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Derivatization in Gas Chromatography of Lipids

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Derivatization is performed as part of gas chromatographic (GC) analysis of lipids primarily to extend the spectrum of substances that can be determined by this method. Derivatization in general converts less volatile and thermally labile substances into compounds that can be analyzed in the gaseous state. One of the most important methods is protection (derivatization) of polar groups such as –OH, $–NH_2$, –SH, or –COOH. Due to an elevated temperature in a gas chromatograph, the analysis of compounds having free polar groups either suffers from a poor division (formation of tailing peaks) like, e.g., in the case of free fatty acids, or the analysis completely fails, e.g., in the case of saccharides formed after the hydrolysis of glycopeptides (e.g., digalactosyldiacylglycerols) (Table 1).

Another area where it is advantageous to use derivatization involves compounds, the derivatization of which yields derivatives with increased utility value. An example is the derivatization of fatty acids giving rise to nitrogen derivatives, e.g., picolinyl esters which make it possible to identify the position of the double bond(s) in the fatty acid chain using gas chromatography–mass spectrometry (GC–MS) (Harvey 1992). Similarly, it is possible to derivatize the double bond of dimethyl disulfide; the resulting adducts in the mass spectrum then show characteristic fragments (Christie and Han 2010; Christie 1998).

There are thus basically two reasons why derivatization should be carried out. The first is to permit the analysis of compounds that cannot be analyzed in their native state because of thermolability or low volatility, the second is that derivatization aids in increasing the utility value of the analyzed compounds or increases the limit of detection (e.g., higher sensitivity of electron capture detector to pentafluorobenzyl esters of fatty acids). Examples of compounds which cannot be chromatographed without derivatization include diacylglycerols formed after the hydrolysis of phospholipids, glycolipids, or triacylglycerols.

In the case of highly volatile compounds, such as the volatile fatty acids produced by oxidative ozonolysis and subsequent treatment of unsaturated fatty acids, esterification with pentafluorobenzyl bromide gives less volatile esters.

One of the methods most frequently used in gas chromatography is silylation that gives rise to silyl ethers. In essence, silylation is a replacement of hydrogen in a polar group such

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as –OH, $–NH_2$, –SH, or –COOH that yields silyl derivatives that have much higher volatility than the starting compounds and are more thermostable. The disadvantage is their susceptibility to hydrolysis caused by even traces of atmospheric moisture.

All these pros and cons underlie some restrictions and recommendations that have to be observed when performing derivatization. The first is the exclusion of even traces of water and alcohols (e.g., methanol after lipid extraction by the method of Bligh and Dyer (1959)). Furthermore, all silylating agents are toxic and burn skin and mucous membranes, and derivatization thus has to be carried out in a well-ventilated fume cupboard.

The following sequence of reactions should be performed by experienced organic chemists rather than for routine analytical practice.

After silylation, gas chromatography of a monosaccharide (galactose) formed by the hydrolysis of a glycolipid (e.g., digalactosyldiglyceride) gives four peaks, i.e., α-galactopyranose, β-galactopyranose, α-galactofuranose, and β-galactofuranose. The situation can be simplified by alkylation of the oligosaccharide by methyl iodide (labeling free –OH groups), reduction of acetals (ketals) by $NaBD_4$ (labeling carbon no. 1 of the galactose), and esterification by trifluoroacetic acid anhydride (marking –OH groups forming the bond with a neighboring sugar, or a pyranose or furanose cycle) (Biermann and McGinnis 1988). For more details see the Fig. 1.

Below are examples of derivatizing methods and agents suitable for individual groups (classes) of compounds having free polar groups such as –OH, $–NH_2$, –SH, or –COOH. One should note, however, that the advancing instrumental techniques shift the focus of analysis of the less volatile and thermolabile compounds with higher molecular weight from gas chromatography to HPLC. Steroids can be mentioned as example; the description of their derivatization in a 40-year-old publication Ahuja (1976) covers almost one sixth of the review concerned with their analysis. At present (according to the Web of Science) analysis of steroids by HPLC in 2015 has been performed five times more often than using gas chromatography.

The derivatives most often analyzed by gas chromatography are fatty acid esters. Fatty acids, most often in the form of methyl esters, are obtained by hydrolysis of complex lipids, both polar (e.g., phospholipids or glycolipids) or nonpolar, e.g., triacylglycerols, followed by derivatization. Methyl esters are most often prepared either by the reaction of free acid with esterifying agent, e.g., BF_3-methanol or HCl-methanol, or by the reaction of acid with diazomethane. All of these methods (see the brief list of reactions below) have their pros and cons described in many reviews and textbooks and also in other chapters of this compendium. It should be noted that as with any chemical reaction carried out in organic chemistry, two undesirable phenomena may occur. The first is that the yield of the desired methyl ester is not 100 %, and the second is the formation of by-products, artifacts that may in many cases make it difficult to identify the desired methyl esters or even may lead to wrong conclusions. Another important group that is being derivatized in lipidomic analysis includes saccharides. Because five entries in this compendium are devoted to methyl esters, we shall focus on other derivatives.

The compounds used most often for determining the position of the double bond are either nitrogen derivatives – picolinyl esters (esters of 3-pyridylcarbinol, correctly nicotinyl esters because picolinyl alcohol is 2-pyridylcarbinol), dimethyloxazoline, and pyrrolidide derivatives. It is difficult to say which derivatives are given preference since all have some drawbacks as well as advantages. Dimethyloxazoline derivatives have the best chromatographic properties of all three groups and are suitable for conjugated systems of double bonds, e.g., conjugated linoleic acid. Conversely, picolinyl esters are eluted from the column at a temperature about 40 °C higher than the methyl esters, but their mass spectra are most readily identified including the use of the gaps of 26 and 40 Da. Their further advantage is that they can be easily analyzed by HPLC with UV detector (Christie and Stefanov 1987).

Derivatization in Gas Chromatography of Lipids, Table 1 Names of conventional derivatizing agents

Method	Reagent	Common name
Silylation	BSA	Bistrimethylsilylacetamide
	BSTFA	Bistrimethylsilyltrifluoroacetamide
	HMDS	Hexamethyldisilane
	MSTFA	N-methyl-trimethylsilyltrifluoroacetamide
	MTBSTFA	N-methyl-N-t-butyldimethylsilyltrifluoroacetamide
	TMCS	Trimethylchlorosilane
	TMS-DEA	Trimethylsilyldiethylamine
	TMSI	Trimethylsilylimidazole
Acylation	HFBA	Heptafluorobutyric Anhydride
	HFBI	Heptafluorobutyrylimidazole
	MBTFA	*N*-Methyl-bis(trifluoroacetamide)
	PFBCI	Pentafluorobenzoyl Chloride
	PFPA	Pentafluoropropionic Anhydride
	PFPI	Pentafluoropropionylimidazole
	TFAA	Trifluoroacetic Anhydride
	TFAI	Trifluoroacetylimidazole
Alkylation	BF_3 (BCl_3)	in methanol or butanol
	DMA	dimethylacetal
	PFBBr	Pentafluorobenzyl bromide
	TBH	Tetrabutylammonium hydroxide

Preparation of these derivatives is described in Christie (1989) only briefly: The acid is heated with thionyl chloride for about 10 min at 100 °C, the excess reagent is evaporated in nitrogen flow and 3-pyridylcarbinol in acetonitrile is added. The mixture is again heated for 1 min at 100 °C and may then be injected directly onto the GC or further purified (Fig. 2). For the identification and interpretation of the mass spectra, see Harvey (1992).

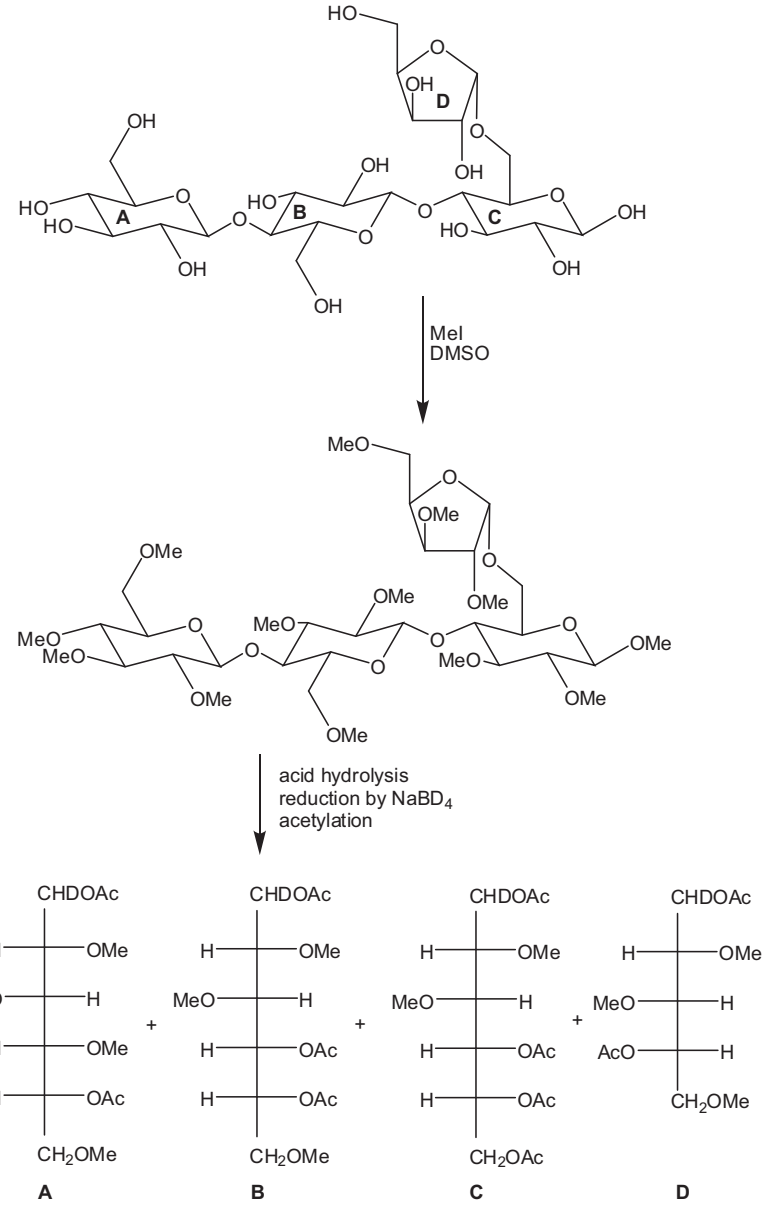
When acids are present, mostly polyunsaturated or containing other functional groups (hydroxyl groups, epoxy group, etc.), then it is preferable not to heat the reaction mixture and use carbonyldiimidazole and 3-pyridylcarbinol, see the Fig. 3.

Dimethyloxazolines are prepared by one step synthesis (Fig. 4), see the scheme below. In the case of free fatty acids, the reaction of a fatty acid with 2-amino-2-methylpropanol (commercially available) takes place for two hours in a nitrogen atmosphere at 180 °C, while in the case of methyl esters or intact lipids the reaction time has to be extended to more than 10 h. However, such high temperature results in a partial degradation of heat-sensitive acids. The reader will find the interpretation of their mass spectra, e.g., Harvey (1992).

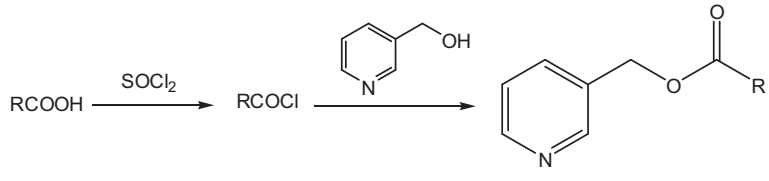
An alternative procedure (Fig. 5) can be used to eliminate degradation at higher temperatures. Acid chlorides, prepared as in the case of 3-pyridylcarbinol esters (see above), are left to react with 2-amino-2-methylpropanol in dichloromethane. This solution is added to a solution of acid chlorides under ice-cooling and the reaction mixture is left for 1 h at room temperature. CH_2Cl_2 is removed in a nitrogen stream, trifluoroacetic anhydride is added and the mixture is either heated for 1 h at 50 °C or is allowed to stand for 3 h at room temperature. Hexane-water mixture is added; the hexane layer is separated, dried, and the solution is ready for GC analysis.

The last derivatives are pyrrolidides, which are prepared by reacting methyl esters with pyrrolidine in the presence of acetic acid, see the Fig. 6.

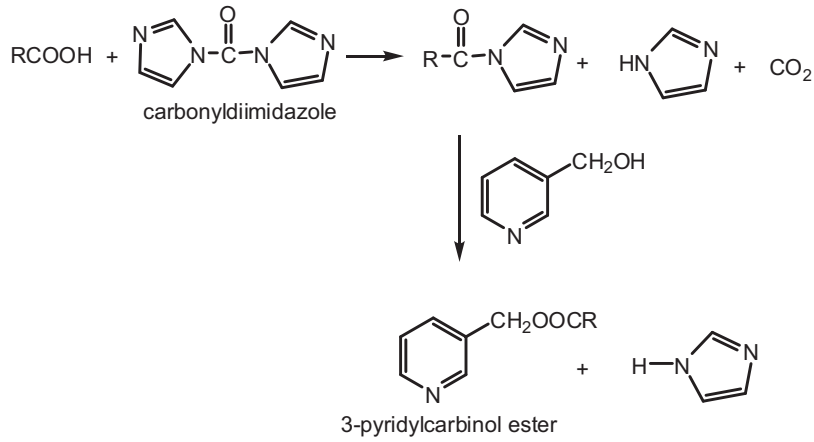
A mixture of methyl esters, pyrrolidine, and acetic acid is heated at 100 °C for 1 h, excess



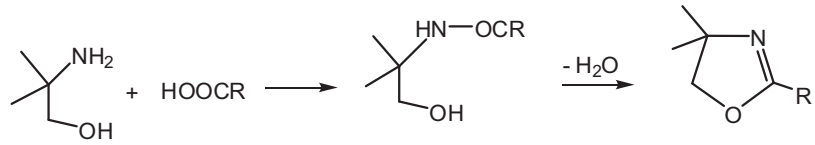
Derivatization in Gas Chromatography of Lipids, Fig. 1 Determining the structure of an oligosaccharide



Derivatization in Gas Chromatography of Lipids, Fig. 2 Synthesis of 3-pyridylcarbinol esters



Derivatization in Gas Chromatography of Lipids, Fig. 3 Mild synthesis of 3-pyridylcarbinol esters



Derivatization in Gas Chromatography of Lipids, Fig. 4 Synthesis of dimethyloxazolines

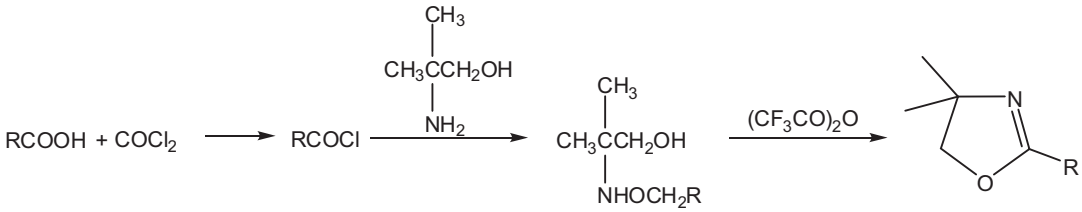
pyrrolidine is evaporated in nitrogen flow and the mixture is diluted with water and extracted with hexane-diethyl ether. After drying it can be analyzed by GC. Triacylglycerols or free fatty acids may be used instead of the methyl esters.

Further derivatization methods, though somewhat different, include many procedures such as oxidation with the permanganate-periodate reagent (frequently termed "von Rudloff oxidation"), and ozonolysis followed by oxidative or reductive cleavage of the ozonide. These methods, however, are completely beyond the normal derivatizations, and their merits and shortcomings are treated in specialized review (Christie 1989).

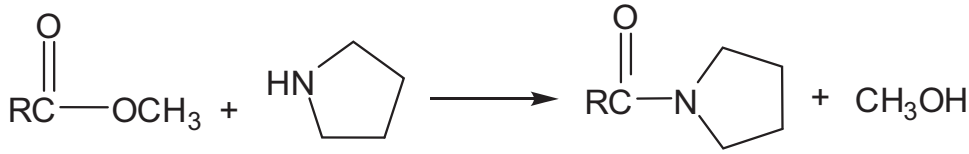
We believe that hydroxylation of double bonds has only historical significance at present. These bonds can be oxidized to vicinal diols by, e.g., alkaline potassium permanganate and osmium tetroxide. The same applies to epoxidation and deuteration. These methods are now hardly ever used and are being replaced by the technique developed by Brenna (2006). Covalent adduct chemical ionization (CACI) mass spectrometry was developed as a very fast method for determination of double bond(s) by a direct derivatization in mass spectrometer. Its principle is based on the addition of acetonitrile, or better stated the addition of (1-methyleneimino)-1-ethenylium (MIE) formed from acetonitrile in the mass spectrometer and giving rise to an adduct, see the Fig. 7. A great advantage of this method is in avoiding the necessity of derivatizing methyl esters outside the mass spectrometer. The analysis can be directly carried out and the data from MS or tandem MS can be readily interpreted by Brenna (2006) based on the presence of alpha or omega ions. Reaction of MIE in an antiparallel sense followed by analogous dissociation yields the ω-diagnostic ion.

Dimethyl disulfide addition was described by Christie and Han (2010). Under iodine catalysis, dimethyl disulfide reacts with a double bond(s) of fatty acids to form a derivative(s) according to the following Fig. 8.

Fatty acids (ideally only monoenes) are dissolved in dimethyl disulfide, and a solution of iodine in diethyl ether is added dropwise. The reaction mixture is stirred for 24 h at room temperature and the reaction is terminated by addition of hexane-aqueous solution of sodium thiosulfate. The hexane layer is separated, dried, and is ready for GC analysis.



Derivatization in Gas Chromatography of Lipids, Fig. 5 Mild synthesis of dimethyloxazolines



Derivatization in Gas Chromatography of Lipids, Fig. 6 Synthesis of pyrrolidides

The method is much less suitable for the analysis of dienes to polyenes due to the occurrence of (often major) side-products (Fig. 9).

The use of Diels-Alder (MTAD) adducts for conjugated double bonds is also at present minor. The principle consisted reaction of the fatty acid methyl ester with the 4-methyl-1,2,4-triazolin-3,5-dione, to form the adduct as shown in Fig. 10.

Most of hydroxy fatty acids including eicosanoids may exist as enantiomers (*R*/*S*). For instance, 2-hydroxypalmitic acid (see Fig. 11) can be separated in the form of diastereomeric fatty acid derivatives (Beneytout et al. 1986), e.g., L-(−)-menthyloxycarbonyl, methoxytrifluoromethylphenyl acetate, (*R*)-(+)-1-phenylethyl urethane and D-phenylpropionate derivatives.

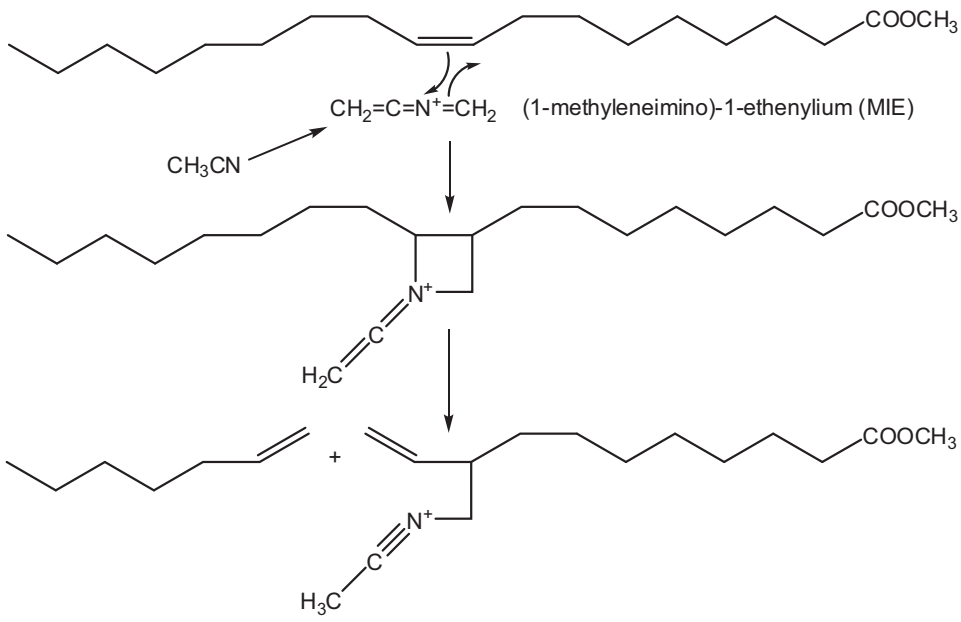
Diastereomeric methoxytrifluoromethylphenyl acetate derivatives of methyl 2-hydroxypalmitate and 2-hydroxystearate were separated by GC on fused silica columns (25 m x 0.32 mm) coated with OV-1 phase. The column was temperature programmed from 180 to 240 °C at 2 °C/min, and the carrier gas was helium (Beneytout et al. 1986).

An example of a fine analysis is the resolution of a chiral branched-chain fatty acids, which may be done as a separation of L-(−)-menthol esters (Fig. 12) of eight possible diastereomers of pristanic acid. Their separation was and still is difficult; however, it has been proved that a halophilic bacterium contained DDD isomer, while other sources contain mixtures of diastereoisomers the proportions of which depend on the biological material.

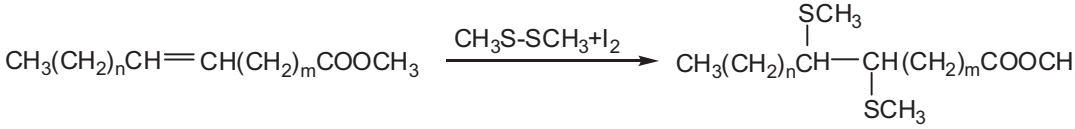
Summary and Conclusion

The choice of suitable derivatization methods useful for the analysis of lipids, mainly fatty acids, using the GC is dependent on many factors (Blau and King 1979; Knapp 1979). The derivatives have to be suitable, detectable, and efficient for GC or better for GC–MS analysis. Today, with a few exceptions, it is preferable to use GC–MS, and relevant derivatives should therefore give appropriate fragmentation patterns of compounds and thus provide useful information on the structure of molecules. Non-derivatized lipids, e.g., free fatty acids or long-chain free alcohols, can also be analyzed by GC as well, but during their chromatography they will tend to tail because of the adsorption and non-specific interaction with the column.

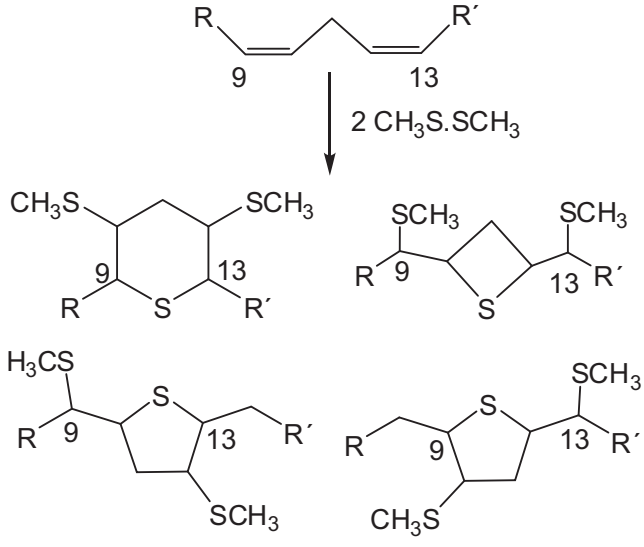
Virtually all functional groups pose problems in GC. It should be borne in mind that the GC method has been developed and, at the beginning also used, only for the analysis of hydrocarbons from oil and oil products (petrol, diesel). Therefore, it is advisable to use derivatization by the almost universal but less specific silylation. Silyl



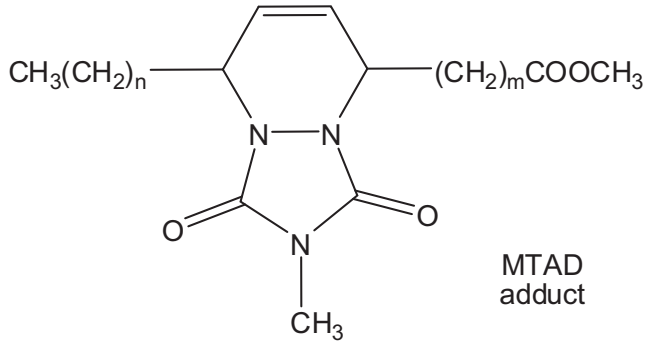
Derivatization in Gas Chromatography of Lipids, Fig. 7 Covalent adduct chemical ionization mass spectrometry



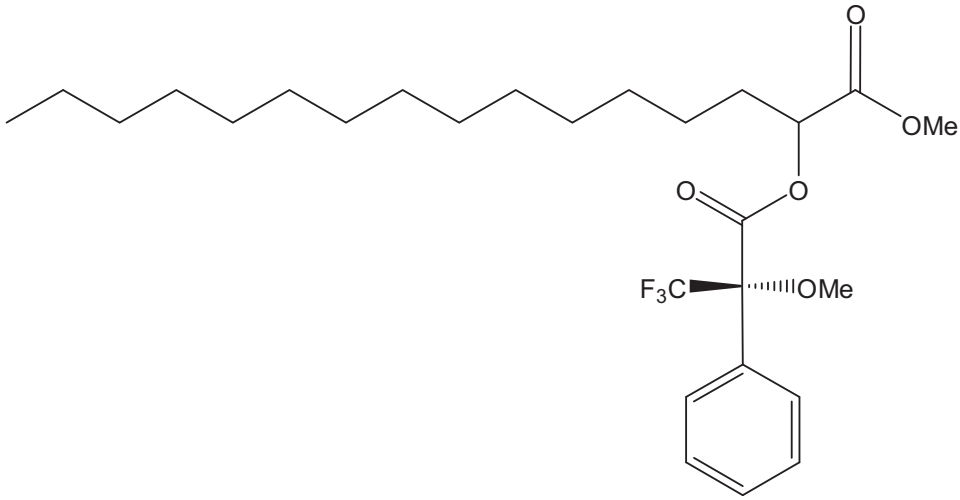
Derivatization in Gas Chromatography of Lipids, Fig. 8 Synthesis of dimethyl disulfide adducts



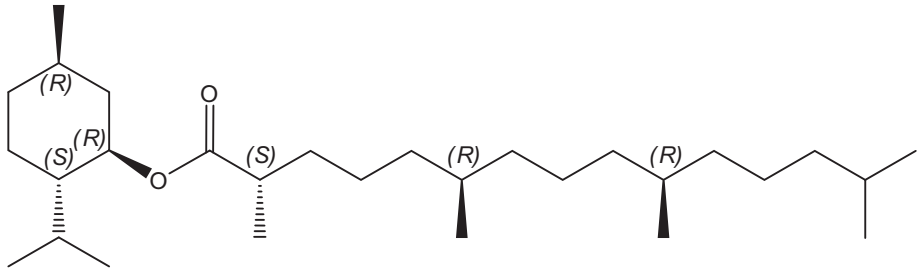
Derivatization in Gas Chromatography of Lipids, Fig. 9 By-products of dimethyl disulfide addition



Derivatization in Gas Chromatography of Lipids, Fig. 10 Structure of Diels-Alder adduct



Derivatization in Gas Chromatography of Lipids, Fig. 11 Structure of methoxytrifluoromethylphenyl acetate with 2-hydroxypalmitic acid



Derivatization in Gas Chromatography of Lipids, Fig. 12 (2S, 6R, 10R)-2,6,10,14-tetramethylpentadecanoic (pristanic) acid

derivatives are generally less polar, more volatile, more thermally stable, and they also serve to enhance mass spectrometric properties. They also give the characteristic ions of use in trace analyzes (e.g., ion at 73 Da, i.e., -Si$(CH_3)_3$). In conclusion, we can say that selection of appropriate methods of derivatization can help considerably both in chromatography alone as well as in the identification of lipid compounds by GC–MS analysis.

Cross-References

- ▶ Gas Chromatography of Fatty Acid Methyl Esters: Derivatization
- ▶ Gas Chromatography of Fatty Acid Methyl Esters: Fast Analysis
- ▶ Gas Chromatography of Fatty Acid Methyl Esters: Practical Applications of Fast Gas Chromatography
- ▶ Nomenclature of Fatty Acids

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