Determination of higher fatty acids in blood serum using diazomethane for their methylation

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Original title: Opredelenie vysshikh zhirnykh kislot syvorotki krovi metodom gazo-zhidkostnoi khromatografii s primenieniem diazometana dlya ikh metilirovaniya


Translated by the Translation Bureau (GES)
Multilingual Services Division
Department of the Secretary of State of Canada

Department of the Environment
Fisheries and Marine Service
Halifax Laboratory
Halifax, N.S.

1976
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Laboratornoe delo, No. 2, 1975, pp 90-92(USSR)

Laboratory Work , No2, 1975, pp 90-92(USSR)
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**Laboratornoe Delo** (Laboratory Work), No. 2, 1975, pp 90–92 (USSR)

UDC 616.153.295-074;543.544.45

**DETERMINATION OF HIGHER FATTY ACIDS IN BLOOD SERUM BY GAS-LIQUID CHROMATOGRAPHY USING DIAZOMETHANE FOR THEIR METHYLATION**

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It has become possible to determine the qualitative and quantitative composition of higher fatty acids in biological liquids and tissues through the use of chromatographic methods of analysis. Gas-liquid chromatography has been shown to be the most convenient and reliable of such methods. This approach has been used in recent times to determine the fatty acid composition of blood serum both in experiments and in clinics [1,2] so that it has been possible to obtain more extensive information concerning the state of fat exchange in normal, healthy individuals and in various pathological states.

By combining gas-chromatography with physical-chemical methods of separating general lipids into distinct fractions, it is possible to establish the fatty acid composition of each of them. However, in any case, the determination of fatty acids in a preliminary test-sample using gas-liquid chromatography requires that they initially be converted into methylated ester which possess a higher degree of

*Numbers in the right-hand margin indicate the corresponding pages in the original.*
volatility.

The use of acetyl chloride for the methylation of fatty acids has received wide application in research practice [3,4]. This method is not particularly complex, but does, however, require considerable time and provides a relatively low percentage of methylation. A proposed method of methylation of fatty acids using a mixture of boron fluoride and methylated spirits [5] has not been widely used because of its high toxicity.

In 1960 Schlenk and Gellerman [6] proposed that diazomethane be used for the esterification of fatty acids in order to obtain a high yield of methylated esters. However, work with diazomethane following the method recommended by these authors requires special precautions because of the toxicity of this substance. In consultation with and through the technical aid of the Laboratory of Physical-Chemical Methods of Analysis and Standardization (Director — Candidate of Chemical Sciences V. A. Zakupra) of the Kiev VNIIPK "Neftekhim" (Oil-Chemistry), we developed a relatively simple apparatus for the methylation of fatty acids using diazomethane. With its aid, it is possible to avoid potentially explosive concentrations of diazomethane as it allows us to control the amounts produced. The surplus of diazomethane remaining after the methylation is absorbed in the ether within the system, which is, for practical purposes, a closed one. This system for methylation using diazomethane has demonstrated good productivity and completeness of the methylation of higher fatty acids, while requiring comparatively simple procedures.

The extraction of the general lipids from blood serum and their

1 All-Union Scientific Research Institute of Applied Chemistry.
saponification were conducted according to the method of A. G. Vereshchagin and his co-authors [7]. To a test tube containing 10—20 ml of a mixture of methanol and ether (3:1), 0.5 ml of blood serum was added a drop at a time. The test tube's contents were energetically shaken for 1—2 mins, heated in a water bath for 5 mins at 100 degrees, and filtered through a degreased paper filter. This lipid extraction was repeated twice more. The combined extracts were concentrated by evaporation in a water bath in a nitrogen atmosphere. Then 0.2 ml of 4 N. spirit solution KOH was added to the residue, energetically shaken, and placed in a water bath at 80 degrees for 30 mins. After the saponification 2—3 ml of heptane were added to the mixture (to remove the unsaponified lipid components) and it was let stand for 20 mins. Next the heptanoic layer was suctioned off and discarded. The washing process with heptane was repeated two more times. To the remaining mixture, 0.4 ml of 4 N. sulphuric acid was added, the test tube energetically shaken, and 3 ml of heptane added to extract the fatty acids. The extraction procedure was repeated twice more. The combined heptanoic extract was concentrated in a water bath in a nitrogen atmosphere. The dry residue containing the mixture of fatty acids was transferred quantitatively to the apparatus for methylation.

**Methylation of Fatty Acids.**

The apparatus proposed in this article for the methylation of fatty acids using diazomethane (see diagram) includes a funnel with a ground glass stop-cock (1), a 250 ml flask with a side-branch outlet (4), and two containers with the dimensions 150·30 mm (2,3). The side branch-
Apparatus Diagram for the Methylation of Higher Fatty Acids Using Diazomethane.

Explanation in the text.

Pieces (0.5-0.7 cm diam) of the containers lead downward at right angles and each of them extends almost to the bottom of the receiving container. All the parts of the apparatus are connected using close fitting ground-glass slides.

Initially 3.36 g KOH, 1 ml hydrazine hydrate, and 1 ml dehydrated methanol are placed in container 2. The mixture of fatty acids which are to undergo methylation are transferred to container 3 and diethyl ether is poured into the flask (4). Then the funnel (1) is connected and it is filled with purified chloroform. At this point the apparatus is ready for use. The chloroform from the funnel is carefully added a drop at a time to container 2. (A large quantity of chloroform should not be added all at once since the resulting reaction
could cause the mixture undergoing reaction to be forced into the next container. As a result of the reaction involving the contents of container 2, diazomethane is formed.

\[
3\text{KOH} + \text{NH}_2 = \text{NH}_2 + \text{CHCl}_2 \rightarrow \text{CH}_2\text{N}_2 + 3\text{KCl} + 3\text{H}_2\text{O}.
\]

Through the side-branch outlet the diazomethane flows into container 3 and the methylation of the fatty acids takes place there.

\[
\text{R} - \text{COOH} + \text{CH}_2\text{N}_2 \rightarrow \text{R} - \text{COOCH}_3 + \text{N}_2.
\]

The surplus diazomethane which has not participated in the reaction passes into flask 4 where it is absorbed by the ether. The methylation process is allowed to continue until the test-sample of fatty acids has acquired a stable yellowish-green colouration. The methylation time for a sample mixture of fatty acids does not exceed 5 mins. After the methylation is completed the esters are concentrated in a water bath and the mixture of dry esters is dissolved in 1 ml n-hexane. The amount of hexane required to dissolve the fatty acids can be reduced to 0.5 ml or increased to 5 ml depending on the concentration of fatty acids expected to be involved.

The Separation of Fatty Acids Using Gas-Chromatography.

The analysis of the fatty acid esters takes place in a chromatograph Chrom-3-1K (Czechoslovakia) in a stainless-steel column 240 x 0.6 cm (internal diameter) using chromium sorbin W which has been silanized with hexamethyldisilizane. The particle size is 60--80 mesh. The column temperature is 185 degrees, that of the measuring hopper 300 degrees; the carrier-gas is helium and the rate of its passage through the column is 35 ml/min; a flame ionization detector is used. The velocity of the air and hydrogen (for maintaining the flame) is respectively 950 and
55 ml/min. The sensitivity of the recording instrument is 10 mV. The test sample of 1–3 μl volume is introduced using a Hamilton syringe. The analysis proceeds for 25 mins and is terminated after the arachidonic acid peak has emerged.

The concentration of the separate fatty acids is calculated by comparing the the peak areas obtained in the experiment with the corresponding peak areas in known quantities of standard methylated ethers/esters of fatty acids.

An investigation of the higher fatty acids in the blood serum of 13 healthy people between the ages of 20 and 30 gave the following results: the amount of palmitic acid was $110 \pm 4.8$ mg %, of stearic $62.8 \pm 2.6$ mg %, of oleic $141.4 \pm 4.2$ mg %, and of arachidonic $54.1 \pm 3.1$ mg%.

In special experiments related to the use of diazomethane to methylate a mixture of standard fatty acids, it was found that their methylation reached $98.7 \pm 0.6$ in comparison with the data for methylation using acetyl chloride referred to in the literature [3] of $95.4 \pm 1.8$ %.

Consequently, it can be concluded that the recommended method using diazomethane for the methylation of fatty acids gives a high percentage of methylated ester yield and that, thanks to the apparatus described above, its application in practice is simple, rapid, and safe.

Bibliography.


Жирные кислоты, метилирование, диазометан

Поступила 8/Х 1973 г.