made of the usual parameters of malabsorption. Although the mechanism by which neomycin lowers serum cholesterol is still subject to some difference in interpretation of the data from various laboratories, the evidence favors a reciprocal relationship between the amount of bile acids excreted and levels of serum cholesterol. This could be a part of a mild "malabsorption syndrome" although the choice of words in this case may be unfortunate. The unfavorable consequences of a doubling of fat excretion may have to be balanced against the consequences of a doubling of bile acid excretion and its concomitant decrease of serum cholesterol.

The generalized impression that neomycin sterilizes the gut should be corrected. There is a sharp decrease in some organisms and an increase in the numbers of other organisms which are apparently normal organisms since there have been no reports of side effects due to pathogenic bacteria.

The primary side effect is an initial diarrhea which usually subsides within two weeks. Seldom is the diarrhea severe enough to cause withdrawal of the drug. The mild "malabsorption syndrome" produced by 2 g neomycin/day appears to be medically inconsequential, whereas the decrease in serum cholesterol appears to be significant.

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Thin-Layer Chromatography of Lipids[1]

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Abstract

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Introduction

IN THE LAST FEW YEARS, TLC has become widely accepted and is now considered an indispensable tool in many laboratories. Monographs on this technique have been written or edited by Bobbitt (35), Marini-Bettolo (273), Randerath (341), Stahl (394) and Truter (430). Several chapters on TLC have been published in a book edited by Morris and James (296). In another volume, an article providing a survey of equipment and material used in TLC has just been printed (261). A publication of von Arx and Neher (7) is also of interest in this connection.

Two reviews (267,323) on applications of TLC in lipid analysis were published almost simultaneously in 1961. Progress in the development of TLC was discussed more recently in articles by Hofmann (165) and by Lines (255). These two publications appeared in a volume which also contains several other contributions pertaining to the subject to be discussed here.

Chapters reviewing the use of TLC and other chromatographic techniques for the analysis and isolation of lipids have been prepared by Morris (292) and by Schlenk (366). Applications of chromatographic techniques to a more specific problem, the study of the biosynthesis of fatty acids, have been discussed by Pascaud (320).

Reference is made here to the treatise on lipid analysis that has been written by Entenman (102), to the first volume of a new serial publication on the same subject which has been edited by Boekenoogen (36) and to Neher's (301) well-known monograph on steroid analysis. The second edition of the latter work has just been released.

[1] This review is an extension of an article included in AOCS Lectures of the 12th Annual Short Course on *Newer Lipid Analyses*, Chicago, 1961. It is based on talks given at the International Course on *Chromatographic Methods in Lipid Research* conducted at Milano, Italy, 1962 and at the Symposium: *Drugs in Lipid Metabolism*, AOCS Meeting, Minneapolis, 1963.

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The present article has been written to briefly summarize the vast amt of literature on uses of TLC in biochemical studies of lipids and, furthermore, to point out new trends in the application of this method to the analysis of mixtures of lipophilic compounds. A few new developments in technique have been considered also, whereas references pertaining to the utilization of TLC in chemical syntheses of lipids have been omitted. Generally only publications that appeared during the years 1962, 1963 and early 1964 have been included in this article. Earlier applications of TLC in the lipid field have been well described in the above-mentioned books and reviews.

Technique

Until two years ago, TLC had been used almost exclusively as an adsorption technique, but a few applications of reversed-phase partition TLC had been explored. Recently, TLC on polyamides has been described and chromatography on layers containing chelate-forming ions or other complexing agents (145, 146,293,295) has permitted separations that previously could not be accomplished by any simple method.

Great advances in lipid analysis have been made by the use of adsorbents impregnated with silver nitrate (452,453). Unsaturated lipids form π-complexes with silver nitrate in the course of chromatography, and these complexes migrate at a slower rate than do saturated compounds of the same class. Thus, methyl esters of saturated fatty acids can be separated from the esters of mono-unsaturated acids and from the esters of di-unsaturated acids. *cis,trans*-Isomers also can be resolved by chromatography on adsorbents containing silver nitrate (452,453). This new principle of chromatography has been rapidly adapted to the thin-layer technique (14,293,294,454). A silica gel layer can be easily impregnated by spraying an aqueous solution of silver nitrate onto it (293). It is more convenient, however, to apply the adsorbent to the plates as a slurry with a solution of silver nitrate in water (14,294,454), or to place freshly coated and briefly air-dried chromatoplates on edge in a shallow layer of an aqueous silver nitrate solution which is allowed to travel the length of the plates (70).

TLC of the methyl esters of unsaturated fatty acids as their acetoxymercurimethoxy compounds has been applied for prefractionating complex mixtures of esters, prior to GLC, into groups having a uniform degree of unsaturation. This technique has also been used to characterize esters eluted from gas-chromatographic columns (91,190,239). Recently, it has been realized that the same type of separation, i.e., fractionation according to the number of acetoxymercurimethoxy groups/molecule, can be achieved under identical conditions with many other lipids, regardless of the functional groups(s) of the parent compounds (270).

This principle of fractionation is complementary to adsorption chromatography, which separates mixtures of lipids primarily according to classes of compounds having the same type and number of functional groups. It is also complementary to reversed-phase partition chromatography, which fractionates not only according to chain length, but also to degree of unsaturation. Chromatography on adsorbents containing silver nitrate can be used to separate saturated and unsaturated members on one class of compounds according to their degree of unsaturation; however, saturated and unsaturated components of different classes overlap. Thus, TLC of mercuric acetate adducts also complements chromatography on layers containing silver nitrate (270,454).

Aqueous-alcoholic solutions of 2′,7′-dichlorofluorescein and Rhodamines have been employed frequently for visualizing lipids on adsorbent layers and on paper (383); plain distilled water has been recommended also (133). The use of these nondestructive indicators permits recovery and isolation of lipid fractions from thin layers. Iodine appears to have become the most popular reagent for visualizing lipids, especially unsaturated ones. A few seconds exposure to iodine vapors suffices for detecting unsaturated lipids on an adsorbent layer. Even esters of polyunsaturated fatty acids are not altered by this treatment (101). Considerable losses of polyunsaturated lipids are encountered, however, when plates bearing them remain exposed to air and iodine vapors for several min (305, 448).

Radioactive lipids can be detected by autoradiography (6,113,269,344,379,390,392) and by scanning the layers with a Geiger-Müller or a gas-flow counter (6,68,351,364,373). The application of a gelatinous scintillation liquid to plates has been recommended to facilitate detection of material of low activity (352). Suspension counting of labeled lipids is a most valuable technique for the detection and quantitative estimation of fractions scraped off adsorption layers (6, 390,391). This method works equally well with layers impregnated with silicone (428).

Thin-Layer Chromatography as an Analytical Tool

Qualitative Analysis. TLC has been proven to be useful for characterizing complex lipophilic natural products through their "chromatographic patterns," i.e., the number, shapes and relative positions of the spots they form, and also the colors the various alicyclic constituents yield with corrosive spray reagents. Naturally-occurring neutral lipids such as fats, oils and waxes, balsams and resins, have been fractionated not only by adsorption-TLC but also by reversed-phase partition-TLC and by chromatography on adsorbent layers containing silver nitrate. The patterns of separation of castor oil, for example, are different on the three types of thin layers but they are, in each case, more or less characteristic for castor oil.

TLC has contributed to facile diagnosis of disease in organisms, organs and parts of organs. Moreover, spoilage (110) and adulteration of commercial products such as fats and oils (151,180,324) and egg powder (1) have been revealed by TLC analysis.

Separations of the "total lipids" of animal and human tissues have been, and still are, of particular interest. A systematic chromatographic analysis of such lipid extracts should logically proceed in the following three steps: first, the separation of the mixture into classes of compounds, which should be followed by a subfractionation of each of these classes and, eventually, a determination of the constituent fatty acids and other long-chain moieties as well as water-soluble constituents of the various subfractions. In the analysis of mixtures of steryl esters, glycerides, phospholipids and other ester lipids, the second step is usually omitted, although methods for the efficient fractionation of steryl esters and of glycerides are now available (e.g., 159, 205,286; see also 36,102,292, 366). Instead, the various lipid classes isolated by adsorption chromatography are hydrolyzed and the constituent fatty acids are resolved by partition methods. This procedure yields no information, of course, about the structures of individual glycerides and phospholipids.

TLC and GLC are often applied consecutively as two complementary separation procedures (e.g., 39, 61,62,101,137,432,434,460). By use of a single plate, 20/20 cm, coated with a silicic acid layer 0.25 mm thick, sufficient amt of a lipid class may be obtained for the gas-chromatographic analysis of the constituent fatty acids as methyl esters. After visualization with a non-destructive reagent, such as 2′,7′-dichlorofluorescein, lipid fractions can be eluted and isolated from the coating material for the preparation of methyl esters (84,278; see also 326). This procedure has the advantage that the purity of each lipid class can be confirmed by TLC before it is reacted further. It is much simpler, however, and in most cases satisfactory, to transesterify the various lipid fractions without their prior elution from the silicic acid by treating scrapings of the plate with methanol-hydrochloric acid (127,434) or with methanol-sulfuric acid-benzene (39,61). In addition, gas-chromatographic analysis of fatty acids in the form of the corresponding alcohol acetates (176) can be undertaken on fractions eluted from chromatoplates.

Adsorption-TLC has been recommended for the separation of the methyl esters of fatty acids, as a class, from the dimethyl acetals of aldehydes (101,432,434) prior to gas chromatographic analysis of either or both of these types of compounds. TLC has been used also to reduce the complexity of mixtures of methyl esters which were fractionated first into groups of esters having the same degree of unsaturation, each of which was then resolved further by GLC (21,91,168,190,239, 338,460).

Unusual lipids have been detected by TLC in extracts of animal and human tissues. The same technique has been employed, usually in combination with column chromatography, for isolating these compounds (78,80,212,313,416,422,432,434) and also for identifying their hydrolysis products, such as polyalcohols other than glycerol (57,98,225), and long-chain alcohols, ethers, and aldehydes (101,137,304,313, 432,434). Paper partition chromatography has been used for analyzing water-soluble moieties of the complex lipids such as sugars (220,221,242) and amino acids (179,259). The aldehydic fragments formed during deterioration of fats and oils, and also in the course of the reductive ozonolysis of unsaturated lipids, have been fractionated and identified by TLC. The non-volatile aldehydic cores of unsaturated lipids have been chromatographed as such (336), whereas volatile aldehydes and ketones have been fractionated in the form of their dinitrophenyl hydrazones. Reference is made to a series of publications describing the chromatography of derivatives of short-chain aldehydes and ketones on plain adsorbent layers (4,13, 37,172,272,281,298,362,429) and on adsorbents containing silver nitrate (13,196,440). Methyl mercaptan has been identified as a constituent of cheddar cheese by TLC of its dinitrobenzoate (254). Separations of homologous short-chain carboxylic acids as well as of homologous dicarboxylic acids, which are formed by oxidative cleavage of the double bonds in unsaturated fatty acids, have been accomplished by TLC (42, 223,224,321,329). These procedures have been utilized in the elucidation of the structures of unsaturated fatty acids (143,459).

Quantitative Analysis. Publications describing methods for the quantitative estimation of spots of lipids on TLC have appeared recently. These procedures are based upon a *visual comparison* (461) or *precise measurement of spot sizes or spot areas* on the chromatoplate, or on photostats thereof (180,339,340), also, upon *photodensitometry* of plates, photographs, or X-ray prints in transmitted light (33,268,269, 335,336,475,476) or reflected light (15,197,255,) with instruments built for stepwise advancement and scanning (15,33,197,255,268) or with devices that permit measuring the density of an entire spot all at once (58; see also 261). In all of these methods, it is mandatory to chromatograph two or more model mixtures alongside the unknown sample on the same plate (15,33,268,340) because it is not possible to combine the results from different chromatograms. Corrosive spray reagents are often used for charring lipid fractions on adsorbent layers. The composition of these reagents and the mode of heating the plates are crucial factors if quantitative evaluation of chromatograms is intended (255,268,335).

Quantitative determinations of fractions after elution from the plates have been based upon *weighing* after evaporation of the eluting solvent (209,229), upon *colorimetry* after chemical reaction (22,188,256, 276,310,330,347,348,382,398,448), *UV-spectrophotometry* (30,49,276,309,397), *IR-spectrophotometry* (235), *fluorimetry* (248,309), *bioassay* (358,400) and *radiometric techniques* (6,47,98,125,152,153,304,308,390, 391,472).

The errors encountered in the various methods of quantification are usually claimed to be within ±5%; however, it appears that a method that works well in one laboratory cannot always be reproduced in another. Therefore, a comparison and evaluation in *one* laboratory of methods of quantification, using identical samples for each method, would be highly desirable. (Such a comparative study has just been reported for GLC: Horning et al., J. Lipid Res. 5, 20, 1964.).

Adsorption-TLC has been used to isolate lipid fractions whose composition was then quantitatively determined by a method based on partition, such as GLC (e.g., 9,21,61,62,91,338,434) and reversed-phase paper chromatography (269; see also 183,184).

Thin-Layer Chromatography as a Preparative Tool

TLC has been used as an analytical auxilliary in combination with preparative separation techniques. In addition, this technique has been found applicable as a preparative method.

Adsorption-TLC has been used to select a suitable solvent for adsorption chromatography on columns (e.g., 55,63,71,279,433) and the use of reversed-phase partition TLC has been helpful in choosing a solvent system for countercurrent distribution (56). Conversely, the result of preparative isolation procedures has been conveniently assessed by TLC of fractions. Thus, adsorption-TLC has been applied for monitoring the eluate collected from adsorbent and partition columns (e.g., 79,155,185,195,240,303,413,433) and also for cross-checking the efficiency of separations on ion-exchange columns (220), reversed-phase partition columns (159,178) and of countercurrent distribution experiments (215).

Chromatoplates measuring 20/100 cm (147) and coatings as thick as 2 mm have been advocated for *preparative TLC.* However, separations on "thick" layers are not nearly as sharp as those achieved on layers of 0.25-mm thickness. If minor constituents are to be isolated from a complex mixture, it is most economical to prefractionate that mixture on a column or on a layer 0.5 or 1-mm thick, and then to purify the desired fractions by rechromatographing

them on thin layers. Several investigators have employed TLC for purifying "hot" lipid preparations (6,269,327,344; see also 54), including those labeled with H^3 by the Wilzbach technique (201,328,373,465). TLC has been used also for recovering radioactively labeled sterols from liquid scintillation "cocktails" (346).

In preparative work, an alcoholic solution of 2′,7′-dichlorofluorescein is most frequently used as a spray reagent for visualizing lipid fractions on chromatoplates.

It must be realized that a fraction isolated by adsorption-TLC, or any other chromatographic technique, is not necessarily an individual compound. The homogeneity of such a fraction has to be ascertained by techniques that are based on different chromatographic principles, such as chromatography on adsorbents impregnated with silver nitrate, reversed-phase partition chromatography and/or GLC. The combined use of adsorption-TLC and GLC has been reported for the analysis of ester lipids (see above), steroids (177,173,193), and terpenes (307,312,435,436, 437,438), as well as other types of compounds (59, 183,184).

The term "chromatographically pure" is meaningful only if various complementary chromatographic methods have not yielded further fractionation. Chemical reactions and physical constants are still indispensable for the characterization and conclusive identification of substances isolated by chromatography.

Pure compounds or mixtures of compounds isolated by TLC have been characterized further by their melting points (e.g., 337,345). Determinations of critical solution temp (CST-values) by use of the microscope have been found valuable for characterizing less than 1 mg polymorphic compounds and liquid substances (368,369). Fractions have been recovered from chromatoplates for determining their color spectra (73, 96,97), UV spectra (30,422,424,449), or IR spectra (85,235,299,337,449); isomeric ozonides have been isolated by TLC to record their NMR spectra (345), and the optical rotations of gangloisides have been determined (409). Mixtures of biologically active compounds have been also resolved by TLC and the activities of the eluted fractions were determined (358, 400). Furthermore, mass spectrometry has been used to elucidate the structures of prostaglandins (94,358) and other compounds (158) that were recovered from thin-layer plates. The purification of small amt of compounds by TLC for elemental analysis has become a convenient and widely practiced procedure (e.g., 337,345).

Applications

Analysis of Lipids in Microoraginisms, Plants and Laboratory Animals

Fats, Oils, Waxes and Their Hydrolysis Products. Adsorption-TLC has been used for fractionating naturally occurring fats and oils (209,229,262,264,278) and waxes (139,208) into classes of compounds. The deterioration of vegetable oils (110,166) as well as adulteration of vegetable oils with mineral oils (180) or animal fats (151,324) has been detected by the same technique; adulteration of egg powder with vegetable oils has been similarly ascertained by TLC (1). Hydrocarbons isolated from fish oils have been chromatographed on adsorbent layers before and after their catalytic hydrogenation (250).

Human skin fat and sebum have been analyzed by adsorption-TLC (26,138,211). Total lipid extracts of human and animal blood sera, as well as various organs, have been fractionated by TLC according to classes (e.g., 60,61,62,90,91,155,162,209,213,214,229, 235,325,403,432,434,443,450).

The possibility of characterizing fats and oils by adsorption-TLC after ozonization is of great interest (336); the potential usefulness of this method has certainly not yet been exhausted.

Chromatography on thin layers of silica gel containing silver nitrate has been used for fractionating vegetable oils and animal fats (14,15,44) as well as human skin lipids (139). The steryl esters isolated from human blood serum have been well resolved by the same technique (139,294).

Reversed-phase partition TLC has been employed for characterizing fats and oils (5,205,206,207) and their constituent triglycerides (205,206,207,286). Synthetic mixtures of wax esters (208) and of cholesteryl esters (286) and also of steryl esters isolated from beef liver (209) and human urine (209) have been resolved by reversed-phase partition TLC.

Small amt of glycol esters recently have been found in beef lung (57; see also 77). However, it has been stated (57) that these compounds cannot be distinguished by adsorption-TLC from triglycerides. Methyl esters of higher fatty acids have been detected in animal (78,258) and human (212,434) tissue lipid extracts. Some authors have claimed that these esters are genuine tissue constituents (78,212), but others have suggested that they may be artifacts produced during extraction and storage of lipids (258,434).

Classes of neutral hydrolysis products of naturally occurring lipids such as diglycerides, monoglycerides and glyceryl ethers (149,236), also alcohols (208), have each been resolved by reversed-phase partition TLC. The separation of α-monoglycerides from β-monoglycerides on layers of hydroxyl apatite (161, 163) is of interest since both adsorption-TLC on silica gel and reversed-phase partition TLC fail to resolve mixtures of these isomers.

Adsorption-TLC has been used to separate non-oxygenated fatty acids, as a class, from epoxy acids, hydroxy acids and dihydroxy acids (208,216,217,378, 408). Short-chain fatty acids have been fractionated according to chain length by adsorption-TLC, whereas mixtures of esters of non-oxygenated long-chain fatty acids have been resolved according to their degree of unsaturation on adsorbent layers containing silver nitrate (21,293,460). These compounds also have been fractionated according to degree of unsaturation *and* chain length on layers impregnated with a hydrophobic agent such as paraffins (21,205,206,208). "Critical pairs" of acids or esters have been resolved by reversed-phase partition TLC after hydrogenation or bromination of the unsaturated partners (205,206); bromination during reversed-phase partition TLC has been reported also (205,206). Saturated and unsaturated epoxy acids as well as hydroxy acids have been resolved in the form of methyl esters by chromatography on silica gel-silver nitrate (293,295). In addition, the *threo* and *erythro* forms of dihydroxy fatty acids have been separated on layers of silica gel impregnated with boric acid or other complexing agents (293,295). Adsorption-TLC on silica gel containing both boric acid and silver nitrate has been used for separating stereo isomers of methyl esters of saturated and unsaturated dihydroxy acids (295).

Reference is made here to two recent reviews of the various chromatographic techniques used for fractionating mixtures of acids or their methyl esters (182, 468).

Reactions of unsaturated fatty acids with water (361) and the UV-photolysis of fatty acids (359) have been studied with the help of TLC.

Adsorption chromatography on silica gel layers has aided in developing procedures for the elucidation of the structures of unsaturated fatty acids by ozonolysis (143,218,334,337,338,345,415) and other methods of degradation (459,472); applications of TLC in the analysis of fragments of unsaturated acids have been reported also (143,459).

TLC has become a very useful auxilliary tool in gas-chromatographic analyses of the constituent fatty acids of various lipid classes isolated from microorganisms (72,103,104,143,181,253,285,332,350), plants (29,44,252,269,278,303,459), laboratory animals (46, 61,62,63,132,302) and human subjects (see below).

Several reports have appeared on the application of TLC to studies of fat absorption in laboratory animals (8,203,318,467) and in humans (27,127,433), of the lipolysis of glycerides *in vitro* (46,114,162,164, 191,192,357,376), and of the biosynthesis of triglycerides (3,48,322). The same technique has been employed in work concerned with the incorporation of epoxy and hydroxy acids into animal lipids (60,144, 439) and of radioactively labeled non-oxygenated fatty acids into microorganisms (284), plants, animal and human tissues (74,99,144,219,266,288,401, 402,403,404). Also, recent studies of interconversions of fatty acids in microorganisms (103,316,317), plants (153) and laboratory animals (32,34,60,152, 168) have been greatly facilitated by the use of TLC. Applications of this technique in nutritional studies have been also reported (62,213,214,291).

Prostaglandins and their methyl esters have been chromatographed on silica gel, on mixtures of silica gel with silver nitrate, and on hydrophobic layers (94,358). The biological assay of prostaglandins isolated from chromatoplates also has been reported (358). The structures of the methyl esters of prostaglandins isolated through TLC have been determined by mass spectrometry (94,358).

Phospholipids, Sulfolipids and Glycolipids. Experimental conditions for the separation of the common phospholipids by TLC have been described (165,177, 210,268,297,306,385,387,388) and these methods have been applied to the analysis of phospholipid mixtures isolated from plant and animal tissues (64,66,86,132, 169,170,171,179,185,241,268,271,278,290,297,306,343, 349,353,381,385,387,388,421,423,426,427,450). New phospholipids have been discovered and isolated by use of TLC in combination with column chromatography (128,179,181,259,290,333). TLC has been employed in studies on the biosynthesis of phospholipids (11,53,98,287,425), their enzymatic hydrolysis (75, 76,140,289,427) and chemical cleavage (45). Procedures for the quantitative evaluation of TLC of phospholipids have been perfected (86,330,347). The non-volatile aldehydic cores obtained by reductive ozonolysis of lecithins have been resolved by chromatography on layers impregnated with silicone oil (336). Lysolecithin and non-lipid hydrolysis products of lecithins and other phospholipids, such as glyceryl phosphoryl choline, phosphoryl choline and choline, also ethanolamine, serine, inositol and their glyceryl phosphoryl and phosphoryl derivatives have been separated by TLC (86,210,385; see also 98); ceramides (122) and mixtures of sphingosines (226,356) or their derivatives (56,227) have been fractionated by the same technique. Phosphatidic acids, α-glyceryl phosphate, glycerol (385) and mixtures of inorganic phosphates (348) have been chromatographed on thin layers.

TLC has been utilized for fractionating sulfolipids (187,274,279,280,405) and glycolipids, such as cerebrosides (226,263,409,410,416; see also 31). Structural studies on the gangliosides, another class of glycolipids, have been facilitated by the use of the TLC technique (118,194,220,221,242,243,244). The hydrolysis products of glycolipids have been analyzed by TLC (51,108,122,129,220,356,463), paper chromatography (220,221,222,242,244) and GLC (243). The gangliosides of animal tissues (71,232,466) and healthy and diseased human tissues, especially of the brain, have been characterized by TLC (120,121,186, 230,231,232,233,355,411,412,413,414). The biosynthesis of glycolipids has been studied (50,122). Hitherto unknown glycolipids have been detected in brain (313) and kidney (416) and their structures have been determined. In addition, work on mucolipids has involved the use of TLC (251,473).

Steroids, Including Bile Acids. Several authors have described procedures for the fractionation of estrogens, androgens and other C_{18}- and C_{19}-steroids (9,49, 65,81,88,89,123,157,234,248,256,342,383,389,399,407, 445). The C_{21}-steroids, including the corticosteroids (9, 17,49,65,81,88,124,234,257,309,342,370,375,389,397,398, 444) and C_{27}- and C_{28}-steroids (9,10,19,40,52,324,389, 444) have also been investigated. The following coating materials have been employed: Silica gel G (e.g., 9,65,123), starch-bound silica gel (389), mixtures of silica gel with Celite (445), alumina (399), magnesium silicate (374) and cellulose (260, saponins); also, silica gel containing silver nitrate (454) and adsorbents impregnated with formamide (444) or a hydrophobic agent have been utilized (324,444,469).

An early pregnancy test, which is based on a semiquantitative estimation of pregnanediol in urine has been improved upon (461,462) and is being widely employed in Europe (167,462). Methods for the quantitative determination of various steroids separated by TLC have been worked out (2,22,30,49,248,276,309, 310,382,397,398), and a procedure for the estimation of free and esterified cholesterol in lipid extracts has also been described (446). Some methods of quantification involve the use of radioactively labeled steroids as internal standards (382,447). It has been found that non-radioactive estradiol on a silica gel layer can produce a faint spot on an X-ray film (344).

TLC has been used for purifying steroids labeled with H^3 by the Wilzbach technique (201,328,373) and for recovering labeled steroids from liquid scintillation solutions (346).

Applications of TLC in studies of the biosynthesis of steroids (20,198) and steroid-conjugates (314,315) have been described. The interconversions and catabolism of steroids (116,141,246,384) have also been studied by the use of TLC. Recent work on the moulting hormone, ecdysone, constitutes a fine example of the usefulness of TLC in various aspects of biochemical investigations (199,200,201,202).

The consecutive use of adsorption-TLC and gas-liquid partition chromatography has led to the discovery of cholesterol in higher plants (193). It has been emphasized that a combination of TLC and GLC yields more accurate analyses of steroid mixtures than either of these methods used singly, or indeed any other method of fractionation (117,173).

1,4-Diphenylbutane-diol-2,3, an unusual lipophilic constituent of yet unknown physiological significance, recently has been isolated from a sterol fraction of

testicular tissues of bulls and rats; a method for the determination of this compound involves the use of TLC (300).

Procedures for the fractionation of mixtures of bile alcohols (204) and of bile acids on chromatoplates have been described (16,100,115,148,150,154,160,165, 178,238,375,441,456) and TLC has been used in metabolic studies (38,148,282).

A few publications on TLC of sapogenins and saponins (18,260,275,418) have appeared. The thin-layer technique has been used for studying the biosynthesis of these compounds (20).

Mixtures of steroid alkaloids also have been fractionated by TLC (247).

Reference is made here to work on the separation of cardiac glycosides (396).

Terpenes, Including Carotenoids, Balsams and Resins. Adsorption-TLC has been used extensively for fractionating monoterpenes, sesquiterpenes and diterpenes (307,393,360). It has been found that essential oils from the rhizomes of the diploid, triploid and tetraploid forms of a plant have different compositions (471). Furthermore, it has been shown that the oils from the dorsal and ventral surfaces of the leaves of each of these plants also exhibit different patterns when chromatographed on a silica gel layer (471).

Recent developments in technique have brought about significant new applications. Thus, chromatography on layers of silica gel containing silver nitrate has been found useful for fractionating sesquiterpenes (136) and diterpenes (312). Reversed-phase partition TLC has been employed for separating monoterpene alcohols and also sesquiterpene alcohols (394).

Partition-TLC and adsorption-TLC have been applied in combination (307,435,436,437,438). With these two techniques, farnesol has been identified as the sex attractant of the male bumblebee (400). The "juvenile hormone" of the silk worm has been found to behave chromatographically like farnesol and farnesal (367).

Mixtures of carotenoids have been fractionated by adsorption-TLC (73,93,95,96,97,142,422). A scheme for the separation of as many as 30 different carotenoids has been worked out recently. This procedure involves the use of layers of calcium hydroxide, magnesium phosphate, silica gel and a mixture of calcium hydroxide with silica gel (395). Adsorption-TLC has been utilized in studies of the biosynthesis of carotenoids in plants (73,95,96,283) and the findings have been discussed in several reviews (126,134,135). The degradation of isoprenoids has also been investigated with TLC as an analytical tool (130,377). Procedures for the fractionation of bile pigments (420) and of chlorophylls have been described (93,189). Reference is made to papers on the fractionation of fat-soluble vitamins and biologically active quinones, many of which are carotenoids. These publications are mentioned later in this text.

Methyl esters of resin acids have been separated on silica gel layers containing silver nitrate (312). Balsams and resins have been efficiently fractionated by adsorption-TLC (197). Countercurrent distribution has been used to separate gram amt of these complex natural products into more simple fractions whose constituents could then be characterized by adsorption chromatography on layers of silica gel and by GLC of derivatives (215).

Fat-Soluble Vitamins and Biologically Active Quinones. Several authors have described procedures for the fractionation of vitamins A,D,E and K and their isomers by adsorption-TLC (228,249,311,331,406,455); reversed-phase partition-TLC has also been employed (41,228). A new method for the quantitative estimation of the tocopherols has been described (83) and TLC has been employed in studies of the metabolism of the tocopherols (67,69,82,87,156,464; see also 277, 372,380,386).

Adsorption-TLC has been useful in isolating ubiquinones (43,424,457,458). The same technique has been employed in a study of the biosynthesis of the quinone ring in ubiquinones (43). Mixtures of the various ubiquinones and plastoquinones have been resolved by reversed-phase partition TLC (92,354,395, 424,457,458), and a method for the quantitative determination of these compounds has been described (457).

p-Benzoquinone and other simple quinones have been identified by TLC to be the defensive repellents used by certain insects (e.g., 363; see also 365).

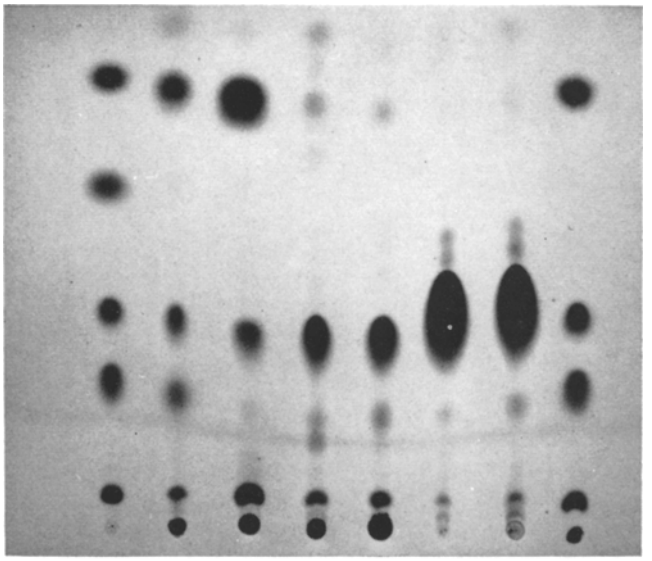

FIG. 1. TLC of total lipid extracts of various tissues of a normal individual (434). Total lipids of: S, serum; AP, aortic plaque (stage III); L, liver; K, kidney; PF, perinephric fat; BM, bone marrow.

Standard mixtures of: 1) Cholesterol, oleic acid, triolein, methyl oleate, cholesteryl oleate; 2) lecithin, cholesterol, oleic acid, triolein, cholesteryl oleate.

Adsorbent: Silica Gel G.

Solvent: Petroleum hydrocarbon-diethyl ether-acetic acid, (90/10/1, v/v/v).

Indicator: Charring with chromic sulfuric acid solution.

Topographic Lipid Analysis of Human Tissues

Thudichum, who was credited with having originated most of our knowledge of the chemical constitution of the brain, died at the turn of the century just a few years before Tswett's first publication on chromatography appeared. When Knoop, more than 60 years ago, set about to take a first look at intermediary metabolism, he did not have at his disposal any radioactively tagged compounds and, thus, he fed fatty acids labeled with phenyl groups; he did not know, at that time, about the advent of chromatography and, thus, he was obliged to use classical methods of preparative organic chemistry to isolate and identify the metabolites of the compounds he had administered. The contributions of these researchers and their contemporaries are all the more creditable if consideration is given to the painstaking work involved. With the benefit of present-day techniques,

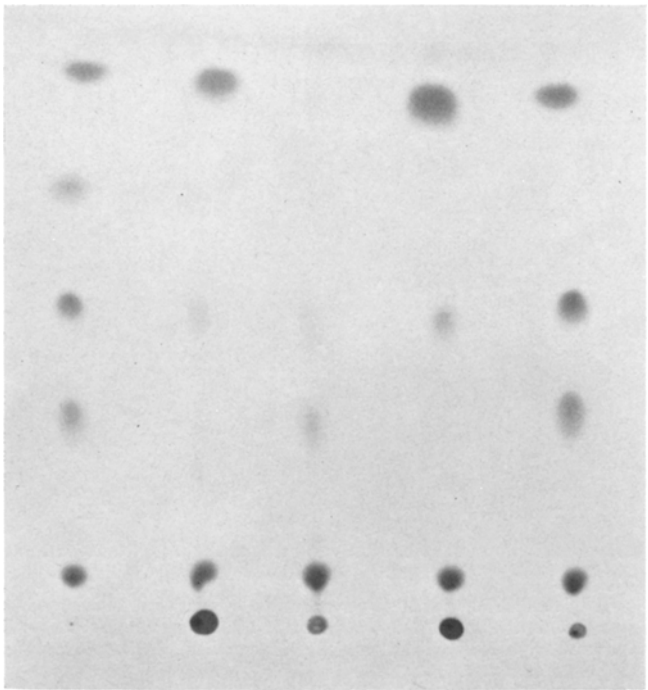

FIG. 2. TLC of total lipid extracts of aortae from an adult human and a newborn baby (434). I) Age 36, male, died of brain hemorrhage. a) Lipids of unaffected intima; b) lipids of atheromatous plaques. II) Six-hr-old baby boy, died during surgery for correction of omphalocele. Lipids of the adventitia-free aorta. 1 and 2 standard mixtures. Experimental conditions as in Figure 1.

investigations of the type undertaken by these workers should be possible on a broader scale.

Adsorption chromatography in columns was the first chromatographic technique to find application to lipid analysis. It permitted the resolution of complex mixtures into classes of compounds.

The availability of efficient gas-chromatographic equipment has brought about an intensive study of the fatty acid composition of human tissue lipids. In early work, the fatty acids of total lipid extracts were analyzed, but it soon became apparent that the various lipid classes have different fatty acid composition. Consequently, changes in the pattern of fatty acids of total lipids could be merely a reflection of changes in the ratios of the lipid classes. Therefore, complex mixtures of lipids were later fractionated by column chromatography into classes of compounds, and the constituent fatty acids of each of these classes were then determined.

Most investigators have concentrated their efforts on a thorough fatty acid analysis of one lipid class from one tissue, e.g., the triglycerides isolated from the adipose tissue, the bone marrow or the liver of several individuals. Comparison between the fatty acid composition of the steryl esters, triglycerides and the total phospholipids or the three major phospholipid classes from sera of healthy and/or atherosclerotic individuals with the fatty acid composition of the corresponding lipid classes from unaffected and/or diseased aortae or parts of aortae of human autopsy material have been reported.

With the exception of serum, most interest has been focused in the past on the analysis of the constituent fatty acids of various lipid classes in *diseased* tissues that were obtained from autopsy material. Despite great efforts, chromatographic techniques of lipid analysis cartainly have not yet become diagnostic tools of the clinician. This may be due to two factors: first, data on the composition of lipids from normal healthy humans and physiological variations of these compositions are not available; and secondly, possibly as a consequence of the first reason, the types and extent of changes of lipid composition in pathological states have not been substantiated and expressed in terms of chromatographic data.

Lipid Patterns in Healthy Humans. Comparatively few analyses have been made of the tissue lipids of healthy humans. Not any report has become known to the author of a comparison of the distribution of lipid classes in various tissues of *one* healthy individual and of fatty acid analyses of each of these classes. Neither have comparisons been made of the patterns of lipid classes in tissues of a healthy male with those of a healthy female adult human. Also, comparisons of the lipids of a healthy adult with those of a healthy adolescent, infant and/or newborn baby have apparently not been made. Is it not paradoxical that pathological conditions have been analyzed *before* healthy human tissues have been studied and "normal ranges" of values have been established?

It appears highly desirable to know with as much detail and accuracy as possible the lipid patterns of normal individuals of both sexes and of different ages. This knowledge would permit a more accurate evaluation of dietary effects and other exogenous influences on the lipids of man.

The application of adsorption-TLC in conjunction with GLC has made it possible to analyze many samples in a very short time. In most applications, it is no longer necessary to pool samples from different individuals. Indeed, the amt of lipid required for an analysis is so minute that small individual organs, such as the thymus of an infant or even parts of a single human organ, can be thoroughly investigated.

Several authors have proven that satisfactory results can be obtained by GLC of methyl esters prepared by transesterification of lipid fractions that had been isolated from thin-layer chromatograms (39,61, 127,155,434).

With regard to the distribution of lipids in healthy human tissues, the following generalizations can be deduced from the analyses known so far:

1) Human tissues contain *minor lipid classes* which have not previously been detected. For example, new types of galactolipids have been demonstrated in brain (313); sterols less polar than cholesterol have been detected in adult human aortae (434), whereas lipid extracts of baby aortae were found to contain two fractions which could not be found, so far, in the aorta or any other organ from adult humans (434).

2) The *pattern of lipid classes* of various organs of *adult humans* with no abnormality in lipid metabolism is constant and characteristic for each organ (155, 434). Permanent gross changes in these patterns are indicative of pathological conditions. This is *not to imply,* of course, that all diseases are expressed in the lipid pattern of one or more tissues.

The *lipid class patterns* of *baby* tissues are markedly different from those of the corresponding adult tissues (155,434). (A publication describing changes in the patterns of lipid classes during postnatal development of the rat has appeared recently: Dobiášová et al., Biochim. Biophys. Acta *70,* 713, 1963.)

3) The *fatty acid composition* of lipid classes is characteristic for each class in many, but not necessarily all, tissues of "healthy" adult individuals (155). The patterns of the constituent fatty acids in the various lipid classes of adult and baby organs are different (155). (Fatty acid analyses of cerebroside

sulfates from infant whole brains and adult human cerebral gray and white matter have just been published: O'Brien et al., J. Lipid Res. *5*, 109, 1964)

Figure 1 demonstrates the characteristic class patterns of the total lipids from human serum, aortic plaque, liver, kidney, perinephric fat and bone marrow.

The striking differences in the pattern of lipid classes of a baby aorta and that of an adult male are shown in Figure 2. A surprisingly close similarity is evident in the composition of the lipids of unaffected intima and atheromateous plaques obtained from the same aorta.

Pronounced changes in the total lipid content as well as the lipid composition of maturing mouse brain have been observed (112,442) before chromatographic methods for the detailed analysis of these changing patterns became available. Later, desmosterol has been detected in chick embryo (111) and the same substance has been identified by GLC in developing rat brain (237). Very recently, the major phospholipids of immature and adult rat organs, and subcellular fractions thereof, have been analyzed by conventional methods. It has been found in these studied that there occurs an over-all decrease of phosphatidyl ethanolamines with a concomitant increase in phosphatidyl cholines. This change takes place without alteration in the total phospholipid content (28).

Table I shows the approx fatty acid composition of steryl esters, triglycerides and total phospholipids from various human tissues.

Analyses of the fatty acid composition of individual phospholipid classes from human serum (347), cerebrospinal fluid (330), placenta (90) and brain (24) have been published. From these data, it appears that each of the major phospholipid classes in adult humans exhibits a distinct fatty acid pattern.

It has been demonstrated in microorganisms and in laboratory animals, that the patterns of lipid classes, the fatty acid composition of these classes and even the structures of unsaturated fatty acids can be influenced by the nutrient media (253) and rather unphysiological diets (338). The observation has been made that the addition of small amt of a simple derivative of a naturally occurring lipid to a diet can alter the fatty acid composition of lipid classes in laboratory animals (265). It has also been shown that even some normal food constituents such as cholesterol—also alcohol—if fed over prolonged periods can change the lipid class pattern in certain tissues as well as the fatty acid composition of classes (62). Studies in microorganisms have proven that differences in temp (285) and light conditions (104, 350) can very significantly influence the fatty acid composition of their lipids. In contrast, human beings living in widely separated parts of the world on rather diverse diets show very little differences in the lipid patterns of their tissues. Small differences in fatty acid composition of, e.g., the steryl esters from sera

TABLE I

Approx Fatty Acid Composition of Lipid Fractions from the Total Lipid Extracts of Human Tissues (434)

(Serum, Atherosclerotic Aorta, Liver, Kidney, Perinephric Fat, Bone Marrow)

	Steryl esters	Triglycerides	Phospholipids
	%	%	%
Saturated fatty acids	25 ± 5	40 ± 5	55 ± 5
Unsaturated fatty acids	75 ± 5	60 ± 5	45 ± 5

of healthy Americans (434) and healthy Germans (371) are certainly within the limits of variation that may be introduced by differences in sampling and analytical techniques (see Table II). No significant differences have been found in the fatty acid composition of sera from healthy Americans living in the Midwest of the U.S. and healthy Japanese, despite the drastically different diets of these two population groups (431). It is apparent from Table II that the fatty acid composition of steryl esters, triglycerides and total phospholipids of sera from healthy and atherosclerotic humans (417) do not differ significantly.

Lipid Patterns in Pathological Cases. Pathological conditions involving disturbances of lipid metabolism can be manifested in changes of the total lipid content of one or more tissues, in unusual patterns of lipid classes in one or several tissues and/or the fatty acid several tissues. Figure 3 shows a thin layer chromatogram of normal brain and brain tissues from children that had suffered from Tay-Sachs disease. These cases serve as good examples of unusual patterns of lipid classes.

The patterns of lipid classes in various brain tissues of normal individuals and multiple sclerosis patients also have been compared. Pronounced differences have been found in the lipid composition of demyelinating areas and the surrounding white matter from normal white matter (169,170,470).

Fabry's disease, which has formerly been considered a dermatological disorder, has been characterized as a sphingoliposis (416). Fractions of the polar lipids of kidney have been isolated and their major component has been partially characterized as a glycolipid (416) which may be identical with a minor constituent of normal human plasma (410).

It has been found that normal adrenals contain less phospholipids than organs which were overstressed (91).

Significant differences in the steroid composition of adrenals from normal persons and patients with Cushing's syndrome have been demonstrated by adsorption-TLC (342). The changes in the hormone content of the adrenals in guinea pigs after diphtheria toxin poisoning (23,370) are particularly interesting in this connection.

A recent study of the sterols in the skin of normal and Triparanol-treated rats is quoted here as an example of the profitable application of TLC and GLC

TABLE II

Fatty Acid Composition of Steryl Esters, Triglycerides and Phospholipids from Human Sera (Percentage)

Fatty acids	9 Atherosclerotic Americans (417)			15 Healthy Germans (371)			12 Healthy Americans (434)		
	Steryl esters	Triglycerides	Phospholipids	Steryl esters	Triglycerides	Phospholipids	Steryl esters	Triglycerides	Phospholipids
14:0	tr.	1.4 ± 1.0	tr.	1.0 ± 0.2	1.7 ± 0.1	tr.	tr.	1.1 ± 0.2	tr.
16:0	15.9 ± 1.9	30.0 ± 3.0	39.2 ± 3.5	12.0 ± 0.8	28.9 ± 1.3	30.7 ± 1.1	14.8 ± 1.1	29.5 ± 1.6	32.1 ± 1.8
16:1	4.6 ± 1.0	3.8 ± 0.7	2.2 ± 0.3	8.4 ± 0.6	7.7 ± 0.5	3.6 ± 0.6	6.2 ± 0.4	4.9 ± 0.4	1.8 ± 0.2
18:0	2.9 ± 0.7	6.6 ± 1.6	12.6 ± 2.9	3.1 ± 0.2	4.3 ± 0.6	12.1 ± 0.7	3.0 ± 0.2	5.2 ± 0.8	13.1 ± 1.1
18:1	26.2 ± 1.1	43.8 ± 2.6	24.5 ± 2.5	20.7 ± 1.0	36.0 ± 0.8	15.1 ± 0.4	25.4 ± 1.2	39.4 ± 1.9	16.2 ± 1.2
18:2	40.8 ± 2.4	11.6 ± 2.2	11.4 ± 2.0	44.9 ± 1.3	11.3 ± 0.5	20.7 ± 0.6	39.8 ± 1.8	12.0 ± 1.1	10.1 ± 0.9
18:3	tr.	tr.	tr.	1.0 ± 0.1	1.0 ± 0.1	1.3 ± 0.1	tr.	tr.	tr.
20:3	tr.		tr.				tr.	1.0 ± 0.1	1.0 ± 0.1
20:4	6.3 ± 1.0	tr.	2.4 ± 0.5	3.9 ± 0.4	2.4 ± 0.4	8.7 ± 0.4	5.2 ± 0.9	1.9 ± 0.3	3.6 ± 0.6

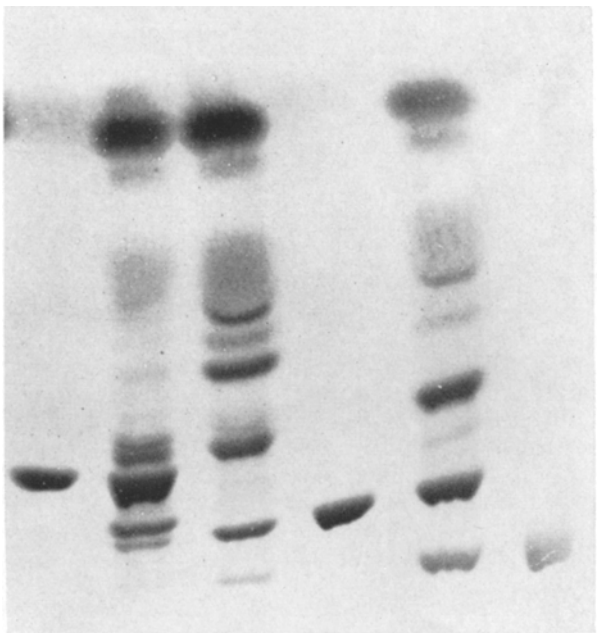

FIG. 3. TLC of lipid extracts of healthy and diseased human brain tissues, each of which had been preserved in formalin for 26 years (186). 1) Ganglioside Tay-Sachs; 2) total lipid extract of the brain cortex in a case of infantile amaurotic idiocy; 3) total lipid extract of normal brain cortex; 4) ganglioside A (Klenk); 5) total lipid extract of the brain cortex in a special case of late infantile amaurotic idiocy; 6) neuraminic acid—free residue of ganglioside A.

Adsorbent: Silica Gel G.

Solvent: *n*-Propanol-conc aqu. ammonia-water, (60/20/10, v/v/v).

Indicator: Anis aldehyde sulfuric acid in acetic acid (Reagent of Kägi-Miescher).

in investigations of the effects of drugs on the lipid composition of tissues (173).

Minor differences in the fatty acid composition of normal and diseased human tissues have been reported in several instances, but contradictory statements have been made by different authors. Is not the similarity in fatty acid composition of, e.g., the steryl ester fractions from the sera of healthy Americans and from atherosclerotic Americans more striking—considering the accuracy of the method of analysis and the human factor involved—than the small differences of the relative percentage of a particular fatty acid, especially if it represents a minor component? To date, there are indeed not many pathological states known in which clear-cut changes in the fatty acid composition of any lipid class of tissue lipids occur. The present author is aware of only two cases: a dramatic increase of palmitoleic acid has been observed in a not too well known obstetrical disease (175); and 3,7,11,15-tetramethyl hexadecanoic acid(phytanic acid) has been found as a major constituent of the steryl esters and triglycerides from the liver and the triglycerides from the kidney of a young girl with Refsum syndrome (222). The ratio of the classes of neutral lipids in the liver of this patient indicates a moderate increase of the triglyceride fraction. Phytanic acid has been identified also in the total serum lipids of three patients with the same disease (222).

Changes of the fatty acid pattern in the steryl ester fraction of serum have been observed in patients with liver carcinoma (474), whereas, in idiopathic hypercholesterolaemia, analyses have shown a normal fatty acid composition (477). The same is true for many other diseases, even if the cholesterol level of the blood is significantly different from normal (474).

Both TLC and GLC, and certainly any combination of these two techniques, and also thin-layer electrophoresis (105,106,107) should become useful tools for diagnosing physiological variations, effects of drugs and pathological states that are manifested in the lipid composition of blood serum, cerebrospinal fluid and other body fluids.

Many recent investigations have been concerned with the effects of drugs on the biosynthesis of sterols and bile acids (119,174,319). In contrast, very little is known about the effects of various compounds on the metabolism of steryl esters, triglycerides and phospholipids. This may, to some extent, be due to a lack of methods for the analysis of these types of lipids.

There can be little doubt that chromatographic techniques although they have already been utilized extensively in lipid analysis will still find wider application in the future.

Conclusion

A few years ago, the main advantages of TLC were believed to be its *simplicity*, its *speed* and *efficiency*, its *sensitivity* and its high *capacity* (267). More recently, the great *versatility* of TLC has become apparent and it now constitutes a valuable tool, not only for the fractionation of lipophilic substances, but also for the resolution of mixtures of water-soluble compounds. Certainly, many of the spectacular results obtained by TLC could have been achieved by other techniques, or combination of techniques; however, there is no doubt that many investigators have saved much time and effort by chromatographing on plates covered with a thin layer of the material they formerly used in columns.

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