Characterization of γ-Linolenic Acid Geometrical Isomers in Borage Oil Subjected to Heat Treatments (Deodorization)

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Heating of borage oil, either under vacuum as a model or during steam-vacuum deodorization, produces artifacts that are geometrical isomers of γ-linolenic acid (*cis*-6,*cis*-9, *cis*-12 18:3 acid). In a first approach, we have studied the behavior of these fatty acids in the form of either methyl or isopropyl esters on two capillary columns (CP-Sil 88 and DB-Wax). From this study, it appears that the DB-Wax capillary column is the best suited analytical tool to study in some detail γ-linolenic acid geometrical isomers. In a second approach, the structure of these isomers was formally established by combining several analytical techniques: Argentation thin-layer chromatography, comparison of the equivalent chainlengths with those of isomers present in NO_2-isomerized borage oil on two different capillary columns, partial hydrazine reduction, oxidative ozonolysis, gas chromatography coupled with mass spectrometry and gas chromatography coupled with Fourier transform infrared spectroscopy. The two main isomers that accumulate upon heat treatments are the *trans*-6,*cis*-9, *cis*-12 and *cis*-6,*cis*-9,*trans*-12 18:3 acids with minor amounts of *cis*-6,*trans*-9,*cis*-12 18:3 acid. One di-*trans* isomer, supposed to be the *trans*-6,*cis*-9,*trans*-12 18:3 acid, is present in low although noticeable amounts in some of the heated oils. The content of these artificial fatty acids increases with increasing temperatures and duration of heating. The degree of isomerization (DI) of γ-linolenic acid is less than 1% when the oil is deodorized at 200°C for 2 h. Heating at 260°C for 5 h increases the DI up to 74%. Isomerization of γ-linolenic acid resembles that of α-linolenic (*cis*-9, *cis*-12,*cis*-15 18:3) acid in several aspects: The same kinds and numbers of isomers are formed, and similar degrees of isomerization are reached when the octadecatrienoic acids are heated under identical conditions. It seems that the reactivity of a double-bond *vis-à-vis cis-trans* isomerization is linked to its relative position, central or external, and not to its absolute position (Δ6, 9, 12 or 15).

KEY WORDS: Borage oil, deodorization, geometrical isomers, heated oil, α-linolenic acid, γ-linolenic acid, *trans* fatty acids.

In a recent series of papers, it was shown that α-linolenic acid (*cis*-9,*cis*-12,*cis*-15 18:3 acid) geometrical isomers are common constituents of fully refined α-linolenic acid-containing oils (1-3) and of foods containing such oils (1,4). It has also been reported by a few authors that the formation of α-linolenic acid geometrical isomers is linked to heat treatments (5-9), generally deodorization (5,6), performed at temperatures equal to or higher than 190-200°C. Under the most common conditions used for industrial oil deodorization in Europe (1-4) and North America (5), the major isomers that accumulate have the structures *cis*-9,*cis*-12, *trans*-15 and *trans*-9,*cis*-12,*cis*-15 (85-90% of total *trans* isomers). Two other minor constituents having the structures *cis*-9,*trans*-12,*cis*-15 and *trans*-9,*cis*-12,*trans*-15 are also generally present (10-15%).

In the present study, we have investigated the effect of heat treatments (simple heating under vacuum used as a model on the one hand and deodorization under vacuum in the presence of steam on the other hand) on the structure of γ-linolenic acid (in the text, γ-linolenic acid or all-*cis* 18:3n-6 isomer will refer to the *cis*-6,*cis*-9,*cis*-12 18:3 acid). Deodorization is sometimes applied to γ-linolenic acid-containing oils (10), for example to remove potential pesticide residues. Moreover, the study of γ-linolenic acid isomerization may be of some help to understand the reaction mechanisms underlying geometrical isomerization of the more common α-linolenic acid. In the present study, we have focused our attention on borage oil, which contains *ca.* 25% of its fatty acids as γ-linolenic acid.

EXPERIMENTAL PROCEDURES

Heating of oils. Aliquots of a given oil (2 to 2.5 mL) are introduced in ready-to-seal glass ampoules. Each ampoule is connected to a water vacuum aspirator with a stopcock in the line. The ampoule is dipped in a water bath at 50°C and left under vacuum for approximately 15 min with occasional shaking. The stopcock is closed and disconnected with the ampoule from the aspirator before sealing the ampoule. The oil samples are heated in an oven at the desired temperature (±5°C) for a given duration (2,9).

Deodorization of oils. Refined borage oil is deodorized in a laboratory-scale deodorizer under reduced pressure (0.1-0.7 kg/cm^2) for two hours. The amount of stripping steam is 3-5% water by weight of oil per hour.

Fatty acid methyl esters (FAME) and isopropyl esters (FAIPE) preparation. FAME are prepared by transesterification of oil triglycerides (or by direct esterification when free fatty acids are used) according to Morrison and Smith (11). In screw-capped tubes containing two drops of oil (or a few mg of free fatty acids), are added 1.5 mL of BF_3 in methanol (12%, wt/vol). The resulting mixture is homogenized with benzene until complete dissolution of the oil droplets. The tubes are tightly capped and left in a boiling waterbath for 1 h (0.5 h for free fatty acids). After adding 1.5 mL water, FAME are extracted twice with 2.5-mL portions of hexane.

FAIPE are prepared essentially as described by Wolff and Fabien (12). Two drops of oil (or a few mg of free fatty acids) are introduced into screw-capped tubes, and 1.5 mL hexane, 2.8 mL isopropanol and 0.25 mL concentrated H_2SO_4 are added to the oil. At the end of the reaction (100°C for 1 h), 5 mL water is added, and the upper layer is withdrawn after thorough mixing and decantation. A second extraction with 2.5 mL hexane is performed.

Nitrous isomerization of oils. The oils are geometrically isomerized as described previously (13). To 0.25-1.25 mL of the oil are added, successively, 1 mL 2 M aqueous sodium nitrite and 0.8 mL 6 M nitric acid. The reaction is performed at 50°C for 25 min in screw-capped tubes under vigorous magnetic stirring. At the end of the

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reaction, water is added, and the reaction products are extracted with hexane. After removal of hexane, FAME (or FAIPE) are prepared as described above. Most of the dark-colored material is removed by adding silica gel H to the hexane extracts. The supernatant is used as such for further chromatographic analyses.

Partial hydrazine reduction of unsaturated fatty acids. Oils (or methyl esters) (5–50 mg) are saponified in screw-capped tubes in the presence of 3 mL 0.5 M ethanolic KOH (100°C, 1 h). After acidification with 1.2 M aqueous HCl, free fatty acids are extracted after addition of water and hexane. After removal of hexane, free fatty acids are dissolved in 95% ethanol (5 to 50 mL, depending on the initial weight of fatty material) to which is added 0.2 to 1.6 mL hydrazine hydrate (64% hydrazine; Sigma Chemical Co., St. Louis, MO). The reaction is performed at 40–45°C for 90 min with gentle magnetic stirring under a light stream of O_2 onto the surface of the solution. At the end of the reaction, the solution is acidified with 3 M aqueous HCl, and the fatty acids are immediately extracted with hexane. The fatty acids are then transformed into either FAME or FAIPE.

Ozonolysis of FAME. Methyl esters of fatty acids partially reduced with hydrazine are fractionated by argentation thin-layer chromatography (Ag-TLC) to isolate *cis* and *trans* monoenes (see below). After elution from the gel, the two fractions are submitted to oxidative ozonolysis according to the procedure of Ackman *et al.* (14) as modified by Grandgirard *et al.* (8). A micro-ozonizer (Supelco Inc., Bellefonte, PA) is used for this purpose. A few mg of FAME are dissolved in 2 mL of a 7% (wt/vol) solution of BF_3 in methanol into a screw-capped tube. Ozone in oxygen is bubbled through the solution (10 mL/min) for 12 min. The tube is tightly capped and heated at 100°C for 2 h. After cooling, water is added, and the resulting products [monomethyl esters (MME) and dimethyl esters (DME)] are extracted with chloroform.

Ag-TLC. FAME or FAIPE are separated according to the number and geometry of their double bonds by TLC on $AgNO_3$-impregnated silica-gel plates, Ag-TLC. Commercial precoated plates (DC-Vertigplatten Kieselgel H; Merck, Darmstadt, Germany) are soaked in a 5% (wt/vol) $AgNO_3$ solution in acetonitrile for 20 min, partially dried in air for *ca.* 10 min and activated for 30 min at 120°C. The migration solvents are mixtures of hexane and diethyl ether: 90:10 (vol/vol) for the separation of *cis* and *trans* monoenes, 85:15 (vol/vol) for the separation of octadecatrienoic acid geometrical isomers, and occasionally 70:30 (vol/vol) for a second purification of *trans*-trienoic acids. At the end of chromatographic runs, plates are briefly air-dried for a few minutes and then sprayed with a 0.2% solution of 2′,7′-dichlorofluorescein in 95% ethanol. After visualization under ultraviolet light, the bands are scraped off with a cutter in an aluminum foil, and the gel is transferred into test tubes. FAME or FAIPE are then extracted in the following way: 1.5 to 2.5 mL methanol (depending on the quantity of silica gel) is added to the gel, followed by 2.5 mL hexane and 1 to 2 mL 5% aqueous NaCl solution. Thorough mixing follows each addition. After decantation and withdrawal of the upper phase, the lower phase is extracted a second time with hexane (2.5 mL).

Gas–liquid chromatography (GLC). Analyses of FAME and FAIPE by GLC are carried out on Carlo Erba 4130 and HRGC chromatographs fitted with flame-ionization detectors and split injectors (Carlo Erba, Milano, Italy). The first chromatograph is equipped with a CP-Sil 88 capillary column (50 m × 0.33 mm i.d., 0.24 μm film; Chrompack, Middleburg, The Netherlands). This column is operated at 160°C for 45 min; the temperature is then increased to 195°C at a rate of 7.5°C/min and held at this temperature until completion of the analysis. The carrier gas is helium (inlet pressure, 1 kg/cm^2). The second chromatograph is fitted with a DB-Wax capillary column (30 m × 0.32 mm i.d., 0.5 μm film; J & W Scientific, Folsom, CA). The temperature of the column is maintained at 180°C for 25 min and then raised at a rate of 7.5°C/min up to 215°C, where it is left until the end of the chromatographic run. The inlet pressure of the carrier gas (hydrogen) is 0.9 kg/cm^2. In both cases, the split-injection ports and the detectors are maintained at 250°C. Quantitative analyses are performed with SP 4290 and Chromjet integrators (Spectra Physics, San Jose, CA). Equivalent chainlengths (ECLs) are calculated according to Ackman (15) after addition of 16:0, 18:0 and 20:0 acid methyl or isopropyl esters when necessary.

GLC coupled with mass spectrometry (GLC–MS). MME and DME are analyzed by GLC–MS with a Hewlett-Packard 5890 gas chromatograph coupled with a 5970 Mass Selective Detector (Hewlett-Packard, Palo Alto, CA). A DB-Wax column (30 m × 0.25 mm i.d., 0.5 μm film) is used for the separation of MME and DME. Temperature programming is the same as that previously described (16).

GLC coupled with Fourier transform infrared spectroscopy (GLC–FTIR). The gas phase infrared spectra are obtained with a Brucker IFS 85 FTIR spectrometer (Brucker Spectrospin, Wissembourg, France). This is connected to a Carlo Erba 5160 gas chromatograph equipped with an on-column injector and a flame-ionization detector. A BPX 70 capillary column (50 m × 0.22 mm i.d., 0.25 μm film; SGE Scientific Pty Ltd, Melbourne, Australia) is used. The oven temperature is programmed from 30 to 240°C at a rate of 10°C/min. Other conditions are as described elsewhere (17).

RESULTS AND DISCUSSION

Optimized gas–liquid chromatographic conditions for the analysis of γ-linolenic acid isomers. In a first approach, we studied the gas–liquid chromatographic behavior of γ-linolenic acid isomers present in NO_2-isomerized and in vacuum-heated samples of borage oil. Nitrous isomerization of polyunsaturated fatty acids leads exclusively to geometrical isomerization, with no significant positional shift of double bonds (18,19). Heating of α-linolenic acid leads almost exclusively to geometrical isomerization (8,9,19). On the other hand, the effect of heating on γ-linolenic acid has not been studied, and it is not known whether heating of this fatty acid may give rise to positional and/or geometrical isomerization. However, and for the sake of clarity, we will consider in the following that heating of γ-linolenic acid generates geometrical isomers only. This will be formally demonstrated later.

Analysis of FAME on a CP-Sil 88 capillary column. Analyses of FAME prepared with NO_2-isomerized borage oil on a CP-Sil 88 capillary column allow the resolution of five peaks (instead of eight peaks corresponding to the

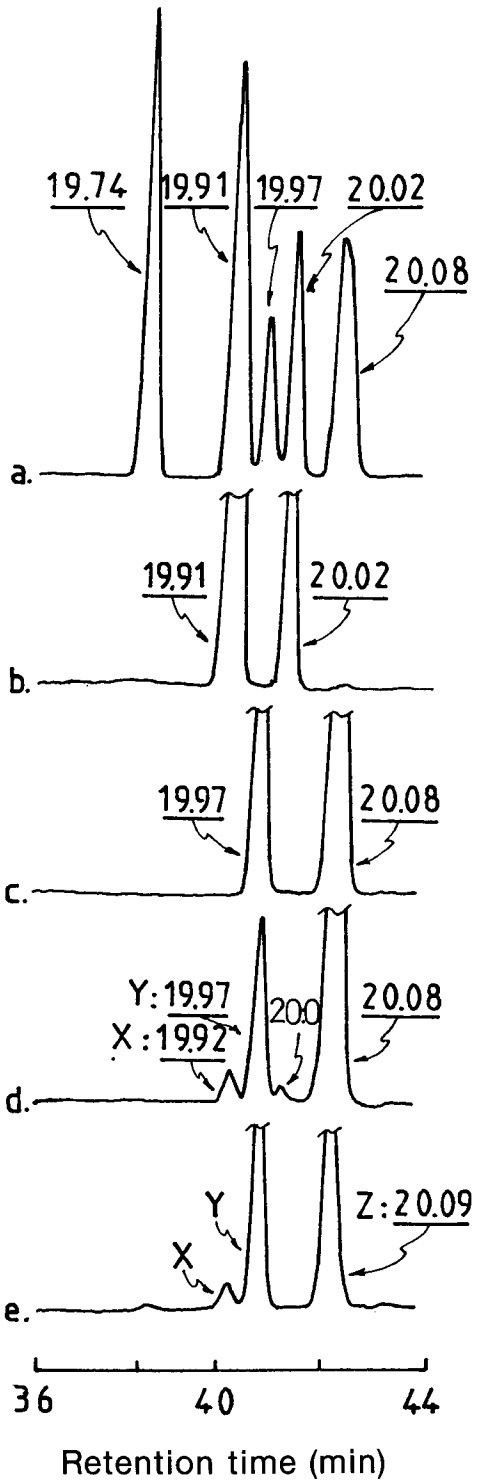

FIG. 1. Partial chromatograms (region of 18:3n-6 acid isomers) obtained on a CP-Sil 88 capillary column (Chrompack, Middleburg, The Netherlands) with fatty acid methyl esters prepared with: a., NO_2-isomerized borage oil; b., di-*trans* fraction isolated from a.; c., mono-*trans* fraction isolated from a.; d., borage oil heated under vacuum at 240°C for 6 h; e., *trans* fraction isolated from d. Underlined numbers correspond to equivalent chainlengths.

eight theoretical geometrical isomers) (Fig. 1). Fractionation by Ag-TLC of this mixture of FAME indicates that the first eluting well-resolved peak (ECL = 19.74) is the all-*trans* isomer (fraction not presented in Fig. 1). Inside each of the mono- and di-*trans* isomer fractions of γ-linolenic acid, isomers are poorly resolved. Both fractions give only two peaks instead of three (Fig. 1), one peak containing a single component, the other containing two unresolved isomers. The number of components under a peak can be easily determined, because nitrous elaidination gives families (mono- or di-*trans* isomers) in which each member represents approximately one-third of the total (19). Fortunately, there is no overlap between the constituents of the two fractions. However, the two unresolved mono-*trans* isomers (ECL = 20.09) co-elute with the all-*cis* 18:3n-6 acid (Fig. 1). A supplementary drawback is that two isomers, one mono-*trans* (ECL = 19.97) and one di-*trans* (ECL = 20.02) are badly separated from 20:0 acid.

Analysis of FAME prepared with a sample of borage oil that was heated under vacuum at 240°C for 6 h showed that two supplementary peaks are present as compared to a sample of cold-pressed borage oil (peaks X and Y in Fig. 1). The first small peak (X) has an ECL value (19.92) identical to that of one of the di-*trans* peaks (containing two isomers) present in the di-*trans* fraction of NO_2-isomerized borage oil. Any shift of the double bond from position 6 to position 5 would have led to components (with all *cis* and *trans* combinations being taken into account) that would have had ECLs between 19.34 and 19.87 [Wolff R.L., unpublished observations made with NO_2-isomerized *cis*-5,*cis*-9,*cis*-12 18:3 (pinolenic) acid]. As no peaks were detected in this chromatographic zone, it is unlikely that positional isomerization, at least in the direction of the carboxylic group, may have taken place during heating. The second peak (Y) has an ECL value (19.97) identical to that of one of the mono-*trans* isomers present in the mono-*trans* fraction of NO_2-isomerized borage oil (Fig. 1). Analysis of the *trans*-triene fraction isolated by Ag-TLC from FAME of the heated sample of borage oil shows a third peak (peak Z in Fig. 1) that previously co-eluted with γ-linolenic acid in unfractionated FAME. This supplementary peak has an ECL value (20.09) identical to that of the peak containing two mono-*trans* isomers. No traces of the all-*trans* isomer could be detected.

From the preceding observations, it appears that heated borage oil may contain a maximum of three mono-*trans* and two di-*trans* isomers of γ-linolenic acid (Table 1). Peak X is a di-*trans* isomer (or a mixture of two di-*trans* isomers), peak Y contains only one mono-*trans* isomer and peak Z (masked by the all-*cis* isomer) a mixture (or one out) of two mono-*trans* isomers. However, analysis of these isomers as FAME on the CP-Sil 88 column does not allow a direct quantification of all *trans* isomers. A complementary fractionation by Ag-TLC is necessary to remove the interfering all-*cis* 18:3n-6 acid. Consequently, gas-chromatography of FAME on the CP-Sil 88 capillary column is inadequate for routine analyses.

Analysis of FAIPE on a CP-Sil 88 capillary column. Analyses of FAIPE prepared with NO_2-isomerized borage oil also resulted in the resolution of five peaks only (Fig. 2). However, the di-*trans* fraction isolated by Ag-TLC from this modified oil showed three fairly well-resolved peaks with ECLs 19.54, 19.58 and 19.66. It is highly probable that the two first distinct di-*trans* isomers obtained with FAIPE correspond to the splitting of the FAME peak that contained two unresolved di-*trans*

TABLE 1

Equivalent Chainlengths (ECLs) of Fatty Acid Methyl Esters (FAME) and Fatty Acid Isopropyl Esters (FAIPE) Prepared with NO_2-Isomerized Borage Oil, Fractionated by Argentation Thin-Layer Chromatography (Ag-TLC) and Analyzed on CP-Sil 88 and DB-Wax Capillary Columns

	FAME CP-Sil 88		FAIPE CP-Sil 88		FAME DB-Wax	
Ag-TLC fraction	NO_2-isomerized	Heated[a]	NO_2-isomerized	Heated	NO_2-isomerized	Heated
All-*trans*	19.74	–	19.43	–	19.18	–
Di-*trans*	19.91*[b]	+	19.54	–	19.18	–
	20.02	–	19.58	+	19.21	+
			19.66	–	19.29	–
Mono-*trans*	19.97	+	19.58	+	19.08	+
	20.08*	+	19.72*	+	19.18	+
					19.22	+
All-*cis*	20.08	+	19.66	+	18.98	+

[a]Samples of borage oil that were heated under vacuum in sealed ampoules at 240°C for 6 h. A plus sign in columns concerning the isolated *trans* or all-*cis* fractions of heated borage oil indicates that a peak with the corresponding ECL is present in this fraction, a minus sign that it is absent.
[b]ECL values with an asterisk correspond to peaks containing two components.

isomers (peak with ECL = 19.91 in Fig. 1). On the other hand, there is no improvement in the resolution of mono-*trans* isomers, which still give two peaks only [ECL = 19.58 (one component) and ECL = 19.72 (two components)]. Moreover, one of the di-*trans* isomers (ECL = 19.66) co-eluted with γ-linolenic acid (fraction not presented in Fig. 2). The intermediary peak of the three di-*trans* isomers (ECL = 19.58) co-eluted with the first eluting mono-*trans* isomer (Fig. 2).

Analysis of FAIPE prepared with heated (240°C, 6 h) borage oil resulted in the resolution of the main γ-linolenic acid peak (ECL = 19.66) from two supplementary peaks with ECLs equal to 19.58 and 19.72. Analysis of the *trans*-triene fraction isolated from these FAIPE by Ag-TLC gave the same two peaks, with no component having the same ECL as γ-linolenic acid. Since the second peak (Z′) has the same ECL as that of the two unresolved mono-*trans* isomers, it should correspond to peak Z (Fig. 1). Consequently, the first peak (Y′ in Fig. 2) is a mixture of components X and Y (Fig. 1). The all-*trans* isomer was not detected.

Hence, analysis of FAIPE on a CP-Sil 88 capillary column is a convenient means to accurately quantitate the content of geometrical isomers of γ-linolenic acid in heated borage oil. However, the first eluting peak (ECL = 19.58) may contain one mono-*trans* and one di-*trans* isomer, while the late-eluting peak (ECL = 19.72) may contain two mono-*trans* isomers (Table 1).

Combining results of analyses of FAME and FAIPE on the CP-Sil 88 capillary column indicates that heated borage oil may contain, as main artifacts, two or three mono-*trans* and one di-*trans* isomers of γ-linolenic acid (Table 1). The di-*trans* isomer can be quantitated if FAME are used. Two of the three mono-*trans* isomers remain combined under the same peak, in admixture with γ-linolenic acid (FAME), or well separated from this last isomer (FAIPE). Partial data concerning individual *trans* isomers (as FAIPE on the CP-Sil 88 capillary column) can be obtained in this way: the first peak corresponds to the sum of one mono- and one di-*trans* isomers, the second to the sum of the two other mono-*trans* isomers.

Analysis of FAME on a DB-Wax capillary column. Analysis of FAME prepared with NO_2-isomerized borage oil on a DB-Wax capillary column gives four peaks only (Fig. 3) with ECLs 18.98, 19.08, 19.18 and 19.28. Fractionation by Ag-TLC of FAME from this oil gave mono-*trans* and di-*trans* fractions in which three peaks are present. The first eluting triene in unfractionated FAME from NO_2-isomerized borage oil is the all-*cis* 18:3n-6 isomer (ECL = 18.98; fraction not presented in Fig. 3). The second peak (ECL = 19.08) is exclusively made up by one mono-*trans* isomer species. The third eluting peak (ECL = 19.18) contains two mono-*trans*, two di-*trans* and the all-*trans* isomers (the fraction containing this last isomer is not presented in Fig. 3). The late-eluting component is exclusively composed of one di-*trans* isomer.

Analysis of the mono-*trans* triene fraction isolated by Ag-TLC from the heated (240°C, 6 h) sample of oil shows three peaks. The first eluting peak (ECL = 19.08) is exclusively made up by one mono-*trans* isomer (Fig. 3). The second (ECL = 19.18) and the third peaks (ECL = 19.22) are also mono-*trans* isomers, but a peak having the same ECL as the third peak (19.22) was found in the linoleic acid fraction that migrates together with di-*trans* isomers of γ-linolenic acid. Thus, the late eluting peak is a mixture of one mono-*trans* and one di-*trans* isomers. There was neither improvement nor diminution in the resolution of γ-linolenic isomers when FAIPE were used instead of FAME (results not shown). Analyses of γ-linolenic acid isomers are more informative when they are performed with either FAME or FAIPE on a DB-Wax capillary column. Combining all data from gas-chromatography analyses of FAME and FAIPE on the CP-Sil 88 and DB-Wax capillary columns allows us to conclude that heating of borage oil leads to the formation of three mono-*trans* and one di-*trans* isomers (Table 1). Analyses of FAME (or FAIPE) on the DB-Wax capillary column seem therefore to be the best means to determine the content of γ-linolenic acid isomers in heated borage oil. Moreover, analyses are faster on this column than on the CP-Sil 88 capillary column (18 min *vs.* 43 min for the

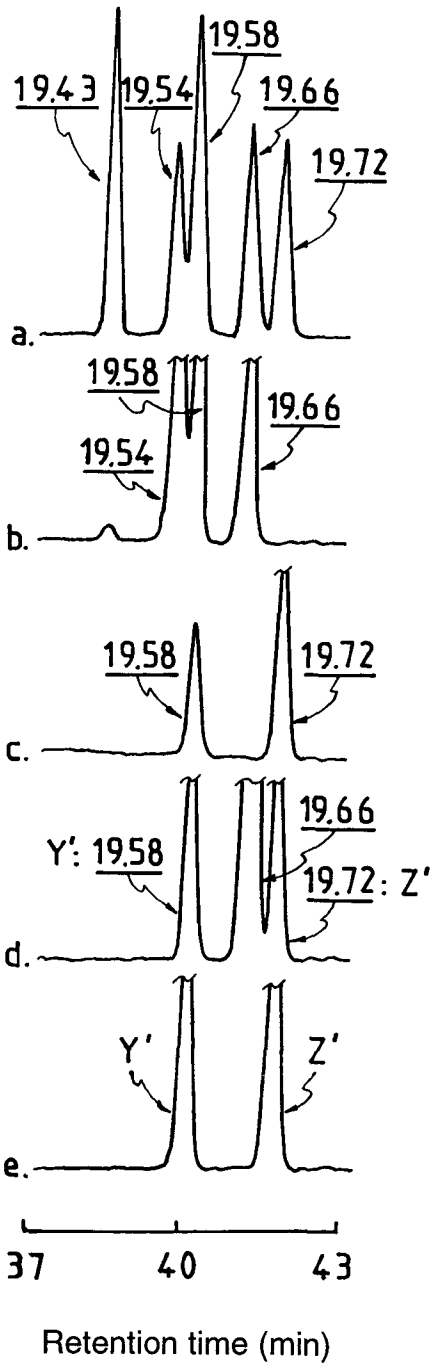

FIG. 2. Partial chromatograms (region of 18:3n-6 acid isomers) obtained on a CP-Sil 88 capillary column with fatty acid isopropyl esters prepared with: a., NO_2-isomerized borage oil; b., di-*trans* fraction isolated from a.; c., mono-*trans* fraction isolated from a.; d., borage oil heated under vacuum at 240°C for 6 h; e., *trans* fraction isolated from d. Underlined numbers correspond to equivalent chainlengths. See Figure 1 for company source of CP-Sil 88.

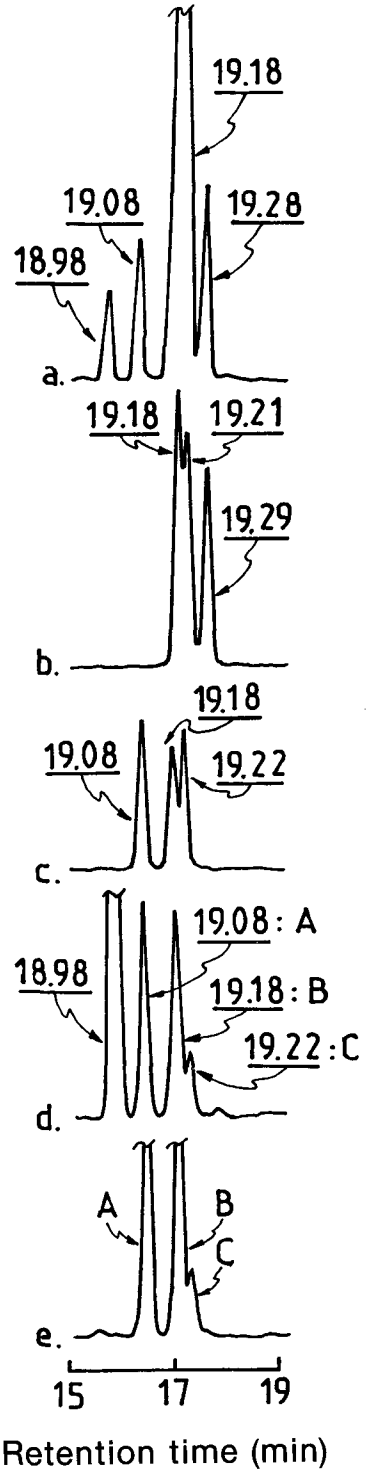

FIG. 3. Partial chromatograms (region of 18:3n-6 acid isomers) obtained on a DB-Wax capillary column (J&W Scientific, Folsom, CA) with fatty acid methyl esters prepared with: a., NO_2-isomerized borage oil; b., di-*trans* fraction isolated from a.; c., mono-*trans* fraction isolated from a.; d., borage oil heated under vacuum at 240°C for 6 h; e., *trans* fraction isolated from d. Underlined numbers correspond to equivalent chainlengths.

elution of the last isomer of γ-linolenic acid; compare Figs. 2 and 3).

Isomers of γ-linolenic acid that appear upon heat treatment have systematically ECLs that are identical to those of certain of its geometrical isomers. This is independent of the kind of analysis of fatty acids, as methyl or isopropyl esters on the CP-Sil 88 capillary column or as methyl esters on the DB-Wax capillary column. There is thus little chance that any positional isomerization of

γ-linolenic acid may have occurred during heating. This is evidenced in the following.

Structure determination of artifact fatty acids in heated borage oil. Starting with *ca.* 45-mg aliquots of borage oil that was heated under vacuum in sealed ampoules at 260°C for 5 h, FAME and FAIPE were prepared and analyzed by GLC on the DB-Wax and CP-Sil 88 capillary columns, respectively (Fig. 3). On both columns, artifact fatty acids accounted for *ca.* 14% of total fatty acids. This corresponds to a degree of isomerization (DI, ratio of total *trans* isomers on total octadecatrienoic acids of a given metabolic series times 100) of γ-linolenic acid of about 74%. Fatty acids (either as isopropyl or as methyl esters) of the heated oil present three main supplementary peaks immediately following the γ-linolenic acid peak on the DB-Wax column and corresponding to peaks A, B and C in Figure 1 [ECLs (methyl esters): 19.08, 19.18 and 19.22, respectively]. ECLs of these peaks are the same as those determined for mono-*trans* isomers isolated from NO_2-isomerized borage oil or from borage oil heated at 240°C for 6 h. When FAIPE are analyzed on the CP-Sil 88 column, they show two supplementary peaks, one before, and one just after the γ-linolenic acid peak. They correspond to peaks Y′ and Z′ in Figure 2, having ECLs equal to 19.58 and 19.72, respectively, exactly as for borage oil heated at 240°C for 6 h. Consequently, borage oil heated at 260°C for 5 h is a good starting material for the further characterization of γ-linolenic acid isomers.

FAME were further fractionated by preparative Ag-TLC (8 plates). The fraction migrating between γ-linolenic acid and linoleic acid, and having the same R_f as mono-*trans* isomers of γ-linolenic acid was localized, scraped off together with γ-linolenic acid and eluted from the silica gel. The all-*cis* 18:3n-6 acid fraction was collected because it may contain nonmethylene-interrupted isomers (other than conjugated isomers) if some positional isomerization has occurred. Analysis by GLC on the DB-Wax column of FAME present in this mixture shows the following composition: *cis*-9,*cis*-12 18:2 acid, 4.1%; *cis*-6,*cis*-9,*cis*-12 18:3 acid, 23.9%; artifact A, 23.8%; artifact B, 25.6%; artifact C, 15.0%. Artifacts A and B present the same ratio between them before and after fractionation by Ag-TLC. As compared to artifacts A and B, artifact C shows a relative decrease of 30% after Ag-TLC fractionation. This indicates that peak C initially contained at least two components with different R_fs, one of these co-migrating with linoleic acid and certainly being a di-*trans* isomer (results not shown).

The fraction that had been isolated by preparative Ag-TLC was saponified and transformed into free fatty acids prior to partial reduction with hydrazine. An aliquot of the resulting mixture was transformed into FAIPE and analyzed by GLC on the DB-Wax and CP-Sil 88 capillary columns. Stearic acid represented 13.3% of total fatty acids, octadecenoic acids accounted for 34.9%, octadecadienoic acids for 35.8% and unreacted γ-linolenic acid plus artifacts for 12.5%. Accumulation of stearic acid upon partial hydrazine reduction clearly demonstrates that artifacts A, B and C contain 18 carbon atoms. The remaining free fatty acids were methylated, and *cis* and *trans* monoenes were separated by Ag-TLC. Each band content was analyzed by GLC–FTIR on a BPX70 capillary column. In both cases, two peaks were resolved, the first one certainly containing the Δ6 and Δ9 monoenes and the second one the Δ12 monoene. The two *trans* monoene peaks present in the fast-moving monoene band during Ag-TLC clearly showed absorption at 970 cm^{-1}, which is characteristic of *trans* ethylenic bonds (results not shown). Each fraction was further analyzed by GLC on the CP-Sil 88 column, because this column is well adapted for the separation of petroselinic (*cis*-6 18:1) acid and oleic (*cis*-9 18:1) acid (20) (Fig. 4). Three main peaks, accounting together for 95.3 and 98.5% of total components, were detected in the *cis*- and *trans*-monoene fractions, respectively. Since hydrazine treatment has no effect on the geometry of double bonds, the presence of *trans* monoenes indicates that each of artifacts A, B and C initially contained one *trans* double bond. The fact that three components are present in the two monoene fractions demonstrates that artifacts A, B and C contain three double bonds at the most. The *cis* monoenes have ECLs identical

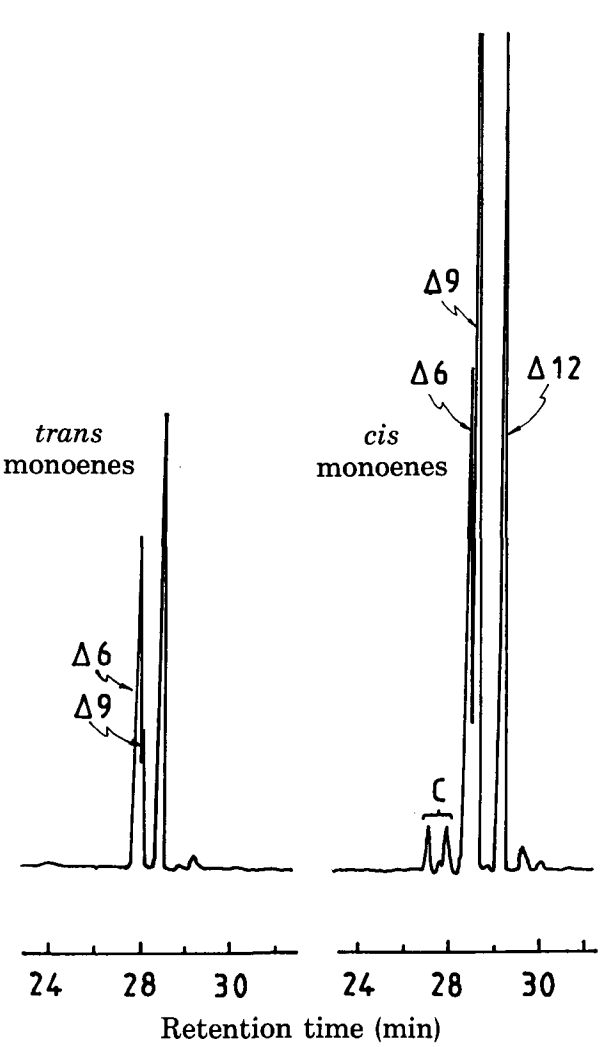

FIG. 4. Partial chromatograms of *cis*- and *trans*-octadecenoic acid methyl esters (on a CP-Sil 88 capillary column) isolated by argentation-thin layer chromatography (Ag-TLC) from a concentrate of octadecatrienoic acid methyl esters that was submitted to partial hydrazine reduction. The concentrate was itself obtained by Ag-TLC of fatty acid methyl esters prepared with a sample of borage oil heated at 240°C for 6 h. C, contaminants present throughout all of the experiment. See Figure 1 for company source of CP-Sil 88.

to those of *cis*-6 18:1 (ECL = 18.53), *cis*-9 18:1 (ECL = 18.57) and *cis*-12 18:1 (ECL = 18.73) acids prepared by partial hydrazine reduction of fatty acids derived from fresh cold-pressed borage oil. The two first-eluting peaks of the *trans*-monoene fraction have ECLs identical to those of *trans*-6 18:1 (ECL = 18.38) and *trans*-9 18:1 (ECL = 18.42) acids prepared by nitrous isomerization of celery seed oil and olive oil. The third peak, anticipated to be *trans*-12 18:1 acid, had an ECL of 18.51.

The *cis* and *trans* monoene fractions were then submitted to ozonolysis in BF_3-methanol. The resulting MME and DME were analyzed on a DB-Wax capillary column and characterized by MS. For each fraction, DME and MME with 6, 9 and 12 carbon atoms were observed as major constituents. These identifications conclusively demonstrate that heating of borage oil under vacuum leads mainly to geometrical isomerization of double bonds in γ-linolenic acid, without any significant positional shifts. This also indicates that the third *trans* monoene (ECL = 18.51) is indeed *trans*-12 18:1 acid.

Analysis of the *trans*-monoene fraction on the CP-Sil 88 column (Fig. 4) indicates that *trans*-9 18:1 acid accounts for only 11.8% of *trans* monoenoic acids. From this relative dearth, it can be deduced that the initial *cis*-6, *trans*-9,*cis*-12 18:3 acid was low in the mixture of mono-*trans* isomers of γ-linolenic acid isolated by preparative Ag-TLC. Consequently, artifact C on the DB-Wax column, which is the less abundant of the three mono-*trans* isomers, contains the *cis*-6,*trans*-9,*cis*-12 isomer of γ-linolenic acid. Artifacts A and B are the two other mono-*trans* isomers of γ-linolenic acid. However, it cannot be decided on the sole basis of our data which peak corresponds to *trans*-6,*cis*-9,*cis*-12 18:3 acid or to *cis*-6,*cis*-9,*trans*-12 18:3 acid. Despite this ambiguity, it is clear that the Δ9 double bond is the least reactive double bond with regard to geometrical isomerization. In α-linolenic acid, the least reactive ethylenic bond is Δ12 (5,9). In both cases, the less reactive double bond is that located in the central position. Consequently, the isomerization rate of double bonds is linked to their relative position (central or external) rather than to their absolute position (Δ6, 9, 12 or 15).

Tentative identification of individual γ-linolenic acid geometrical isomers. The preceding observations allowed to conclude that three mono-*trans* (two major and one minor components) and one di-*trans* geometrical isomer of γ-linolenic acid accumulate as main artifacts upon heat treatment of borage oil. The same observations were previously made with α-linolenic acid (5,8,9). It was thus decided to compare the isomerization rate of these two octadecatrienoic acids. For this purpose, samples of borage oil and linseed oil were heated under vacuum in sealed ampoules under the same conditions, at the same time. The calculated DIs of octadecatrienoic acids presented in Table 2 clearly show that α- and γ-linolenic acids isomerize to approximately the same rates. These similarities (the same kinds and numbers of isomers and the same rates of isomerization) seem to indicate that γ-linolenic acid isomerizes in the same manner as α-linolenic acid. Because data concerning the elution order of γ-linolenic acid geometrical isomers on the DB-Wax or other related capillary columns are lacking in the literature, we then tried to correlate the relative percentages of individual γ-linolenic acid isomers (determined with FAME chromatographed on the DB-Wax column) to those of α-linolenic acid isomers [easily accessible through gas chromatography of FAME performed on the CP-Sil 88 capillary column (12)]. Because we know that there is only one di-*trans* isomer (which coelutes with *cis*-6,*trans*-9,*cis*-12 18:3 acid on the DB-Wax column, peak C), it can be proposed that this di-*trans* isomer is *trans*-6,*cis*-9,*trans*-12 18:3 acid (Table 3). Concerning the two other main peaks, we know that they correspond to the two other mono-*trans* isomers exclusively. If the isomerization mechanism of γ-linolenic acid is the same as that of α-linolenic acid, and if the resulting geometrical isomers present the same relative order of *cis* and *trans* double bonds along the hydrocarbon chain, comparison of the relative percentages of the two mono-*trans* peaks (Table 3) would indicate that the first eluting mono-*trans* isomer (peak A) corresponds to *trans*-6,*cis*-9,*cis*-12 18:3 acid and the second one (peak B) to *cis*-6,*cis*-9,*trans*-12 18:3 acid. On the DB-Wax capillary column, the elution order of isomers should thus be *cis*-6,*cis*-9,*cis*-12, *trans*-6, *cis*-9,*cis*-12, *cis*-6,*cis*-9,*trans*-12 and *cis*-6,*trans*-9,*cis*-12 plus *trans*-6,*cis*-9,*trans*-12 18:3 acids. We did not find any other means of reasoning to solve this rather difficult problem of identification. Complementary work is needed to verify our hypothesis.

TABLE 2

Comparison of the Isomerization Rates (expressed as degrees of isomerization) of γ- and α-Linolenic Acids in, Respectively, Borage and Linseed Oils Heated Under Vacuum in Sealed Ampoules

Temperature (°C)	Duration (h)	Oil	*trans*[a] content (%)	Total 18:3[b] (%)	DI[c] (%)
240	6	Borage	7.6[d]	23.0	33.0
240	6	Linseed	14.1	44.3	31.8
260	5	Borage	14.3	19.4	73.7
260	5	Linseed	29.4	41.8	70.3

[a]Sum of *trans* isomers of either γ- or α-linolenic acids.
[b]Sum of all isomers including the all-*cis* isomer of either 18:3n-6 or 18:3n-3 acids.
[c]Degree of isomerizatioin: ratio of total *trans* isomers on total 18:3 (n-3 or n-6) isomers times 100.
[d]Figures are means of values obtained through analyses of fatty acid methyl esters on a DB-Wax capillary column and of fatty acid isopropyl esters on a CP-Sil 88 capillary column.

TABLE 3

Comparison of the Relative Proportions of Individual γ-Linolenic Acid and α-Linolenic Acid Geometrical Isomers in Heated Oils

γ-Linolenic acid isomers[a]	Proportions in heated borage oil[b]		α-Linolenic acid isomers[c]	Proportions in heated linseed oil	
	240°C, 6 h	260°C, 5 h		240°C, 6 h	260°C, 5 h
A	42.7	32.5	*t,c,c*[d]	40.3	30.6
B	45.1	35.2	*c,c,t*	46.5	37.6
C	12.2	32.2	*c,t,c* + *t,c,t*	13.2	31.9

[a]Peak lettering as in Figure 3 (analyses on the DB-Wax capillary column).
[b]Proportions of individual *trans* isomers are relative to their total. Analyses on the DB-Wax capillary column.
[c]Individual isomers analyzed on the CP-Sil 88 capillary column.
[d]*c,cis; t,trans.* Structures of double bonds are given in the order 9, 12 and 15.

Effect of high-temperature deodorization of borage oil on γ-linolenic acid structure. Deodorization of borage oil at high temperatures (200°C and more) induces the appearance of artifact fatty acids in chromatograms that present the same characteristics as those appearing in heated borage oil (peaks A, B and C for FAME or FAIPE on the DB-Wax column; peaks Y′ and Z′ for FAIPE on the CP-Sil 88 column) (Fig. 5). Heating under vacuum at 240°C for 4 h, a sample of borage oil that was first deodorized at 240°C for 2 h increases the proportion of these extra peaks from 3.9 up to 7.2%. This corresponds to an increase in DI from *ca.* 15.6 up to 29.6% (Fig. 6). This last value compares well with that of borage oil heated under vacuum at 240°C for 6 h (Table 2). It would thus appear that the main effect of deodorization at high temperature is also to geometrically isomerize double bonds in γ-linolenic acid. However, to be sure of this effect, we have isolated an almost pure fraction of mono-*trans* isomers of 18:3 acids by preparative Ag-TLC (10 plates) from FAME prepared with a sample (*ca.* 80 mg) of oil that was deodorized at 240°C for 2 h. In this oil, artifacts with the same ECLs as peaks A, B and C accounted for 3.9% of total fatty acids (DI = 15.6%). The first fractionation of *trans* isomers led to a mixture of fatty acids with the following composition (analyses of FAME on the DB-Wax column): *cis*-9,*cis*-12 18:2 acid, 12.6%; *cis*-6,*cis*-9,*cis*-12 18:3 acid, 51.8%; artifact A, 15.9%; artifact B, 16.7%; artifact C, 2.8%. A second Ag-TLC fractionation of the preceding mixture allowed a purification higher than 98% of artifacts A, B and C. The final concentrate has the following composition: *cis*-9,*cis*-12 18:2 acid, 1.6%; *cis*-6,*cis*-9,*cis*-12 18:3 acid, 0%; artifact A, 41.8%; artifact B, 48.2%; artifact C, 7.6%. FAME of this purified fraction were then converted into free fatty acids and submitted to partial hydrazine reduction. FAME were then prepared and *cis* and *trans* monoenes were isolated by Ag-TLC. Analyses of these fractions by GLC on the CP-Sil 88 capillary column showed three main peaks for each monoene band (purity higher than 95%), with ECLs (18.38, 18.41 and 18.51 for *trans* monoenes and 18.52, 18.57 and 18.73 for *cis* monoenes) identical to those of monoenes isolated by Ag-TLC from partially reduced *cis* and *trans* octadecatrienoic acids prepared with FAME of borage oil heated at 260°C for 5 h. The *cis* and *trans* monoenes have thus their ethylenic bonds in positions 6, 9 and 12. Consequently, the parent *trans* octadecatrienoic acids generated by deodorization at high temperature are geometrical isomers of the precursor γ-linolenic acid. Because *trans*-9 and *cis*-9 18:1 acids are the least and most abundant isomers, respectively, in the *trans* and *cis*

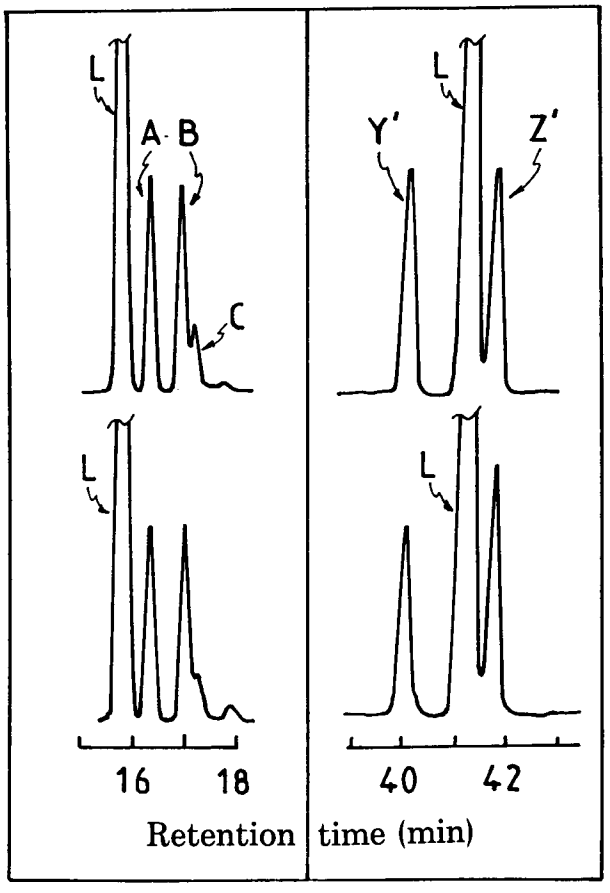

FIG. 5. Comparison of partial chromatograms of fatty acids from borage oil heated under vacuum at 240°C for 6 h (upper tracings) and from borage oil deodorized at 240°C for 2 h (lower tracings). Injections were made at different loads. Left chromatograms, analyses of fatty acid methyl esters on a DB-Wax capillary column; right chromatograms, analyses of fatty acid isopropyl esters on a CP-Sil 88 capillary column. Lettering of peaks is the same as in Figures 2 and 3, except for L which stands for γ-linolenic acid. See Figures 1 and 3 for company sources.

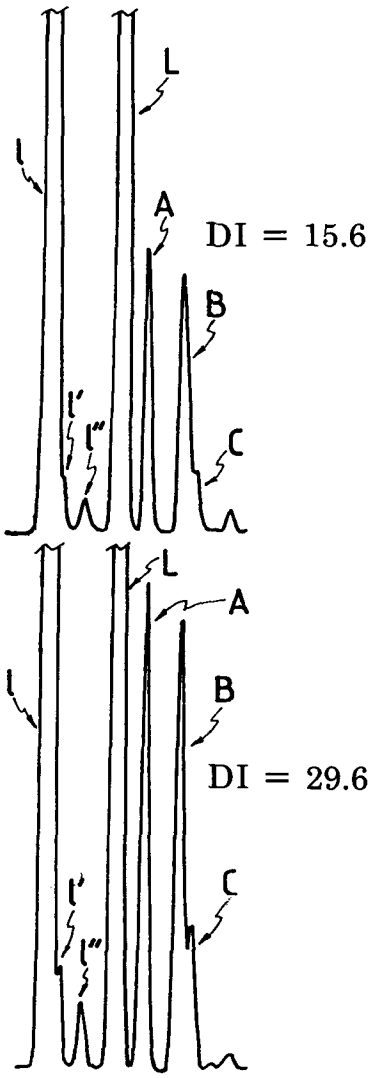

FIG. 6. Comparison of partial chromatograms obtained on a DB-Wax capillary column with fatty acid isopropyl esters (FAIPE) prepared with a sample of borage oil that was deodorized at 240°C for 2 h (upper tracing) and then heated under vacuum in sealed ampoules at 240°C for 4 h (lower tracing). Injections of the same quantities of FAIPE in both cases. Lettering of peaks as in Figure 5 except for l, which designates *cis*-9,*cis*-12 18:2, and l′ and l″, which designate mono-*trans* isomers of linoleic acid. DI, degree of isomerization. See Figure 3 for company source.

TABLE 4

Fatty Acid Composition of Borage Oil Deodorized at Different Temperatures as Determined by Gas–Liquid Chromatography of Fatty Acid Isopropyl Esters on a 30-m Long DB-Wax Capillary Column (data are given as peak area percentages)

Fatty acid	Native oil[a] (n = 2)[b]	200°C 2 h (n = 2)	220°C 2 h (n = 2)	240°C 2 h (n = 2)
14:0	0.07	0.07	0.07	0.07
16:0	9.41	9.07	9.32	9.37
16:1n-9	0.11	0.11	0.11	0.10
16:1n-7	0.26	0.25	0.26	0.26
18:0	2.57	2.67	2.53	2.57
18:1n-9	14.03	14.06	14.04	14.23
18:1n-7	0.56	0.56	0.56	0.57
c,c-18:2n-6[c]	38.63	38.63	38.60	38.72
c,t-18:2n-6[d]	—	trace[e]	trace	trace
t,c-18:2n-6	—	trace	0.05	0.19
Sum 18:2n-6	38.63	38.63	38.65	38.91
c,c,c-18:3n-6	25.40	25.04	24.05	21.00
t,c,c-18:3n-6	—	0.09	0.53	1.72
c,c,t-18:3n-6	—	0.13	0.63	1.97
c,t,c-18:3n-6[f]	—	trace	trace	0.20
Sum 18:3n-6	25.40	25.26	25.21	24.89
18:3n-3	0.20	0.18	0.18	0.14
18:4n-3	0.17	0.17	0.15	0.08
20:0	0.14	0.14	0.14	0.14
20:1	4.00	4.15	4.06	4.27
22:0	0.08	0.09	0.09	0.08
22:1	2.17	2.60	2.47	2.20
24:0	trace	trace	trace	trace
24:1	1.45	1.37	1.30	1.30
Others	0.75	0.62	0.95	0.82

[a]Refined borage oil that was not deodorized and further used for deodorization experiments.
[b]Number of analyses.
[c]*c, cis; t, trans.* The configurations of double bonds are given in the order 9 and 12 for 18:2n-6 acids and 6, 9 and 12 for 18:3n-6 acids. Identification of peaks corresponding to *trans* isomers is tentative only (see text).
[d]Peak fused with the trailing edge of *cis*-9,*cis*-12 18:2 acid (shoulder 1′ in Fig. 6).
[e]Peaks (or shoulders) visible on chromatograms, but not taken into account by the integrator.
[f]Probably includes some *trans*-6,*cis*-9,*trans*-12 18:3 acid. Generally too low to be properly taken into account by the integrator.

monoene fractions, this indicates that the mono-*trans* *cis*-6,*trans*-9,*cis*-12 18:3 isomer is the least abundant of the three mono-*trans* isomers. The same observation was previously made with borage oil that was simply heated under vacuum.

Influence of deodorization temperature on borage oil fatty acids. Table 4 gives the fatty acid compositions of borage oil that were steam-deodorized at 200, 220 and 240°C for 2 h. At these temperatures, total *trans*-18:3n-6 isomers account for 0.2, 1.2 and 3.9% of total fatty acids, respectively. These values correspond to DIs of 0.9, 4.6 and 15.6%, respectively. Mono-*trans* isomers of linoleic acid also appear to some extent during heating or deodorization (Fig. 6). For the sample deodorized at 240°C, analyses of FAME on the CP-Sil 88 capillary column indicate that the *cis*-9,*trans*-12 and the *trans*-9,*cis*-12 isomers account for 0.23 and 0.15%, respectively, of total fatty acids. This corresponds to a DI of linoleic acid of about 1%. The main benefit of the deodorization process is the destruction of oxidation products, such as peroxides, and the removal of unwanted volatile components. Among components removed are oxidation products such as aldehydes, hydrolysis products such as free fatty acids, residual extraction solvents and potential pesticide residues. The higher the temperature, the better the quality in this respect. However, from the deodorization experiments with borage oil, it is clear that the γ-linolenic geometrical isomer content increases with temperature, a well-established phenomenon that was observed with α-linolenic acid in α-linolenic acid-containing oils (9). As for these oils (5,9), the temperature of deodorization of borage oil should be less than 200°C if geometrical isomers of γ-linolenic acid are to be completely avoided. If higher temperatures are used, the all-*cis* 18:3n-6 acid

is unstable. One should also note the influence of high-temperature deodorization on the content of 18:3n-3 and 18:4n-3 acids, mainly in the sample that was deodorized at 240°C. The presence of linoleic and γ-linolenic acid geometrical isomers thus constitutes a good means to detect whether γ-linolenic acid-containing oils have been submitted to steam-vacuum deodorization at temperatures equal to or higher than 200°C. On the other hand, by the use of capillary column GLC procedures presented in this study, the deodorization process can easily be controlled in this respect. Due to the high price of these oils, this quality aspect should not be neglected.

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