Estimation of Conjugated Octadecatrienes in Edible Fats and Oils

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Interest in conjugated-diene fatty acids in foods has recently been increased by discovery of their antioxidant and anticarcinogenic properties. Conjugated octadecatrienes (COTs), members of another group of fatty acids, are also present in foods. COTs are formed during the processing of vegetable oils as the result of the dehydration of secondary oxidation products of linoleic acid. Little information is available concerning the occurrence and nutritional properties of COTs in edible oils. Levels of COTs, determined in 27 vegetable oils by ultraviolet (UV) spectroscopy, ranged from not detected (<0.001) to 0.2%. Determination of COTs by gas chromatography of the methyl esters, obtained by transesterification at room temperature with sodium methoxide/methanol, gave lower levels (not detected, 0.051%) than did determination by UV spectroscopy. Methylation with boron trifluoride produced COTs from naturally occurring moieties in the oils and, therefore, is not recommended.

KEY WORDS: Boron trifluoride, conjugated linoleic acid, conjugated trienes, methylation.

Certain isomers of conjugated octadecadienoic acid have been reported to have antioxidant and anticarcinogenic properties (1–4). These isomers, which are sometimes referred to as conjugated linoleic acid (CLA), also have been identified as food constituents (3,5). The research described here was concerned with determining whether other fatty acids similar to CLA in structure are also present in edible fats and oils. We found conjugated octadecatrienes (COTs) in most of the processed vegetable oils that we examined. Estimating the levels of COTs in the oils is a necessary step in determining the significance of the presence of these compounds in the oils.

Although a variety of conjugated fatty acid components of lipids have been found in processed vegetable oils, this work estimated the presence of fully conjugated trienes only, *e.g.*, 9,11,13-octadecatrienoate. COTs are considered unusual fatty acid components that are generally found above trace levels only in rare oilseeds (6). These compounds have physiological effects (*e.g.*, interference with prostaglandalin synthesis) at low levels (7), and above the trace level they are not generally considered acceptable components of food oils (8). It has been known for some time that these compounds are present in edible oils (9), but the levels have not been established. The current interest in CLA prompted us to estimate the levels of COTs typically present in edible fats and oils.

During our investigation of fats and oils, we found compounds that were converted to conjugated fatty acid esters when subjected to heat in the presence of acid, *e.g.*, boron trifluoride. A general scheme, beginning with linoleate and ending with COT, was established from published data (10,11) and is shown in Figure 1. The autoxidation of linoleic acid to hydroperoxide is well established. In addition to the hydroperoxide shown, 9-hydroperoxy-10-*trans*-12-*cis*-octadecadienoate is also produced during oxidation. Peroxides can be converted to hydroxides by several mechanisms, *e.g.*, reduction by metals, and hydroxy fatty acids may also occur naturally in vegetable oils. When hydroxides are exposed to acid, they can be converted to conjugated acids. To avoid this class of reactions, we used a transmethylation procedure with sodium methoxide/methanol to produce methyl esters for gas chromatographic (GC) analyses.

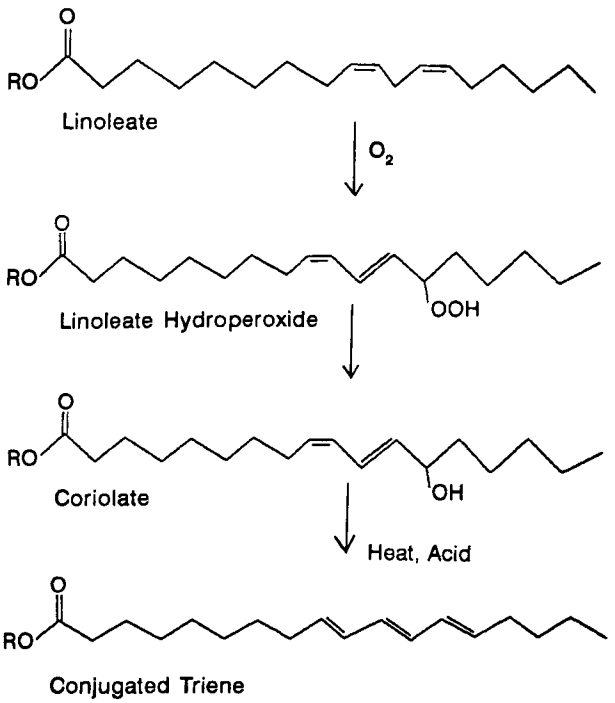

FIG. 1. A possible scheme for formation of conjugated trienes in oils during processing or analysis if acid-catalyzed methylation is used.

Results obtained by ultraviolet (UV) spectroscopy of diluted fats and oils in hexane were compared with the GC results. Results are given for the analysis of 27 vegetable oils obtained from local grocery stores around Washington D.C. and from a commercial food laboratory (Libra Labs. Inc., Metuchen, NJ).

MATERIALS AND METHODS

Materials. Representative vegetable oils were purchased from local grocery stores around Washington, D.C. Tung oil was purchased from a local hardware store. Oat bran oil was obtained by extraction of oat bran with hexane. Bacon fat was collected from the pan after frying. Other oils, used in a variety of frying applications, were obtained from a commercial testing laboratory (Libra Labs). Beef fat was extracted from hamburger (12). Coriolic (13-hydroxy-9-*cis*-11-*trans*-octadecadienoic) acid was synthesized by $NaBH_4$ reduction of the peroxide formed from the reaction of linoleic acid and lipoxidase (13). Hexane was high-performance liquid chromatography (HPLC) grade; all other solvents were redistilled from glass. α-Eleostearic (9-*cis*,11-*trans*,13-*trans*-COT) acid, 13 g, m.p.

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48°C, was recrystallized from commercial tung oil (14). β-Eleostearic (*all-trans* 9,11,13-COT) acid was synthesized from 3 g of the alpha isomer by adding a trace of I_2 to a solution in hexane as previously described (15); the reaction product was recrystallized from 90% EtOH to obtain pure product, m.p. 72°C. Diazomethane was prepared from MNNG (1-methyl-3-nitro-1-nitrosonguanidine) according to instructions obtained from a commercial kit (Aldrich Chemical Company, Milwaukee, WI). The diazomethane was used to methylate coriolic acid, and α- and β-eleostearic acids derived from tung oil. All other reagents in the procedures cited were used as specified.

Instrumentation. GC was performed by using a Hewlett-Packard (Palo Alto, CA) 5890A instrument under the following conditions: CP-Sil-88 capillary column, 50 m × 0.25 mm i.d.; helium carrier gas; flame-ionization detector (FID); injector 220°C; detector 280°C; column 75°C for 2 min, then 20°C/min to 185°C and hold for 33 min, then 4°C/min to 225°C. Analyses were run in both split and splitless modes.

Low-resolution electron ionization (EI) GC/mass spectrometry (GC/MS) analyses were obtained with a Hewlett-Packard 5890 series II gas chromatograph, coupled to a Fissons VG (Wytheshawe, United Kingdom) Autospec Q mass spectrometer and OPUS 2000 data system. The GC/MS system used version 1.6C software.

The same capillary GC column was used to obtain FID and GC/MS data. Adjusting the capillary GC column head pressure to 10 psi gave GC data that were comparable to the GC FID data. The GC/MS conditions were splitless injection, with helium sweep restored 1 min after injection; injector and transfer lines 250°C; oven 75°C for 2 min after injection, then 20°C/min to 185°C and hold for 15 min, 4°C/min to 225°C and hold for 5 min.

The mass spectrometer was tuned to a resolution of 1000 (5% valley) by observing *m/z* 305 in the EI mass spectrum of perfluorokersene (PFK). The mass scale was calibrated with PFK for magnet scans from 440 to 44 daltons at 1 s per decade. Filament emission was 200 μA at 70 eV. Ion source temperature was 250°C.

A Mattson Instruments (Madison, WI) Model Sirius 100 Fourier transform-infrared (FTIR) spectrometer, equipped with a matrix isolation (MI) Cryolect interface operated at 12K under vacuum, was used with GC to obtain IR spectra. This system, which was used with a CP-Sil-88 capillary column, has been described in detail (16). UV spectra in hexane were obtained by using a Beckman DU-7 spectrophotometer (Beckman Instruments, Fullerton, CA).

Procedures. In the first methylation procedure, approximately 25 mg oil was heated with 1.5 mL 0.5 N NaOH/MeOH to 100°C for 7 min, followed by heating with 2 mL (14%) boron trifluoride/MeOH at 100°C for 5 min. This procedure, used for marine oils, has been described in detail (17). Tricosanoic ($C_{23:0}$) fatty acid methyl ester (FAME) was used as an internal standard. This method is referred to as the BF_3 procedure.

The second procedure is as follows: 300 mg fat or oil was added to a 50-mL centrifuge tube containing 0.1 mg $C_{23:0}$ FAME; then 10 mL 0.5 N NaOMe/MeOH was added, and the tube was vortexed several times, as needed, to ensure that the entire test portion was dissolved; the tube was purged with argon, sealed and left at room temperature in the dark overnight; then 10 mL hexane was added to the tube, and the contents were vortexed for 1 min; the hexane layer was removed with a pipette, washed with an equal volume of water, and then dried over sodium sulfate. A 2-μL aliquot was injected into the gas chromatograph by splitless injection. This methylation is referred to as the NaOMe procedure.

Ethyl esters were obtained by adding 1 g oil to 10 mL 0.5 N potassium ethoxide in absolute ethanol; after 8 h, 10 mL isooctane was added. When 10 mL water was added, the solution separated into two phases; the isooctane layer was isolated, water-washed and dried with Na_2SO_4. Oxazoline derivatives were prepared by reacting the appropriate fatty acid with 2-amino-2-methylpropanol at 170°C for 1 h, as previously described (18). UV measurements of COTs in oils and of reference materials were made by dilution in hexane, using the formula $E_{1\%} = 1710$ at 270 nm (8), where $E_{1\%}$ is the absorptivity of a 1% solution. This formula is specific for 9-*cis*,11-*trans*,13-*trans*-COT, but was arbitrarily chosen as a way to estimate the total COTs present.

RESULTS AND DISCUSSION

Figure 2 shows a gas chromatogram obtained from a splitless injection of FAME produced by exposing a test portion of used cottonseed oil to NaOMe/MeOH. Peak C23 is an internal standard of tricosanoic acid methyl ester. COT FAMEs and tetracosanoic acid methyl ester (C24), which was present in low amounts (<<0.5%) in most vegetable oils that we examined, elute in the same region. Figure 3A shows the same chromatogram with a time scale of 36–40.4 min. Figure 3B shows a chromatogram with the same time scale for the reaction product of coriolic acid and boron trifluoride. Figure 3C shows a chromatogram for a co-injection of the components from Figure 3A plus Figure 3B. External standards are not very useful for analyses with the splitless injection technique in the system that we used. The chromatographic pattern of synthetic COTs is similar to that of the cottonseed oil, except for the occurrence of the C24 FAME. Quantitation was made by dividing the total area counts found in the retention region shown in Figure 3A (not including the response at the C24 retention time) by the area counts for the C23 FAME, and then multiplying this ratio by the percent of C23 added to the cottonseed oil.

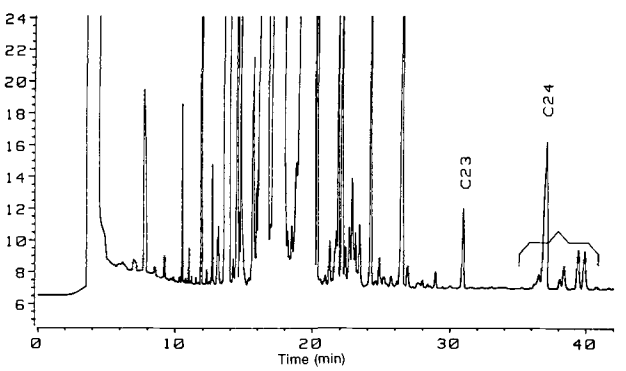

FIG. 2. Flame-ionization detector chromatograms of heated cottonseed oil fatty acid methyl esters (FAMEs) on CP-Sil-88. C23 (tricosanoic FAME) is added internal reference. C24 (tetracosanoic FAME) is low-level constituent of oil.

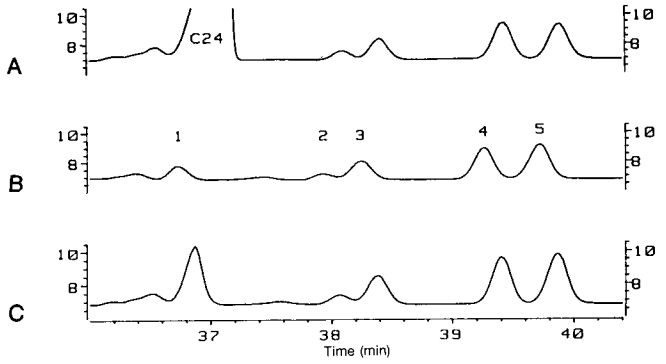

FIG. 3. A, same chromatogram as in Figure 2, but with time scale of 36–40.4 min. B, chromatogram of methyl coriolate, reaction product of BF_3 and coriolic acid (13-hydroxy-9-*cis*-11-*trans*-octadecadienoic acid). C, chromatogram obtained from co-injection of solutions that produced A and B.

The peaks in Figure 3B are numbered 1–5. A mixture of compounds, primarily COT FAMEs, elutes at each of these retention times. A large number of test samples, as well as the synthetic COTs, were examined by GC/MI/FTIR. Methyl and ethyl esters of α-eleostearic acid, 9-*cis*,11-*trans*,13-*trans*-octadecatrienoic acid and β-eleostearic acid, *all-trans* 9,11,13-octadecatrienoic acid, derived from tung oil, were used as reference standards. The interpretation of IR bands for COT FAMEs at given retention times is outlined in Table 1. Figure 4 shows the GC/MI/FTIR spectra obtained at the retention time of peak 4 in Figure 3B. This value corresponds to the retention time of β-eleostearic acid methyl ester. The IR spectrum from cottonseed oil at the retention time of peak 4 (Fig. 3A) is shown in Figure 4B. The bands at wavenumber 3020 are due to =C-H stretching; those at wavenumber 998 are due to =C-H out-of-plane deformation. The IR spectra thus support the identification of peak 4 as a β-eleostearate. Figures 5 and 6 are GC/MI/FTIR spectra of ethyl esters derived from tung and sunflower oils. In Figure 5A, the GC/MI/FTIR spectrum of ethyl α-eleostearate is characterized by out-of-plane deformation bands at wavenumbers 995, 968 and 730. Figure 5B shows the GC/MI/FTIR spectrum at the retention time of ethyl α-eleostearate in a test portion of esterified sunflower oil. The spectra of these ethyl esters would correspond to those of the FAMEs at the retention time of peak 1 in Figure 3; thus, peak 1 in Figure 3 is consistent with identification as an α-eleostearate. In Figure 6, COTs eluting at the retention time of ethyl β-eleostearate are characterized in much the same way as were the FAMEs in Figure 4.

Figure 7A presents the EI mass spectrum from GC/MS

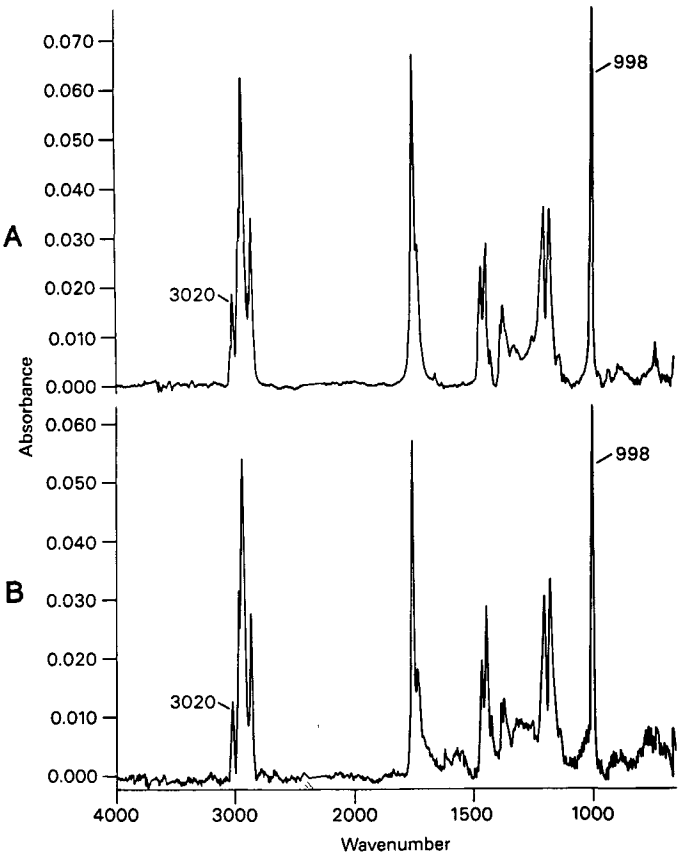

FIG. 4. Gas chromatography/matrix isolation/Fourier transform-infrared spectra of fatty acid methyl esters (FAMEs) at retention time of peak 4 in Figure 3. A, β-eleostearic acid methyl ester derived from tung oil; B, cottonseed oil FAME.

of the oxazoline derivative of a major COT (corresponding to peak 5 in Fig. 3B) resulting from the reaction of BF_3 with coriolic acid; Figure 7B presents the EI mass spectrum from GC/MS of the oxazoline derivative, at the same retention time, obtained from fatty acids of hydrolyzed heated cottonseed oil. The interpretation of the fragmentation is shown at the top of the figure; *viz.*, from the parent ion at *m/z* 331 a loss of 15 daltons occurs, followed by successive losses of 14 daltons until the loss of 12 daltons occurs, going from *m/z* 246 to 234. The spectrum of the reaction product is interpreted as an 8,10,12-COT. Because the GC/FTIR data show only *trans* double bonds, this chemical moiety is completely characterized. The EI mass spectrum obtained at the retention time corresponding to peak 4 (Fig. 3B) is essentially identical to that of the α-eleostearate oxazoline derivative (19); however, the IR spectrum shows only *trans* double bonds, and the compound has the same retention time as β-eleostearate oxazoline. Therefore, peak 4 is identified as a β-eleostearate. The EI GC/MS and GC/MI/FTIR spectra corresponding to peak 1 in Figure 3 (for both the cottonseed oil and the reaction product of coriolic acid and BF_3) show only partial resolution from a complex mixture, but the interpretation is consistent with an α-eleostearate.

GC analysis of FAMEs of oat bran oil, prepared by the BF_3 methylation procedure, indicated the presence of 2.1% COTs. This result was not confirmed by direct UV

TABLE 1

Characteristic Infrared Band Positions (cm^{-1}) for Conjugated Trienes[a]

COT	=C-H stretch	=C-H deformation		
α-*c,t,t*[b]	3026 (3005, sh)	995	968	730 (broad)
Other *c,t,t*	3021 (3005, sh)	997	969	729 (broad)
β-*t,t,t*	3020 3002	998	—	

[a]Four cm^{-1} resolution.
[b]*c, cis; t, trans.*

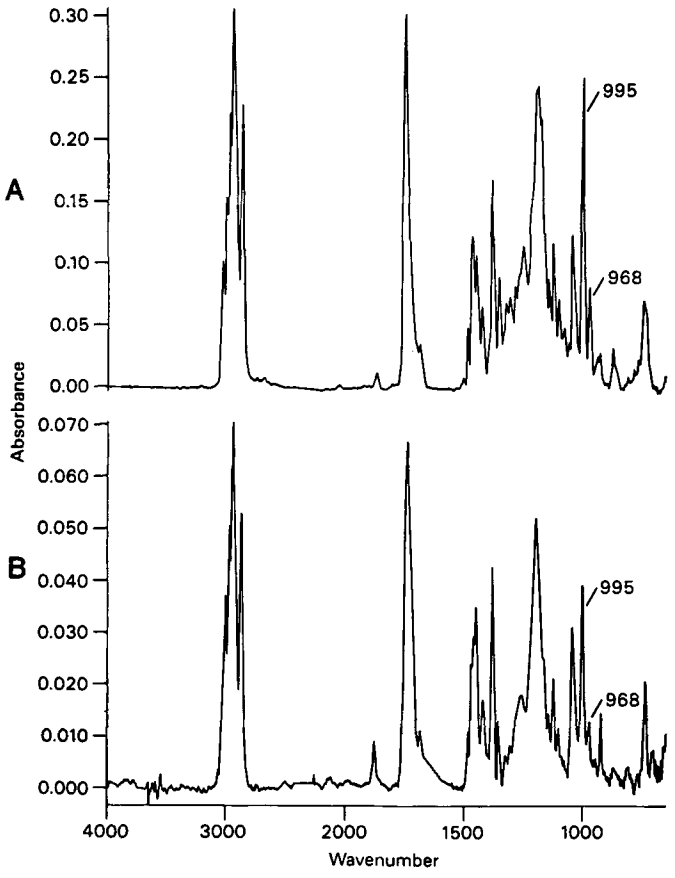

FIG. 5. Gas chromatography/matrix isolation/Fourier transform-infrared spectra of ethyl esters corresponding to the retention time of peak 1 in Figure 3. A, α-eleostearic acid ethyl ester derived from tung oil; B, sunflower oil ethyl ester.

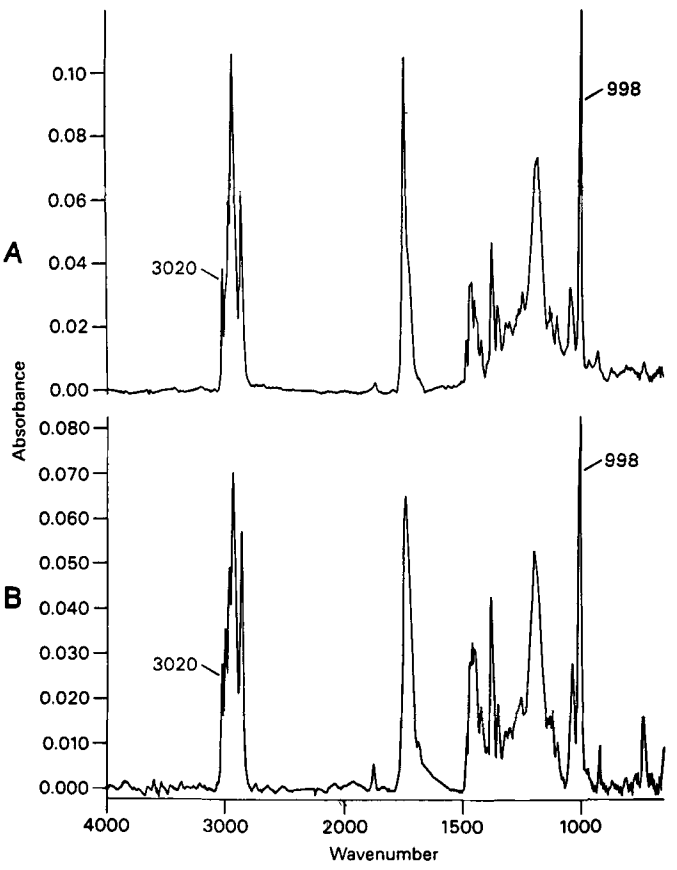

FIG. 6. Gas chromatography/matrix isolation/Fourier transform infrared spectra of ethyl esters corresponding to the retention time of peak 4 in Figure 3. A, β-eleostearic acid ethyl ester derived from tung oil; B, sunflower oil ethyl ester.

analysis of the unmethylated oil. The NaOMe methylation procedure showed no presence of COTs; thus, we concluded that the COTs are produced by the BF_3 procedure. In addition to changing our methylation procedure to NaOMe, we also discovered a likely (10,11) precursor of the COT, coriolate. The chromatogram in Figure 8A demonstrates that the BF_3 procedure does convert methyl coriolate to COT, and Figure 8B shows that NaOMe methylation does not create COT artifacts.

Figure 9 shows the UV pattern exhibited by COT isomers produced by reaction of BF_3 with coriolic FAME and the oat bran oil after treatment with BF_3. Figure 10 shows that COTs are not produced by NaOMe methylation, and provides no evidence of COTs in the unmethylated oat bran oil.

To supplement the quantitation of COTs by GC of methyl esters, UV measurements of the oils and fats diluted with hexane were obtained. A tangent skim measure of the absorbance of the three maxima characteristic of COTs was compared with the reported value of $E_{1\%} = 1710$ for α-eleostearic acid (8). In Figure 11, an example of quantitative measurement is shown for rice bran oil. This pattern of COT absorbance was typical for many unused vegetable oils. Figure 12 shows that the COT pattern is difficult to measure in heated oils.

For recovery studies, oat bran oil or bacon fat was spiked with tung oil, and the resulting UV absorption and GC response for α-eleostearate were measured. Recoveries from bacon fat, as determined by GC quantitation of the methyl ester for α-eleostearate, were [% spike (% recovered)] 0.008 (111); 0.04 (95); 0.08 (79) and 0.8 (92). Recoveries at the lower spiking levels tended to be higher because of the inclusion of area counts of non-COT compounds in the COT retention time range. The procedure gave acceptable results over the spiking range. UV recoveries from oat bran oil were essentially 100%, as would be expected, because in the procedure the reference tung oil was merely diluted with an oil that has no chromophore at the wavelength of interest.

Presentation of the findings is provided in three parts. In general, low levels of COTs were found in all the processed oils. Table 2 shows results for unheated products. Levels of FAMEs varied from not detected (*ca.* < 0.002%, UV) for oat bran oil and beef fat that did not undergo any heat processing, to 0.035% for corn oil. The UV results tend to be higher than those determined by GC. Some of the reasons for this include (i) the calculation for UV spectroscopy is based on a specific α-eleostearate isomer, which is a minor component of the mix of COTs present; (ii) the conjugated triene moiety present as free fatty acid or polymer is not converted to FAMEs by the sodium methoxide methylation procedure; and (iii) in quantitation of COTs, the FAMEs eluting near tetraecosanoic acid are not included in the GC calculation. Table 3 gives

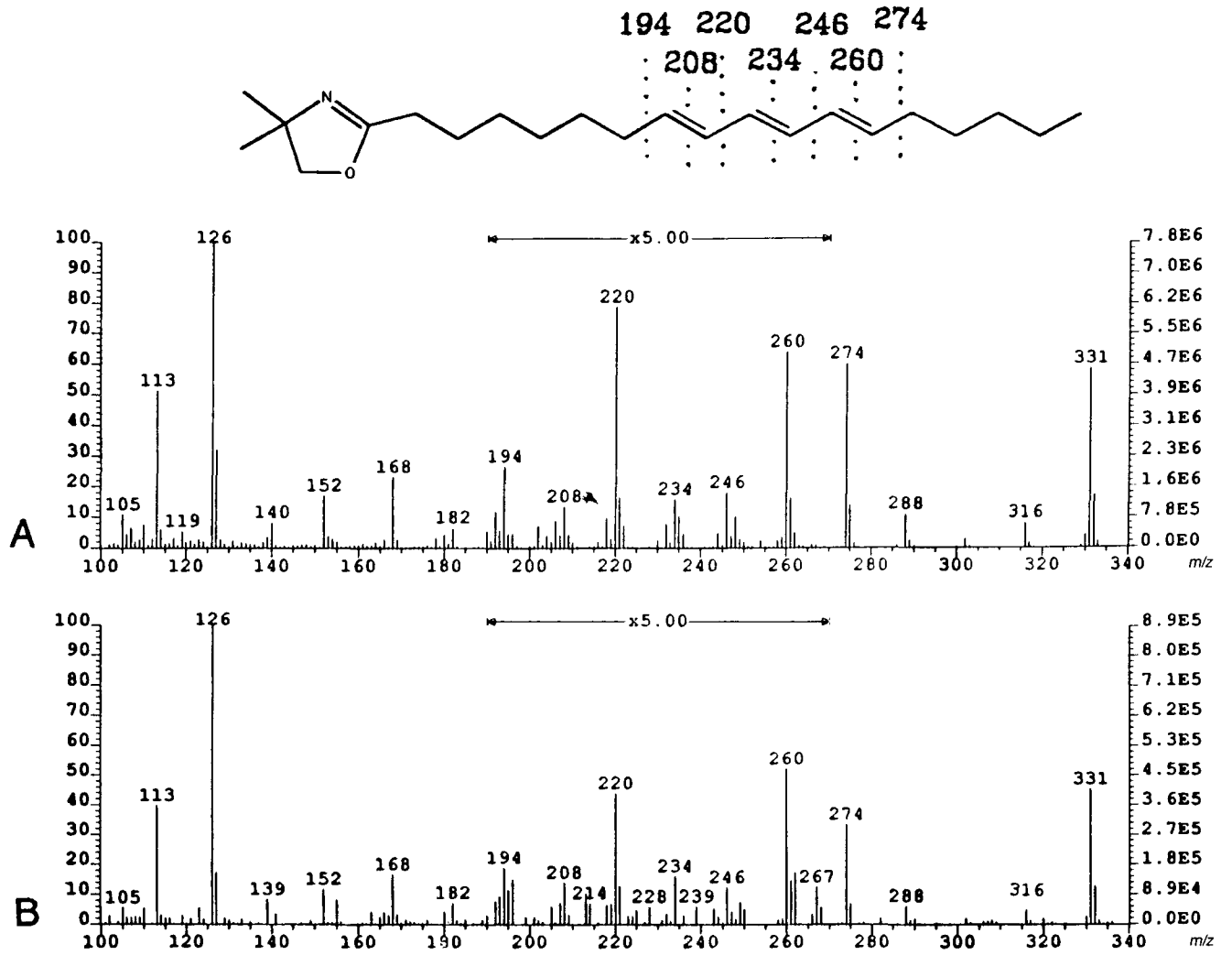

FIG. 7. Mass spectra from gas chromatography/mass spectrometry of oxazoline derivatives of compounds eluting at retention times corresponding to peak 5 in Figure 3. A, reaction product of coriolic acid with BF_3; B, cottonseed oil.

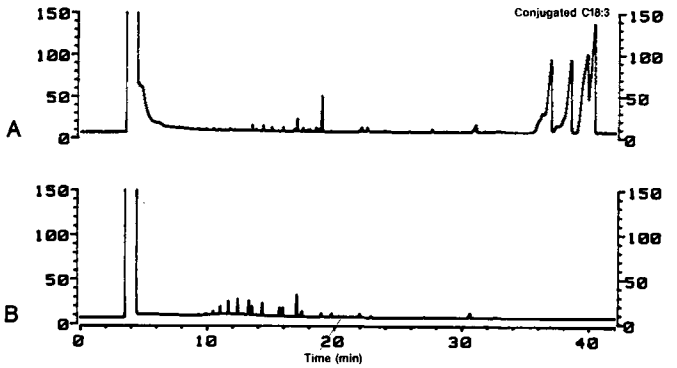

FIG. 8. Chromatograms of methyl coriolate treated by A, BF_3 procedure; B, NaOMe procedure.

TABLE 2

Conjugated Trienes in Unheated Fats and Oils[a,b]

Fat or oil	Level (%)	
	Gas chromatography	Ultraviolet
Oat bran	n.d.[c]	n.d.
Beef	n.d.	n.d.
Corn	0.035	0.23
Rice bran	0.024	—[d]
Soybean[e]	0.026	0.23
	0.012	0.19
Soybean[f]	0.009	0.017

[a]Quantitation of fatty acid methyl esters after methylation with sodium methoxide.
[b]Quantitation of 268 nm maxima determined by tangent skim.
[c]Not detected.
[d]Not determined.
[e]Partially hydrogenated.
[f]Heavily hydrogenated.

the results for drippings of cooked bacon fat and an arbitrary selection of oils used in specific product preparations. The highest FAME findings among the vegetable oils (0.043, 0.051%) were found for cottonseed oils used to cook fish; the lowest findings (0.005–0.01%) were for heavily hydrogenated soybean oils. Table 4 shows results of findings for a process used to cook onion rings. In this process, fresh oil was added to make up for volume losses

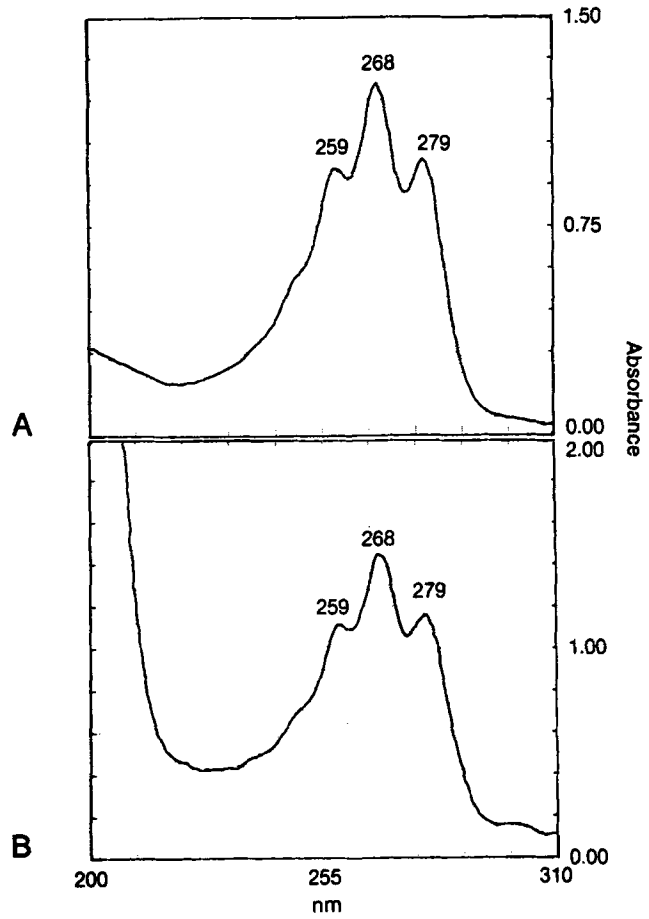

FIG. 9. Ultraviolet spectra of A, methyl coriolate; and B, oat bran oil; both were methylated by BF_3 procedure.

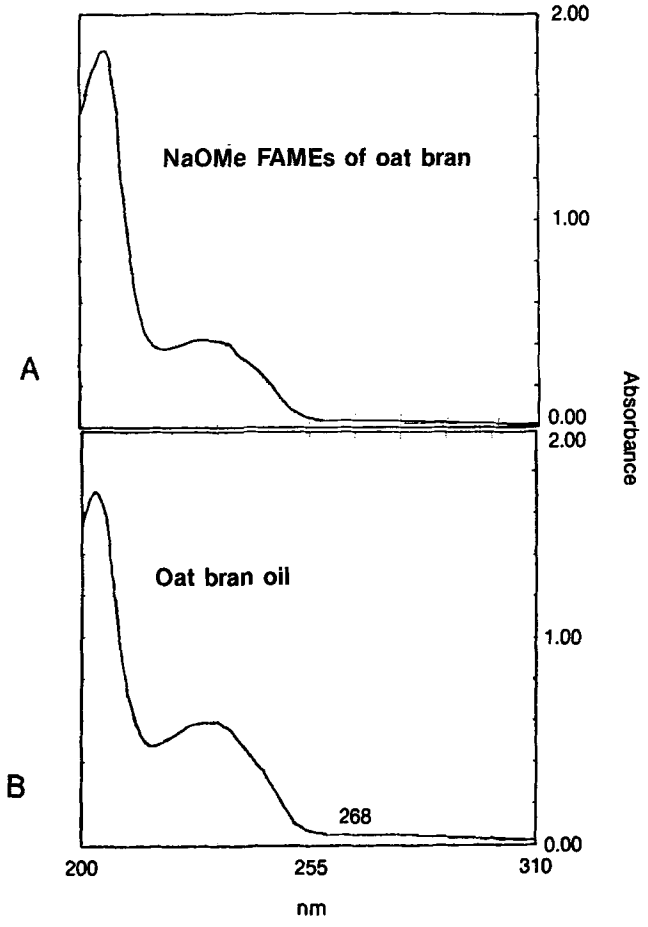

FIG. 10. Ultraviolet spectra of oat bran oil treated with NaOMe/MeOH; and B, oat bran oil. A, fatty acid methyl esters.

TABLE 3

Conjugated Trienes in Used Cooking Fats and Oils[a,b]

Fat or oil	Level (%)	
	Gas chromatography	Ultraviolet
Bacon	n.d.[c]	n.d.
Cottonseed	0.051	0.11
	0.043	0.15
Safflower	0.019	0.078
	0.008	0.059
Soybean[d]	0.028	0.053
	0.035	0.091
	0.040	—[e]
	0.051	0.027
	0.023	0.067
Soybean[f]	0.005	0.013
	0.010	0.020
	0.008	0.050

[a]Quantitation of fatty acid methyl esters after methylation with sodium methoxide.
[b]Quantitation of 268 nm maxima determined by tangent skim.
[c]Not detected.
[d]Partially hydrogenated.
[e]Not determined.
[f]Heavily hydrogenated.

from cooking. Although no wide differences in levels are apparent, it is possible that COTs are being produced and/or degraded in the cooking process.

In the process of isolating the precursor to COTs for use in this study, we speculated that the same type of precursor may exist in CLA analyses. We confirmed this by exposing a test sample of methyl oleate to air for several

TABLE 4

Conjugated Trienes in Soybean Oil Under Different Cooking Conditions

Description	Level (%)	
	Gas chromatography	Ultraviolet
1, Fresh oil	0.018	0.013
2, Fresh oil, low heat	0.011	0.009
3, #2 brought to 180°C	0.012	0.027
4, After 30 min of production	0.016	0.016
5, After 60 min	0.017	0.018
6, After 120 min	0.019	0.017
7, After 240 min	0.016	0.013
8, After 480 min	0.017	0.014
9, Maximum use oil	0.019	0.016

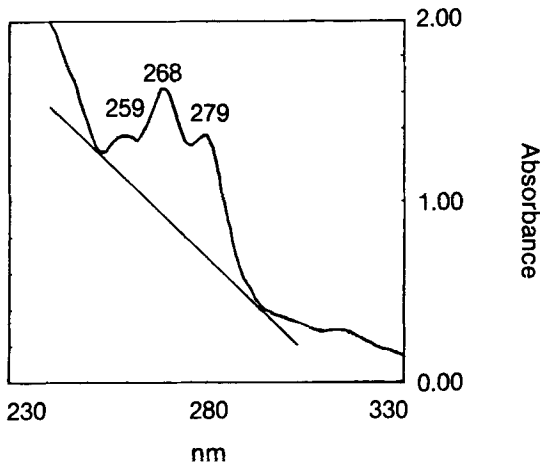

FIG. 11. Ultraviolet spectra of rice bran oil diluted with hexane; numbered wavelengths are maxima for conjugated octadecatrienes.

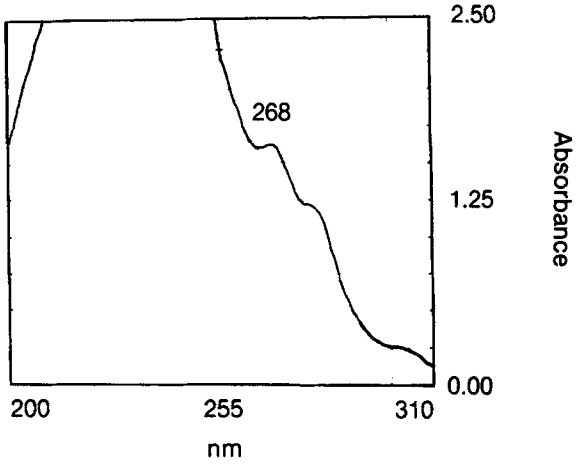

FIG. 12. Typical ultraviolet spectra obtained from heated oil diluted with hexane.

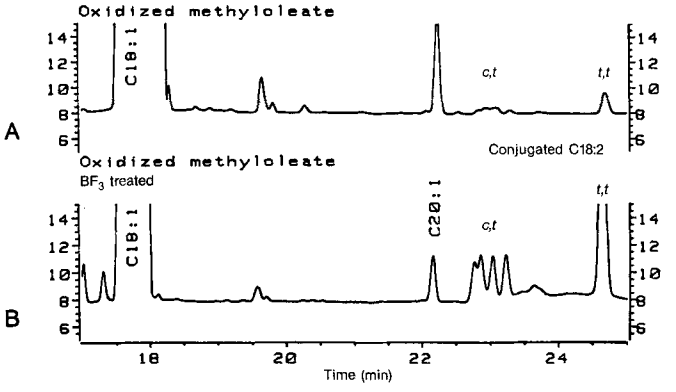

FIG. 13. Flame-ionization detector chromatograms of oxidized methyl oleate (A) and oxidized methyl oleate treated by the BF_3 procedure (B).

months. Figure 13 shows chromatograms of oxidized methyl oleate before and after treatment of the test sample with BF_3; a greater than tenfold increase in CLA was found as a result of the BF_3 procedure.

Some COT isomers have biological activity at very low levels (7). In the data presented here, levels as high as 0.051% have been found. At this time we do not know what level of conjugated triene would constitute health concerns. We also do not know whether conditions exist under which COTs may have anticarcinogenic activity, as has been shown for conjugated dienes (2). The reduced peroxides of oleic and linoleic acid moieties convert to conjugated dienes and trienes, respectively, when samples are methylated with procedures that employ heat and acid.

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