## Changes in fatty acid composition during preparative thin-layer chromatography*

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» Thin-layer chromatography (TLC) is being used increasingly for the separation of the lipid classes (1, 2) prior to the analysis of their fatty acids by gas-liquid chromatography (GLC). Malins and Mangold (3) have described the separation of the methyl esters of fatty acids of menhaden oil from other lipid constituents and stated "the gas chromatogram of the total methyl ester fraction agreed in all details with a chromatogram obtained from a large scale preparation of these methyl esters." This suggests that little if any autoxidation occurs during TLC. Identification of the spots is, of course, crucial to the eluting of the separate lipids. The use of iodine vapor is a favorite method (2, 3). It occurred to us that iodine may react chemically with unsaturated lipids, thus invalidating GLC studies of the eluted spots. The present study establishes this fact by showing a partial loss of polyunsaturated fatty acids after staining of the TLC plates with iodine vapor.

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Thin-layer plates of Silica Gel G[1], prewashed with ether and chloroform and air-dried, were spread with a commercial applicator set to provide a thickness of 500 $\mu$. A solution of lipid in hexane was applied across the plate (1 in. from the bottom), giving a streak about $^1/_4$ in. wide, and the plate was developed in a solvent system composed of hexane–diethyl ether–acetic acid 80:20:1.5 (v/v). After air-drying briefly, the developed plate was placed in a tank containing iodine vapor, and the brown streak associated with the cholesterol esters was marked. Iodine was allowed to evaporate from the plate, the silica gel was scraped onto a filter paper, and the cholesterol esters were eluted with diethyl ether. One unstained plate was developed simultaneously, the stained plate serving as a template for the unstained plate. For comparison, a purified fraction of cholesterol esters was also prepared by column chromatography on silicic acid[2] according to the procedure of Horning, Williams, and Horning (4).

Methyl esters of the fatty acids were prepared from the lipids by methanolysis with concentrated sulfuric acid for 15–18 hr at 76° in a sealed tube. The methyl esters were chromatographed on an ethylene glycol-adipate polyester column (15% on Chromosorb W, 80–100 mesh) at 191° and 20 psi, argon inlet pressure, using the Barber-Colman Model 10 gas chromatograph. Areas were calculated as the product of the peak height and width at half height. The results are reported in terms of area percentage.

Table 1 shows the composition of methyl esters from dog adrenal cholesterol esters and human serum choles-

[1] Obtained from Brinkmann Instruments, Inc., Great Neck, Long Island, N. Y.

[2] Unisil, Clarkson Chemical Co., Williamsport, Pa.

TABLE I. ANALYSIS OF METHYL ESTERS OF FATTY ACIDS OF DOG ADRENAL AND HUMAN SERUM CHOLESTEROL ESTERS

| | | Dog Adrenal | | | | | Human Serum | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Cholesterol Esters Thin-Layer Chromatography | | | | | Cholesterol Esters TLC | | |
| Fatty Acid* | Column | No $I_2$ | $I_2$(3 min) | $I_2$(5 min) | R6G | Δ%† | No $I_2$ | $I_2$ | Δ% |
| 14:0 | 0.7‡ | 0.8 | 1.0 | 0.9 | 0.8 | +43 | 0.8 | 1.2 | +50 |
| 16:0 | 7.8 | 8.6 | 10.6 | 11.2 | 8.9 | +36 | 15.5 | 19.0 | +23 |
| 16:1 | .. | .. | .. | .. | .. | .. | 2.3 | 2.5 | +9 |
| 18:0 | 1.7 | 1.4 | 1.7 | 1.9 | 1.4 | .. | 1.1 | 1.6 | +46 |
| 18:1 | 34.0 | 34.0 | 36.2 | 37.3 | 36.2 | +6 | 19.6 | 17.0 | −7 |
| 18:2 | 13.4 | 13.4 | 12.5 | 12.4 | 12.7 | −7 | 52.7 | 49.4 | −6 |
| 20:3 | 7.7 | 7.2 | 6.1 | 5.6 | 7.9 | −21 | .. | .. | .. |
| 20:4 | 10.2 | 9.7 | 7.2 | 7.3 | 11.2 | −29 | 7.1 | 5.9 | −17 |
| 22:4 | 12.1 | 12.0 | 9.8 | 7.8 | 12.4 | −19 | .. | .. | .. |
| Others | 4.7 | 5.2 | 6.1 | 5.0 | 6.1 | .. | 1.0 | 3.2 | .. |

* C:X indicates carbon chain length and degree of unsaturation.

† Δ% is given as the difference between the 3-min $I_2$ stained plate and the column.

‡ Composition has been expressed in terms of area percentage.

TABLE 2. RADIOACTIVITY RETAINED BY CHROMATOGRAPHED TRIGLYCERIDES EXPOSED TO $I^{131}$ VAPOR

| Expt. No.* | $I^{131}$ Added | Tri-palmitin | Tri-stearin | Tri-olein | Tri-linolein |
|---|---|---|---|---|---|
| | *μc* | | *cpm* | | |
| 1 | 3.5 | 35 | .. | 308 | 488 |
| 2 | 12.5 | .. | 47 | ... | 5467 |

* In Experiment 1, 1 mg each of the specified triglycerides was used. In Experiment 2, 2 mg of each of the specified triglycerides was used.

terol esters, isolated by column chromatography, TLC with iodine staining, TLC without iodine staining, and TLC with plates prestained with 0.5% Rhodamine 6G (5). It is evident from these results that staining of the plates with iodine vapor, prior to the removal of the lipid and transmethylation, leads to a relative decrease in polyunsaturated fatty acids with a proportional increase in the more saturated fatty acids. It is also apparent that the loss of polyunsaturated fatty acids is roughly proportional to the total number of double bonds, since the relative losses of linoleate, eicosatrienoate, and arachidonate were 7%, 21%, and 29%, respectively, when compared with a sample isolated by column chromatography. The fact that the results obtained with unstained plates or plates prestained with Rhodamine 6G are nearly identical with those obtained by column chromatography may be taken as evidence that little autoxidation of polyunsaturated fatty acids occurs during thin-layer chromatography, as suggested previously (3).

To shed additional light on the mechanism of the selective loss of polyunsaturated fatty acids, an experiment was designed to test whether $I_2$ vapor containing $I^{131}$ would result in the permanent addition of $I^{131}$ to the lipid. A solution of tri-iodide ($I_3^-$) was made by mixing 0.5 g $I_2$ crystals and 0.25 g NaI in 50 ml of $H_2O$ (6). A small amount of this solution was placed in a petri dish; $NaI^{131}$ was added just prior to staining the plate. Thin-layer plates spotted with pure triglycerides were developed as previously described and stained with the $I^{131}$. The plates were then allowed to stand in the open for 18 hr until no $I_2$ was visible on the plate and the lipid-containing areas of the plate gave no blue color with starch solution. The areas of the plate containing saturated triglycerides were stained very lightly, whereas the areas containing unsaturated triglycerides were strongly stained. Silica gel from appropriate areas was removed with a spatula and eluted with diethyl ether, and the extracts were taken to dryness. Radioactivity was determined in a Packard Tri-Carb Liquid Scintillation Counter using toluene containing 2,5-diphenyloxazole as a phosphor. The results are summarized in Table 2. The small uptake of iodine by the saturated triglycerides is not fully explained. In Experiment 1, a small amount of oleic acid was detected by GLC in the transmethylated esters derived from the tripalmitin standard. No such impurity was noted in the tristearin used in Experiment 2. It is probable that small amounts of iodine may be taken up by adsorption on saturated lipids.

These studies suggest that the loss of polyunsaturated fatty acids from the TLC plates stained with $I_2$ vapor results from the iodination of the double bonds. They also indicate that the loss of polyunsaturated fatty acids is roughly proportional to the total number of double bonds they contain, which is in accordance with the easier iodination of polyunsaturated fatty acids. Iodine vapor is thus to be avoided in detecting lipid spots on thin-layer plates if the lipids are to be subsequently analyzed by GLC. Rhodamine 6G is a suitable noninterfering substitute.

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