Thin-layer chromatography is an analytical method applying techniques of paper chromatography to a thin layer of adsorbents normally used in column chromatography. This is a relatively new method of adsorption and partition chromatography on a micro scale. Thin-layer chromatography is an excellent supplement to the previously known methods of column, paper, ion-exchange and gas liquid chromatography.

Historically, the first attempts to use a thin adsorbent layer for chromatographic separations were described by Izmailov and Schraiber (8) over twenty years ago. These workers separated mixtures of organic compounds on layers of adsorbent powder, examining the rings produced under ultraviolet light. After its introduction, very little work was done with thin-layer chromatography until the period of 1949 to 1951. At this time several workers (Meinheiser and Hall (14); Kirchner, et al. (9) ) described the use of binding agents to fix the adsorbent rigidly to the glass plate. Actually the method remained in obscurity until 1956 when Stahl described equipment and procedures for the preparation of chromatoplates, and demonstrated the potential usefulness of thin-layer chromatography in the fractionation of a wide variety of compounds. Since equipment has become commercially available, thin-layer chromatography has suddenly gained wide recognition. Very few laboratories, especially those engaged in lipid research, are without this valuable tool.

**Apparatus.** The glass plates are usually about 20 x 20 cm, but can be any size depending on the dimensions of your applicator and developing tank. Thickness of the plate is very important with most types of applicators except the one based on the design of Mutter and Hofstetter (22).

The uniform coating of glass plates with a thin-layer of adsorbent requires the use of a special applicator. At the present there are at least three types of applicators on the market\(^a\),\(^b\),\(^c\). The equipment described by Stahl is probably the most widely used, but the applicator described by Mutter and Hofstetter (22) is advantageous in at least one respect. These latter workers have designed an apparatus that permits the

---

\(^b\)C. Desaga Gm. b. H. Hauptstrasse 60, Heidelberg, Germany; U.S. representative: C. A. Brinkmann and Co., Inc., 115 Cuttermill Road, Great Neck, Long Island, New York.
\(^c\)Research Specialties Co., 200 South Garrard Blvd., Richmond, Calif.
regulation of the thickness of the adsorbent (about 250 microns desirable) regardless of the thickness of the glass plates. Thus, inexpensive, good quality window glass may be used instead of plate glass. Other applicators require the glass plates to be of uniform thickness if the adsorbent layer is to be the same on all plates. Preparative chromatograms may also be obtained with this applicator. A more versatile model of Stahl’s applicator has been produced, which permits one to obtain uniform thin layers of any desired thickness between 250 μm and 2mm. This modified applicator is finding application in preparative work, but it also requires the glass plates to be of uniform thickness, if layers from plate to plate are to be uniform.

In addition to the glass plates and applicator, a chromatographic developing chamber, spotting template, desiccated storage cabinet and a 100°C drying oven are essential equipment.

Adsorbents used are about the same as those used in column chromatography, except in most cases a binder has been added. Adsorbents commercially available contain 5 to 15% binder (usually calcinated calcium sulfate), however, adsorbents can be obtained that contain no binder. Work in our laboratory indicates that a binder is necessary in the adsorbent only when the thicker, preparative type, plates are being used.

Particle size and uniformity of the adsorbent powder are much more critical in thin-layer chromatography than in standard column chromatography. A standardized 200 mesh adsorbent is preferable. The most widely used adsorbent is silica, silica gel or silicic acid. These terms are often used synonymously in the literature. They refer to powdered solids of the general formula SiO₂·XH₂O, which consists of porous, three dimensional siloxane (tetrahedral Si-O-Si) structures with surface silanol (Si-OH) groups. There is usually a mono or multimolecular layer of adsorbed water on the adsorbent particles. During the activation process, which is merely heating the chromatoplates at about 105°C-120°C, this adsorbed "free water" is reversibly removed. Silicic acid plates should not be exposed to temperatures above 170°C, because at this temperature the silanol groups will begin condensing, liberating the "fixed water". The degree of thermal degradation of the silanol groups increases with rising temperatures until at about 1100°C only SiO₂ remains. This thermal degradation is rather irreversible, because it destroys pore structure and permanently impairs adsorbent properties by reducing the number of active sites available for hydrogen bonding (26).

Silicic acid is most frequently used for the separation of neutral and acidic lipids. The silicic acid is slurried with distilled water in a ratio of 1:2 W/V and applied to the plates in a uniform layer. Stahl (21) prepared acidic silicic acid chromatoplates by using aqueous 0.5 N oxalic acid solution instead of water in making the slurry. This same worker also prepared alkaline chromatoplates in a similar manner utilizing aqueous 0.5 N KOH. Mangold and Kammermeck (13) used chromatoplates of silicic acid containing about 10% ammonium sulfate for the separation of phospholipids and strongly acidic fatty acid derivatives. These layers prepared in basic or acidic solutions do not adhere well to the glass plates. It is recommended that these plates be dried at room temperature for several hours before they are placed in the drying oven.
Diatomaceous earth or Kieselguhr is especially suited for partition separations of substances which are strongly hydrophilic or Amphoteric. After impregnation, it can also be used as an inert carrier for the separation of hydrophobic substances. Like silicic acid, diatomaceous earth is applied to the plates as a slurry (1:2 W/V) made with distilled water. Weill and Hanke (24) used this adsorbent for the fractionation of Malto-oligosaccharides.

Aluminum oxide (alumina) is rarely used for the chromatography of complex mixtures because it causes hydrolysis of ester linkages and isomerizations of double bonds. However, alumina is superior to any other adsorbent for the chromatographic fractionation of the Vitamin A group, hydrocarbons and various classes of lipids, especially the basic ones. It is also often used for the chromatography of sterols (16).

Other adsorbents such as magnesium oxide, magnesium carbonate, calcium hydroxide, calcium carbonate, dicalcium phosphate, florisil, cellulose and many others have been described in the literature (12). Almost any adsorbent used in column chromatography may be adapted to thin-layer chromatography.

Reversed-phase partition TLC has been used in resolving a lipid class into its individual constituents. This is done by impregnating thin-layers of adsorbent with silicone (11) or some other hydrophobic substance.

Ion exchange TLC has not been used as extensive as adsorption TLC, however, I can see no reason why the present ion exchangers could not be adapted to this technique. Ecteola cellulose bound to glass plates by collodion was used by Randerath (20) to separate various purine and pyrimidine nucleotides.

Selection of the solvent is based upon the well known principles of adsorption chromatography. Polar solvents cause a greater migration than non-polar solvents or the \( R_f \) value (Distance sample spot travels) of a substance increases with increasing polarity of the solvent. Frequently, it is convenient to adjust the polarity of the solvent by adding small amounts of a polar solvent to a non-polar solvent. Generally this is the case, but it is recommended that these solvent mixtures be kept as simple as possible for better reproducability (12).

The solvent required for good separation depends on the type and number of functional groups in the compounds being separated. Hydrocarbons are adsorbed very lightly and require a low polarity solvent for their separation. If functional groups are introduced into a hydrocarbon, the adsorption affinity increases in the following sequence: \( R - \text{cH}_2, R-\text{O-alkyl}, R-C = O, R - \text{NH}_2, R - \text{OH}, R - \text{COOH} \). Within each of the above classes, saturated compounds are eluted easier than unsaturated compounds. Polyunsaturated compounds with isolated double bonds are more easily eluted than those containing a conjugated system of double bonds. Cis-isomers are more easily eluted than their trans counterparts.

The actual selection of a solvent system to meet a particular need may be accomplished by using a simple spot test and a thorough knowledge of
the order of solvents. The spot test is carried out by placing a drop of
sample on the layer and after drying, slowly adding solvent from a micro-
pipette. The movement of the sample is then observed. In the case of an
unknown mixture, the spot test is started by using a solvent of medium
polarity. If the sample remains in the vicinity of the starting spot, then
a stronger solvent must be used. If migration is too fast then a weaker
solvent mixture must be employed. By this method one can select the proper
solvent mixture with a minimum of extra work.

Visualization and Identification. Many chromogenic spray reagents,
commonly employed in paper chromatography, can also be applied to TLC. In
addition, a number of corrosive sprays such as concentrated H₂SO₄ and HNO₃,
which cannot be used on paper, can be utilized on these inert coated plates.
After spraying with these oxidizing acids, the chromatoplate is heated and the
compounds present can be seen as dark spots on a light background. Spray
reagents such as 1, 4 dichlorofluorescein or Rhodamine 6 G may be used in
conjunction with an ultraviolet lamp. Fluorescent chemicals and oxidizing
acids may be incorporated in the adsorbent layer for visualizing spots, and
this eliminates the necessity of spraying.

Autoradiographs are readily obtained when chromatoplates con-
taining radioactive labeled substances are exposed to No-Screen Medical
X-Ray Safety Film.

Documentation of Chromatograms. Many people consider documenta-
tion to be a major disadvantage with this technique. We have not found this
to be true. We record chromatograms by tracing them onto acetate paper or
tracing them directly into a laboratory manual. This gives a permanent
record of all chromatograms.

Other methods of documentation that are being used include
photography, radiography and dipping the chromatograms into a solution of
label glaze. After dipping a chromatogram in label glaze the adsorbent layer
is pulled off the glass plate as a film.

Quantitative Thin Layer Chromatography. Present methods for
quantitative evaluation of thin-layer chromatograms leave much to be desired.
Few of the methods are exact and others are impractical.

Rough estimations are obtained by eluting and weighing. This method
is practical only if individual spots contain from 20 to 50 mg. of material.
Spectrophotometry and colorimetry may also be used for quantitative estima-
tions after the sample is eluted from the adsorbent. When using these
techniques, care must be taken to assure removal of all the adsorbed substance.
As the polarity of a compound increases elution becomes more difficult.

Photodensitometry after charring as applied by Privett et al. (18)
(19) gives good results in a range 5 to 35 μg of material. The spots are
measured in a Photovolt Photodensitometer with a stage attached for semi-
automatic plotting of curves. The quantitative estimation is made by measur-
ing the area under the curve. Individual standard curves must be prepared
for all compounds that do not give spots of the same densitometrically
determined response (18).
Measuring spot areas may be useful in supplementing the photodensitometric method, as it can be applied to amounts of 30 to 100 ug with an accuracy of ± 5%.

Applications. Although it has been stated that the greatest usefulness of TLC is for the separation of lipids and essential oils, a brief review of the literature reveals that it has extremely wide scope.

In the separation of lipids, TLC has been investigated quite extensively. Good resolutions have been obtained in the separation of steroids (7)(10), fat soluble vitamins (2), phospholipids (23) and crude preparations of various fats, oils and waxes. Basic fractionations between the various classes (aldehydes, ketones, acids etc.) of compounds have been obtained. These classes may be further separated according to degree of unsaturation, chain length, and isomeric forms.

Thin-layer chromatography has also been employed in the fractionation of carbohydrates, amino acids, and various proteins. This technique has been applied to the study of various sulfur-containing compounds such as sulfa drugs (25) and nucleotides (20).

Thin-layer chromatography is also being employed in the detection of food additives such as antioxidants and coloring agents. Other fields such as toxicology and pharmacology are using this technique for the rapid detection of impurities and poisons.

Summary. Thin-layer chromatography is achieved by first making a slurry of the adsorbent. The slurry is usually made with two parts of distilled water and one part adsorbent, but use could be made of various other solvents. This slurry is then applied uniformly to glass plates with the aid of a standard applicator. After the adsorbent has been applied, the plates are allowed to dry at room temperature, usually this requires 5 to 15 minutes. The plates are then placed in a drying oven (usually 105-120°C) for activation. Degree of activation depends on the length of time the plates are allowed to remain in the oven and the temperature of the oven. The activated plates are now stored in a desiccated container, until they are used. If too much moisture is adsorbed, the plates may be reactivated. The materials to be chromatographed are applied to the adsorbent with a micropipette or a microsyringe. The spotted plates are then placed in an equilibrated developing chamber containing the solvent. The solvent ascends in the adsorbent layer and separates the compound mixture as it moves.

The advantages of this method are quite evident. Development is much faster for thin-layer chromatography than for column or paper chromatography. The separating time, depending on solvent used and layer thickness, ranges from 20 to 40 minutes for most applications. Only in rare cases, such as for amino acids, are longer developing times required. Simplicity is another major advantage of this technique. With a minimum of orientation good results can be obtained by inexperienced personnel.

Sensitivity and capacity are also very important attributes of this technique. A minimum of 0.5 ug and up to 500 ug, can be applied to one spot.
Among the relatively few disadvantages is the fact that $R_f$ values are not reproducible, which suggests the running of standards alongside the sample for comparison purposes. The quantitative removal and extraction of individual spots from the plate may at times be more tedious than elution from paper chromatograms.

**Glass-Paper Chromatography**

This is a technique that combines paper chromatography and thin-layer chromatography into one technique. It makes use of a glass filter paper impregnated with an inert phase such as silicic acid. Development time is much faster than paper chromatography and $R_f$ values are more reproducible than those of TLC.

**Chromatogram Preparation.** Sheets of glass fiber paper* of a size suitable for available equipment is freed of organic impurities by heating in an oven at 600°C for one hour. Dieckert et al. (3) accomplished this by placing the paper in a borosilicate glass container with a loose fitting lid. This container was then placed in an annealing oven and heated at 600°C for one hour.

The impregnating solution is a potassium silicate solution, which is prepared by the addition of concentrated KOH to a slurry of silicic acid and water. Muldrey et al. (6) have successfully used sodium silicate for the separation of some phosphorus containing lipids, and these workers omitted the HCl treatment. The HCl treatment will be explained later. After the silicic acid has dissolved, the solution is filtered through a sintered glass filter to remove insoluble debris. The filtered solution is then diluted to about 0.4% (15) potassium silicate. This solution has a useful life of about one week, because it readily absorbs CO$_2$. If CO$_2$ is absorbed, it is held until the HCl treatment of the chromatogram and at this time is released as a gas. This gas will form undesirable pockets in the chromatogram (5).

The sheets of glass paper free of organic impurities are then dipped in the 0.4% potassium silicate solution and allowed to dry in an oven. Some workers stroke the dipped glass paper with a glass rod to remove the excess impregnating solution. After the impregnated strips are thoroughly dry, they are treated with 4 N HCl for 5 minutes. The acid treatment precipitates the potassium silicate as silicic acid. The impregnated chromatogram is washed with distilled water until all acid is removed. The chromatogram is then dried in a 100°C oven and stored in a closed container until used (3).

The basic principles of this technique are quite similar to those discussed earlier for TLC. Selection of a solvent is about the same as that for TLC or paper chromatography. Visualization of the developed chromatogram is also very similar to TLC, however, it has the definite advantage over paper chromatography in that the use of corrosive sprays is possible.

* H. Reene Angel and Co., 52 Duene St., New York, N.Y.
Applications. Most literature concerning the use of glass-paper chromatography deals with the separation of lipids, however, Dieckert et al. (3)(4) have successfully separated sugars as well as lipids with this technique. This same group of workers have reported the separation of glycerides, cholesterol and cholesteryl esters by glass-paper chromatography (5)(4). Cry (17) has also reported the separation of glycerides of mixed fatty acid composition by glass paper chromatography. Dieckert et al. (6), and Muldrey et al. (15) have used this technique for the fractionation of phosphorus containing lipids.

Very little work has been reported on the glass-paper chromatographic separation of proteins. Arimura and Dingman (1) have reported quite good success with the technique when it is used as an assay for vasopressin and oxytocin.

References


---------------------

MR. PEARSON: This morning our discussion so far has centered upon chromatographic techniques. Inasmuch as we want to break up the morning somewhat more evenly, we would like to go ahead with the next paper which is quite a different topic—and probably not so new—in that it has been used for a great many years. It deals with Spectrophotometric Methods. This paper is prepared by Dr. Derrel E. Goll and Dr. Harry E. Snyder of Iowa State University, and I am going to ask Dr. Goll to give the paper.

###