Identification of Conjugated Fatty Acids by Gas Chromatography–Mass Spectrometry of 4-Methyl-1,2,4-triazoline-3,5-dione Adducts

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ABSTRACT: 4-Methyl-1,2,4-triazoline-3,5-dione was reacted with conjugated fatty acid methyl esters to form Diels-Alder cycloaddition products. The electron impact mass spectra of conjugated octadecadienoates and 9,11,13-octadecatrienoate were simple and informative and allowed the positions of the double bonds to be determined. The dienes gave single adducts whereas the triene formed four products that corresponded to two stereoisomers of the adducts of the 9,11-diene system and two of the 11,13-diene system. The method can be used to complement other methods for identifying conjugated fatty acids. *JAOCS 75*, 137–142 (1998).

KEY WORDS: Conjugated fatty acids, Diels-Alder adducts, gas chromatography–mass spectrometry, 4-methyl-1,2,4-triazoline-3,5-dione adducts.

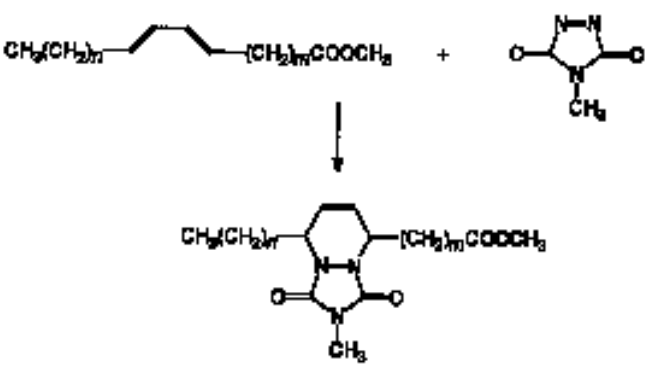
There is currently a great interest in isomers of conjugated linoleic acid (CLA) because of their anticarcinogenic properties (1,2). They are formed in ruminants by anaerobic bacteria during biohydrogenation of linoleic acid (3) and during partial hydrogenation of vegetable oils (4). They are present in cow's milk (5), dairy products (6,7), human milk (6), and meat from ruminants (7–9). CLA fed to rats can be elongated and desaturated in the liver to conjugated octadecatrienoic (10) and eicosatrienoic (10,11) acids. Although conjugated arachidonic acid was not detected in one study (10), it was unambiguously identified in another (11). All three metabolites were detected in the livers of lambs that had a high CLA content naturally in their diet (12).

Among other sources of conjugated fatty acids are the seeds of some plants (13). These include dienes, e.g., 18:2(10*t*,12*t*) from *Chilopsis linearis*; trienes, e.g., 18:3(9*c*,11*t*,13*c*) (punicic acid) from some genera of the Punicaceae and Cucurbitaceae; tetraenes, e.g., 18:4(9*c*,11*t*,13*t*,15*c*) (parinaric acid) from *Parinarium* and *Impatiens*; and acids with oxygen functions, e.g., 18-OH-18:3(9*c*,11*t*,13*t*) (kamolenic acid) from the kamala tree, *Mallotus philippinensis*.

Measurements of conjugated fatty acids often exploit their ultraviolet (UV)-absorbing properties after separation by high-performance liquid chromatography (HPLC) (12,14). From second-derivative UV spectra, conjugated linolenic, conjugated eicosatrienoic, and conjugated arachidonic acids were detected in lamb liver (12). Gas chromatography (GC) has been used to identify the methyl esters of conjugated octadecadienoic acids (15). The positions of the double bonds in conjugated dienes and trienes can often be verified by mass spectrometry of dimethyloxazoline (DMOX) derivatives (16).

Conjugated double bonds react specifically with certain dienophiles to form Diels-Alder cycloaddition products. Maleic anhydride has been used extensively to determine conjugated fatty acids of *E,E* configuration (13). 4-Phenyl-1,2,4-triazoline-3,5-dione (17) and 4-methyl-1,2,4-triazoline-3,5-dione (MTAD) (18) readily form stable adducts with conjugated dienes (Scheme 1). The mass spectra of both types of adducts are simple and informative. The positions of the double bonds can be deduced from ions that result from fragmentation on either side of the ring (17,18), but only MTAD adducts are sufficiently volatile to be analyzed by GC. A related reagent, 4-[3-(1-propenyl)propyl]-1,2,4-triazoline-3,5-dione, has been used for sensitive fluorescence detection of CLA in human serum by HPLC (19).

The present study is concerned with investigating the use of MTAD for identifying conjugated fatty acids by GC–mass spectrometry (MS).



SCHEME 1

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EXPERIMENTAL PROCEDURES

1,3-Hexadiene and mixtures of isomers of CLA, as free acids and as methyl esters (Sigma Chemical Co., Poole, United Kingdom), and MTAD (Aldrich Chemical Co., Gillingham, United Kingdom) were obtained. Solvents were HPLC- or Distol-grade (Fisher Scientific UK, Loughborough, United Kingdom). The total lipids from seeds of *Trichosanthes kirilowii* were extracted under an atmosphere of nitrogen in a darkened room according to the method of Bligh and Dyer (20). Triacylglycerols were purified on Isolute^{TM} silica solid-phase extraction columns (International Sorbent Technology Ltd., Mid Glamorgan, United Kingdom) with hexane/acetone (99:1, vol/vol; 30 mL).

Formation of adducts. The method was modified from that of Young *et al.* (18). Solutions of fatty acid methyl esters, containing known amounts of conjugated fatty acid methyl esters, and MTAD in dichloromethane were mixed in a test tube at 0°C by agitating for less than 10 s. CLA methyl ester (220 μg, 1.15 mM) and MTAD (425 μg, 5.79 mM) in dichloromethane (650 μL), and 9,11,13-octadecatrienoic acid methyl ester (44 μg, 1.16 mM; in total fatty acid methyl esters from *T. kirilowii* triacylglycerols) and MTAD (85 μg, 5.79 mM) in dichloromethane (130 μL) were used; the molar ratio of conjugated fatty acid methyl ester to MTAD was 1:5. The reactions were immediately stopped by addition of a twofold molar excess (relative to MTAD) of 1,3-hexadiene, followed by agitation for a few seconds. The mixtures were taken to dryness, by blowing under nitrogen at 30°C, and redissolved in dichloromethane to give approximately 0.1% (wt/vol) solutions of the fatty acid methyl ester adducts. They were analyzed directly by GC–MS on a nonpolar capillary column.

Other derivatization procedures. Free fatty acids were converted to methyl esters by sulfuric acid-catalyzed esterification, and fatty acid methyl esters were released from triacylglycerols by sodium methoxide-catalyzed transesterification (21). DMOX derivatives were prepared from methyl esters according to Fay and Richli (22).

GC–MS. Fatty acid methyl ester MTAD adducts and fatty acid DMOX derivatives, in dichloromethane, were analyzed by GC–MS on a Hewlett-Packard 5890 Series II Plus gas chromatograph (Stockport, United Kingdom), fitted with a split/splitless injector (split ratio 20:1) and a DB-5MS^{TM} (30 m × 0.25 mm i.d., 0.25 μm film thickness; J&W Scientific, Folsom, CA) capillary column, connected to a Hewlett-Packard 5989B MS Engine quadropole mass spectrometer. The oven temperature was held for 160°C for 3 min and was temperature-programmed to 350°C at 5°C/min. Helium was used as carrier gas at a flow rate of 1 mL/min, and pressure-programming was used in constant-flow mode. The mass spectrometer was operated in electron impact mode at an ionization energy of 70 eV and emission current of 300 μA. The ion source and interface temperatures were at 250°C, and the scan range was 35–750 a.m.u. at 0.44 scans/s.

RESULTS AND DISCUSSION

The reaction between MTAD and conjugated fatty acids is rapid, and hexadiene was added almost immediately to pre-

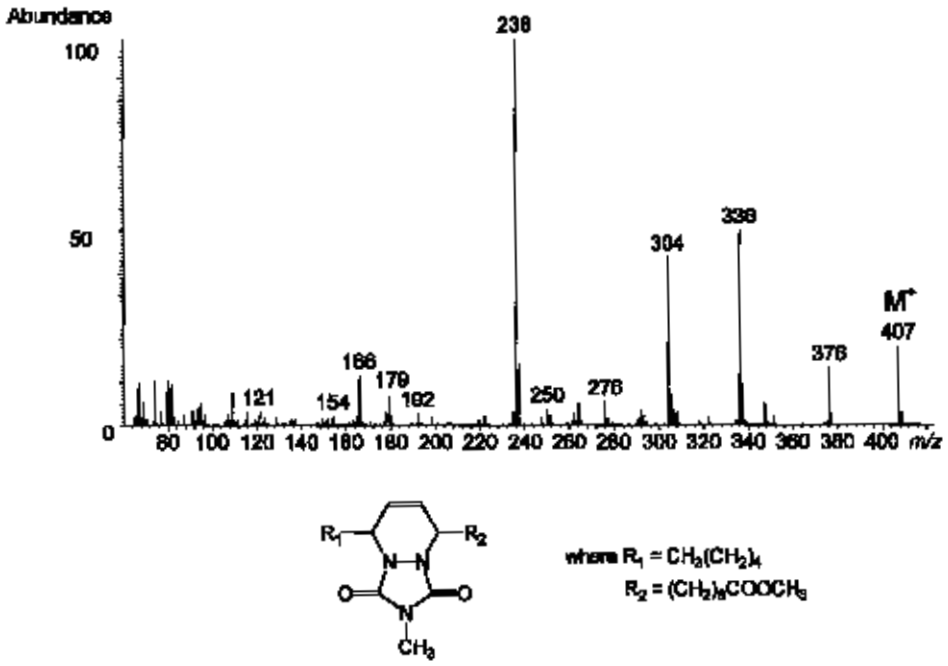


FIG. 1. Electron impact mass spectrum of 4-methyl-1,2,4-triazoline-3,5-dione (MTAD) adduct of 10,12-18:2 methyl ester.

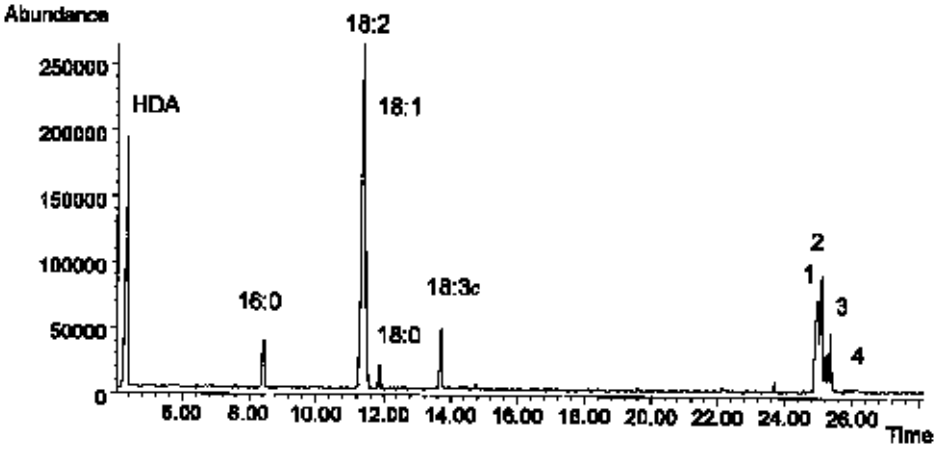


FIG. 2. Total ion chromatogram of products from reaction of MTAD with total fatty acid methyl esters from the triacylglycerols of *Trichosanthes kirilowii*. For conditions, see the Experimental Procedures section. HDA = 1,3-hexadiene MTAD adduct; 16:0 = methyl palmitate; 18:0 = methyl stearate; 18:1 = methyl oleate; 18:2 = methyl linoleate; 18:3 = nonadducted 9*c*,11*t*,13*c*-18:3 methyl ester; 1,3 = 9,11,13-18:3 methyl ester MTAD adduct, of 9,11-diene system; 2,4 = 9,11,13-18:3 methyl ester MTAD adduct, of 11,13-diene system. See Figure 1 for other abbreviation.

vent by-product formation. MTAD specifically forms Diels-Alder adducts with conjugated systems (18). However, under harsh conditions, for example when there is a large excess of MTAD, methylene-interrupted unsaturated fatty acids may react with the reagent to some extent to form unidentified polar products. Fortunately, these do not interfere with the analysis of adducts of conjugated fatty acids.

The MTAD adduct of the commercial CLA methyl ester mixture was analyzed by GC–MS and could only be eluted on a nonpolar (DB-5MS) capillary column. There was a single peak (with a retention time of 25.4 min, compared to 12.3 min for the nonadducted methyl ester), the mass spectrum (Fig. 1) of which was simple and readily interpretable as the adduct of octadec-10,12-dienoic methyl ester. The observed ions were analogous to those seen for the MTAD adducts of conjugated diene-containing hydrocarbons and acetates (18). There was a strong M^+ ion (*m/z* 407), which confirmed that a 1:1 adduct had formed between the fatty acid methyl ester and reagent. The alkyl chain on one side of the ring was defined as R_1, and the ester chain on the other side was R_2 (Fig. 1). The position of the ring between C-10 and C-13 of the C_{18} chain (and hence the location of the 10,12-diene system in the parent fatty acid) was determined from either of two abundant ions, $[M - R_1]^+$ (*m/z* 336) and $[M - R_2]^+$ (*m/z* 236), resulting from cleavages alpha to the ring. Thus, *m/z* 336 corresponded to a methyl ester of a saturated C_9 acid unit (R_2) attached to the ring, and 71 amu (M – 336) was the mass of a C_5 saturated alkyl unit (R_1). Similarly, *m/z* 236 corresponded to the alkyl unit attached to the ring, and 171 amu (M – 236) was the mass of the ester unit. The $[M - R_2]^+$ ion lost methyl isocyanate to produce an ion (*m/z* 179) of low abundance. Two prominent ions (*m/z* 376 and 304) were accounted for by the loss of a methoxy radical and methanol (from the methyl ester group) from the molecular ion and $[M - R_1]^+$ ion, respectively. The ion at *m/z* 166 ($[M - R_1 - R_2 + H]^+$) resulted from loss of both side chains.

The adduct of the 8,10-diene was unresolved from and at the leading edge of the main component, and the expected ions (diagnostically $[M - R_1]^+$ and $[M - R_2]^+$ ions at *m/z* 308 and 264, respectively) were observed in the mass spectrum. Overlapping with the 8,10-diene, there was evidence for a small amount of a 9,11-diene ($[M - R_1]^+$ and $[M - R_2]^+$ ions at *m/z* 322 and 250, respectively).

GC–MS of the MTAD adducts of the methyl esters of the commercial CLA-free acid mixture also verified the expected composition. There was a series of overlapping GC peaks with mass spectra indicative of MTAD adducts of 8,10-, 9,11-, 10,12-, and 11,13-18:2 methyl esters. The diagnostic $[M - R_1]^+$ and $[M - R_2]^+$ ions occurred at *m/z* 308 and 264, 322 and 250, 336 and 236, and 350 and 222, respectively.

In confirmation, the compositions of the CLA free acid and methyl ester mixtures were determined by GC–MS as the DMOX derivatives. By comparison to published spectra (16), the main component of the methyl ester mixture had a 10,12-18:2 structure, and there was a minor 8,10-18:2 component. The free acid mixture contained substantial amounts of 8,10-, 9,11-, 10,12-, and 11,13-18:2 acids (P. Juaneda, personal communication).

Punicic acid [18:3(9*c*,11*t*,13*c*)] and two other geometrical isomers [18:3(9*c*,11*t*,13*t*) and 18:3(9*t*,11*t*,13*c*)] were 38 and 3%, respectively, of the total fatty acid methyl esters from the seed oil of *T. kirilowii* (23).

Four closely eluting MTAD adducts were observed by GC–MS (Fig. 2). The mass spectra (Fig. 3) of two of the peaks, 1 and 3, were similar and were interpreted as adducts of the 9,11-diene system, with the remaining double bond in

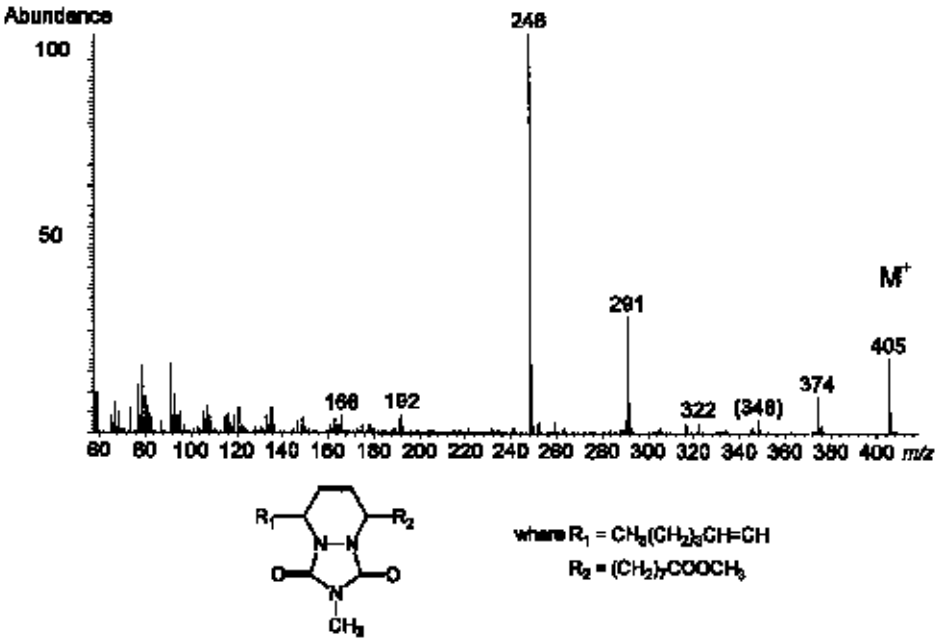


FIG. 3. Electron impact mass spectrum of 9,11,13-18:3 methyl ester MTAD adduct of 9,11-diene system; peak 1, Figure 2. (*m/z* 316 and 348 are derived from the MTAD adduct of the 11,13-diene system; Fig. 4). See Figure 1 for abbreviation.

the R_1 alkyl side-chain, as described below. Likewise, the mass spectra (Fig. 4) of the other two peaks, 2 and 4, were similar and corresponded to the adducts of the 11,13-diene system with the remaining double bond in the R_2 ester side-chain. Hence, MTAD reacted with the triene as if it had two independent diene systems (Scheme 2) and unequivocally proved that it was an 18:3(9,11,13) acid.

Analogous to the mass spectra of the MTAD adducts of the CLA methyl esters, the mass spectra (Fig. 3) of the adducts of the 9,11-diene system had ions at M^+ (*m/z* 405) and $[M - CH_3O]^+$ (*m/z* 374), confirming that a 1:1 adduct had formed between the fatty acid methyl ester and reagent. The location of the ring between C-9 and C-12 of the C_{18} chain (and therefore a 9,11-diene system in the parent fatty acid), and the de-

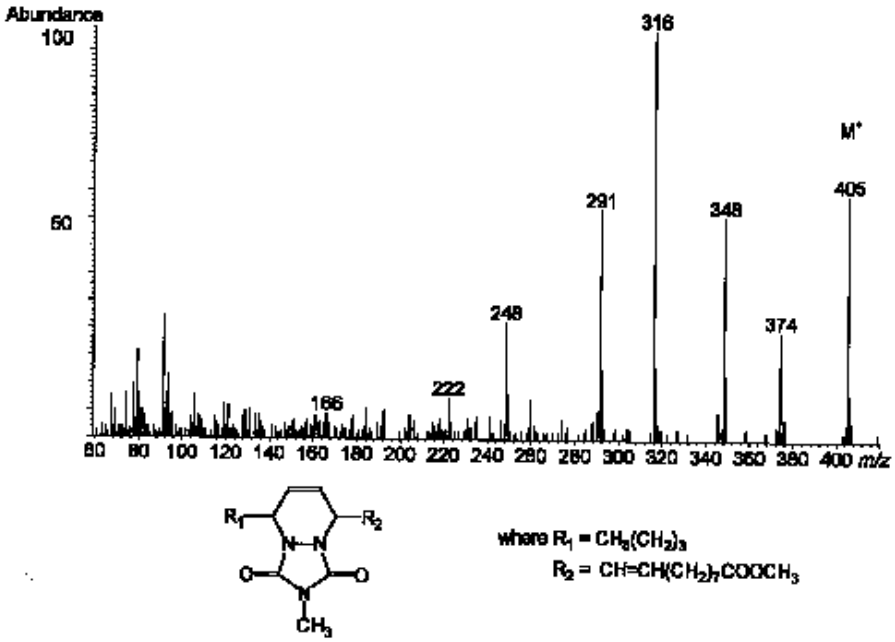
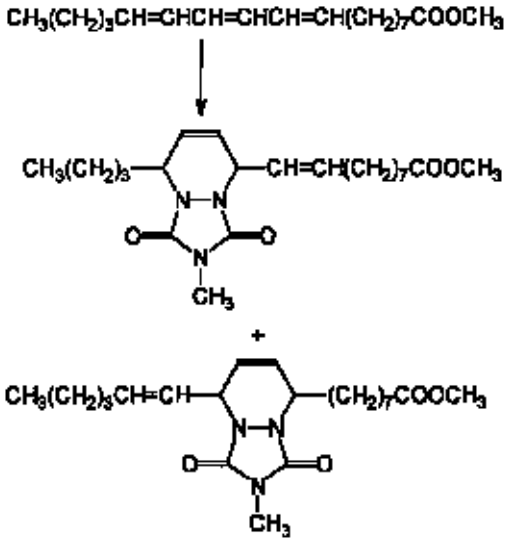


FIG. 4. Electron impact mass spectrum of 9,11,13-18:3 methyl ester MTAD adduct of 11,13-diene system; peak 2, Figure 2. See Figure 1 for abbreviation.



SCHEME 2

termination that the remaining double bond was in the R_1 alkyl chain, were revealed by either of the ions ($[M - R_1]^+$ at *m/z* 322 and $[M - R_2]^+$ at *m/z* 248) in which one of the side-chains had been lost. For example, *m/z* 248, the base peak, corresponded to a C_6 monounsaturated alkyl unit attached to the ring (the exact position of the double bond could not be verified from this ion), and 157 amu (M – 248) was the mass of a methyl ester of a saturated C_8 acid. In contrast to the mass spectrum of the CLA methyl ester adduct, $[M - R_1]^+$ lost a methoxy radical $[M - R_1 - CH_3O]^+$ (*m/z* 291) rather than methanol. Also, the abundance of ions that had lost the R_1 chain (especially the $[M - R_1]^+$ ion) was reduced, suggesting that the double bond in the alkyl chain had a stabilizing effect. There was also an $[M - R_2 - CH_3NCO]^+$ ion [*m/z* 191/(192)] of low abundance.

The mass spectra (Fig. 4) of the adducts of the 11,13-diene system had ions at M^+ (*m/z* 405) and $[M - CH_3O]^+$ (*m/z* 374), again confirming that a 1:1 adduct had formed between the fatty acid methyl ester and reagent. The $[M - R_1]^+$ ion (*m/z* 348) located the ring between C-11 and C-14 of the C_{18} chain (and therefore an 11,13-diene system in the parent fatty acid), and this time, the remaining double bond was in the R_2 ester chain. Hence *m/z* 348 corresponded to a methyl ester of a monounsaturated C_{10} acid (R_2) attached to the ring, and 57 amu (M – 348) was the mass of a C_4 saturated alkyl unit (R_1). The $[M - R_1 - CH_3OH]^+$ ion (*m/z* 316) was the base peak and, in contrast to the mass spectrum of the 9,11-diene adduct, there was an abundant $[M - R_1–CH_3NCO]^+$ ion at *m/z* 291. The $[M - R_2]^+$ ion (*m/z* 222) was of low abundance and difficult to discern, although it was the base peak in the spectra of the other adducts, and the $[M - R_2 - CH_3NCO]^+$ (*m/z* 165) was of similar low abundance to many surrounding peaks. Therefore, again it appears that the double bond in the chain had a stabilizing effect. An ion at *m/z* 248 was probably derived partly from contamination with the adduct of the 9,11-diene system.

There were two possible explanations for the occurrence of pairs of adducts with similar mass spectra. Either they corresponded to different stereoisomers of the chains about the ring or they differed in the geometry of the remaining double bond in the chain. The former explanation seems unlikely because only one adduct was observed for each of the positional isomers of CLA methyl esters. It is more probable that the pairs were *Z* and *E* isomers of the double bond in the chain. The remaining double bond in both the 9,11- and 11,13-diene adducts of punicate was expected to be of *Z* configuration. The adducts of the other two naturally occurring geometrical isomers would give *E* as well as *Z* isomers, but their amounts were too small to account for the observations. It is likely that the reaction conditions for adduct formation caused geometrical isomerization of the double bonds in either punicate or its adduct to give the observed products. Such isomerization is known to occur under relatively mild conditions (23).

Mass-spectral analysis of MTAD adducts has previously been used only for nonfatty acid conjugated compounds (18). In this report, the mass spectra of MTAD adducts have been shown to be useful for determining the position of conjugated systems in fatty acids. The approach is therefore an alternative or complementary tool to methods based on mass spectra of, for example, DMOX derivatives (16), and indeed has the advantage in that adduct formation is selective for conjugated systems (18).

Apart from their mass-spectral properties, the adducts have potential for detecting unknown conjugated fatty acids in fatty acid methyl ester mixtures by using GC only. Conversely, this approach was used to suggest that a cyclohexenyl dienoic fatty acid, formed in heated vegetable oils, did not have conjugated double bonds in the ring (24).

The classical Diels-Alder reaction of conjugated fatty acids with maleic anhydride was specific for *E,E* dienes under most conditions (13). MTAD reacts readily with conjugated dienes of different geometrical configurations under mild conditions (18). Under certain conditions, MTAD reacts more readily with 9*c*,11*t*-18:2 than with 9*c*,11*c*-18:2 (O. Berdeaux, unpublished data), suggesting that there is potential for differentiating between different geometrical isomers.

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