A Paper Chromatographic Procedure for Separating 1-Mono-, 1,3-Di-, and Triglycerides, Cholesterol, and Cholesteryl Esters[1]

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IN ORDER TO STUDY the enzymatic synthesis of the glycerides, efficient micro procedures were needed for the separation and identification of these substances and their possible intermediates. In a preliminary report Dieckert and Reiser (1) stated that silicic acid-impregnated glass fiber filter paper could be used to separate saturated glycerides, cholesterol, and cholesteryl acetate. In the present study this procedure was extended to include the unsaturated glycerides and cholesteryl palmitate. Visualization of the lipides by the dichromate-sulfuric acid reagent mentioned in the first report (2) was found to be of limited usefulness because a) the yellow-orange background color interfered with the location of those lipides comprising only a small percentage of the lipide mixture, and b) this reagent did not permit a differentiation between cholesterol- and noncholesterol-containing lipides. Various reagents were investigated to overcome these problems.

During the process of screening reagents for detecting the lipides it was also learned that the glass paper contained carbonaceous impurities which interfered with the visualization of the lipides on the chromatogram. Procedures were investigated for removing these materials from the paper.

Experimental

Preparation of Samples. A stock solution, containing 1 mg. of sample per ml. ACS grade chloroform, was prepared for each of the following reference compounds: cholesteryl acetate (Eastman Organic Chemicals), cholesteryl palmitate (Sigma Chemical Company), cholesterol (Fisher Scientific Company), tripalmitin (prepared in this laboratory), unsaturated triglycerides (Wesson Oil), 1,3-dipalmitin, 1,3-diolein, 1-monopalmitin, and 1-monoolein. The mono- and diglycerides were generously donated (see acknowledgments).

Preparation of the Silicic Acid-Impregnated Glass Fiber Paper. One hundred grams of Mallinckrodt silicic acid (reagent grade) were slurried with 200 ml. of distilled water. To the slurry were added 100 ml. of saturated potassium hydroxide solution. Upon solution of the silicic acid the silicate solution was filtered through a sintered glass filter to remove insoluble debris. The filtered solution was then diluted with 600 ml. of distilled water.

Although the silicate solution is quite stable, its useful life is markedly limited by the rate of absorption of atmospheric carbon dioxide. After a period of time the carbonate concentration increases to a point where a large amount of carbon dioxide gas is liberated during the hydrochloric acid treatment (see next paragraph) of the strip. The gas creates pockets in the glass paper. These irregularities, if large enough, cause uneven development of the chromatogram. On the basis of this criterion the useful life of the silicate solution is about one week.

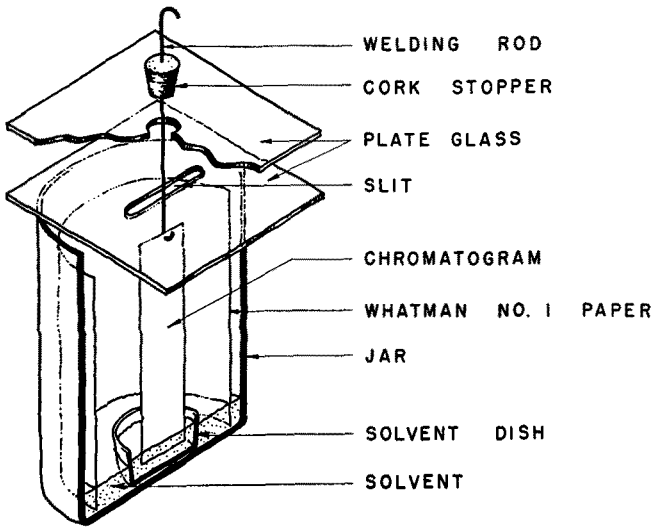

FIG. 1. Schematic drawing of apparatus for ascending chromatography.

A series of three 12 x 13-in. Pyrex glass dishes fitted with plate glass covers to keep out dust were arranged in a row. Dish No. 1 contained the potassium silicate solution, dish No. 2 contained 4 N hydrochloric acid, and dish No. 3 contained distilled water. Sheets of glass fiber filter paper (No. X-934-AH), obtained from H. Reeve Angel and Company, 52 Duane street, New York City 7, were dipped into the silicate solution. The excess silicate was removed from the impregnated sheet by repeatedly stroking each side of the sheet with a glass rod. Care in handling was required at this stage to prevent the tearing of the paper. When stroking ceased to remove more silicate, the impregnated sheet was immersed in the hydrochloric acid bath for 5 min. to precipitate the silicate as silicic acid. The sheet should not be allowed to remain in the acid bath any longer than necessary, otherwise an excessive loss of silicic acid will occur through the leaching action of the hydrochloric acid.

The impregnated sheets were taken from the acid bath, and the excess acid was removed with a glass rod as before. The sheet was then immersed in the distilled water bath for exactly 5 min. During this time the bath was not agitated as this caused too much silicic acid to be washed out of the paper. The water-washing step was repeated three times to assure the removal of the residual acid and salt. Inadequate washing of the impregnated sheets resulted in the streaking of the lipides during the development of the chromatogram. The washed sheet was drained of excess water and covered with methanol (ACS grade). The sheet was allowed to soak without agitation for 5 min. The methanol washing step was repeated once. Next the strip was soaked twice in ethyl ether (ACS grade) for 5 min. each time. The

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bulk of the water and most of the carbonaceous material were removed from the paper during the methanol-ethyl ether treatment. The ether moist sheet was hung in a covered glass tank and allowed to dry.

Preparation of the Chromatographic Tank. Figure 1 is a diagrammatic sketch of the assembled chromatographic tank, showing its constituent parts. A battery jar about 8 in. in diameter and 18 in. deep was used as the chromatographic tank. A shallow glass dish was placed in the bottom of the tank to hold the solvent used to develop the chromatogram. A 14 x 22-in. sheet of Whatman No. 1 filter paper was held along the inside of the tank but outside of the solvent by pieces of masking tape. It was found that without this paper the tank failed to reach equilibrium even after being sealed for 24 hours. This condition manifested itself in the telescoping of the lipide-containing spots on the chromatogram.

A top, consisting of an 11 x 11-inch glass plate with a 1 x 6-in. slit symmetrically located with respect to the sides was sealed on the tank with modeling clay. Over the slit was placed a 11 x 11-in. glass cover with a centrally located 1-in. hole fitted with a rubber stopper. The stopper was pierced by a 12-in. length of 1/16 in. welding rod having a hook on one end to hold the chromatogram.

To equilibrate the system the cover was removed, and the solvent system used was introduced through the slit into the solvent dish and into the bottom of the tank outside of the dish. The solvent on the outside of the dish bathed the bottom of the Whatman paper sheet and helped to establish and maintain equilibrium in the tank. The glass cover minus the chromatogram was replaced and fastened to the bottom plate with spring clamps. Approximately 8 hrs. were allowed for the tank to equilibrate before a chromatogram was introduced. At the end of that time the cover was again removed and the chromatogram was placed on the hook and introduced into the tank. This operation was done swiftly in order to disturb the equilibrium of the tank as little as possible. The chromatogram was allowed to equilibrate for 1 hr. before lowering it into the solvent mixture.

When several 3/4 x 12-in. chromatograms were run at one time, the welding rod hook was enlarged to form a large "L." The chromatograms were then spaced along the foot of this "L," and the collection was introduced into the tank as a unit and equilibrated as described above.

Preparation of Chromatograms. In the initial phases of this work 6 x 12-in. glass paper sheets impregnated with silicic acid were used. All compounds were spotted on a line 1 in. from the bottom of the sheet. The five different initial spots were placed at 1-in. intervals along this line, beginning 1/2 in. in from the edge. Later, however, it was found that the initial spots could be located 5/8 in. apart with good results. This observation permitted the use of 4 x 12-in. sheets while maintaining the same five chromatographic lanes.

In the early studies 10 to 20 μgm. of each substance were applied to the origin of a chromatographic lane. Thus the origin at which the mixture was spotted contained 10 to 20 μgm. of each of the compounds of the mixture. If a single substance was applied at the origin, then this spot contained 10 to 20 μgm. of that substance. Later the amount of each compound applied to a given origin was reduced to 5 to 10 μgm. All substances were applied with glass capillary tubes.

A typical experiment with the 4 x 12-in. sheets was set up as follows. At the origin of lane No. 1 a mixture containing 1-monopalmitin, 1,3-dipalmitin, tripalmitin, cholesterol, and cholesteryl palmitate was pipetted. At position No. 2 1-monopalmitin was placed, at position No. 3 cholesterol, at position No. 4 1,3-dipalmitin, and at position No. 5 tripalmitin. Similar studies were carried out in which the mono-, di-, and tripalmitin were replaced by 1-monoolein, 1,3-diolein, and Wesson oil, respectively. Other experiments were conducted in which a mixture of the saturated series of glycerides, cholesterol, and cholesteryl palmitate, and a mixture containing the unsaturated series of glycerides plus cholesterol and cholesteryl palmitate were chromatographed on adjacent lanes of the same chromatogram. To learn the effect of fatty acid chain length on the chromatographic behavior of the sterol esters, experiments were conducted by substituting cholesteryl acetate for cholesteryl palmitate.

When 3/4 x 12-in. strips were used, each of the strips represented one chromatographic lane. Otherwise the experiments were run as described in the previous paragraph.

Solvent Systems. Three solvent systems were used in the present experiments. The first consisted of 1% (V/V) ethyl ether (ACS grade) in isooctane, the second 2% (V/V) ethyl ether in isooctane, and the third 5% (V/V) ethyl ether in isooctane. The ethyl ether was used without purification. The isooctane was purified by passage through a silicic acid column.

Detection of Spots. A 50% (V/V) aqueous sulfuric acid reagent was used to visualize the lipides on the chromatogram and to differentiate between cholesterol-containing lipides and noncholesterol-containing lipides. The reagent was sprayed evenly over the chromatogram, using a De Vilbiss all-glass nebulizer actuated by a few pounds of pressure from a nitrogen cylinder. The treated chromatogram was gently heated over an electric heater. The appearance of the pink spot indicated the presence of a cholesterol-containing lipide. If too much heat was applied, the cholesterol-containing lipides charred before giving the pink spot. These spots were outlined with a soft lead pencil. The chromatograms were then heated more strongly. The glycerides and the sterols charred, yielding brown to black spots on a white background. The new spots were marked with a soft lead pencil. To observe and mark the spots, the treated chromatogram was placed on a clean glass plate and viewed by transmitted light. The glass plate served as a solid surface for marking the outlines of the spot while the chromatogram was viewed at the same time.

Results and Discussion

The R_f values obtained with the various solvent systems appear in Tables I, II, and III. All R_f values except those in Column 1, Table III, are averages of at least six values. In all systems tried, the order of the compounds on the chromatogram from origin to solvent front were: 1-monoglycerides, 1,3-diglycerides, cholesterol, triglycerides, and cholesteryl esters. This is the same order obtained by Fillerup and Mead (2) and Borgström (3) for silicic acid columns. This consistent order of movement of compounds in the two systems suggests that silicic acid impregnated glass paper could be used as a rapid means for deter-

TABLE I

R_f Values Obtained with the 2% (V/V) Ethyl Ether in Isooctane

Substance	Mixture	(Mixture)[a]	Single
1-Monopalmitin	0.03	0.01	0.03
1-Monoolein	0.03	0.01	0.04
1,3-Dipalmitin	0.17	0.03	0.15
1,3-Diolein	0.18	0.02	0.16
Tripalmitin	0.73	0.04	0.72
Unsaturated glycerides (Wesson oil)	0.67	0.05	0.70
Cholesterol	0.43	0.05	0.42
Cholesteryl acetate	0.94	0.02	.....
Cholesteryl palmitate	0.94	0.01	0.94

[a] Standard deviation about the mean of a given lipide when chromatographed in mixture.

TABLE II

R_f Values Obtained with 5% (V/V) Ethyl Ether in Isooctane System and Behavior of Lipides Toward Spray Reagent

Substance	Mixture	(Mixture)[a]	Single	H_2SO_4[b]	H_2SO_4[c]
1-Monopalmitin	0.11	0.02	0.11	−	+
1-Monoolein	0.11	0.03	0.11	−	+
1,3-Dipalmitin	0.51	0.02	0.53	−	+
1,3-Diolein	0.46	0.05	0.48	−	+
Tripalmitin	0.94	0.03	0.93	−	+
Unsaturated glycerides (Wesson oil)	0.90	0.01	0.89	−	+
Cholesterol	0.67	0.04	0.67	+	+
Cholesteryl acetate	0.96	0.01	.....	+	+
Cholesteryl palmitate	0.92	0.02	0.93	+	+

[a] Standard deviation about the mean.
[b] Warmed gently without charring: A bright pink spot on a white background is a positive test.
[c] Heated until charring occurs. A black spot on a white background is a positive test.

mining the behavior of any series of compounds on a silicic acid column with a given solvent system.

Unsaturation in the fatty acid moiety of the glycerides and chain length in the fatty acid moiety of the cholesteryl esters had no effect on the R_f values of the respective substances in any of the solvent systems (Tables I and II). In all systems the lipides traveled as well defined, slightly elliptical spots (Figure 2). In the 2 and 5% ethyl ether in isooctane systems, the reproducibility of the R_f values (Tables I and II), as indicated by the standard deviation about the mean, was good.

The effect of increasing the concentration of ethyl ether in the solvent system appears in Table III. Cholesteryl esters moved at the solvent front in all cases. The glycerides and cholesterol showed increasing R_f values with increasing concentrations of ethyl ether in isooctane.

TABLE III

The Effect of Various Concentrations of Ethyl Ether in Isooctane on the R_f Values of the Glycerides, Cholesterol, and Cholesteryl Esters

Substance	% Ethyl Ether in Isooctane		
	1%	2%	5%
1-Monopalmitin	0.0	0.03	0.11
1,3-Dipalmitin	0.10	0.17	0.51
Cholesterol	0.30	0.43	0.67
Tripalmitin	0.45	0.73	0.94
Cholesteryl acetate	0.95	0.94	0.96

The sulfuric acid-water reagent, plus strong heat, was quite sensitive for locating lipides on the chromatogram. For example, 5 μgm. of tripalmitin or cholesteryl palmitate were easily detected on a chromatogram even though these substances had traveled 10 to 11 in. from the origin. The sulfuric acid-water reagent, plus mild heat, was slightly less sensitive for the cholesterol-containing lipides. Care had to be exercised to locate 5 μgm. of cholesterol palmitate when this amount of the substance had traveled 10 in. from the origin.

The technique for removing the carbonaceous impurities from the silicic acid impregnated glass fiber paper, described under "Experimental," was adequate unless the paper was very dirty. When the paper was "clean," at most only a very slight band of carbonaceous material appeared at the solvent front.

Summary

A reproducible paper chromatographic procedure has been developed for resolving a mixture consisting of 1-mono-, 1,3-di-, and triglycerides, cholesterol, and cholesteryl esters. The separation is accomplished, using silicic acid impregnated glass fiber filter paper as the chromatographic medium and various ethyl ether-isooctane mixtures as the solvent systems. A reagent consisting of 1:1 concentrated sulfuric acid: water has been introduced for visualizing the lipides on the chromatogram and for distinguishing between glycerides and cholesterol-containing lipides. Unsaturation in the fatty acid moiety of the mono-, di-, and triglycerides had no detectable effect on the R_f values of these substances. Fatty acid chain length did not affect the R_f of the cholesteryl esters with the solvent systems studied. Increasing the percentage ethyl ether in the solvent system caused an increase in the R_f values of each lipide not moving at the solvent front.

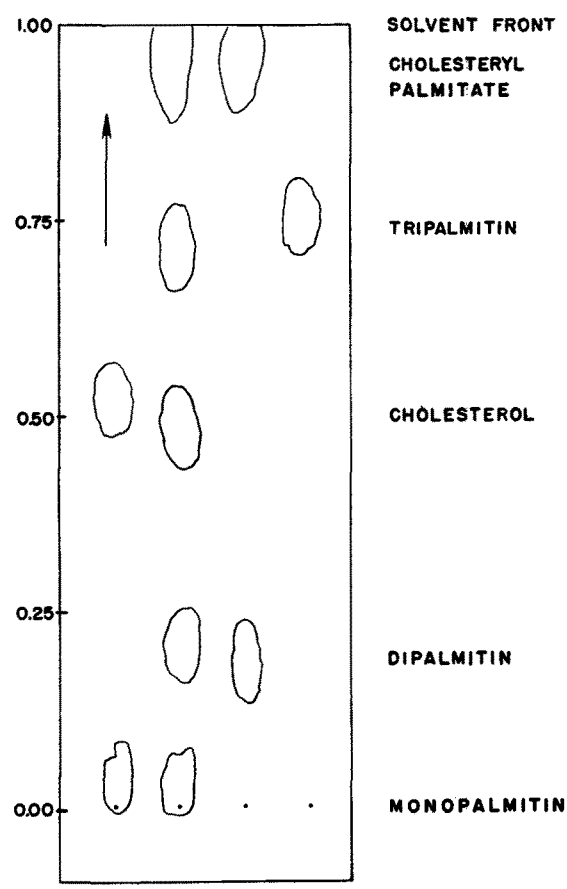

FIG. 2. Tracing of a chromatogram showing separation of neutral lipides.
Solvent: 2% (V/V) ethyl ether-isooctane.

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Periodate-Permanganate Oxidations. IV. Determination of the Position of Double Bonds in Unsaturated Fatty Acids and Esters[1, 2]

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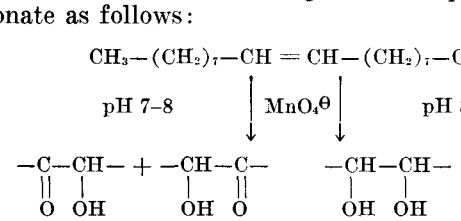
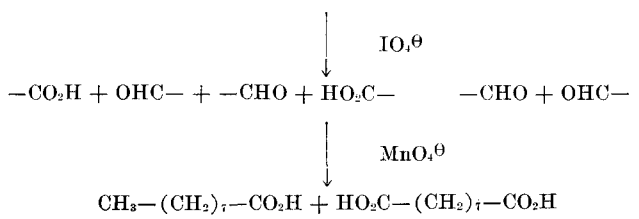
THE POSITION of double bonds in unsaturated fatty acids, and alkenes in general, has been determined mainly by permanganate oxidation or by ozonolysis. In 1950 Haverkamp and co-workers (2) demonstrated that these oxidation reactions do not adhere strictly to the following expected reaction scheme:

$$R{-}CH{=}CH{-}(CH_2)_n{-}CO_2H + 4\,O \longrightarrow R{-}CO_2H + HO_2C{-}(CH_2)_n{-}CO_2H \qquad (I)$$

By means of an elegant chromatographic method for determining homologous dicarboxylic acids in the C_7 to C_{13} range, these authors found that lower homologues are produced in addition to the expected dicarboxylic acid. The much more tedious method of hydroxylating the unsaturated compound with peracid, followed by periodate or lead tetraacetate cleavage and subsequent oxidation of the aldehydes, also did not lead to quantitative results. Best results were obtained with potassium permanganate in glacial acetic acid (according to Armstrong and Hilditch [1]) or with ozonolysis, but 5 to 8% of lower degradation products were still obtained.

The presence of 5% or more of lower homologous dicarboxylic acids can be misleading in the qualitative and quantitative interpretation of such oxidative results, and a method which adheres strictly to reaction I is highly desirable. The results obtained in this work show that such a quantitative oxidation is possible with the periodate-permanganate reagent (3, 4). This reagent, which consists of a dilute solution of sodium *meta*-periodate containing catalytic amounts of potassium permanganate, was shown to react with oleic acid in the presence of potassium carbonate as follows:





Although two different reaction routes occurred, the end-products were the same. The very mild conditions at pH 7–8 appear to be conducive to the quantitative oxidation of the intermediate aldehydes to carboxylic acids since practically theoretical yields of azelaic and pelargonic acids were obtained after complete oxidation (3). The over-all reaction may be written as

$$R{-}CH{=}CH{-}(CH_2)_n{-}CO_2H + 4IO_4\theta \xrightarrow{MnO_4\theta} R{-}CO_2H + HO_2C{-}(CH_2)_n{-}CO_2H + 4\,IO_3\theta$$

which is equivalent to reaction I. The crucial feature of the reaction is that, in the pH range, from about 5 to 10 periodate is capable of regenerating the permanganate. The limiting reaction conditions under which this reaction scheme was strictly adhered to were: temperatures below 40°C., a pH range of about 6 to 9, reaction times of less than 1 week's duration, and concentrations not higher than twice those described in the experimental part.

Since Haverkamp *et al.* (2) have shown by means of partition chromatography that lower homologues were formed in permanganate oxidations, the analysis of the oxidation products obtained from the periodate-permanganate oxidation of oleic acid was repeated by this method. The method was extended to cover the analysis of monocarboxylic acids as well. Where tailing occurred (which could, of course, be due to the presence of small amounts of the next lower homologue), the respective fractions were rechromatographed. These experiments showed conclusively that, within the error of the method (± 2%), the expected acidic end-products were formed quantitatively and that no lower degradation products were formed.

In order to show that the method is a more general one, the reaction with several other unsaturated fatty

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[2] Issued as Paper No. 214 on the "Uses of Plant Products" and as N.R.C. No. 3882.