Gas Chromatographic Analysis of Diels-Alder Adducts of Geometrical and Positional Isomers of Conjugated Linoleic Acid

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ABSTRACT: This research demonstrates the gas chromatographic analysis of the 4-methyl-1,2,4-triazoline-3,5-dione (MTAD) adducts derived from standards of *cis,trans*-9,11-octadecadienoic acid, *trans,trans*-9,11-octadecadienoic acid, and *cis,cis*-9,11-octadecadienoic acid. Methyl *cis,trans*-9,11-octadecadienoate and methyl *trans,trans*-9,11-octadecadienoate formed Diels-Alder addition products with MTAD to produce adducts with similar mass spectral fragmentation patterns but different retention times determined by gas chromatography/ mass spectrometry. Methyl *cis,cis*-9,11-octadecadienoate reacted slowly and produced two adducts with similar fragmentation patterns and different retention times. These results were comparable to those reported for an analogous series of conjugated hexadienes. Based on hexadiene reactions, methyl *cis,trans*-9,11-octadecadienoate produced a *trans* adduct as a major product while methyl *trans,trans*-9,11-octadecadienoate formed a *cis* adduct. Methyl *cis,cis*-9,11-octadecadienoate reacted slowly under the conditions used leaving mostly unreacted material. Of the adducts observed from this isomer, a major *trans* adduct and a minor *cis* adduct were formed.

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KEY WORDS: Conjugated linoleic acid, Diels-Alder reaction, GC/MS, 4-methyl-1,2,4-triazoline-3,5-dione.

Definitive analysis of the positional isomers of methyl esters of conjugated fatty acids and other conjugated compounds has been reported by mass spectrometry (MS) of Diels-Alder adducts. 4-Methyl-1,2,4-triazoline-3,5-dione (MTAD) and 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) have been shown to be useful adduct-forming agents for analyses of conjugated fatty acid methyl esters and insect pheromones (1–5). The adduct presents a simple mass spectrum that allows determination of the position of conjugation (3–5). Electron-impact fragmentation patterns of MTAD adducts of conjugated fatty acids are typically dominated by cleavage fragments that include the ring formed during adduct synthesis (Fig. 1). Young *et al.* (1) synthesized a series of Diels-Alder adducts of insect pheromones using PTAD derivatives. Although the PTAD derivatives produced simple and informative mass spectra, the derivatives were not sufficiently volatile to accomplish useful gas chromatography (GC). Subsequently, Young *et al.* (2) prepared MTAD derivatives of insect pheromones and studied the characteristics of the products by GC/MS. Although they recognized that the derivatives of *trans,trans*-2,4-hexadiene and *trans,cis*-2,4-hexadiene produce chemically distinct compounds, they reported the retention times and mass spectra for the short-chain MTAD adducts as being indistinguishable. Read *et al.* (3) prepared novel 1,3,4-triazoline-2,5-dione adducts of methyl *cis,trans*-9,11-octadecadienoate and methyl *trans,trans*-9,11-octadecadienoate and measured the retention times of the products by reversed-phase high-performance liquid chromatography. Under the conditions reported, baseline separation of the two adducts was accomplished.

Using a combination of capillary GC and selective ion monitoring of MTAD-derivatized esters, Christie *et al.* (4) demonstrated that commercial samples of conjugated linoleic acid (CLA) contained four positional isomers. Although Christie *et al.* (4) indicated that the esters contained *cis,trans* and *trans, trans* isomers of octadecadienoic acid, they reported that the retention time of the MTAD derivatives was similar in both products. In another study with conjugated trienes, Dobson (5) produced adducts of methyl esters of oil from *Trichosanthes kirilowii* and separated the products using nonpolar capillary GC. Pairs of adducts with similar mass spectra were observed.

Recently, pure standards of three geometrical isomers of 9,11-octadecadienoic acid have become available. We report here the reaction of these isomers with MTAD and examine the adduct by GC/MS.

EXPERIMENTAL PROCEDURES

Source of chemicals. MTAD, anhydrous sodium sulfate, dichloromethane, 1,3-hexadiene, and hexane (all ACS grade) were purchased from Aldrich Chemical Company (Oakville, Canada). CLA standards of *cis,trans*-9,11-octadecadienoic acid, *cis,cis*-9,11-octadecadienoic acid, and *trans,trans*-9,11-octadecadienoic acid were purchased from Matreya, Inc. (Pleasant Gap, PA). The standard of *trans,cis*-10,12-octadeca-

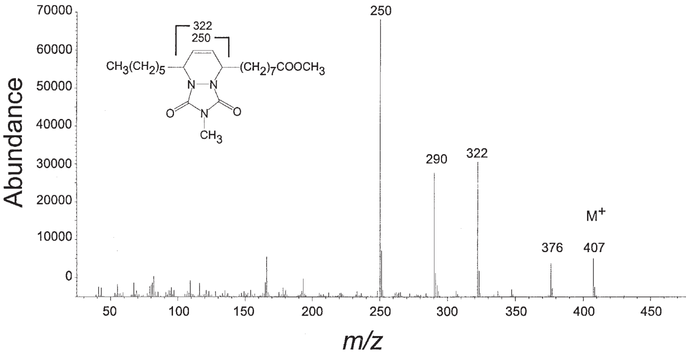

FIG. 1. Mass spectrum of the 4-,methyl-1,2,4-triazoline-3,5-dione (MTAD) adduct of methyl *cis,trans*-9,11-octadecadienoate showing major fragments.

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dienoic acid was a gift from Natural Lipids (Hovdebygda, Norway). The CLA isomer mixture was obtained from Sigma (St. Louis, MO). Boron trifluoride/methanol complex (14%) was obtained from VWR CanLab (Edmonton, Canada).

Derivatization procedures. Samples of CLA were methylated using boron trifluoride/methanol. Free acid (*ca.* 1 mg) was transferred to a clear glass test tube equipped with a Teflon-lined screw cap (8 mL; Kimax, Baxter-Canlab, Mississauga, Canada) and a small magnetic stir bar. Boron trifluoride/methanol complex (1 mL) was added using a glass pipette. The mixture was purged with argon gas, the tube capped, and the mixture vortexed for 2 min. After magnetically stirring the mixture for 30 min at room temperature, hexane (2 mL) and water [1 mL; from a Millipore Super-Q (Marlborough, MA) water purification system] were added. After a 2-min vortex, the layers were allowed to separate. The top organic layer was transferred (disposable glass pipette) to another test tube containing anhydrous sodium sulfate (100 mg). After 5 min, a portion of the clear hexane solution of CLA methyl esters (*ca.* 200 µL) was transferred with a disposable glass pipette to a test tube at 0°C containing MTAD in dichloromethane (85 µg in 130 µL). The MTAD derivatives of CLA methyl esters were obtained by Dobson's procedure (5).

GC/MS conditions. Mass spectra were obtained with a Hewlett-Packard (HP) (Palo Alto, CA) model 5989A mass spectrometer coupled to an HP model 5890 Series II gas chromatograph. Chromatographic separations were achieved using a HP-5MS fused-silica column (30 m × 0.25 mm i.d., 0.25 µm film thickness). Typical gas chromatographic conditions were: injector, 250°C; transfer line, 280°C; helium carrier gas (1.2 mL/min) using constant flow mode; temperature program 160°C for 3 min, 160–290°C at 5°C/min, hold for 5 min. Mass spectral conditions were: electron impact mode, 70 eV; full scan (35–500 *m/z*) with high-energy dynode on; ion source temperature, 275°C; quadrupoles, 100°C.

RESULTS AND DISCUSSION

The chemical reactions of simple conjugated dienes with triazoline diones have been the subject of intense investigation. We have used these studies to interpret reactions of the more complex conjugated dienes of CLA. Jensen and Foote (6) prepared the PTAD adducts of *cis,cis*-2,4-hexadiene, *cis,trans*-2,4-hexadiene, and *trans,trans*-2,4-hexadiene in chloroform. The reaction of *cis,trans*-2,4-hexadiene and *trans,trans*-2,4-hexadiene produced the expected Diels-Alder products (Fig. 2A,B). The *cis,trans*-2,4-hexadiene produced a *trans* adduct while the *trans,trans*-2,4-hexadiene produced a *cis* adduct. However, *cis,cis*-2,4-hexadiene produced a mixture of the *cis* and *trans* adduct ring structures in chloroform. Foote and colleagues (6,7) described a theoretical framework to understand the reaction of PTAD with the three hexadiene isomers. PTAD reacted slowly with *cis,cis*-2,4-hexadiene, and its reaction rate was six times greater with *cis,trans*-2,4 hexadiene and 600 times faster with *trans,trans*-2,4-hexadiene. Using computer-based modeling, Foote and coworkers supported the argument that the more reactive dienes readily adopted the s-*cis* conformation (Fig. 2A,B) and reacted through either typical or modified Diels-Alder transition states. Other reaction intermediates have been proposed for the formation of Diels-Alder adducts from *cis,cis*-2,4-hexadiene (6,7). Jensen and Foote concluded that for dienes where the s-*cis* conformation was feasible, the concerted pathway dominated and Diels-Alder products will retain stereochemistry. Hindered dienes yielded Diels-Alder products with mixed stereochemistry.

In light of the current knowledge of the chemistry of PTAD and hexadiene, MTAD would react in a similar fashion with CLA isomers producing two major products. Methyl *trans,trans*-9,11-octadecadienote would be expected to rapidly form the *cis* Diels-Alder adduct (Fig. 2A) while methyl *cis,trans*-9,11-octadecadienoate would form the trans Diels-Alder adduct at a slower rate (Fig. 2B). The methyl *cis,cis*-9,11-octadecadienoate would react slowly, if at all, and the final product could include both the *cis* and *trans* adducts (6,7).

As expected, total ion chromatograms of the MTAD-derivatized standards for the three highly enriched CLA standards demonstrated different responses of each compound to the derivatizing agent. The reaction of the methyl *cis,trans*-9,11-octadecadienoate was not complete, and a small residual peak

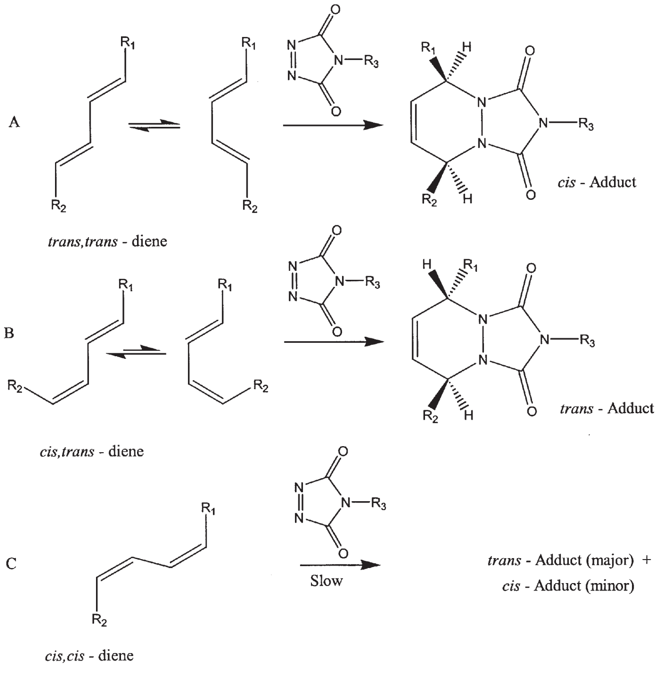

FIG. 2. Reaction of conjugated dienes with a triazoline dione. (A) The s-*trans* and s-*cis* conformations of *trans,trans*-diene are in equilibrium. The s-*cis* reacts rapidly with the triazoline dione to form the *cis* Diels-Alder adduct. (B) The s-*cis* conformation of the *cis,trans* diene is hindered, and reaction with the triazoline dione is slowed. A small amount of residual unreacted ester is observed (6). The reaction product is a *trans* Diels-Alder adduct. (C) The s-*cis* conformation is not feasible for *cis,cis* dienes. An alternative pathway occurs, leading to the formation of both *cis* and *trans* adducts (6,7). Note: $R_1 = CH_3$; $R_2 = CH_3$; $R_3 = Ph$ for hexadiene reactions with 4-phenyl-1,2,4-triazoline-3,5-dione. $R_1 = (CH_2)_7COOCH_3$; $R_2 = (CH_2)_5CH_3$; $R_3 = CH_3$ for reactions of MTAD with methyl 9,11-octadecadienoates. See Figure 1 for abbreviation.

was observed near 14 min. A major adduct peak was observed at 27.2 min (Fig. 3A) and assigned as the *trans* Diels-Alder adduct. Methyl *trans,trans*-9,11-octadecadienoate reacted to completion, and only one adduct peak was observed with a retention time of about 26.9 min (Fig. 3B). This product was interpreted to be the *cis* Diels-Alder adduct. The methyl *cis,cis*-9,11-octadecadienoate MTAD reaction was incomplete with large amounts of unreacted material observed in the total ion chromatogram (Fig. 3C). The chromatogram indicated a large peak of unreacted diene at 14.5 min as well as peaks for *cis* and *trans* adducts at 26.8 and 27.1 min, respectively. The adduct at 27.1 min was the dominant product. The mass spectra of all adducts were very similar (with no noticeable diagnostic differences). Observed rates of reaction were consistent for those reported for the simpler substituted hexadienes (6).

Derivatized standards were then mixed and injected in pairs to confirm differences in retention times mentioned above. The mixtures of methyl *trans,trans*-9,11-octadecadienoate adduct with either the methyl *cis,trans*-9,11- (Fig. 3D) or methyl *cis,cis*-9,11-octadecadienoate (not shown) yielded two peaks in the adduct region with baseline separation. The mixture of methyl *cis,trans*-9,11-octadecadienoate adduct with methyl *cis,cis*-9,11-octadecadienoate adduct yielded a minor peak at 26.8 min and a major adduct peak at 27.1 min (Fig. 3E). It therefore appeared that the reaction products of PTAD with a series of hexadiene isomers were similar to the reaction products of MTAD with 9,11-octadecadienoate isomers.

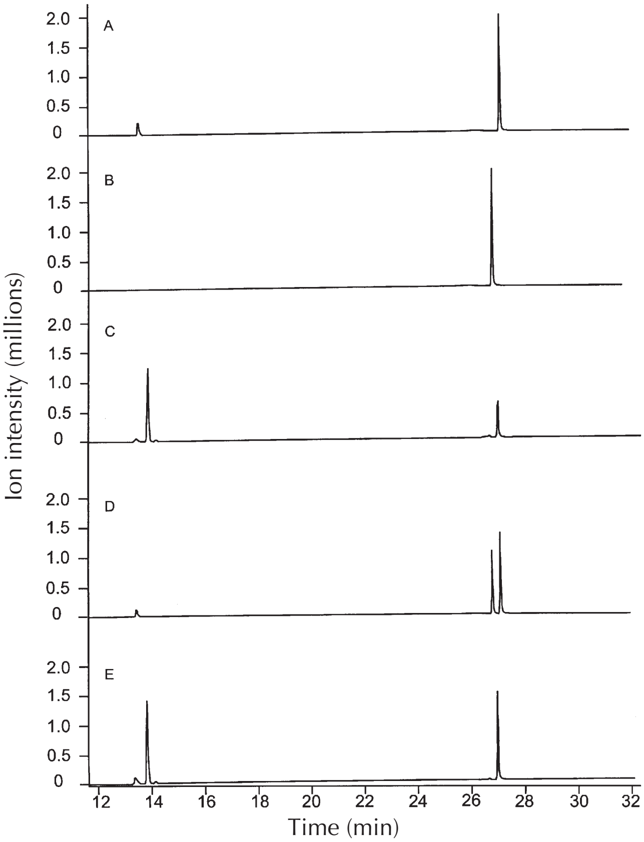

FIG. 3. Gas chromatography/mass spectrometry (GC/MS) (total ion traces) of products from reaction of MTAD with fatty acid methyl esters of standards of 9,11-octadecadienoic acids. (A) Methyl *cis,trans*-9,11-octadecadienoate. (B) Methyl *trans,trans*-9,11-octadecadienoate. (C) Methyl *cis,cis*-9,11-octadecadienoate. (D) Co-injection of standards from A and B. (E) Co-injection of standards from A and C. See Figure 1 for other abbreviations.

Extracted ion traces of the diagnostic ion *m/z* 250 for adducts of conjugated methyl 9,11-octadecadienoate geometrical isomers further confirmed the results of the total ion chromatogram. The methyl *cis,trans*-9,11-octadecadienoate standard adduct was noted to contain a single peak at 27.1 min corresponding to the same peak identified in the total ion chromatogram (Fig. 4A). Similarly the selective ion chromatograms of methyl *trans,trans*-9,11 (Fig. 4B) and methyl *cis,cis*-9,11 octadecadienoate (Fig. 4C) reflect the total ion chromatogram in the same region. Notably the CLA isomer mixture contained the expected peaks for the *cis* and *trans* Diels-Alder adducts (Fig. 4D).

Analytical standards were available for the aforementioned 9,11 series of geometrical isomers and *trans,cis*-10,12-octadecadienoic acid. However, some mixed CLA standards and commercial CLA can contain positional isomers corresponding to 8,10-, 9,11-, 10,12-, and 11,13-octadecadienoic acids (4). GC/MS was performed on the MTAD adducts of the CLA isomer mixture using the diagnostic ions $[M - R_1]^+$ 264, 250, 236, and 222 amu (8) for the 8,10-, 9,11-, 10,12-, and 11,13-octadecadienoic acid methyl esters, respectively (Fig. 5). Pairs of peaks were observed in chromatograms of 264, 250, and 236 amu. The trace of 222 amu revealed a peak with a leading shoulder. In all cases the first peak eluting was smaller. Retention times of MTAD derivatives of enriched standards allowed the identification of three of the peaks by both retention time and diagnostic ions. Methyl *cis,trans*- and methyl *trans,trans*-9,11-octadecadienoates were observed as two peaks at 250 amu. Adduct of methyl *trans,cis*-10,12-octadecadienoate had a retention time of 27.2 min and a diagnostic ion of 236 amu (data not shown). *Cis* adducts of *trans,trans* geometrical isomers eluted before adducts of *cis,trans* or *trans,cis* isomers. By assuming that the remaining four compounds had the same chromatographic properties observed for the standards, it was possible to tentatively assign the remaining peaks. We have found the elution of four major peaks in the same pattern as previously reported by Christie *et al.* (4) with considerable overlap between the elution of the 9,11 and 8,10 adducts and some separation of the 10,12 and 11,13 adducts. These major peaks are derived from the *cis,trans* and *trans,trans* isomers. The *cis,cis* and *cis,trans* isomers formed the *trans* adduct.

While MTAD analysis remained useful as a tool for determining the position of conjugation in fatty acids, the current study revealed limitations of MTAD analysis of isomer mixtures. As *cis,cis* and *cis,trans* dienes produced the same *trans* adduct, it was impossible to quantify these isomers when they were in a mixture. The propensity of *cis,cis* dienes to form the *cis* Diels-Alder adduct as a minor product limited the usefulness of this technique to analyze the *trans,trans* diene. The amount of *cis* adduct formed cannot therefore be used to quantify the *trans,trans* diene in a mixture of diene isomers. Incomplete reactions will lead to underestimates of *cis,cis* dienes.

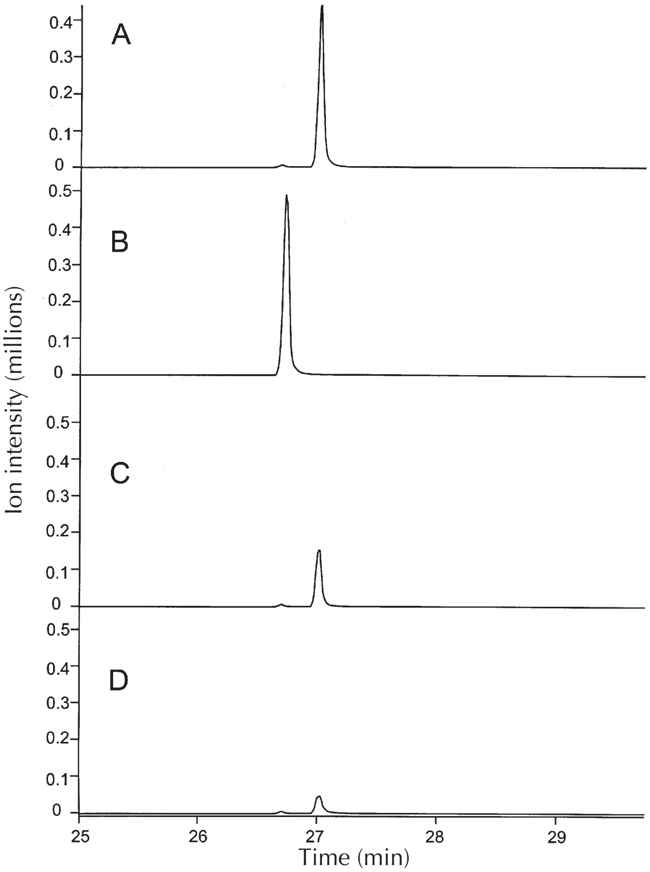

FIG. 4. GC/MS (extracted ion traces) of *m/z* 250 fragments of MTAD derivatives. (A) Methyl *cis,trans*-9,11-octadecadienoate. (B) Methyl *trans,trans*-9,11-octadecadienoate. (C) Methyl *cis,cis*-9,11-octadecadienoate. (D) Conjugated linoleic acid (CLA) methyl ester isomer mixture. See Figures 1 and 3 for other abbreviations.

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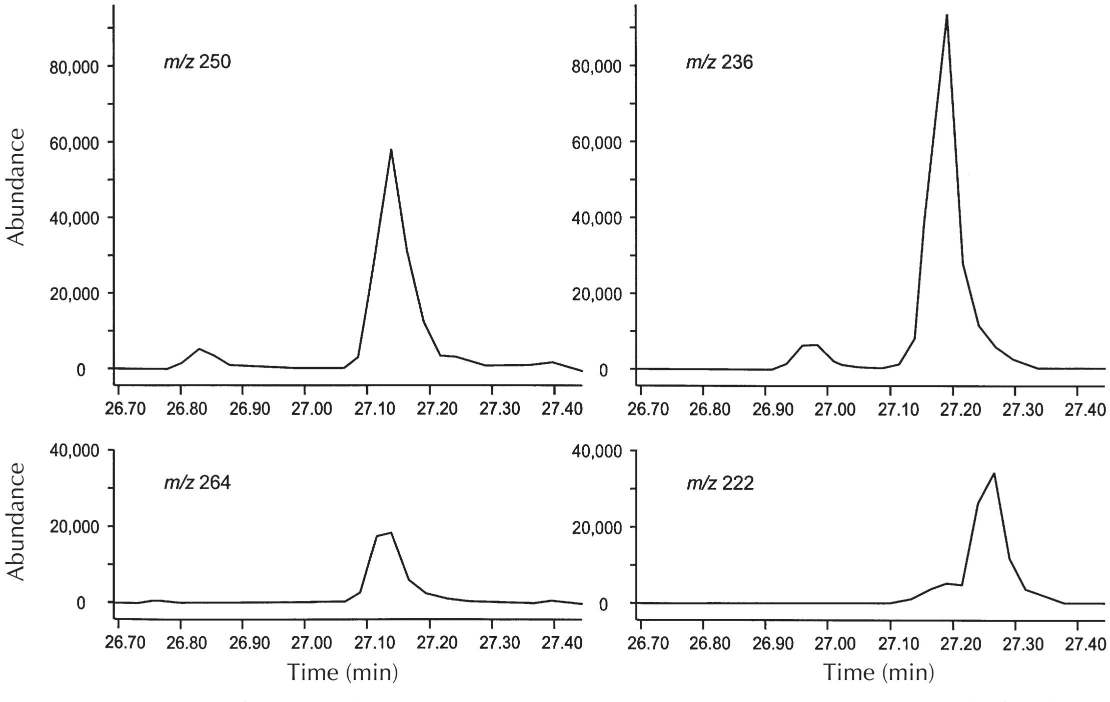

FIG. 5. GC/MS (extracted ion traces) of CLA methyl ester isomer mixture derivatized with MTAD. See Figures 1, 3, and 4 for abbreviations.