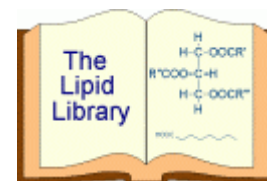


## MASS SPECTRA OF METHYL ESTERS OF FATTY ACIDS



### Part 7. Derivatization of the Double Bonds

Preparation of methyl esters *per se* is described on its own web page, and there is more information on this subject in the “**Selected topics in the analysis of lipids**” section of this website. Sometimes it is necessary to derivatize methyl esters of fatty acids by reaction at the double bonds in order to locate these by GC-MS. The useful techniques of hydrogenation and deuteration are described below, together with preparation and mass spectral properties of 4-methyl-triazoline-3,5-dione (MTAD) and of dimethyl disulfide (DMDS) adducts.

#### Hydrogenation

Catalytic hydrogenation is a simple procedure that provides invaluable structural information regarding fatty acid identity, when combined with GC or GC-MS analysis. It is best carried out with methyl esters, but then there may be considerable advantages in conversion to picolinyl ester or DMOX derivatives for mass spectrometry.

Some needlessly complex procedures involving high pressures and temperatures are sometimes described in the literature, but details of a convenient practical procedure are described here [1].

**Protocol:** The unsaturated ester (1-2 mg) in a test-tube is dissolved in methanol (1 mL) and Adams' catalyst (platinum oxide; 1 mg) is added. The tube is connected via a two-way tap both to a reservoir of hydrogen (e.g. in a balloon or football bladder) at or just above atmospheric pressure, and to a vacuum pump. The tube is alternatively evacuated and flushed with hydrogen several times to remove any air, then it is shaken vigorously while an atmosphere of hydrogen at a slight positive pressure is maintained for 2 hr. At the end of this time, the hydrogen supply is disconnected, the tube is flushed with nitrogen and the solution is filtered to remove the catalyst. The solvent is evaporated under reduced pressure, and the required saturated ester is taken up in hexane or diethyl ether for GLC analysis.

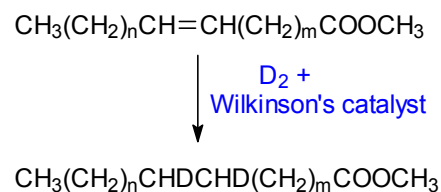
At its simplest, hydrogenation is used merely to determine the chain length of components. By eliminating all unsaturated centres in fatty acid methyl esters from most samples of natural origin, a simple set of peaks is obtained for the saturated even-numbered homologous series and these can be compared with authentic standards. However, the presence of anomalous peaks may be an indication of novel structures. In samples of animal origin, small amounts of odd-chain fatty acids may be detected in the GC trace, together with methyl-branched fatty acids, usually *iso*- closely followed by *anteiso*-isomers, which are best identified as the picolinyl ester or pyrrolidide derivatives.

#### Deuteration

While hydrogenation eliminates unsaturation, and aids identification and location of other functional groups, selective deuteration will assist both in locating unsaturation and characterizing other moieties. Indeed, deuteration has been used since the early days of mass spectrometry of lipids as a means of locating double bonds, and for unravelling fragmentation mechanisms, but the value of

the procedure for structure determinations was limited with methyl ester derivatives as the wide range of rearrangement ions formed led to some scrambling of the deuterium atoms in the alkyl chain. However, by using nitrogen-containing derivatives, which give clean radical-induced fragmentations with minimal rearrangement, most problems have been eliminated. Again, the reaction is best carried out with methyl esters prior to conversion to picolinyl ester or DMOX derivatives for mass spectrometry.

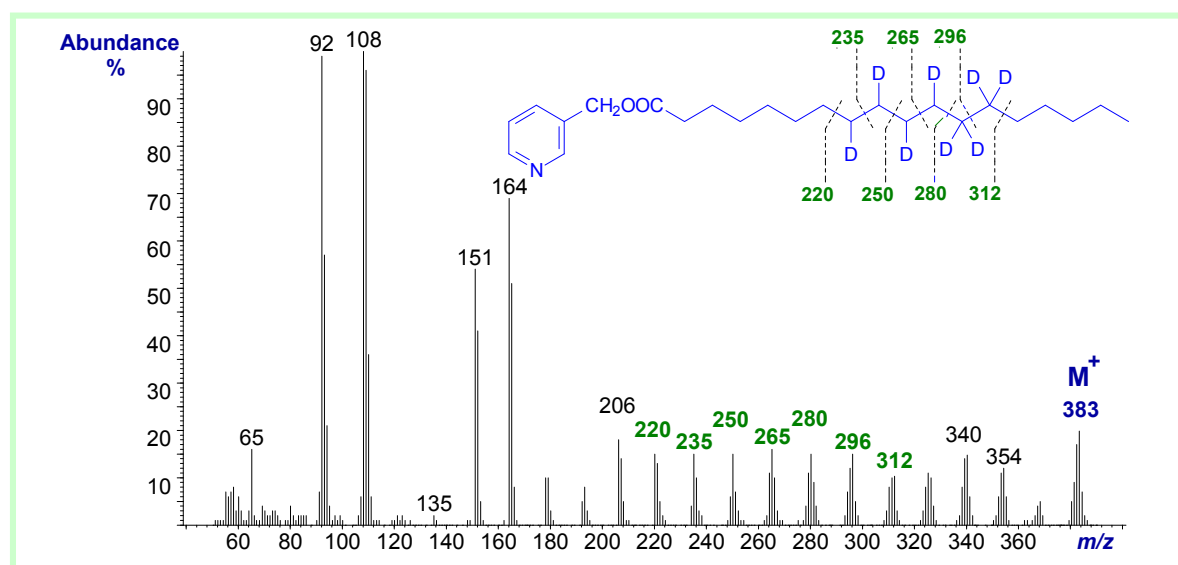
Deuteration with deuterium gas and Wilkinson's catalyst (tris(triphenylphosphine)-rhodium(I) chloride) is usually employed for the purpose, and can be recommended. Gaseous deuterium is available commercially in small cylinders, or it can be generated *in situ* by reaction of deuterium chloride with sodium borodeuteride. It is essential to have a good excess of deuterium so that the reaction goes rapidly to completion, otherwise some isomerization of the double bonds and scrambling of the hydrogen atoms is possible (author, unpublished). The following method is based on that of Dickens *et al.* [2].



**Protocol:** Methyl esters of unsaturated fatty acids are subjected to deuteration with deuterium gas and Wilkinson's catalyst. The fatty ester (up to 2 mg) and Wilkinson's catalyst (5 mg) in dioxane (1 ml) are degassed with helium in a tube fitted with a septum. The vessel is purged with five volumes of deuterium with constant stirring, and then is left with an atmosphere of deuterium at 60°C for 2h. The solvent is removed in a stream of nitrogen and the required ester is obtained by adsorption chromatography on a small column of Florisil™ (0.5 g), eluted with hexane-acetone (96:4, v/v).

The technique was first used in conjunction with pyrrolidide derivatives, and has also been used with DMOX derivatives, but picolinyl esters appear best for the purpose, as they give cleaner radical-induced fragmentations with fewer rearrangement ions. Also, they give more abundant ions of high molecular weight with saturated fatty acids. On mass spectral analysis, clear diagnostic ion fragments are obtained that permit the determination of the positions of the original double bonds in the alkyl chain.

As an example, an usual fatty acid with two double bonds and a triple bond in conjugation in the seed oil of *Tanacetum corymbosum* was identified as octadeca-8,10-dien-12-ynoic acid by deuteration of the methyl ester derivative prior to conversion to the picolinyl ester for analysis by GC-MS [3]. The spectrum is –

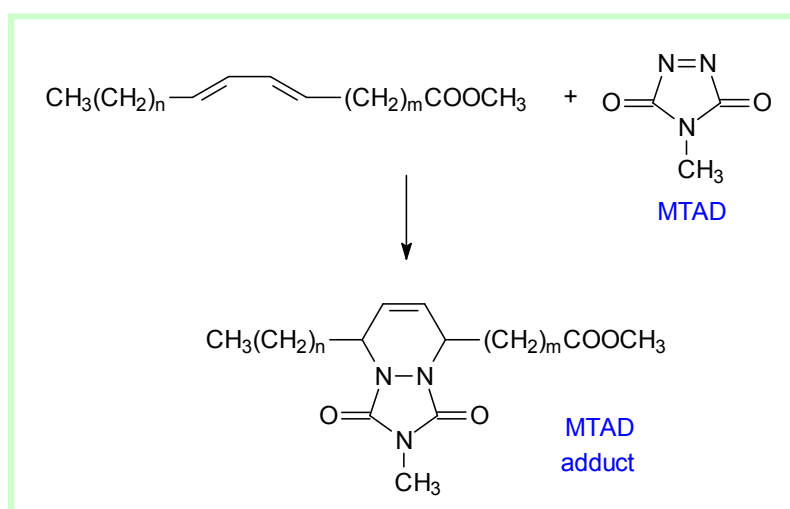


Mass spectra of picolinyl esters are described elsewhere in these web pages, suffice for the moment to point out that one deuterium atom is added to each carbon of the double bonds and two to each carbon of the triple bonds, and these are easily identified from the mass spectrum.

A further example was the proof of structure of 12-oxo-octadec-9-enoic acid from milk fat [4]. Methodology of this kind has enabled us to identify a large number of different fatty acids of marine, plant and animal origins.

### Diels-Alder (MTAD) Adducts for Conjugated Double Bonds

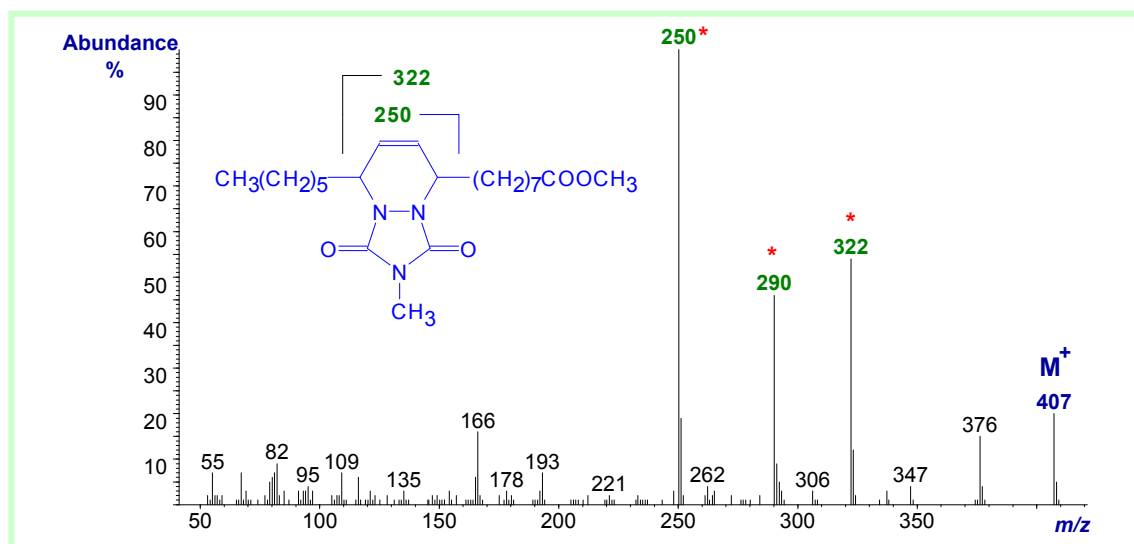
A useful derivative specific for determination of double bond positions in conjugated dienes is to form the Diels-Alder adduct of the fatty acid methyl ester by reaction with the reagent, 4-methyl-1,2,4-triazoline-3,5-dione (MTAD). Such derivatives have excellent mass spectrometric properties, enabling determination of structures in such samples as commercial conjugated linoleic acid (CLA) and the metabolites formed from this in animal tissues.



Reaction occurs almost instantaneously at room temperature and must be stopped immediately by adding 1,3-hexadiene [5].

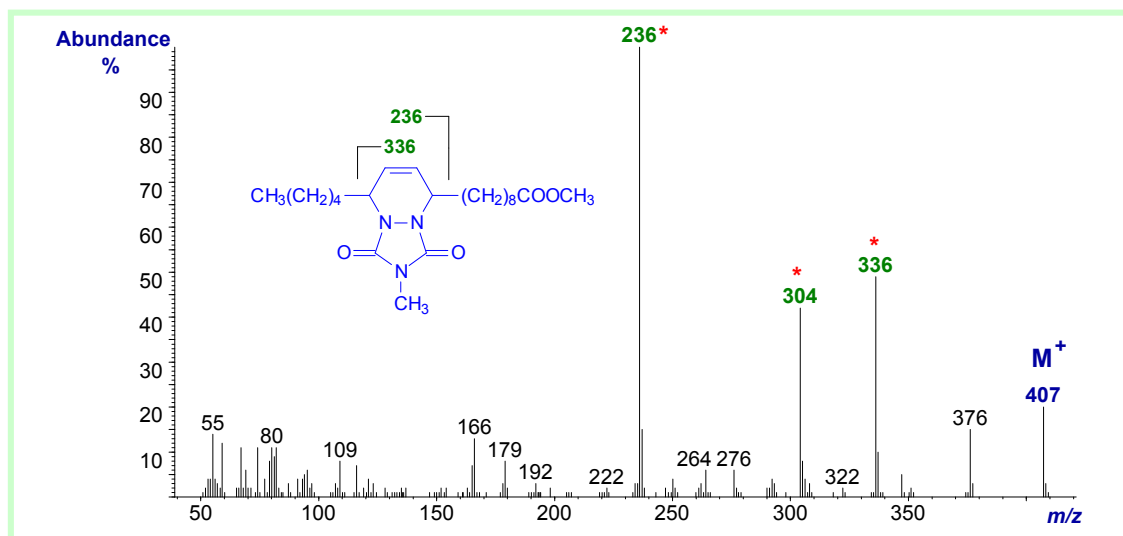
**Protocol:** The CLA methyl ester (220  $\mu\text{g}$ ; 1.15mM) and MTAD (425  $\mu\text{g}$ ; 5.8 mM) in dichloromethane (650  $\mu\text{L}$ ) are mixed in a test-tube at 0°C by agitating for less than 10 seconds. The reaction is immediately stopped by addition of 1,3-hexadiene, followed by agitation for a few seconds. Excess reagents are removed a stream of nitrogen at 30°C, and the sample is redissolved in dichloromethane for analysis by GC-MS.

The mass spectrum of the **MTAD adduct of methyl 9-cis,11-trans-octadecadienoate** is illustrated below –



Cleavage occurs on either side of the six-membered ring, enabling simple location of the carbons that originally constituted the conjugated double bond system, *i.e.* at  $m/z = 250$  and  $322$ . Confirmatory evidence comes from the ion representing loss of methanol from the ion containing the carboxyl moiety, *i.e.* at  $m/z = 290$  in this instance. Indeed, these ions can be used with selective ion monitoring to quantify positional isomers in the presence of mixtures.

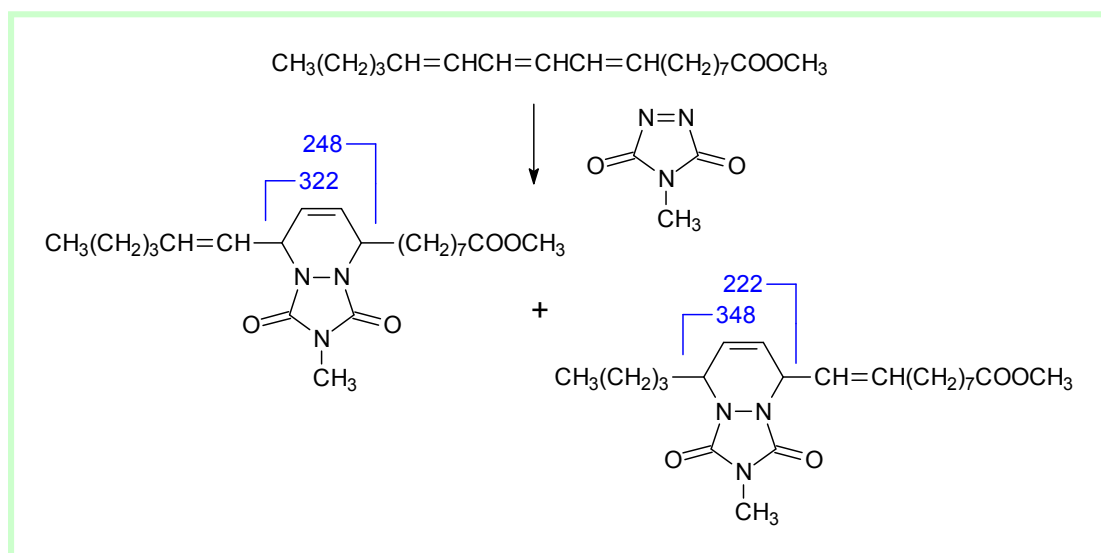
As an example, the spectrum of the **MTAD adduct of the 10,12-18:2** isomer follows –



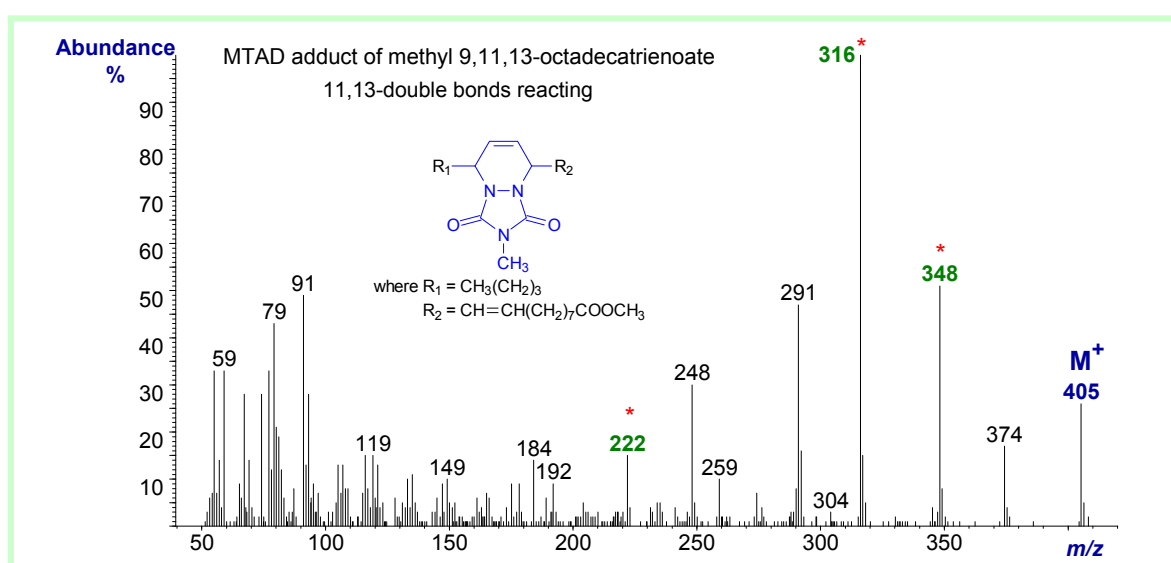
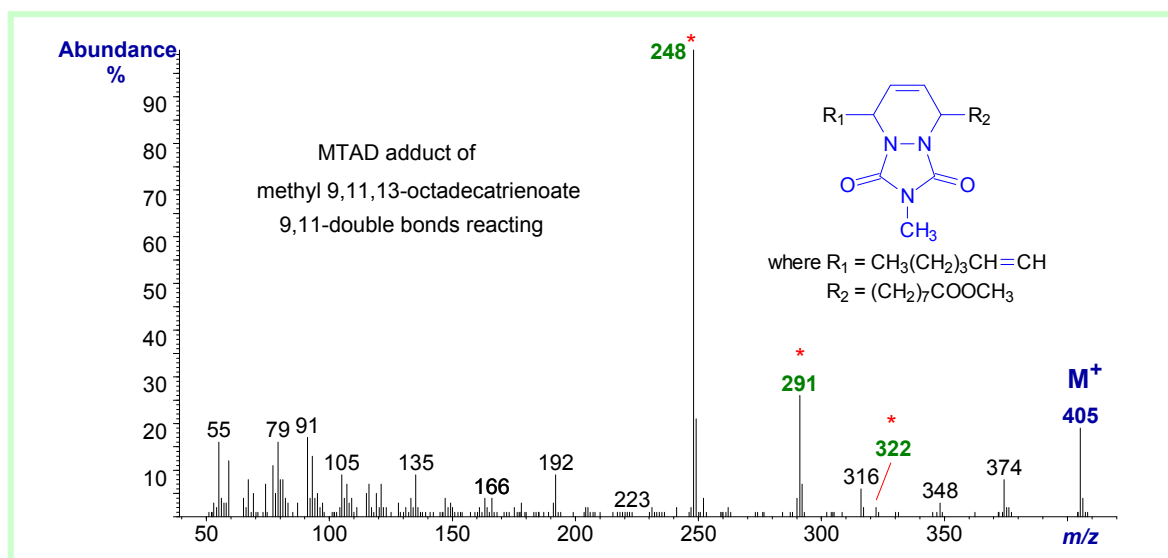
In this instance, the carbons that were part of the original conjugated double bond system can be located by the ions at  $m/z = 336$  and  $236$ , with that at  $304$  representing loss of methanol from the carboxyl-containing ion.

Spectra of the MTAD adducts of the 11,13- 12,14- and 13,15-18:2 isomers are in the **Archive** Section (but without interpretation).

When the reagent is reacted with a conjugated triene, such as punicic acid (9,11,13-octadecatrienoate), two possible products are formed.



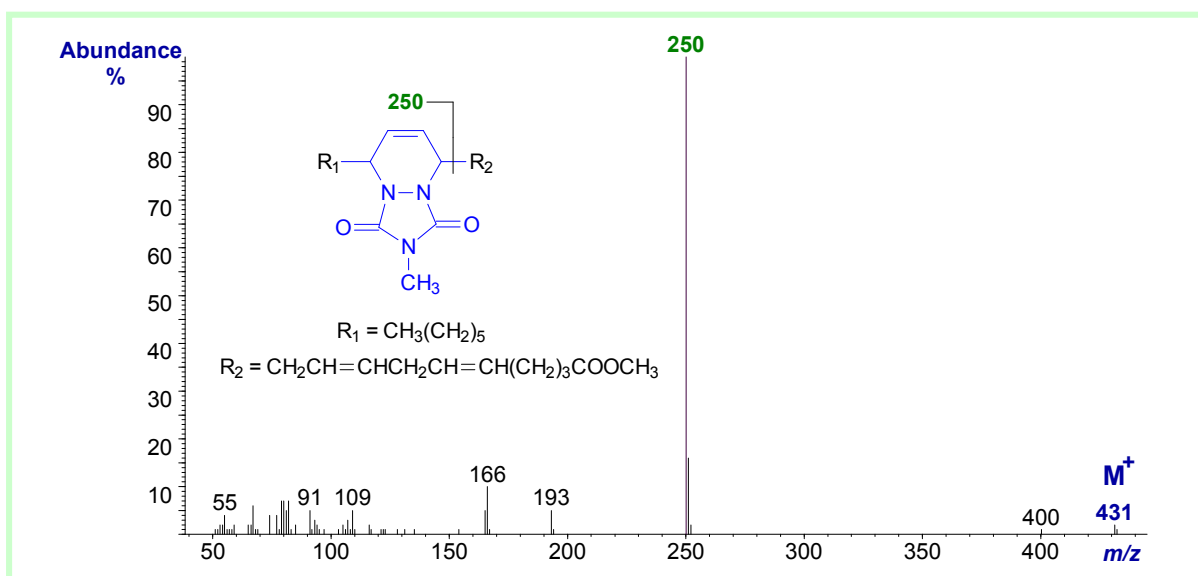
Both of these products are separated by GC and give diagnostic spectra, first with the 9,11-double bonds reacting and then with the 11,13-double bonds reacting –



In the first, The key diagnostic ions are expected to be at  $m/z = 248$  and  $322$ , but with the latter further fragmentation has occurred with loss of a methoxyl group, so that the ion at  $m/z = 291$  helps to define the structure.

With the second, interpretation is less straight forward, and the original article by Dobson [5] should be consulted. However, the important diagnostic ions at  $m/z = 222$  and  $348$  are present, as is the latter less the elements of methanol ( $m/z = 316$ ).

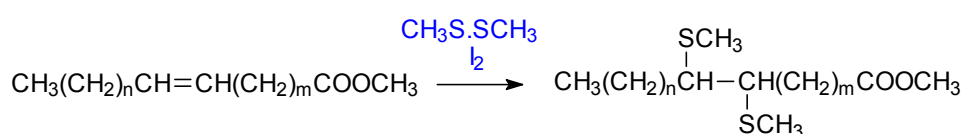
The method can also be used with care to locate conjugated dienes in the presence of non-conjugated double bonds, e.g. for 5,8,11,13-eicosatetraenoate [6], and the spectrum is -



However, the reagent is best used with fractions enriched in conjugated fatty acids if possible, as a small amount of reaction with methylene-interrupted double bonds, moving them into conjugation, can sometimes occur.

### Dimethyl Disulfide Adducts

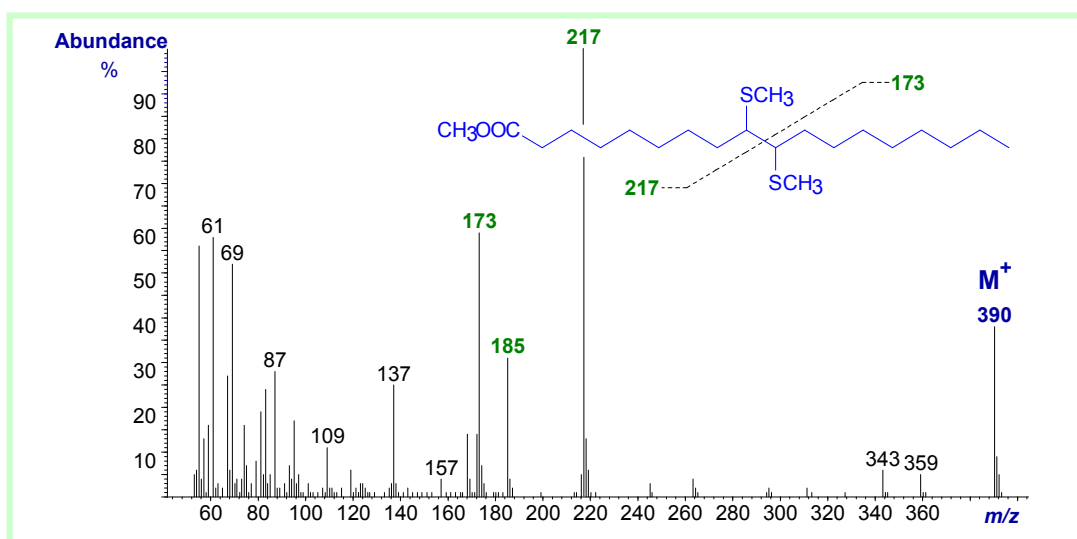
To get round the problem of locating double bonds, it is possible to prepare specific derivatives of unsaturated fatty acids that 'fix' the double bond. The most useful of these for monoenes are the dimethyl disulfide adducts, as they have excellent mass spectrometric properties and are prepared in a simple one-pot reaction (Francis [7]). It has become evident that the reaction may have much more to it than many have considered, and a number of interesting publications describing its use have appeared. These have been detailed in a substantial review elsewhere, and this publication should be consulted for many of the relevant references [8]. Indeed, there have been more than 80 publications using aspects of this method for fatty acids alone, and many more for pheromones, hydrocarbons, terpenes, etc. The adduct adds substantially to the molecular weight of the original ester, and tends to elute at a temperature about  $40^\circ C$  higher than the latter from a GC column containing a non-polar silicone phase.



Adduct formation has been shown to be entirely stereospecific, presumably by *trans* addition, so that *threo*- and *erythro*-derivatives are formed from *cis*- and *trans*-isomers respectively. Although the different geometrical isomers have indistinguishable mass spectra, they are eluted separately from GC columns containing either polar or non-polar phases, with that derived from the *cis*-isomer eluting first. There may be potential, therefore, to use this as an additional method for determining *trans* fatty acids. The most widely used method is outlined below (although a more rapid method has recently been described [9]).

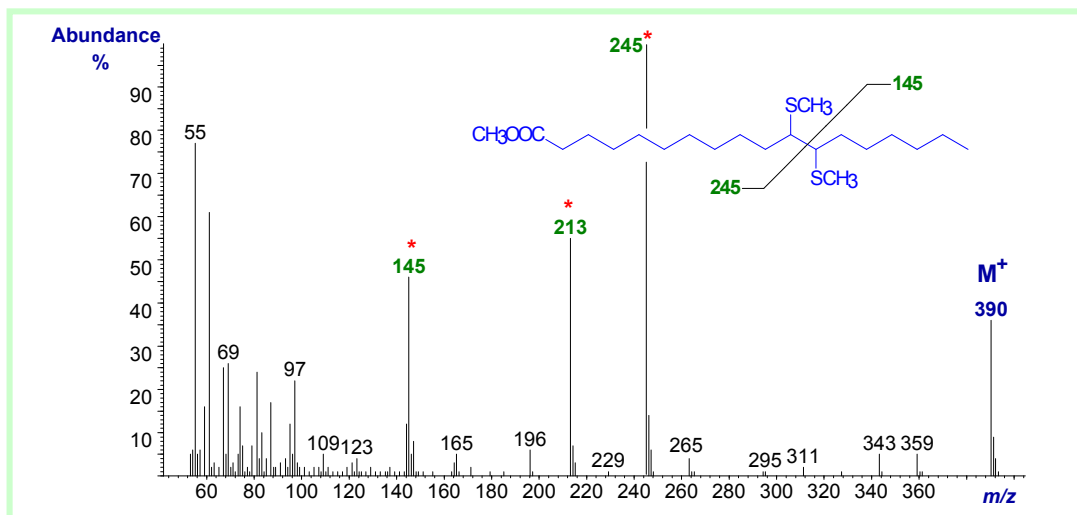
**Protocol:** The monoenes (1 mg) are dissolved in dimethyl disulfide (0.2 mL) and a solution (0.05 mL) of iodine in diethyl ether (60 mg/mL) is added. The mixture is stirred for 24 hours, then hexane (5 mL) is added, and the mixture is washed with dilute sodium thiosulfate solution, dried over anhydrous sodium sulfate and evaporated to dryness. The product is taken up in fresh hexane for injection directly onto the GC column.

The mass spectrum of the **dimethyl disulfide adduct of methyl oleate** is illustrated first -



Cleavage occurs between the carbons that originally constituted the double bond to yield two substantial fragment ions, i.e. that containing the terminal methyl part of the molecule at  $m/z = 173$  and that with the carboxyl group at  $m/z = 217$ . A further prominent ion at  $m/z = 185$  corresponds to the latter fragment with the loss of the elements of methanol.

The mass spectrum of the **dimethyl disulfide adduct of methyl 11-octadecenoate** follows -



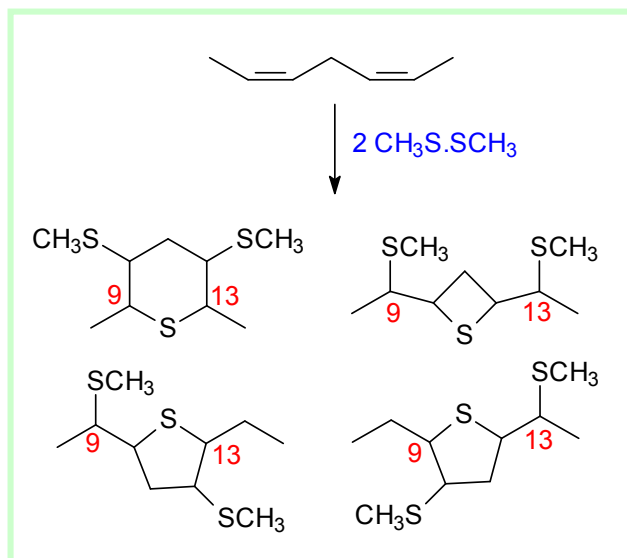
The spectrum differs from the previous one in that the diagnostic ions are shifted by 28 amu as expected.

Also, DMDS adducts can be resolved better than the unchanged esters on GC columns, and this property has been used to separate and quantify petroselinic, oleic and vaccenic acids in seed oils [10]. While simpler alternatives may be available for this specific purpose, it may be worth keeping it in mind for confirmation or for other difficult analyses.

**Dienoic fatty acids** present more of a problem than monoenes for the technique. The considerable increase in molecular weight means that rather high temperatures are required for GC analysis. Also, when the two double bonds are in close proximity, complications can arise in the reaction with dimethyl disulfide. There is no problem when double bonds are separated by more than four carbon atoms, but this is a relatively rare occurrence in nature. However, 9,15-octadecadienoic acid (with four methylene groups between the double bonds) from mango pulp was characterized simply as the *bis*-DMDS derivative [11]. Similar types of dienoic fatty acids have been characterized from sponges in Carballeira's laboratory in the same way.

When the double bonds are closer together, a variety of products is possible. Most natural dienoic fatty acids have methylene-interrupted double bonds. When dimethyl disulfide was reacted under mild conditions (30 minutes reaction, 35°C) with methyl linoleate, only one double bond reacted [12]. Thus, an equimolar mixture of methyl 9,10-*bis*(methylthio)octadec-12-enoate and methyl 12,13-*bis*(methylthio)octadec-9-enoate was formed, and again distinctive mass spectra were obtained, which permitted location of the double bonds. This technique has also been used with ether lipids.

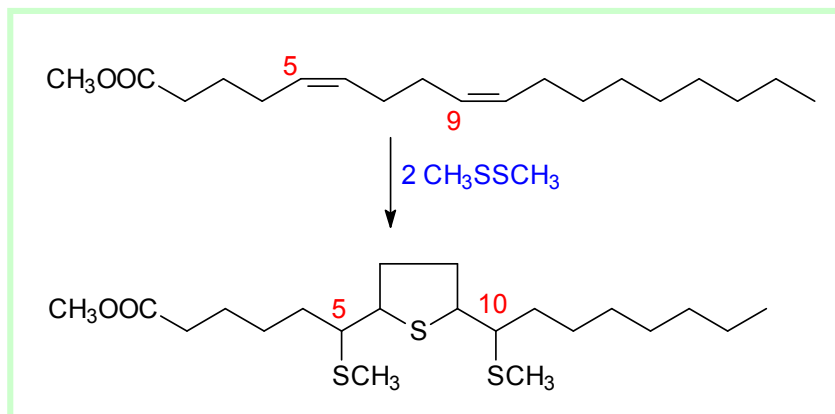
When higher temperatures (up to 60°C) and longer reaction times (40 hours) were employed, a second mole of dimethyl disulfide was added, and cyclization occurred giving heterocyclic compounds with thietane, tetrahydrothiophene and tetrahydrothiopyran structures (4-, 5- and 6-membered rings), as illustrated [13,14] -



The reaction has been studied in a number of laboratories, but mainly in that of Carballeira. These heterocyclic compounds can give characteristic and diagnostic spectra also, so that the technique continues to have some practical value. In one report, only thietane formation was observed, but in more systematic studies four distinct products were obtained in proportions that varied according to the reaction conditions [6]. Di-*cis* and di-*trans* forms of linoleate gave products with distinct

stereochemistry and different chromatographic properties, suggesting again that the technique might have value for determining the geometry of double bonds in such fatty acids [13].

Long-chain fatty acids with 5,9-diene systems are common constituents of marine sponges. Reaction of these with dimethyl disulfide under appropriate conditions gives a 5-membered cyclic thioether substituted with two alkyl chains, each containing a methylthio group on the carbons immediately adjacent to the ring as illustrated [12,15] -



These compounds also give characteristic mass spectra permitting location of the double bonds, and many different demospongiac acids of this type have been identified in this way, including some containing bromine atoms and methyl branches in addition to the 5,9-double bond system.

**Polyunsaturated fatty acids.** It is possible to generate monoenes from polyunsaturated fatty acids in order to use the reaction. Thus, heneicosapentaenoic acid (21:5(n-3)) from eel lipids was first isolated by silver ion TLC, before being subjected to partial hydrogenation with hydrazine to a mixture of monoenes; these were then converted to DMDS derivatives for structural analysis by GC-MS [16]. 5,9,12-18:3 and 5,9,12-17:3 fatty acids have been identified by this means from such odd natural sources as mites and slime moulds.

In addition to the difficulties with dienes and polyenes, I have not found DMDS derivatives suitable for determining the position of the double bond in a cyclopentene ring, although confirmation of the positions of double bonds in the aliphatic chain of natural fatty acids with this structural feature was obtained. It has been used successfully for hydroxy, methoxy, branched-chain and benzene ring-containing fatty acids with one double bond in the aliphatic chain.

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