Positional and Geometrical Isomers of Linoleic Acid in Partially Hydrogenated Oils

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The geometrical and positional isomers of linoleic acid of a partially hydrogenated canola oil-based spread were isolated and identified. Through partial hydrazine reduction and mass spectral studies, *cis*-9,*trans*-13 octadecadienoic acid was identified as the major isomer. Other quantitatively important isomers characterized were *cis*-9,*trans*-12; *trans*-9,*cis*-12 and *cis*-9,*cis*-15. These four were also the major isomers in margarine based on common vegetable oils. A number of minor isomers were detected and some structures identified were *trans*-9,*trans*-12; *trans*-8,*cis*-12; *trans*-8,*cis*-13; *cis*-8,*cis*-13; *trans*-9,*cis*-15; *trans*-10,*cis*-15 and *cis*-9,*cis*-13. The proportions of the various isomers are given for some margarines in the Canadian retail market. The amounts of *trans*-9,*trans*-12 isomer in Canadian margarines were generally below 0.5% of the total fatty acids.

KEY WORDS: Gas-liquid chromatography of *trans*-dienes, linoleic acid isomers, lipoxidation, margarine fatty acids, octadecadienoic acid isomers, partially hydrogenated oils, *trans* fatty acids, *trans*-octadecadienoic fatty acids, *trans*-polyunsaturated fatty acids.

Many geometrical and positional isomers of linoleic acid (*c*9,*c*12-18:2) are formed during partial hydrogenation of vegetable oils. Significant levels of these 18:2 isomers (refers to all the geometrical and positional isomers of octadecadienoic acid, except linoleic acid) are present in margarines and other food fats containing such oils. It appears that the 18:2 isomers of partially hydrogenated oils are mainly geometrical isomers of linoleic acid (1–4) and positional isomers of linoleic acid usually involving one carbon migration of the double bond (4). Even though structures of most of the isomers have been identified or proposed (4), it is extremely difficult to identify them accurately in food fats, because of their complexity and the lack of suitable reference standards. The relative elution order of these isomers in gas-liquid chromatography (GLC) has not been clearly established, which makes identification of the isomers more difficult, especially for those workers who rely solely on GLC for analysis of fatty acids in food fats for nutritional information.

Reference standards of the three geometrical isomers of linoleic acid, namely, *t*9,*t*12; *c*9,*t*12 and *t*9,*c*12, are obtainable from chemical suppliers or could be easily synthesized by geometrical isomerization of linoleic acid (5,6). Hence, for an experienced chromatographer, identification of the above three isomers should not pose a problem (6–10). Nevertheless, it is strongly emphasized here that *t*9,*t*12-18:2 can easily be confused with the major 18:2 isomer peak of partially hydrogenated oils, because in many GLC columns, including the polar cyanosiloxane capillary columns, the isomers have the same retention time (6,7,9,10). The major 18:2 isomer peak of GLC has not been characterized yet. In the past, probably because of the lack of information on the structure and the chromatographic properties of the 18:2 isomers, a report erroneously identified the major 18:2 isomer peak in GLC as *t*9,*t*12-18:2 and reported that some margarines contain higher levels (3%) of this di-*trans* fatty acid (11). However, other workers found less than 1% of this isomer in margarines (8,9,12,13).

The *t*9,*t*12-18:2 isomer is suspected to interfere with the metabolism of linoleic acid (14–16). It suppressed the levels of arachidonic acid and eicosanoids in laboratory rats fed diets containing low levels of linoleic acid (16). Because of the possible physiological effects of *t*9,*t*12-18:2, it has been recommended that the *trans,trans*-octadecadienoic acids in margarines sold in Canada should be less than 1% of the total fatty acids (17). The other *trans* isomers of octadecadienoic acid may also effect essential fatty acid metabolism (18).

It is therefore of practical importance to know the types and levels of octadecadienoic isomers present in food fats containing partially hydrogenated oils. In the present study, the major isomers and some of the minor 18:2 isomers present in a sample of spread, which was a blend of partially hydrogenated canola oil (major component), palm oil and butter, were characterized through a combination of techniques, which involved silver nitrate-thin-layer chromatography ($AgNO_3$-TLC), capillary GLC, partial hydrazine reduction and oxidative ozonolysis. The structure of the major isomer was identified as *c*9,*t*13-18:2 and the positions of the double bonds were confirmed by gas-liquid chromatography-mass spectroscopy (GLC-MS) of the picolinyl ester derivative. The 18:2 isomer levels in 50 commercial margarines were also determined. Another purpose of our investigation was to provide the equivalent chainlength (ECL) values of the 18:2 isomers on the polar SP-2560 (a cyanopropylsiloxane liquid phase) capillary column, which is one of the GLC columns frequently used for *cis-trans* isomer separation.

MATERIALS AND METHODS

Common fatty acid methyl ester standards were purchased from Sigma Chemical Company (St. Louis, MO). Several uncommon *cis* and *trans*-C_{18} unsaturated fatty acid methyl esters required for GLC identification of peaks were prepared from authentic linoleic and α-linolenic acids through partial hydrazine reduction with subsequent geometrical isomerization with p-toluenesulfinic acid (6). The margarines were purchased from retail stores in major cities in Canada.

Methylation. The fat was extracted and methylated as described previously (6). Free fatty acids were converted to fatty acid methyl esters (FAME) by heating with 7% BF_3-MeOH.

$AgNO_3$-TLC. Preparative $AgNO_3$-TLC plates were prepared as described previously (6). FAME were applied to the $AgNO_3$-TLC plates in hexane solution with a TLC streaker (Applied Science Laboratories, State College, PA). The plates were developed in toluene at −25°C. The separated bands were visualized under UV radiation (254

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nm) after spraying with 0.2% 2′,7′-dichlorofluorescein in ethanol. The bands were scraped from the plate and extracted with hexane/$CHCl_3$ (1:1, v/v).

GLC. The GLC of FAME was carried out with a Hewlett-Packard 5880 gas chromatograph equipped with a flame ionization detector and Hewlett-Packard Chemstation (Hewlett-Packard, Co., Palo Alto, CA). The GLC column used was a SP-2560 flexible fused silica capillary column (100 m × 0.25 mm i.d., 20 μm film thickness; Supelco, Inc., Bellefonte, PA). The column was operated isothermally (180°C) or the oven temperature was programmed at a rate of 1°C per min from 150 to 200°C and held at the final temperature for 25 min. The detector and injector port temperature was 250°C. Hydrogen was used as the carrier gas at a pressure of 20 psig. The ECL values reported in Table 1 were for FAME analyzed isothermally at 180°C.

For analysis of the short-chain FAME (monomethyl esters), which were produced by oxidative ozonolysis of monounsaturated FAME, the column oven temperature was programmed from 100 to 150°C at a rate of 1°C per min. The dimethyl ester products were analyzed isothermally at 180°C. The other GLC conditions were the same as described above.

Liquid chromatography. The liquid chromatography (LC) system consisted of Waters Associates (Milford, MA) Model 510 solvent delivery system, R401 refractive index detector, 712 WISP sample injection system and a 740 data module. The LC column was a Waters PrepPak 25 mm × 10 cm cartridge (Bondapak C_{18}, 125°A, 15–20 μ) used in the RCM 25 × 10 compression module. The FAME, as solutions in 100% MeOH, were separated according to their partition numbers by means of MeOH/H_2O (95:5, v/v) at 8 mL/min. The total methyl octadecadienoate (18:2) fraction was collected. Recovery of FAME from MeOH/H_2O eluent was achieved by extracting with hexane.

Hydrazine reduction. Fatty acids were partially reduced with hydrazine as described by Ratnayake *et al.* (19). In a typical experiment, fatty acids (1–10 mg) were dissolved in 30 mL of 95% EtOH. The solution was heated to 40°C and 1 mL of anhydrous hydrazine was added. The reduction reaction was allowed to proceed for 1 hr at 40°C with gentle stirring with a spin bar magnet. Throughout the reaction a steady stream of oxygen (1–2 mL/min) was maintained just above the surface of the solution. The reaction was quenched by the addition of 3N HCl to pH 4–5, and the fatty acids were extracted with hexane. The fatty acids were converted to FAME for analysis.

Oxidative ozonolysis. Oxidative ozonolysis of the monounsaturated FAME was executed in BF_3-MeOH as described by Ackman *et al.* (20), with recovery of the mono- and dimethyl ester products by extraction with hexane. Ozone was generated in a Model T-408 Polymetrics Laboratory Ozonator (Polymetrics, Inc., San Jose, CA). The dimethyl esters of dicarboxylic acids required for GLC identification were prepared by subjecting the readily available C_{18} monounsaturated fatty acids (*c*6-18:1; *c*9-18:1; *c*12-18:1; *c*15-18:1) to oxidative ozonolysis. Other dimethyl esters were identified with semi-log plots of GLC retention times with carbon numbers of the above generated dimethyl esters.

Enzymatic oxidation. A slightly modified procedure of Dolev *et al.* (21) was followed for the enzymatic oxidation of *cis,cis*-methylene interrupted polyunsaturated fatty acids.

The FAME fraction (~10 mg) obtained after LC and $AgNO_3$-TLC fractionation (band 4, see below) was hydrolyzed (KOH/EtOH and subsequent acidification) to fatty acids. The recovered fatty acids were dissolved in 10 mL of 95% EtOH and added to 80 mL 1M potassium borate buffer (61.9 g H_3BO_3 + 25.0 g KOH in 1000 mL H_2O, adjusted to pH 9.0). The enzyme (100 mg dry lipoxidase powder, Sigma Chemical Company) was added and the solution was slowly stirred for 30 min. Throughout the reaction a steady stream of oxygen (5 mL/min) was maintained just above the surface of the solution. The reaction was terminated by adjusting the pH to 5.0 with 6N HCl, and the solution was extracted immediately with hexane. The recovered fatty acids were converted to FAME with BF_3-MeOH.

Gas-liquid chromatography-mass spectrometry. The picolinyl ester derivatives were prepared by reaction of the mixed anhydride of trifluoroacetic acid and the fatty acid with 3-(hydroxymethyl)-pyridine (22). Mass spectra of picolinyl esters were obtained on a VG Analytical MS system (Model 7070EQ), equipped with an 11/250 data system and interfaced to a Varian GC (Model Vista 6000) operated at an ionization energy of 70 eV (Varian Associates, Palo Alto, CA). The GLC column used was a fused silica capillary column (30 m × 0.2 mm, 0.25 μ film thickness) coated with DB-5. Helium was the carrier gas. The column oven temperature was programmed from 80 to 220°C at 10°C per min.

RESULTS

A commercial spread, made from a blend of 20% butter and 80% of a mixture of partially hydrogenated canola oil and unhydrogenated palm oil, was selected for the study of the 18:2 isomers. The GLC profile of the C_{18} region of the spread's fatty acids, as their FAME, is shown in Figure 1. There were two reasons for selecting this spread—first, it had relatively low levels of linoleic acid and, second, the concentrations of the 18:2 isomers, especially those major isomers designated as peaks 20, 23 and 25, were relatively higher than in other spreads and margarines that we examined in a previous study (8).

Table 1 summarizes the identification of the 18:2 isomers based on the analysis of each peak. Also given in Table 1 are the experimental ECL values of the 18:1 and 18:2 isomers. The 18:1 isomer elution pattern on the SP-2560 column was established as described in our previous publications (6,7) and by comparison with the GLC profile of FAME of human adipose tissue lipids published by Hudgins *et al.* (10). The ECL values of the 18:2 isomers, calculated from the summation of the fractional chainlength (FCL) of the corresponding 18:1 FAME and the base value of 18:0, are also given in Table 1.

Reversed-phase LC was used first to fractionate the FAME according to their partition numbers, *i.e.*, the chainlength of the fatty acid minus two times the number of ethylenic bonds in the molecule. The reversed-phase LC allowed the isolation of a pure 18:2 fraction with no other degree of unsaturation or chainlength. Peaks 12, 14 and 16–29 shown in Figure 1 constituted the 18:2 fraction. This fraction was further fractionated by preparative $AgNO_3$-TLC with development in toluene at −25°C. The

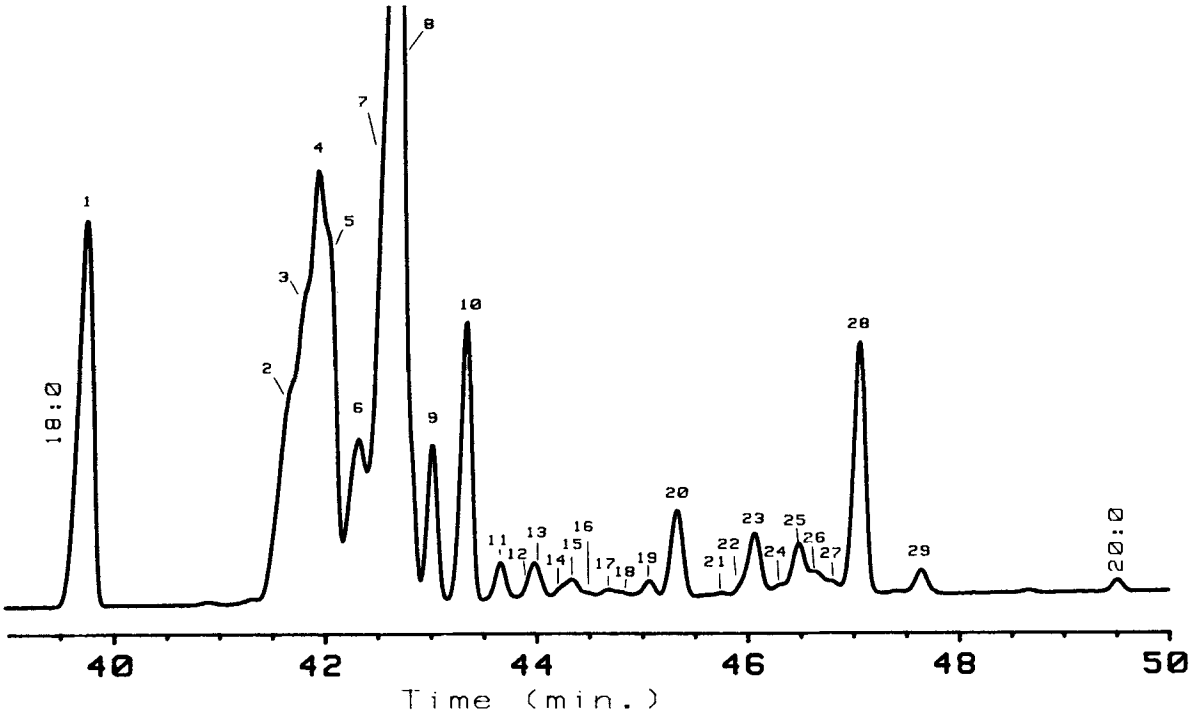

FIG. 1. The C_{18} region of the gas chromatogram of the FAME of canola/palm/butter spread. Analysis on a SP-2560 flexible fused silica capillary column. The column oven temperature was programmed as described in the text. See Table 1 for peak identifications. Peaks 12, 14 and 16–29 are the 18:2 isomers.

LC and $AgNO_3$-TLC fractionations were repeated several times to obtain sufficient material for further analyses.

The $AgNO_3$-TLC resulted in five bands of R_f values 0.38 (band 1), 0.28 (band 2), 0.21 (band 3), 0.16 (band 4) and 0.14 (band 5). Figure 2 shows the GLC profiles of the five bands. It is known that 18:2 isomers not only separate according to the geometry of the double bonds on $AgNO_3$-TLC, but also according to the positions of the double bonds (12,23,24). The diethylenic fatty acids whose two double bonds are separated by one methylene group (MID) generally have a higher mobility on $AgNO_3$-TLC than those diethylenic fatty acids whose double bonds are separated by two or more methylene groups (NMID). Thus, combining this information with the type of the major component(s) found in each band (Table 1 and Fig. 1), band 1 could be classified as *tt*-MID, band 2 as *ct/tc*-MID + *tt*-NMID, band 2 as *cc*-MID, band 4 as *ct/tc*-NMID and band 5 as *cc*-NMID.

Peaks 12, 14, 16–19, 23, 25 and 28. Peak 19 was mainly concentrated in $AgNO_3$-TLC band 1 (Fig. 2a), which had the same mobility as that of reference *t*9,*t*12-18:2. On GLC, peak 19 co-chromatographed with *t*9,*t*12-18:2. Hence, peak 19 was identified as *t*9,*t*12-18:2. Peaks 23 and 25 were concentrated in band 2 (Fig. 2b). This band had the same R_f value as those of synthetic *c*9,*t*12-18:2 and *t*9,*c*12-18:2. On GLC, peak 23 co-eluted with *c*9,*t*12-18:2 and peak 25 with *t*9,*c*12-18:2. Partial hydrazine reduction of band 2 FAME resulted in the formation of four major 18:1 isomers of almost equal proportions (not shown). They were identified through GLC retention-time comparison with standards of *c*9-18:1, *c*12-18:1, *t*9-18:1 and *t*12-18:1. Peak 23 was identified as *c*9,*t*12-18:2 and peak 25 as *t*9,*c*12-18:2 from these data.

The minor components designated as peaks 12, 14 and 16–18 in the Figures were not sufficiently concentrated in any of the bands for complete structural elucidation. Nevertheless, from their GLC elution order and behavior on $AgNO_3$-TLC, they could be tentatively characterized as *tt*-NMID isomers. This was based on the fact that on GLC these isomers eluted before *t*9,*t*12-18:2 (peak 19), which is an MID. Generally, on polar GLC columns *tt*-dienes have lower ECL values than *ct/tc* and *cc* dienes and the NMID elute before MID (25–27); hence, peaks 12, 14, 16, 17 and 18 are probably *tt*-NMID. Furthermore, these isomers did not migrate with *t*9,*t*12 isomer on band 1 and peaks 14, 16, 17 and 18 were found in bands 2 (Fig. 2b) and 3 (Fig. 2c), while peak 12 was found only in band 3 (Fig. 2c). The low mobilities of peaks 12, 14, 16, 17 and 18 as compared to *t*9,*t*12-18:2 are consistent with the $AgNO_3$-TLC properties of the *tt*-NMID structures (23).

Peak 28 was identified as linoleic acid from its GLC retention time data and its mobility on $AgNO_3$-TLC by comparison with a standard. Linoleic acid was chiefly concentrated in band 3 (Fig. 2c).

Peaks 20, 21, 22 and 26. The major 18:2 isomer in the margarine was peak 20, which was mainly in band 4 (Fig. 2d). Peak 20 eluted slightly after *t*9,*t*12-18:2 on the SP-2560 capillary column. In a previous study we have shown that on the SP-2340 flexible fused silica capillary column, peak 20 almost co-eluted with the above di-*trans* isomer (6). It has been speculated by several researchers (2,3,6,7,9,12,23) that this major 18:2 isomer in partially

TABLE 1

Structures of 18:1 and 18:2 Isomers in Spread and Their ECL Values on SP-2560 at 180°C

Peak no.[a]	Expt. ECL	Isomer	Cal. ECL	ΔECL[b]
18:1 isomers				
2	18.43	*t*8	—	—
3	18.46	*t*9	—	—
4	18.48	*t*10	—	—
5	18.52	*t*11	—	—
6	18.55	*t*12; *c*8	—	—
7	18.61	*t*13	—	—
8	18.63	*c*9	—	—
9	18.73	*c*11	—	—
10	18.80	*c*12	—	—
11	18.88	*c*13	—	—
13	18.97	*c*14	—	—
15	19.00	*c*15	—	—
18:2 isomers				
12	18.96	*tt*-NMID	—	—
14	18.99	*tt*-NMID	—	—
16	19.08	*tt*-NMID	—	—
17	19.09	*tt*-NMID	—	—
18	19.13	*tt*-NMID	—	—
19	19.16	*t*9,*t*12	19.01	0.15
20	19.24	*c*9,*t*13	19.24	0
		*t*8,*c*12	19.23	0.01
21	19.30	*tc*/*ct*	—	—
22	19.31	*t*8,*c*13 ?[c]	19.31	0
		cc-MID	—	—
23	19.36	*c*9,*t*12	19.18	0.18
24	19.43	*c*8,*c*13 ?[c]	19.43	0
25	19.45	*t*9,*c*12	19.26	0.19
26	19.46	*t*9,*c*15	19.46	0
		*t*10,*c*15 ?[c]	19.48	−0.02
27	19.49	*c*9,*c*13 ?[c]	19.51	−0.02
28	19.58	*c*9,*c*12	19.43	0.15
29	19.63	*c*9,*c*15	19.63	0

[a]See Figures for peak numbers.

[b]Δ ECL = experimental ECL − calculated ECL.

[c]Tentative identification.

hydrogenated oils is a NMID with one of the double bonds being in the *trans* configuration. Band 4 also contained several other isomers; including linoleic acid, which was the second largest component in this band. The presence of a large proportion of linoleic acid always interfered in our preliminary attempts to identify peak 20, as well as other isomers present in this band, through degradative and GC-MS studies. Thus, the next step in the identification process of peak 20 was to eliminate linoleic acid from the FAME mixture of band 4. It is known that lipoxidase enzyme, present in a number of plant tissues, selectively oxidizes certain polyunsaturated fatty acids to hydroperoxides (28,29). The only known substrates are the polyunsaturated fatty acids, containing a *cis,cis*-1,4-pentadiene group (*i.e.*, methylene-interrupted ethylenic bonds), such as linoleic and linolenic acids. As expected, treatment of the fatty acids of band 4, in the unesterified form, with lipoxidase (21) completely eliminated linoleic acid (Fig. 3). Not only was linoleic acid eliminated or oxidized, but comparison of Figures 2d and 3 shows that a major portion of peak 22 was also oxidized by lipoxidase. This suggests the presence of another *cis,cis*-methylene interrupted diene in band 4. The isolation of band 4 and the subsequent enzymatic reaction was repeated several times to obtain enough material for structural elucidation. A portion of the fraction obtained after lipoxidation was converted to the picolinyl ester derivative and subjected to GC-MS analysis (22). Another portion was subjected to partial hydrazine reduction (19).

The reconstituted total ion chromatogram of the picolinyl esters showed only a single peak (not shown) for the various 18:2 isomers present in the lipoxidase-treated band 4. However, since peak 20 is the dominant component (92%, Fig. 3), the mass spectrum of this single GC-MS peak is essentially that of the components in peak 20. The mass spectrum (Fig. 4) gave prominent ions at *m/z* 91, 92, 108, 151 and 164, which are characteristic of picolinyl ester derivatives (22). The molecular ion appeared at *m/z* 371, which is in agreement with the molecular weight for the picolinyl ester derivatives of 18:2 acids. There were significant ions at *m/z* 356 ($M-CH_3$) and [with gaps of 14 atomic mass units (amu) separating ions] at *m/z* 342, 328 and 314, representing cleavage at successive methylene groups. This was followed by a gap of 26 amu to *m/z* 288. In picolinyl ester derivatives of unsaturated fatty acids the gap of 26 amu represents a cleavage at either side of the double bond (22,30). Thus the 26 amu gap between *m/z* 314 and 288 is due to the presence of a double bond at the C_{14}-C_{13} position. After the significant ion at *m/z* 288, further ions (*m/z* 274 and

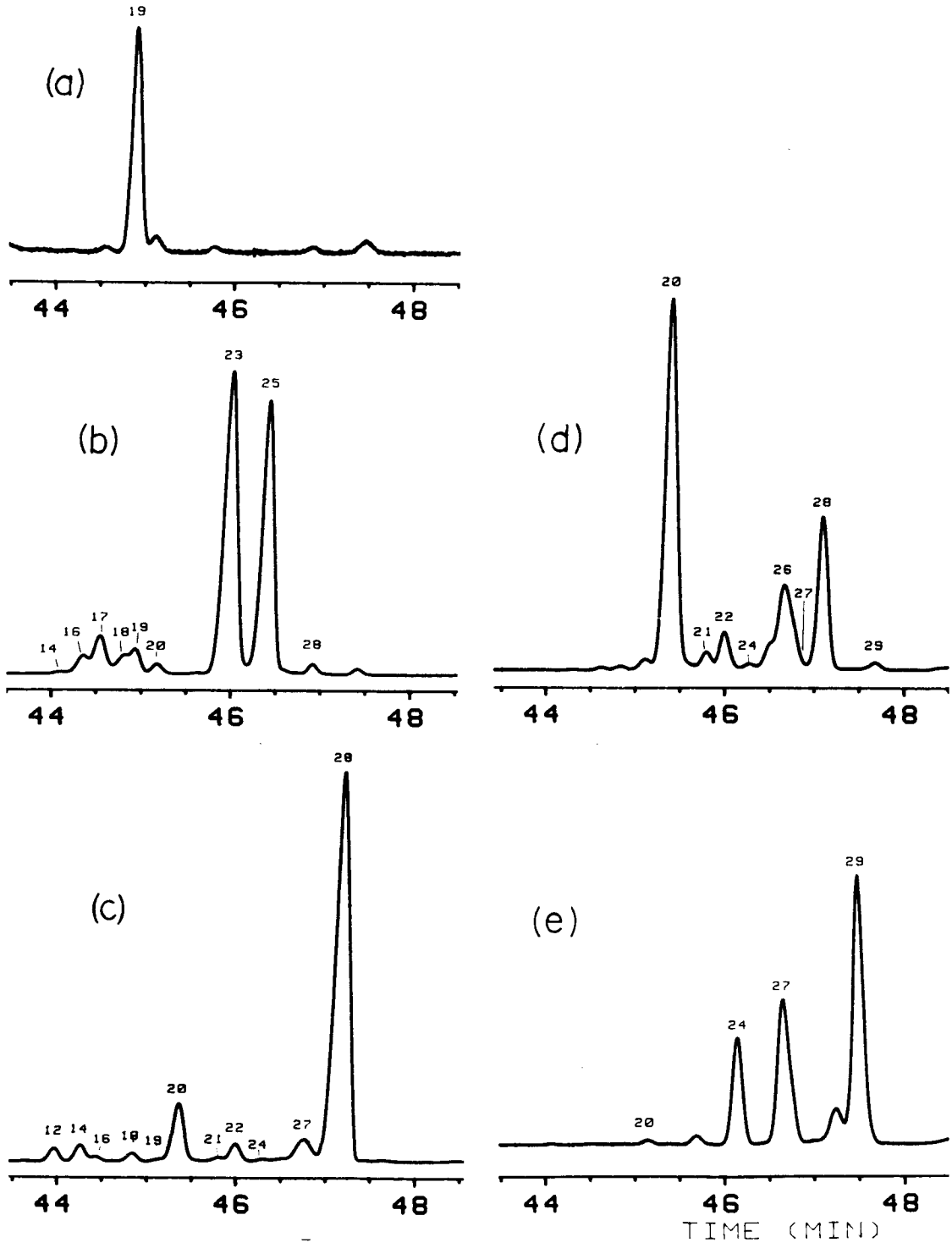

FIG. 2. Capillary gas chromatograms of the 18:2 FAME fractions of the canola/palm/butter spread obtained from $AgNO_3$-TLC fractionation of the total 18:2 FAME isolated using LC. (a) Band 1, (b) band 2, (c) band 3 (d) band 4, and (e) band 5. GLC operating conditions were the same as for Figure 1. See Table 1 for peak identifications.

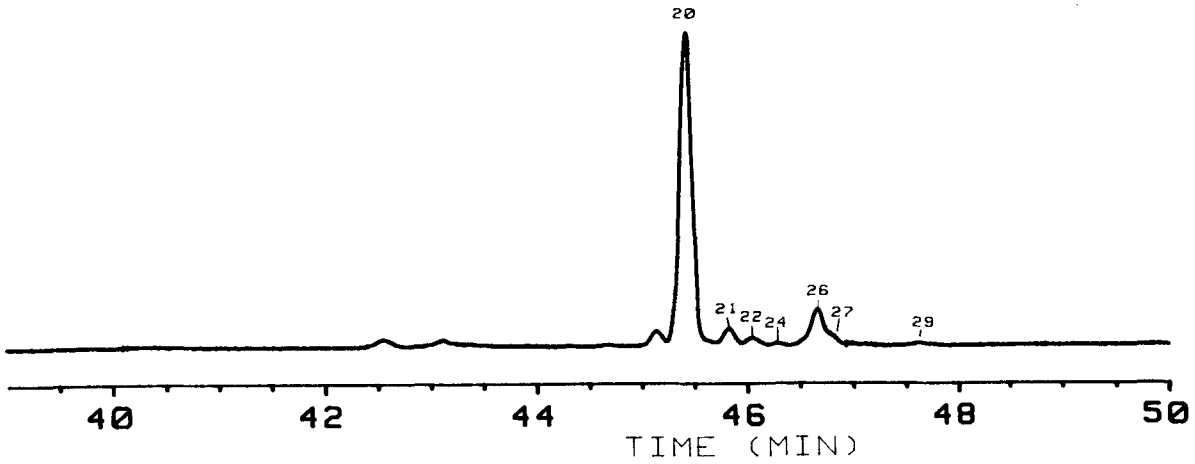

FIG. 3. Capillary gas chromatogram of the unreacted portion of 18:2 FAME of the $AgNO_3$-TLC band 4 illustrated in Figure 2d after lipoxygenase treatment. GLC operating conditions were the same as for Figure 1. See Table 1 for peak identifications. (It should be noted here that the hydroperoxide products of lipoxygenation were not detected in this gas chromatogram. Probably the hydroperoxide were decomposed during methylation of the fatty acids with BF_3-MeOH.)

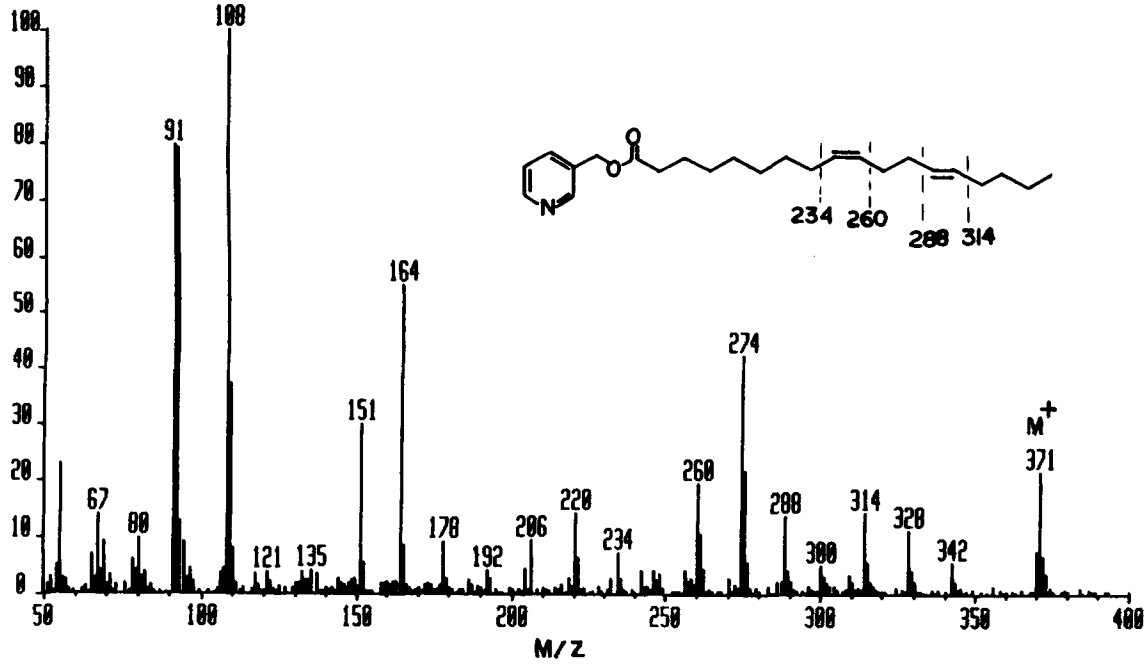

FIG. 4. Electron ionization mass spectrum of the picolinyl ester derivative of the 18:2 fraction illustrated in Figure 3. The mass spectrum is essentially of *c*9,*t*13-18:2.

260) appeared 14 amu apart. This was followed by another gap of 26 amu to *m/z* 234, which shows that the second double bond is at the C_{10}-C_9. Thus peak 20 is mainly 9,13-18:2.

The hydrazine product (as FAME) of the fatty acid fraction obtained after lipoxidase treatment (Fig. 3) is shown in Figure 5. Partial reduction has some special effects. Hydrazine reduces ethylenic bonds without affecting the position of the geometry of the remaining ethylenic bonds in the polyunsaturated molecule (19). Therefore, a study of the structures of the unsaturated fatty acids formed during hydrazine reduction, particularly the monounsaturates, will reveal the complete structure of the parent polyunsaturated fatty acid. The GLC retention times of the new peaks 1, 3, 8, 10 and 12 (Fig. 5), formed after reduction, were identical to the retention times of reference standards of 18:0, *t*9-18:1, *c*9-18:1, *c*12-18:1 and *c*15-18:1, respectively. Peaks 2, 4, 7, 11 and 13 were tentatively identified as *t*8-18:1, *t*10-18:1, *t*13-18:1 and *c*14-18:1, respectively. The tentative identifications were based on the comparison of the GLC retention times with the total FAME of the starting spread (Fig. 1 and Table 1), and with

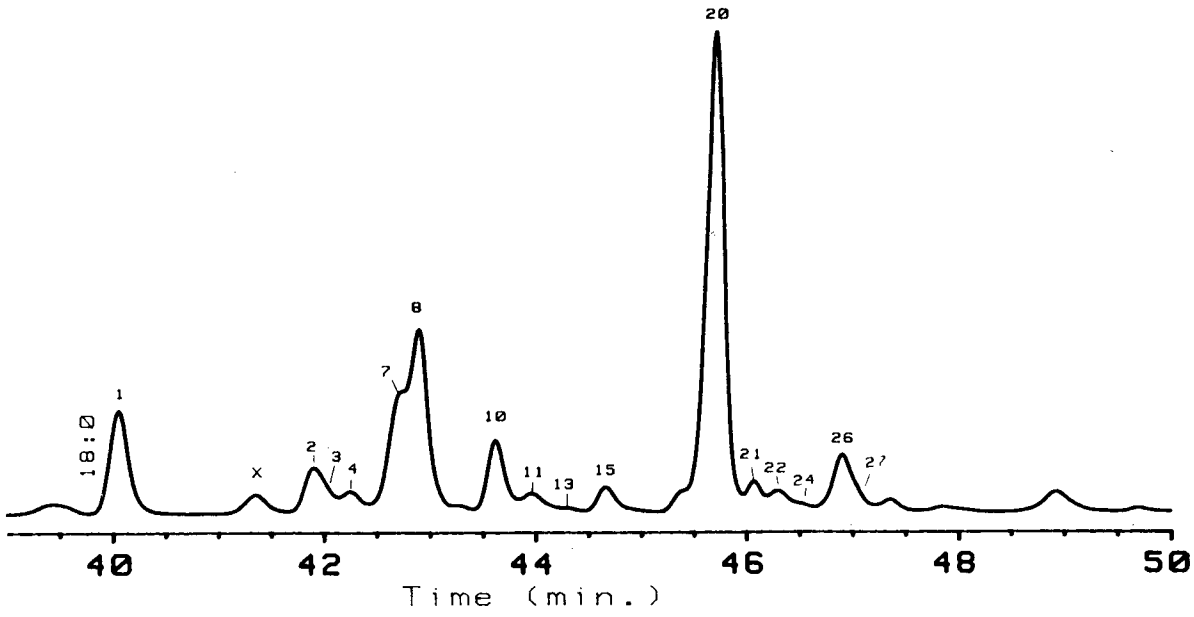

FIG. 5. Capillary gas chromatogram of the partial hydrazine product (as FAME) of the 18:2 fraction illustrated in Figure 3. GLC operating conditions were the same as for Figure 1. See Table 1 for peak identifications. Peaks marked with x are most likely environmental contaminants or components left over on the GLC column from previous runs.

those elution orders published in the literature (7,10) for the SP-2560 column. It should be noted here that, on the SP-2560 column, generally, most of the *trans*-18:1 isomers elute before the *cis* isomers. However, a few of the *trans* isomers with high delta values (*e.g.*, *t*13-18:1) co-elute with some of the *cis* isomers (*e.g.*, *c*9-18:1; see Fig. 1). The tentative identifications were confirmed by oxidative ozonolysis of the monounsaturated fatty acids isolated by using $AgNO_3$-TLC (described below).

The $AgNO_3$-TLC of the hydrazine-reduction product developed in toluene at −25°C for 2 hr showed 6 bands. The top band (band 1, R_f 0.72) contained only 18:0 (peak 1), whereas the least mobile band (band 6, R_f 0.16) was composed of all the unreacted 18:2 isomer peaks 20, 21, 22, 24, 26 and 27. The monounsaturated FAME were distributed in the four bands, 2–5. The *trans* 18:1 isomers appeared in two bands. The uppermost *trans* band (band 2, R_f 0.60; Fig. 6a) had the same mobility as that of reference *t*9-18:1. It contained *t*13-18:1 as the major component, with moderate amounts of *t*10, *t*9 and *t*8 isomers. The second *trans* band (band 3, R_f 0.55; Fig. 6b) contained only *t*8-18:1. The *cis* isomers also separated into two bands. The *cis* band with the highest mobility (band 4, R_f 0.46; Fig. 6c) had *c*12-18:1 as the major component, and *c*15-18:1 was present at a moderate level; *c*11-; *c*13- and *c*14-18:1 isomers were present as minor components. The *cis* band with the lowest mobility (band 5, R_f 0.29; Fig. 6d) was composed of *c*9-18:1 with minor levels of another isomer on the leading edge of its shoulder. This isomer was most likely *c*8-18:1. Oxidative ozonolysis (20) of each 18:1 band with O_3/BF_3-MeOH produced the expected dimethyl ester and monomethyl ester products that confirmed the correctness of the proposed double bond positions of the 18:1 isomers. The elution order of the *cis* and *trans* isomers on $AgNO_3$-TLC observed in the present study was consistent with that published by Gunstone *et al.* (31) for a series of synthetic *trans* and *cis* methyl octadecenoates. Therefore, the $AgNO_3$-TLC data presented here support the proposed identities of the 18:1 isomers (Fig. 5).

The final step in the identification of the 18:2 isomer peaks 20, 21, 22, 24, 26 and 27 is to correlate the amounts of these 18:2 isomers with the type and amounts of 18:1 isomers produced *via* partial hydrazine reduction (Fig. 5). *c*9- And *t*13-18:1 were the major products and these two were present in approximately equal proportions. Thus, *c*9- and *t*13-18:1 should have originated from peak 20, because it is the predominant GLC peak in the starting 18:2 fraction (Fig. 3). Earlier it was shown by GC-MS analysis that the two double bonds in the 18:2 isomer of peak 20 are in the Δ9 and Δ13 positions. Thus, the diene isomer in peak 20 was identified as *c*9,*t*13-18:2. The ECL data presented in Table 1 confirmed the proposed structure. The calculated ECL for *c*9,*t*13-18:2 [FCL of *c*9-18:1 (0.63) + FCL of *t*13-18:1 (0.61) + 18.0] was 19.24, which is equivalent to the experimentally determined ECL for peak 20.

The second largest pair of 18:1 isomers produced *via* hydrazine reduction was *t*8 (peak 2) and *c*12 (peak 10) (Fig. 5). The occurrence of these two in nearly equal proportions indicated the presence of *t*8,*c*12-18:2 in the parent 18:2 fraction (Fig. 3). The calculated ECL value of *t*8,*c*12-18:2 was 19.23, which shows that this 18:2 isomer overlaps with *c*9,*t*13-18:2 in peak 20. The *c*9,*t*13-18:2 has to be the dominant isomer in peak 20, because the GC/MS data (discussed above) gave no indication of the presence of ethylenic bonds at Δ8 and Δ12 positions. The mass spectrum was dominated by ions due to the *c*9,*t*13 isomer (Fig. 4). The

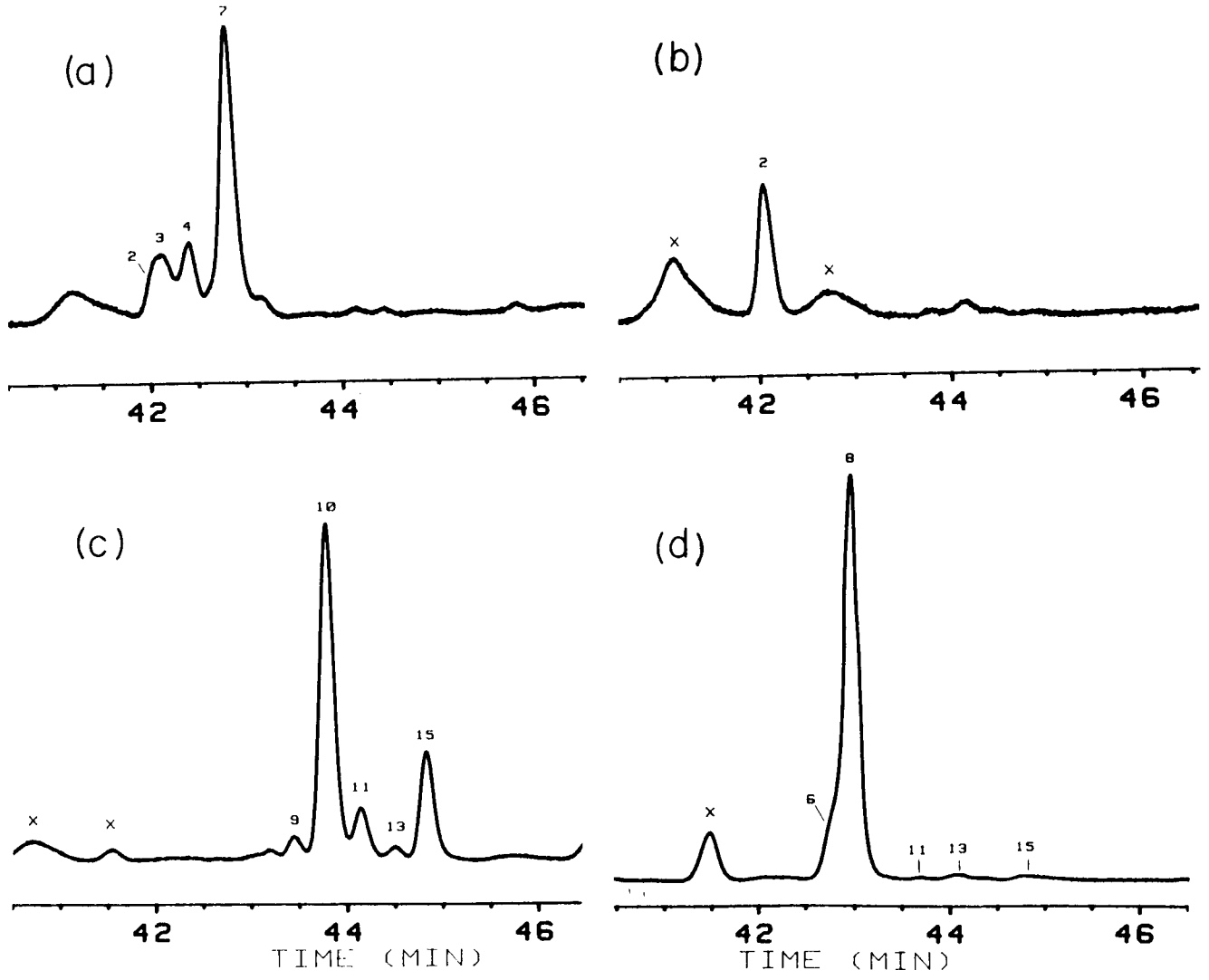

FIG. 6. Capillary gas chromatograms of the 18:1 FAME isomer fractions obtained by $AgNO_3$-TLC fractionation of the partial hydrazine product illustrated in Figure 5. (a) Band 2, (b) band 3, (c) band 4, and (d) band 5. GLC conditions were the same as for Figure 1. See Table 1 for peak identifications. Peaks x; see Figure 5.

relatively larger proportions of the *c*9- and *t*13-18:1 isomers as compared to *t*8- and *c*12-18:1 in the hydrazine product (Fig. 5) further supports *c*9,*t*13-18:2 as the major diene in peak 20.

The presence of low levels of *t*9-, *t*10- and *c*15-18:1 in the hydrazine reduction product (Fig. 5) and the ECL calculations (Table 1) are strongly suggestive that peak 26 is composed of *t*9,*c*15-18:2 and *t*10,*c*15-18:2. The ECL value of 15.46 for *t*9,*c*15-18:2 was confirmed by comparison with a synthetic standard, which was prepared from *c*9,*c*15-18:2 through geometrical isomerization with p-toluenesulfinic acid (6). Partial hydrazine reduction of α-linolenic acid and subsequent $AgNO_3$-TLC fractionation provided *c*9,*c*15-18:2. Due to unavailability of standards, the proposed structure of *t*10,*c*15-18:2 could not be confirmed. From ECL calculations (Table 1), the structure of peak 22 could be proposed as *t*8,*c*13-18:2. The presence of minor levels of *t*13-18:1 in the hydrazine reduction product may support this structure. The low concentration of peak 21 in the 18:2 fraction did not permit the identification of the component(s) in this peak, but the mere presence of this peak in band 4 suggests that the components are mono-*trans*-18:2. The identifications of peaks 24 and 27 are discussed below.

Peaks 24, 27 and 29. Peaks 24, 27 and 29 were mainly concentrated in the $AgNO_3$-TLC band 5 (Rf 0.14; Fig. 2e) of the LC-isolated total 18:2 fraction of the starting spread. This band had the same mobility as that of reference *c*9,*c*15-18:2. Peak 29 was co-chromatographed on GLC with reference *c*9,*c*15-18:2 and was identified as such. The calculated ECL (Table 1) of *c*9,*c*15-18:2 was equivalent to the experimental ECL value of peak 29, which further confirmed the identity of peak 29. Through ECL calculations, the 18:2 isomers of peak 24 and 27 were tentatively identified as *c*8,*c*13-18:2 and *c*9,*c*13-18:2, respectively. A partial hydrazine reduction product (Fig. 7) of the FAME of band 5 was unfortunately contaminated with a number of minor impurities, but it indicated the presence of *c*8-, *c*9-, *c*13- and *c*15-18:1 isomers, which are compatible with the proposed structures for the 18:2

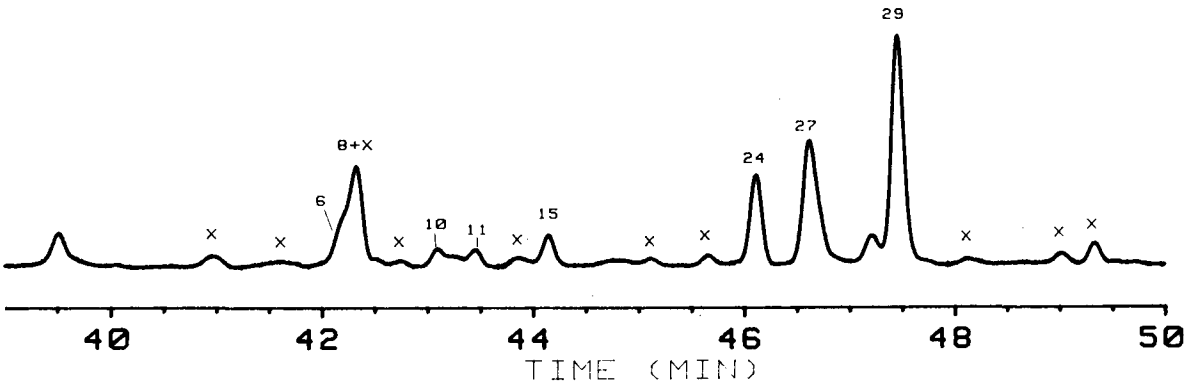

FIG. 7. Capillary gas chromatogram of the partial hydrazine product (as FAME) of the 18:2 fraction of the $AgNO_3$-TLC band 5 illustrated in Figure 2e.

isomers of band 5. The possible presence of *c*12-18:1 isomer in the hydrazine product (Fig. 7) is difficult to account for, but may suggest the presence of an 18:2 isomer in band 5 with a *c*12 double bond.

18:2 isomers in margarine. As an extension of this work, we studied the 18:2 isomer composition of 50 margarines purchased from retail stores in Canada. The results are given in Tables 2 and 3. The details of the other fatty acids are given in a previous publication from this laboratory (8). The canola/palm/butter spread, used above for the 18:2 isomer identification, served as the reference fatty acid mixture for GLC identification of the 18:2 isomers in the 50 margarine samples.

DISCUSSION

We have identified the major 18:2 isomer in a sample of spread, of which the major fat component was partially hydrogenated canola oil, as *c*9,*t*13-18:2. On the SP-2560 flexible fused silica capillary column, the *c*9,*t*13 isomer co-eluted with the *t*8,*c*12 isomer, and these two together formed the major 18:2 isomer peak in all the margarines that we have examined. The *c*9,*t*13- and *t*8,*c*12-18:2 structures are not a novel finding. They had been previously reported in partially hydrogenated soybean (32,33) and corn (4) oils. But in these reports, the details of the structure elucidation and, most importantly, the chromatographic properties of the two isomers were lacking. Probably partly because of this lack and partly due to unavailability of reference standards, the existence of *c*9,*t*13- and *t*8,*c*12-18:2 isomers in partially hydrogenated oils escaped the attention of those researchers who examined the biological and nutritional implications of *trans*-polyunsaturated fatty acids in food fats (34). Analytical chemists also failed to notice them in partially hydrogenated oils, margarines and other food fats containing partially hydrogenated oils (2,3,6,9,11,12,23). On many GLC columns, *t*9,*t*12-18:2, reference standards of which are readily available, overlaps with *c*9,*t*13- and *t*8,*c*12-18:2. This may have been the main reason for the failure of the previous workers to notice the presence of the above two mono-*trans* dienes in partially hydrogenated oils. The ECL values and the elution order of the 18:2 isomers presented in this study for the SP-2560 column, which is one of the polar columns frequently used for *cis-trans* isomer separation, should be helpful to future researchers for accurate identification of the 18:2 isomers in partially hydrogenated oils.

As demonstrated in the present study for the canola/palm/butter blend spread, *c*9,*t*13-18:2 is most likely the dominant component in the major 18:2 isomer peak of the margarines examined. The *c*9,*t*13 isomer, together with the two mono-*trans* geometrical isomers of linoleic, form the main *trans*-polyunsaturated fatty acids in margarine and other food fats made from partially hydrogenated oils. Our study shows that margarines with low levels of linoleic acid are enriched with the above mono-*trans* 18:2 isomers, for example, print margarines made from soybean, canola and canola/palm oils. The *c*9,*t*12 and *t*9,*c*12 isomers are not known to have any important biochemical or nutritional consequences, provided essential fatty acids are in adequate supply (34). Nevertheless, they are devoid of essential fatty acid activity (14). Therefore, the margarines with a high ratio of mono-*trans*-18:2 to essential fatty acid should be of concern. The nutritional and biochemical properties of *c*9,*t*13-18:2 are not known. The Δ6-desaturase is active only on fatty acids with a *cis*-9 double bond and, hence, it may be expected that the *c*9,*t*13-18:2 is converted to a 20:4 with a *trans* bond, similar to the metabolism of *c*9,*t*12-18:2 (14,35). A recent publication by Hudgins *et al.* (10) describes the presence of an unidentified 18:2 isomer in the adipose tissue of free-living adult men. These authors suspected it to be a mono- or di-*trans* isomer. A comparison of the GLC elution pattern of the 18:2 isomers presented here with that of Hudgins *et al.* (10) on the same SP-2560 column suggests that the unidentified isomer in the adipose tissue fatty acids is indeed *c*9,*t*13-18:2. Perhaps this may indicate the long-term intake and ready absorption of *c*9,*t*13-18:2 by humans from dietary partially hydrogenated oils.

The *t*9,*t*12-18:2 isomer has been implicated in interfering with the metabolism of essential fatty acids (14–16,18,34,35). Fortunately, it is not present in appreciable amounts in our food supply (12,32–34). Our data show that most Canadian margarines contain less than

TABLE 2

Octadecadienoic Isomer Composition and Totals of Fatty Acid Classes in Canadian Print (Hard) Margarines and Spreads

Fat base:[a]	SB	V	V/P	C	V/L	V/P/B
n:	3	5	7	1	1	2
18:2 Isomers						
tt-NMID	0.56 ± 0.71	0.21 ± 0.19	0.17 ± 0.08	0.10	0.51	0.29
*t*9,*t*12	0.65 ± 0.67	0.29 ± 0.27	0.28 ± 0.15	0.10	0.92	0.32
*c*9,*t*13+*t*8,*c*12	2.14 ± 0.46	1.07 ± 0.17	1.07 ± 0.52	0.57	1.07	1.05
*c*9,*t*12	1.98 ± 0.57	0.65 ± 0.21	0.64 ± 0.12	0.62	1.13	0.82
*t*10,*c*15+*t*9,*c*15	0.19 ± 0.12	0.08 ± 0.07	0.07 ± 0.09	0.03	0.08	0.24
*t*9,*c*12	1.76 ± 0.43	0.62 ± 0.27	0.51 ± 0.69	0.47	0.82	0.65
*c*8,*c*12	0.12 ± 0.02	0.03 ± 0.04	0.01 ± 0.01	0.05	0.03	0.07
*c*9,*c*13	0.05 ± 0.03	0.03 ± 0.02	0.04 ± 0.01	0.10	0.01	0.13
*c*9,*c*15	0.26 ± 0.12	0.27 ± 0.13	0.19 ± 0.15	0.38	0.02	0.58
Other fatty acids						
Saturates	24.0 ± 6.6	19.6 ± 1.8	22.7 ± 5.4	16.4	17.6	34.7
c-18:1	28.0 ± 4.5	36.4 ± 4.8	36.2 ± 5.6	24.7	35.8	35.9
t-18:1	31.1 ± 8.7	30.1 ± 5.2	32.9 ± 3.5	26.4	37.2	20.6
Linoleic	8.3 ± 6.2	8.1 ± 2.9	4.6 ± 0.9	30.0	4.8	2.9
Linolenic	0.6 ± 0.6	2.1 ± 1.4	0.5 ± 0.3	0	0.6	0.5
t-18:3	0.3 ± 0.2	0.4 ± 0.3	0	0	0	0.4
Total *trans* (IR)	38.8 ± 10.0	33.4 ± 5.8	35.2 ± 2.6	28.2	42.2	25.0

[a]SB, soybean oil; V, unspecified vegetable oil, fatty acid data indicates canola oil as the major component; P, palm oil; C, corn oil; L, lard; B, butter; and n, number of samples.

TABLE 3

Octadecadienoic Isomer Composition and Totals of Fatty Acid Classes in Canadian Tub (Soft) Margarines

Fat base:[a]	SB	V	V/P	C	SO/P	O
n:	13	10	2	3	2	1
18:2 Isomers						
tt-NMID	0.07 ± 0.04	0.13 ± 0.15	0.09	0	0	0
*t*9,*t*12	0.13 ± 0.09	0.23 ± 0.24	0.11	0	0	0
*c*9,*t*13+*t*8,*c*12	0.51 ± 0.23	0.58 ± 0.41	0.91	0.40 ± 0.38	0.45	0.02
*c*9,*t*12	0.32 ± 0.21	0.47 ± 0.22	0.84	0.26 ± 0.21	0.18	0.08
*t*10,*c*15+*t*9,*c*15	0.02 ± 0.01	0.08 ± 0.16	0.04	0.02 ± 0.02	0.03	0
*t*9,*c*12	0.32 ± 0.18	0.33 ± 0.15	0.72	0.26 ± 0.19	0.11	0.09
*c*8,*c*12	0.01 ± 0.01	0.01 ± 0.01	0.02	0.02 ± 0.01	0.02	0
*c*9,*c*13	0.02 ± 0.03	0.03 ± 0.01	0.03	0	0	0
*c*9,*c*15	0.04 ± 0.08	0.27 ± 0.11	0.15	0.02 ± 0.03	0.16	0
Other fatty acids						
Saturates	19.9 ± 4.9	15.0 ± 2.6	15.8	21.1 ± 7.2	22.3	22.4
c-18:1	24.2 ± 4.5	39.4 ± 6.2	34.6	25.8 ± 4.8	18.6	48.3
t-18:1	16.4 ± 3.3	23.5 ± 3.0	17.9	15.9 ± 7.4	6.7	19.2
Linoleic	33.1 ± 4.6	14.6 ± 6.2	23.8	35.5 ± 9.0	51.3	9.7
Linolenic	3.5 ± 1.4	4.2 ± 1.6	3.8	0.7 ± 0.1	0.1	0.3
t-18:3	1.0 ± 0.6	1.4 ± 0.9	1.3	0	0.2	0
Total *trans* (IR)	18.7 ± 3.9	26.4 ± 3.8	35.2	16.8 ± 7.8	7.2	19.4

[a]SO, sunflower; O, olive oil; see Table 2 for other abbreviations.

0.5% *t*9,*t*12-18:2, and only in one print margarine did the level exceed 1%, which is the maximum level recommended in margarines sold in Canada (17). Oils that have been hydrogenated to a greater extent may contain this di-*trans* as the major 18:2 isomer (23).

An examination of the structures of the 18:2 isomer characterized in the present study reveals that the Δ9 double bond position prevails in almost all 18:2 isomers. Furthermore, the Δ9 double bond is in the *cis* configuration in the majority of the isomers. This may indicate that the Δ9 double bond is the least reactive center of unsaturation during hydrogenation. In case the Δ9 bond is attacked, it results only in the geometric isomerization of the *cis* to the *trans* configuration. There is no migration of the double bond position. The *c*9,*t*13-18:2 isomer is most likely formed from linoleic acid involving the migration and isomerization of the *c*12 double bond. This implies that the Δ12 double bond is more reactive than the Δ9 double bond in linoleic acid. However, in α-linolenic acid the Δ12 bond is the least reactive (36), because it is more sterically crowded than the Δ9 and Δ15 double bonds. The hydrogenation of any position in a fatty acid will undoubtedly be modified by the steric environment, and this may account for the differences in the reactivity of the Δ12 double bond in linoleic and linolenic acids. In linoleic acid, the absence of a Δ15 double bond probably makes

the Δ12 bond more exposed than the Δ9 bond. The proximity of the Δ9 bond to the carboxylic group and the glycerol backbone, may protect sterically the Δ9 bond from attack by hydrogen.

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