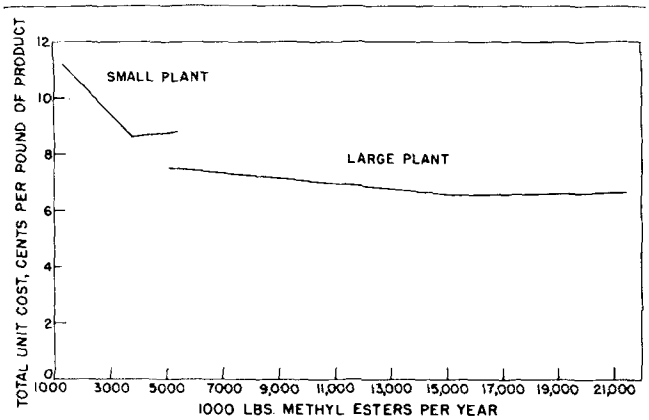

FIG. 2. Manufacturing costs for producing methyl esters from acidulated cottonseed foots.

tions in a hypothetical plant with a capacity of 60,000 lbs. of acidulated foots daily.

The cost of the raw materials, although only 3.4¢ per pound of product and chiefly the cost of foots, is the largest single item of unit cost in producing methyl esters; and, for the higher productions covered by this study, raw materials' cost accounts for more than one-half of total unit manufacturing cost. Surplus cottonseed foots can be economically converted into a low-cost feed additive with improved nutritional and handling properties. The process is already a commercial success.

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Analysis of Complex Lipid Mixtures by Thin-Layer Chromatography and Complementary Methods

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THIN-LAYER ADSORPTION chromatography on silicic acid according to Stahl (11) has been used for the resolution of lipid extracts of blood (12), acetylated lipids (3,4), and fats, oils, and waxes (7). The present contribution is concerned with further applications of this technique to lipid separations. In addition, the preparation and some applications of "siliconized chromatoplates" for the fractionation of classes into their constituents by reversed-phase partition chromatography in conjunction with thin-layer chromatography are also described.

Experimental

Preparation of Chromatoplates. Thin layers (250 to 275 μ) of silicic acid on glass (20 x 20 cm.) were prepared according to Stahl (11), using "Silica Gel G"[2] a fine grade (60μ or about 250 mesh) of silica gel containing 1% of plaster of Paris. "Silica Gel G" and the apparatus for its application were purchased from C. Desaga G.m.b.H., Heidelberg, Germany.[3]

Siliconized chromatoplates were obtained by slowly immersing the silicic acid plates at room temperature into a solution of 5% silicone[4] ("Dow Corning 200 fluid," viscosity 10 cs.) in diethyl ether. Plates may be used immediately after evaporation of the solvent.

Solvents and Indicators. Thin layers of silicic acid were developed with mixtures of petroleum ether, B.P. 60–70°C., diethyl ether, and acetic acid.

Separation within classes of lipids on siliconized chromatoplates was achieved with systems of acetonitrile-acetic acid-water or acetic acid-water. After having been sprayed with 0.2% 2',7'-dichlorofluorescein[5] in ethanol, all lipids on untreated silicic acid films were manifested as a bright yellow-green fluorescence by ultraviolet light.[6] Unsaturated lipids were detected as brown spots on a light yellow background with iodine vapors.

Dichlorofluorescein was found to be unsatisfactory for locating the position of lipids on siliconized chromatoplates because the entire plates fluoresced. The indicators α-cyclodextrin-iodine however revealed monochain saturated lipids as white spots on a purple background (8). Unsaturated lipids were detected, in the usual manner, with iodine vapors alone. The substances were amenable to further fractionation since no evidence was found which suggested that the indicators reacted chemically with the lipids to any marked extent.

Separation of Lipids into Classes. Complex lipid mixtures may be fractionated rapidly into classes of compounds by thin-layer chromatography on silicic acid. Figure 1 demonstrates the different migration rates of a variety of lipids, ranging from hydro-

[1] Supported in part by the U. S. Public Health Service National Institutes of Health Research Grant 5817, and the National Science Foundation.

[2] E. Merck A. G., Darmstadt, Germany.

[3] "Silica Gel G" may be obtained in the U.S.A. from Terra Chemicals Inc., 500 Fifth avenue, New York 36, N. Y., or from C. A. Brinkmann and Company Inc., 115 Cutter Mill road, Great Neck, L. I., N. Y. The later firm is also the U. S. representative of C. Desaga G.m.b.H., Heidelberg, Germany.

[4] Dow Corning Corporation, Midland, Mich.

[5] Eastman Kodak Company, Rochester 3, N. Y.

[6] "Mineralight," Ultra-Violet Products Inc., San Gabriel, Calif.

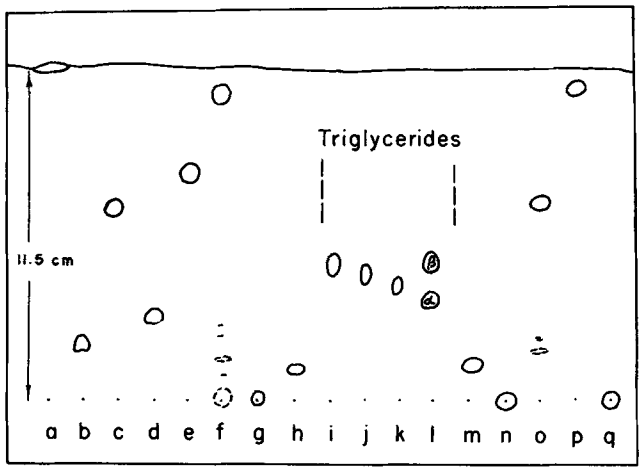

FIG. 1. Thin-layer adsorption chromatography of lipid classes on silicic acid.

Solvent system: 90 volumes of petroleum ether, B.P. 60–70°C., 10 volumes of diethyl ether, 1 volume of acetic acid.
Development time: 40 min.
Indicator: Dichlorofluorescein, 0.2% in ethanol.
Amounts: 20 γ, each.
a—Octadecene-9, b—Oleyl alcohol, c—Oleylaldehyde, d—Oleic acid, e—Methyl oleate, f—Cholesteryl oleate, g—Monoolein, h—Diolein, i—Triolein, j—Trilinolein, k—Trilinolenin, l—Tributyrin (α) plus Tristearin (β), m—Cholesterol, n—Selachyl alcohol, o—Chimyldiolein, p—Oleyl oleate, q—Dioleoyl lecithin.

carbons to phosphatides. The separation of these substances on silicic acid is effected by virtue of their differences in polarity, which depend primarily upon the type and number of functional groups in the molecules. Subfractionation of triglycerides derived from fatty acids of widely different chainlengths (1) and from unsaturated acids of the C_{18}-series (i,j,k,) is also presented. Homologous compounds, such as triglycerides, are only slightly separated from each other. Unsaturation however has a more pronounced effect on the subfractionation of lipid classes. The spots of triolein, trilinolein, and trilinolenin demonstrate the diminution in migration rate because of increasing unsaturation. These subfractionations, although perceptible with pure compounds, usually are not sufficient to interfere with the separation of classes. Larger amounts of a substance result in a slightly increased migration rate. This increased rate and the undetermined factors account for a lack of close reproducibility of R_f values. The pattern of separation of a mixture however is reproducible and reliable.

Polar substances, such as monoglycerides, glyceryl-ethers, and phospholipids, which do not migrate under the conditions stated in Figure 1, were fractionated with a more polar solvent. A mixture of 30 volumes of petroleum ether, 70 volumes of diethyl ether, and 1 volume of acetic acid served to segregate monoolein or selachyl alcohol, both R_f 0.27, from dioleoyl lecithin, which remains at the starting point.

Resolution Within Classes on Siliconized Chromatoplates. Classes of lipids were resolved into their constituents by reversed-phase partition chromatography on siliconized silicic acid plates. Figure 2 demonstrates the separation of reference mixtures and of a C_{18}-fraction from a distillation of methyl esters derived from menhaden oil. By comparison of the spots with those of reference compounds the components of this fraction were assumed to be octadecanoic, otadecenoic, octadecadienoic, octadecatrienoic, and octadecatetraenoic acids. After iodine vapors were used for staining, the unsaturated esters were scratched off the plate and eluted from the adsorbent with diethyl ether. The identity of the polyunsaturated fatty acids was confirmed by their ultraviolet spectra after alkaline isomerization. A constituent of unknown structure was evident in the chromatogram, and comparison with reference compounds, alkaline isomerization, and gas-liquid chromatography all failed to reveal the identity of this substance.

As in chromatography on siliconized paper (8) and on columns of silicone-Celite (2), aqueous acetic acid yielded favorable separations of lipids on thin layers of siliconized silicic acid. The system of 85 volumes of acetic acid and 15 volumes of water was used to separate C_{18}-acids from each other within 3 to 4 hrs. at room temperature. The corresponding methyl esters were resolved under the same conditions. Acids and esters of chainlengths other than C_{18} were separated in solutions of acetic acid and water in different proportions.

In reversed-phase partition chromatography at ambient temperatures the rate of migration of a lipid with one double bond is about equal to that for a saturated analog shorter by two methylene groups. Consequently palmitic acid is not separated from oleic acid. Such pairs were resolved by low-temperature chromatography (10) on siliconized chromatoplates. Palmitic acid, R_f 0.0, and oleic acid, R_f 0.1, were separated in the solvent system of 40 volumes of formic acid, 40 volumes of acetic acid, and 20 volumes of water within 8 hrs. at 4–6°C.

Saturated and unsaturated acids or methyl esters were also resolved by the use of solvents in which part of the acetic acid was replaced by peracetic acid (5). By this procedure saturated substances were still separated from each other on siliconized chromatoplates in 3 to 4 hrs. at room temperature while all unsaturated compounds migrated to the solvent front as oxygenated derivatives. A mixture of 10 volumes of peracetic acid,[7] 75 volumes of acetic acid, and 15 volumes of water served as the developing solvent.

[7] Becco Chemical Division, Food Machinery and Chemical Corporation, Buffalo 7, N. Y.

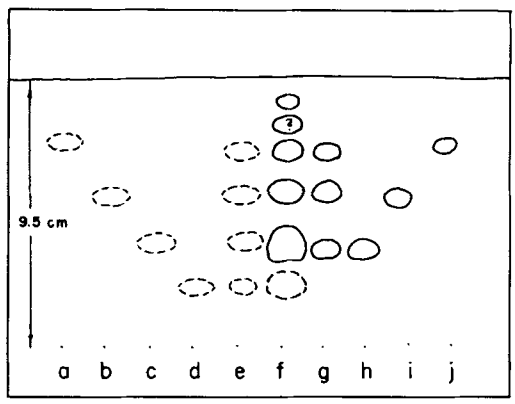

FIG. 2. Reverse-phase partition chromatography of methyl esters on a siliconized chromatoplate.

Solvent system: 70 volumes of acetonitrile, 10 volumes acetic acid, 25 volumes of water.
Development time: 40 min.
Indicators: Iodine (solid lines), followed by α-cyclodextrin, 1% in 30% aqueous ethanol and iodine (dotted lines).
Amounts: 20 γ of individual compounds.
a—Methyl laurate, b—Methyl myristate, c—Methyl palmitate, d—Methyl stearate, e—Mixture of saturated methyl esters, f—Fraction derived from menhaden oil (150 γ), g—Mixture of unsaturated C_{18}-esters, h—Methyl oleate, i—Methyl linoleate, j—Methyl linolenate.

Combined Methods. Adsorption chromatography of lipid mixtures yields classes of compounds that may be further resolved by standard methods of partition chromatography. In our experience thin-layer chromatography gives separations that are more discrete and more rapid than those obtained by column chromatography. Thin-layer adsorption chromatography on silicic acid is an ideal supplement and often a desirable preliminary for other separation techniques on a micro-scale, such as paper chromatography and gas-liquid chromatography.

Lipid classes, after having been isolated on silicic acid, were scratched off the plate and eluted from the adsorbent. All substances presented in Figure 1 were eluted with diethyl ether except the phospholipid, which was partially recovered with methanol. The appropriate eluant for lipids may be determined by chromatographing these substances with different solvents on silicic acid. Solvents that carry a compound with the solvent front are suitable as eluants for that substance.

Thin-layer adsorption chromatography on silicic acid was used for the purification of small amounts of lipids. About 10 mg. of impure linolenyl aldehyde, for example, were dissolved in diethyl ether, and the solution was applied along one side of a plate, 2 cm. from the edge. The aldehyde was separated within 20 min. from linolenic acid, nonadecatrieneol-2, and nonadecatrienediol-1,2 with the solvent system of 95 volumes of n-hexane and 5 volumes of diethyl ether at room temperature. A narrow vertical strip of the chromatogram was sprayed with dichlorofluorescein solution. All contaminants were located close to the starting-point. The pure aldehyde, R_f 0.65, was scratched off the plate and eluted from the adsorbent with diethyl ether. Thin-layer chromatography of the recovered aldehyde indicated that it was pure.

The combination of thin-layer chromatography with reversed-phase paper chromatography is illustrated by the following example. Ratfish liver oil was saponified and acidified, and the glyceryl ethers were separated as a class from the fatty acids, Vitamin A, sterols, and hydrocarbons. The system of 70 volumes of petroleum ether, 30 volumes of diethyl ether, and 1 volume of acetic acid was used as the developing solvent. The substances were located with dichlorofluorescein. After elution from the adsorbent with diethyl ether, the glyceryl ethers were resolved into their constituents by chromatography on siliconized paper with the solvent system of 60 volumes of tetrahydrofuran and 40 volumes of water at 30°C. for 18 hrs. (6). Batyl alcohol, R_f 0.38, and selachyl alcohol, R_f 0.45, were identified as the major constituents of this fraction.

Fatty acids were analyzed by combining thin-layer chromatography with gas-liquid chromatography. A Beckman "GC-2 Gas Chromatograph" [8] was used in this investigation. Methyl esters of fatty acids derived from menhaden oil were separated from the unsaponifiable constituents by thin-layer adsorption chromatography on silica gel with a mixture of 90 volumes of petroleum ether and 10 volumes of diethyl ether. The esters appeared, after exposure to iodine vapors, as two spots, R_f 0.80 and 0.89, which were eluted with diethyl ether and analyzed by gas chromatography (1). Saturated constituents were found mainly in the upper spot whereas highly unsaturated esters were enriched in the lower. 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.

Many vegetable oils and some waxes and bacterial lipids contain hydroxylated acids that are altered during gas-liquid chromatography (9) so that an analysis by this method alone is difficult and often unsatisfactory. Classes of hydroxylated acids may be separated by thin-layer chromatography from the nonhydroxylated acids, and the latter may be eluted from the chromatogram and analyzed by gas-liquid chromatography. About 10 mg. of the total acidic fraction from castor oil, for example, were separated into dihydroxystearic acid, ricinoleic acid, and nonhydroxylated acids with the solvent system of 70 volumes of petroleum ether, 30 volumes of diethyl ether, and 1 volume of acetic acid. The nonhydroxylated acids, which comprise about 10% of the total, were eluted, esterified with diazomethane, and further resolved into their constituents by gas-liquid chromatography. Palmitic, stearic, and three unsaturated C_{18}-acids were identified as major components of this fraction.

In all kinds of column chromatography one depends upon analysis of the eluant, and substances remaining on the column are not detected. In thin-layer chromatography ("open columns") and in paper chromatography all components can be visualized and measured.

Conclusion

Further work has been done on the application of thin-layer adsorption chromatography to the fractionation of complex lipid mixtures into classes.

New methods, the use of siliconized silicic acid plates and the application of thin-layer adsorption chromatography combined with the complementary techniques of gas-liquid chromatography and paper chromatography, are presented for the resolution of classes of lipids into their constituents.

In contrast to such elaborate conventional techniques as column chromatography, analyses using the methods reported in this paper can be performed rapidly in large numbers on a routine basis.

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[8] Beckman Scientific and Process Instruments Division, Fullerton, Calif.

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