Conversion of Allylic Hydroxy Oleate to Conjugated Linoleic Acid and Methoxy Oleate by Acid-Catalyzed Methylation Procedures

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Conjugated linoleic acid (CLA), a term describing a group of conjugated octadecadienoic acids that are both naturally occurring and formed during food processing, is the subject of considerable current research because of the recently reported antioxidant and anticarcinogenic properties of these compounds. Allylic hydroxy oleates (AHOs), secondary products of lipid autoxidation, have also been found in foods. By means of high-performance liquid chromatography with ultraviolet detection, gas chromatography/mass spectrometry and gas chromatography/matrix isolation/Fourier transform infrared spectroscopy, we determined that currently used acid-catalyzed methylation procedures convert AHOs to CLA and other products that potentially yield high values in determination of CLA in foods. A mixture of AHOs, containing mainly (8- and 11-)hydroxy-9-octadecadecenoates, was synthesized and tested by methylation procedures with the following catalysts: BF_3, HCl, NaOMe and tetramethylguanidine. Both the BF_3 and the HCl procedures converted AHOs to CLA. The base-catalyzed procedures did not convert AHOs to CLA.

KEY WORDS: Allylic hydroxy oleates, boron trifluoride, conjugated linoleic acid, methylation, tetramethylguanidine.

Conjugated linoleic acid (CLA) is a term used to describe a group of octadecadienoic acids that contain two conjugated double bonds. The determination of CLA as a food component (1,2) has been a subject of growing interest because of the recently reported antioxidant (3) and anticarcinogenic (3-6) properties of these compounds.

Allylic hydroxy oleate (AHO) is a secondary oxidation product of oleic acid, and it occurs naturally in foods, as do other hydroxy fatty acids (7,8). The presence of AHO in food products can create problems in CLA analysis because AHO undergoes reactions during exposure to strong acid, forming a number of procedural artifacts, particularly CLA.

Although we found no specific publications in the literature that report conversion of AHO to CLA, this result is not unexpected as similar chemical reactions have been reported (9-11). It is important to spell out the details of this reaction because many recent publications (1,12,13) that have reported CLA findings have used acid-catalyzed methylation procedures without stating whether AHO was present in the products being analyzed.

With regard to methoxy fatty acid methyl esters (FAMEs), specific reports (14-16) show that these compounds are artifacts formed during BF_3-catalyzed methylation procedures; however, these results involve high temperatures, long reaction times and questions about O_2 exposure, and are probably due to an undescribed mechanism that allows addition of methanol to double bonds. Another means by which methoxy compounds are formed during methylation procedures is conversion of hydroxy fatty acids to methoxides. Although nonallylic hydroxy fatty acids, e.g., ricinoleate, are not altered by BF_3 to form methoxy compounds (17), allylic hydroxy fatty acids easily form methoxides when HCl/methanol is used (18).

Data presented here describe high conversions of AHO to CLA and methoxy oleate by two different acid-catalyzed methylation procedures, and no similar conversions by two different base-catalyzed procedures.

MATERIALS AND METHODS

Materials. BF_3 methanol, 14%, 3 N methanolic HCl and 2,2-dimethoxypropane were obtained from Supelco, Inc. (Bellefonte, PA); methyl oleate, sodium borohydride, 2-amino-2-methyl-1-propanol, 25% sodium methoxide (NaOMe)/methanol and 1,1,3,3-tetramethylguanidine (TMG) were obtained from Aldrich Chemical Co. Inc. (Milwaukee, WI). CLA, 9,11-octadecadienoic acid and other fatty acid and methyl esters were obtained from Nu-Chek-Prep, Inc. (Elysian, MN). BSTFA (38833), a special formulation of N,O-*bis*(trimethylsilyl)trifluoroacetamide, catalyzed with 1% trimethylchlorosilane, was obtained from Pierce Chemical Co. (Rockford, IL). Organic solvents were redistilled from glass quality.

AHO synthesis and concentration. Hydroperoxides of methyl oleate were prepared (19) by exposing 7.17 g methyl oleate to 100 mL air/min and to 366 nm light for 87 h. The peroxidized methyl oleate was reduced to its hydroxy derivatives (AHO) by reaction with 200 mg $NaBH_4$ in 100 mL methanol for 45 min. After reduction the mixture was acidified to pH 2 with 0.5 M citric acid, diluted with 600 mL water and extracted with 100 mL petroleum ether. The petroleum ether was dried with Na_2SO_4 and reduced to a volume of 30 mL with a stream of argon. A large portion of the unreacted oleate was removed as follows: Four 1-min extractions of the product solution with 30 mL 80% methanol/20% water were combined in a 1-L separatory funnel containing 600 mL water and 100 mL petroleum ether; the funnel was shaken for 1 min, and the petroleum ether was dried over Na_2SO_4. Aliquots of this solution were taken to dryness and used as needed. After most of the methyl oleate was removed, analysis by gas chromatography (GC) and by GC/mass spectrometry (GS/MS) of silylated products showed that the product contained 80% AHO, primarily 8- and 11-hydroxy isomers. AHO was silylated by heating with BSTFA for 5 min in a sealed vial at 100°C. Injections were made directly from the reaction solution.

In the methylation procedures described here, tricosanoic acid methyl ester ($C_{23:0}$) was used as an internal standard, and 0.1 or 1.0 mg was added to each test portion before methylation. The final solvents were dried with Na_2SO_4. Hexane was used for ultraviolet (UV) analyses, and isooctane was used for GC analyses. However, only the use of isooctane in the final solutions in the procedures is described.

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BF_3. The procedure for marine oils was used (20). Briefly, the oil (25 mg) was treated with 0.5 N NaOH/methanol for 7 min, followed by BF_3/methanol for 5 min, both at 100°C. BF_3 was neutralized with saturated NaCl/water, and FAMEs were extracted into isooctane.

Methanolic HCl. The procedure was based on that recommended by Supelco. A mixture of 7 mL ethyl ether, 0.5 mL 2,2-dimethoxypropane and 2.5 mL 3 N methanolic HCl was added to ≤100 mg oil in a 15-mL test tube with a Teflon liner. The tube was purged with argon and placed in the dark overnight at room temperature. To extract the FAMEs, the reaction mixture was added to 100 mL water and 10 mL hexane and shaken for 1 min in a 250-mL separatory funnel; the aqueous layer was removed, and the hexane was washed with water and dried over anhydrous Na_2SO_4. The solvent was evaporated under argon, and the residue was redissolved in isooctane.

TMG. The methylation was based on a published procedure (21). Briefly, the oil (50–100 mg) was added to a reaction tube containing 2 mL 20% TMG/methanol, and the tube was sealed and heated to 100°C for 2 min. The reaction product was transferred to a tube containing 20 mL saturated NaCl/water and vortexed for 1 min; then 5 mL hexane or isooctane was added to the tube, and the tube contents were vortexed for 1 min. Isooctane was dried over Na_2SO_4 before analysis.

Sodium methoxide. Ten mL 0.5 N NaOMe/methanol was added to ≤100 mg oil in a 15-mL test tube with a Teflon liner. The tube contents were vortexed, and the tube was briefly immersed in hot water to dissolve the oil and then purged with argon and placed in the dark overnight at room temperature. The methyl esters were extracted with two 5-mL portions of isooctane.

4,4-Dimethyloxazoline derivatives for GC/MS were prepared in a manner similar to that reported previously (22). Briefly, 5–10-mg portions of FAMEs were placed in a microscale reaction vial, an amount of 2-amino-2-methyl-1-propanol equal to *ca.* five times the weight of the FAMEs was added, and the vial was purged with argon and suspended in a 170°C wax bath for 6 h. The reaction mixture was then transferred by pipette to a 250-mL separatory funnel containing 40 mL petroleum ether and 50 mL water and was shaken; saturated NaCl was added to break the emulsion, and the aqueous layer was removed. The petroleum ether was rewashed with water and then dried over anhydrous Na_2SO_4 and concentrated to an appropriate volume with argon.

Instrumentation. GC was performed with 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 raised 20°C/min to 185°C and held for 33 min, then raised at 4°C/min to 225°C. Analyses were run in both split and splitless modes.

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

The same capillary GC column was used to obtain both 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 10K under vacuum, was used with GC to obtain infrared spectra. This system, which was used with a CP-Sil-88 capillary column, has already been described in detail (12,23).

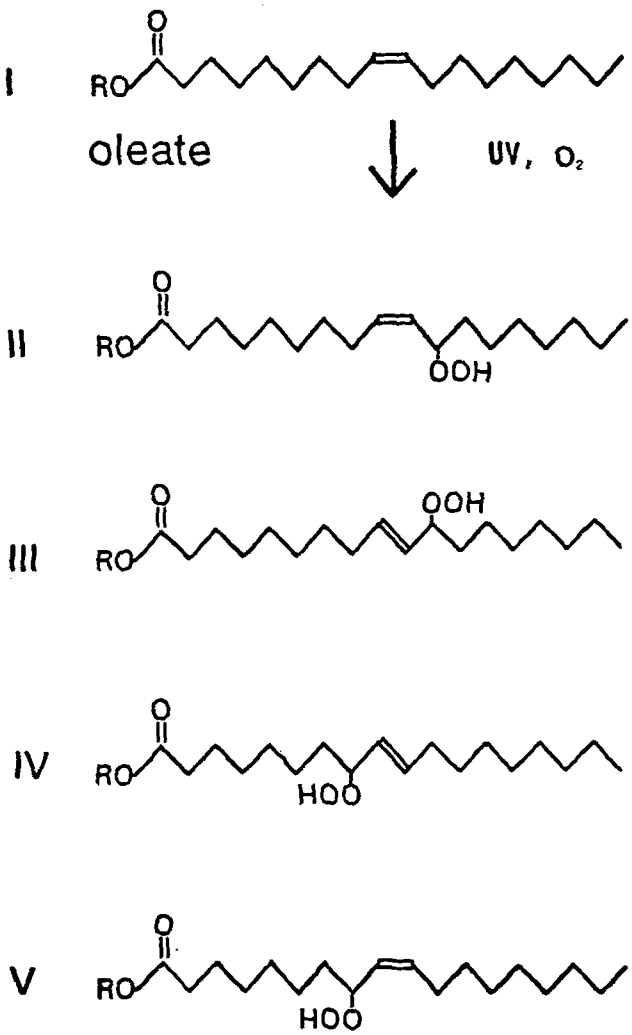
UV spectra in hexane were obtained with a Beckman DU-7 spectrophotometer (Beckman Instruments, Fullerton, CA).

RESULTS AND DISCUSSION

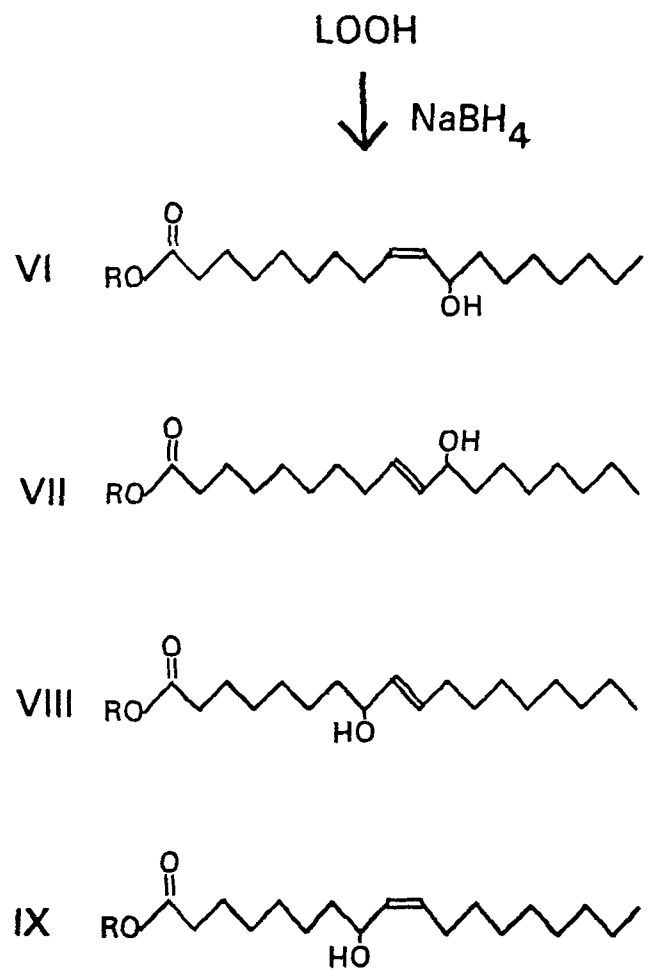
Scheme 1 shows some possible hydroperoxides (LOOHs) produced by the photolysis of methyl oleate (**I**) in the presence of air. Other LOOHs, with the -HC=CH- bond shifting to the 8 or 10 carbon (7,24), were also present to a lesser extent in the synthetic product. In this work a total peroxide yield of 15.8% was determined by iodimetry (25). The hydroperoxides primarily form at the allylic position on either side of the double bond (-HC=CH-) with (**III,IV**) or without (**II,V**) a change in *cis/trans* configuration. Upon reduction of the LOOHs with $NaBH_4$, the four isomers of lipid hydroxides (LOHs) shown in Scheme 2 were expected. The product LOHs were confirmed to be mainly a mixture of 8- and 11-hydroxy-9-octadecenoates (AHOs) by comparing the EI mass spectra of their silylated derivatives with previously published spectra (7). AHOs with double bonds in the 8 and 10 positions were also present, but they are not shown in Scheme 2.

After most of the methyl oleate was removed from the reaction mixture, 1–2 mg of the AHO was methylated by four different procedures. Chromatograms of reaction products obtained for the four methylation procedures, catalyzed by BF_3, NaOMe, HCl and TMG, are shown in Figures 1–4, respectively.

In general, the *cis/trans* configurations were determined by GC/MI/FTIR. The double bond positions were established by EI GC/MS determinations of the 4,4-dimethyloxazoline derivatives. Detailed IR and MS data are presented below. In Figure 1, the components eluting from 36.5 to 38 min were identified as primarily *cis/trans* isomers of 7,9- and 9.11-octadecadienoate (Scheme 3). Other compounds were also present in this retention range, but they have not been further characterized. The response at 40.6 min was primarily a mixture of *trans,trans* 7,9- and 9,11-octadecadienoate. Evidence that the peaks occurring from 41 to 43 min were a mixture of 8- and 11-methoxyoctadecenoates was obtained by interpretation of high-resolution GC/MS fragmentation according to basic principles. No response in the retention time region of 36.5–43 min was due to single components.



SCHEME 1



SCHEME 2

A base-catalyzed methylation was performed to determine whether any conversion of AHOs produced responses at the CLA retention times. The chromatogram in Figure 2, which was obtained by injection of an extract that was methylated with sodium methoxide/methanol, shows no peaks at the retention times of CLA. This procedure, however, will not convert free fatty acids to methyl esters, although it will transmethylate triglycerides to methyl esters (26).

An acid-catalyzed procedure under milder temperature conditions, which would esterify free fatty acids as well, was also tried. This procedure, in which HCl/methanol is used at room temperature with or without 2,2-dimethoxypropane, yielded extracts from which we obtained the chromatogram shown in Figure 3. GC/MS and GC/MI/FTIR examination showed many of the same compounds seen in the BF_3 procedure (Fig. 1), but in different ratios. The absorbance at 234 nm, measured by UV, was 2.4 AU/mg/mL of reacted AHO in the HCl/methanol procedure, as compared to 15 AU/mg/mL of reacted AHO in the BF_3 procedure. We have no explanation for the magnitude of the difference in UV absorbance. Measurements of GC/FID peaks relative to the methyl tricosanoate internal standard indicate that, in the HCl/methanol procedure, 72% of the AHO was converted to compounds eluting in the 36.5–43 min region. The BF_3 procedure yielded 78% for the conversion.

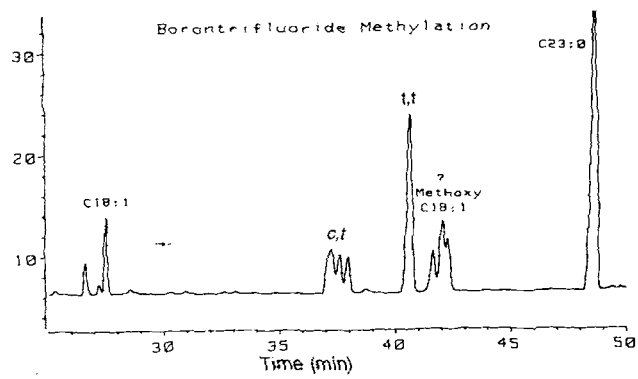


FIG. 1. Partial chromatogram, 25–50 min, showing products of reaction of allylic hydroxy oleate with BF_3/methanol: conjugated linoleic acid *c/t* isomers (36.5–38 min), *t,t* isomers (40.6 min) and methoxy oleates (41–43 min). Residual methyl oleate and methyl triconsanoate (C23:0), which was used as an internal standard, are also present.

A base-catalyzed methylation procedure, which would also esterify free fatty acids, was tried. Figure 4 shows

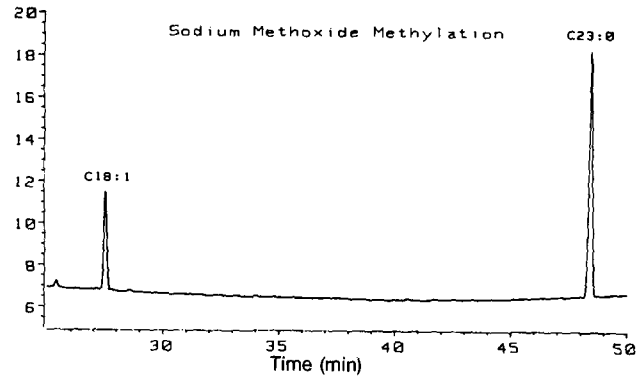


FIG. 2. Partial chromatogram, 25–50 min, showing products of reaction of allylic hydroxy oleate with NaOMe/methanol. Residual methyl oleate and methyl tricosanoate (C23:0), which was used as an internal standard, are also present. Negligible responses are present at the conjugated linoleic acid retention time region, 36.5–40.6 min.

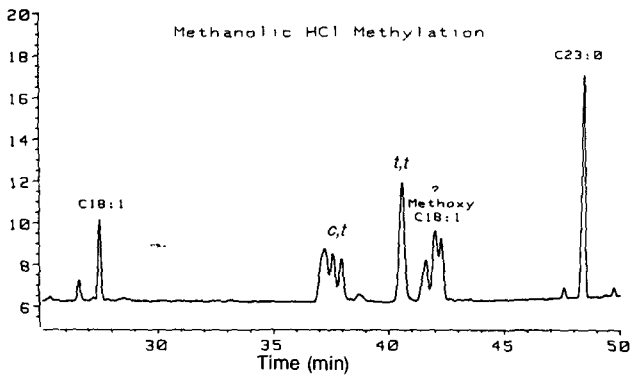


FIG. 3. Partial chromatogram, 25–50 min, showing products of reaction of allylic hydroxy oleate with HCl/methanol: conjugated linoleic acid *c/t* isomers (36.5–38 min), *t,t* isomers (40.6 min) and methoxy oleates (41–43 min). Residual methyl oleate and methyl tricosanoate (C23:0) are also present.

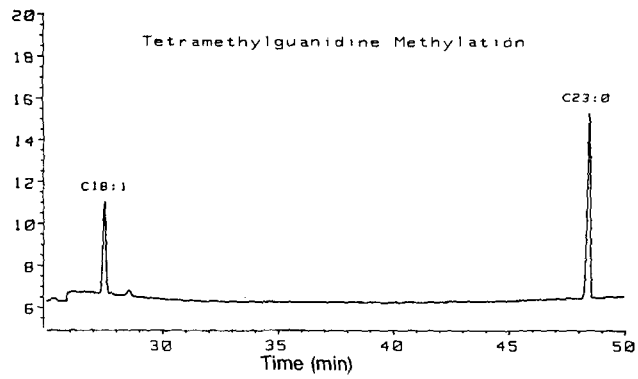
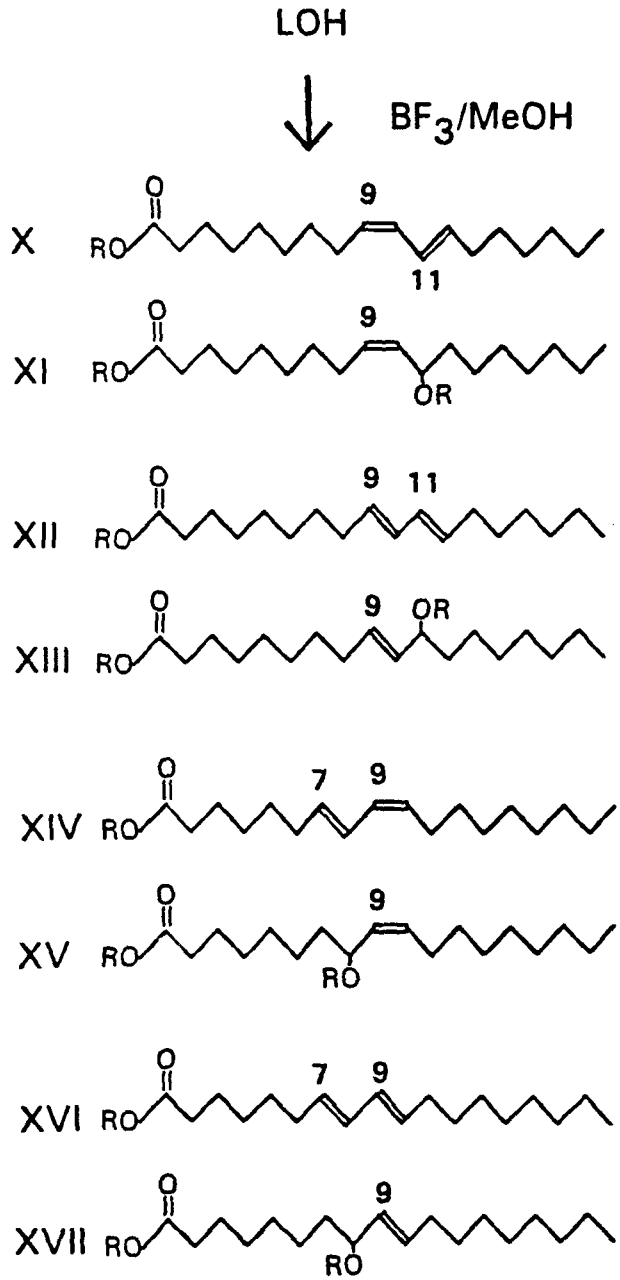


FIG. 4. Partial chromatogram, 25–50 min, showing products of reaction of allylic hydroxy oleate with 1,1,3,3-tetramethylguanidine/methanol. Residual methyl oleate and methyl tricosanoate (C23:0), which was used as an internal standard, are also present. Negligible responses are present at the conjugated linoleic acid retention time region, 36.5–40.6 min.

the resultant chromatogram obtained by injecting an extract of the AHO reaction mixture methylated with TMG. No peaks at appreciable levels were seen in the CLA retention region.



SCHEME 3

Scheme 3 shows structures for some of the compounds produced from the reaction of AHOs with either BF_3/methanol or HCl/methanol. Dehydration (**X, XII, XIV, XVI**) with the formation of a *trans* double bond or methylation of a hydroxy group (**XI, XIII, XV, XVIII**) was determined by interpretation of MS and IR spectra. Identification or quantitation of specific isomers could not be confirmed further because of a lack of pure individual reference compounds in addition to a lack of chromatographic resolution.

For three of four olive oils tested, a GC pattern similar to that seen in Figure 1 was observed after BF_3 methylation, but not after TMG methylation. Figure 5 illustrates the effect of different methylation procedures on the

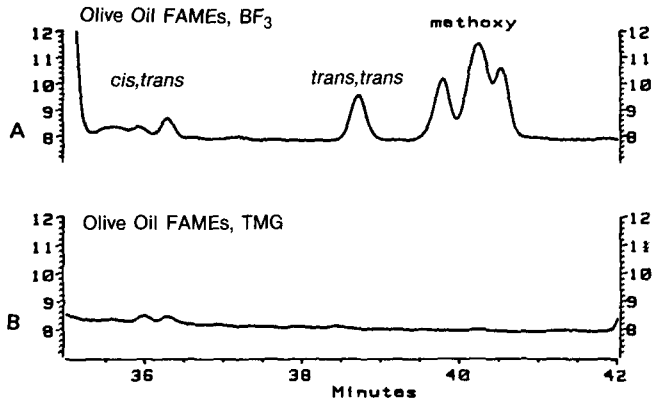


FIG. 5. Partial chromatograms (35–42 min) of the same olive oil methylated with either (A) BF_3 or (B) 1,1,3,3-tetramethylguanidine (TMG). FAMEs, fatty acid methyl esters.

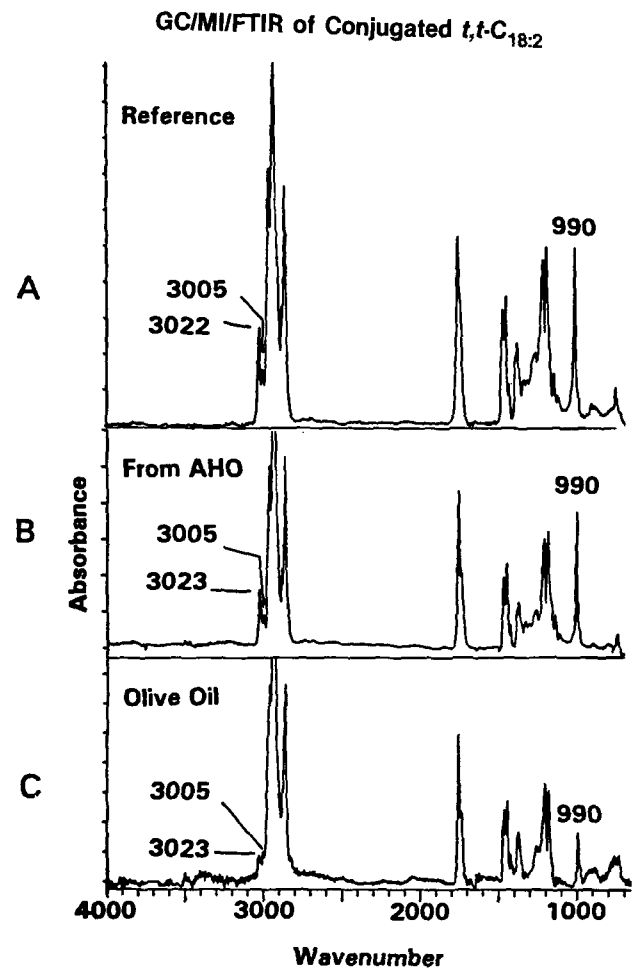


FIG. 6. Gas chromatography/matrix isolation/Fourier transform infrared (GC/MI/FTIR) spectra, resolution 4 cm^{-1}, of t,t-$C_{18:2}$ compounds: (A) from a commercial reference material; (B) products of the reaction of allylic hydroxy oleate (AHO) with BF_3; (C) olive oil fatty acid methyl esters derived from BF_3.

findings of CLA in one of the olive oils. BF_3 methylation produced *trans,trans* CLA (Fig. 5A); based on a spiked internal reference, tricosanoic acid methyl ester, the amount was calculated to be 0.2% of the oil. No *trans,*

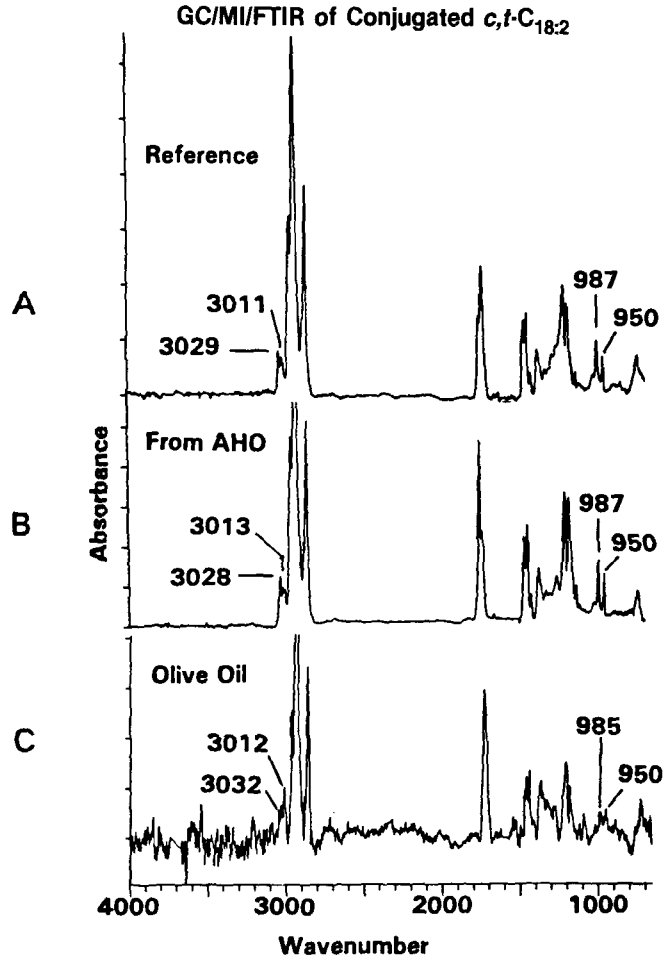


FIG. 7. GC/MI/FTIR spectra, resolution 4 cm^{-1}, of c,t-$C_{18:2}$ compounds: (A) from a commercial reference material; (B) products of the reaction of AHO with BF_3; (C) olive oil FAMEs derived from BF_3. Abbreviations as in Figures 5 and 6.

trans CLA was found (<0.01%) when the same material was methylated by TMG. The *trans,trans* CLA was confirmed by GC/MI/FTIR as shown in Figure 6. The IR spectra at the *trans,trans* retention time are presented for (A) a reference 9,11-CLA obtained commercially, (B) the reaction of AHO with BF_3 and (C) an olive oil. Resolution is 4 cm^{-1} for the spectra shown. Bands at wave-numbers 3005 and 3022 represent =C-H stretch, and the band at 990 represents the =C-H out-of-plane deformation. Confirmation of *cis/trans* double bond isomers is shown in Figure 7 at the same resolution for the same injected portion. Bands near wavenumbers 3011 and 3032 represent =C-H stretch, and those near 985 and 950 represent out-of-plane deformation of =C-H.

An example of interpretation of -HC=CH- location is illustrated in Figure 8, which shows the EI mass spectra of (A) the 4,4-dimethyloxazoline derivative of the 9,11-CLA isomer obtained from the reaction of AHO with BF_3 and, at the same retention time, (B) the 4,4-dimethyloxazoline derivative obtained by TMG methylation from the fat extracted [Ref. 25, sec. 970.52 L(e)] from cooked hamburger. Identical spectra at the same retention time were also seen for the 9,11-CLA reference ma-

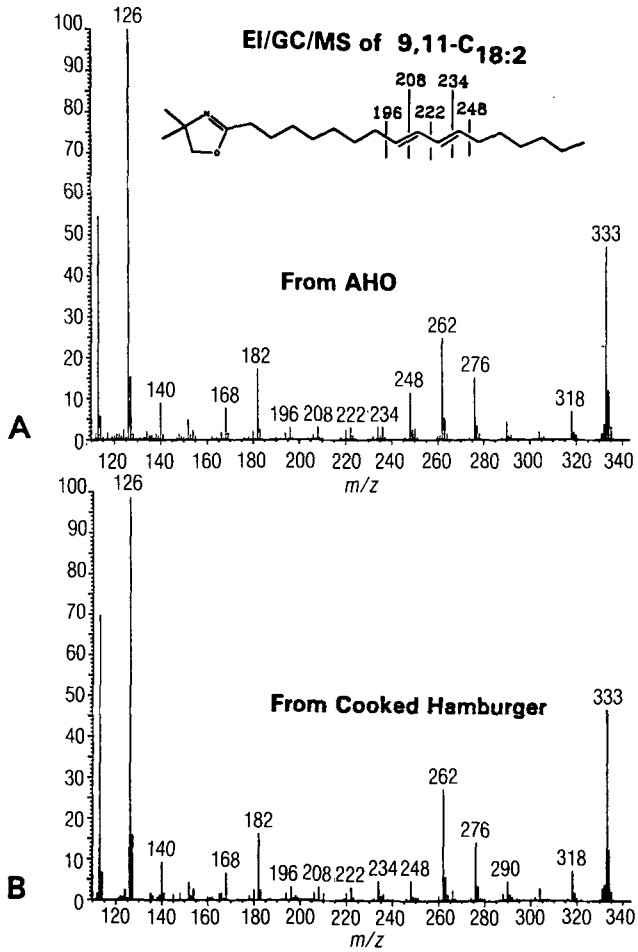


FIG. 8. Mass spectra from electron ionization (EI)/GC/mass spectrometry (MS) of 4,4-dimethyloxazoline derivatives of 9,11-$C_{18:2}$: (A) from reaction of AHO with BF_3; (B) product obtained from TMG methylation of fat from cooked hamburger. Abbreviations as in Figures 5 and 6.

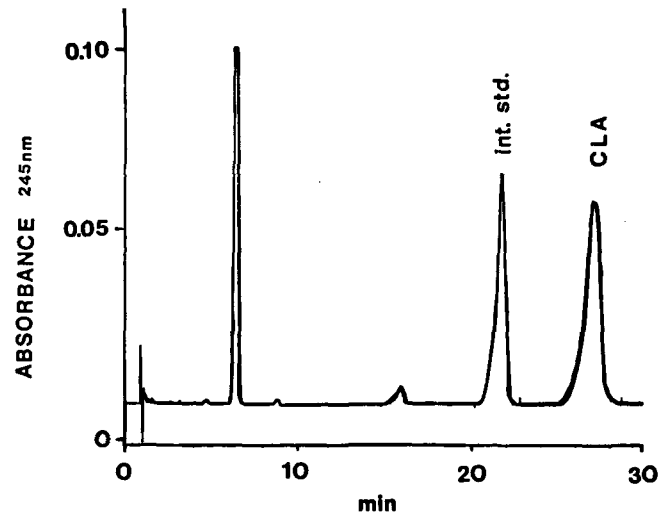


FIG. 9. Tracing of a high-performance liquid chromatogram indicating the retention volume of congugated linoleic acid (CLA). Response: absorbance at 245 nm.

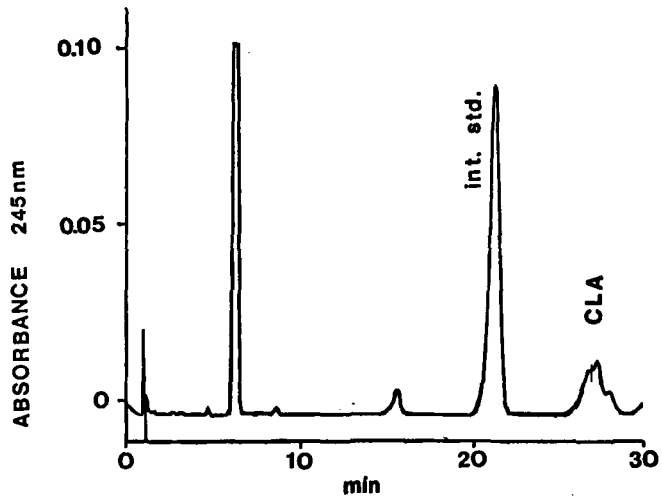


FIG. 10. Tracing of a high-performance liquid chromatogram of the reaction products of allylic hydroxy oleate with HCl/methanol. Response, absorbance at 245 nm, is detected at the retention volume of conjugated linoleic acid (CLA).

terial. After a loss of 15 *m/z* from M^+, successive differences of 14 *m/z* are present until the loss of both double-bond carbons, indicated by a difference of 12 *m/z* as shown at the top of Figure 8.

The conversion of AHO to CLA was also confirmed by a previously reported procedure (1) in which methanolic HCl methylation and high-performance liquid chromatography (HPLC) with UV detection are used. The results are presented in Figures 9 and 10. In this procedure an ODS column eluted with 80% acetonitrile/water is used, with UV detection at 245 nm. Figure 9 shows that CLA elutes at 28.5 min; Figure 10 shows that the reaction product of AHO and HCl/methanol, which yields CLA and other products, will interfere with CLA determination.

Thus, conversion products of AHO with acid-catalyzed methylation have been demonstrated to interfere in CLA determination by GC/MS, GC/MI/FTIR and HPLC/UV. Acid-catalyzed procedures were previously shown to isomerize CLA (1).

The biologically active form of CLA is thought to be the *cis*-9,*trans*-11 isomer (1). This isomer can be produced enzymatically from linoleic acid (1), but it can also be produced as a minor product from the dehydration of AHO-like precursors. The presence of the *cis*-9,*trans*-11 isomer as an artifact of acid-catalyzed methylation is small relative to other CLA isomeric artifacts. In many foods that have been examined, the *cis*-9,*trans*-11 was the major CLA isomer present (1); therefore, its presence in these foods is not in question.

The information presented above should be considered in reviewing previously reported findings of CLA. This information is applicable when acid-catalyzed procedures have been used and the product analyzed has a likelihood of containing AHO-like components, especially those products that have undergone oxidation and contain appreciable amounts of oleic acid. Methylation with TMG has not been sufficiently studied to validate its general use for methylation of small (mg) amounts of oils.

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