THE CHROMATOGRAPHY OF LIPIDS IN TEST TUBES COATED WITH A THIN LAYER OF SILICIC ACID

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The analytical separation of lipids by thin layer silicic acid chromatography has proven to be a useful tool. This method has involved the use of glass plates coated with a suitable stationary phase[1–4]. In the present investigation the thin layer technique is applied to chromatography with test tubes which are coated on the inner surface with a thin layer of silicic acid. This communication includes a description of the details of the method, a discussion of its relative merits and suggestions concerning the possible extension of the method to special problems by simple modifications of the procedure. The application of the system to studies on lipids from fungi is also included as an example of its use.

MATERIALS AND METHODS

Reagents

The silicic acid powder, ethyl ether and the methyl alcohol used in these studies were reagent grade products from the J. T. Baker Chemical Company. The chloroform was a Baker and Adamson reagent grade solvent which contained 0.75 % ethyl alcohol as a preservative, and the *n*-hexane was from the Eastman-Kodak Company (boiling range 66–69°). The silicic acid was put through a 140 mesh screen prior to use. The ethyl ether was shaken with an aqueous solution of ferrous sulfate, dried and freshly distilled prior to use.

Coating of tubes with silicic acid

Test tubes with an inside diameter of 13.5 mm and a length of 150 mm proved to be a convenient size for routine use, and the chromatographic data presented here are based on this vessel. These tubes must be cleaned thoroughly in a dichromate–sulfuric acid cleaning solution before they are coated with a stationary phase. A suspension consisting of one part silicic acid and two and a half parts chloroform (w/v)** is used to deposit a thin layer of silicic acid on the inside of the tube. The coating suspension is triturated with the side of a thick stirring rod against the flat surface of a glass container (Erlenmeyer flask) to ensure homogeneity of the mixture.

The test tube to be coated is filled with the suspension and allowed to stand about

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five seconds in a vertical position. The contents are then slowly poured out of the tube in one continuous and slow tilting motion. It is helpful to rest the open end of the tube on the lip of the receiving vessel during this part of the procedure. After most of the slurry has been discharged, the vessel is held in an inverted position for about 2 to 3 sec, or until almost all the draining overflow of suspension has stopped its downward motion. The contents of the test tube should not be allowed to drain too long in the inverted position since this can result in a cracking of the thin-layer deposit. The tube is then slowly tilted, without rotation, to a horizontal position and placed on a level surface where it remains until most of the solvent has evaporated from the deposit. After the coated vessel has air dried in the horizontal position, the top side is marked for future reference since it is the linear surface at this part of the circumference which is employed for the chromatography. It has been found convenient to prepare the coating of the tubes with the manufacturer's mark designating the side which is opposite to that employed for chromatography of lipids. The deposit of silicic acid at the surface used for chromatography has a thickness of 0.14 $\pm$ 3 mm.

An additional coating of silicic acid is deposited at the lip of the test tube. This second surface deposit is essential for a reproducible level rise of the solvent during chromatography. The open end of the previously coated tube is immersed at a 45° angle to a depth of about 5 mm in the chloroform–silicic acid suspension. In this position it is rotated slowly through one revolution whereby an additional thin layer of silicic acid is formed on both sides of the glass. The vessel is placed with the open side up in a suitable rack for storage. These coated tubes are heated for one hour at 110° just prior to use[2,4].

Mobile phases

A two-solvent system was employed to demonstrate the use of the test tube chromatography for the more polar lipids. One component of the solvent system is a mixture composed of 93 vol. methyl alcohol, 5 vol. water and 2 vol. concentrated ammonium hydroxide (28 % NH_3)*. This solvent (NH_3–MeOH) was combined with varying amounts of chloroform to form mobile phases which were profitably employed in the screening of *Neurospora crassa* mutants for lipid abnormalities. The three most useful solvents are 100 % chloroform and 20 or 30 % ammoniacal methanol (NH_3–MeOH) in chloroform (v/v). Mixtures of ethyl ether and *n*-hexane are used for those lipids which travel too fast in mobile phases based on chloroform and methyl alcohol as solvents.

Spotting of samples and chromatography

For individual compounds 5 μg of the substance in 1 μl of a suitable solvent is applied with a micropipette to the silicic acid layer within the tube at a position 1 cm from the lip. In some instances the iodine staining method[5] used here was found to be sensitive enough to detect 1 μg of material. Thirty to fifty μg samples are applied in this manner when the total-lipids from the tissues are being investigated. For the ascending chromatography the mouth of the tube is immersed in 1.5 ml of the mobile phase, contained in a 19 mm i.d. shell vial, and allowed to develop at 25° to a solvent

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height of 10 cm above the point of the spot application. A tin foil shield at the upper surface of the vial is adequate to minimize evaporation of the solvent. Even without this precaution evaporation is not a serious problem since the chamber enclosing the fluid exposes a small liquid surface to the atmosphere for a relatively short experimental time. The slight compression of air in the test tube during this procedure makes the solvent rise to a 16 mm height in the thin space between the outer wall of the chromatography tube and the solvent vial. The average time required for a 10 cm solvent rise is about 40 min. When the solvent reaches the predetermined height the chromatographic tube is removed from the solvent, air dried and exposed to iodine vapor according to WHITEHOUSE *et al.*[5]. These workers have shown that most lipids will concentrate iodine vapor in sufficient quantities to make their detection possible by the resulting contrast in color due to the absorbed halogen. This spotting procedure is readily accomplished by attaching a 15 × 50 mm shell vial, containing an iodine crystal, to the open end of the chromatographic tube; a gum rubber gasket is satisfactory for this attachment (Figs. 1 and 2). The iodine spots begin to develop within a few hours and can be observed over a period of several days. Acids present in the solvents used for the mobile phase, even in trace amounts, tend to decrease the sensitivity of the iodine spotting method. When this factor becomes a problem, the acids must be either removed by evaporation in a vacuum desiccator or else neutralized

TABLE I

APPROXIMATE R_F VALUES IN SOME SOLVENTS AND SOLVENT MIXTURES

Substance	*Solvent**					
	n-Hexane	*Ethyl ether–n-hexane (3:97)*	*Ethyl ether–n-hexane (15:85)*	*Chloroform*	*NH_3·MeOH–chloroform (20:80)*	*NH_3·MeOH–chloroform (30:70)*
Cholesterol oleate	0.15	0.45	0.87	0.91		
Cholesterol palmitate	0.20	0.47	0.83	0.96		
Methyl stearate	0.17	0.40	0.72	0.99		
Tripalmitin	0.00	0.15	0.62	0.99		
Stearic acid	0.03	0.13	0.43	0.99		
Myristic acid	0.01	0.10	0.41	0.99		
Cholesterol acetate			0.65	0.63		
β-Sitosterol acetate			0.69	0.55		
Triolein		0.10	0.57	0.43**	0.95	
Cholesterol				0.28	0.95	
β-Sitosterol				0.26	0.92	
Oleic acid		0.05	0.35	0.26**	0.92	0.95
Ergosterol				0.27	0.86	0.92
Phosphatidyl-ethanolamine				0.00	0.35	0.60
Erythro-sphingosine				0.00	0.34	0.50
Erythro-dihydro-sphingosine				0.00	0.30	0.47
Dipalmitoyl-phosphatidylcholine				0.00	0.29	0.48
Sphingomyelin				0.00	0.25	0.42

* Abbreviations: NH_3·MeOH = methyl alcohol–water–28% conc. ammonium hydroxide, 93:5:2 (v/v/v). All solvent ratios in the Table are on a volume basis.

** These unexpected values were obtained repeatedly on purified samples from different sources.

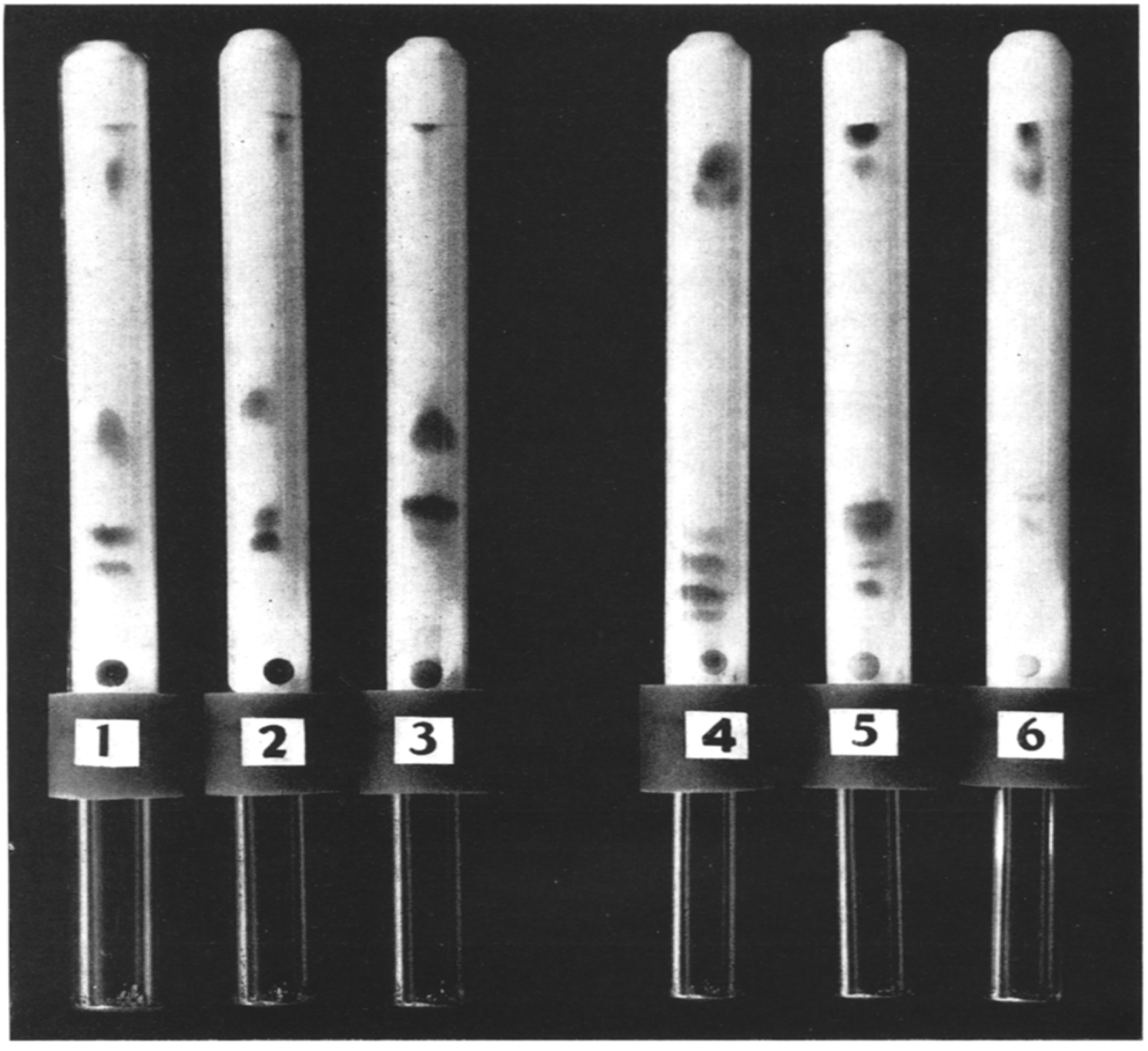

Fig. 1. Shows the iodine spots that are visible for the components in the total-lipid extracts of strains 1 A (tubes 1 and 4), 47904 (tubes 2 and 5) and 319a (tubes 3 and 6). Chloroform was the mobile phase for tubes 1, 2 and 3; 20 % NH_3–MeOH in cloroform was the mobile phase for tubes 4, 5 and 6. Samples weighing 50 μg were used in each chromatogram.

with ammonia vapor. The ammonia vapor is also removed under vacuum in a desiccator before the iodine staining is attempted.

RESULTS

In Table I are presented the data on the chromatographic behavior of several lipids representing various types with respect to chemical structure and solubility. The R_F values are taken at the concentration centers of the spots which seldom exceed 6 mm in their vertical dimension. Figs. 1 and 2 show the simple iodine chamber employed for vapor spotting. They also illustrate an application of the method to the chromatography of the total lipids obtained from three strains of *Neurospora crassa*, strains 1 A

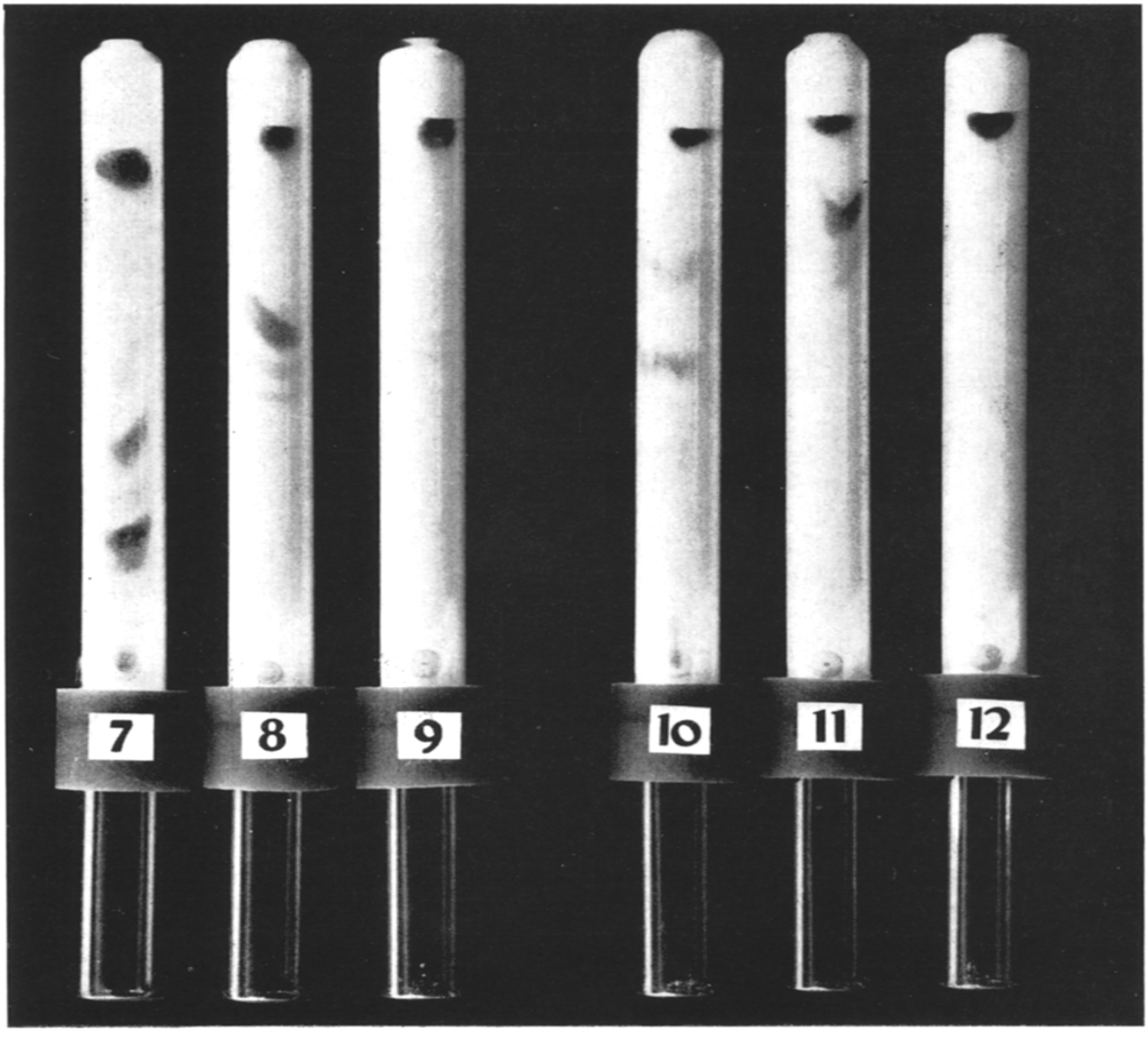

Fig. 2. Shows the iodine spots that are visible for the components in the total-lipid extracts of strains 1 A (tubes 7 and 10), 47904 (tubes 8 and 11) and 319a (tubes 9 and 12). Tubes 7, 8 and 9 were developed with 30 % NH_3–MeOH in chloroform as the mobile phase; 40 % NH_3–MeOH in chloroform was used for tubes 10, 11 and 12. Samples weighing 50 μg were used in each chromatogram.

(normal "wild type"), 47904[6,7] and 319a[8]. It is known that strains 1 A and 47904 differ in their phospholipid composition[9,10]. The chromatographic patterns (Figs. 1 and 2) show the expected qualitative and quantitative differences at the positions corresponding to R_F ranges of 0.10–0.30 and 0.20–0.70 with 20 % and 30 % NH_3–MeOH in chloroform, respectively. Mutant 319a is a morphological mutant[8] which is also employed here to illustrate the use of the test tube chromatographic technique because this organism gives a unique pattern in which one unknown lipid accounts for a large part of the total extractable lipids. The presence of this substance in high concentration is apparent by the dense iodine spots with R_F values of 0.30 and 0.92 in the 100% chloroform and the 20% NH_3–MeOH in chloroform solvents, respectively.

This preliminary observation on strain 319a will be used as the basis for future studies concerned with the biochemical significance of this lipid abnormality.

DISCUSSION

The intent in these studies was the development of a simple, inexpensive and reproducible means of separating lipids by thin layer silicic acid chromatography. The present test tube method makes use of apparatus which is normally stocked in most laboratories. This procedure has been used successfully to screen mutant strains of *Neurospora crassa* for lipid abnormalities. Some advantages of the test tube chromatographic method which might predispose it to acceptance and use by other workers are summarized as follows:

1. The method employs test tubes which serve as their own vapor chambers for both chromatography and the development of iodine spots.

2. The coating of the thin-layer stationary phase is accomplished in a simple and reproducible manner without need for any special apparatus.

3. Since the system lends itself to use with test tubes of varying sizes, it can be adapted to specific needs by employing variations of readily available glassware. The method was successfully employed with an ascending solvent rise up to 30 cm.

4. The cylindrical character of the thin-layer deposit tends to promote undistorted solvent fronts. The level rise of the ascending mobile phase facilitates the reliable comparison of unknown lipids with authentic samples. In such comparisons the two samples are applied next to each other in the same chromatographic tube.

5. Since the only terminal edge of the thin-layer deposit is outside of the test tube, the chromatograms are durable and resistant to damage by handling. It is not necessary to add a binding substrate to the silicic acid to decrease its fragility. The tubes may be conveniently manipulated for purposes of observation since the iodine vapor chamber is an attached part of the closed system.

6. Preliminary experiments indicate that two or more solvents developing the chromatograms in the same ascending dimension may be utilized for the separation of complex lipid mixtures. In a typical experiment the less polar lipids are partially separated by development with a moderately polar solvent as the mobile phase. The solvent is allowed to ascend to half the length of the tube. A change to a more polar solvent is then made as quickly as possible to minimize evaporation of the original solvent moistening the silicic acid. As the second mobile phase ascends, it continues the movement of the original solvent front and also effects the movement of the more polar lipids which are still near the point of the sample application at the time of the solvent change. A detailed study on the infinite combinations possible with such a system was not attempted here since the basic information included in this communication is adequate to facilitate further empirical studies with multiple solvents systems.

7. Although aqueous solvent systems tend to increase the fragility of silicic acid thin-layers on flat glass plates[2], solvent systems containing up to 20% water (acetic acid–water, 8:2) were used successfully with the test tube method.

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SUMMARY

Test tubes, coated on the inner surface with a thin layer of silicic acid, are employed as the stationary phase for the ascending chromatography of various lipids. The chromatographic behavior of a number of purified lipids has been determined with mobile phases based on mixtures of chloroform with methyl alcohol and *n*-hexane with ethyl ether. The use of this system for the chromatography of lipid mixtures is illustrated with lipids extracted from different strains of *Neurospora crassa*.

REFERENCES

1 J. E. MEINHARD AND N. F. HALL, *Anal. Chem.*, 21 (1949) 185.
2 J. G. KIRCHNER, J. M. MILLER AND G. J. KELLER, *Anal. Chem.*, 23 (1951) 420.
3 R. H. REITSEMA, *Anal. Chem.*, 26 (1954) 960.
4 E. STAHL, G. SCHROTER, G. KRAFT AND R. RENZ, *Pharmazie*, 11 (1956) 633.
5 M. W. WHITEHOUSE, A. E. BRESLER AND E. STAPLE, *J. Chromatog.*, 1 (1958) 385.
6 N. H. HOROWITZ, D. BONNER AND M. B. HOULAHAN, *J. Biol. Chem.*, 159 (1945) 145.
7 N. H. HOROWITZ, *J. Biol. Chem.*, 162 (1946) 413.
8 S. EMERSON AND M. EMERSON, unpublished.
9 M. O. HALL AND J. F. NYC, *J. Am. Chem. Soc.*, 81 (1959) 2275.
10 M. O. HALL AND J. F. NYC, *J. Lipid Research*, 2 (1961) 321.