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Some Practical Aspects of Thin-Layer Chromatography of Lipids

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Abstract

This report describes methods for the thin-layer chromatography (TLC) of lipids and some practical aspects of the methods.

In order to present some basis for choosing the correct powder for particular separations, some properties of several widely used silica gel powders are compared. The effect of binder material such as calcium sulfate in silica gel is studied. The three systems, silica gel as a polar phase, silver nitrate-impregnated silica gel, and reversed phase systems are described with application to neutral lipids. Also included are the applications of TLC to the polar lipids, such as phospholipids, cerebrosides, sulfatides, sphingomyelin and other glycolipids from various sources. The pitfalls and precautions involved in these separations are discussed in detail.

Introduction

THIS REPORT DESCRIBES methods for the qualitative separation of lipids by thin-layer chromatography (TLC) and shows some of the ways the methods can be applied. The technique is based on the principle of adsorption and partition chromatography first described by Izmailov and Shraiber (1). It is a rapid method and presents one of the most effective means of analytical separation. The real impetus came through the work of Stahl (2) who in 1956 demonstrated the first practical method of applying thin layers of adsorbents to glass plates and applied the technique to the separation of a great number of substances. There are many publications on the TLC of lipids with good reviews by Mangold (3) and Fontell (4) and discussions of various aspects in the books by Truter (5), Bobbitt (6) and Randerath (7).

The proper use of TLC requires an understanding of its limitations and variables. Some basis is needed for choosing the correct TLC powders. Some of the widely used silica gel powders on the market were subjected to emission spectroscopy, pH analysis, neutron activation, gamma spectrometry, and comparative results are shown. The effects of binder material such as calcium sulfate and of silver nitrate impregnation in TLC silica gel powders are also shown.

It should be clearly understood that using only silica gel in chromatography does not provide a reversed phase system and separates only chemical *classes* of lipids with limited subfractionation. Silver nitrate-impregnated silica gel, however, provides some separation within chemical classes differing in the de-

TABLE I

Trace Constituents of TLC Silica Gels by Emission Spectroscopy; pH Measurements of a 5% Distilled Water Slurry

Elements present	Adsorbosil-2		Adsorbosil-S-2		Silica Gel H	Camag	Woelm
	Raw materials	Final product	Raw materials	Final product	Final product	Final product	Final product
Fe as Fe_2O_3	0.02	+	+	+	+	+	+
Al as Al_2O_3	0.03	+	+	+	+	+	+
Ti as TiO_2	0.05	+	+	+	+	+	+
Ca as CaO	0.06	+	+	+	+	+	+
Na as Na_2O	0.10	+	+	+	+	+	+
Mg as MgO	0.21	+	+	+	+	+	+
Sn	<0.01	+	+	+	+	+	+
Cd	<0.01	+	+	+	+	+	+
pH	4.6	7.0	5.2	5.1	7.8	7.2	6.1

+ Indicates element was present in approximately same proportion as in ADSORBOSIL-2 raw material.

gree of unsaturation and the separation of *cis-trans* isomers. On the other hand, reversed phase systems fractionate within chemical classes on the basis of both carbon number and unsaturation. The three systems, silica gel as a polar phase, silver nitrate-impregnated silica gel, and reversed phase systems, such as paraffin oil- or undecane-impregnated diatomaceous earth as a nonpolar phase, will be reviewed and discussed, and some of the ways the TLC method can be applied to neutral lipids will be shown. Also shown will be separations of phospholipids and of the more polar lipids such as cerebrosides and major glycolipids of plant tissue.

Characteristics of Adsorbents Used

Spectroscopic Analysis

Qualitative emission spectroscopy was employed to compare the metallic impurities of the following TLC adsorbents: Adsorbosil-2, Adsorbosil S-2, Silica Gel H, Woelm, and Camag. Iron was used as a calibration standard throughout the study. The results were recorded on photographic film and read by densitometer. The percentages of metallic impurities in the first column of numbers in Table 1 are taken from the manufacturer's label for the raw material for Adsorbosil-2. The emission spectra of the various powders showed general agreement, within one order of magnitude, with this analysis, for both raw and finished materials. Apparently there are no major differences between the various products, as far as these metal impurities are concerned. Also, the methods of treatment used to obtain the finished products cause little change in the amts of these impurities.

pH Analysis

An important property of silica gels, but less known, is the acidic or basic nature of the powder. A measurement of the pH on a 5% by weight slurry of the various silica gels with distilled water was obtained on a Beckman Zeromatic pH Meter. The results are shown in Table 1. The indicated variations in pH can in part explain the different TLC separations with different brands of silica gel. Commercial products tested showed variations in pH of 5.1 to 7.8.

TABLE II

Effect of Acid Treatment of TLC Silica Gels as Measured by Neutron Activation

Trade Names	Background Counts, cpm/g			
	Original Sample	Acid Washed		
		Aqua Regia	HCl	HNO_3
Adsorbosil-S-2	915	1220	1550	1090
Silica Gel H	1535	.....	.....	.....
Woelm	16,110	6253	.....	.....
Biosil-A	1740	1210	1478	1120
Mallinckrodt	2590	1268	1550	2070

Activation Analysis

Activation analysis by thermal or fast neutrons is a very sensitive and easy method of detecting phosphorus- and sulfur-bearing compounds by TLC. Chromatographic plates can be exposed to the reactor as long as one likes and it should be possible to reach sensitivities of 10^{-10}g P or 10^{-8}g S. The biggest problem with commercial silica gels is the presence of impurities such as a binder material, chloride ion, silicates and trace elemental impurities. All of these give off high background counts which interfere appreciably with the actual analysis. To get a better idea about this, different commercial silica gel powders were first subjected, as purchased, to neutron activation. In the next step, each was washed first with aqua regia, next with reagent grade HCl and then with HNO_3. After each acid treatment they were rinsed thoroughly with deionized water, tested for neutrality and subjected to neutron activation. This consisted in exposure to the fast neutrons in a reactor for 1 hr and then allowing to decay for a week. Table II shows the results in background counts (cpm/g) of the various silica gels. Adsorbosil S-2, a supposedly pure silicic acid, gave only 915 cpm/g right out of the bottle. Mallinckrodt (screened for TLC) gave 2590 cpm/g, and Silica Gel H gave 1535 cpm/g.

It is obvious that more tests like this need to be made in an effort to begin to understand the different

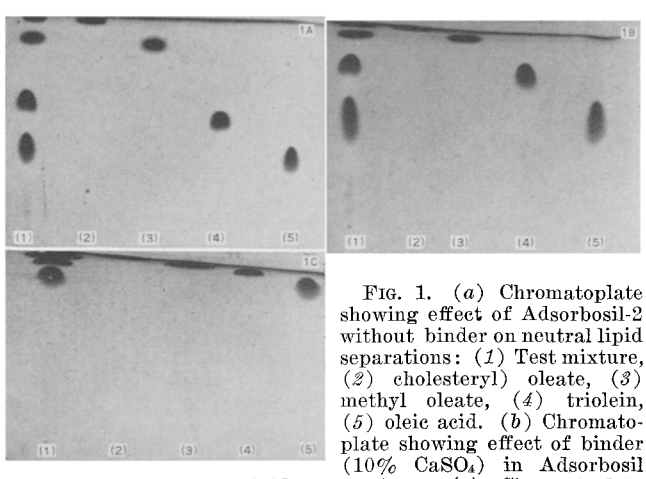

FIG. 1. (*a*) Chromatoplate showing effect of Adsorbosil-2 without binder on neutral lipid separations: (*1*) Test mixture, (*2*) cholesteryl) oleate, (*3*) methyl oleate, (*4*) triolein, (*5*) oleic acid. (*b*) Chromatoplate showing effect of binder (10% $CaSO_4$) in Adsorbosil on the same neutral lipid separations. (*c*) Chromatoplate showing effect of binder (25% $CaSO_4$) in Adsorbosil on the same neutral lipid separations. The plates were developed in petroleum ether, ethyl ether and acetic acid, 90:10:1 v/v/v, and visualized with a 50% sulfuric acid-dichromate spray solution.

behavior found for most of the silica gels used for TLC. So far, Adsorbosil S-2 (silicic acid) gave the lowest background count and is probably a most desirable product for neutron activation work, but on the other hand may not have the separation characteristics of some of the impure products shown here.

The acids cleaned up a great deal of the impurities in most of the powders except in the case of Adsorbosil S-2, which picked up contamination from the reagent grade acids that were used in the above treatment. This difficulty points to further problems that can be encountered.

Gamma Spectroscopy

Two widely used adsorbents, Adsorbosil-2 and Silica Gel-H, were qualitatively studied by gamma spectroscopy immediately upon removal from the reactor. A 400-channel gamma spectrometer (Technical Measurements Corporation, North Haven, Connecticut) equipped with a 3 × 3 in. sodium iodide crystal was used in the analysis. Both Adsorbosil-2 and Silica Gel-H powders showed large silicon gamma peaks and the presence of sodium and chloride ions. Upon decay of the gamma activity, about 10 days, the absorption curve showed P^{32} as the major isotope impurity. Copper, a prime suspect as an oxidizing agent in TLC powders, was not detected by gamma spectrometry. Cupric ion is largely a positron emitter accompanied by a weak gamma ray with energies close to those of silicon. These peaks were hidden by the silicon peak. It can be pointed out, however, that sodium and chloride ions were clearly detected in both samples along with phosphorus.

A quantitative study is under way to characterize TLC powders as to their elemental analysis using gamma and emission spectrometry. The work is becoming increasingly important because of effects that could interfere with quantitative techniques.

Effects of Calcium Sulfate as a Binder

Stahl (8) reported in 1958 that the addition of anhydrous calcium sulfate makes little or no difference in the adsorptive activity of thin layers of silica gel. It can be shown by actual experiments that there is an appreciable effect on lipid separations when calcium sulfate is used as a binder. A quantitative mixture of pure neutral lipids of cholesteryl oleate, methyl oleate, triolein and oleic acid was developed on chromatoplates with various amts of calcium sulfate. The same care was taken in the preparation of all the chromatoplates that were used in this test. The plates were developed in petroleum ether, ethyl ether and acetic acid, 90:10:1 v/v/v, and visualized with a 50% sulfuric acid-dichromate spray solution. Figure 1 shows a comparison of chromatoplates that had no calcium sulfate with others having 10% and 25%. Figure 2 is a curve showing the R_f values of oleic acid plotted against % $CaSO_4$. Note the linearity of the curve. This illustrates that, for the samples investigated, the amt of calcium sulfate used as a binder *does* affect the separation characteristics by increasing R_f values. It is also shown in Figure 2 that the amt of silver nitrate impregnated on the powder gives the opposite effect in R_f values for methyl ester separations.

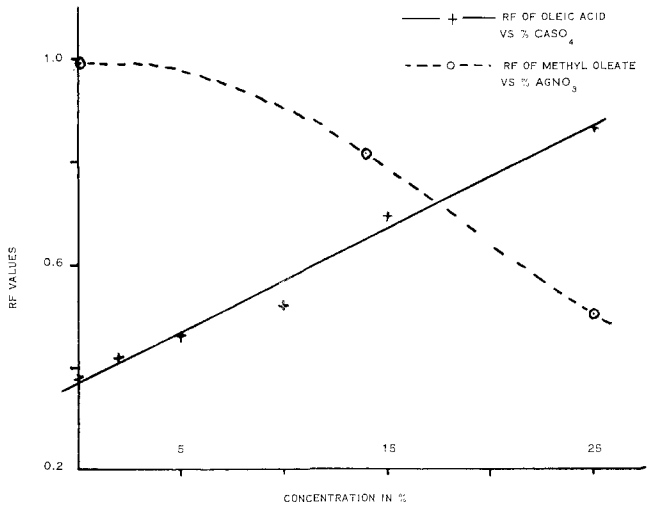

FIG. 2. Curves showing the effect of (+) calcium sulfate as binder, and (O) impregnated silver nitrate, both in Adsorbosil.

Neutral Lipids

Silica Gel as a Polar Phase

The separation of lipids into chemical classes by TLC has become routine in the past five years. Mangold (3) has illustrated many of these separations in a table given at the AOCS Short Course in 1961. Instances were shown where subfractionation within chemical classes could be obtained. The separation of compounds having short and long chains such as tricaproin and tristearin, or cholesteryl acetate and cholesteryl oleate, showed this behavior. These separations within classes are due to the changes of polarity caused by large differences in chain length and unsaturation. Figure 3 shows such a complete separation of cholesteryl acetate and oleate. Figure 4 shows other neutral lipid separations of interest, such as methyl esters and triglycerides.

Silver Nitrate-Impregnated Silica Gel

Silica gel impregnated with silver nitrate is useful for lipid separation. Not only are components separated according to the degree of unsaturation and the *cis-trans* configuration, but also components of the same type of unsaturation of varying chain length.

Barrett el al. (9) and Morris (10) described procedures and results with triglycerides of C_{18} acids and with methyl esters of fatty acids. Easy separations were obtained between the methyl esters of stearate, oleate, and linoleate, and a large separation between oleate and elaidate. de Vries (11) in 1962 achieved the same results in a modified solvent system and also

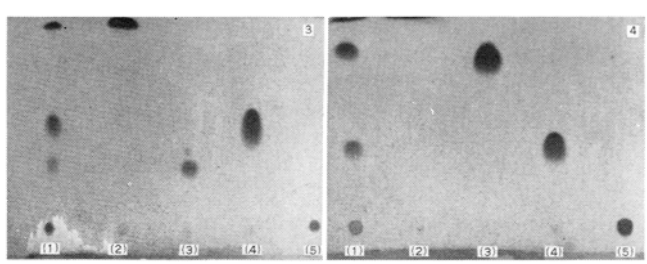

FIG. 3. Chromatoplate monitor for a cholesteryl ester reaction mixture; (*1*) Mixture, (*2*) cholesteryl oleate, (*3*) cholesteryl acetate, (*4*) methyl oleate, and (*5*) cholesterol. Note subfractionation between cholesteryl oleate and cholesteryl acetate. Developed in n-hexane:benzene, 60:40 v/v, on Adsorbosil-1. Visualized with a 50% sulfuric acid-dichromate spray solution.

FIG. 4. Neutral lipid separations on Adsorbosil-1. Developing solvent n-hexane:benzene, 60:40 v/v: (*1*) Mixture, (*2*) 1-octadecene, (*3*) cholesteryl oleate, (*4*) methyl oleate, and (*5*) triolein. Visualized with a 50% sulfuric acid-dichromate spray solution.

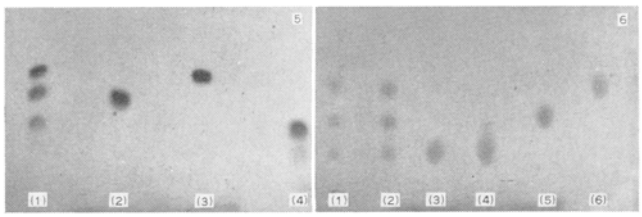

FIG. 5. Separation of closely related sterols on 25% silver nitrate-impregnated Adsorbosil-1: (*1*) Mixture, (*2*) cholesterol, (*3*) cholestanol and (*4*) desmosterol. Developing solvent: redistilled chloroform. Visualized with a 50% sulfuric acid-dichromate spray solution.

FIG. 6. Reversed-phase separation of monoene methyl esters on Reversil-3 impregnated with 15% n-decane in n-heptane. (*1*) and (*2*) 5 μg and 10 μg, respectively, of a mixture of methyl oleate, eicosenoate, erucate and nervonate, (*3*) methyl erucate and nervonate, (*4*) methyl nervonate, (*5*) methyl eicosenoate, and (*6*) methyl oleate. Developing solvent, acetic acid: acetonitrile, 1:1 v/v, 70% saturated with n-decane. Spots made visible with iodine vapor.

the separation of certain triglycerides differing either in total unsaturation or in the steric configuration of the component fatty acids.

According to Barrett, separations on silver nitrate-impregnated silica gel depend upon the formation of coordination complexes of olefinic linkage with silver ion. The complexes formed have been investigated extensively by Lucas (12) and found to be easily broken when samples were eluted from silica gel with appropriate solvents. Barrett stated that the quantitative analysis of triglyceride mixtures revealed an error of less than 3%.

The separation of closely related sterols was demonstrated by use of silver nitrate-impregnated silica gel (13) and is shown in Figure 5. Cholesterol, cholestanol, and desmosterol are shown to be completely separated from one another on 25% silver nitrate-impregnated Adsorbosil-1.

Recently Kaufmann (14) analyzed lecithins and the hydrolytic cleavage products of phosphatides. The homologs of natural lecithins were fractionated on silver nitrate-impregnated silica gel plates, and nine fractions from soya-lecithin and seven from egg-lecithin were found. The compounds cleavable by acid hydrolysis from phosphatides (choline, ethanolamine, serine, threonine, and inositol) were also separated. This work of Kaufmann's could not be duplicated in our laboratory.

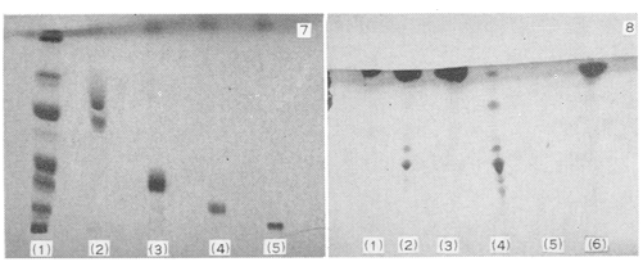

FIG. 7. Polar lipid separations on Adsorbosil-1. (*1*) Crude soy plant extract, (*2*) sulfatides, (*3*) lecithin, (*4*) sphingomyelin and (*5*) lysolecithin. Developing solvent, CMW 60:30:5 by volume, and visualized with a 50% sulfuric acid-dichromate spray solution.

FIG. 8. Cochromatogram of a rich neutral lipid sample. (*1*) Mono-, di-, and triglycerides, (*2*) rich neutral lipid mixture with less than 2% polar lipids, (*3*) soybean oil, (*4*) crude egg phosphatides, (*5*) phosphatidyl ethanolamine (cephalin) and (*6*) cholesterol. First developing solvent, benzene; second, CMW 70:25:5 by volume, at 38C. Visualized with a 50% sulfuric acid-dichromate spray solution.

Reversed Phase Thin-Layer Chromatography

Reversed phase partition TLC may be used to resolve a lipid class into its individual molecular species. In this type of chromatography, a nonpolar liquid phase such as a hydrocarbon or silicone is coated on a suitable support like diatomaceous earth or silica gel treated with dichlorodimethylsilane. The solvent or mobile phase passes over the coated support or stationary phase and carries the substances to be separated along with it. The components of the sample are thus partitioned between the two phases. Solvent systems used in this type of chromatography are highly polar, such as methanol, acetic acid and water. They are always saturated beforehand with the nonpolar material used as the stationary phase, to prevent elution of the stationary phase by the developing solvent.

Malins and Mangold (15) coated thin layers of Silica Gel G by slowly immersing the plates at room temp in a 5% solution of silicone (Dow Corning 200) in diethyl ether and used these chromatoplates to separate methyl esters of saturated and unsaturated fatty acids.

Winterstein (16) used paraffin as the stationary phase in the separation of a mixture of carotenals consisting of molecules of 20 to 40 carbons in length. The coating procedure was essentially the same as that used by Mangold, but with a solvent consisting of light petroleum containing 5% liquid paraffin. The light petroleum was subsequently removed at 120C.

Kaufmann and Makus (17) used Silica Gel G impregnated with a 15% solution of undecane in light petroleum and also separated methyl esters, but of long chain types such as the stearate, arachidate and behenate, as well as a mixture of the esters of oleic, linoleic, linolenic and erucic acids. They observed certain critical pairs that could not be separated, such as linolenate from laurate, linoleate from myristate, and oleate from palmitate. They also separated long chain fatty alcohols (C_{16}-C_{22}), and diglycerides and triglycerides. In all cases polar solvent systems were used. For the resolution of cholesteryl esters Kaufmann et al. (18) used liquid paraffin as the stationary phase. Kaufmann and Ko (19) separated triglycerides, hydroxy acids, keto acids, and lactones using tetradecane-impregnated Kieselguhr G. Kaufmann and Khoe (20) have recently separated free fatty acids on thin layers of calcium sulfate impregnated with undecane. The layers in this procedure could be rinsed with water like paper chromatograms.

Recently, Wolfman and Sachs (21) employed the Kaufmann method (17) in the separation of desmosterol and cholesterol, which differ only by the presence of a double bond. They used Adsorbosil-1 impregnated with 15% undecane in petroleum ether. They obtained excellent results using acetic acid-acetonitrile (1:1) 70% saturated with undecane.

Reversil-3 for TLC

The use of reversed phase systems for the separation of neutral lipids was repeated and the work of Kaufmann and Makus (17) and Malins and Mangold (15) was confirmed. With Reversil-3, a ready-made reversed phase powder, a mixture of relatively pure C_{18} = to C_{24} = monoene methyl esters (5 μg samples) showed even sharper separations, and maximum sensitivity. The use of Reversil-3 allows rapid development of the chromatoplates, and requires less labor in their preparation.

Reversil-3 is a 10-μ range silica gel that has been acid washed, solvent extracted, and treated with di-

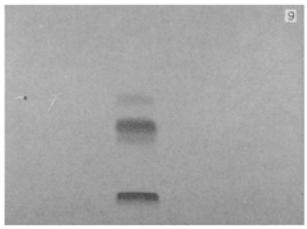

FIG. 9. Separation of beef brain cerebrosides and sulfatides on Adsorbosil-1. Developing solvent, CMW 90:10:1 by volume, and visualized with a 50% sulfuric acid-dichromate spray solution.

methyldichlorosilane as described by Howard and Martin (22). The silane treatment renders the powder hydrophobic (nonwetted by strongly polar solvents) and inert. In the case of the monoene methyl ester separations, chromatoplates were spread in the usual manner by slurrying Reversil-3 with a 15% mixture of n-decane in n-heptane instead of water and allowing the plates to air-dry for several hours. Samples of 5 μg and 10 μg were applied to the chromatoplate and developed in acetic acid:acetonitrile (1:1) 70% saturated with n-decane. Figure 6 shows these separations visualized with iodine vapors. The methyl oleate spot moved the farthest from the origin, followed by methyl eicosenoate, methyl erucate and methyl nervonate. The methyl erucate and nervonate spots were not completely resolved by this solvent, but the other spots were well defined.

More work is needed in this area, especially in testing new phases. It is not enough to be content with the present methods for reversed phase chromatography. Both polar and nonpolar phases, as in gas chromatography, need to be critically evaluated. For example, a phase like diethylene glycol succinate (DEGS), a very successful liquid phase in gas chromatography, might be used in a similar manner for reversed phase TLC. Whether these methods can be worked out for resolving difficult mixtures of heat-unstable, high-molecular weight lipids such as the ketosteroid metabolites, mixed glycerides, critical pairs and other closely related chemicals, only time will tell. This type of study is becoming more important because of the increasing amt of work published on quantitative TLC analysis of lipids. Better separations and newer approaches to lipid analysis are needed.

Polar Lipids

Use of Neutral Silica Gel

The separation of biologically important compounds, such as phospholipids, cerebrosides, sulfatides, and other glycolipids, is obtained on neutral silica gel powders and requires polar solvent systems generally consisting of chloroform, methanol and water (CMW). A number of spots can be seen in lipid extracts such as those from soybean. Figure 7 shows the separation obtained on samples of a crude plant extract from soybean on Adsorbosil-1. The plant extract at the left edge of the plate shows 11 spots. The fast-moving components in this mixture are neutral lipids (top two spots), followed by digalactosyl diglyceride, unknown, phosphatidyl ethanolamine, sulfolipid, monogalactosyl diglyceride, lecithin, phytosphingomeylin, lysolecithin and phosphatidyl serine and other polar compounds. The other samples shown on the rest of the plate were used as reference compounds. They are the two sulfatide spots, lecithin, sphingomyelin and lysolecithin. Note the presence of spots at the solvent front. These are either hydrolysis products or artifacts.

Recently demonstrated was a technique for detecting phospholipids in an extract rich in neutral lipids

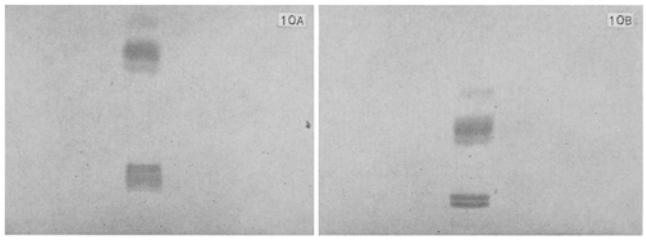

FIG. 10. Separation of beef brain cerebrosides and sulfatides on Adsorbosil-1. Developing solvent, (a) CMW 90:20:2 by volume; (b) CMW:NH_4OH 90:20:2:1 by volume. Visualized with a 50% sulfuric acid-dichromate spray solution.

(23). Less than 2% polar lipids could be detected by this method. Basically the method involved the development of chromatoplates in benzene or chloroform, and after 10 min, cochromatographing the same plate in a chamber saturated with a solvent system of CMW, 70:25:5 by volume, at 38C. The lipid sample used in this analysis was an extract of a synthetic fatty emulsion. The emulsion had been prepared from plant oils, egg phosphatides and water-soluble materials. The phospholipid separation was compared with that obtained on a crude egg lecithin on the same chromatoplate. Figure 8 shows the neutral lipids at the solvent front. The second sample is the emulsion in question and shows minor amts of polar lipids. The fourth sample is the crude egg lecithin extract. Lecithin is the intense middle spot on both samples.

Beef brain cerebrosides and sulfatides can be separated into their components by TLC with use of conventional CMW solvents, but the two sulfatide spots are not always distinguishable. These separations can be illustrated in a series of chromatoplates by showing the effect solvent systems can have on certain compounds. In Figure 9, CMW 90:10:1 brings out the three cerebroside spots with R_f's of 0.39, 0.31, 0.24 and one sulfatide spot which barely moves off the origin. When CMW 90:20:2 is used, the R_f's of the cerebrosides increase to 0.90, 0.73, 0.63, and one sulfatide has an R_f of 0.25 with tailing (Fig. 10a). By adding just one part 28% ammonia to the same solvent system, three cerebroside bands and two sulfatide bands (with no tailing) having R_f's of 0.55, 0.42, 0.36, and 0.14 and 0.11 can be shown (Fig. 10b).

The illustrations cited emphasize that no one system or method can be used for a final analysis of a given mixture of components. It is usually worthwhile to use other separation procedures such as column chromatography or preparative TLC to conc components into smaller samples or into particular classes of compounds.

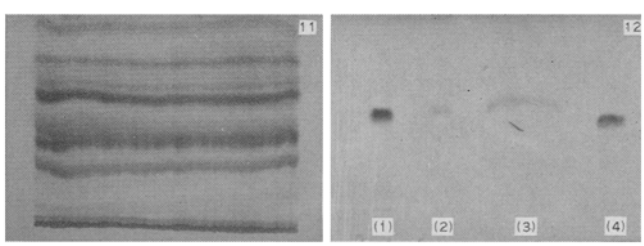

FIG. 11. Preparative chromatoplate of polar lipids on Adsorbosil-2. Developing solvent CMW 60:30:5 by volume; spots made visible with iodine vapor.

FIG. 12. Rechromatographed phosphatidyl ethanolamine (U)-C^{14} "prepped" from chromatoplate. (1) and (4) standard phosphatidyl ethanolamine (PE); (2) and (3) "prepped" PE (U)-C^{14}. Visualized with a 50% sulfuric acid-dichromate spray solution.

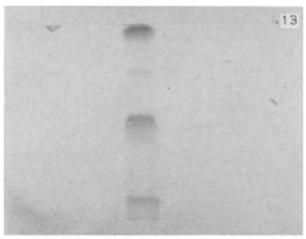

FIG. 13. Separation of mono- and digalactosyl diglycerides from spinach leaf extract on Adsorbosil-1. Developing solvent, chloroform:methanol: acetic acid, 100:25:8 by volume, and visualized with a 50% sulfuric acid-dichromate spray solution.

Radioactive Materials

A good example of the use of TLC for the detection and preparation of radioactive materials is the procedure used in our laboratory for the purification of radioactive phosphatidyl ethanolamine (PE). The radioactive PE is prepared by growing *Chlorella pyrenoidosa* with a mixture of mineral nutrients and labeled carbon dioxide ($C^{14}O_2$) in a closed system. The labeled algae are extracted with solvents and the C^{14}-labeled lipids subsequently chromatographed on a thin layer plate of Adsorbosil-2. The plate is developed in CMW 60:30:5 and is shown in Figure 11. The spots representing PE are detected by iodine vapor, scraped off the plate into a filter funnel and extracted with a solvent mixture of ethanol, chloroform, water and acetic acid. Figure 12 shows the rechromatographed results on the pure C^{14}-labeled PE.

This method is useful for preparing laboratory amts of C^{14}-labeled compounds and is specially useful for the repurification of tritiated lipids.

Glycolipids

The best characterized and major glycolipids of plant tissue are monogalactosyl diglycerides (g-gal) and digalactosyl diglycerides (g-gal-gal). These lipids were first identified by Carter and coworkers (24–26) using chemical and IR studies. G-gal was obtained pure by combination of solvent fractionations and silicic acid chromatography. Recently, Allen (27) reported the complete separation of the two galactosyl diglycerides by use of a diethylaminoethyl (DEAE) cellulose column prepared by Rouser's method (28) after a prior separation by countercurrent distribution. O'Brien and Benson (29) have also succeeded in separating the galactosyl lipids on columns of Florisil and DEAE cellulose. Steim (30) purified monogalactosyl diglyceride by the passage of an acetone-soluble fraction of beef brain lipids through a column of Adsorbosil (140/200 mesh) with diethyl ether to remove the nonpolar lipids and elution with acetone. The pure g-gal was subsequently prepared by a final pass through another column of Adsorbosil (140/200 mesh) with a solvent system of chloroform having a linear gradient of 0 to 4% methanol.

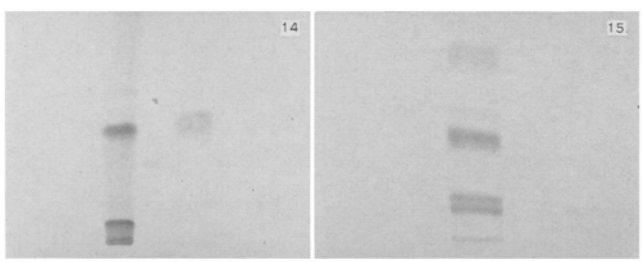

FIG. 14. Autoradiogram of monogalactosyl diglycerides from C^{14}-labeled Chlorella superimposed on chromatoplate showing beef brain monogalactosyl diglyceride on Adsorbosil-1 that was made visible by a 50% sulfuric acid-dichromate spray solution. Developing solvent, CMW 90:10:1 by volume.

FIG. 15. Separation of beef brain galactosyl diglycerides, cerebrosides and sulfatides on Adsorbosil-1. Developing solvent, CMW 90:20:2 by volume, and visualized with a 50% sulfuric acid-dichromate spray solution.

For analytical purposes, however, superior separations have been obtained by TLC on silica gel. Nichols (31) worked out solvent systems for glycolipids and other plant lipids on Silica Gel G. In our laboratory, a modified Marinetti solvent with carefully controlled water content (diisobutyl ketone, acetic acid and water, 40:25:3.7 by volume) was used and found to be as satisfactory as the CMW 65:25:4 system. Good separations of g-gal and g-gal-gal lipid were obtained on crude spinach extracts using chromatoplates of Adsorbosil-1. Figure 13 shows a chromatogram of these separations from spinach leaves. Not shown in this figure, however, are the chlorophylls, carotenoids, sterols and other neutral lipid spots that were removed from the sample by first chromatographing the plate in redistilled acetone. It was then cochromatographed in chloroform:methanol:acetic acid, 100:25:8 by volume. The farthest moving spot, and the most intense, is the monogalactosyl diglyceride. The tailing of the middle spot (digalactosyl diglyceride) was contributed to by the presence of phosphatidyl ethanolamine, just faintly visible. The spot near the origin is lecithin.

Autoradiography can be used to locate the major glycolipids in C^{14}-labeled chlorella. TLC separations of the radioactive compounds are made on a chromatoplate. The spots are visualized by exposing the plates to an x-ray film for a certain length of time and then developing the film. Compounds exhibiting radioactivity show up as dark spots on a transparent background. The greater the activity of the compound, the darker the spot on the film. Spots can be evaluated by densitometry (32).

Figure 14 is an autoradiogram of monogalactosyl diglyceride (from C^{14}-labeled chlorella) superimposed on a chromatoplate showing the same compound, nonradioactive, from beef brain. The streaky appearance of the autoradiogram is common in our laboratory with radioactive separations. The spot near the origin is lecithin, with more polar compounds above it. The solvent system was CMW 90:10:1.

Figure 15 shows the relationship of galactosyl diglycerides to cerebrosides and sulfatides in beef brain lipids. The separation was obtained on Adsorbosil-1 with use of CMW 90:20:2. Individual components are, from above down, monogalactosyl diglyceride (R_f 0.61), three cerebroside bands (R_f 0.41, 0.34, 0.31), followed by the two sulfatides (R_f 0.14, 0.11) and the origin. The variation in R_f values for the cerebroside bands from those previously shown could be attributed to the water content of the solvent system. However, good agreement was obtained with the sulfatides.

Pitfalls and Precautions

There are many ways in which erroneous conclusions can be drawn from thin layer chromatography. Rouser et al. (28) reported on the degree of variability of results with TLC and concluded that the technique must be used with great care. They encountered variations in migration of lipids on chromatoplates spread with different amts of adsorbents and with different silica gel preparations, variations according to the method of activation and cooling of the plates and the method of maintaining saturation in the developing chamber. All of these variables could lead to deception.

Another source of difficulty is the autoxidation of labile lipids. A particularly labile lipid is phosphatidyl ethanolamine, because it contains the bulk of the

polyunsaturated fatty acids of a natural mixture whereas other phospholipids are more likely to have a 1:1 ratio of saturated to unsaturated acids, usually palmitic, oleic and linoleic. An exception to this is the phosphatidyl ethanolamine of *Escherichia coli*. This lipid is relatively stable because the unsaturated fatty acids are mostly monoenes (33). Air oxidation is the main reason for difficulties. For instance, if a polyunsaturated sample stands on a plate before being developed, autoxidation and hydrolysis can easily occur. These effects show up as extra spots or streaking of the sample. Some autoxidation products fail to move from the origin, but not all spots remaining at the origin are due to autoxidation.

Still another source of difficulty may be introduced by humidity and fumes in the laboratory. Such conditions can interfere with lipid separations. Silica gel is an adsorbent with the ability to adsorb water quickly when it is very active. The presence of fumes in the laboratory can change the pH of the adsorbent layer and also cause discoloration of the adsorbent. Laboratory conditions such as these are the prime reason why layers flake or fall off plates.

The water content in solvent systems must also be controlled. Too much water will move most lipids close to the solvent front, with streaking, and too little will have the opposite effect. The ratio of the water in the solvent system also has an effect on the separation of certain lipids. This behavior is particularly pronounced with phosphatidyl ethanolamine and the sulfatides. These compounds may have close R_f values, but they can be separated by varying the amount of water in the system (28).

Artifacts may be introduced by using impure solvents and dirty silica gel powders. A good deal of information may be obtained by visual inspection. A means for testing dirty silica gel is to extract the powder with a pure polar solvent such as methanol and subsequently remove the solvent by evaporation. Another method of checking the quality of both solvents and powder is by two-dimensional TLC using the same solvent in both directions. Discolored solvent fronts in both directions are an indication of an impure solvent, and a conc of a discolored substance at one edge of the plate points to an impure powder.

It is very important that the solvent be pure. It is best to use a solvent that has a known molecular formula. For example, petroleum ether, which is a mixture of low-boiling hydrocarbons, may not always give reproducible results. Most solvents should be redistilled. Some solvents are sufficiently purified by passage through a column of aluminum oxide. Stock solutions of the purified solvent systems are usually prepared and stored for future use. Approx 200 ml of the system is enough to saturate a developing chamber (12 in. × 10 in.), and for development of chromatoplates. Solvent systems should be changed in such chambers every day to insure reproducibility.

It is important to control ambient temps in the laboratory and the developing chamber. Increased temps will reduce the adsorptive capacity of the adsorbent and cause poor resolution. The effect may also be due to volatilization of the developing solvent causing the solvent to ascend rapidly. The effect is more marked if the chamber is not adequately sealed. Decreased temps have little effect on separations but the rate of development is very slow. The difference of opinion regarding the effects of temp may possibly originate in the differences in the solvents used for development.

Confusion can arise on separations of unknown lipid materials. For example, a single spot on a chromatoplate may represent more than one substance, or, several spots may represent only one substance. Lipids such as cerebrosides give three spots and possibly more, sulfatides show two spots, and sphingomyelin also shows two spots. If these are suspected, they should always be identified by using authentic standards or a well-characterized natural mixture. These multiple separations show clearly the presence of different classes of compounds. The slower moving components of cerebrosides and sulfatides have hydroxy-type fatty acids, and the faster moving spots are due to the components with nonhydroxy-fatty acids (34).

A simple factor which can influence results is nonhomogeneity in the powder mixture. The blending of binder material with silica gel is important and care should be used in this process. In some cases it is necessary to adjust the activity of the adsorption media or even their pH value. The significance of this is not truly realized. TLC powders are being supplied with little information to the consumer as to their physical properties and chemical composition, and very little knowledge on which to base standards for these. More fundamental work is needed.

Most importantly, the interplay of adsorbents, solvents, and the behavior of the functional groups characteristic of the particular lipids under study need to be understood. These three factors must be coordinated in order to achieve the desired separations. When the reproducibility of lipid separations and their major variables in thin layer chromatography have been explored qualitatively, quantitative analysis can be undertaken with more assurance.

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