Heat Treatment of Vegetable Oils I. Isolation of the Cyclic Fatty Acid Monomers From Heated Sunflower and Linseed Oils[1]

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Linseed and sunflower oils were heated at 275 C for 12 hr under nitrogen. The sunflower oil was also heated in a commercial fryer at 200 C for 48 hr using a 2-hr daily cycle. The cyclic fatty acid monomers (CFAM) formed during the heat treatment of the linseed oil were isolated by a combination of saponification, esterification, column chromatography on silicic acid and urea fractionation. The isolated CFAM fraction was 99% pure, the balance being some 18:2ω6. Another step was necessary to isolate the CFAM from heated sunflower oils. The urea adduct fractionation resulted in the isolation of a nonurea adduct fraction which contained a mixture of CFAM and 18:2ω6. These were further separated using high performance liquid chromatography (HPLC) on a C18 reverse phase column. Each fraction was analyzed by gas liquid chromatography and hydrogenated to determine the content of the C18 straight chain fatty acids.

Many chemical reactions including oxidation, polymerization, hydrolysis, isomerization and cyclization occur during deep fat frying (1,2). These reactions lead to the formation of fatty acid geometrical isomers (3), dimers (4,5), cyclic fatty acid monomers (6-10) and polymeric triglycerides (11-13), as well as some oxidative components (4,14). Among all these components, cyclic fatty acid monomers (CFAM) have shown a possible toxic effect (1,15-18). The objectives of this work were, on one hand, to find a suitable method to isolate CFAM in view of toxicological studies and, on the other hand, to compare the cyclic monomers formed from linolenic acid with those arising from linoleic acid. For this purpose, we have selected two types of oils, one rich in linolenic acid, linseed oil, and one rich in linoleic acid, sunflower oil. These oils were heated at 275 C for 12 hr under nitrogen. Sunflower oil was also heated at 200 C in the presence of air in order to compare the CFAM which could be formed at a lower temperature (conditions usually used during frying processes) with those formed at higher temperatures in the absence of oxygen.

A combination of column chromatography and urea adduct fractionation resulted in the isolation of CFAM from a heated linseed oil. However, one more step (HPLC on a C18 reverse phase column) was necessary to isolate the CFAM from the heated sunflower oils.

MATERIALS AND METHODS

Purification of solvents. All the solvents were redistilled before use.

Heating conditions. A linseed oil purchased from Robbe-Hyfran (France) was heated at 275 C for 12 hr under nitrogen, in a 10-l round-bottomed flask. A sunflower oil (Venus, Hungary) was heated at 275 C for 12 hr under nitrogen in a one-l round-bottomed flask. A sunflower oil obtained from Lesieur Cotelle (France) was heated in a commercial fryer (Calor 08). The volume of the oil in the aluminum coated tank was 3.0 l. This oil was heated at 200 C for 48 hr using a 2-hr daily cycle.

Gas liquid chromatography (GLC). All the GLC analyses were effected on a Becker-Packard 420 chromatograph fitted with a flame ionization detector

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TABLE 1

Fatty Acid Composition (wt %) of Sunflower and Linseed Oils

	Linseed	Sunflower
16:0	5.0	6.4
18:0	3.5	4.8
18:1ω9+7	18.6	21.5
18:2ω6	18:1	65.6
18:3ω3	53.9	0.2
Others[a]	0.9	1.2

[a]Includes some 14:0, 15:0, 17:0, 16:1, 20:0, 20:1 and 24:0.

TABLE 2

Column Chromatography on Silicic Acid of Heated Linseed and Sunflower Oils (wt %)[a]

Solvent	Linseed 275 C/N_2	Sunflower 275 C/N_2	Sunflower 200 C/air
800 ml PE/Et_2O[b]	61.5	83.5	55.1
200 ml PE/Et_2O[b]	13.6	2.4	13.6
120 ml PE/Et_2O[b]	1.3	-	2.1
100 ml PE/Et_2O[b]	0.3	-	0.4
800 ml MeOH	22.7	14.1	28.4
400 ml MeOH	0.6	-	0.4

[a]Average of 3 columns.
[b]PE/Et_2O, mixture of petroleum ether:diethyl ether (95:5).

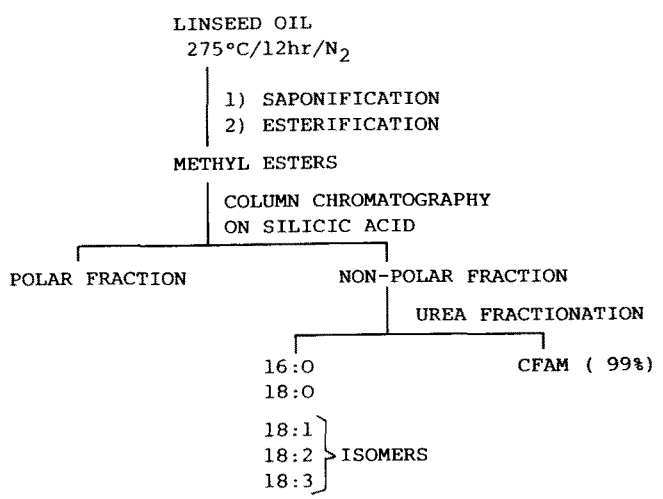

FIG. 1. Flow chart for the isolation of the CFAM from a linseed oil heated at 275 C for 12 hr under nitrogen.

(FID) and a Ros injector (19). The analyses were performed on capillary columns, coated with either Silar 10 C (50 m long and 0.50 mm i.d.) or Carbowax 20 M (25 m long and 0.30 mm i.d.) and on CP SIL 88 (50 m long and 0.32 mm i.d.). All quantitative analyses were effected using a Vista CDS 401 (Varian) or an Autolab System 4 (Spectraphysics) integrator.

Preparative high performance liquid chromatography (HPLC). The preparative HPLC was carried out on a reverse phase column (Waters, 5.7 cm i.d., 30 cm) using a Waters Prep LC 500. The sample (up to 10 g) was dissolved in acetone. The solvent systems used were a mixture of acetonitrile/water (90:10) or pure acetonitrile at 150-200 ml/min, depending on the separation desired.

A C18 reverse phase column (Merck, Lichrosorb, 7 mm i.d., 25 cm) was used for smaller quantities (up to 50 mg). Pure methanol or acetonitrile were used at 4 ml/min.

Preparation of fatty acid methyl esters. Oils were saponified with KOH, and unsaponifiables were removed by AOCS procedure Ca-6a-40. The recovered fatty acids were converted to methyl esters by refluxing for 5 hr with 4 volumes of 1% H_2SO_4 in MeOH (20). The esters were recovered with petroleum-ether and washed free of acids.

Fractionation of methyl esters by column chromatography on silicic acid. The method used for separation of triglycerides (21,22) was slightly modified as follows (23): Silicic acid (70-200 mesh) was dehydrated at 160 C for 6 hr. After cooling, about 220 g of silicic acid was hydrated at 5%. The column used was 55 cm long and 4 cm i.d., and 40 g fatty acid methyl esters (FAME). were fractionated at once. The nonpolar fraction was

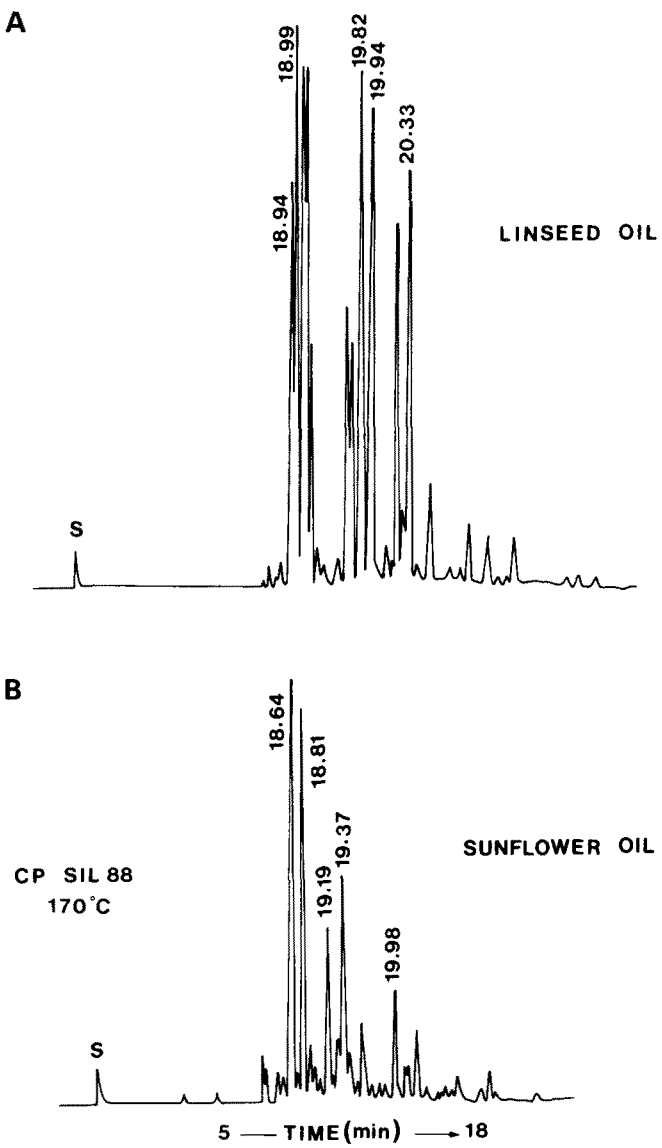

FIG. 2. Gas liquid chromatographic analyses on fused silica column (CP SIL 88), 50 m long and 0.32 mm i.d., of the CFAM isolated from (A) a linseed oil heated at 275 C for 12 hr under N_2, and (B) a sunflower oil heated at 725 C for 12 hr under N_2.

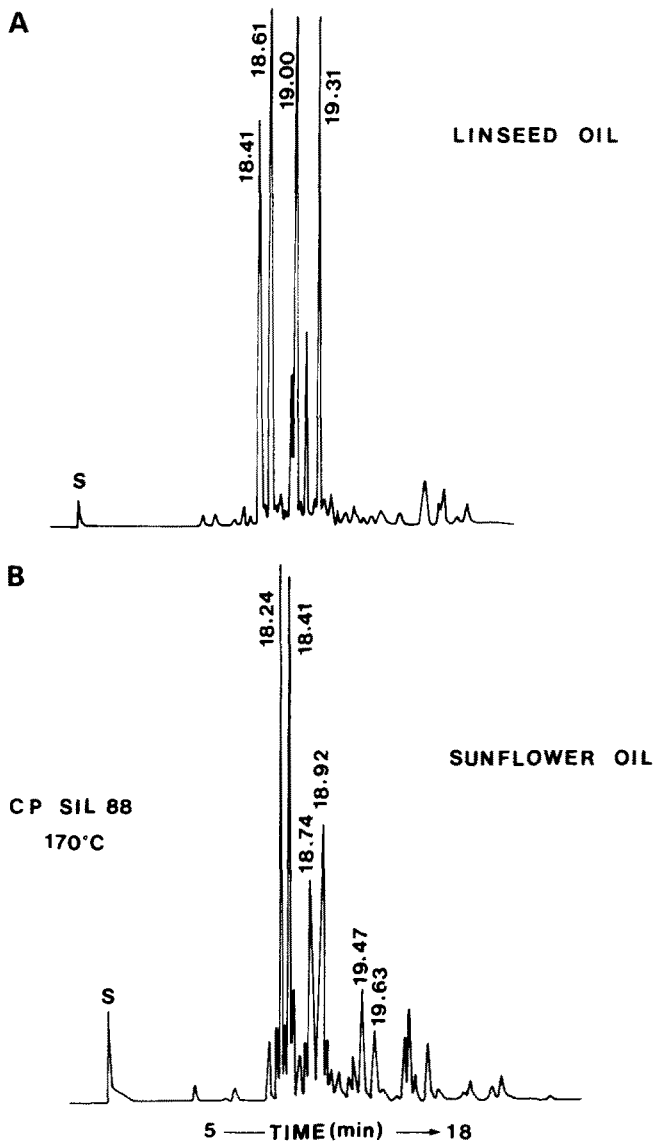

FIG. 3. Gas liquid chromatographic analyses on fused silica column (CP SIL 88), 50 m long and 0.32 mm i.d., of the hydrogenated CFAM from (A) a linseed oil heated at 275 C for 12 hr under N_2, and (B) a sunflower oil heated at 275 C for 12 hr under N_2.

eluted with one l of a mixture of petroleum ether:diethyl ether (95:5). The purity of the nonpolar fraction was checked by thin layer chromatography (TLC). The TLC plates (Merck 5721, 0.25 mm thickness) were developed with a mixture of hexane:diethyl ether:acetic acid (80:20:1), and the spots were detected by spraying with a solution of 2'7' dichlorofluorescein in ethanol.

Urea fractionation of fatty acid methyl esters. A saturated solution of urea in methanol was prepared by dissolving 400 g urea in 1.6 l methanol. This mixture was heated until complete dissolution of urea, and ≃140 g methyl esters were added. The resulting solution was cooled under nitrogen at room temperature and remained at 4 C overnight. The adducts were separated by filtration from the nonurea adduct fraction which contained the cyclic fatty acids. Two l of H_2O and 30 ml HCl were added to the filtrate, and the fatty acid methyl esters were extracted by petroleum ether.

Two l H_2O and 30 ml HCl were added to the crystals (urea adduct fraction), and the fatty acid methyl esters (straight chain saturates, monoenes, dienes and trienes) were extracted as previously described. Both fractions were analyzed by GLC on Carbowax, CP Sil 88 and Silar-10C phases.

Hydrogenation of cyclic fatty acid monomers (CFAM). The hydrogenation was effected using platinum oxide as catalyst in 10 ml of a mixture of chloroform and methanol (2:1) as solvent and a hydrogen pressure of 2 bars. Each reaction was allowed to proceed 4 hr. The catalyst was removed by filtration and the saturated methyl esters were extracted with chloroform after the addition of H_2O. The quantity of CFAM was determined by GLC analyses on CP Sil 88 using 17:0 as internal standard.

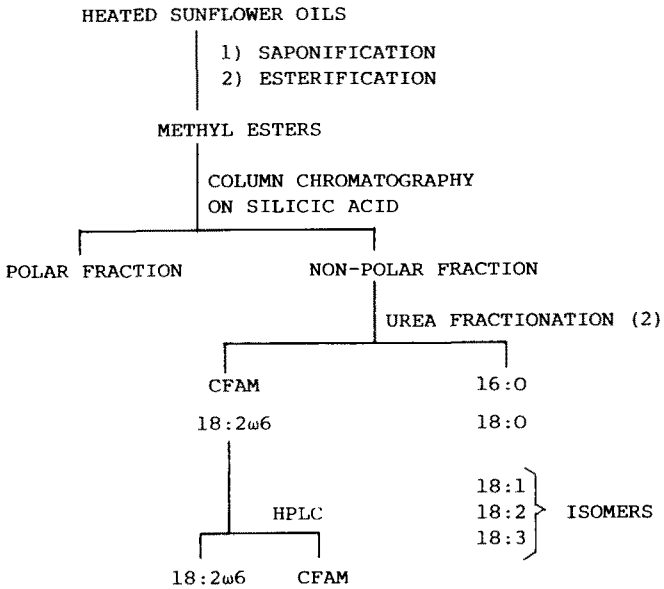

FIG. 4. Flow chart for the isolation of the CFAM from a sunflower oil heated at 275 C for 12 hr under N_2 and a sunflower oil heated at 200 C for 48 hr in a commercial fryer.

RESULTS AND DISCUSSION

Degree of alteration of the heated oils. The major fatty acids of linseed and sunflower oils are 16:0, 18:0, 18:1ω9, 18:2ω6 and 18:3ω3 (Table 1). Linseed oil is rich in linolenic acid (≃54%), while sunflower oil is rich in linoleic acid (66%). The interesting feature of sunflower oil is that it contains only minor amount of 18:3ω3 (0.2%). The degree of alteration of the three heated oils

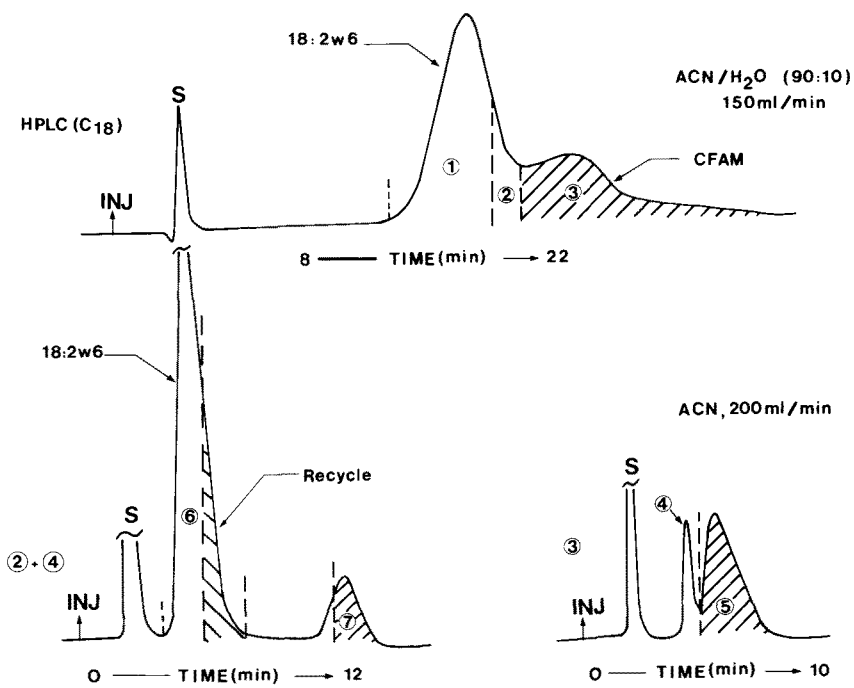

FIG. 5. HPLC fractionation on a C18 reverse phase column (30 cm long and 5.7 cm i.d.) of a mixture of CFAM and 18:2ω6 isolated from a sunflower oil heated at 275 C for 12 hr under nitrogen.

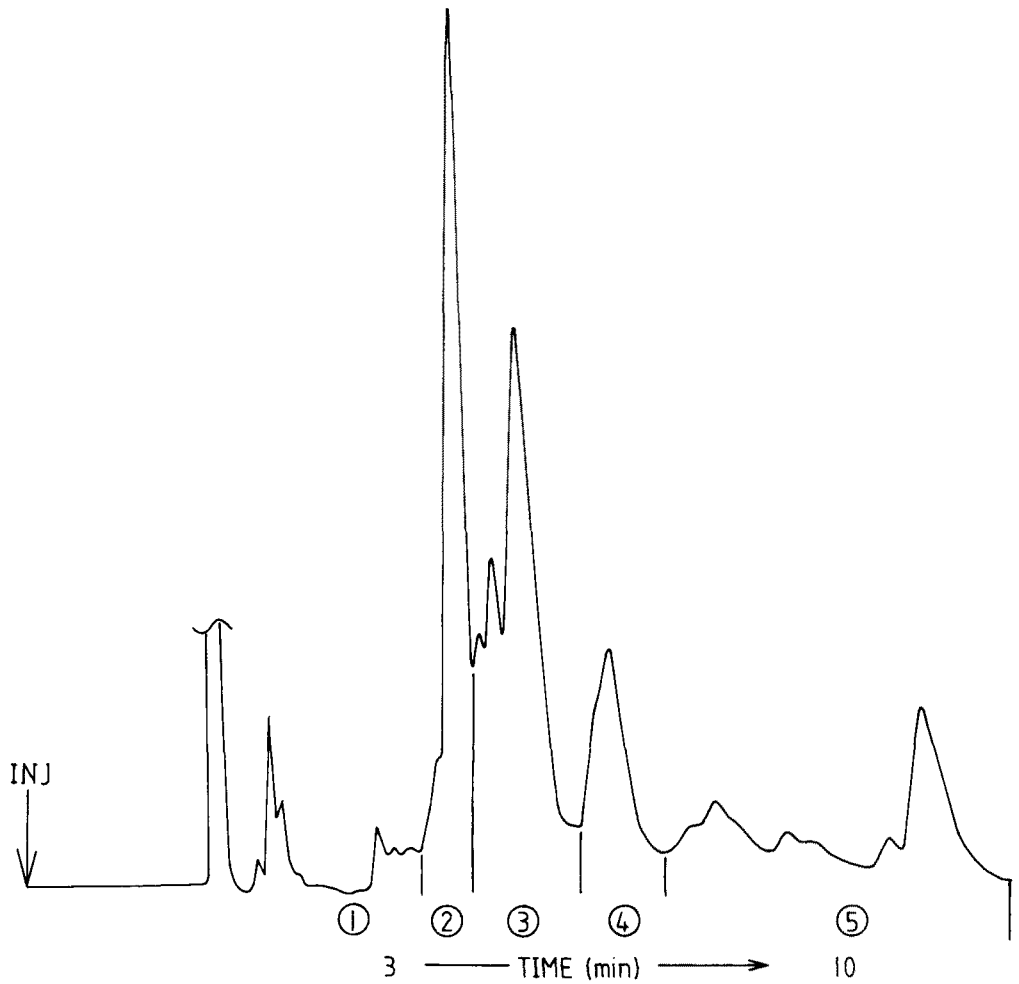

FIG. 6. HPLC fractionation on a C18 reverse phase column (25 cm long and 7 mm i.d.) of a mixture of CFAM and 18:2ω6 isolated from a sunflower oil heated at 200 C for 48 hr in a commercial fryer.

(linseed oil and sunflower oil at 275 C for 12 hr under N_2 and sunflower oil at 200 C and 48 hr) was studied by column chromatography. Six fractions were collected. The first and second fractions (Table 2) were the nonpolar fractions. These show only one spot when analyzed by TLC. The third and fourth fractions contained a mixture of polar and nonpolar components, while the fifth and sixth fractions eluted by methanol contained only polar material. However, the third and the fourth fractions represented less than 2.5% of the total methyl esters. The heated linseed oil contained less nonpolar material (75.1%) than the corresponding sunflower oil (85.9%). However, the sunflower oil heated under nitrogen at 275 C was less altered than the oil heated at 200 C in the presence of air (Table 2).

Isolation of CFAM from a heated linseed oil. The method used to isolate the CFAM is outlined in Figure 1. The esterification was carried out in the presence of H_2SO_4 as described elsewhere (20). A similar reaction was carried out with BF_3/MeOH and HCl-MeOH in order to verify that no artifact could be formed when using H_2SO_4 instead of BF_3 or HCl. The total fatty acid methyl esters contained 11.1% CFAM. Two urea fractionations were necessary to isolate a pure fraction of CFAM, as the first nonadduct fraction still contained some linolenic acid. Urea fractionation is a very useful reaction to isolate components having a ring. This reaction already has been used extensively to study heated fats (7,24). The GLC analysis on a CP Sil 88 phase of the resulting fraction is shown in Figure 2A. The major CFAM had equivalent chain lengths (ECL values) between 18.94 and 20.33. This complex mixture was hydrogenated on PtO_2. The original fraction (Fig. 2A) was transformed into a mixture of four components of ECL values of 18.41, 18.61, 19.00 and 19.31 and two other components of smaller quantities (Fig. 3A). Only traces of 18:0 were observed after hydrogenation, which seems to indicate that the isolated CFAM fraction contains only minor amounts of the straight chain C18 unsaturated fatty acids (principally some 18:2ω6). Similar hydrogenated cyclic fatty acid profiles (4 major hydrogenated components and 2 minor isomers) already were observed in earlier studies on heated linseed oils (9,25). In a recent study (25), we showed the possibility of further fractionating the CFAM in three groups using the formation of methoxy bromomercuric adducts. However, due to the toxicity of mercury, it would not be possible to carry out this reaction in a preparative scale for animal feeding studies. The CFAM mixture isolated by the method described in Figure 1 was run on a C18

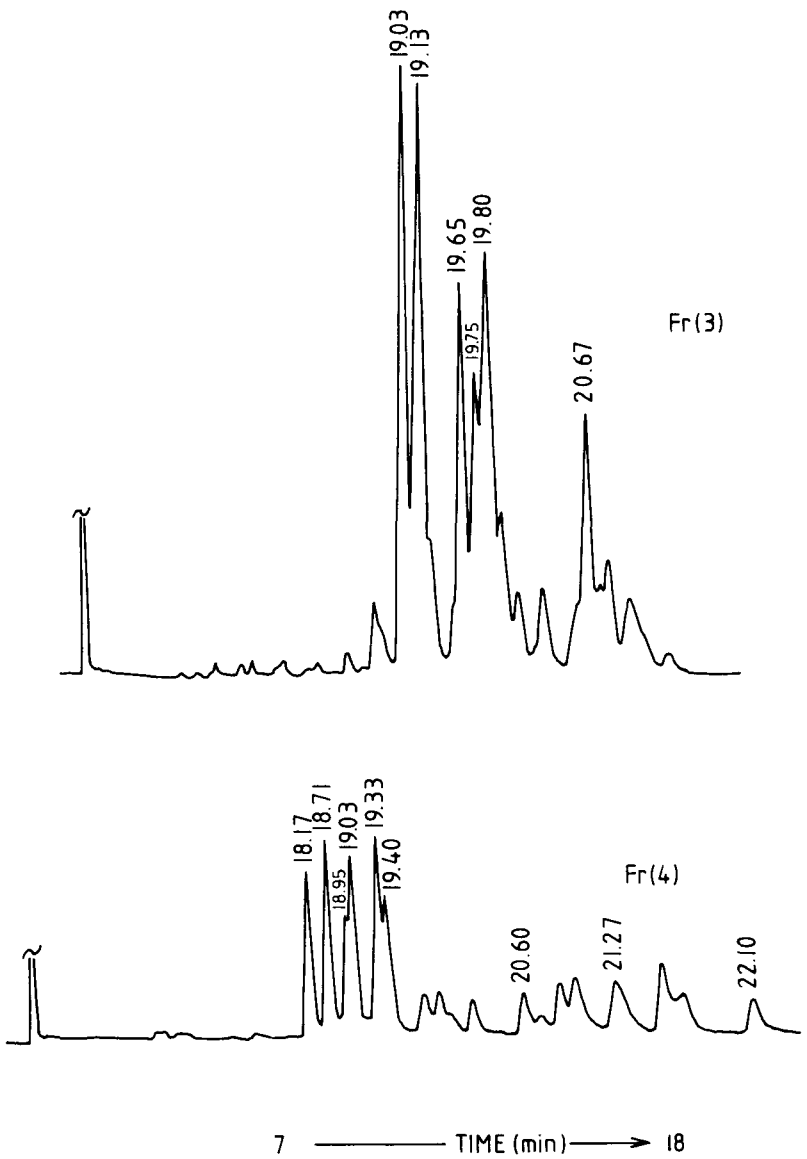

FIG. 7. Gas liquid chromatographic analyses on fused silica column (CP SIL 88), 50 m long and 0.32 mm i.d., of the CFAM fractions isolated from a sunflower oil heated at 200 C for 48 hr in a commercial fryer.

reverse phase column in order to study the possibility of further fractionating this complex CFAM mixture using HPLC. However, only one peak which had the same retention volume as 18:2ω6 was observed. This result seems to indicate that all the CFAM formed after the heat treatment of linseed oil could be dienoic fatty acid isomers.

Isolation of CFAM from heated sunflower oils. The method used to isolate the CFAM is outlined in Figure 4. The sunflower oil heated under nitrogen contained 1.0% CFAM (10 times less than for linseed oil), while the sample heated 48 hr at 200 C contained 0.5% of these cyclic components. The urea fractionation carried out on the nonpolar fraction gave two fractions. The urea adduct fraction was a mixture of 16:0, 18:0, 18:1, 18:2 and 18:3 isomers, and the other nonadduct fraction was a mixture of CFAM and 18:2ω6. A second urea complexation only slightly improved the purity of the CFAM fraction. Further complexation did not permit elimination of the 18:2ω6 in the CFAM fraction. This could be due to the amount of 18:2ω6 in the starting sunflower oil if compared to linseed oil (Table 1). After the third urea complexation, some CFAM was found even in the urea adduct fraction. To obtain a pure CFAM fraction, the mixture of 18:2ω6 and CFAM was further fractionated by HPLC on a C18 reverse phase column. This type of phase usually gives a good separation of fatty acids of the same chain length having different unsaturations. Two types of HPLC analyses were used. A preparative HPLC analysis was used when large quantities of CFAM were needed for toxicological studies. A semi-preparative column was used to isolate small quantities of material for further chemical analyses.

Preparative HPLC was used to recover the CFAM from the sunflower oil heated at 275 C for 12 hr under

nitrogen. The mixture of CFAM and 18:2ω6 (after urea fractionation, Fig. 4) was injected on a C18 reverse phase column using a mixture of ACN/H_2O (90:10) at 150 ml/min (Fig. 5). Three fractions were then collected. Fraction 1 contained pure 18:2ω6. Fraction 3, which still contained some 18:2ω6 mixed with the CFAM, was reinjected using ACN at 200 ml/min. Two fractions were collected. Fraction 5 contained 96% CFAM and 4% 18:2ω6. Fraction 4 and Fraction 2, which still contained some CFAM, were reinjected using the recycle mode. The resulting Fraction 7 showed the same CFAM profile as Fraction 5 when analyzed by GLC, but was not as pure (75%). Fraction 5 was used for all further structural analyses.

A semi-preparative column was used to isolate the CFAM from a sunflower oil heated at 200 C for 48 hr. The mixture of CFAM and 18:2ω6 was fractionated using MeOH at 4 ml/min (Fig. 6). Five fractions were

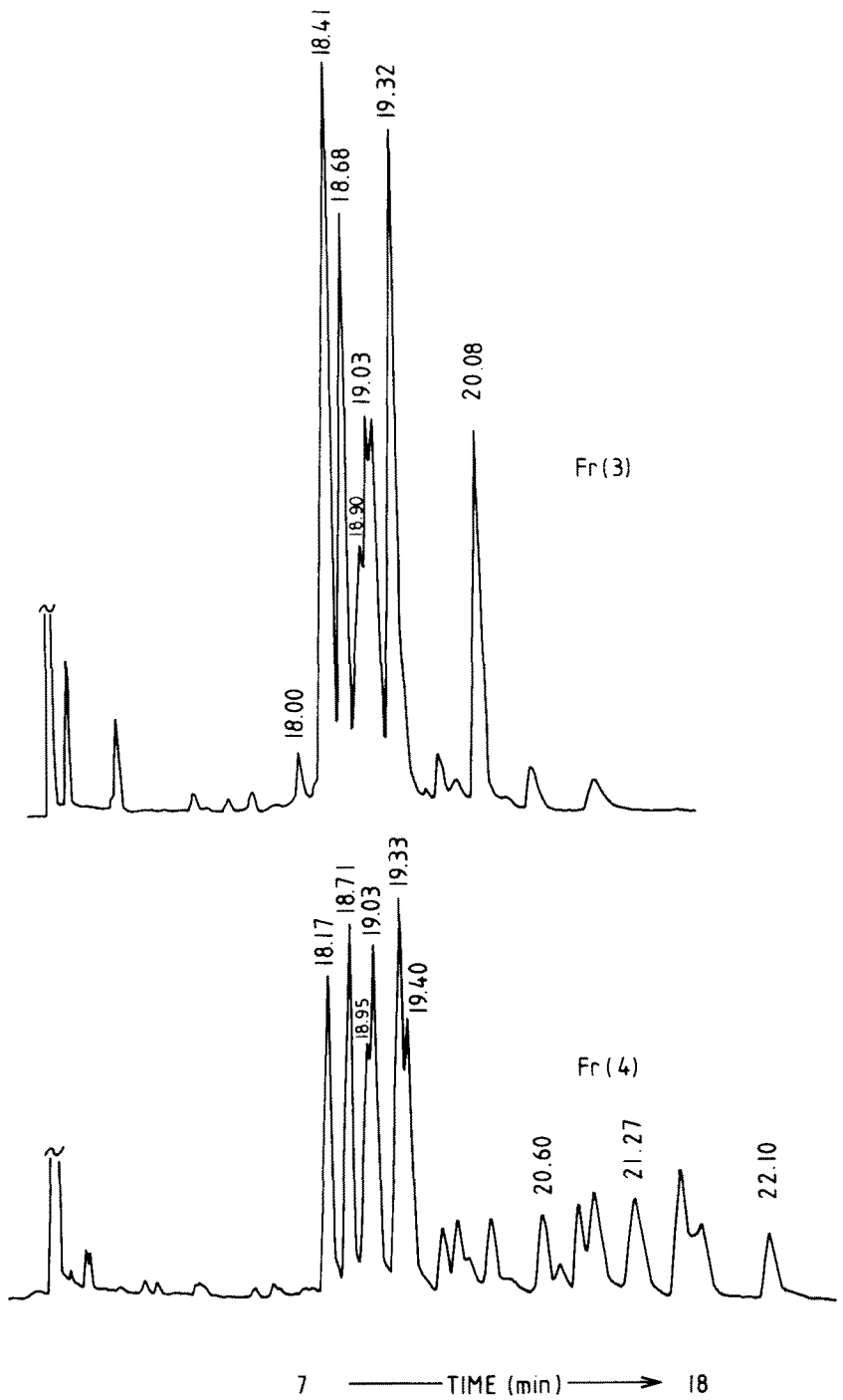

FIG. 8. Gas liquid chromatographic analyses on fused silica column (CP SIL 88), 50 m long and 0.32 mm i.d., of the hydrogenated CFAM fractions from a sunflower oil heated at 200 C for 48 hr in a commercial fryer.

collected. Fraction 1 contained minor peaks with ECL values shorter than 14.00 on CP Sil 88. These components with shorter retention time could indicate a slight oxidation which took place during the isolation procedure. Fraction 2 contained the 18:2ω6, and Fractions 3 and 4 contained the CFAM. The GLC analysis of Fraction 5 on CP Sil 88 did not show any components in the C18, C20 and C22 areas. The components detected by HPLC in Fraction 5 also were detected in the urea used in the fractionation. Fraction 4 had the same retention volume as 18:0 and the major peak of Fraction 3 the same as 18:1. It is interesting to note that the isolated CFAM from the sunflower oil heated at 275 C under N_2 had a similar HPLC profile on the semi-preparative column with lower quantities of Fraction 4 and Fraction 5. However, as previously outlined, the HPLC analysis of the CFAM isolated from the heated linseed oil showed only the presence of one peak with the same retention volume as 18:2ω6. From these observations, the CFAM from linseed oil could be anticipated to be a mixture of dienoic fatty acids while the CFAM from sunflower oil would be a mixture of monoenoic and saturated acids.

The GLC analyses of the CFAM fractions isolated from heated sunflower oils are presented in Figure 2B and Figure 7. The major CFAM found in heated sunflower oils had shorter ECL values than those observed in heated linseed oil (Fig. 2A). Each fraction was then hydrogenated and further analyzed by GLC (Figs. 3B and 8). No changes were observed in Fraction 4 before and after hydrogenation (Figs. 7 and 8). Fraction 4 therefore includes the saturated components. This confirms the retention volume observed for the HPLC analyses. The hydrogenation of the CFAM isolated from both heated sunflower oils gave components of shorter ECL values. However, the GLC profile of the hydrogenated fractions was somewhat similar to those observed prior to hydrogenation (Figs. 2B and 7). This was not the case for linseed oil, where the isolated CFAM were transformed into a mixture of four major components (Figs. 2A and 3A) upon hydrogenation.

The method described in Figure 1 proved to be satisfactory to isolate a fraction of CFAM from a heated linseed oil. About 450 g of the CFAM mixture (Fig. 2A) was isolated using this method. This mixture was used to carry out toxicological studies on rats. The results will be published elsewhere (26).

The GLC analyses of the isolated CFAM fractions from sunflower and linseed oils seem to indicate that the CFAM formed from linolenic acid are different compared to those formed from linoleic acid. Furthermore, 10 times less CFAM are formed in heated sunflower oil than in linseed oil. This indicates a large difference in the reactivity of linoleic and linolenic acids. In order to elucidate the structures of the CFAM, we will still have to determine the size of ring, the degree of unsaturation of the molecules, and the position and geometry of the ethylenic bond(s). As a first step in this study, GC-FTIR and GC-MS analyses of these CFAM fractions will be found in another paper (27).

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