t*	21.10	0.11	20.74	0.19
14*c*,17*c*	21.87	0.10	21.04	0.09
14*t*,17*c*	21.65	0.11	21.07	0.15
14*c*,17*t*	21.55	0.12	20.97	0.17
14*t*,17*t*	21.33	0.13	20.94	0.17

[a]Chromatographic conditions are described in the experiment section.
[b]Diethylenic Interaction Correction = ECL (obs.) $-\Sigma$ FCL.
[c]Position of the ethylenic bond with respect to the carboxyl group.
[d]*c* and *t* Denote *cis* and *trans* respectively, for example, 20:2Δ5*t*,8*c* denotes 20:2-*trans*-Δ5, *cis*-Δ8.

(30). Considering the high degree of unsaturation in the esters being examined, such small amounts of conjugated esters could have easily arisen from autoxidation, and can be considered insignificant.

The IR spectrum of EPAI showed absorption at 970 cm^{-1}, confirming the presence of *trans* unsaturation in the artifacts. Treatment of authentic EPA methyl ester with *p*-toluenesulfinic acid for 15 min under conditions where strictly *cis-trans* isomerization occurs (22), furnished a product (Fig. 6a) which was strikingly similar to that formed by heat treatment of EPA at 220°C (Fig. 5a and Fig. 1b and c). When the isomerization reaction was allowed to continue for 30 min, additional artifacts were observed. On SUPELCOWAX-10, they eluted on the shoulders of the peaks B, C and D, and as a cluster of peaks (denoted F) after peak D (Fig. 6b). When the isomerization was carried out for one hr, the cluster F increased in intensity relative to peaks B, C and D (Fig. 6c). Furthermore, it was found that products similar to that in cluster F could also be made when EPA is heated for the same length of time as before, but at an elevated temperature of 250°C.

DISCUSSION

In a previous study, Ackman and coworkers (12) identified *cis-trans* isomers of α-linolenic acid in steam deodorized vegetable oils; this process subjects the oil to temperatures in excess at 200°C. More recently, Grandgirard and coworkers showed that heat treatment of rapeseed and soybean oils at 240°C results in the geometrical isomerization of α-linolenic acid (13). Later, they also showed that such C_{18} artifacts are elongated to EPA geometrical isomers in animals (31). None of these studies found evidence for any positional isomerization of the ethylenic bonds.

There are 30 possible geometrical isomers of EPA; some of these could be expected to exhibit the same physicochemical properties. Therefore, resolution of EPA *cis-trans* isomers from each other is a complex problem, particularly in the absence of authentic standards for comparison. However, tentative structures for these isomers could be deduced by comparison of their equivalent chain lengths (ECL) on polar capillary columns with those calculated from fractional chain length (FCL) values of the corresponding *cis* and *trans*

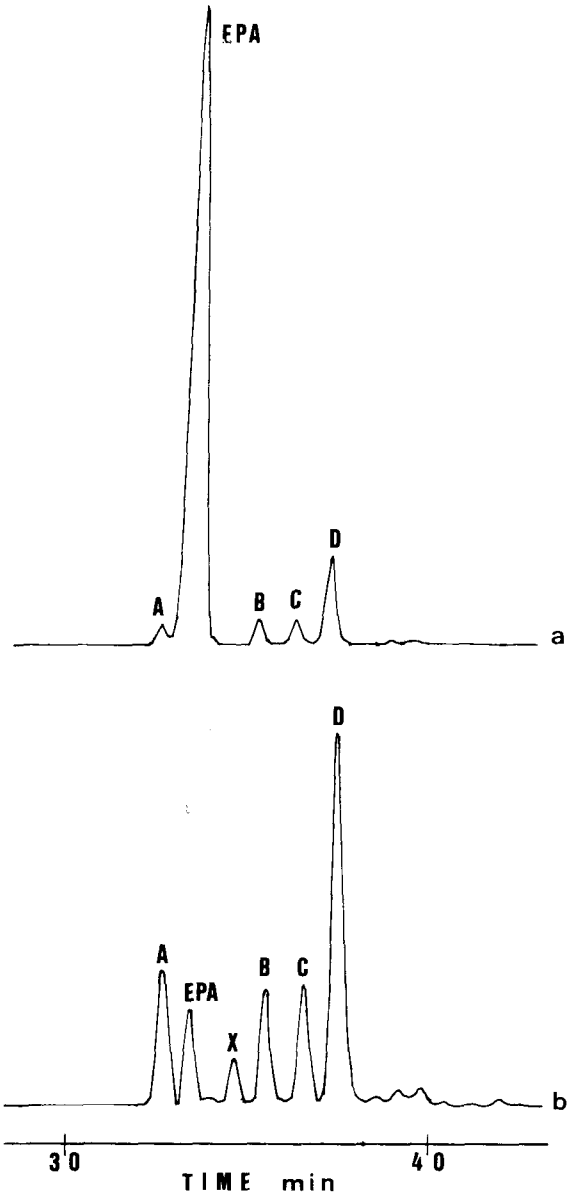

FIG. 4. GLC profile of the C_{20} monoethylenic fraction obtained from the partial hydrazine reduction product of menhaden oil EPA artifacts (EPAA; Fig. 1c) after purification by reversed phase HPLC. Analysis on a SP-2340 (60 m) capillary column operated isothermally at 200 C. Identifications: 1 = 20:1-Δ5*t*; 2 = 20:1-Δ8*t*; 3 = 20:1-Δ5*c*+20:1-Δ11*t* 4 = 20:1-Δ8*c*+20:1-Δ14*t*; 5 = 20:1-Δ11*c*+20:1-Δ17*t*; 6 = 20:1-Δ14*c* and 7 = 20:1-Δ17*c*.

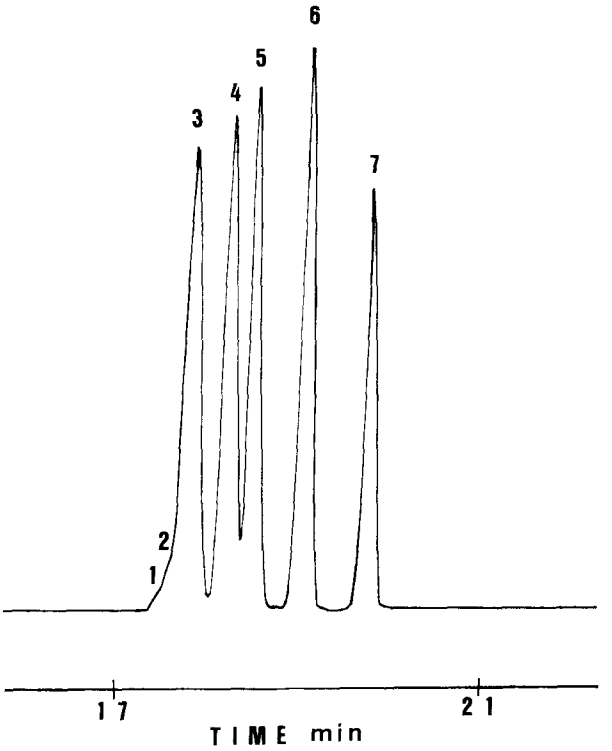

FIG. 5. GLC profiles of: (a) methyl ester of EPA after heating the free acid at 220°C for five hr, and (b) EPA artifact methyl esters (EPAI) isolated by $AgNO_3$-TLC. Analyses on a SUPELCOWAX-10 capillary column operated isothermally at 195°C. A,B,C, and D correspond to the artifact ethyl esters shown in Figure 1, b and c. Note the coelution of peaks D and E and the appearance of a new peak X. Peak X is an environmental contaminant, probably a phthalate.

monoethylenic isomers. This approach has been successfully applied to identification of C_{20} all *cis*-polyunsaturated fatty acids (19) and some *cis* and *trans* C_{18} isomers (20).

Table 1 shows ECL values of the *cis-trans* monoethylenic and methylene-interrupted diethylenic derivatives of EPA on two different GLC phases. Also shown are values for "diethylenic corrections", which is the difference between the observed ECL and that calculated by summation of FCL values. The ECL values of possible EPA isomers were calculated by addition of the relevant diethylenic corrections to the sum of the FCL values. However, the ECL values so obtained for EPA methyl ester (20:5-Δ5*c*,8*c*,11*c*,14*c*,17*c*) on both phases were found to be higher than the experimental values; the differences for SP-2340 and SUPELCOWAX-10 being 0.14 and 0.12, respectively. Therefore, this difference (error value) was subtracted from the calculated values to obtain the final ECL values. For example, for 20:5-Δ5*c*,8*c*,11*c*,14*c*,17*t* methyl ester on SP-2340, the calculation is 20.00 + 0.47 + 0.57 + 0.62 + 0.78 + 0.65 + (0.03 + 0.08 + 0.15 + 0.12) − (0.14) = 23.33.

Table 2 shows the ECL of the EPA isomers (EPAI) determined on SUPELCOWAX-10 (moderately polar) and SP-2340 (more polar) liquid phases, the chromatograms are shown in Figures 5b and 7, respectively. Also shown in Table 2 are some of the possible *cis-trans* EPA isomers for each of the observed ECL values. When making these assignments, only peaks agreeing with the experimental ECL values within ± 0.02 units have been included. On SUPELCOWAX-10 the ECL of peak A (Fig. 5b, Table 2) matches with the calculated ECL value of the mono-*trans* isomer 20:5-Δ5*c*,8*c*,11*c*,14*c*,17*t* as well as with that of the di-*trans* isomer 20:5-Δ5*c*,8*c*,11*c*,14*t*,17*t*. However, the latter was not found when EPAI was chromatographed on SP-2340 (Fig. 1, Table 2), and therefore peak A was identified as a mono-*trans* isomer. This same isomer is the first to elute on SP-2340 as well, as

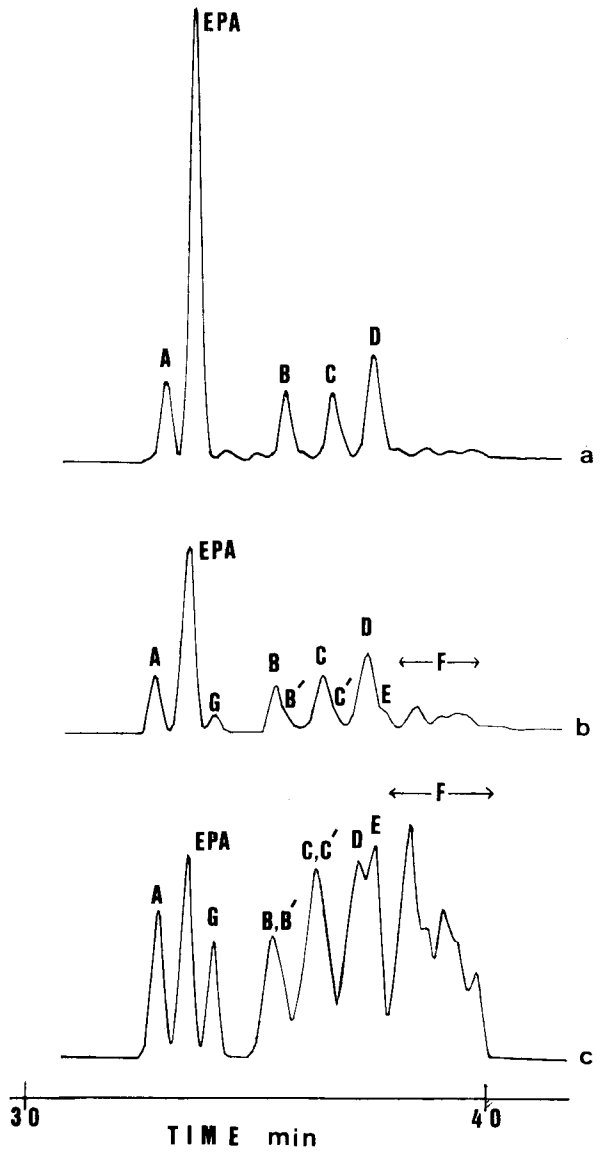

FIG. 6. GLC profiles of EPA methyl ester subjected to *cis-trans* isomerization with *p*-toluenesulfinic acid for: (a) 15 min, (b) 30 min, and (c) one hr. Analysis on a SUPELCOWAX-10 capillary column operated isothermally at 195°C. Note the development of new peak G and shoulders on peaks B, C, D as well as a cluster of peaks (denoted F) after peak D.

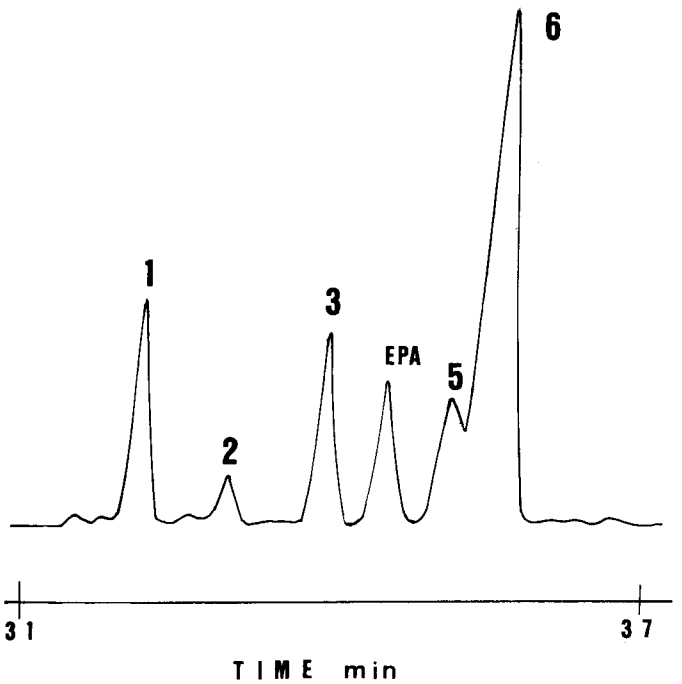

FIG. 7. SP-2340 GLC profile of the methyl esters of EPA artifacts (EPAI) isolated from EPA free acid heated at 220°C for five hr; isothermal operation at 200°C. Peaks 1, 2, 3, 5 and 6 are geometrical isomers of EPA.

indicated by the more or less equal area percent. The peak denoted EPA (Fig. 6b) was augmented when coinjected with authentic EPA methyl ester, and is due to residual EPA. The peak denoted X, though not visible in the GLC profile of heated EPA total esters (Fig. 5a), appears as a distinct peak in the artifact concentrate obtained from $AgNO_3$-TLC (Fig. 5b). However, GC/MS on the Finnigan MAT Ion Trap Detector of EPAI revealed that the mass spectrum of X was different from those of the other component artifacts, all of which exhibited very similar fragmentation patterns. This observation, coupled with the fact that it is not shown in the GLC of the total reaction product before fractionation (Fig. 4a) suggested that X is not an artifact of EPA, but an environmental contaminant. Based on its mass spectral fragment ions at m/z 370, 259, 147, 129 and 111, X is probably a phthalate ester, and it was not examined further. By comparison of observed and calculated ECL values, peaks B and C were tentatively identified as due to 20:5-Δ5*t*,8*c*, 11*c*,14*c*,17*c* and 20:5-Δ5*c*,8*t*,11*c*,14*c*17*c*, respectively. However, it can be seen from Table 2 that there is much overlap between peaks due to mono-*trans* and poly-*trans* isomers of EPA. Furthermore, it has been shown that while the polyunsaturated esters containing a single *trans* ethylenic bond may be identified by consideration of predicted ECL values, the method is less satisfactory when applied to systems with more than one *trans* unit (32). Therefore, the occurrence of some poly-*trans* structures cannot be ruled out altogether.

It is noteworthy that no evidence for the Δ11*t* isomer was found. Resistance to geometrical isomerization by the central double bond in the all-*cis* polyunsaturated system has been noted before. For example, while the *trans*-Δ9 and *trans*-Δ15 isomers of α-linolenic acid (18:3-Δ9*c*,12*c*,15*c*) were found in heated soybean and rapeseed oils (31), and also in partially hydrogenated soybean oil (33), no *trans*-Δ12 isomer was detected in either study.

The artifacts in menhaden oil ethyl esters (Fig. 1b and c) were identified by comparison of their GLC characteristics with that of EPAI. Comparison shows that: A is 20:5-Δ5*c*,8*c*11*c*,14*c*,17*t*, B is 20:5-Δ5*t*,8*c*,11*c*, 14*c*,17*c*, and C is 20:5-Δ5*c*,8*t*,11*c*,14*c*,17*t*.

Artifact A is recognized easily in any fish oil as there is no component normally present in SUPELCOWAX-10 GLC analysis on the front edge of the 20:5n-3 peak (Fig. 1a). Artifact B is also usually visible, but the rest are ob-

TABLE 2

Gas-Chromatographic Data for the EPA Artifacts (EPAI) Isolated from Heated Eicosapentaenoic Acid

Peak[a]	% Area	ECL (obs)	Possible Structures[b] (ECL predicted)[c]
SUPELCOWAX-10			
(A)	14.3	21.73	17*t*,(21.71); 14*t*,17*t*(21.75)
	10.3	21.78	EPA
(X)	5.6	21.86	14*t*(21.88); 11*t*,14*t*,17*t*(21.88)
(B)	12.5	21.97	5*t*(21.99); 8*t*,17*t*(21.96); 11*t*,17*t*(21.97); 5*t*,14*t*,17*t*(21.96); 8*t*,11*t*,14*t*,17*t*(21.96)
(C)	13.5	22.05	8*t*(22.03); 11*t*(22.04); 5*t*,8*t*,17*t*(22.05); 8*t*11*t*,17*t*(22.05); all *trans* (22.05)
(D)	43.8	22.15	8*t*,14*t*(22.13); 5*t*,8*t*,11*t*,17*t*(22.14)
SP-2340[d]			
1	13.6	23.32	17*t*(23.33); 8*t*,17*t*(23.34); 5*t*,8*t*,14*t*(23.33)
2	3.5	23.45	14*t*(23.43); 8*t*,11*t*(23.47); 8*t*,14*t*(23.44)
3	12.8	23.57	5*t*(23.59); 5*t*,8*t*(23.55)
4	9.9	23.65	8*t*(23.66); EPA
5	11.5	23.73	?
6	48.7	23.79	?

[a]The letters shown in parenthesis refer to peaks shown in Figure 5b.
[b]All the structure shown are geometrical isomers of 20:5-Δ5*c*,8*c*,11*c*,14*c*,17*c*, (EPA); for simplicity the 20:5 and *cis* ethylenic bonds are omitted. For example, 17*t* = 20:5-Δ5*c*,8*c*,11*c*,14*c*,17*t*.
[c]The predicted ECL values are shown in parenthesis.
[d]The SP-2340 peak numbers refer to peaks shown in Figure 7.

scured by the natural isomers of 22:1 (n-11 and n-9).

We have not been successful in identifying the two of the major artifact components, namely D and E (Fig. 5b, peak D). Although poly-*trans* structures such as 20:5-Δ5*c*,8*t*,11*c*,14*t*,17*c* are possible on the basis of ECL values on SUPELCOWAX-10, this identification could not be confirmed by SP-2340 ECL calculations. The possibility of artifacts D and E being cyclic compounds was also investigated as the occurrence of cyclic fatty acid monomers in heated polyunsaturated vegetable oils has been reported (34–36). Hydrogenation of EPAI over platinum oxide gave a product containing 20:0 (72%) and three other components (28%), which were tentatively identified by GC/MS as C_{20} cyclic monomers (15). Considering that artifact D (Fig. 5b) accounts for 43% of the unhydrogenated EPAO, it is not possible that D is entirely due to cyclic compounds. We believe, rather, that D contains predominately EPA geometric isomers with more than one *trans* ethylenic bond. From that it is clear that the artifacts A-E is heated menhaden oil ethyl esters are composed mainly of geometric isomers of EPA. Whether these have biological activity or interfere with the biochemistry of the corresponding all-*cis*-fatty acids is not known.

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