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Analysis of Long-Chain Fatty Acids by Gas-Liquid Chromatography

Micromethod for Preparation of Methyl Esters

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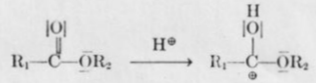
▶ **An essential prerequisite for the analysis of lipide mixtures of biological origin by gas-liquid chromatography (GLC) is the quantitative formation and isolation of the constituent methyl esters. A micromethod is described involving interesterification with methanol and hydrochloric acid. By sublimation the methyl esters are isolated from the reaction products in a pure form ready for gas-liquid chromatography. The methylation and sublimation can be done with ease on a large number of samples. This method eliminates the use of alkali and diazomethane, which may lead to isomerization or pyrazoline formation.**

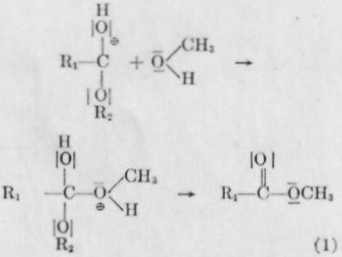
A RELIABLE method for formation of methyl esters of long-chain fatty acids necessarily precedes analysis by gas-liquid chromatography. The requirements of an optimal micromethod are: quantitative yield, absence of change in double-bond structure of highly unsaturated acids, and technical convenience.

Saponification of fatty acids esterified as glycerides, phosphatides, and sterol esters can be followed by methylation with diazomethane. However, this procedure suffers some disadvantages—yields may be poor because of the formation of addition products of diazomethane at ethylene bonds (pyrazolines) (*4*); structural changes in double bonds may occur during saponification (*1*); and the isolation of soaps from nonsaponifiable contaminants is rarely completely satisfactory.

Formation of fatty acid methyl esters by interesterification, on the other hand, has proved in this laboratory to be technically simpler, milder, and more quantitative than diazomethanolysis. Yields of methyl esters from glycerides, phosphatides, and cholesterol esters are nearly quantitative, alterations in double bond structures are avoided, and the technical aspects are relatively simple. Completeness of interesterification demands an acid medium and the absence of water. In an acid medium,

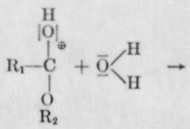


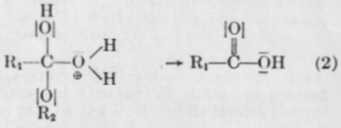
the electron-donating capacity of the oxygen in water, H—Ö—H, is greater than that of the oxygen in methanol, CH_3—Ö—H, and, therefore, methylation demands strictly anhydrous conditions. In the absence of water,



(1)

However, if water is present,





(2)

As Reaction 2 has preference over Reaction 1, methyl ester formation by interesterification is hindered in the presence of water.

In the method described below, any nonsaponifiable contaminants which may be present are eliminated by sublimation of the methyl esters. This feature assumes considerable importance when methyl esters are formed from cholesterol esters; the mixture of methyl esters applied to the gas-liquid column can be evaluated quantitatively only if free from cholesterol. Similarly, the triglycerides isolated by silicic acid chromatography from mixtures of naturally occurring lipides (*2*) are frequently contaminated by free cholesterol; the methyl esters formed by interesterification are conveniently freed of cholesterol by sublimation.

The procedure has proved satisfactory for interesterification of safflower oil glycerides, soybean phosphatides, and cholesterol palmitate, as well as for glycerides, sterol esters, and phosphatides isolated from human serum by silicic acid chromatography (*2*). Absence of changes in polyenoic acids was verified by ultraviolet and infrared spectrophotometry and by degradation studies (*5*).

METHOD

Reagents. Dry hydrochloric acid (5%) in superdry methanol (*6*).

Sodium sulfate–sodium bicarbonate mixture, reagent grade, anhydrous, 4 to 1 mixture by weight.

Petroleum ether, 30° to 60° C., Merck, reagent grade, redistilled.

Benzene, Merck, reagent grade, dried over sodium and distilled.

Apparatus. Microinteresterification assembly, 19/38 (*3*), consisting of round-bottomed test tubes, Liebig condensers, cold fingers, and six-place manifold, with nitrogen inlet. (Available *in toto* from Metro Industries, 11-38 31st Ave., Long Island City 6, N. Y., catalog No. ME-517, or piecemeal.)

McLeod gage.

High vacuum pump, cold-trapped.

Interesterification. The esters or acids to be methylated (1 to 10 mg.) are dissolved in 4 ml. of 5% hydrochloric acid in superdry methanol and 0.5 ml. of dry benzene in a 15-ml. microsublimation tube to which a condenser with a calcium chloride moisture trap is connected. The mixture is refluxed in a silicone bath at 80° to 100° C. for 2 hours, with frequent shaking at the start to dissolve the lipide mixture. After cooling to room temperature, two volumes of water are added, and the methyl esters are extracted three times with 3 ml. of petroleum ether. The pooled extracts are simultaneously neutralized and dried over sodium sulfate–sodium bicarbonate mixture for 1 hour. The esters are then quantitatively transferred with petroleum ether to a second microsublimation tube and the solvent is evaporated to dryness at reduced pressure in a 40° C. water bath.

Microsublimation. After the microsublimation tube is fitted to the cold finger, a vacuum of 0.2 ± 0.15 mm. of mercury is produced. The tube is then lowered into a silicone bath at 60° ± 2° C. for 60 minutes. The assembly is disconnected after cooling, and the sublimed methyl esters are rinsed off with petroleum ether into a glass-stoppered tube. After evaporation of solvent, the preparation is now ready for application to the gas-liquid chromatography column.

The sublimation technique is described for use with 1 to 5 mg. of methyl esters. If greater quantities are sublimed, an increased sublimation time may be required. Use of a manifold permits several sublimations to be carried out simultaneously.

COMMENTS

In pilot experiments with soybean phosphatides, safflower oil, and cholesterol palmitate, the recovery of methyl esters was better than 95% of theory. When free cholesterol was added to the methyl esters prior to sublimation, it remained quantitatively as residue in the microsublimation tube, and the methyl esters were Liebermann-Burchard negative. However, sublimation at 85° C. and 0.2 mm. of mercury permits traces of cholesterol to collect on the cold finger, more at higher temperatures. In the case of soybean phosphatides, the entire phosphorus content of the aliquot selected for interesterification remained in the water washes and yields of methyl esters were quantitative, indicating completeness of methylation.

Benzene is incorporated in the interesterification mixture in order to assure complete solution of the lipides. This is essential in the case of the cholesterol esters which are not completely soluble in methanol. Unless the lipides are completely dissolved, interesterification is incomplete. When these precautions are observed during the interesterification of cholesterol esters, the recoveries of methyl esters on the cold finger and of free cholesterol in the residue are quantitative.

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