notes on methodology

A source of contamination in the ultramicro analysis of methyl esters of fatty acids by gas–liquid chromatography

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SUMMARY Contaminants which could be erroneously identified as methyl esters of fatty acids on gas–liquid chromatographic (GLC) analysis were traced to anhydrous methanolic HCl used for methanolysis. Further studies indicated that the artifacts are not esters of carboxylic acids even though they mimic them on GLC analysis.

IN OUR ANALYSES of microgram quantities of brain lipid fatty acid methyl esters by gas–liquid chromatography (GLC) we have encountered extensive contamination which appears to be introduced during the methanolysis of the lipid.

The methanolysis procedure employed was a modification of that of Stoffel et al. (1) which was suitably scaled down for use with 6–10 μg of material. The instrument in use was a Pye Argon Gas Chromatograph. Chromatograms of blanks which had been carried through our entire procedure gave a number of GLC peaks which could be erroneously identified as methyl esters of commonly occurring fatty acids. The source of this contamination was traced to the anhydrous methanolic HCl (CH_3OH–HCl) used for methanolysis of the lipid. While diethyl ether extracts of the "residue" from as much as 50 ml of analytical reagent (Analar) grade methanol gave no peaks on GLC analysis, extracts of 0.25 ml of CH_3OH–HCl gave a number of peaks. Redistillation of the methanol prior to preparation of the acid solution in an all-glass apparatus did not prevent the appearance of the artifacts. Some contamination was encountered in freshly prepared 4–6 N CH_3OH–HCl both before and after refluxing for 4–6 hr; however, it increased greatly if the solution was stored for 48 hr or more in a refrigerator. Analytical reagent grade methanol from two manufacturers (Hopkin & Williams Ltd. and British Drug Houses Ltd.) and from several different batches have been used. Since any commercial HCl gas is liable to be contaminated by grease from the vessel and valves, we also prepared batches of CH_3OH–HCl by passing HCl gas, prepared in the laboratory from analytical reagents in an all-glass apparatus, into anhydrous methanol. All samples gave similar results. In Fig. 1 two chromatograms of the major artifacts are shown. A number of these peaks have the same retention volumes as the methyl esters of commonly occurring fatty acids. Several other peaks were observed which had carbon numbers (2) up to 27.8. These peaks were reproducible humps on the baseline (see peak 9) at the temperatures employed, but they became sharper at higher temperatures (190°) and would represent a serious hazard to investigators interested in trace components. It will be noted that on storage of the CH_3OH–HCl solution peak 8 increased greatly. We have found consistently that this artifact increases on storage to such an extent that it is detectable in extracts from 1 ml of solution at lower sensitivity settings. On the other hand when fresh solution is refluxed the artifacts appear to be produced in more nearly equal amounts.

We have been unable to collect sufficient quantities of the contaminants for detailed analysis. However, some simple tests have been performed. Spot tests for a carboxyl ester linkage (3) were negative. Thin-layer chromatography (TLC) employing Silica Gel G as the adsorbent revealed that the contaminants moved only when a polar solvent system was used (chloroform–methanol–acetic acid–water 65:25:8:4). The ether extract of 20 ml of 4 N CH_3OH–HCl which had been refluxed for 4 hr gave varying numbers of spots, depending on the detection method used. Charring after spraying with 50% H_2SO_4 revealed two spots, one at the solvent front and another with an R_F of 0.58. Exposure to iodine vapor revealed another spot (R_F 0.31) and Rhodamine 6G a fourth spot (R_F 0.86). In all cases the spot with R_F 0.58 was the most prominent. Several less polar solvent systems normally used for chromatography of fatty acid methyl esters (4) failed to move the material from the origin.

Samples of the contaminants were run on a column with a nonpolar stationary phase (Apiezon L) at 210°. It was found that while a particular sample gave the same number of peaks as on a polar polyethylene glycol adipate (PEGA) column, their retention volumes corresponded to different fatty acid methyl esters. Thus the major peak 8 (20:3)[1] on the chromatogram in Fig. 1 has the same retention volume on Apiezon as methyl palmitate (16:0). Other peaks on an Apiezon column with the same retention volumes as 14:0, 18:0, 18:1, 20:3, 20:4 were also obtained.

[1] The figure before the colon denotes number of carbons in chain; that after it, the number of double bonds.

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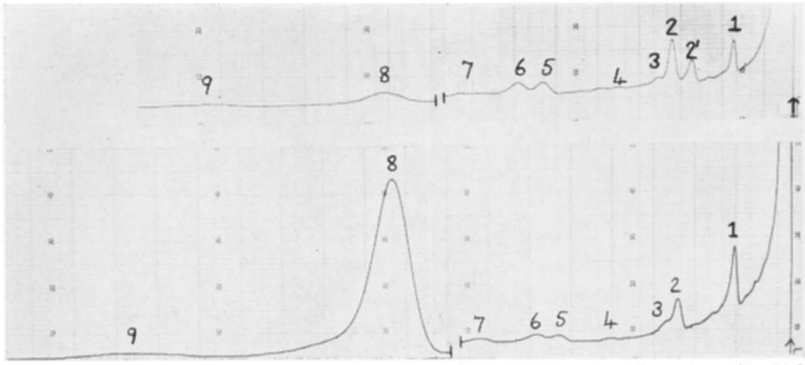

FIG. 1. Gas–liquid chromatograms of diethyl ether extracts of methanolic HCl using a polyethylene glycol adipate column. *Top:* extract from 0.25 ml of freshly prepared 4 N CH_3OH–HCl refluxed 4 hr. Total retention time to apex of peak 8, 82.6 min. Gap in tracing, 35 min. Area of peak 8 corresponds to approximately 0.5–1 μg of a methyl ester. *Bottom:* extract from 2 ml of 4 N CH_3OH–HCl stored for 1 week at 4°. Total retention time to apex of peak 9, 108.7 min. Gap in tracing, 30 min. Area of peak 8 corresponds to approximately 10–15 μg.

Two different PEGA columns, both at 160°, detector voltage 1500, argon flow rate 40 ml/min. Possible erroneous identities of peaks: *1*, 14:0. *2*, 16:0. *2'*, ? (carbon number 15.5). *3*, 16:1. *4*, 17:1?. *5*, 18:0. *6*, 18:1. *7*, 18:2. *8*, 20:3. *9*, 20:4.

From the accumulated evidence it may be deduced that these contaminants are not esters of carboxylic acids even though they mimic them on GLC analysis. We believe that they may be due to oxidative breakdown of methanol followed by condensation reactions of the products. However, it is also possible that the contaminants are due to trace impurities in the methanol which are not removed by conventional purification and redistillation and which are eluted from the column as methylated products.

Lindgren et al. (5) have drawn attention to a number of potential sources of contamination in the GLC analysis of methyl esters. We have confirmed their observations and during our studies observed strict precautions to prevent the intrusion of contaminants from glassware, fingerprints, and other sources. The use of rubber and plastic stoppers and stopcock grease was avoided. Solvents were redistilled and operations were carried out under an atmosphere of oxygen-free nitrogen. Although we endorse the recommendation that solvents should be redistilled before use, it appears that when the commonly preferred method of methanolysis is employed on an ultramicroscale this is an inadequate precaution. Although under the GLC conditions used, freshly prepared weaker solutions of 1 N CH_3OH–HCl show only traces of pseudo fatty acid methyl esters 14:0 and 20:3, we believe that this procedure is still hazardous. Furthermore, when fatty acids are amide-linked, a prolonged heat treatment in an acid–alcohol solution is frequently employed, thus increasing the possibility of artifacts from this source.

Methods of avoiding contamination may be suggested. Since on TLC with less polar solvent systems, the contaminants do not move from the origin, methyl ester preparations may be purified by TLC prior to GLC analysis. Alternatively, the free fatty acids may be obtained via alkali hydrolysis or aqueous acid hydrolysis, whichever is appropriate, and methylated by the boron trifluoride procedure (6), although it has recently been shown (7) that this method too can give rise to artifacts (methoxy derivatives). If an instrument with a flame ionization detector is available, it should be possible in most cases to chromatograph the free acids directly (8). It should be emphasized that whatever procedure is employed, blanks carried through the entire operation should be performed.

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