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Part III

Thin-Layer Chromatography of Lipids

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A METHOD for chromatographic adsorption analysis on thin layers of adsorbent was described as early as 1938 by Izmaĭlov and Shraĭber (68). A decade later Williams (226) used a layer of adsorbent between horizontal glass plates, the upper one containing a small hole through which samples and solvents were introduced. Meinhard and Hall (120) developed "surface chromatography" on a powdered adsorbent, fixed rigidly to a microscope slide by means of a suitable binder. At the same time, Kirchner and Keller (81) described a procedure for impregnating filter paper with silicic acid. In 1951 Kirchner, Miller, and Keller (82) extended the use of adsorption chromatography to glass strips, 0.5 x 5.25 in., or sheets coated with a layer of adsorbent which contained starch or plaster of Paris as binding agent. Miller and Kirchner (125) developed an apparatus for coating glass strips with the adsorbent more rapidly than could be accomplished by hand. The same group of workers applied the new technique extensively to the separation and identification of terpenes and oxygenated terpenes and to their isolation from essential oils (82, 83, 84, 85, 86, 122, 123, 124).

Through the work of Kirchner, Miller, and Keller the chromatostrip method became well known and it was also used by other workers for the analysis of terpenes (21, 51, 67, 90, 142). With this method Vannier and Stanley (212) were able to detect the addition of grapefruit oil to lemon oil. These workers (193) and Bernhard (12) applied the chromatostrip technique to the detection and isolation of substituted coumarins from lemon oil. Stanley and Vannier (192) used chromatostrips for the quantitative estimation of biphenyl in citrus fruits. Onoe (143) utilized the same technique for the fractionation of 2,4-dinitrophenyl hydrazones of aliphatic aldehydes; Fukushi and Obata (45) analyzed azulenes; and Maruyama, Onoe, and Goto (115) separated organic peroxides.

A modification and extension of the chromatostrip method was developed by Reitsema (157, 158). He prepared chromatoplates, 5 x 7 in., to characterize various essential oils by chromatography. The use of chromatoplates instead of narrow strips permits running reference compounds alongside the mixtures of unknowns. Rigby and Bethune (159) used chromatoplates for separating and characterizing hop oils, and Wagner (218) analyzed phenols similarly.

Further application of the chromatostrip and chromatoplate techniques was reported by Lagoni and Wortmann (91, 92), who developed a method for the detection of fat-soluble pigments. These techniques were also used by Mottier (131, 132, 133, 134, 135), who carefully described methods for the analysis of food colors and of amino acids. Demole (34) separated phytol and isophytol, and Röchelmeyer (161) described the fractionation of alkaloids on thin layers of adsorbent.

It is amazing that the elegant technique of chromatographing on open columns, i.e., on thin layers of an adsorbent, was not applied to other lipids after it had become such a prominent method in terpene research.

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Actually the method remained in obscurity until 1956 when Stahl (182, 183, 184) described equipment and procedures for the preparation of chromatoplates, and demonstrated the potential usefulness of thin-layer chromatography in the fractionation of substances other than terpenes. Thin-layer chromatography (TLC) has suddenly gained recognition and the technique is now being applied to the analysis of a great variety of substances. Simultaneously the method itself has been developed further and supersedes all other separation techniques in many laboratories, especially those engaged in lipid research.

Adequate equipment for work with thin-layer chromatography, such as spreaders for the uniform coating of glass plates, and standardized adsorbents, have become commercially available.

Several reviews on the application of TLC have appeared recently (35, 36, 73, 130, 168, 188, 190, 232, 233). Vioque (213) and Fontell, Holman and Lambertsen (44) discussed specific applications of TLC for the analysis of lipids. Brenner and Pataki (18) studied some theoretical aspects of TLC. (See also 40, 117, 234.)

The present review provides detailed descriptions of apparatus and techniques used in TLC. Applications of the method to lipids and related substances are summarized briefly.

Lipid fractions resolved by TLC may be isolated and further analyzed by complementary methods, such as paper chromatography (PC) and gas-liquid chromatography (GLC). Examples of the application of TLC in conjunction with PC and GLC are presented here.

Apparatus and Techniques

The Applicator and Other Equipment. The uniform coating of glass plates with a thin layer of powdered material requires the use of a special applicator.

At present there are three types of applicators on the market.[2,3,4] The equipment designed by Stahl (182, 183, 186) is the oldest and most widely used, and therefore it is described in detail.

A plastic board 22 x 113 cm., with retaining ledges 1.8 cm. wide along a short and a long side (Fig. 1), is placed on a laboratory bench so that the long edge faces the worker. The short retaining ledge is now on the right side of the board. A glass plate, 5 x 20 cm., and five carefully cleaned square glass plates of uniform thickness, 20 x 20 cm., and another plate, 5 x 20 cm., are placed closely together in a row, on the board. A drop of water under each plate will prevent the glasses from sliding on the board. The applicator (Fig. 1) is placed on the small plate in the "open" position. The operator should grasp the applicator with both hands and move it slowly, in one stroke, across the plates to assure that they are well aligned. In the author's laboratory it was found useful to attach a piece of cork about 0.5 cm. high with adhesive tape to the leading edge of the applicator's guide bar. This permits one to move the applicator more smoothly across the row of plates.

A slurry of adsorbent is made by thoroughly mixing in a mortar 25 g. of Silica Gel G, a commercial

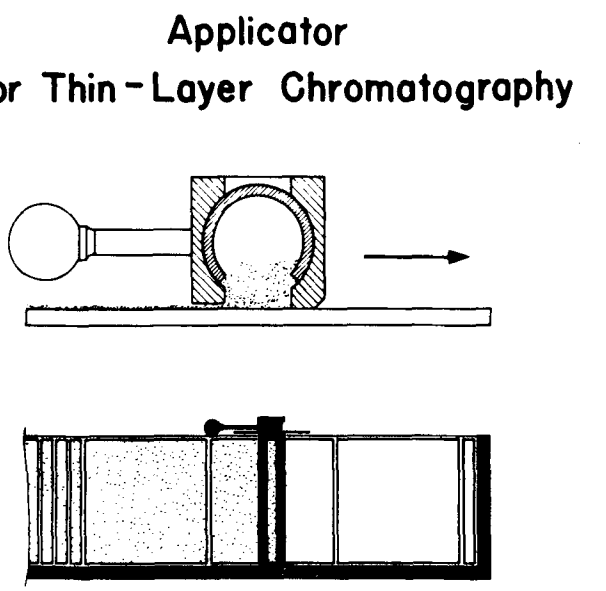


FIG. 1.

preparation containing calcinated calcium sulfate (or another adsorbent containing the same binding material[5,6]), with 50 ml. of distilled water. The slurry must be of uniform consistency and free of air bubbles. The time required for preparing the slurry should not exceed 1–1½ min. The mixture is poured into the applicator and the lever turned 180° in the direction of the red arrow on top of the instrument, to permit the slurry to run out of the slit as the applicator is moved smoothly across the row of glass plates on the board. The entire operation, from the addition of water to the silica gel until the spreading of the thin layer, must be finished in about 4 min. before the slurry hardens. The chromatoplates are air-dried for 10–20 min. to allow the binder to set and then are placed in a rack and activated by heating in an oven to 110–120°C. for 1–2 hr. The adsorbent layer thus produced is about 250–275 μ thick and very uniform in appearance in both reflected and transmitted light. According to Stahl (186) the activity of the adsorbent layer corresponds to grades II to III of the Brockmann activity scale (20). The chromatoplates may be kept in a storage cabinet to protect them from dust and laboratory fumes.

The adsorbent layer absorbs water from the air and thus slowly becomes deactivated. Adsorbents of different activities produce different rates of migration of various substances during chromatography. Therefore, R_f values usually are not highly reproducible in thin-layer adsorption chromatography. The pattern of separation, however, is constant and reproducible.

Plates, whose efficiency of separation is affected as a result of too much moisture, may be reactivated by heating. In very humid laboratories it is advisable to store the activated chromatoplates in a desiccator over freshly activated silica gel. Seiler and Seiler (174), working on the separation of inorganic cations, recommend further drying of the air-dry chromato-

[2] C. Desaga G.m.b.H., Hauptstrasse 60, Heidelberg, Germany; U. S. representative: C. A. Brinkmann and Company, Inc., 115 Cutter Mill Road, Great Neck, Long Island, New York.

[3] Camag A. G., Homburger Str. 24, Muttenz, B.L., Switzerland; U. S. representative: Kensington Scientific Corporation, 1717 Fifth Street, Berkeley 10, California.

[4] Research Specialties Co., 200 South Garrard Blvd., Richmond, California.

[5] E. Merck, A. G., Darmstadt, Germany; U. S. representative: Terra Chemicals Inc., 500 Fifth Avenue, New York 36, New York.

[6] Fluka, A. G., Buchs, S. G., Switzerland; U. S. representative: Gallard-Schlesinger Chemical Manufacturing Corp., 1001 Franklin Avenue, Garden City, New York.

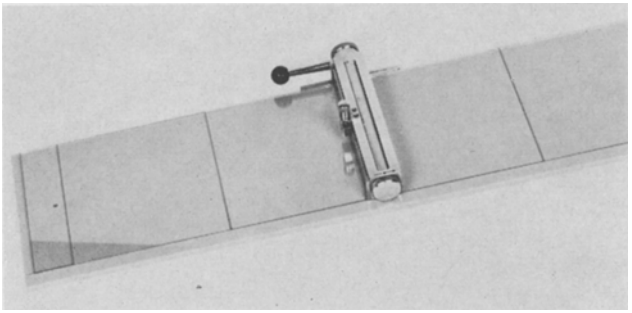


FIG. 2. Applicator "Model S" according to Stahl. Desaga G.m.b.H.[2]

plates over calcium chloride in a desiccator overnight. Thereafter the plates should be stored in a cabinet.

Recently a versatile model of Stahl's applicator has been produced which permits one to obtain uniform thin layers of any desired thickness between 250 μ and 2 mm. This applicator, Model S, as shown in Figure 2, is commercially available.[2] Stahl's new spreader may find application especially in preparative work where the use of plates of higher capacity is desired.

A rather different applicator for the preparation of chromatoplates has been designed by Mutter and Hofstetter (137). This relatively new apparatus[3] and its use have been adequately described by Wollish, Schmall, and Hawrylyshyn (232). They have given detailed instructions for the building of a similar instrument, as well as auxiliary equipment, in any small machine shop. The instrument is shown in Figure 3. It produces uniform layers of adsorbent in adjustable thicknesses on glass plates, 3.5 x 4.5 in.

An important advantage of this instrument, according to Wollish *et al.* (232), is that the glass plates used need not be uniform in thickness; thus one can easily buy inexpensive window glass cut to size in any glass shop.

Another new applicator for thin-layer chromatography has just become commercially available.[4] It is a simplified version of Stahl's original applicator, as shown in Figure 4. It yields fine layers about 275 μ thick. Layers of different thickness cannot be produced with this apparatus.

In many cases inexpensive, self-made applicators may be adequate, such as those described by Barbier, Jäger, Tobias, and Wyss (7), Černy, Joska and Lábler (25), Machata (98), Miller and Kirchner (125), Mottier (134), and Vioque (213). The author, however, believes that the purchase of a commercial applicator, standardized and uniform glass plates, and commercially available adsorbents is a good investment because they will save the many hours of experimentation that would be required to build a satisfactory instrument and produce a suitable adsorbent.

Peifer (145) has recently developed a method for TLC on lantern slides, 8 x 10 cm., on microscopic slides, 2.5 x 7 cm., and even on microscope cover slips, 2.5 x 3 cm. The glass plates are covered with adsorbent by dipping them into a suspension of Silica Gel G in chloroform or chloroform-methanol. The microchromatoplates may be used after drying over a hot plate for 1–3 min. This rapid and simple technique does not require the use of applicators, glass plates of specific dimensions, or prolonged drying periods. Therefore, it will become a useful modification of TLC even though it is not suited for resolving complex mixtures or larger samples.

Adsorbents, Stationary Phases for Partition Chromatography, and Ion Exchangers

Silica Gel (Silicic Acid). This adsorbent is most frequently used for the separation of neutral and acidic lipids (234) in columns (29), on impregnated cellulose paper (163), or on impregnated glass fiber paper (54). Fine grade (about 80 μ or 200 mesh) silica gel, containing up to 15% calcinated calcium sulfate (gypsum, plaster of Paris) as a binder, is widely used in TLC. Such preparations may be purchased as "Silica Gel G for thin-layer chromatography according to Stahl."[2,5] If very fine layers are desired, the commercial product should be run through a 200-mesh sieve before use. Silica Gel G is slurried with distilled water in a ration 1/2, w/v, and applied to the glass plates as described above.

For special purposes it may be necessary to remove small amounts of ferric ion from the adsorbent. Seiler and Seiler (174) accomplished this by washing 500 g. of Silica Gel G with 1000 ml. of concentrated hydrochloric acid-water, 1/1, v/v. After removing of the acid by decanting, the adsorbent was washed with three to four portions of 1000 ml. of water, and filtered through a Büchner funnel. After drying in a stream of air the adsorbent was washed with 300 ml. of benzene and then dried in an oven at 120°C. for 1–2 hr. Because the acid treatment removes a great part of the calcium sulfate, it is necessary to add 2 g. of this material to the 500 g. of washed adsorbent before preparing the chromatoplates.

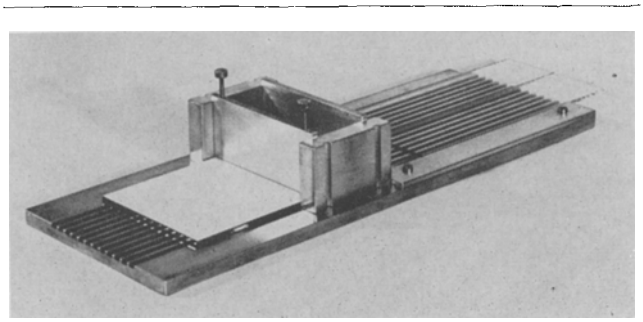


FIG. 3. Applicator according to Mutter and Hofstetter. Camag A.G.[3]

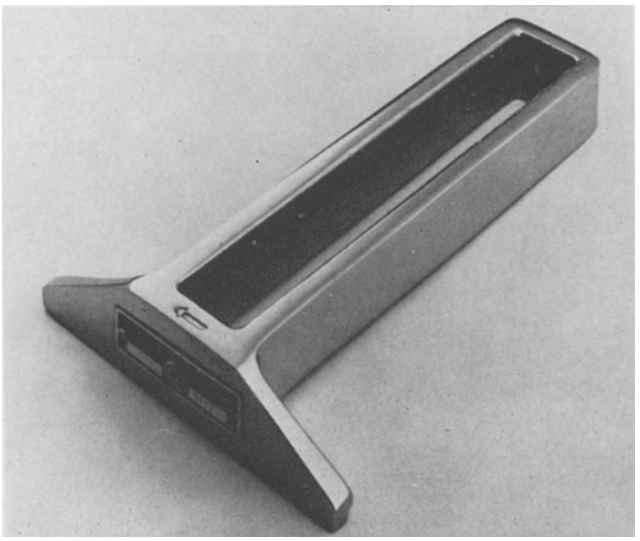


FIG. 4. Applicator of Research Specialties Co.[4]

A product closely resembling the commercial materials may be obtained by thoroughly mixing; e.g. Mallinkrodt Silica Gel,[7] 200 mesh, (154) with 10–15% calcinated sulfate of the same grain size (145). Onoe (143) prepared thin layers of silicic acid on glass strips from a slurry of 23 g. of silica gel, 200 mesh, and 30 ml. of a 2.5% solution of polyvinyl alcohol to which a few drops of ethanol were added to prevent foaming.

Several workers have recommended solvents other than water for preparing slurries of silicic acid. Müller and Honerlagen (136) claim that more uniform layers are obtained if 16 g. of Silica Gel G is mixed with 30 g. of acetone. Peifer (145) and Peifer, Muesing and Janssen (146) suspended 35 g. of Silica Gel G in 100 ml. of either chloroform or a mixture of chloroform-methanol, 3/2, v/v, for the preparation of microchromatoplates by the dipping technique. The use of nonaqueous solvents results in shorter drying periods for the wet chromatoplates. The adsorbent layer does not adhere very well to the glass and therefore, it may be necessary to expose the plates to steam (145).

It may be advantageous, especially in preparative work, to chromatograph on thin layers of powdered silica gel which do not contain calcinated calcium sulfate or other binders Such loose layers can easily be obtained by applying the dry adsorbent with any spreader which will produce layers of any specific thickness from 0.5 to 2 mm.

Hernandez, Hernandez, Jr., and Axelrod (57) have described procedures for activating and standardizing silicic acid preparations used for chromatographing so that all Brockmann activity grades are obtainable and reproducible with this adsorbent. In TLC it is usually not necessary to know the activity of the adsorbent layer, as long as satisfactory separations can be obtained. Since the R_f-values are not reproducible in TLC, they may be referred to the position of standard dyes on the chromatogram. Stahl (183) recommends developing a mixture of 50 mg. each of p-dimethyl amino benzene (butter yellow), sudan red G, and indophenol, in 50 ml. of benzene,[2] concurrently with the samples to be separated. However, this test mixture can be applied only when benzene, methylene chloride, chloroform, or solvent mixtures of similar eluting power are used. Azulene is used with less polar solvents, such as petroleum hydrocarbon, toluene, or carbon tetrachloride.

Seher (171) has found that constant R_f values are obtained on Silica Gel G plates if the adsorbent is conditioned by developing the plate with chloroform before the sample itself is chromatographed.

Modified Silicic Acid Layers. For the separation of acidic compounds, Stahl (185) prepared acidic silicic acid chromatoplates, by using aqueous 0.5 *N* oxalic acid solution instead of water in making the silicic acid slurry. Peifer (145) and Peifer, Muesing, and Janssen (146) added 2.5 ml. of concentrated sulfuric acid to 100 ml. of chloroform-methanol, 3/2, v/v. This mixture was slurried with 35 g. of Silica Gel G, as described above, for the preparation of microchromatoplates. The sulfuric acid in the adsorbent layer has the added advantage that the spraying of the plates after chromatography can be circumvented. The microchromatoplates may be placed on a hot plate right after developing, and the sulfuric acid in the adsorbent suffices for charring the organic matter in the individual spots.

Stahl (185) also described the preparation of alkaline chromatoplates. He used aqueous 0.5 *N* KOH solution instead of water in making the slurry of Silica Gel G. The use of such alkaline silica gel layers has not yet been described for TLC of lipids. This author assumes that such plates might be suitable for achieving separations of the kind described by Rouser, Bauman, Kritchevsky, Heller, and O'Brien (162) on silicic acid-silicate-water columns.

Winterstein, Studer, and Rüegg (229) separated carotenals on chromatoplates that were prepared from a slurry of 5 g. Silica Gel G and 20 g. calcium hydroxide in 50 ml. of water.

Honegger (63), in his work on thin-layer ionophoresis and combined thin-layer ionophoresis-TLC, described the use of adsorbent layers containing sodium citrate buffer. Honegger prepared chromatoplates with a slurry of 25 g. of Silica Gel G (or Kieselguhr G, or Aluminum Oxide G) in 50 ml. of 0.1 M sodium citrate buffer of pH 3.3. Spraying of chromatoplates with buffer solution also yielded useful layers. Nürnberg (141) added a buffer to the developing solvent instead of incorporating it into the adsorbent layer.

Mangold and Kammereck (111) used chromatoplates of Silica Gel G containing about 10% ammonium sulfate for the separation of phospholipids and strongly acidic fatty acid derivatives. Such weakened plates were prepared from a slurry of 25 g. of Silica Gel G in a solution of 2.5 g. ammonium sulfate in 60 ml. of water. This slurry has to be applied to the glass plates very quickly, as it hardens even faster than do slurries of plain Silica Gel G.

Adsorbent layers containing acids, bases, or salts do not adhere well to the glass plates and tend to flake off. It is recommended that such plates be dried at room temperature for several hours.

Diatomaceous Earth (Kieselguhr). This adsorbent may be purchased already mixed with calcinated calcium sulfate as Kieselguhr G, Merck, for thin-layer chromatography according to Stahl.[25] Diatomaceous earth may be useful for separations of polar lipid where a very weak adsorbent is desired. It is applied like Silica Gel G, as a slurry in water, 25 g. per 50 ml. Stahl and Kaltenbach (191) used it for the fractionation of sugars by TLC.

Aluminum Oxide (Alumina). This adsorbent is rarely used for the chromatography of complex lipid mixtures because it causes hydrolysis of ester linkages and isomerization of double bonds (14, 15, 204, 205, 206). Still, alumina is superior to any other adsorbent for the chromatographic fractionation of the A-vitamins, also of hydrocarbons and other classes of lipids, especially of basic ones. It is often used for chromatographing sterols (138).

Two brands of alumina for TLC are commercially available: Aluminum Oxide G Merck, for thin-layer chromatography according to Stahl,[5] and Fluka Alumina for coated-glass chromatography.[6] Both preparations contain around 5% of calcinated calcium sulfate as a binder. The product manufactured by Merck is slurried with water in a ratio of 25 g./50 ml., whereas Fluka Alumina is mixed in a ratio of 20 g./65 ml. of water. The manufacturer of the latter material states that a high activity stage, approximately corresponding to Brockmann activity grade II (20), is obtained by heating the chromatoplates for at least 4 hr. to 200–220°C. The plates are cooled and stored

[7] Mallinckrodt Chemical Works, St. Louis 7, Missouri.

in a desiccator containing alumina of Brockmann activity grade I (Fluka Alumina with moisture indicator [6]). If plates with an adsorbent layer of lower activity (III or IV) are desired, the plates are dried at a temperature of 150–160°C. for 4 hr. In any case, the activated plates must be stored for at least 24 hr. over alumina of the corresponding Brockmann grade for setting the required activity, according to the producer's [6] specifications.

Alumina may also be obtained by mixing, e.g., Alcoa Activated Alumina [8] 200 mesh with 5% of calcinated calcium sulfate of the same grain size (108).

Hermánek, Schwarz, and Čekan (56) applied a layer, about 0.6 cm. thick, of plain alumina powder to glass plates using Mottier's procedure (134) and they separated a great many steroids on such chromatoplates. Černy, Joska, and Lábler (25) used loose alumina layers, 3–4 mm. thick, for TLC of steroids also.

Stahl (189) has recently described a convenient technique for determining the activity of alumina according to the Brockmann scale (20). The gadget and the test dyes required for this procedure are now commercially available.[2]

Other Adsorbents. All adsorbent materials used in column chromatography may also be applied in TLC, either as a loose layer of powdered material (153), or, with a binder, as a solid film (121). Kirchner, Miller, and Keller (82) have already described the preparation of thin layers of magnesium oxide, magnesium carbonate, calcium hydroxide, calcium carbonate, dicalcium phosphate, Anex, Filtrol, Florisil, Talc, and Starch. These workers used starch (5%) or calcinated calcium sulfate (20%) as a binding agent.

Florisil,[9] which has recently been adapted for the fractionation of complex lipid mixtures on columns (24), appears to be especially promising. Peifer (145) has described a procedure for coating microchromoplates with a thin layer of this adsorbent.

Sodium carbonate (156), charcoal (62), and sugar (75) have been used in paper and column chromatography of lipids but the author is not aware of any applications of these adsorbents in TLC.

Cellulose. The preparation and use of chromatoplates covered with cellulose was first described by Teichert, Mutschler, and Röchelmeyer (203) who separated alkaloids. Several brands of cellulose powder for TLC are now commercially available.[10,11,12] The oldest product of this kind [10] contains calcinated calcium sulfate as a binder. Only 8 g. of this material, slurried with 55 ml. of water, suffice for coating 5 standard size square plates with a fine layer of cellulose. The coated plates are dried in air for a few minutes, and then in an oven for ½ hr. at 100°C. Plain cellulose powder [12] can also be applied as thin layers on glass plates (145).

To this reviewer's knowledge, cellulose chromatoplates have not yet been used successfully in the lipid field. They might become very helpful for the separation of lipids from non-lipids by adapting the procedures described by Lea and Rhodes (95) and by Westley, Wren, and Mitchell (224).

Teichert, Mutschler, and Röchelmeyer (203) impregnated cellulose chromatoplates with a 20% solution of formamide in acetone. Such plates may be useful for TLC of phospholipids (219).

Layers for Reversed-Phase Partition TLC. Thin layers of Silica Gel G can be impregnated with silicone (101), undecane (79) or higher paraffins (80, 229) or with squalene (26). They are then hydrophobic and can be used for the resolution of a lipid class into its individual constituents by reversed-phase partition TLC. Malins and Mangold (101) coated thin layers of Silica Gel G by slowly immersing the plates at room temperature into a 5% solution of silicone (Dow Corning 200 fluid viscosity 10 cs)[13] in diethyl ether. Such a silicone solution is now also commercially available in an aerosol package [4] for impregnating chromatoplates by spraying. Wintersteín, Studer, and Rüegg (229) dipped Silica Gel G plates for 2 min. into a 5% solution of paraffin oil in petroleum hydrocarbon. The same procedure was later applied by Kaufmann and Makus (79) and by Kaufmann, Makus, and Deicke (80). These authors also used undecane as a temporary impregnating agent. In all these procedures it is important that the chromatoplates to be impregnated be of the same temperature as the silicone or paraffin solutions and, also, that the plates be immersed into these solutions very slowly. Disregarding these precautions will lead to crumbling of the thin layers. The impregnated plates may be used immediately after evaporation of the solvent.

The author has impregnated thin layers of cellulose [10] and of diatomaceous earth [5] with silicone. Such plates, however, proved to be inferior to impregnated Silica Gel G plates for lipid separations.

Polyethylene powder (Hostalen S)[14] as used in columns (231) has also been used successfully for the preparation of chromatoplates which yield favorable separations of fatty acids or their methyl esters. Hostalen S, 20 g. 250 mesh, is slurried with 50 ml. of aqueous ethanol (96%), 1/1, v/v, and applied to glass plates with a commercial spreader. The chromatoplates are dried at room temperature for several hours and are then ready for use. Davídek and Davídeková (33) have recently reported the preparation of thin layers of polyamide on glass plates, and the separation of flavonoids on such chromatoplates. Stahl (188) mentioned the use of Perlon as a stationary phase in TLC.

Ion Exchangers. Only one publication has appeared so far on the use of ion exchangers in TLC. Randerath (155) used Ecteola Cellulose [12] bound to the glass plates by collodion to separate various purine and pyrimidine nucleotides. Undoubtedly, materials like the various modified cellulose ion exchangers applied on columns by Rouser, Bauman, Kritchevsky, Heller, and O'Brien (162) will also be useful if applied in the TLC technique.

Ion exchange chromatography has so far found little application in the lipid domain. It can be expected that the current wide use of TLC will also lead to extensive utilization of ion exchange materials for the separation of polar lipids.

Various Coating Materials. Urea has been used for separating straight-chain from branched-chain fatty acid in columns (62) and on paper (3). It can be expected that similar separations are possible on

[8] Aluminum Ore Company, East St. Louis, Illinois.
[9] Floridin Co., Tallahassee, Florida.
[10] Excorna o.H.G., Mainz, Germany; U. S. representative: C. A. Brinkmann and Company, Inc. (see 2).
[11] Macherey, Nagel & Co., Düren, Rhld., Germany; U. S. representative: C. A. Brinkmann and Company, Inc. (see 2).
[12] H. Reeve Angel & Co., Ltd.; U. S. representative: H. Reeve Angel & Co., Inc., 52 Duane Street, New York 7, New York.
[13] Dow Corning Corp., Midland, Michigan.
[14] Farbwerke Hoechst A. G., Frankfurt-M., Germany.

glass plates which are coated with a thin layer of urea or another clathrate-forming material. Molecular sieves should be tried also.

The Sample [15]

Neutral lipids such as fats, oils, and waxes are applied to the plates with a micropipette or better, with a microsyringe, 10–50 λ,[16,17] as 0.1–1% solutions in petroleum hydrocarbon, b.p. 60–70°C. More polar solvents such as diethyl ether should be avoided as they effect chromatographic separations at the point of application which results in wider spots and poor resolution. Phospholipids, sulfolipids, glycolipids, and other very polar compounds are applied as chloroform or chloroform-methanol solutions, 1/1, v/v. Mercuric acetate adducts of unsaturated lipids *(vide infra)* are also dissolved in chloroform.

The samples must always be applied as single spots, not in a streak across the plate, as is frequently done in paper chromatography, because the developing solvent will not migrate across such a cut in the adsorbent layer. To avoid irregularities in migration of the solvent, the chromatoplates should always be developed against the direction of application of the layer. To achieve optimal resolutions the spots should not spread more than about 0.5 cm. in diameter. This is accomplished by applying the solutions in several small portions in a stream of nitrogen. Several firms [2,3,4] offer special transparent template with side rails that permit covering a chromatoplate without touching the adsorbent layer. Such a template facilitates spotting of samples; it may also be used to draw a line across the plate, 10–15 cm. from the starting points, to mark the level to which the solvent will be allowed to travel. The use of the template as designed by Stahl is schematically illustrated in Figure 5.

In adsorption chromatography, between 10 and 20 different samples, 50–500 γ of each, may be applied on one plate in a row of spots along one side of a plate, about 2 cm. from the edge. Several mg. of one complex lipid mixture may be fractionated on one plate by applying its solution along one side of a plate. Even up to 50 mg. of less complicated mixtures may be separated on one plate if the constituents are widely different in polarity, such as fatty acids and their methyl esters.

Only 1–10 mg. of lipids or lipid derivatives like mercuric acetate adducts of unsaturated lipids can be separated on one plate through partition chromatography by the thin-layer technique.

Selection of Solvents

Lipids may be separated by adsorption into classes of compounds, and classes may be resolved by reversed-phase partition chromatography. Fractionation is also possible according to degree of unsaturation. The choice of solvents depends on the type of separation desired. Solvent mixtures, rather than individual solvents, are usually applied, but the mixture should be kept as simple as possible for better reproducibility. Also, multicomponent systems tend to produce secondary solvent fronts, due to demixing during chromatography. A binary or ternary mixture of solvents can always replace a developing system consisting of five or six components.

Separating Lipids Into Classes. Brockmann and Volpers (19) found that a solvent elutes organic compounds from adsorbent columns according to the type and number of functional groups in these compounds. Hydrocarbons leave the column first, followed by ethers, ketones, alcohols, and acids, in this sequence. Within each class, saturated compounds are eluted before unsaturated ones. Polyunsaturated compounds with isolated double bonds are more easily eluted than are those containing a conjugated system of double bonds; *cis*-compounds are more easily eluted than their *trans*-isomers.

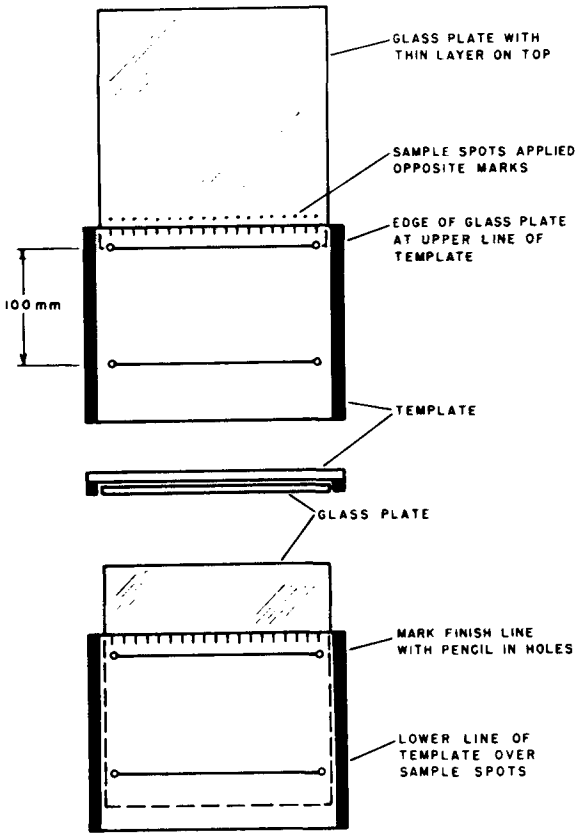


FIG. 5. Use of the template.[1]

These rules apply also in TLC: hydrocarbons migrate further on a chromatoplate than do ethers, ketones, or alcohols, and within each spot the saturated components of a class are concentrated in the front part of this spot. These subfractionations are quite pronounced with, e.g., the methyl esters of fatty acids derived from fish oils, where great differences exist in chain length and degree of unsaturation of the individual constituents. However, they never interfere with separation according to classes.

Trappe (204, 205, 206) established a series of solvents of increasing elution power. This Eluotropic Series of Solvents was extended, and other series were described, e.g., by Strain (199), and by Knight and Groennings (88). The tabulation of eluotropic series of solvents (Table I) is very helpful in choosing suitable solvents for adsorption chromatography on columns or on thin layers. Izmailov and Shraiber (68) and other workers (145, 183) have described a spot

[15] Lipid standards for TLC may be obtained from The Hormel Foundation, Austin, Minnesota.

[16] Hamilton Company, Inc., Whittier, California.

[17] Disposable self-filling, self-measuring dilution micropipettes (Gerarde Pipettes) are recommended for work with radioactive lipids. "Unopette," Becton-Dickinson, Rutherford, New Jersey.

test for choosing a suitable solvent for chromatography: drops of a solution of the sample to be fractionated are placed on a thin layer of the adsorbent to be used and different solvents are applied to these spots. The various solvents will yield concentric rings of the constituents of the mixture and the solvent effecting the clearest separation by this test is also most suitable for chromatographing the mixture in a larger scale on a chromatoplate or on a column.

TABLE I

Eluotropic Series of Solvents

Trappe (204)	Strain (199)	Knight and Groennings (88)
Light petroleum	Light petroleum 30–50°	Heptane
Cyclohexane	Light petroleum 50–70°	Diisobutylene
Carbon tetrachloride	Light petroleum 70–100°	Benzene
Trichloroethylene	Carbon tetrachloride	Isopropyl chloride
Toluene	Cyclohexane	Diisoproply ether
Benzene	Carbon disulfide	Diethyl ether
Dichloromethane	Anhydrous diethyl ether	Ethyl acetate
Chloroform	Anhydrous acetone	sec. Butyl alcohol
Diethyl ether	Benzene	Ethyl alcohol
Ethyl acetate	Toluene	Water
Acetone	Esters of organic acids	Acetone
n-Propanol	1,2-Dichloroethane	Methanol
Ethanol	Alcohols	Pyruvic acid
Methanol	Water	
	Pyridine	
	Organic acids	
	Mixtures of acids or bases, water, alcohols, or pyridine	

Mixtures of two or three solvents of different polarities may be chosen for optimum resolution, instead of a single solvent. Schroeder (169) found mixtures of petroleum hydrocarbon with diethyl ether to be especially useful. This solvent pair is now preferred in column chromatographic separations of natural lipid mixtures according to classes of compounds (e.g. 42, 58, 118, 119, 181) and also in TLC (e.g. 107, 108, 126, 149). Ethyl acetate may be used to replace diethyl ether in such mixtures.

Classes of non-polar lipids, such as hydrocarbons, ethers, cholesteryl esters, and alkyl esters are best separated, as classes, by TLC with nonpolar solvents such as petroleum hydrocarbons, carbon tetrachloride or benzene or mixtures of two solvents containing largely one of these solvents (Table II). More polar lipids are resolved with mixtures of petroleum hydrocarbon and diethyl ether in different ratios (Table II). The same solvent mixtures with 1–2% of glacial acetic acid serve for resolving complex mixtures of natural lipids (Table II). The addition of acetic acid is essential if free fatty acids are present, in order to prevent their streaking.

Very polar nitrogenous lipids of industrial importance, like amines, amides, and nitriles, are well resolved with a mixture of chloroform-methanol-aqueous ammonia. Chloroform is equilibrated, at 20°C., with aqueous ammonia, 10/1, v/v, and 97 volumes of this ammoniacal chloroform and 3 volumes of methanol are used as the developing solvent system (111).

Acidic lipids, such as sulfates and sulfonates, phosphates and phosphonates, are separated by TLC on Silica Gel G containing 10% of ammonium sulfate, with mixtures of chloroform-methanol-aqueous sulfuric acid. Methanol containing 5% of 0.1 *N* sulfuric acid is mixed with chloroform, depending upon the polarity of the lipids to be resolved (111). Typical solvent systems are chloroform-acidic methanol-sulfuric acid, 97/3 and 8/2, v/v. The latter solvent mixture is especially suitable for the separation of natural phospholipids, such as lecithins, cephalins, and sphingomyelins on modified Silica Gel G plates. This acidic system will split, however, any aldehydogenic phospholipids.

Solvent systems for the separation of phospholipids, sulfolipids, and glycolipids on Silica Gel G plates are listed in Table II. Typical solvent systems for the separation of steroids, of bile acids, and of triterpenoic acids are listed in the same table.

Solvents for Fractionating Classes by Reversed-Phase Partition TLC. The solvent systems used in chromatography on silicone-impregnated paper or on paraffin-impregnated paper can also be applied in reverse-phase partition TLC. Chakrabarty (26) has used the same developing solvents on silicic acid plates that had been impregnated with squalane.

TABLE II

Solvents for Thin-Layer Chromatography of Lipids on Silica Gel[a]

	References
Fats, Oils, and Waxes, Oxygenated Acids and Their Esters	
Tetralin-Hexane, 1/1, 1/3	(80)
Petroleum hydrocarbon[b]-diethyl ether, 95/5, 90/10, 80/20, 50/50	(101,108,149)
Petroleum hydrocarbon diethyl ether-acetic acid, 90/10/1, 80/20/1, 70/30/2	(101,108,128)
Diethyl ether	(79)
Diisopropyl ether	(79,80)
Diisopropyl ether-acetic acid, 100/1.5	(79,80)
n-Propanol-conc. aqueous ammonia, 2/1, followed by chloroform-benzene, 3/2, followed by carbon tetrachloride	(65,222)
Phospholipids, Sulfolipids, and Glycolipids	
Chloroform-methanol-water, 70/22/3, 65/30/5, 65/25/4, 60/35/8	(164,219,220)
Chloroform-methanol-aqueous ammonia, 3/1 with 40 ml. of conc. aqueous ammonia/liter; 1/3 with 40 ml. of conc. aqueous ammonia/liter	(30,64,111)
Chloroform-methanol-aqueous sulfuric acid Chloroform-methanol (containing 5% of 0.1 *N* sulfuric acid), 97/3, 4/1	(111)[c]
n-Propanol-water, 7/3	(89)
n-Propanol-aqueous ammonia (12%), 4/1	(72,223)
n-Propanol-conc. aqueous ammonia, 7/3	(72)
n-Propanol-aqueous ammonia (12%), 4/1, followed by ethylene chloride-methanol, 49/1, followed by chloroform-acetic acid (96%), 95/5	(72)
n-Butanol-pyridine-water, 3/2/1	(87)
Steroids	
Benzene, toluene, cyclohexane, hexane, dichlorethane	(25,31,56)[d]
Benzene-ethyl acetate, 9/1, 2/1	(31)
Benzene-ethanol, 49/1, 95/5, 9/1	(25)
Cyclohexane-benzene, 4/1, 1/1	(31)
Cyclohexane-ethyl acetate, 19/1, 17/3, 9/1, 7/3	(7,31)
Diethyl ether	(56)[d]
Bile Acids	
Upper phase of the system, toluene-acetic acid-water 10/10/1, 5/5/1	(48)
n-Butanol-acetic acid-water, 10/1/1	(48)
Triterpenoic Acids and Esters	
Benzene, cyclohexane, methylene chloride, diisopropyl ether, ethyl acetate, butyl acetate	(207,210)
Cyclohexane-toluene, 4/1, saturated with formic acid	(210)
Diethyl ether-hexane, 1/1	(214)
Diisopropyl ether-acetone, 5/2	(210)
Diisopropyl ether-acetone, 5/2, with 5% of pyridine	(210)
Ethyl acetate-methanol-diethyl amine, 14/4/3	(210)
Chlorobenzene-acetic acid, 9/1	(210)
Methylene chloride-pyridine, 7/2	(210)

[a] All ratios are v/v.
[b] Usually petroleum hydrocarbon, b.p. 60–70°C. (mainly hexane).
[c] On silica gel G containing 10% of ammonium sulfate.
[d] On alumina.

Suitable solvent systems for reversed-phase partition PC or TLC of a given class of lipids can be chosen after studying the behavior of this lipid in adsorption chromatography (111). Typical solvent systems are given in Table III.

Compounds differing by two methylene groups and one double bond, such as palmitic and oleic acids, or palmitonitrile and oleonitrile, myristonitrile and linoleonitrile, lauronitrile and linolenonitrile, overlap in reversed-phase partition chromatography. Such critical pairs are separated by low-temperature chromatography. Palmitic and oleic acids may be resolved with a mixture of acetic acid-formic acid-water, 2/2/1,

TABLE III

Solvents for Reversed-Phase Partition Thin-Layer Chromatography

Solvents[a]	Ratios v/v	Stationary phases[b]	Lipid classes fractionated	Ref.
Methanol	.....	Higher paraffins	Carotenals	(229)
Acetic acid-water	24/1	Undecane	Fatty acids	(79)
.....	3/1	Silicone	Fatty acids and their methyl esters	(101)
.....	17/3	Silicone	Fatty acids and their methyl esters and aldehydic cores derived from lecithins	(101) (152)
.....	17/3	Squalane	Fatty acids and their methyl esters	(26)
Acetic acid-acetonitrile	1/1	Undecane	Fatty acids	(79)
Acetic acid-acetonitrile water	2/14/5	Silicone squalane	Methyl esters of fatty acids	(101) (26)
Methyl ethyl ketone-acetonitrile	7/3	Higher paraffins	Cholesteryl esters of fatty acids	(80)
Chloroform methanol-water	5/15/1	Undecane	Diglycerides	(79)
Acetone-acetonitrile	7/3	Undecane	Triglycerides	(79)
Acetone-ethanol-water	6/1/3	Polyethylene	Methyl esters of fatty acids	.....
Acetic acid formic acid water[c]	2/2/1	Silicone	Fatty acids saturated/unsaturated	(101)
Acetic acid-peracetic acid-water	15/2/3	Silicone	Fatty acids saturated/unsaturated	(101)

[a] All solvent mixtures must be saturated with the stationary phase.
[b] Silica Gel G served as a carrier for the stationary phases. Only polyethylene was applied directly to the plates.
[c] Chromatographed at 4–6°C. (All others at room temperature.)

v/v/v, at 4–6°C., on siliconized Whatman #1 paper [12] (165) or on a siliconized Silica Gel G plate (101). An alternative method of resolving critical pairs is to chromatograph with oxidizing solvents (101, 105, 107). Saturated and unsaturated nitriles can be resolved on siliconized paper or on a siliconized chromatoplate with solvent systems consisting of 75 volumes of acetic acid and 25 volumes of water, or 70 volumes of acetonitrile and 30 volumes of water. A mixture of 65 volumes of acetic acid, 10 volumes of peracetic acid [18] and 25 volumes of water, or of 70 volumes of acetonitrile, 10 volumes of perhydrol, and 20 volumes of water separates saturated nitriles from one another, whereas all unsaturated lipids are quantitatively oxidized and migrate in bulk to the solvent front.

Solvents for Separating Lipids According to Unsaturation. Saturated and unsaturated lipids can be separated by chromatography after reacting the latter with mercuric acetate in methanol. A procedure for forming acetoxymercuri-methoxy compounds of unsaturated lipids is described later in this text.

Saturated and the acetoxymercuri-methoxy derivatives of unsaturated lipids, e.g., hydrocarbons, methyl esters, or nitrils, are resolved by TLC on Silica Gel G with a mixture of petroleum hydrocarbon-diethyl ether, 4/1, v/v. The derivatives of the unsaturated lipids are then fractionated according to the degree of unsaturation, regardless of chain length, in a solvent consisting of n-propanol-glacial acetic acid, 100/1, v/v. After isolation by TLC, the original monounsaturated, diunsaturated, and triunsaturated lipids can each be recovered by treating their complexes with methanolic hydrochloric acid.

Developing

Chromatostrips and chromatoplates are usually developed at room temperature in ascending technique, i.e., they are placed on edge in a covered jar containing a 0.5–1 cm. layer of solvent which then travels up the length of the plate until it reaches a pre-set line. Jars for use with standard chromatoplates are commercially available.[2,4,19] Several chromatoplates may be simultaneously developed in one jar. To obtain straight solvent fronts, the adsorbent should be scraped off the edges of the plates before chromatography, and the chromatoplates should not touch the sides of the jar. For the same reason, it is advantageous to line the inside of the jars with filter paper which acts as a wick to assure that they are uniformly saturated with the solvent vapors (185). This gives a straight solvent front, rounder spots and it shortens the developing time by about ⅓. It may be noted that chromatography in lined jars requires somewhat more polar solvent than in plain jars. For example, to obtain the same kind of separation one will have to use a system of petroleum hydrocarbon, b.p., 60–70°C., diethyl ether, glacial acetic acid, 80/20/1, on a jar lined with filter paper, but the same solvents in a ratio of 90/10/1, v/v/v, in a jar that does not contain such a wick. Generally the separations achieved in a saturated chamber are not quite so sharp as those obtained in plain chambers.

Chromatoplates may also be developed step-wise, running the same solvent once or twice or different solvent systems consecutively in the same direction (multiple development). Stahl (184) has utilized Matthias' wedge strip technique (116) in TLC, by scratching a row of wedge-tipped strips in the adsorbent layer.

Dhopeshwarkar and Mead (38), also Chakrabarty (26), have used a gradient-elution scheme for TLC of lipids. The developing solvent mixture is continuously being diluted with a more polar solvent. This technique permits fractionation of lipids of widely different polarity on a single plate.

Brenner and Niederwieser (17) extended the scope of TLC by developing a simple arrangement which forces solvents to run over. This method was applied to the separation of substances that could not be separated otherwise. The same technique should be useful for collecting fractions from an overflowing chromatoplate. An apparatus for use with chromatostrips has been devised by Stanley, Ikeda, and Cook (194).

Chromatoplates may be run in two-dimensional technique. A single sample is placed in a corner of the plate, and the plate is then developed, consecutively, in two directions with two different solvent systems. Using the same solvent in both directions yields little improvement in resolution but indicates alteration of components if the resulting spots are not uniformly on a line bisecting the plate. For example, in TLC of tissue lipids it can be observed that the phospholipid fraction remains where it had been after developing in the first dimension (211). Stahl (187) has applied two-dimensional TLC for studying chemical reactions on the plate of the compounds fractionated by chromatography in the first direction (Separation-Reaction-Separation; SRS Technique). Miller and Kirchner (124) had already described carrying out oxidations, reductions, dehydration, and hydrolysis reactions, and formation of derivatives on the plate. Kaufmann and Makus (79) have performed adsorption chromatography and reversed-phase partition chromatography

[18] Becco Chemical Division, Food, Machinery and Chemical Corp., Buffalo 7, New York.

[19] A. H. Thomas Company, Vine Street at Third, Philadelphia 5, Pennsylvania.

of a sample, consecutively, on the same plate. The lipid mixture was first fractionated according to classes by adsorption chromatography. Then the unused major portion of the chromatoplate was impregnated, and classes were resolved into individual compounds by reversed-phase partition chromatography in the second direction.

The possibility of reacting parts of a complex mixture in the course of chromatography has been illustrated by chromatography in oxidizing solvents (101, Table III).

Chromatoplates may be developed horizontally. This is mandatory if loose layers of adsorbent, free of binding agent, are applied (e.g., 25, 56, 91, 92, 131, 153). It is also used in radial development, i.e., Circular-TLC. The sample is placed in the center of a plate and developed by slowly dripping solvent onto it from a pipette (91, 92, 226) or by supplying solvent from a reservoir through a wick (184). Chakrabarty (26) has found centrifugally accelerated TLC useful.

Visualization and Identification of Lipids

Most indicators used in PC of lipids (103, 113) may also be applied in TLC, provided washing of the chromatograms is not required. These indicators are usually non-destructive, thus permitting the individual fractions to be eluted from the plates for further fractionation and identification. In addition, corrosive spray reagents, such as oxidizing acids, may be used for charring organic matter on the chromatoplates over a hot plate. Such indicators have the great advantage that all compounds that may not be detected by more specific reagents are visualized unless they are volatile. The author prefers chromic sulfuric acid solution. With this agent, unsaturated lipids appear as light brown spots on a white background, even before heating. Heating the plates yields further information on the chemical nature of the various compounds as different colors appear at increasing temperatures.

A list of indicators used in TLC of lipids is presented in Table IV.

The consecutive use of two or several indicators on one chromatoplate increases the probability that no substance remains undetected. The author prefers using iodine vapors on one plate, and after evaporation of the iodine (5 min.), dichlorofluorescein, followed by chromic sulfuric acid solution (111). The dichlorofluorescein on the plate does not interfere with the subsequent charring process, neither is silicone on reversed-phase plates charred when heated with chromic-sulfuric acid. Dichlorofluorescein, however, cannot be used on reversed-phase layers, as the whole plate fluoresces when sprayed with this indicator (101).

Fluorescent chemicals may be incorporated in the adsorbent layer for visualizing spots in U.V. light after chromatography. Reitsema (157) added about 0.004% of Rhodamine 6 G to his chromatoplates, Stahl (183) used 0.04% sodium fluorescein, Černy, Joska, and Lábler (25) mixed morin into loose layers of alumina. Kirchner, Miller, and Keller (82), following a suggestion by Sease (170), incorporated about 0.75% of each zinc silicate and zinc cadmium sulfide as fluorescent agents into silicic acid layers on glass. The reviewer adds 1% of the commercial Luminescent Chemical #601 [22] to Silica Gel G plates, in

[20] Eastman Kodak Company, Rochester 3, New York.
[21] "Mineralight," Ultra-Violet Products Inc., San Gabriel, California.
[22] E. I. du Pont de Nemours & Co. (Inc.), Photo Products Department, Wilmington 98, Delaware.

TABLE IV
Indicators Used in Thin-Layer Chromatography of Lipids

Reagent	Color of spot	Substances visualized	Sensitivity	Ref.
Daylight or U.V. light	Various colors, white background	Colored compounds. Fluorescent substances	0.1–10 γ	(37) (225) (228)
Fluorescent dye or mineral in adsorbent layer	Various colors, colored background	Aromatic derivatives of lipids. Conjugated unsat. fatty acids	1 γ	(47) (82) (157)
Radioisotopes	An autoradiograph is taken	All radioactive labeled materials	<0.1 γ	(107) (112) (195)
Iodine vapors or iodine, 1% in methanol [a]	Brown, white or yellow background	All unsaturated lipids, also some saturated nitrogenous lipids	<1 γ	(101) (107) (108)
α-Cyclodextrin, 1% in aqueous ethanol, 30% followed by iodine vapors	White, purple background	Monochain lipids	5 γ	(101) (107)
2′,7′-Dichlorofluorescein, 0.2% in ethanol, 96% [a]	Green, purple background in U.V. light	Nonpolar lipids, saturated and unsaturated	1–5 γ	(101) (108) (111)
Rhodamine B, 0.05% in ethanol, 96%	Purple, rose background	Most lipids	1 γ	(79) (80) (220)
Bromothymol blue 0.04 mg. in 100 ml. 0.01 *N* aqueous NaOH	Yellow on blue background	Most lipids	0.1–1 γ	(52) (71) (72)
Fluorescein-bromine 0.04% sodium fluorescein in water followed by bromine vapors	Yellow, pink background, best in U.V. light	Unsaturated compounds	1 γ	(4) (82) (183)
Wet hydroxylamine impregnated paper, followed by 5% ferric chloride in 0.5 *N* aqueous HCl	Purple, white background	Esters	5–10 γ	(35) (40)
Antimony trichloride or antimony pentachloride or stannic chloride. Saturated solution in chloroform or carbon tetrachloride	Various colors in daylight and U.V. light	Aliphatic lipids, steroids	1–10 γ	(7) (31) (222)
o-Phosphoric acid 40% in water	Gray-blue	Pregnandiol	.....	(221)
Phosphomolybdic acid, 5–20% in ethanol, 96%	Blue, white, or yellow-green background after heating to 100°C.	Neutral lipids Antioxidants	<0.5 γ 1 γ	(7) (80) (171)
Aqueous sulfuric acid, 1:1, v/v, or conc. sulfuric acid, or saturated chromic-sulfuric acid solution	Various colors during heating, eventually gray or black	Most nonvolatile organic compounds	<1 γ	(82) (112) (128)
Ninhydrin [a] 0.2% in n-butanol-aqueous acetic acid 10%, 95/5	Red-purple, white background after heating to 105°C.	Aminophosphatides	10 γ	(164) (219) (220)
Dragendorff reagent a) 1.7 g. basic bismuth nitrate in 100 ml. aqueous acetic acid (20%); b) 40 g. potassium iodine in 100 ml. water. Reagent: 20 ml. of a) with 5 ml. of b) and 70 ml. of water	Orange or red-orange	Cholin-phosphatides	10–20 γ	(164) (219) (220)
Diphenyl amine 20 ml. 10% diphenylamine in ethanol, 96%, plus 100 ml. conc. HCl and 80 ml. acetic acid	Blue-gray, light gray background	Glycolipids	4–10 γ	(87) (219) (220)
2,4-Dinitrophenyl hydrazine, 0.5% in 2 *N*-HCl	Yellow or orange	Aldehydes and ketones	1–5 γ	(7) (157) (158)
Potassium iodide-starch, 5 ml. 4% KOH with 20 ml. acetic acid, after 5 min.: 1% aqueous starch solution	Blue, white background	Peroxides	.....	(183)
Ammonium thiocyanate 0.2 g. in 15 ml. acetone and 0.4 g. ferrous sulfate in 10 ml. water	Red and red-brown, white background	Peroxides	.....	(183)
2,6-Dichloroquinone chlorimide 1% in ethanol	Various colors, white background	Antioxidants	1 γ	(171)
s-Diphenylcarbazone 0.1% in ethanol, 95%	Purple and blue, light rose background	Mercuric acetate adducts of unsaturated lipids	10–20 γ	(110)

[a] Available as aerosol package[1].

TLC of derivatives of short-chain alcohols, aldehydes and ketones, acids, amines and mercaptans.

Thin-layer chromatograms of radioactively labeled lipids are recorded by taking autoradiographs on x-ray film, as described below. Still, it is to be emphasized that chemical indicatars should always be used also. Application of both photographic and chemical detection methods yields information regarding both radiochemical and chemical purity of a substance.

Documentations of Chromatograms

Because thin-layer chromatograms are relatively small, they can readily be copied by tracing them with a pencil directly into a laboratory journal.

Thin-layer chromatograms can be preserved if a stable indicator was used for visualizing the individual spots. This may be done by covering the chromatoplates with a glass plate of the same dimensions; the two plates protecting the adsorbent layer are sealed along the edges with tape. Barrollier (9) recommended dipping chromatoplates into a dilute solution of label glaze. The adsorbent layer is firmly held together by the label glaze and can be pulled off the glass as a film. The reviewer has found Fisher Label Glaze [23] suitable for Barrollier's procedure. This technique requires, of course, that an indicator be used which is insoluble in the solvent used for diluting the label glaze. Meinhard and Hall (120) demonstrated that the adsorbent layer can be stripped off on adhesive tape and pasted into a laboratory journal.

Black and white or color photography of chromatoplates is easily done in daylight or in U.V. light. Bürki and Bolliger (23) recommend Sylvania F8T5/BLB, Blacklite Blue tubes [24] and a yellow filter for taking black and white photographs in U.V. light. A photograph of a thin-layer chromatogram taken in daylight is shown in Figure 10.

Chromatoplates may be copied by exposing them to sheet film or photographic paper in an enlarger. Seher (e.g. 171) and Morris, Holman, and Fontell (e.g. 126) prepared photostats [25] of chromatoplates (Fig. 11). This technique has been applied also for quantitative analysis (74, 172, 173). It is simple and rapid, but it has the disadvantage that small or weakly stained bigger spots are easily lost. Hefendehl (55) recommended spraying the chromatoplates with a solution of paraffin in ether (1/1, w/v) to render them transparent. These plates yield better photostats than do untreated ones.

Chromatoplates containing radioactively labeled substances yield fine x-ray prints (107, 112). Autoradiographs are obtained by exposing the plates in the dark to No-Screen Medical X-Ray Safety Film [20] for a few hours to one week, depending upon the type of radiation and the activities of the resolved compounds. The films are developed with Supermix Developer [26] for 4 to 6 min., and they are fixed for 30 min. with Acid Fixer.[20] Autoradiographs of commercial preparations of C^{14}-labeled lipids are presented in Figures 18 and 19.

Elution and Recovery of Lipids

One of the striking features of TLC is the high capacity of the plates. It is possible to separate by adsorption TLC up to 50 mg. of a lipid mixture on a single chromatoplate, 20 x 20 cm., coated with a thin layer of Silica Gel G of 250–275 μ thickness. About 5 mg. of lipid may be separated on such a plate by partition and reversed-phase partition TLC. Lipids, after having been isolated by TLC, may be scraped off the plates and extracted from the carrier material. The appropriate eluans for lipids may be determined by chromatographing these substances with different solvents on plates. Solvents that carry a compound with the solvent front are suitable as eluans for that substance (101). Care must be taken, however, that the eluans does not dissolve large amounts of the calcium sulfate, and thus aqueous solvents cannot be used.

A novel technique of recovering substances from a chromatoplate is to sublime the material from the adsorbent layer onto a cooled glass plate held 1 mm. above it (39).

TLC has been used to analyze commercial preparations of lipids labeled with C^{14}, H^3, or I^{131}, and for the purification and isolation of milligram amounts of compounds (112). Winterstein, Studer, and Rüegg (229) isolated unusual carotenoids by TLC, stating that no other method allowed the preparation of some of these materials (230).

With polar lipids, the yields of recovered material are usually very poor. This may be due to the presence of calcinated calcium sulfate in the solid adsorbent layer; and if so, better yields may be expected with loose layers of plain adsorbents. The use of sugar (75) as an adsorbent may be helpful. Even though it has a rather low capacity, its solubility in water may facilitate recovery of lipids. Thicker layers of adsorbents may be applied to preparative work. However, the thickness of the coating should not exceed 1 mm. to avoid distortion by uneven migration rates on the surface and inside the adsorbent layer.

The yields are improved by using the less polar derivatives of lipids containing free functional groups. Thus, alcohols and acids are more easily recovered as acetates and methyl esters, respectively (112). The nonpolar derivatives are especially important in TLC with isotopically labeled lipid derivatives *(vide infra)* where quantitative recovery is crucial.

Recovering various substances from a chromatostrip (194) or from a chromatoplate (17) is also possible by letting the developing solvent run over.

Esters of unusual fatty acids are often found by GLC in mixtures of methyl esters derived from natural lipids. Curves from such analyses can be correctly interpreted only if the sample subjected to GLC was free of compounds that are not methyl esters of fatty acids. The absence of such contaminants is not routinely checked. It appears to be necessary to analyze each sample of methylation products by TLC prior to GLC for substances other than methyl esters. Pure methyl esters may be isolated by TLC (211).

Quantitative Thin-Layer Chromatography

The methods that have been suggested for the quantitative evaluation of thin-layer chromatograms are summarized in Table V.

Few of the above methods are exact and the better ones are particularly impractical. Four of these methods involve elution of the various lipids from the plate. A suitable eluans for a given lipid is found by

[23] Fisher Scientific Company, 633 Greenwich Street, New York 14, New York.
[24] Sylvania Electric Products Inc., 500 Fifth Avenue, New York, New York.
[25] Copease Corporation, 425 Park Avenue, New York, New York.
[26] General Electric X-Ray Department, Milwaukee 1, Wisconsin.

TABLE V

Methods of Quantitative Thin-Layer Chromatography

Method	Compounds analyzed	Range of sample size (per spot)	Error %	Ref.
Eluting, weighing	Neutral lipids from feces and fecaliths	20–50 mg. (per plate)	semi-quant-	(225)
Eluting and U.V. spectrophotometry	Esters of p-hydroxy benzoic acid	20–70 γ	2–3	(47)
Eluting, colorimetry of hydroxamic acid-Fe complexes	Methyl esters of fatty acids and of oxygenated acids, mono-, di-, and triglycerides	0.1–1 mg.	± 5	(216)
Eluting with aqueous sulfuric acid, colorimetry after heating	Bile acids	10–60 γ	± 3	(48)
Eluting and radiometry	Labeled derivatives of acids, hydroxy and amino compounds	0.1–100 γ	± 5	(107, 112)
Charring followed by photodensitometry	Mono-, di-, and triglycerides	2–30 γ	± 5	(150)
	Glycerides and "aldehydic cores" derived from unsaturated glycerides	2–30 γ	± 5	(149)
	Lecithins and "aldehydic cores" derived from unsaturated lecithins	2–30 γ	± 5	(152)
Measuring of spot area	Tocopherols	30–100 γ	± 5	(172,173)
	Industrial lipids	30–130 γ	± 5	(74)
Mineralization, colorimetry of phosphate	Phospholipids	0.2–25 γ	± 3	(52)

chromatographing it in various polar solvents as described in the preceding paragraphs.

Semiquantitative analysis by weighing eluted material is possible only with nonpolar lipids that can easily be recovered from the adsorbents. The same limitations apply to the hydroxamic acid method; however, the colorimetric determination is much more exact than weighing of small amounts. In view of its general applicability to a wide range of compounds having various functional groups (28) the method will, in the reviewer's opinion, become a preferred procedure for the quantitative evaluation of thin-layer chromatograms. So far, the hydroxamic acid method has been applied only to the quantitative analysis of esters of common and oxygenated fatty acids derived from seed oils. Extension of the method to the analysis of mono-, di-, and triglycerides has been suggested (216). The method described for the elution and quantitative colorimetry of bile acids with sulfuric acid may be of general applicability. The isotopic derivative technique is, in the author's experience, very precise, one reason being that the lipids are separated as derivatives which are more easily quantitatively recovered than are the more polar original compounds.

Direct quantitative determination of lipids on the plates would be desirable, especially if a method could be found that would not involve destruction of the compounds measured. Most methods suggested so far require the use of destructive reagents for visualizing the lipids on the plate, or derivatization before chromatography. Photodensitometry after charring of the lipid materials as applied by Privett and Blank (149), Privett, Blank, and Lundberg (150) gives good results in a range of 5–35 γ of lipid This method has been most extensively applied by the last-mentioned authors, and has yielded valuable information. The spots are measured in a Photovolt photodensitometer Model 520A, 52-C,[27] with a stage attached for semi-automatic plotting of curves. The areas under the densitometer curves are directly proportional to the amount of sample in a range of about 5–30 μg. of carbon (Fig. 14). This applies to saturated compounds. Different classes of saturated lipids give different curves. Also, unsaturated lipids are more deeply stained than are the saturated members of the same class. Therefore, individual standard curves must be prepared for all compounds that do not give spots of the same densitometrically determined response (Fig. 14) (149). Mixtures of known composition should be run alongside the sample to be analyzed. The densitometer mentioned is easily adapted to fit square plates. Complex mixtures of natural lipids containing both saturated and unsaturated fatty acid moieties can be measured only if the whole mixture is measured after catalytic hydrogenation. Unfortunately, hydrogenation often involves not only the desired saturation of the double bonds, but changes the functional groups, and thus also the pattern of the mixture to be analyzed.

The method of measuring spot areas on photostats of chromatoplates supplements the photodensitometric method, as it can be applied to amounts of 30–130 γ of lipid per spot. In the author's laboratory this method has been found useful for the analysis of synthetic lipid mixtures with an error of ± 5% (174).

Preparation of Radioactively Labeled Lipid Derivatives

The isotopic derivative technique, i.e., the method for detecting and analyzing components of mixtures after reaction of their functional groups with a radioactively labeled reagent, has so far found little application in the lipid field, due to lack of efficient fractionation procedures. TLC allows the rapid separation of complex lipid mixtures and thus constitutes an ideal auxiliary tool for the analysis of lipids by the isotopic derivative technique.

Fatty acids may be labeled by esterifying them with radioactive diazomethane, whereas lipids containing hydroxy or amino groups are labeled by acetylation with radioactive acetic anhydride. p-Tolylsulfonyl methyl-C^{14}-nitrosamide (198), a precursor for the preparation of "hot" diazomethane, and acetic anhydride-1-C^{14} and acetic anhydride-H^3 are all commercially available. The latter two reagents are available in license-exempt packages.

Radioactive diazomethane, $C^{14}H_2N_2$, is prepared by reacting a solution of 10 mg. (0.05 millimoles) of p-tolylsulfonyl-methyl-C^{14}-nitrosamide, specific activity of 0.6 millicuries per millimole [28] in 1 ml. diethyl ether with 2 ml. of an ice-cold solution of 0.1 g. of NaOH in ethanol-water, 10/1, v/v, in a micro gas generator. Diazomethane and ether are distilled in a stream of nitrogen by heating the reaction vessel to 60–70°C. The diazomethane is collected in two test tubes in series, each containing 1–2 ml. of ether which is maintained at 0–5°C. The diazomethane solutions are combined at the end of the distillation and aliquots are added to solutions of 2–20 mg. fatty acids (0.01–0.1 millimoles) in diethyl ether–methanol 9/1, v/v (166, 198). Excess diazomethane is avoided in order to conserve this expensive reagent and also to prevent the formation of polymethylenes that may interfere

[27] Photovolt Corporation, 95 Madison Avenue, New York 16, New York.

[28] New England Nuclear Corporation, 575 Albany Street, Boston 18, Massachusetts.

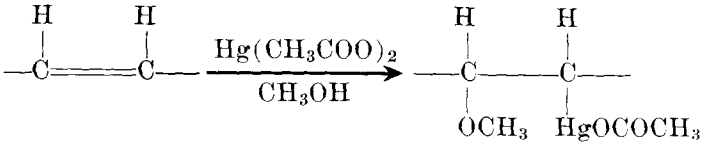
in subsequent analyses. The diazomethane can also be distilled directly into a solution of the fatty acids to be esterified in diethyl ether–methanol, 9/1, v/v (166).

Lipids (10–20 mg.) containing hydroxy or amino groups may be labeled by reaction with a 1/10 solution of acetic-1-C^{14} anhydride, specific activity of 0.6 millicuries per millimole [29] in pyridine. The reaction is conducted in a 5 x 150 mm. sealed tube at 100°C. for 30–60 min., using about 20% excess of reagent. After cooling, the tubes are opened, the reaction mixture is diluted with 10 ml. *N*-sulfuric acid, the acetylated lipids are extracted with diethyl ether, washed with water, and dried over sodium sulfate. Monoglycerides and diglycerides undergo acetolysis to a small extent; and therefore, if quantitation of lipids containing ester-bonds is desired, this method must be applied with caution (107).

Reaction with radioactive ketene (2) may yield cleaner acetylation products.

Preparation of Mercuric Acetate Addition Compounds

Unsaturated lipids react with mercuric acetate in methanol, at room temperature, to give acetoxymercuri-methoxy compounds in quantitative yields:



cis-Compounds react ten times faster than their *trans* isomers (70). The original lipid may be regenerated by cleaving the addition compound with hydrochloric acid.

Chromatography of acetoxymercuri-methoxy compounds of unsaturated lipids on silicic acid effects their fractionation according to degree of unsaturation.

The acetoxymercuri-methoxy compounds of lipids are best obtained by the method published by Jantzen and Andreas (70) for the preparation of adducts of methyl esters of unsaturated fatty acids. This procedure is described here:

The reagent is a solution of 14 g. mercuric acetate in 250 ml. methanol, 2.5 ml. water, and 1 ml. glacial acetic acid. About 25 to 50 ml. of this solution is added to 1 g. of esters and allowed to react in a stoppered flask in the dark at room temperature. After 24 hr., the methanol is evaporated at <30°C. *in vacuo* or by a stream of nitrogen, and the dry residue is taken up with 50 ml. chloroform. This chloroform solution is washed with five 25-ml. portions of water to remove excess mercuric acetate, and then dried with sodium sulfate. Saturated lipids do not react with mercuric acetate, unless they contain a reactive functional group.

The unsaturated lipids are recovered after chromatography by shaking the silicic acid containing the adducts in a test tube, with 10 ml. methanol and 0.5 ml. conc. HCl. After most of the adsorbent has settled, the supernatant is decanted and filtered. The residual silicic acid is treated once more with methanol-hydrochloric acid. The combined filtrates of the two extractions are diluted with 25 ml. water and extracted consecutively, once with 25 ml., and four times with 10 ml. of diethyl ether. The combined ether extracts are washed with water, dried over sodium sulfate, and evaporated.

[29] Radioactive Acetic Anhydride, either labeled with C^{14} or H^3, is available from several companies. The author has used preparations of the Picker X-Ray Corporation, 25 South Broadway, White Plains, New York.

Applications

In the lipid field, the following types of separations have been performed by TLC:

Fractionations according to classes of compounds (Fig. 6). Long-chain hydrocarbons, alcohols, aldehydes, acids, monoglycerides, diglycerides, triglycerides, and other lipids are separated as classes according to the type and number of functional groups, irrespective of chain lengths and degree of unsaturation, by adsorption TLC on silicic acid. Subfractionations within classes are perceptible but generally do not interfere with class separations.

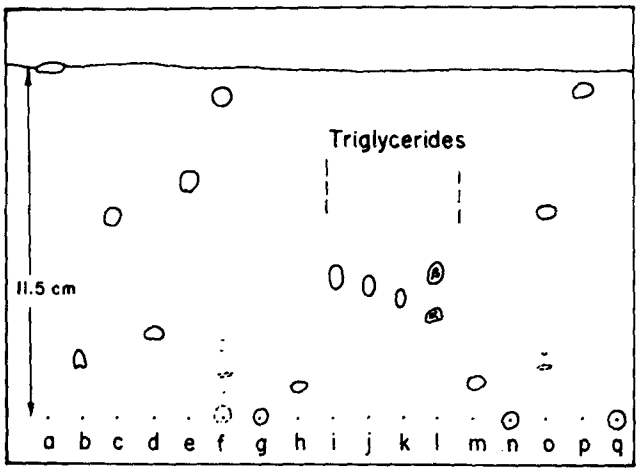


FIG. 6. Thin-layer adsorption chromatography of lipid classes on Silica Gel (101). Solvent: Petroleum hydrocarbon-diethyl ether-acetic acid, 90/10/1, v/v/v. Development time: 40 min. Indicator: Dichlorofluorescein. Amounts: 20 γ, each. a) octadecene-9, b) oleylalcohol, c) oleylaldehyde, d) oleic acid, e) methyl oleate, f) cholesteryl oleate, g) monoolein, h) diolein, i) triolein, j) trilinolein, k) trilinolenin, l) tricaproin (α) and tristearin (β), m) cholesterol, n) selachyl alcohol, o) selachyl diolein, p) oleyl oleate, q) dioleoyl lecithin.

Fractionations within homologous series (Fig. 7 and 8). a) Compounds of low chain lengths (C_1–C_6) are separated within classes by TLC of their derivatives. Alcohols, for example, are separated as 3,5-dinitrobenzoates on silicic acid. With the same adsorbent, aldehydes and also ketones are fractionated as 2,4-dinitrophenyl hydrazones, and acids are separated as anilides.

b) Long-chain compounds (C_{12}–C_{20} lipids) are fractionated within classes by reversed-phase partition TLC on, e.g., siliconized silicic acid. Higher fatty acids, for example, are separated according to chain length and unsaturation.

Overlapping critical partners, such as palmitic and oleic acids, are well separated by chromatography at low temperatures, by chromatography with oxidizing solvents, or by the following technique:

Fractionation according to degree of unsaturation (Fig. 9). Saturated and various unsaturated members of a homologous-vinylogous series are separated according to their degree of unsaturation by TLC of the mercuric acetate addition compounds formed with unsaturated lipids. As an example, mixtures of methyl esters of naturally occurring fatty acids are rapidly and quantitatively separated by TLC, in the

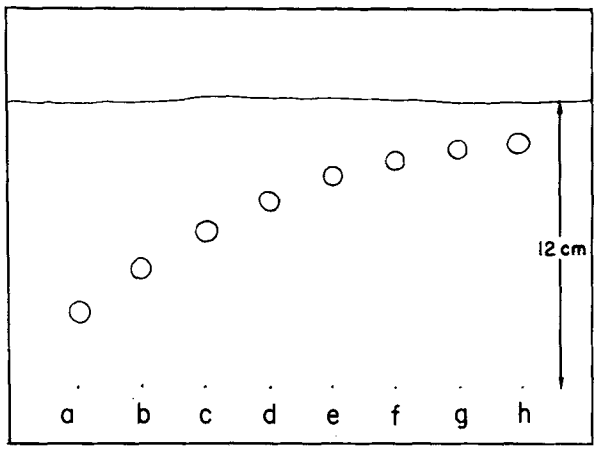


FIG. 7. Thin-layer adsorption chromatography of derivatives of short-chain compounds of a homologous series. Solvent: Petroleum hydrocarbon-diethyl ether, 70/30, v/v. Development time: 40 min. Indicator: 1% of "Fluorescent Chemical No. 601"[22] in the silica gel layer. Observed in U.V. light. Amounts: 5 γ, each. 2,4-dinitrophenyl derivatives of a) methyl amine, b) ethyl amine, c) propyl amine, d) butyl amine, e) amyl amine, f) hexyl amine, g) heptyl amine, h) octyl amine.

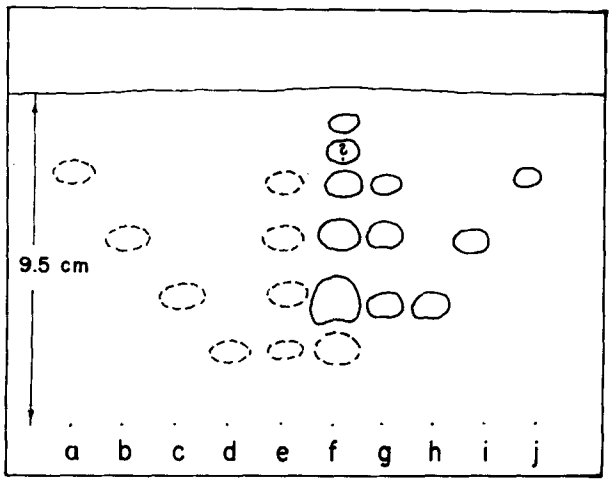


FIG. 8. Reversed-phase partition chromatography on a siliconized chromatoplate (101). Solvent: Acetonitrile acetic acid-water, 70/10/25, v/v/v. Development time: 40 min. Indicators: Iodine (solid lines), followed by α-cyclodextrin-iodine (dotted lines). Amounts: 20 γ of individual compounds. a) methyl laurate, b) methyl myristate, c) methyl palmitate, d) methyl stearate, e) mixture of saturated methyl esters, f) C_{18}-Fraction derived from menhaden oil, g) mixture of unsaturated C_{18} methyl esters, h) methyl oleate, i) methyl linoleate, j) methyl linolenate.

form of their acetoxymercuri-methoxy derivatives, into saturated esters, monoenoates, dienoates, and trienoates. The original esters can be recovered after separation of their addition compounds.

Fractionation of positional isomers and fractionation of cis-trans *isomers is also possible in certain cases (128, 147) (Fig. 11).*

The fractions obtained by one of these methods are amenable to further resolution by either or several of the other techniques, or by complementary methods. Combinations of adsorption-TLC with reversed-phase PC and GLC have proven to be especially useful. Lipids which cannot be resolved by adsorption chromatography (TLC) may well be separated by partition chromatography (PC, GLC).

Fats, Oils and Waxes

Procedures for the fractionation of neutral lipids—naturally occurring crude vegetable fats, marine oils, and insect waxes, and their hydrolysis products, such as fatty acids, and also alcohols, glyceryl ethers and other nonsaponifiables have been described. Adsorption-TLC effects sharp separations of chemically very similar classes of lipids, such as long-chain alkyl esters, steryl esters, and polyenol esters of fatty acids; even triglycerides and glyceryl ether diesters may be resolved.

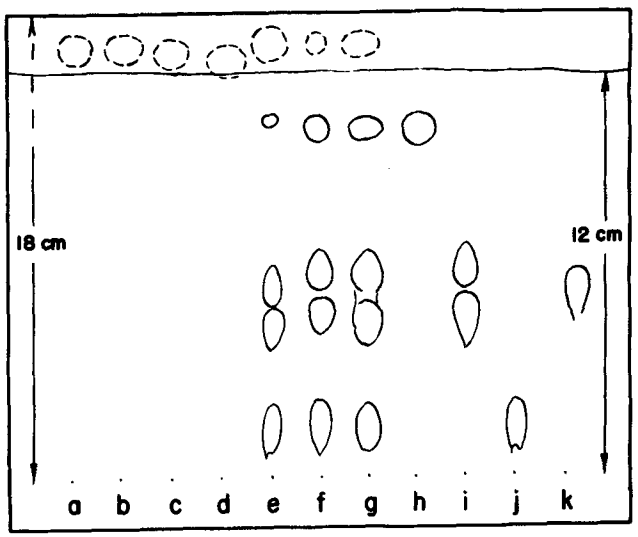


FIG. 9. Thin-layer chromatography of mercuric acetate adducts on Silica Gel (See 110). First solvent: Petroleum hydrocarbon-diethyl ether, 4/1, v/v. Second solvent: n-Propanol-acetic acid, 100/1, v/v. Development times: First Solvent, 1.5–2 hours, Second Solvent, 3–4 hours. Indicators: s-diphenyl carbazone (solid lines), followed by iodine (dotted lines). Amounts: 20 γ of esters, 50–100 γ of mercuric acetate adducts. a) methyl stearate, b) methyl oleate, c) methyl linoleate, d) methyl linolenate; e–j) Mercuric acetate adducts of e) C_{16} fraction from *Chlorella*, f) C_{18} fraction from *Chlorella*, g) total methyl esters from *Chlorella Pyrenoidosa*, h) methyl oleate, i) methyl linoleate, j) methyl linolenate, k) mercuric acetate.

Among the vegetable lipids analyzed are jojoba oil, castor oil, olive oil, oiticica oil (108, 112), and wheat (140). The following animal lipids have been investigated: lanolin, liver oils of soupfin shark, basking shark, and ratfish, also fur seal blubber oil (108) and dogfish liver oil (102) (Fig. 10).

The last-mentioned publication refers to work of Malins on the analysis of the constituent fatty acids of each of the triglyceride and the glyceryl ether diester fractions of dogfish liver oil. This commendable work would not have been possible by any other means than TLC and GLC.

Weicker (222), Huhnstock and Weicker (65), and van Dam (32) applied TLC to the analysis of human serum lipids. Williams, Sharma, Morris, and Holman (225) analyzed human feces and fecaliths by TLC and GLC. The same two techniques have also been applied, consecutively, to the analysis of human tissue lipid extracts, by Mangold and Tuna (109). The total lipids of human serum and of the following tissues were analyzed: bone marrow, liver, kidney, perinephric fat, spleen, and atheromatous plaques of the aorta, TLC was used to obtain lipid classes, the fatty acid

patterns of which were then determined by GLC of the methyl esters. Lipid classes not previously found in man were detected in several human tissues. Some of these lipids, such as methyl esters of fatty acids, are most likely artefacts produced during extraction. Others, like glyceryl ether diesters, must be regarded as genuine constituents of human tissues (211). Stein and Stein (195) used TLC in studying the selective *in vivo* labeling of epididymal fat of the rat with radioactively labeled fatty acids. Dhopeshwarkar and Mead (38), and Fulco and Mead (46) used TLC extensively in nutritional and metabolic investigations.

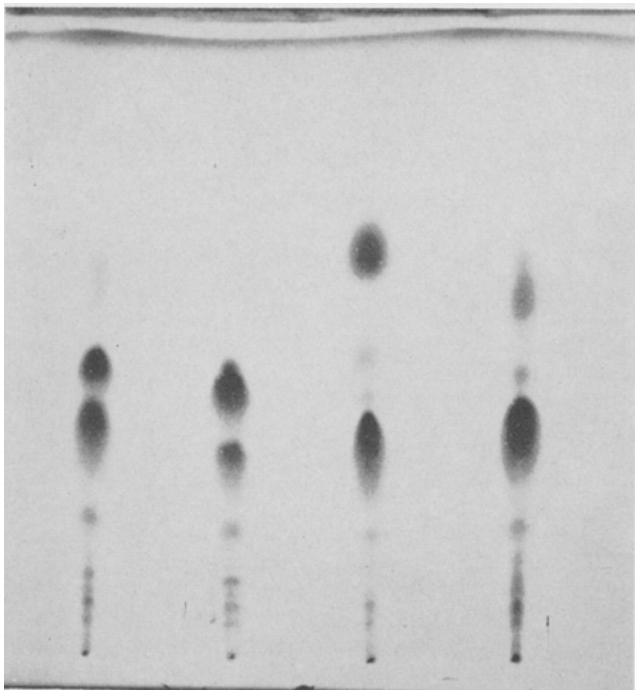


FIG. 10. Fractionation of shark liver oils on silica gel. Solvent: Petroleum hydrocarbon-diethyl ether-acetic acid, 90/10/1, v/v/v. Indicator: Iodine, reproduced by photographing. Amounts: 300 γ, each. Liver oils of a) Dogfish, b) Ratfish, c) Basking shark, d) Soupfin shark. The chromatoplate was developed in saturated atmosphere.

Naturally occurring oxygenated fatty acid were studied by Morris, Holman, and Fontell (126, 128), and by Morris, Hayes, and Holman (129). These workers found two isomeric acids, namely 9-hydroxy-*trans*-10-*cis*-12- and 13-hydroxy-*cis*-9-*trans*-11-octadecadienoic acids, and also at least three distinct epoxy acids in various seed oils (Fig. 11). Vioque, Morris, and Holman (215) found *trans*-9,10-epoxystearic acid in orujo oil. Chalvardjian, Morris, and Holman (27) applied TLC in nutritional studies with epoxy acids. TLC of oxygenated fatty acids was recently also reported by Kaufmann and Makus (79) and by Applewhite, Diamond, and Goldblatt (4).

A method, based on TLC, for the quantitative determination of mono-, di-, and triglycerides in mixtures has been reported by Privett, Blank, and Lundberg (150) (Fig. 12). These workers could quantitatively estimate as little as 0.1% of tripalmitin in monopalmitin.

Procedures for the quantitative analysis of α- and β-monoglycerides after splitting of the former with periodate, and for the analysis of 1,2- and 1,3-diglycerides were also presented. These new methods yielded exact results with synthetic mixtures and were then further applied to the analysis of monoglyceride preparations from safflower oil, menhaden oil, and lard. Periodate titration and molecular distillation served for checking the accuracy of these analyses.

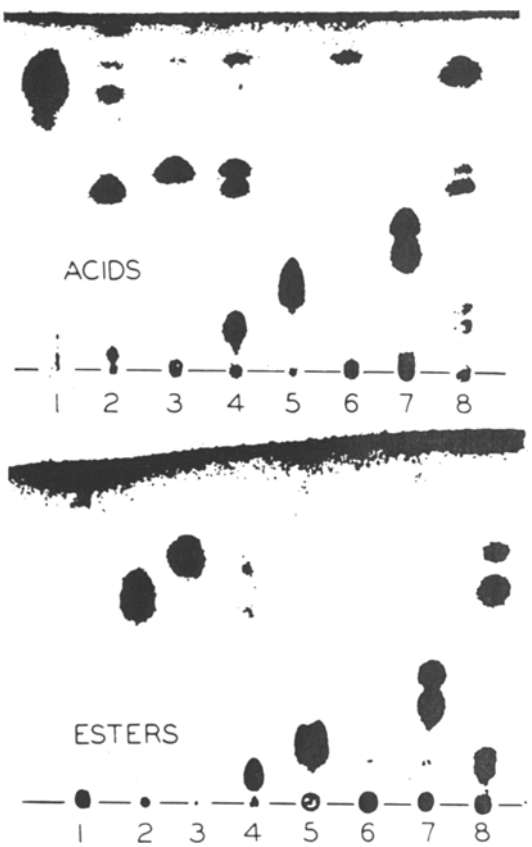


FIG. 11. Thin-layer chromatography of oxygenated fatty acids and their methyl esters (128). Solvent: Petroleum hydrocarbon-diethyl ether, 9/1, v/v (for esters). Petroleum hydrocarbon-diethyl ether-acetic acid, 90/10/1, v/v/v (for acids). Development time: 40 min. Indicator: Charring with aqueous sulfuric acid, 1/1, v/v. Reproduced by photocopying. Amounts: About 50 γ, each. 1) palmitoleic and oleic, 2) *cis*-9:10 epoxystearic, 3) *cis*-12:13-epoxyoleic, 4) *cis-cis*-9:10, 12:13-diepoxystearic plus two monoepoxy impurities, 5) 12-hydroxyoleic, 6) *threo*-12, 13-dihydroxyoleic, 7, *threo*-12,13-chlorohydroxyoleic and *threo*-13,12-chlorohydroxyoleic, 8) *Artemisia absinthium* mixed acids and mixed esters.

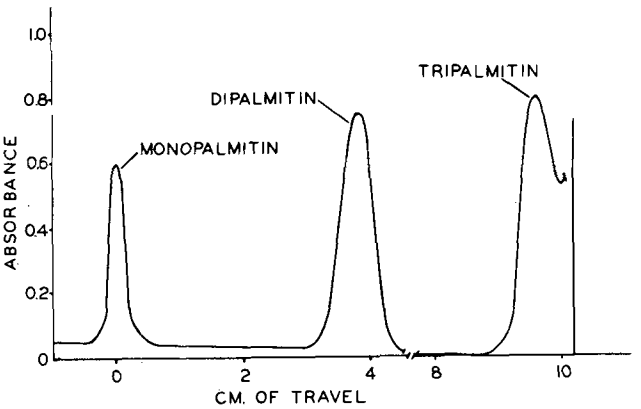
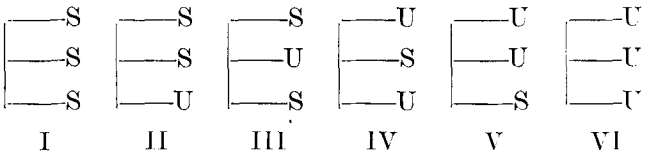


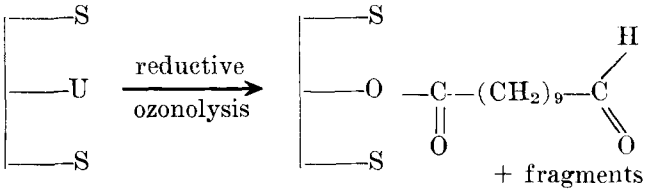
FIG. 12. Densitometric analysis of a mixture of mono-, di-, and tripalmitin (150). Solvent: Petroleum hydrocarbon-diethyl ether, 7/3, v/v. Development time: 40 min. Indicator: Charring with aqueous sulfuric acid, 1/1, v/v.

Privett and Blank (149) have published an elegant method for the determination of mono-, di-, and triglycerides varying in fatty acid constituents in regard to unsaturation. It is based upon reductive ozonolysis of these types of compounds, followed by separation and quantification of the glyceryl residues by TLC. This ozonization-reduction-thin-layer chromatographic method (149) of analysis permits analyzing the four possible types of monoglycerides, six of the seven diglyceride types, and four of the six triglyceride types. Since this method has such wide potential applications, it is explained here in more detail, with the example of the structural analysis of triglycerides.

There are six types of triglycerides, *viz.*,



S and U in these models denote saturated (S) and unsaturated (U) fatty acid moieties. Reductive ozonolysis will leave triglycerides of type I untouched, whereas all other triglyceride types will yield glyceryl residues—aldehydic cores—of the kind demonstrated with a triglyceride of type III:



The saturated fatty acid constituents of a type III triglyceride are not affected by ozonolysis, whereas the long chain of the unsaturated acid is ruptured to yield a structure as depicted above, i.e., the unsaturated-disaturated triglyceride has become a compound of a polarity which is widely different from that of the unchanged fully saturated triglyceride (type I). Consequently, the aldehydic cores resulting from triglycerides of type III and from the other triglycerides containing one or more unsaturated fatty acid moieties, are easily resolved, as classes, by TLC on Silica Gel G. An example of such separations is presented in Figure 13. Saturated and unsaturated triglycerides give very different standard curves, but the aldehydic cores resulting from unsaturated triglycerides by reductive ozonolysis yield identical curves as saturated triglycerides, if correction is made for the different percentages of fissioned carbon lost in the various types of compounds. The quantitative evaluation of chromatograms is demonstrated in Figure 14.

Privett (151) utilized TLC in studies on the autoxidation of unsaturated fatty acids and esters. Figure 15 serves as an example to illustrate the kind of separations possible.

TLC has been used to check the course of synthesizing procedures and the purities of final products (49, 50, 94, 100, 217). In checking by means of adsorption-TLC it is not necessary to start a sequence of reactions with an individual compound, say oleic acid, as is necessary with reversed-phase paper partition chromatography (106). The use of adsorption-TLC permits working with a lipid class, e.g., a mixture of fatty acids derived from fish oils (49, 50).

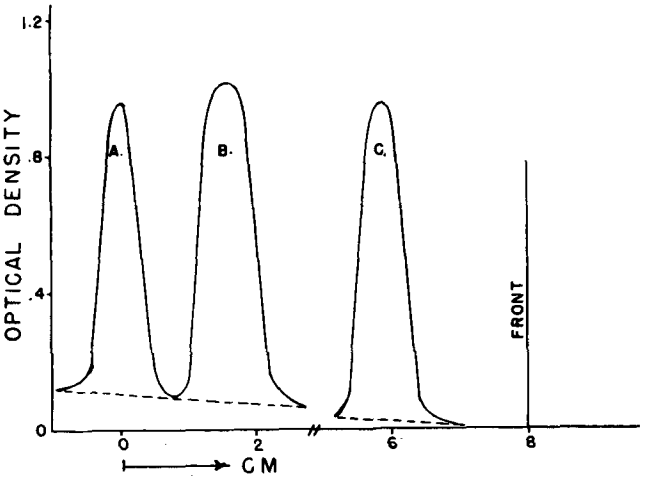


FIG. 13. Densitometric analysis of the aldehyde cores derived from A. Triolein, B. Palmitodiolein, C. Oleodipalmitin (149). Solvent: Petroleum hydrocarbon-diethyl ether, 65/35, v/v. Development time: 40 min. Indicator: Charring with aqueous sulfuric acid, 1/1, v/v.

Methods for analyzing polar lipids of industrial importance, such as amines, amides, and nitrils—also alkyl sulfates and sulfonates, alkyl phosphates and phosphonates—have recently been worked out (111).

Several investigators have applied TLC to check the efficiency of other separation techniques (59, 129, 211). Morris, Holman, and Fontell (127) have found, by the use of TLC, that the methyl esters of some long-chain fatty acids are altered during GLC. Conjugated trienoates undergo *cis-trans* isomerization. The esters of vicinally unsaturated hydroxy acids are dehydrated and unsaturated hydroperoxides are similarly altered to more highly unsaturated derivatives. Mangold and Kammereck (110) fractionated mixtures of the methyl esters of saturated fatty acids and the acetoxymercuri-methoxy compound of unsaturated ones by TLC into groups, according to the degree of unsaturation in the original esters (Fig. 9). Each group of esters was recovered and after cleavage of the addition compounds, it was resolved into its components by GLC, according to chain length. Pairs of esters which are not resolved by GLC are well separated by TLC of their adducts and *vice versa*. The successive application of TLC and GLC results in more reliable identification of the various compounds

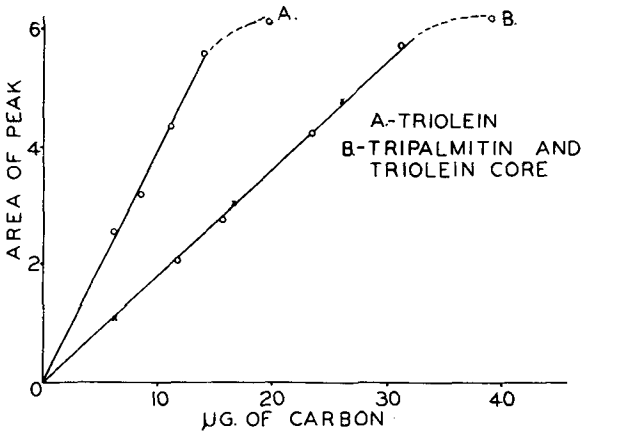


FIG. 14. Standard curves, thin-layer chromatographic analysis of triolein, tripalmitin, and the aldehydic core derived from triolein (corrected for fissioned carbon) (149).

recorded in GLC. In addition, this combination of methods yields more trustworthy quantitative analyses than does GLC alone.

Phospholipids, Sulfolipids, and Glycolipids

Application of TLC in this domain have been especially fruitful (30, 52, 64, 71, 72, 87, 89, 111, 164, 219, 220, 223). The solvents applied are usually the same as those used for resolving phospholipids by chromatography on silicic acid-impregnated paper (113) or on columns of silicic acid (95, 113). The capacity of the chromatoplates is much less with such polar developing solvents than with mixtures of petroleum hydrocarbon and diethyl ether.

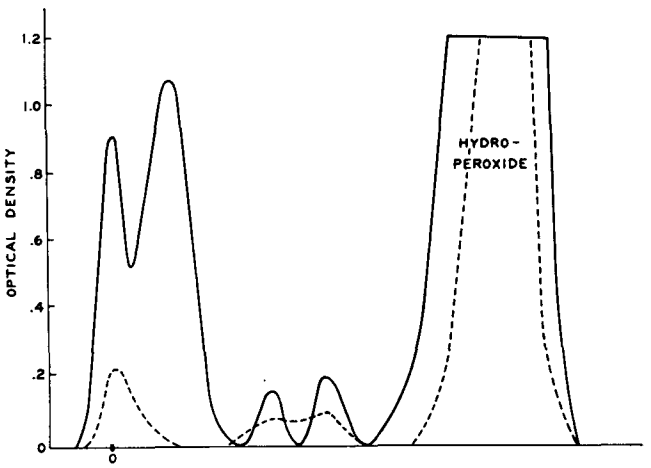


FIG. 15. Densitometric analysis of a chromatogram of autoxidized methyl linoleate (151). Solvent: Petroleum hydrocarbon-diethyl ether, 4/1, v/v. Development time: 40 min. Indicator: Charring with aqueous sulfuric acid 1/1, v/v. Unchanged methyl linoleate migrates to the solvent front.

Figure 16 shows a chromatogram of various phospholipids, as presented by Wagner (219) and Wagner, Hörhammer, and Wolff (220).

Privett and Blank (152) have extended their ozonization-reduction-thin-layer chromatographic method by applying it to lecithins. The four types of lecithins can be distinguished and quantitatively determined. In this case the aldehydic cores derived by reductive ozonolysis are separated by reversed-phase partition TLC on siliconized Silica Gel G plates (101).

Figure 17 shows photodensitometer curves obtained from chromatograms of aldehydic cores derived from various lecithin preparations as obtained by Privett and Blank (152).

TLC has become an important tool in research concerned with the structure of gangliosides (87, 89, 219, 220). With this method Kuhn, Wiegandt, and Egge (89) isolated four crystalline gangliosides from beef brain, each containing one mole of galactosamine and three moles of glucose plus galactose.

Steroids and Bile Acids, Triterpenoic Acids

Linford (97) reported that sterols are chemically altered when they are chromatographed on columns of dry silicic acid, and he recommended that sterols should be separated by partition chromatography on silicic acid containing between 35 and 40% of water. Other workers (e.g., 15, 204) had previously observed that sterols are altered when chromatographed on alumina. Reference is made to a comprehensive discussion of the problem by Neher (138).

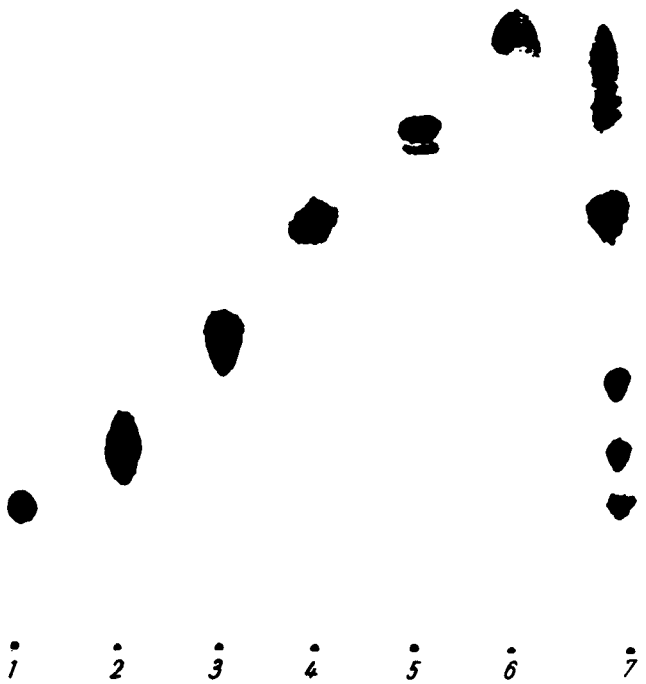


FIG. 16. Separation of phospholipids on Silica Gel. (219, 220). Solvent: chloroform-methanol-water, 65/25/4, v/v/v. Development time: 2 hours. Indicators: Rhodamin B and Dragendorff reagent. Amounts: 50–100 γ, each. 1) lysolecithin, 2) sphingomyelin, 3) lecithin, 4) cephalin, 5) cerebroside, 6) cardiolipid, 7) mixture.

Several recent publications described the use of TLC for resolving steroids by adsorption or partition chromatography on silicic acid; alumina was used as an adsorbent also (1, 7, 25, 31, 32, 69, 100, 139, 201, 202, 221).

Especially remarkable is the resolution of fifteen different cholesteryl esters by two-dimensional reversed-phase partition TLC on paraffin-impregnated Silica Gel G plates, as reported by Kaufmann, Makus, and Deicke (80).

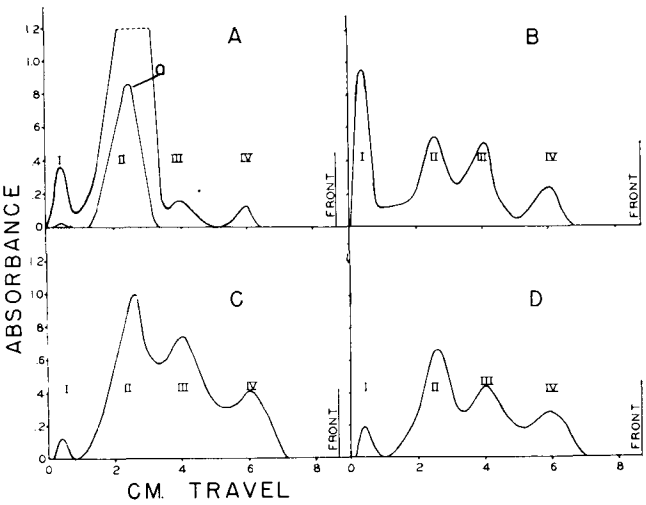


FIG. 17. Densitometric analysis of the aldehydic cores derived from A) egg lecithins, B) beef spinal cord lecithins, C) soybean lecithins, D) wheat lecithins (152). I. Disaturated lecithins, II. aldehydic cores derived from α-saturated-β-unsaturated lecithins, III. aldehydic cores derived from α-unsaturated-β-saturated lecithins, IV. aldehydic cores derived from diunsaturated lecithins. Solvent: acetic acid-water, 85/15, v/v on siliconized Silica Gel G.

Waldi and Munter (221) developed an early pregnancy test which is based upon a semiquantitative determination of pregnandiol in the urine.

Gänshirt, Koss, and Morianz (48) worked out a simple and rapid method for the quantitative determination of bile acids by adsorption TLC. The bile acids were eluted from the plates with aqueous sulfuric acid (65%) and they were quantitatively determined by colorimetry of the red solutions that formed upon heating. The above authors encountered a standard error of only ± 3% in analyses of mixtures of cholic, deoxycholic, and glycodeoxycholic acids, and of glycocholic, taurocholic, and taurodeoxycholic acids (48). TLC of bile acids was also reported by Hofmann (60).

TLC has been extensively applied in work with triterpenoic acids by Tschesche and his co-workers (207, 208, 209, 210). Vioque and Morris (214) reported the isolation of two triterpenoic acids from orujo oil. Stahl (186) demonstrated the use of TLC for the fractionation of tars and naturally occurring balsams and rosins.

Fat-Soluble Vitamins

Winterstein and Hegedüs (227, 228) and Winterstein, Studer, and Rüegg (229) have applied both adsorption and reversed-phase partition TLC to the analysis of vitamin A and the corresponding aldehyde, *viz.*, retinene and other polyenals C_{20}–C_{40}. Winterstein and Hegedüs (227, 228) prepared deeply colored rhodanine derivatives of these aldehydes on the plates, and with this very sensitive indicator technique they were able to detect as little as 0.03 γ of an aldehyde on a plate. The ubiquitary occurrence of retinene in a plant kingdom and its presence in liver was demonstrated. A thorough review on this work has already appeared (230).

Mangold and Malins (108) demonstrated vitamin A esters in various fish oils and free vitamin A in the nonsaponifiable fractions therefrom. Both vitamin A and its ester can readily be recognized by the blue color they yield by heating with sulfuric acid.

Janecke and Maas-Goebels (69) investigated the behavior of vitamins D_2 and D_3 under the conditions usually applied for their assay and they concluded that the D-vitamins are less stable than had generally been believed.

Methods for the quantitative analysis of the E-vitamins were published by Seher (171, 172, 173). The β and γ tocopherols could not be resolved by TLC, but both were well separated from α and from γ tocopherols.

Stoffel and Martius (196) and Billeter and Martius (13) have used TLC in studying the metabolism of K-vitamins. Martius (114) has summarized the results of these investigations in a recent review.

Food Coloring Agents

TLC was applied for analyzing vitamin A, carotenes, and other food coloring agents by Lagoni and Wortmann (91, 92) and also by Mottier (131, 132, 134). Isler, Rüegg, and Schudel (66) have presented a most interesting survey on the use of synthetic carotenoids as food-coloring agents, and in this review they also discussed in detail applications of TLC and other methods in analyzing such substances.

Stanley and Vannier (192) used chromatostrips for detecting biphenyl.

Antioxidants, Prooxidants

Methods for the analysis of synthetic and natural antioxidants by TLC have been described by Seher (171, 172, 173). Complex mixtures of antioxidants in commercial preparations and in oils have been analyzed by two-dimensional TLC (17), mixtures of tocopherols have been quantitatively determined (172, 173). Naturally occurring quinones, which are of significance in this connection, may also be detected by TLC (6). Procedures for quantitative determination of p-hydroxy benzoates, widely used preservatives, have been described by Gänshirt and Morianz (47).

Organic peroxides have been separated by Maruyama, Onoe, and Goto (115) and by Stahl (183). Reference is made again to the work of Privett (151) on the use of TLC for the analysis of autoxidation products (Fig. 15), and to the publications by Seiler and Seiler (174, 175), Seiler and Rothweiler (176) and Seiler and Kaffenberger (177) on the separation by TLC of inorganic cations and anions, many of which are prooxidants.

Minor Constituents of Fats

Because of its sensitivity, TLC is ideal for detecting minor lipid constituents, such as naturally occurring steroids, diterpenoids, triterpenoids, including saponins, aromatic rosins, and fat-soluble vitamins, or additives such as food coloring agents, antioxidants, and insecticides (8, 187).

Methods for analyzing non-lipid material in fats are scarce. The author believes that TLC can be effective in identifying carbohydrates (191, 235), amino alcohols and amino acids (16, 133), as well as short-chain alcohols and aldehydes (37, 143), acids, amines (203), mercaptans and other odoriferous compounds, plasticizers (144), and trace amounts of metals (174, 175, 176).

Thin-Layer Chromatography in Lipid Radio-Chemistry

Methods for analysis of trace amounts of lipids by the use of the isotopic derivative technique in conjunction with TLC and PC have been described by the author (107). The lipid substances were labeled by reacting their functional groups with C^{14}-labeled diazomethane—H^3-labeled diazomethane cannot be used (10, 96)—or with C^{14}- or H^3-labeled acetic anhydride. "Hot" methyl esters, acetylated long-chain alcohols, monoglycerides, diglycerides, glyceryl ethers, amines, and hydroxy ethyl amides of higher fatty acids were fractionated from each other as classes by adsorption-TLC. Their ratios were determined by radiometry. Consecutively, each of the classes of labeled lipid derivatives was further resolved by reversed-phase paper partition chromatography. The ratios of the components of each class were then determined by scanning their respective activities with a radio chromatograph or by evaluating autoradiograms in a photodensitometer. Mangold, Kammereck, and Malins (112) have recently presented an analysis of the fatty acids of castor oil, using these methods.

The same authors have analyzed commercial preparations of radioactively labeled lipids and found that most of them are very impure (112, see also, e.g., 93). Because their indiscriminate use must lead to erroneous results, TLC should be used for purifying such "hot" lipids prior to their application in chemical and metabolic studies (112). Figure 18 shows an

autotradiograph of various commercial fatty acids labeled in the carboxyl group with C^{14}, and Figure 19 represents an autoradiograph of a chromatogram of cholesterol-4-C^{14}. The radioactive sterol preparation was applied in a row across the plate to permit recovering the pure cholesterol. The isolation of C^{14}-labeled phytol from the nonsaponifiable fraction of the alga *Chlorella pyrenoidosa*, grown in an atmosphere of radioactive carbon dioxide (104), was also reported (112).

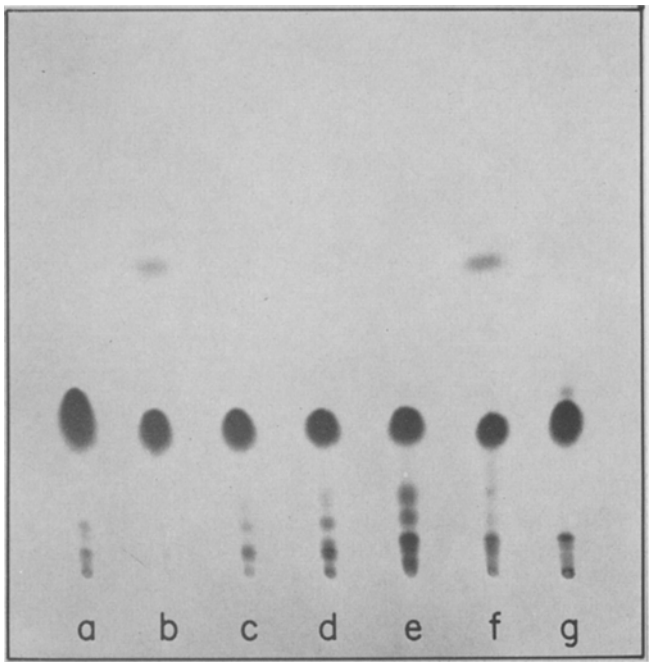


FIG. 18. Autoradiograph of a chromatoplate of commercial fatty acids -1-C^{14}. Solvent: petroleum hydrocarbon-diethyl ether-acetic acid, 90/10/1, v/v/v. Development time: 40 min. a) lauric acid, b) myristic acid, c) palmitic acid, d) stearic acid, e) oleic acid, f) linoleic acid, g, linolenic acid.

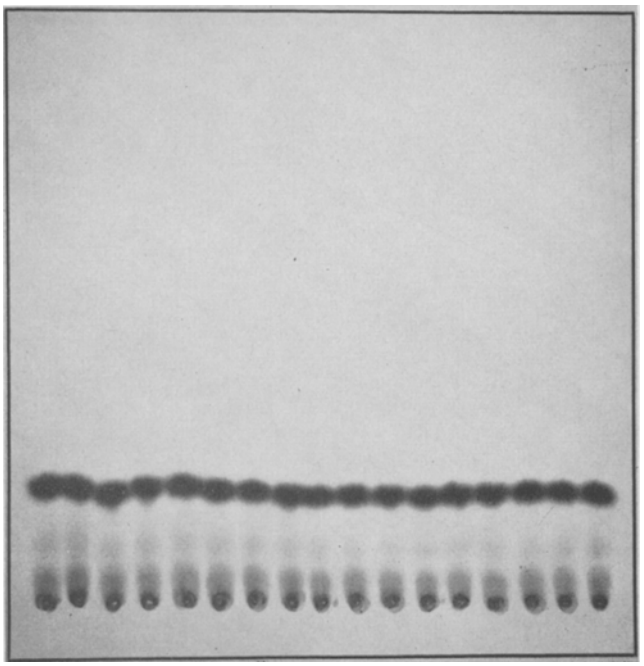


FIG. 19. Autoradiograph of a chromatoplate of cholesterol-4-C^{14}. Solvent: petroleum hydrocarbon-diethyl ether -acetic acid, 70/30/2, v/v/v. Development time: 40 min.

Summary and Discussion

The technique of thin-layer chromatography and its numerous uses in the lipid field have been summarized. The most significant advantages of TLC over existing methods of lipid analysis are, in the reviewer's opinion, the following:

Simplicity: The equipment required can be handled by inexperienced workers.

Speed: Complex lipid mixtures can be separated in 30 min. Less complicated samples can often be resolved in 5 min. All other chromatographic methods require hours or days to achieve similar separations.

Efficiency: Thin-layer chromatography yields separations which are much sharper than the fractionations obtained by column or paper chromatography.

Sensitivity: In many instances, the sensitivity of the detection methods used in paper chromatography are increased one hundred fold when applied on thin-layer chromatograms, because in the latter the individual spots are more discrete and not as diffuse as on paper chromatograms.

Another advantage of thin-layer chromatography is that several indicators may be applied consecutively, on one plate, also corrosive spray reagents, such as chromic-sulfuric acid may be used to char all organic material on the chromatoplate. In other chromatographic techniques frequently the indicator employed for the detection of spots is blind in regard to one component.

Capacity: Up to 50 mg. per plate can be separated and isolated by thin-layer chromatography, whereas only microgram amounts are available, in a reasonable time, through paper chromatography.

Thin-layer chromatography is fully exploited only when it is used in conjunction with other methods. Especially rewarding is the combined use of thin-layer chromatography with the following methods:

Gas-Liquid Chromatography
Paper Chromatography
Column Chromatography
Radioactive Tracers
Derivatization, i.e., the application of chemical reactions prior to chromatography.

Although thin-layer chromatography has been widely applied to lipid analysis and preparation, its usefulness is certainly not yet exhausted.

Refinements of TLC are being designed and new techniques are being developed. Honegger (63) has described ionophoresis on chromatoplates which he combined with TLC in two-dimensional technique for the resolution of amines and amino acids. He found this combination of methods in some respects superior to the separation of amines and amino acids on paper.

Only relatively small amounts of material can be separated on a plate by reversed-phase partition TLC. It may be useful to arrange several impregnated chromatoplates in a block, in a manner similar to that for large-scale paper chromatography recently described by Hall (53).

The isotopic derivative technique involves fractionation of the labeled derivatives on a microscale. TLC is especially applicable to the resolution of such complex lipid mixtures. TLC should be applied in combination with existing methods of labeling sterols (5, 11, 61) and other hydroxy and amino compounds (107, 112) with acetic anhydride-1-C^{14} (or -2-H^3), or

with 3-chloro-anisoyl chloride-3-Cl^{36} (179), or with 4-iodobenzoylchloride-I^{131} (197). Several publications describe procedures for labeling acids and their analysis as methyl-C^{14} esters after esterification with radioactive diazomethane-C^{14} (10, 105, 112, 167, 178, 198), or 4-chloroaniline-Cl^{36} (180). Unsaturated fatty acids were labeled by addition of radio-iodine I^{131} (22, 76, 77). The speed of TLC will be appreciated in work with short-lived isotopes, especially if apparatus can be constructed which will perform automatic advancement of the chromatoplate and graphic or numerical recording of the radioactivity, as in the quantitative evaluation of paper strips and two-dimensional paper chromatograms (148).

Autoradiography of paper chromatograms (43) has in recent years become a prominent method in studies utilizing radioactive tracers (160). Because of its high capacity, adsorption-TLC will certainly also find wide application in isotope studies.

Strickland and Benson (e.g., 200) have been very successful in using neutron activation of phospholipids and sulfolipids on paper chromatograms. It would be desirable to develop techniques for neutron activation TLC analysis. As both calcium and sulfur atoms are activated, calcinated calcium sulfate cannot be applied, and starch will have to be used as a binder. For similar reasons, plastic plates will have to be used instead of glass.

Lipids are well protected against autoxidation if they are chromatographed on silicone-impregnated paper (165). Mahadevan (99) has investigated to what extent unsaturated lipids autoxidize during adsorption TLC, and even with the most sensitive methods he could not find any sign of alteration of unsaturated lipids during chromatography. He demonstrated, though, that TLC is well suited for rapidly purifying oxidized lipids.

This review is concluded with a hint as to the potentials of TLC as a teaching method. The chromatoplate shown in Figure 10 was prepared by a high school student on the first day of his adventures with TLC. It is obvious that this method will find wide application in the training of students.

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