The rapid and inexpensive laboratory procedure described is well established as an adjunct to other physical and chemical techniques for the characterization and identification of organic compounds. Systematic studies on homologous series, as summarized in the present report, may further the knowledge of the relationship between the chemical structures and the CST-values of organic substances. The CST-values determined so far may serve as a basis to predict with sufficient accuracy the critical solution temps of many more pure compounds of various lipid classes. This procedure may be helpful in confirming the structures of synthetic preparations and of materials isolated from the complex natural fats, oils and waxes. Moreover, CST-values of pure compounds may be used for estimating the CST-values of their naturally occurring mixtures. This would be a simple procedure for checking and substantiating the results of quantitative analyses.

ACKNOWLEDGMENTS

B. J. F. Hudson, V. Mahadevan and F. H. Mattson provided various highly purified lipids.

Supported by the U.S. Public Health Service, National Institutes of Health Grants AM 06674 and GM 5817.

REFERENCES

1. Alexejew, W., Ann. physik. Chem. *28*, 305 (1886).
2. AOCS Official Methods Cb 4–35, American Oil Chemists' Society, 35 E. Wacker Drive, Chicago 1, Ill.
3. ASTM Method D611 and D1012, American Society for Testing Materials, 1916 Race Street, Philadelphia 3, Pa.
4. Baumann, W. J., and H. K. Mangold, J. Org. Chem. *29*, 3055 (1964).
5. Baumann, W. J., and H. K. Mangold, J. Org. Chem., in press.
6. Broughton, G., and D. C. Jones, Trans. Faraday Soc. *32*, 685 (1936).
7. Brown, C. W., Ind. Eng. Chem., Anal. Ed., *18*, 739 (1946).
8. Craig, B. M., and N. L. Murty, Can. J. Chem. *36*, 1297 (1958).
9. Crismer, L., Bull. Assoc. Belge de Chim. *9*, 71, 143 (1895).
10. Crismer, L., *Ibid.* *9*, 359 (1896).
11. Crismer, L., *Ibid.* *10*, 312 (1897).
12. Cubero, J. M., and H. K. Mangold, unpublished.
13. Cubero, J. M., and H. H. O. Schmid, unpublished.
14. Discussions Faraday Soc. *15*, 1953. The Equilibrium Properties of Solutions of Non-Electrolytes.
15. Fachini, S., and G. Dorta, Z. dtsch. öl–u. Fettind. *45*, 498 (1925).
16. Fischer, R. W., in Microchemical Techniques (N. D. Cheronis, ed.) Microchem. J. Symp. *2*, 977 (1962).
17. Fischer, R., and J. Horner, Mikrochim. Acta 1953, 386.
18. Fischer, R., and G. Karasek, Mikrochemie ver. Mikrochim. Acta *33*, 316 (1947).
19. Fischer, R., and T. Kartnig, Fette, Seifen, Anstrichmittel *60*, 904 (1958).
20. Fischer, R., and H. Moser, Erdöl Kohle *9*, 377 (1956).
21. Francis, A. W., "Critical Solution Temperatures," No. 31, Advances in Chemistry Series. American Chemical Society, Washington, D.C., 1961.
22. Francis, A. W., Ind. Eng. Chem. *45*, 2789 (1953).
23. Gölles, F., Microchim. Acta 1963, 376 and previous publications.
24. Hildebrand, J. H., and R. L. Scott, "The Solubility of Nonelectrolytes," American Chemical Society Monograph Series, 3rd ed., Reinhold Pub. Corp., New York, 1950.
25. Hoerr, C. W., and H. J. Harwood, J. Phys. Chem. *56*, 1068 (1952).
26. Hoerr, C. W., H. J. Harwood and A. W. Ralston, J. Org. Chem. *9*, 267 (1944).
27. Hoerr, C. W., and A. W. Ralston, *Ibid.* *9*, 329 (1944).
28. Hoerr, C. W., R. S. Sedgwick and A. W. Ralston, *Ibid.* *11*, 603 (1946).
29. Horning, E. C., E. A. Moscatelli and C. C. Sweeley, Chem. Ind. 1959, 751.
30. International Critical Tables, McGraw-Hill Book Co., New York, 1928.
31. Kartnig, T., Sci. Pharm. *29*, 187 (1961).
32. Kaufmann, H. P., "Analyse der Fette und Fettprodukte," Band I, Springer-Verlag, Berlin, Göttingen, Heidelberg, 1958.
33. Kaufmann, H. P., and J. G. Thieme, Fette, Seifen, Anstrichmittel *58*, 585 (1956).
34. Kofler, L., and A. Kofler, "Thermo-Mikro-Methoden zur Kennzeichnung Organischer Stoffe und Stoffgemische," Universitäts-Verlag Wagner, Innsbruck, 1954.
35. Landolt-Börnstein, Physikalisch-Chemische Tabellen; Zahlenwerte und Fünktionen, Springer-Verlag, Berlin, Göttingen, Heidelberg, 1923–1960.
36. Mahadevan, V., and W. O. Lundberg, J. Lipid Res. *3*, 106 (1962).
37. Malkin, T., The *Polymorphism* of Glycerides, in "Progress in the Chemistry of Fats and Other Lipids," R. T. Holman, W. O. Lundberg, and T. Malkin, eds., Vol. 2, Pergamon Press, New York, 1954, pp. 1–50.
38. Mangold, H. K., B. G. Lamp and H. Schlenk, J. Am. Chem. Soc. *77*, 6070 (1955).
39. Mangold, H. K., H. H. O. Schmid and E. Stahl, Thin-Layer Chromatography, in "Methods of Biochemical Analysis," Vol. 12, D. Glick, ed., Interscience, Publ., a division of John Wiley & Sons, Inc., New York, London, Sydney, 1964.
40. McCrone, W. C., "Fusion Methods in Chemical Microscopy," Interscience, Publ., Inc., New York, 1957.
41. Morris, L. J., J. Chromatog. *12*, 321 (1963).
42. Rothmund, V., Z. physik. Chem. *26*, 433 (1898).
43. Schlenk, H., J. L. Gellerman and D. M. Sand, Anal. Chem. *34*, 1529 (1962).
44. Schmid, H. H. O., H. K. Mangold and W. O. Lundberg, Microchem. J. *7*, 287 (1963).
45. Schmid, H. H. O., H. K. Mangold and W. O. Lundberg, *Ibid.* *7*, 297 (1963).
46. Schmid, H. H. O., H. K. Mangold and W. O. Lundberg, *Ibid.*, in press (1965).
47. Sedgwick, R. S., C. W. Hoerr and H. J. Harwood, J. Org. Chem. *17*, 327 (1952).
48. Seidell, A., "Solubilities of Organic Compounds," Vol. II, D. Van Nostrand Co., Inc., New York, 1941; Seidell, A., and W. F. Linke, "Solubilities of Inorganic and Organic Compounds, Supplementary Volume." D. Van Nostrand Co., Inc., New York, 1951.
49. Timmermans, J., "The Physico-Chemical Constants of Binary Systems in Concentrated Solutions," Vols. I–IV, Interscience, Publ., Inc., New York, 1959–1960.
50. Valenta, E., Dinglers Polytechn. J. *252*, 296 (1884).
51. Valenta, E., *Ibid.* *253*, 418 (1884).
52. Zeman, I., and J. Pokorny, J. Chromatog. *10*, 15 (1963).

Lipid Analysis by Quantitative Thin-Layer Chromatography

O. S. PRIVETT, M. L. BLANK, D. W. CODDING and E. C. NICKELL, The Hormel Institute, University of Minnesota, Austin, Minnesota

Abstract

Techniques for the quantitative analysis of lipids using thin-layer chromatography (TLC) are reviewed. The general procedures are divided into two groups on the basis of whether or not the methods involve the recovery of substances from chromatoplates.

Recovery methods are elaborated under detection of spots, recovery of substances and quantification. Methods are described for the recovery of labile compounds from chromatoplates and for the determination of the structures of triglycerides and lecithins.

Methods for the direct quantitative analysis of spots on chromatoplates are reviewed. These include measurements of spot size, reflectance, absorbance of transmitted light, and fluorescence. Details of the photodensitometric method, particularly, spot visualization and instrumentation are described. The analysis of lipid classes using a combination of DEAE cellulose chromatography and TLC by the densitomery of charred spots is illustrated.

Introduction

THE SPEED AND VERSATILITY of thin-layer chromatography (TLC) and its ability to resolve compounds with very minor differences in chemical structure, make it especially valuable as an analytical technique for the quantitative analysis of lipids. In addition to providing a method of analysis for many lipids which cannot be analyzed otherwise, it is also used to facilitate the analysis of many lipid compounds whose quantitative determination pose special problems. Methods for quantitative analysis by TLC may be divided into two broad groups, those which involve recovery of the separated compounds from the chromatoplate followed by an analysis using well-established analytical procedures, and methods based on a direct analysis of the spots on the chromatoplate. The more common techniques employed in quantitative

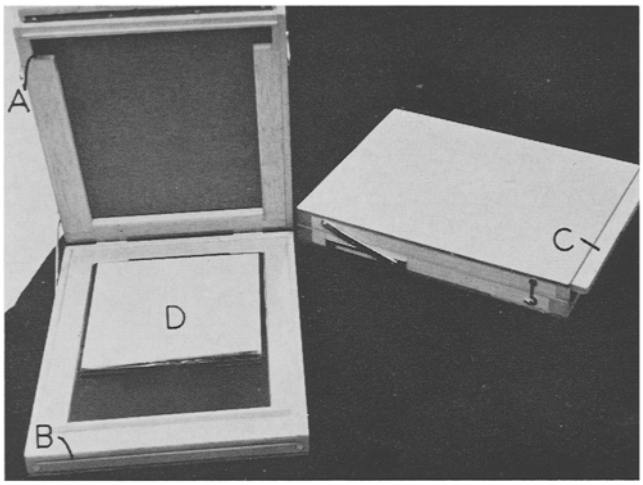

FIG. 1. Film holder for autoradiography. *A*, slot for x-ray film which is loaded in the dark and protected by slide inserted in upper slot *C*. Chromatoplate *D* placed on stage and holder closed as shown on right. Slide is removed and inserted in slot *B* bringing plate into contact with x-ray film.

TLC and selected examples of applications in the field of lipids are elaborated here.

Recovery Methods

Detection Techniques

Recovery methods are the most diverse and most widely applied because, basically, they depend only upon the separation of the constituents. Prior to the recovery of substances from chromatoplates their positions must be located. A host of reagents and techniques have been used for this purpose and a comprehensive discussion of them is beyond the scope of this report. However, there are certain general guides which should be followed in their use for quantitative TLC. The simplest techniques should be employed. For example, many compounds, including phospholipids, bile acids and sterols, may be made visible long enough to be located by spraying the plate with a neutral solvent and viewing it with transmitted light. This technique has formed the basis for the quantitative analysis of menthofurane in lemon oil by combining it with photography and densitometry of the resulting negative (30).

When chromogenic reagents are used as indicators in quantitative applications of TLC, care should be taken to insure that they do not interfere with the subsequent analysis. Common practice is to remove the reagents by extraction or a separate chromatography. Indicators such as bromothymol blue, Rhodamine 6G and 2,7-dichlorofluorescein, which are commonly employed for the detection of lipids, can usually be removed by these methods. Destructive methods of detection, such as charring, may also be employed with recovery methods through the use of a pilot plate prepared under identical conditions as plates used in the analysis. Methods of this type are not recommended when other techniques can be used, however.

Radioactive compounds may be detected for quantitative analyses by scanning devices or by the preparation of a radiogram by exposing the plate to x-ray film. The literature on the use of these techniques has been reported by Mangold (48,49). A simple and highly satisfactory apparatus used in this laboratory (7) for the preparation of radiograms is shown in Figure 1. The plate is placed on a stage which is elevated by a slide arrangement to provide a light but complete contact of the adsorbent layer with the x-ray film which is placed in the slot above it.

Recovery Techniques

The most common recovery method is to scrape the substances from the chromatoplate with a razor blade or similar instrument (25,28,54). Several elaborate techniques have been devised for the removal of spots from chromatoplates, particularly for the recovery of radioactive substances. Vacuum aspiration techniques (31,67,32,5,50,90,51) are commonly employed for the removal of individual spots. Recoveries of the order of 98.7% have been reported for a technique of this type by Hirsh et al. (31). Snyder (89) has described an automatic scraper which removes zones of adsorbent of predetermined size directly into vials for analysis of radioactive lipids by scintillation counting. This technique permits the determination of a distribution pattern of radioactivity over the entire plate and is very useful for the detection of minor components as well as components not well separated from each other.

In some analyses the recovered substances may be extracted from the adsorbent, in others they may be analyzed directly in the presence of the adsorbent. When samples are to be extracted from the adsorbent, care must be taken in the choice of solvents and extraction technique to insure complete recovery of substances. Some compounds, particularly phospholipids, are prone to alteration and require special precautions in order to recover them from chromatoplates unchanged. So unstable are natural lecithins, for example, that they cannot be stored for prolonged periods without some alteration, even at low temps. The best method for the storage of phospholipids is at low temps in dilute solution with air-free solvents. The deterioration of these compounds appears to occur regardless of the degree of unsaturation because even fully saturated lecithins isolated from natural sources, for example, are prone to alteration. It should also be noted that phospholipids generally undergo some alteration during such commonly applied reactions as interesterification or enzymatic hydrolysis. Deterioration of the phospholipids usually may be detected by the formation of products that have higher, as well as lower, Rf values than the parent compounds on TLC. Studies in this laboratory on the nature of the products with the high Rf values formed from lecithin during chemical and enzymatic hydrolysis indicate that they consist mainly of hydrocarbons.

Several procedures have been described for the quantitative recovery of phospholipids. One such satisfactory procedure is as follows. After the plate is developed in a jar in which the air has been displaced with nitrogen it is removed and immediately sprayed with a bromothymol blue indicator dye (0.1%) dissolved in 10% ethanol in distilled water, made basic with NH_4OH. This dye (33,35) is a good indicator for phospholipids and related compounds because it is stable, easy to remove (see below) and a sensitive indicator on wet chromatoplates. Rhodamine 6G (73) and 2,7-dichlorofluorescein (48) are also excellent indicators for phospholipids as well as other lipids. However, these indicators are most sensitive when the plate is dry or near dry and it must be viewed under ultraviolet light conditions which increases the chance of changes in highly labile compounds such as the phospholipids. Chromatoplates should never be allowed to dry in the analysis of phospholipids and all solutions used in the course of the recovery of these substances should be protected from atmospheric oxygen by an inert atmosphere at all times. When the presence of the dye does not interfere with the subsequent analysis, the spots are scraped

into beakers containing about 20 ml of chloroform: methanol:water (65:40:5, v/v/v). Each area from which the adsorbent is scraped is then swabbed with a small piece of cotton wetted with the extraction solvent to insure complete recovery of the sample. The cotton which is pre-extracted with aqueous, as well as organic, solvents is washed with a small amount of chloroform. The slurry of each component is transferred to a sintered glass funnel and washed with several small portions of the same mixture of solvents. The filtrates of each slurry are combined and evaporated to near dryness. The residue is dissolved in a small amount of chloroform. Insoluble matter, such as a binder, if it is employed, is removed at this point by filtration by means of a small cotton plug in the syringe used to make the transfer of the solutions to another flask. This solution is evaporated to near dryness to remove water which evaporates with the chloroform, and then diluted to a known volume for an analysis.

When it is necessary to remove the indicator prior to analysis (particularly for a subsequent gravimetric analysis), a slightly different procedure is followed. The spots are scraped into beakers containing about 20 ml of an aqueous solution of 15% 1:1 (v/v) methanol-acetone. The area of glass from which the absorbent is removed is also swabbed with this solution. The slurry is then transferred to a sintered glass funnel, filtered and washed with two more small portions of this solvent. The filtrate which contains the dye is discarded. The lipid is then recovered by elution, first with a small amount of ethyl ether and then with chloroform:methanol:water (65:40:5, v/v/v). The binder and water are removed as described above. Although we generally use filtration in these procedures, the washing and extraction techniques may be carried out by centrifugation.

Quantitative recoveries of polyunsaturated fatty acids or esters may also be obtained from adsorbents impregnated with silver nitrate by the same general procedure, except that a small amount of HCl should be added to the solutions to insure complete breakup of the silver-fat complexes, and the esters are recovered by a final extraction into petroleum ether which is washed to remove the acid and silver salts. With fatty acids or esters Rhodamine 6G or 2,7-dichlorofluorescein are usually employed to detect the positions of the spots. With plain adsorbents such as silicic acid, iodine staining may be used to locate the positions of the spots of polyunsaturated fatty acids provided they are not left in contact with the iodine vapors for a prolonged period (23). Otherwise, some destruction of the unsaturated fatty acids occurs (101,53).

Descending TLC may also be used to eliminate the problem of alteration in the recovery of phospholipids. The disadvantage of this method is the relatively long time (approx 8 hr) required to elute all the samples from the plate. However, by means of this technique it can be demonstrated that changes in the phospholipids with ascending TLC occur generally during the handling of the sample after development of the plate because by continuing the application of the solvent to the plate in an open-column type of elution all of the substances can be eluted from the plate without any evidences of alteration of their structures. A simple apparatus for descending TLC is shown in Figure 2. The solvent is introduced onto the adsorbent by means of a small stainless steel tray, the open edge of which is placed in contact with the adsorbent layer.

A novel application of the descending technique for the recovery of vitamin A and its derivatives from chromatoplates developed by the normal ascending procedure has been described by Varma et al. (100). In this method the plate is placed in a horizontal position and the spots are eluted individually by applying the solvent by a wick arrangement from the separatory funnel attached to the plate above the spot.

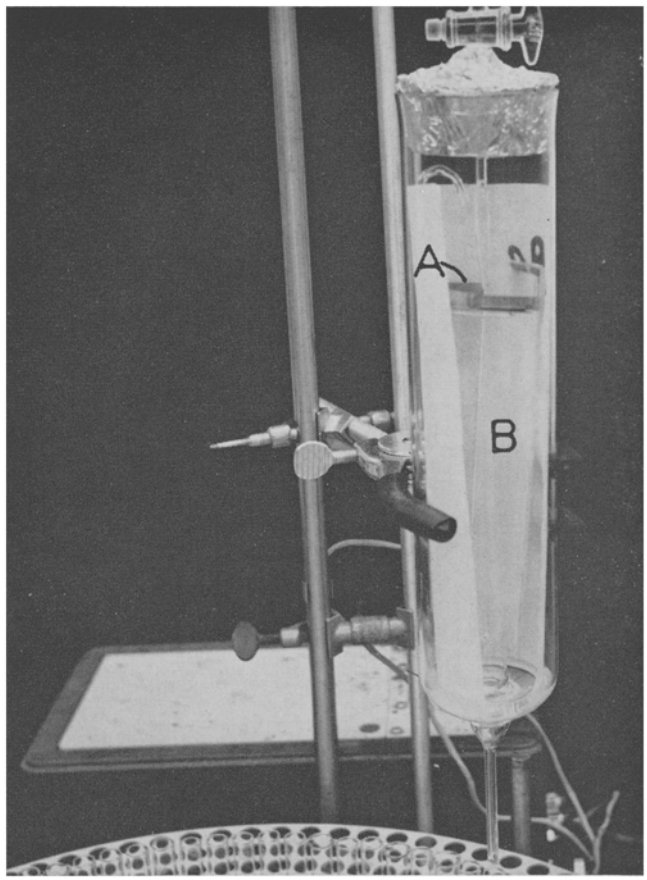

FIG. 2. Apparatus for descending TLC. *A*, tray for introduction of solvent on chromatoplate, *B*, chromatoplate.

Quantification by Gravimetric Analysis

Perhaps the most basic analytical method of quantitative TLC is a gravimetric analysis of the recovered substances. These methods are not generally as accurate as other methods for the analysis of lipids except, perhaps, for the major constituents because of the extremely small amts of substances which must be quantitatively recovered. A major problem in the use of gravimetric analysis in conjunction with TLC has been errors from organic contaminants. A principal source of the organic contaminants is the adsorbent itself as illustrated in Figure 3. The charred material in the right panel of the plate in Figure 3 is the analysis of material extracted from about 6 g of Silica Gel G (Silica Gel G acc. to Stahl, Lot T61044, E. Merg AG, Darmstadt, Germany) in a single batch extraction with ethyl ether. Figure 3 also illustrates the technique of removal of organic contaminants from the adsorbent by a predevelopment of the plate in chloroform prior to application of the sample. A second front (B, Figure 3) was scored for the chromatography of the sample which in this case consisted of material from a batch extraction of Silica Gel G. It is generally good practice in qualitative, as well as quantitative, TLC to extract commercial adsorbents with ethyl ether or chloroform before using them, or to remove the contaminant organic material by development of the plate in chloroform prior to analysis

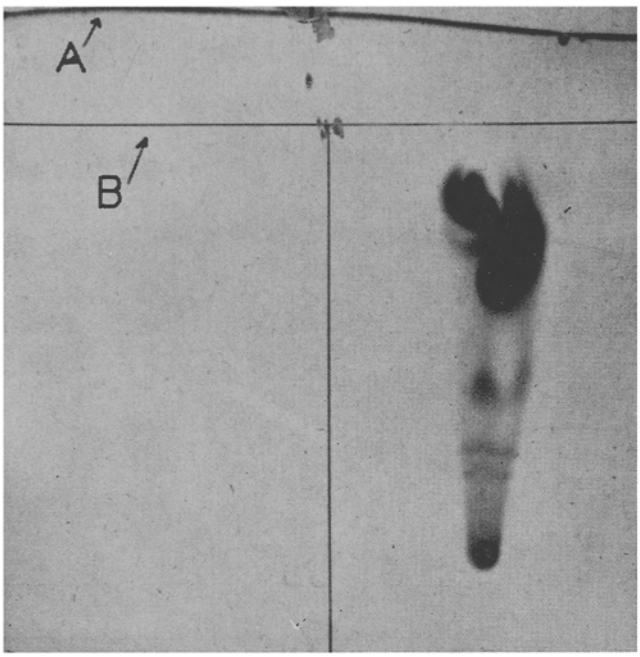

FIG. 3. Chromatoplate of organic contaminants extracted with ethyl ether from 6 g of Silica Gel G (acc. Stahl, Lot T61044, E. Merg AG, Darmstadt, Germany) developed in 85:15:1 petroleum ether:ethyl ether:acetic acid. *A*, solvent front of first development with chloroform; *B*, solvent front for development of sample.

of the sample as shown in Figure 3. Although no organic impurities are generally detectable at the second front in this technique as illustrated in Figure 3, most commercial adsorbents are usually still contaminated with sufficient impurities to cause an interference with an analysis by gravimetric methods. Because of the presence of organic impurities in most commercial preparations of silica gel, we found it expedient for the gravimetric analysis of the major classes of egg lipid (57) to make a special preparation of silicic acid for TLC to insure its freedom from organic contaminants. Details of the method used for such preparations of silicic acid have been described by Blank et al. (7).

Kramer et al. (41) reported that porous glass, which has been employed in column chromatography (45), possessed essentially the same adsorptive properties as silica gel in TLC. This adsorbent (Corning Glass Works) may be treated with nitric acid or hydrogen peroxide, dried, and finally heated to 550C to free it of all organic contaminants without destroying its adsorptive properties as illustrated by the separations shown in Figure 4.

Table I shows the quantitative gravimetric analysis of a mixture of known weights of cholesteryl oleate, triolein, oleic acid and cholesterol on porous glass coated plates, using 2,7-dichlorofluorescein as the indicator and the recovery methods described above. The analysis in Table I was carried out on amts of the individual components of the order of 3 m. Other adsorbents are generally too contaminated to give an accurate analysis on amts of this order of magnitude.

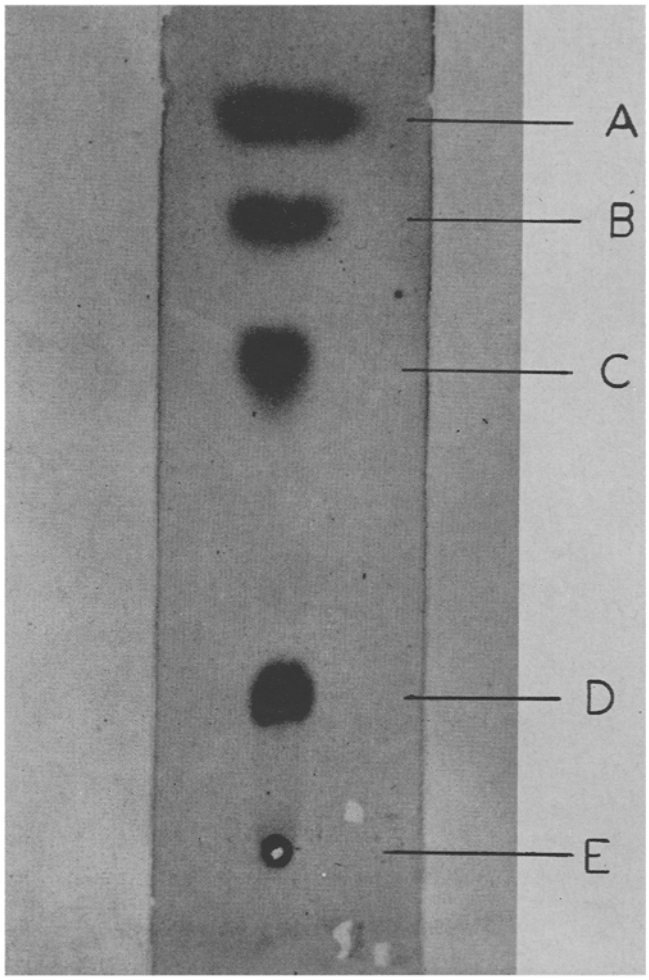

FIG. 4. Separation of a lipid mixture of *A*, cholesteryl oleate; *B*, triolein; *C*, oleic acid and *D*, cholesterol; *E*, hydrogenated egg lecithin on porous glass containing 10% calcium sulfate with 97:3.0:0.02 petroleum ether:ethyl ether:acetic acid.

Recently, Komarek et al. (40) described the use of extra-long plates, thicker layers of adsorbent and development with two solvent systems together with an elaborate extraction technique for use in conjunction with gravitometry. They applied this method to the lipids of bovine semen with a high degree of accuracy. Williams et al. (104) also reported the use of a gravimetric technique for the analysis of the major classes of the lipids of feces and fecaliths. Quantitative analysis by TLC using gravimetric methods has also been applied to lipids by Kaufmann et al. (38). Although the use of porous glass or specially prepared adsorbents eliminates to a large extent the major problem, that of the contamination of organic matter from the adsorbent, gravimetric methods require the utmost in technical proficiency to obtain highly accurate results.

TABLE I

Gravimetric Analysis of Standard Mixture of Lipid Classes via TLC

Lipid	Sample No. 1 (weight mg.)			Sample No. 2 (weight mg.)			% Composition	
	Known	found	recovery %	Known	found	recovery %	Known	Found Avg. of No. 1 & No. 2
Cholesteryl oleate	3.57	3.31	93	3.51	3.68	105	24.9	24.6
Triolein	3.56	3.62	102	3.50	3.35	96	24.8	25.0
Oleic acid	3.61	3.35	93	3.54	3.55	100	25.0	24.0
Cholesterol	3.61	3.86	107	3.55	3.54	100	25.3	26.0
Total	14.35	14.14	98.5	14.10	14.12	100	100	

Spectroscopic Methods

Phosphorus Analysis

Since there are no specific methods for the analysis of phospholipids, a general procedure for their quantification after TLC fractionation is based on a determination of phosphorus after mineralization of the recovered sample. Methods for the analysis of phospholipids by this technique have been described by Wagner (103), Habermann et al. (29), Jatzkewitz (33–35), Doizaki et al. (22) and Davison et al. (21). In the paper by Doizaki et al. (22) this application of the TLC method was shown to be as accurate as paper and column chromatographic methods and could be carried out much faster. However, the method requires the use of standards, and errors due to the suppression by silicic acid of the color reaction in the determination of phosphorus can amount to as much as 20% if not corrected. Whether or not porous glass adsorbents interfere with this color reaction is not known. Since this adsorbent is as efficient as silicic acid, its use in this type of analysis should be investigated.

Ester Analysis

Lipids which are ester derivatives yield hydroxamic acids on reaction with alkaline hydroxylamine and can be estimated colorimetrically by the formation of iron complexes. The reaction has been employed by a number of investigators for the analysis of lipid esters (4,87) and has been studied extensively by Skidmore and Entenman (85). Vioque and Holman (101) demonstrated the use of this reaction in conjunction with TLC for the analysis of simple lipid esters.

Chromic Acid Oxidation

A number of methods have been described for the analysis of lipids based on their oxidation by solutions of chromic acid (12). These methods generally involve the spectrophotometric analysis of the amt of chromic acid that is used up in oxidation of the lipid. Amenta (1) developed a method for the analysis of lipids fractionated by TLC based on the determination of the amt of unreacted chromic acid by the decrease in absorbancy of the solution at 350 mμ. Although the principle of the method is simple, it requires comparison with standards and corrections for reducing substances in the adsorbent which reduces its accuracy and precision.

Glycerol Analysis

The determination of lipids by the chromogenic reaction of chromotropic acid with glycerol has been employed as a general method of glyceride analysis (17, 98,99). Litchfield et al. (44) have used this reaction in conjunction with TLC on chromatoplates coated with silicic acid impregnated with silver nitrate for the determination of triglyceride structure.

Carbonyl Compounds

Despite the existence of very elegant column chromatographic techniques for the fractionation of carbonyl compounds, and the fact that they lend themselves well to gas-liquid chromatography (GLC) their quantitative analysis taxes to the utmost the skill of the analytical chemist. Since elegant techniques have been developed for the fractionation of carbonyl compounds in the form of their dinitrophenylhydrazine (DNPH) derivatives by TLC (79,97), the analysis of complex mixtures of these compounds may be greatly facilitated through the use of TLC in conjunction with the spectrophotometric analysis of the DNPH derivatives.

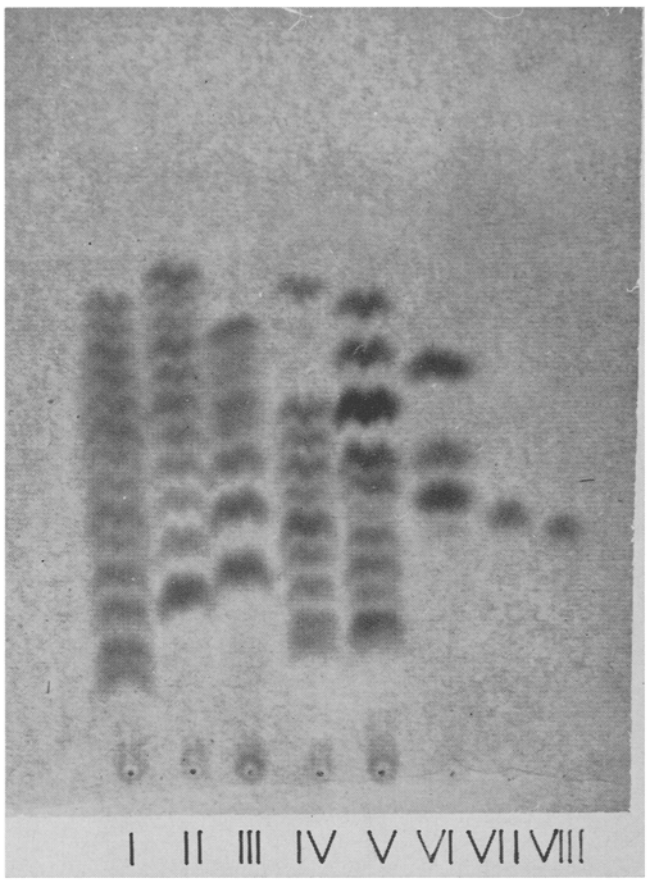

FIG. 5. Separation of members of normal homlogous series of 2,4-dinitrophenylhydrazones of *I*; alkanals C_{1-14}; II, alkan-2-ones C_{3-13}; *III*, alk-1-en-3-ones C_{4-10}; *IV*, alk-2-enals $C_{3-11,16}$; *V*, alka-2,4-dienals $C_{5-12,14,16,18}$; *VI*, alk-3-en-2-ones $C_{6,7,10}$; *VII*, nona-trans-2-trans-6-dienal; *VIII*, nona-trans-2-cis-6-dienal. Carver: Kieselguhr G. Impregnation: 10% phenoxyethanol in acetone. Solvent: light petroleum (bp, 100–120C). Conditions: the mobile solvent was allowed to ascend 9 cm from the starting line three times and 11 cm from the starting line the fourth time. This figure supplied through the courtesy of Gerda Urbach from *J. Chromatog. 12,* 196 (1963).

Methods for the fractionation and analysis of complex mixtures of carbonyl compounds as their DNPH derivatives have been directed primarily to investigations of the composition of odors and flavors and the determination of the structure of unsaturated fatty acids. In these applications they serve well as complimentary methods to GLC. They also provide a means for the determination of compounds with unusual structures that do not lend themselves to GLC.

In the application of TLC of DNPH to the structural analysis of unsaturated fatty acids or other lipids the ozonides may be reacted directly with 2,4-dinitrophenylhydrazine (1a,29a). The procedure employed in this laboratory consists of the preparation of ozonides separately in a nonpolar solvent (65) which provides a high yield of these compounds. The ozonides are purified by TLC with silicic acid coated plates and reacted directly with 2,4-dinitrophenylhydrazine. The products of the reaction are separated by TLC into classes by silicic acid TLC or by the elegant partition method developed by Urbach (97), which separates homologous series of derivatives differing by only one carbon atom as illustrated in Figure 5. Quantification is carried out by spectrophotometry using a derivative of a foreign aldehyde as an internal standard by the same general procedure described below for the analysis of triglycerides. It may be noted that a complete separation of compounds is frequently

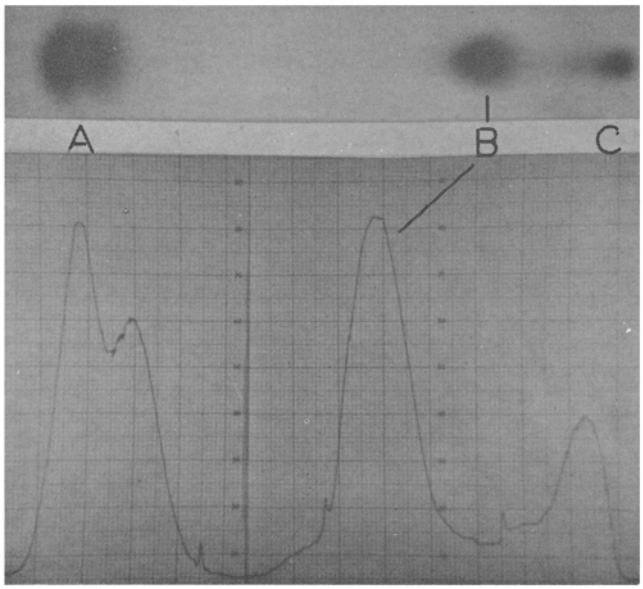

FIG. 6. Analysis of radioactive lipids by autoradiography-densitometry and scintillation counting of the recovered samples. Scintillation analysis: *A*, 39.34% (11,118 CPM); *B*, 40.27% (11,386 CPM); *C*, 20.39% (5,764 CPM). Densitometric analysis (proportionality of peak areas) *A*, 44,32%; *B*, 41.25% and *C*, 14,43%.

not necessary for a quantitative analysis by this procedure.

In summing up, spectrophotometric methods combine very effectively with TLC for the analysis of a wide variety of lipids. Only the more general application of these methods is elaborated here. Obviously, there are many applications of the technique to specific compounds or specific groups of compounds. Among such applications are methods for the analysis of bile acids (26,27,24), sterols (16,18,21), steroids (6,94), vitamin A derivatives (100) and glycolipids (33,34,21).

Radiometric Methods

The determination of radioactivity may be used in conjunction with TLC for quantitative analysis in much the same manner as spectrophotometric methods. Conceivably, the two methods may be combined for a determination of specific activity. General aspects of these methods have been discussed by Snyder (89).

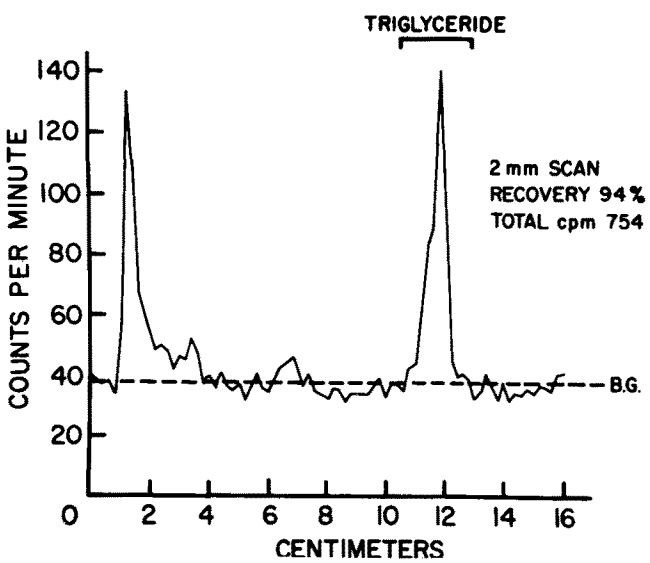

FIG. 7. Carbon^{14} distribution in bone marrow lipids 6 hr after the oral administration of palmitic-1-C^{14} (in corn oil) to an irradiated rat (4 days after 800 r total-body irradiation). This figure supplied through the courtesy of Fred Snyder from *Anal. Biochem. 9*, 183 (1964).

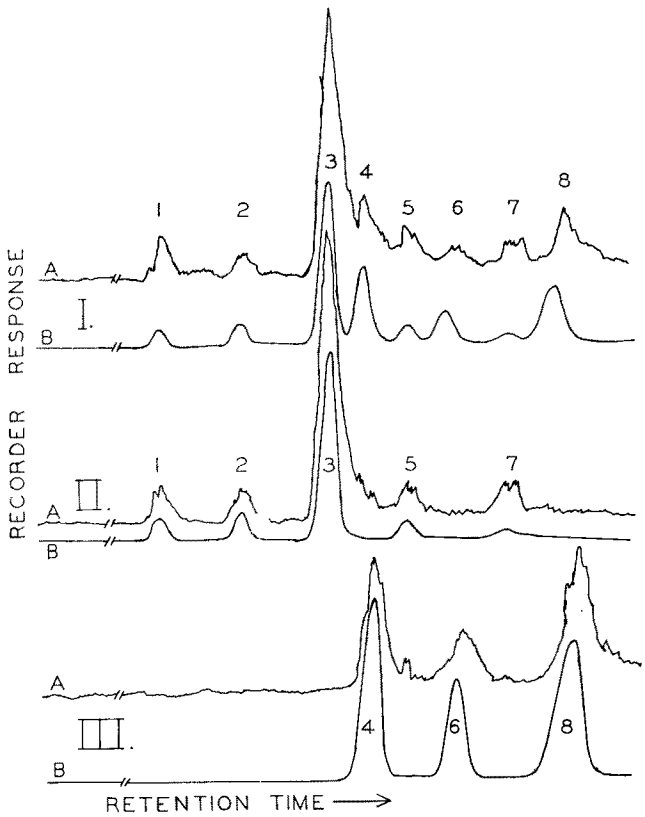

FIG. 8. Determination of fatty acid composition of a species of *Nocardia* cultured on acetate-1-C^{14} by a combination of TLC and radio-GLC (Packard System). *A*, radioactivity analysis (Packard System), *B*, mass analysis, *I*, total lipid methyl esters, *II* and *III*, saturated and monoenoic methyl esters, respectively, separated by $AgNo_3$-TLC; *1*, methyl myristate, *2*, methyl pentadecanoate, *3*, methyl palmitate, *4*, methyl palmitoleate, *5*, methyl heptadecanoate, *6*, methyl heptadecaenoate, *7*, methyl stearate and *8*, methyl oleate.

Scintillation counting is the most widely applied method of analysis used in conjunction with TLC because of its sensitivity and simplicity, but other counting methods may be employed (77,46,48). Techniques for the radioassay of lipids using scintillation counting in conjunction with TLC have been studied by Snyder et al. (88,89), Brown and Johnston (15) and Schulze et al. (77). The methods for the detection of spots and quenching have been the main problems in the quantitative analysis via scintillation counting. The latter has been largely eliminated through the using of scintillation systems containing dioxane or dioxane-water (15,89).

An autoradiographic analysis requires time as dependent on the activity of the sample but it provides a complete analysis and serves as a guide for the application of other analyses to the chromatoplate of the separated components. The application of this technique is illustrated in Figure 6. This figure shows an autoradiogram and a comparison of the densitometric analysis of the spots with that obtained by scintillation counting of the recovered sample. The agreement between the two analyses is not nearly as close as that which can easily be obtained. It is shown to emphasize a precaution that must be taken for an accurate autoradiographic analysis, that is, to avoid oversaturation of the photographic emulsion. When this technique can be applied, it leaves the plate available for a quantitative analysis of nonradioactive compounds by other methods.

Although autoradiographic methods have a number of good features, they are not as sensitive and have a much more limited range compared to direct scintillation counting. These factors, together with the insensitivity of methods based on chromogenic reactions, compared to radiodetection methods, led Snyder (89) to the development of a zonal scraper for the analysis of radioactivity via a distribution curve similar to that which might be obtained from a chromatographic column, for example. In this method, equal zones of adsorbent are automatically scraped from the plate into vials for counting. A typical analysis of this type is shown in Figure 7. Quantitative analysis is determined from the peak areas. Since counting times may be prolonged, the method provides the ultimate in sensitivity. Also, since very narrow zones may be taken, the method frequently will reveal overlapping of spots which would ordinarily be missed.

TLC may be employed in conjunction with radio-GLC for the quantitative analysis of radioactive methyl esters and other compounds which lend themselves to both of these forms of chromatography. A mass and radioactivity analysis by GLC of uniformly labeled methyl esters fractionated by TLC with silver nitrate coated plates is shown in Figure 8 to illustrate the potentialities of the combination of these techniques. TLC is used to separate fatty acids by degree of unsaturation and so simplify the mixture for GLC analysis in this type of application (Fig. 8). With complex mixtures of fatty acids consisting of both odd- and even-membered chain components as well as those having different degrees of unsaturation such a preliminary separation greatly facilitates GLC analysis and permits the use of internal standards for quantitation. By means of plates coated with silicic acid impregnated with silver nitrate, fatty acid esters may be fractionated readily on the basis of the degree and geometry of the unsaturation and in some cases by chain length and position of the double bonds as illustrated in Figure 9.

Through the use of the technique of internal standards described below the combination of radio-TLC and radio-GLC will permit the determination of the structures of radioactive triglycerides and lecithins. The determination of structure of radioactive lecithins may also be made by application of radio-GLC in conjunction with the TLC fraction of these compounds by the method described by Privett and Blank (58) except that a nondestructive method for the detection of the spots would have to be used.

Methods of Internal Standards

When no specific or general analytical methods can be applied, frequently a quantitative analysis can be effected by means of an internal standard. As mentioned above this method may be employed using spectrophotometry for the analysis of carbonyl compounds separated in the form of DNPH derivatives by TLC. It also provides an elegant method for the determination of the composition of triglycerides (8,10,27) and lecithins (9) and for the quantitative analysis of complex mixtures of fatty acids using a foreign methyl ester as an internal standard and analysis by GLC.

The procedure employed in the analysis of triglycerides is determined, to a large extent, by the complexity of the fat. For example, with milk fat, a preliminary direct fractionation on silicic acid may be used to separate the long-chain triglycerides from those containing the short-chain fatty acids (10). However, the main value of TLC in glyceride analysis is its use to fractionate these compounds on the basis

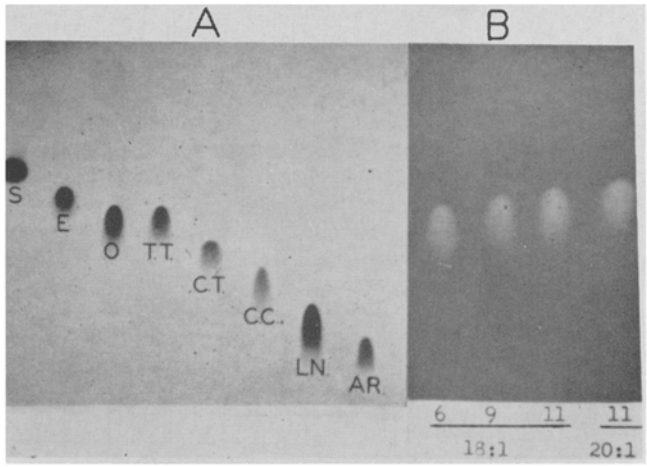

FIG. 9. TLC of fatty acid esters on plates coated with silicic acid impregnated with silver nitrate. *A*, developed with 20% diethyl ether in petroleum ether and spot visualization by charring; *B*, developed with chloroform (sprayed with 2,7-dichlorofluorescein, photographed under UV light). *S*, stearate, *E*, elaidate, *O*, oleate, *T.T.*, linoelaidate, *C.T.*, *cis*-9, *trans*-12-linoleate, cc = *cis*-9, *cis*-12-linolcate, *LN*, linolenate, *AR*, arachidonate.

of the degree of unsaturation with silver nitrate impregnated adsorbents (44,10,36,8,37,3,102). The nature and extent of the separations that may be achieved by this technique is illustrated in Figure 10. With this fat it was possible to separate pairs of triglycerides of the same degree of total unsaturation, that is, there was a separation on the basis of the individual fatty acid constituents. Separations of this type have not generally been recognized, although it has been mentioned by Kaufmann (37). There is some evidence also that glyceride isomers may be fractionated as evidenced by the apparent double bond for P_2O in Figure 11. The relative concn, as well as structure, influence separations using this technique.

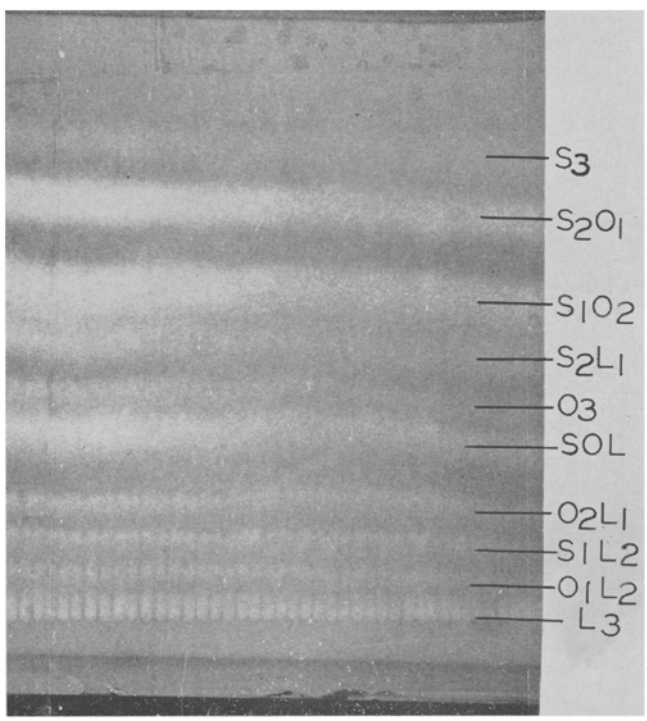

FIG. 10. Fractionation of hog mesenteric fat by TLC with plates coated with silicic acid impregnated with silver nitrate and developed with 0.8% methanol in chloroform. *O*, oleic, *L*, linoleic, *S*, saturated fatty acids.

TABLE II

Analysis of Triglycerides

Trivial name	Shorthand designation	Found wt. %	Calc. wt. %
Tripalmitin	P_3	5.2	4.9
1-Monoleo-2,3-dipalmitin	OPP	10.4	9.5
2-Monoleo-1,3-dipalmitin	POP	4.3	4.8
1-Monopalmito-2,3-diolein	POO	8.4	9.3
2-Palmito-1,3-diolein	OPO	5.0	4.7
Triolein	O_3	4.7	4.6
1-Linoleo-2,3-dipalmitin	LPP	8.7	7.4
2-Linoleo-1,3-dipalmitin	PLP	3.3	3.7
1-Palmito-2-oleo-3-linolein	POL	7.3	7.2
1-Oleo-2-Palmito-3-linolein	OPL	7.4	7.2
1-Oleo-2-linoleo-3-palmitin	OLP	6.8	7.2
1-Linoleo-2,3-diolein	LOO	6.4	7.1
2-Linoleo-1,3-diolein	OLO	3.8	3.6
1-Palmito-2,3-dilinolein	PLL	5.3	5.6
2-Palmito-1,3-dilinolein	LPL	2.7	2.8
1-Oleo-2,3-dilinolein	OLL	5.3	5.5
2-Oleo-1,3-dilinolein	LOL	3.0	2.7
Trilinolein	LLL	2.0	2.1

Therefore, one cannot generalize. However, an elegant feature of the use of a methyl ester as an internal standard and an analysis by GLC is that complete resolution of the triglyceride types is not necessary as illustrated below.

Methyl pentadecanoate serves very well as an internal standard for the analyses of most triglycerides because it is rarely present in more than trace amts in natural fats. The application of the internal standard technique in conjunction with lipase hydrolysis by the method of Blank, Verdino and Privett (8) is illustrated on the standard mixture of triglycerides shown in Table II. The plate on which this analysis is based is shown in Figure 11.

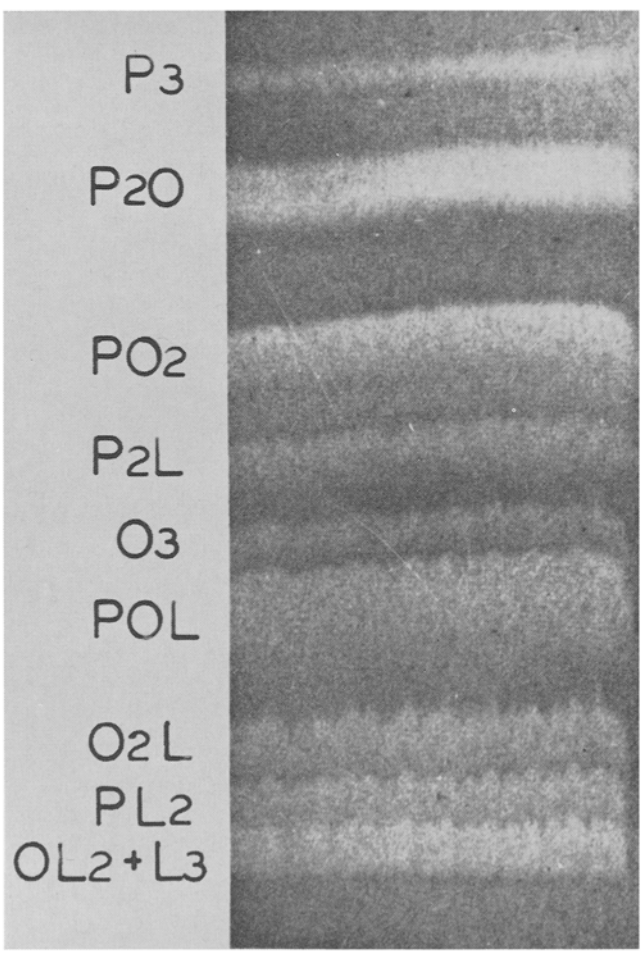

FIG. 11. Fractionation of a standard mixture of triglycerides shown in Table II by TLC with plates coated with silicic acid impregnated with silver nitrate. *O*, oleic, *L*, linoleic and *P*, palmitic acids.

The method of calculation is illustrated in Table III by the analysis of the seventh band shown in Figure 11. Even though the components of this band may be separated by a second chromatography, it is not necessary to perform such a separation because the composition may be calculated from the GLC data. The principle of the method is that from the analysis of the fatty acids, a hypothetical peak area can be calculated for each triglyceride type. From a consideration of the peak areas of the individual triglycerides and the data given by pancreatic lipase hydrolysis a fairly complete picture of the triglyceride composition can be obtained as shown by the analysis in Table II.

Lecithin composition may be determined after reductive ozonolysis via TLC and densitometry of charred spots as described by Privett and Blank (58). The methyl ester internal standard technique may also be used as an instrument of quantification in this method. These investigators (9) have recently developed a new method for the determination of lecithin structure based on a quantitative analysis of the disaturated forms by the internal standard technique after fractionation via mercuric acetate adduct formation. A total fatty acid analysis is followed by determination of the fatty acids residing in the β-position using the phospholipase A hydrolysis technique (68). From these data the composition of lecithins may be determined in terms of the distribution of the saturated and unsaturated fatty acids as follows. The amt of disaturated lecithin is determined directly by TLC as mentioned above. The amt of diunsaturated forms is determined from the total fatty acid composition and the amt of disaturated forms by differenece. In this calculation the amt of the two saturated-unsaturated forms can be determined by the saturated fatty acid composition of the total (all forms) and the amt of the disaturated form. The difference between these values represents the amt of saturated fatty acids in the two saturated-unsaturated forms and permits the calculation of the mole % of these forms in the total. The amt of the diunsaturated forms is then calculated by difference. From the action of phospholipase A the % distribution of saturated and unsaturated fatty acids among the saturated-unsaturated forms can be calculated because the amt of disaturated and diunsaturated forms are known.

The internal standard technique may be well applied with other analytical techniques in addition to GLC. A general technique for the application of TLC in conjunction with GLC, reductive ozonolysis and IR spectral analysis for the analysis of positional and geometric isomers of fatty acids of different chain length is described in reports form this laboratory (60,59,11).

The analysis of unsaturated fatty acids via TLC of their mercuric actate adducts in conjunction with GLC has been described by Mangold et al. (47), and Stearns et al. (93). The latter has employed fractionation by this method after partial hydrogenation in conjunction with ozonolysis for the determination of the positions of radioactive carbon atoms in isotopically labeled unsaturated fatty acids. A similar technique has been employed by Privett and Nickell (64) for the determination of the specific positions of *cis* and *trans* double bonds in polyunsaturated fatty acids. In this method the polyunsaturated fatty acids are partially hydrogenated with hydrazine, the monoethenoid products are isolated by either reversed-phase partition or selective adsorption on silicic acid

impregnated with silver nitrate chromatography as described by Privette and Nickell (65). The monethenoid esters are then separated into their *cis* and *trans* isomers and analyzed by reductive ozonolysis (60,65). The general technique of internal standards may be well applied in these and similar procedures for quantitative analysis of mixtures of fatty acids which could not be analyzed readily otherwise.

Direct Spot Analysis Methods

These methods are generally more rapid and sensitive than recovery methods and they eliminate errors which are associated with recovery of the sample from the chromatoplate. The methods in this category may be divided into those based on the measurement of spot size and those based on the measurement of a function of the size and density of the spot by photometry.

Spot Size Methods

These methods have been studied extensively by Purdy and Truter (66,96) and Seher (80–84). Although Purdy and Truter (66) found that the log weight of the sample versus the square root of spot area comes the closest of all measurements to a linear relationship, it is apparent that these methods are strictly empirical and their precision depends on the artifice of the operator (14,92,96,82). Since the size of the spot varies with the extent of the migration (Rf value) and the structure of the compound, standards of each component of the sample must be chromatographed simultaneously for comparison (66,81,82). The amt of the standard compound employed should also be in the range of that of the sample because of the nonlinearity of the relationship between weight and spot area. Another inherent error in the application of these methods is that of obtaining a precise measure of the size of the spot itself. Seher (81,82) cautions that the spots should be fairly large. However, components may be found at all levels of concn in lipid mixtures, a factor which compounds the difficulties of accurate measure of spot size in the analysis of these compounds. Some investigators, in accordance with the findings of Seher (81,82), have attempted to minimize the error in measurement of spot size by carrying out the measurements on photographs of the spots (48,83,84,2) and Purdy and Truter (66) have devised a number of procedures of varying degrees of complexity for application, in accordance with the information desired and the error which can be tolerated.

In spite of the inherent errors, the method has been applied with a high degree of precision to the analysis of a number of compoonds (96). A most elegant application of a spot size method is that for the analysis of antioxidants reported by Seher (83,84,82). Spot area in this case was measured from contact photographs made of the plates. The quantitative analysis of a large number of antioxidants is described in this work.

Photometric Methods

Photoreflectometric Analysis. Recently, Lines (43) reported a method based on reflectance measurements for the quantitative analyses of lipids by TLC. In this method the spots are charred by heating the plates from room temperature to 200C in 15 to 20 min after spraying them with 10% aqueous H_2SO_4. Such a method is reported to give a linear relationship between the amt of sample spotted on the plate and the light absorbed. Equal weights of cholesterol and monopalmitin gave the same response by this method in spite of the fact that the mol wt of cholesterol is about 84% carbon compared to 69% for that of monopalmitin. Thus, the linear relationship between the amt of sample spotted on the plate and light absorbed must be considered fortuitous, especially since no other compounds were tested. Payne (55) also describes the analysis of lipids by reflectance scanning. In his method the spots are charred by spraying the plate with 50% H_2SO_4 containing a small amt of methyl orange and then heated for 20 min at 160C. Inasmuch as photoreflectometric methods of analysis are widely used in paper chromatography, there is no apparent reason why they cannot be applied equally well in TLC. However, standards and the use of charring conditions which provide as nearly as possible quantitative conversion of the sample to carbon should be employed for the highest accuracy.

TABLE III

Analysis of Triglyceride Bands

Band 7 ($PL_2OL_2L_3$)									
	15:0	16:0	18:1	18:2					
	21.9	11.4	11.7	55.0					
Calc. Peak Area of Trigl.		PL_2	OL_2	L_3					
		(11.4×3)	11.7×3	$55.0 - 46.2$					
		34.2	35.1	8.8					
Lipase Analysis									
$(PL_2 + OL_2 + L_3) = 18.3\%$									
								Fatty Acid Composition	
8.0%		8.3%		2.0%				Total	β-Mono
P, L, L	+ L, P, L	O, L, L	+ L, O, L	+ L, L, L	P			11.4(14.6)	14.7
					O			11.7(15.0)	16.4
					L			55.0(70.4)	68.9
%5.3	2.7	5.3	3.0	2.0	15.0			21.9	

Spectrophotofluorometric Analysis. At present, these methods have been applied mainly in conjunction with the recovery of compounds from chromatoplates (76,75). However, a technique for the direct application of these methods to compounds separated as spots on chromatoplates has been devised by Sawicki et al. (75). In this procedure the chromatogram is cut into sizes suitable for insertion into a solid sample accessory and the natural fluorescence or fluorescence after treatment with appropriate reagent is measured. Since few lipids have a natural fluorescence or undergo fluorescent reactions, this method has only limited application with these compounds. That techniques may be devised for the analysis of lipids, based on quenching of fluorescence seems possible, however.

Photodensitometric Analysis. Of all the techniques of direct spot analysis, methods based on photodensitometric measurements of transmitted light appear to be the most practical. They are simple, fast and accurate; they have wide versatility and may be carried out with densitometers and techniques usually applied in paper chromatography with only little modification. In most applications of these methods a slit just longer than the diam of the spot is employed, as described by Blank et al. (7). The instrument is adjusted so that it gives a reading of 100% transmission when the light is passed through the plate between the spots and zero percent transmission when the shutter in front of the photocell is closed. The passage of each spot over the slit gives a peak of optical density values. The area under the peak (integrated optical density values) should give a linear relationship which passes through the origin with the amt of sample expressed in terms

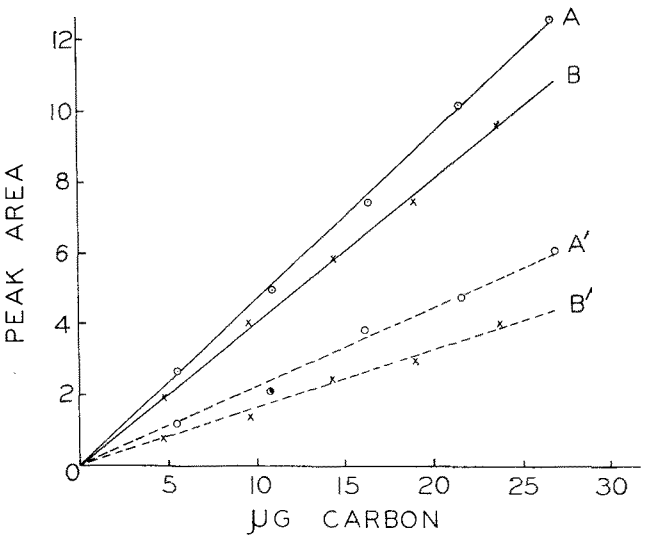

FIG. 12. Standard curve showing relationship of peak area vs µg of carbon; spots charred by heating the plate for 20 min at 180C after spraying it with chromic-sulfuric acid (5). *A* and *B*, methyl palmitate and cholesteryl palmitate respectively; *A'* and *B'*, methyl palmitate and cholesteryl palmitate, respectively, after spraying the plate with methanolic glycerol to make it translucent.

of its carbon content for charred spots as illustrated in Figure 12 if the instrument is properly modified for the analysis of chromatoplates. Commercial densitometers which are normally used for paper chromatography generally fail this test because they are not designed to shield out refracted light from chromatoplates. This problem may be eliminated in most densitometers by equipping them with a narrow slit below the chromatoplate and another above it just in front of the photocell to collimate the beam of light. Our modification of the Photovolt Densitometer Model 52C for use with slits of different sizes and a stage for holding 20 × 20 cm plates is shown in Figure 13. A small motor was also installed in the main housing of this instrument to provide automatic transportation of the stage.

The problem caused by refracted light in the densitometric analysis of charred spots by the usual technique has also been observed by Thomas et al. (95). These investigators attempted to eliminate it by making the measurements on plates made translucent by spraying them with an ethereal solution of mineral oil. Although errors due to refracted light appear to be minimized by the use of a translucent background, this technique greatly reduces the sensitivity of the measurement as noted by Thomas et al. (5) and as illustrated in Figure 12.

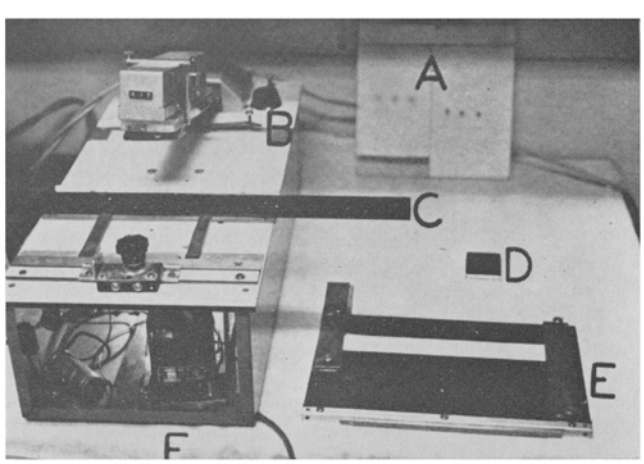

FIG. 13. Photovolt Densitometer Model 52C modified for measurement of chromatoplates. *A*, typical chromatoplate; *B*, height adjustment for photocell; *C*, metal strip containing slits of various sizes; *D*, slit cap for photocell (upper slit) E, adjustable stage for holding chromatoplates; *F*, motor for transportation of stage.

TABLE IV

TLC Analyses of Standard Mixtures of Lipid Classes (wt. %)

	No. 1	No. 2	No. 3	Avg.	Known	Abs. error
Sample 1						
C.E.	19.5	19.0	18.6	19.0	18.8	+0.2
T.G.	19.2	18.8	20.2	19.4	18.8	+0.6
F.F.A.	23.0	23.3	23.4	23.4	23.2	+0.2
Chol.	20.0	20.3	19.8	20.0	20.3	−0.3
H.L.	18.3	19.6	18.0	18.6	20.1	−1.5
Sample II						
C.E.	9.0	8.5	7.0	8.2	7.4	+0.8
T.G.	27.7	26.9	26.8	27.1	26.1	+1.0
F.F.A.	31.6	31.0	30.6	31.1	30.4	+0.7
Chol.	25.6	26.8	28.3	26.9	28.2	−1.3
H.L.	6.1	6.8	7.3	6.7	7.9	−1.2
Sample III						
C.E.	10.0	11.1	9.6	10.2	9.1	+1.1
T.G.	46.0	43.3	45.0	44.8	45.9	−1.1
F.F.A.	11.1	11.8	10.8	11.2	10.7	+0.5
Chol.	10.2	11.7	10.4	10.8	10.0	+0.8
H.L.	22.7	23.1	24.2	23.3	24.3	−1.0

C.E. = cholesterol palmitate.
T.G. = tripalmitin.
F.F.A. = palmitic acid.
Chol. = Cholesterol.
H.L. = hydrogenated lecithin.

The analysis of specific compounds may be performed by the determination of colored spots given in chromogenic reactions by the use of specific filters. However, because lipids are a highly heterogeneous group of compounds, they are usually made visible for photodensitometry by charring them under standard conditions; no filter is employed in the densitometer in this case. Many different charring techniques have been employed to make spots visible. Lines (43), as mentioned previously, employed aqueous 10% H_2SO_4 and heated the plates to 200C. Barret et al. (3) sprayed their plates with an aqueous solution of 50% orthophosphoric acid and then heated them to 340C. Payne (55) used 50% H_2SO_4 containing methyl orange and a charring temp of 160C. Leegwater et al. (42) describes the use of permanganate-sulfuric acid for charring. Kirchner et al. (39) and Morris et al. (52) employed 50% aqueous H_2SO_4 (v/v). Peifer (56) who applied the technique to microplates charred the spots with a sulfuric-perchloric acid mixture. These and many other reagents for charring spots are tabulated by Bobbitt (2) and by Mangold (48). The ideal technique, obviously, is one which gives a quantitative conversion of the compounds to carbon. Because lipids vary considerably in structure and mol wt, no single method can be expected to give a quan-

TABLE V

Analysis of Beef Brain Lipids (86) (wt. %)

Substance	Column	DEAE-TLC	Direct TLC
Cholesterol	20.3	20.4	19.0
"Ceramide"	0.31	0.43	0.43
Cerebroside	13.1	13.5	13.7
Sulfatide	4.0	4.0	4.0
Lecithin	.....	11.3	.....
Sphingomyelin	.....	7.9	.....
Phosphatidyl ethanolamine	14.0	14.0	14.7
Phosphatidyl serine	7.5	7.5	.....
Gangliosides	2.5	2.5	.....
Phosphatidyl inositol	2.0		2.0

titative conversion of all lipid compounds to carbon. With common lipids we have shown (61) that two processes are involved in the amount of conversion of a compound to carbon evaporation which may occur with the highest mol wt lipids and oxidation. The main reason for the frequent observation that unsaturated compounds char quicker or more than saturated compounds under certain conditions (61,62,52) is readily explained by these reactions. In order to limit the loss by evaporation, high temps, that is, above 200C, should be avoided. Strong oxidizing agents should be used to complete the oxidation as quickly as possible but the oxidizing agent and the heating conditions should not be so drastic as to convert the sample to carbon dioxide. The conditions which we have found to be most generally satisfactory consist of heating the chromatoplate at 180C for 25 min after spraying it with an aqueous solution of 70% sulfuric acid (v/v) saturated with potassium dichromate (7).

Although many compounds give the same yields of carbon when the charring is carried out under these conditions, it is generally good practice to employ standards as reference compounds for comparison on the same plate. In addition to correction for yield of carbon, this technique also nullifies the effect of Rf value. As a spot migrates higher on the plate, it becomes larger; the increase in size is compensated generally by a decrease in density, but not completely for spots with widely different Rf values which are frequently encountered in complex lipid mixtures. When standards are not available, only the areas of spots between an Rf value of about 0.3 to 0.7 should be directly compared. However, all of the spots of a lipid mixture can generally be developed in the optimum region of the same plate with different solvent systems via the technique illustrated in Figure 14. In this technique the plate is divided into panels, and a different solvent system is used in each panel. After the spot has ascended the first panel, the adsorbent is removed from the plate to a height just above the level of the solvent in the chromatographic jar so that the solvent cannot reascend it. Then another sample is spotted in the second panel and it is developed in the second solvent

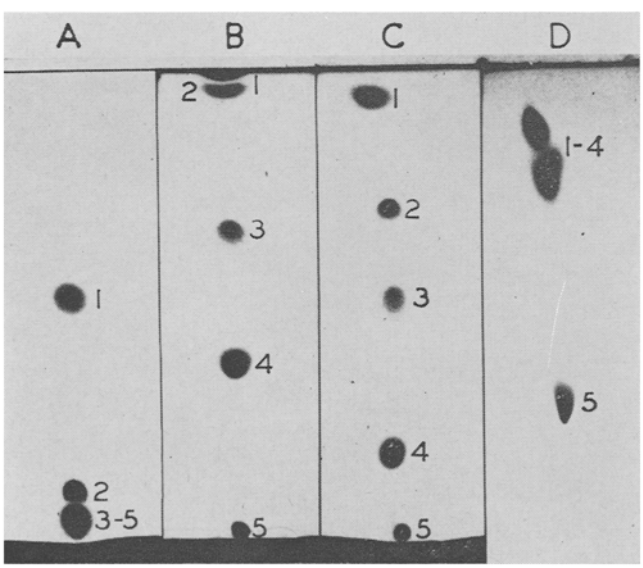

FIG. 14. Chromatoplate of a mixture of lipids in four solvent systems on same plate. *A*, 5% ether in petroleum ether; *B*, 50% ether + 0.5% HAC in petroleum ether; *C*, 15% ether + 1% HAC in petroleum ether; *D*, 70:30:4 $CHCl_3$:MeoH:H_2O. *1*, Cholesterol oleate; 2, triolein; *3*, oleic acid; *4*, cholesterol and *5*, lecithin.

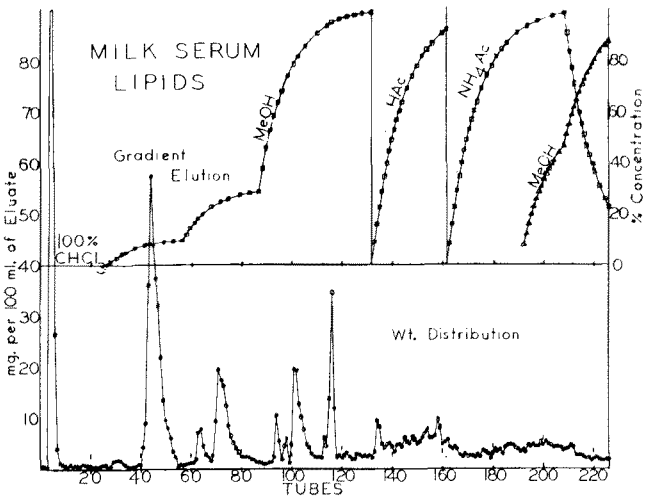

FIG. 15. Weight distribution curve of the fractionation of milk serum lipids by DEAE cellulose column chromatography.

system, one which will bring other spots into the optimum Rf range. The process is repeated until all components of the sample are migrated into the optimum region of the plate. For the utmost in accuracy, a standard may also be included in each panel for comparison.

Peak areas obtained from the densitometric measurements may be determined by means of an automatic integrator, planimetry or by cutting out the peak areas from an automatically recorded chart and weighing the paper. An indication of the accuracy that may be achieved with these methods is given by the analysis of mixtures of various concns of several lipid classes in Table IV.

Applications. Since the technique may be applied generally to organic compounds, its use in many investigations has gone unheralded. We have employed it for the determination of triglyceride and lecithin structure (58), mono-, di- and triglyceride analysis (63,61,62), lipid classes (7) and on ozonides (64). Peifer (56) has applied the method to the determination of cholesterol. Thomas et al. (95) have illustrated that silica gel impregnated with boric acid may be used for the quantitative analysis of 1- and 2-monoglycerides. Barret et al. (2) have employed the same technique for triglyceride analysis by using silicic acid impregnated with silver nitrate as an adsorbent. Demonstration of the technique with this adsorbent, of course, greatly extends its potential application.

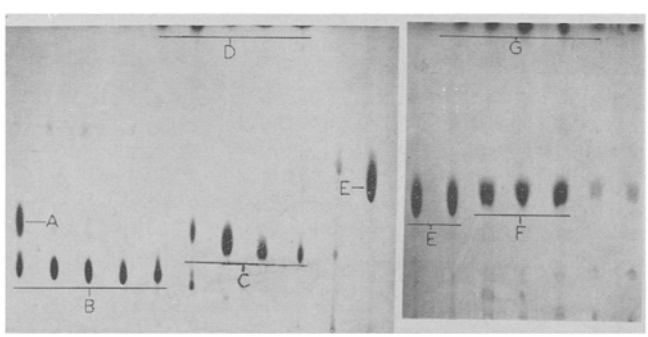

FIG. 16. TLC of fractions of milk serum lipids separated by DEAE cellulose column chomatography. *A*, lecithin (tubes 44–47); *B*, sphingomyelin (tubes 48–54); *C*, unknown (tubes 62–68); *D*, unknown (tubes 62–68); *E*, phosphatidylethanolamine (tubes 70–85); *F*, unknown (tubes 86–99); *G*, unknown (tubes 86–99). Solvent 65:25:4, chloroform:methanol:water.

Analysis of Lipid Classes. One of the most useful applications of the TLC-charring technique is for the determination of lipid class composition, especially of complex lipids. The analysis of these compounds by column methods is long and laborious. The application of quantitative TLC to lipid class analysis involving the major components of the neutral lipids and the polar lipids as a group, the procedure may be carried out by a single chromatography on silicic acid with a solvent system consisting of petroleum ether: ethyl ether:acetic acid (85:15:1, v/v/v) (7). When the phospholipids are of fairly simple composition or information on only the major constituents is required, these compounds may be recovered from chromatoplates in the above analysis and rechromatographed with chloroform:methanol:water (65:25:4, v/v/v). For more complex mixtures of phospholipids, the chromatography may be carried out by two-dimensional techniques along the lines described by Skidmore et al. (85) or Rouser et al. (71), for example.

For a detailed analysis of all components of the polar lipids, a different procedure is followed. The lipid is first fractionated by DEAE cellulose column chromatography as described by Rouser et al. (72,73). The simple mixtures obtained by the fractionation are then analyzed by the TLC densitometry-charring method (7). The complete procedure and various ramifications of it, including the application of two-dimensional TLC, have been described in detail by Rouser et al. (71). This method gave very close agreement with multicolumn methods on beef brain lipids as illustrated in Table V.

The general principle in the analysis of complex lipids is to perform a series of quantitative fractionations and analyses until the sample is reduced to a number of easily resolved simple fractions or pure compounds which lend themselves to quantitative analysis by the TLC-charring method. The DEAE cellulose method fractionates compounds which generally overlap on TLC and also provides a concn of minor components which normally are not detectable by other means. This adsorbent normally fractionates on the basis of its ion exchange properties, but it also has adsorptive properties which may be utilized for a more refined fractionation, that, is within groups. The manner in which DEAE cellulose chromatography complements TLC for the analysis of complex lipids was demonstrated on milk serum lipids. The distribution curve and composition of the solvent in the elution pattern of the fractionation of this fat is shown in Figure 15. Each tube in the distribution pattern in Figure 15 was analyzed qualitatively by TLC and like fractions were combined. The TLC analysis of selected samples of these fractions is shown in Figure 16. Since the higher numbered tubes contained material which was eluted with increasing concns of methanol or ammonium acetate or acetic acid in the solvent, the order of elution of substances from the column was related to their acidity and fractionation was by the principle of ion exchange. The fractionation by TLC (with silicic acid) of the material separated by DEAE cellulose occurred on the basis of polarity. Examination of the results in Figure 16 showed that had not the lecithin, sphingomyelin and phosphatidylethanolamine, for example, been separated by the DEAE cellulose they would have overlapped with unknown compounds to make their analysis by TLC very difficult. Also, the material migrating with the front in tubes 74–99 would certainly complicate the analysis of the neutral lipids by a direct TLC analysis. It is also evident that the substance in tubes 74–99, with an Rf of 0.45, is different from phosphatidylethanolamine which has the same Rf value but was eluted in tubes 70–73.

Conclusions

Thin-layer chromatography has many valuable analytical applications in the field of lipids. Since lipids are an extremely heterogeneous group of compounds and complex mixtures are the rule more than the exception, TLC is especially useful for the analysis of these substances because of its versatility. This versatility extends not only to types of separation but also to techniques of quantitative analysis.

Direct quantitative analysis by means of TLC has many applications and a great expansion of its use in this area may be expected, particularly using photodensitometric methods because of their simplicity, precision and sensitivity.

Recovery methods of analysis, of course, also have wide application and may be coupled with many other methods of analysis. A particular feature of these methods is that frequently it is not necessary to effect a complete separation of all components to obtain a quantitative analysis.

ACKNOWLEDGMENT

This work was supported by the USPHS grant HE-05735 from the National Institutes of Health and a grant from the Special Dairy Board of the National Dairy Council.

REFERENCES

1. Amenta, J. S., J. Lipid Res. *5*, 270 (1964).
1a. Bonner, W. A., J. Chem. Educ. *30*, 452 (1953).
2. Bobbit, M., Thin-Layer Chromatography, Reinhold Pub. Corp. (1963).
3. Barrett, C. B., M. S. J. Dallas and F. B. Padley, Chem. Ind. 1050 (1962); JAOCS *40*, 580 (1963).
4. Bauer, F. C., and E. F. Hirsch, Arch. Biochem. Biophys. *20*, 242 (1949).
5. Beroza, M., and T. P. McGovern, Chemist-Analyst *52*, 82 (1963).
6. Bird, Jr., H. L., H. F. Buckley, J. P. Comer, P. F. Hartsaw and M. L. Johnson, Anal. Chem. *35*, 346 (1963).
7. Blank, M. L., J. A. Schmit and O. S. Privett, JAOCS *41*, 371 (1964).
8. Blank, M. L., B. Verdino and O. S. Privett, JAOCS, in press.
9. Blank, M. L., L. S. Nutter and O. S. Privett, Determination of the Structure of Lecithins, JAOCS, in press.
10. Blank, M. L., and O. S. Privett, J. Dairy Sci. *67*, 481 (1964).
11. Blank, M. L. and O. S. Privett, J. Lipid Res. *4*, 470 (1963).
12. Bragdon, J. H., J. Biol. Chem. *190*, 513 (1951).
13. Bray, C. A., Anal. Biochem. *1*, 279 (1960).
14. Brenner, M., and A. Niedearwiesh, Experientia *16*, 378 (1960).
15. Brown, J. L., and J. M. Johnston, J. Lipid Res. *3*, 480 (1962).
16. Bush, B., and E. V. Truter, "Thin Film Chromatography," E. V. Truter Cleaver-Hume Press Ltd., London (1963) p. 109.
17. Butler, Jr., W. M., H. M. Maleng, M. G. Horning and B. B. Brodie, J. Lipid Res. *2*, 95 (1961).
18. Cerny, V., J. Joska and L. Labler, Collection Czech. Chem. Commun. *26*, 1658 (1961).
19. Corbin, E. A., D. P. Schwartz and M. Kenney, J. Chromatog. *3*, 322 (1960).
20. Csallany, A. S., and H. H. Draper, Anal. Biochem. *4*, 418 (1962).
21. Davison, A. N., E. Graham-Wolfaard, Biochem. J. *87*, 31p (1963).
22. Doizaki, W. M., and L. Zieve, Proc. Soc. Exp. Biol. Med. *113*, 91, (1963).
23. Eng, L. F., Y. L. Lee, R. B. Hayman and B. Gustel, J. Lipid Res. *5*, 128 (1964).
24. Frosch, B., and H. Wagner, Klin. Wochschr. *42*, 192 (1964).
25. Gänshirt, H., Arch. Pharm. *296*, 124 (1963).
26. Gänshirt, H., F. W. Koss and W. K. Morianz, J. Chromatog. *7*, 266 (1962); Arzneimittel. Forsch. *10*, 943 (1960).
27. Gunstone, F. D., F. B. Padley and M. I. Qureshi, Chem. Ind. 483 (1964).
28. Gee, M., J. Chromatog. *9*, 278 (1962).
29. Habermann, E., G. Bandtlow and B. Krusche, Klin. Wochschr. *39*, 816 (1961).
29a. Haagman, P.W., and J. P. Wibault, Res. Trav. Chem. *60*, 842 (1941).
30. Hefendehl, F. W., Planta Medica *8*, 65 (1960).
31. Hirsh, J., and B. Doldrick, J. Lipid Res. *4*, 482 (1963).
32. Janak, J., Nature *195*, 696 (1962).
33. Jatzkewitz, H., Z. Physiol. Chem. *336*, 25 (1964); *326*, 61 (1961).
34. Jatzkewitz, H., Z. Physiol. Chem. *320*, 134 (1960).
35. Jatzkewitz, H., and Mehl, Z. Physiol. Chem. *320*, 251 (1960).
36. Jurriens, G., B. deVries and L. Schauten, J. Lipid Res. *5*, 268 (1964).
37. Kaufmann, H. P., and H. Wessels, Fette, Seifen Anstrichmittel *66*, 13 (1964); *66*, 81 (1964).
38. Kaufmann, H. P., and C. V. Viswanathan, Fette, Seifen Anstrichmittel *65*, 538 (1963).
39. Kirchner, J. G., J. M. Miller and G. E. Keller, Anal. Chem. *23*, 420 (1951).
40. Komarek, R. J., R. G. Jensen and B. W. Pickett, J. Lipid Res. *5*, 267 (1964).

41. Kramer, J. R. G., E. O. Schiller, H. Gesser and A. D. Robinson, Porous Glass—A New Adsorbent for TLC, Anal. Chem., in press.
42. Leegwater, D. C., C. G. Youngs, J. F. T. Spenser and B. M. Craig, Can. J. Physiol. *40*, 847 (1962).
43. Lines, J. G., in "Biochemical Problems of Lipids," Ed. A. C. Frazer, Elsevier Pub. Co., New York, 1963 p. 17.
44. Litchfield, C., M. Farquhar and R. Reiser, JAOCS *41*, 588 (1964).
45. MacDonell, H. L., J. M. Noonan and J. P. Williams, Anal. Chem. *35*, 1253 (1963).
46. Mangold, H. K., R. Kammereck and D. C. Malins, Microchem. J. Symp. Series *2*, 697 (1962).
47. Mangold, H. K., and R. Kammereck, Chem. Ind. 1032 (1961).
48. Mangold, H. K., JAOCS *38*, 708 (1961).
49. Mangold, H. K., JAOCS *41*, 762 (1964).
50. Mathews, J. S., A. L. Pereda and A. Aquilera, J. Chromatog. *9*, 331 (1962).
51. Millet, M. A., W. E. Moore and J. F. Saeman, Anal. Chem. *36*, 491 (1964).
52. Morris, L. J., R. T. Holman and K. Fontell, J. Lipid Res. *2*, 68 (1961).
53. Nechaman, M. Z., C. C. Sweeley, N. M. Oldham and R. E. Olson, J. Lipid Res. *4*, 484 (1963).
54. Pastuska, G., Z. Anal. Chim. *179*, 427 (1961).
55. Payne, S., Proc. Nutri. Soc. (Eng.Scot.) *23*, XV (1964).
56. Peifer, J. J., Mikrochim Acta 529 (1962).
57. Privett, O. S., M. L. Blank and J. A. Schmit, J. Food Sci. *27*, 463 (1962).
58. Privett, O. S., and M. L. Blank, JAOCS *40*, 70 (1963).
59. Privett, O. S., and M. L. Blank, JAOCS *41*, 292 (1964).
60. Privett, O. S., M. L. Blank and O. Romanus, J. Lipid Res. *4*, 260 (1963).
61. Privett, O. S., and M. L. Blank, JAOCS *39*, 520 (1962).
62. Privett, O. S., and M. L. Blank, J. Lipid Res. *2*, 37 (1961).
63. Privett, O. S., M. L. Blank and W. O. Lundberg, JAOCS *38*, 312 (1961).
64. Privett, O. S., and E. C. Nickell, JAOCS *41*, 72 (1964). *Ibid.*
64a. Privett, O. S., and E. C. Nickell, unpublished data.
65. Privett, O. S., and E. C. Nickell, JAOCS *39*, 414 (1962); *40*, 189 (1963).
66. Purdy, S. J., and E. V. Truter, Analyst *87*, 802 (1962); Chem. Ind. 506 (1962).
67. Ritter, F. J., and G. M. Meyer, Nature *193*, 941 (1962).
68. Robertson, and W. Lands. J. Biochem. *1*, (1962).
69. Rosenberg, J., and M. Golgar, Anal. Chem. *35*, 1559 (1963).
70. Rouser, G., G. Kritchevsky, D. Heller and E. Lieber, JAOCS *40*, 425 (1963).
71. Rouser, G., C. Galli, E. Lieber, M. L. Blank and O. S. Privett, JAOCS *41*: 836–840, 1964.
72. Rouser, G., A. J. Bauman, G. Kritchevsky, D. Heller and J. S. O'Brien, JAOCS *38*, 544 (1961).
73. Rouser, G., G. Kritchevsky. D. Heller and E. Lieber, JAOCS *38*, 565 (1961).
74. Rybicka, S. M., Chem. Ind. (London) 1947 (1962).
75. Sawicki, E., T. W. Stanley and H. Thomsen, Direct Spectrophotofluorometric Analysis of Aromatic Compounds, Microchem. J. *8*, (1964).
76. Sawicki, E., T. W. Stanley and J. D. Pfaff, Anal. Chem. *36*, 497 (1964).
77. Schulze, P. E., M. Wenzel, Angew. Chem. Intern. Ed., Engl. I, 580 (1962).
78. Schwartz, D. P. O., W. Parks and M. Kenney, Anal. Chem. *34*, 669 (1962).
79. Schwartz, D. P., and O. W. Parks, Microchem. J. *7*, 403 (1963).
80. Seher, A., Nahrung *4*, 466 (1960).
81. Seher, A., Microchim. Acta Wien 308 (1961).
82. Seher, A., Fette, Seifen Anstrichmittel *61*, 345 (1959).
83. Seher, A., Nahrung *4*, 466 (1960).
84. Seher A., Mikrochim. Acta 308, 1961.
85. Skidmore, W. D., and C. Entenman, J. Lipid Res. *3*, 356 (1962).
86. Skidmore, W. D., and C. Entenman, J. Lipid Res. *3*, 471 (1962).
87. Snyder, F., and N. Stephens, Biochem. Biophys. Acta *34*, 244 (1959).
88. Snyder, F., and N. Stephens, Anal. Biochem. *4*, 128 (1962).
89. Snyder, F., Anal. Biochem. *9*, 183 (1964).
90. Spikner, J. E., and J. C. Towne, Chemist-Analyst *52*, 50 (1963).
91. Squibb, R. L., Nature *198*, 317 (1963).
92. Stahl, E., Z. Anal. Chem. *181*, 303 (1961).
93. Stearns, Jr., E. M., and F. W. Quackenbush, Paper No. 37, AOCS Meeting, Chicago 1964, Radioactivtiy of Individual Carbons in Chains of Unsaturated Fatty Acids from Soybeans.
94. Struck, H., Mikrochim. Acta 634 (1961).
95. Thomas III, A. E., J. E. Scharaun and H. Ralston, Paper No. 75, AOCS Meeting, Chicago, 1964, Quantitative Estimation of Isomeric Monoglycerides by Thin-Layer Chromatography, 1964.
96. Truter, E. V., "Thin Film Chromatography," Cleaver-Hulme Press Ltd., London, 1963, p. 103; Gänshirt, H., F. W. Koss, and W. K. Morianz, J. Chromatog. *7*, 266 (1962).
97. Urbach, G., J. Chromatog. *12*, 196 (1963).
98. VanHandel, E., Clin. *7*, 249 (1961).
99. VanHandel, E., and D. B. Zilversmit, J. Lab. Clin. Med. *50*, 152 (1957).
100. Varma, T. N. R., T. Panalaks and T. T. Murray, Anal. Chem. *36*, 1864 (1964).
101. Vioque, E., and R. T. Holman, JAOCS *39*, 63 (1962).
102. deVries, B., Chem. Ind. 1049 (1962); Fette, Seifen Anstrichmittel *65*, 725 (1963); JAOCS *41*, 403 (1964).
103. Wagner, H., Fette, Seifen Anstrichmittel *63*, 1119 (1961).
104. Williams, J. A., A. Sharma, L. J. Morris and R. T. Holman, Proc. Exp. Biol. Med. *105*, 192 (1960).

Some Practical Aspects of Thin-Layer Chromatography of Lipids

NICHOLAS PELICK, T. L. WILSON, M. E. MILLER and F. M. ANGELONI,
Applied Science Laboratories, Inc., State College, Pennsylvania, and
J. M. STEIM, The Pennsylvania State University, University Park

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-