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MODERN ADVANCES IN THIN-LAYER CHROMATOGRAPHY

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INTRODUCTION

Thin layer chromatography (TLC), an analytical separation technique which can be used at the micro or macro level, was first introduced in 1938 by Izmailov and Shraiber (1) as spot chromatography. Eleven years later (2), surface chromatography was used to fractionate inorganic salts on adsorbent-coated glass plates. In 1951 Kirchner et al (3) used glass strips coated with adsorbents for the separation of terpenes. TLC became popular after Shahl in 1956 (4) introduced a spreader which could prepare uniform and reproducible layers.

Since then, TLC has developed into a powerful analytical tool for sample separation, purification, quantitative determinations, and for the identification and confirmation of a wide variety of complex mixtures.

It is used in biochemistry for separating and screening complex products such as amino acids, nucleic acid bases, peptides, and toxic and carcinogenic compounds and their metabolites; in clinical and toxicological chemistry as a diagnostic procedure for the analysis of urine and blood; in the pharmaceutical industry for the analysis of drugs and their metabolites, vitamins, antibiotics and other products; in environmental studies for the separation, purification and analysis of water, air, and organic particulates for the presence of polycyclic aromatic hydrocarbons and their photooxidation products, pesticides and insecticides; in the cosmetic industry for the analysis of dyes and perfumes; in the study of natural products such as lipids, steroids and plant extracts; in forensic laboratories for the identification of in samples police work and in synthesis to follow organic and inorganic reactions and test the purity of the product.

This review focuses on recent advances in TLC, including modifications of the adsorbent, the solvent and the sample as aids in separating complex mixtures. Quantification methods including densitometry, fluorimetry, radio scanners, TLC/flame ionization detection (FID), and the role of microprocessors in data handling and storage. Developing chambers, including circular and anticircular, linear compressed, and continuous development chamber types, will be compared, and the advantages and disadvantages of each pointed out. A comparison of TLC, HPLC and GLC will also be presented. TLC applications will not be discussed as these can be found elsewhere(5).

ADSORBENTS

Izmailov and Schraiber (1), in their first report suggested that chalk, talc, magnesium oxide, lime, aluminum oxide and similar materials could be used as adsorbents. Today plates precoated with silica gel, alumina, cellulose, polyamide, mixed phases and two-phases side-by-side on the same plate are commerically available on plates ranging in size from 1 X 3 in. to 8 X 16 in. The coatings range in thickness from 100 μm to 1000 μm, and may be hard or soft, with or without a binder or fluorescent indicator, polar or non-polar, neutral, acidic or basic and impregnated with a metal salt or other suitable materials. Some of these supports are used once and discarded; others are permanent, and can be washed after development, dried and reused.

The adsorbent needed depends on by the properties of the sample (polar or non-polar, ionic, or neutral), and other factors, such as the chemical composition of the surface, water content, surface area, and geometrical arrangements of atoms or groups. The adsorbents are also divided according to the type of the chromatographic process involved:partition, adsorption, or ion exchange.

Silica gel is the universal adsorbent and its value has been proven in separations of a wide range of chemical groups (other than strongly polar and ionic mixtures). However, the development of reversed phases (will be discussed later) has grown since it is very effective in separating polar compounds and other chemical mixtures which are poorly resolved on other adsorbents.

As mentioned earlier, plates coated with more than one adsorbent, or gradient phase have also been introduced. For example, plates coated with silanized silica gel and silica gel side-by-side on the same plate have been used to separate oxidation products of cholesterol (6). Whatman Co. (7) introduced a plate in which a narrow reverse phase C_{18} strip is coated side-by-side on a silica gel plate. Stahl et al. (8) recently introduced a pH gradient layer. The advantages of two-phase and gradient layers are many, but their main value is in separating otherwise inseparable mixtures. Two phase plate may also be used for sample clean up on one phase and separation on the other, and for the separation of complex mixtures of varying polarity.

Two phase TLC plates are formed by spreading two different adsorbents side-by-side, by developing a precoated plate in a silylating agent (9), or by grafting two precoated TLC plates together as described by Pandey et al. (10). Grafting TLC is a two-plate system, (using two layers which may or may not be the same) in which plates are clamped together with the edges of their adsorbent layer in contact so that a band on one layer can be transferred to the other. The advantage of such a method is that bands on one plate can be transferred to another plate for further TLC analysis without scraping, eluting and reloading.

Another advance in plate preparation is sintered TLC, introduced in 1973 by Okumura et al. (11). The plate is prepared by mixing silica gel with glass powder. The slurry is then spread on a soda-lime glass plate and the prepared plate placed in an oven

at high temperature to fix the adsorbent layer to the plate. The resultant layer is very hard and can be used repeatedly after cleanup with a chromic/sulfuric acid solution. Okumura (12) wrote an excellent review discussing different applications and plate preparations.

Ion exchange layers of strong cation and anion exchange resins which are spread on poly(ethylene terphthalate) sheets are commercially available (Chromatronix, Inc., Palo Alto, CA). They have been widely accepted and are used to separate amino acids (13) nucleic acid bases (14) peptides and other ionic materials (15) from food and body fluids.

An advance which has also been widely accepted is high performance TLC (HPTLC). In HPTLC, the particle size (3-8 μm) is smaller than in conventional TLC, and the size distribution is kept to a minimum which leads to higher effeciency and less diffusion of the compounds on the plate. As a result the spots are more compact (with better overall resolution and detection limits) and separations are achieved in shorter distances (16) which saves time.

Adsorbent modification: A recent trend has been to modify the adsorbent properties by (a) chemical reaction or (b) physical methods (impregnation of the adsorbent) in order to achieve the separation of otherwise unseparable mixtures.

a) Chemical methods: The adsorbent is modified by a chemical reaction by which a group of interest is bonded to the adsorbent to alter its properties. Silica gel layers have been modified by

silylation to form a more hydrophobic (lipophilic) phase (17-19). Such phases are prepared by bonding an organic group R to the surface of silica to form ≡Si-R or ≡Si-O-Si-R groups. By altering the length of the chain, or the group (R), the properties of the resultant derivatized sorbent are changed. Reversed phases bonded with C_2, C_6, C_8, C_{12} and C_{18}, in the bonded group (R), are commercially available. The group itself may also be changed as in the case of high performance liquid chromatography (HPLC) adsorbents. These derivatization procedures are carried out before a plate is made.

Aringer and Eneroth (9), on the other hand, silylated a commercially available precoated silica gel plate by developing the plate in the very reactive agent hexamethyldisilazane (HMDS). Using derivatized silica gel as the modified adsorbent for TLC allows the migration and separation of polar sample components which would otherwise bind strongly to silica gel. The order of migration of the compounds on silica gel phases which have been bonded is reversed. Many examples have been cited in the literature: a few will be mentioned here. Environmental trace analyses were carried out on C_8 and C_{18} reversed phase plates (20). A mixture of 1-, 2-, and 4-acetylaminofluorene isomers was resolved on reversed phase C_{18} plates but not on silica gel (21). Brinkman and DeVries (22) separated a mixture of dialkylphthalates on C_8 reversed phase plates. A comparison of the resolving power of silica gel, silanized silica gel (HMDS), and C_{18} reversed phase silica gel plates indicated that the latter gave the best resolu-

tion of the oxidation products of cholesterol (21). Van Arx and Faupel (23) separated mixtures of steroids and peptides on reversed phase silica gel and alumina plates which they had prepared. A theoretical treatment of separation on reversed phase and normal phases was published by Martire and Boehm (24).

b) Physical methods (Impregnation): There are different methods for impregnating the adsorbent depending on whether the plates are prepared in the laboratory or are precoated by the manufacturer. In the first case the reagents are added to the slurry. This method gives uniform and controlled concentrations of the reagent. If precoated plates are used, the reagent is introduced by spraying (not very uniform), dipping, developing the plate in the reagent solution or by brushing the reagent solution on the plate. A discussion of impregnation was presented by Halpaap and Ripphahn (25). The adsorbent is normally impregnated with a reagent that forms a complex or an addition compound with the component mixture. It is a selective process in which one component reacts with the reagent while the other does not, whereby separation is achieved. For example, boric acid is used for the separation of vicinal dihydroxy isomers where a cyclic boric acid derivative is formed; silver ions form complexes with olefinic double bonds and cis- and trans- isomers can be separated; metal ions such as zinc, cadmium, manganese, etc. form complexes with nitrogenous bases; picric acid and trinitrobenzene form complexes with polynuclear compounds.

Halpaap and Ripphahn (25) studied the effect of impregnation of hydrophilic and lipophilic stationary phases on R_f values. Stable hydrophilic stationary phases were formed by impregnating precoated silica gel plates with formamide, dimethyl formamide, ethylene glycol, polyethylene glycols, 2-phenoxyethanol and various buffers. Lipophilic stationary phases (for reverse phase chromatography) were obtained by impregnating the silica gel plates with mineral oils, liquid paraffin, undecane, silicone oils, tetradecane or ethyl oleate. They found that when higher R_f values are required, the hydrophilic component of the solvent must be increased in the case of hydrophilic adsorbents, while the lipophilic solvent component must be increased when lipophilic adsorbents are used (25).

Touchstone et al (26) separated a mixture of dihydroxy bile acids on potassium dihydrogen phosphate-impregnated silica gel plates. Impregnation of plates with formamide (27) and boric acid (28) for the separation of isomers of cardenolides was also reported. The separation of N-nitroso-3-methyl-Δ^3-tetrahydropyridine from N-nitro 5-methy Δ^3-tetrahydropyridine was achieved on silver nitrate impregnated silica gel plates by forming a complex between the silver ions and the π-electrons of the double bond on N-nitroso-5-methyl-Δ^3-tetrahydropyridine and not the 3-methyl-isomer (20). Methyl-esters of polyunsaturated fatty acids were also separated by impregnating the plate with silver ions (30).

Other metal ions were used, for example, Martz and Krivis (31) sprayed silicic sheet plates with coper sulphate solution before

spotting to obtain separation of hexosamines and n-acetylhexosamines. Our experience indicates that more uniform impregnation is achieved when precoated plates are dipped or developed in the reagent solution than when sprayed (32).

Yasuda used silica gel plates impregnated with cadmium sulfate (33) cadmium acetate (34) and manganese salts (35) to separate mixtures of aromatic amines. Silica gel and alumina plates impregnated with zinc salts (18,19) calcium oxide (36) and cadmium nitrate (37) were used for various separations including a mixture of nine toxic alkaloids which did not separate on silica gel or alumina (37).

Antonelli et al. (38) used ligand exchange TLC to separate a mixture of α-aminoacids, their β-isomers and peptides, The chelating exchangers used were $Cu(NH_3)_2^+$ on which α-amino acids were retained and $Ni(NH_3)_6^{2}$ which gave better separation of the above three groups of compounds. Ligand Exchange Chromatography (LEC) is a very promising technique. It has been defined (39), "as a process in which interaction between the stationary phase and the molecules to be separated occurs during the formation of coordination bonds inside the coordination sphere of the complex forming ion". Davankor and Semechkin (39) have published a comprehensive review of LEC.

Grant and Meiris (40) mixed Bentone-34, dimethyldioctadecyl ammonium bentonite, with silica gel for the selective separation of some polycyclic aromatic hydrocarbons. They concluded that better separations were achieved with the mixed phase plate than with

silica gel plates. Mixtures of nucleosides, mucleotides, and nucleic acid bases were separated on microcrystalline cellulose layers mixed with chitosan, deacetylated chitin, 2:1 by weight (41).

Soap TLC (42) consists of mixing a detergent with silanized silica gel during the slurry formation. The detergents used were sodium laurylетherosulfate, triethanolamine dodecylbenzenesulfonate, and sodium dodecylhydrogensulfate. The plates were used for the separation of some aliphatic amines. The effect of concentration of detergents on separation and R_f values was also studied and compared with that on layers of strong and weak anion and cation exchangers (43). It was found that the separation of the amines is due to a partition process between the two phases and due to an ion exchange mechanism.

It is important to note that when silica gel layers are modified with hydrophilic or lipophilic reagents, the separation process may change from adsorption to partition chromatography. It is also worth remembering that impregnation of the adsorbent is a selective process and its use depends on the properties of the sample. Impregnation, however, is not limited to silica gel; other adsorbents such as alumina or cellulose may be modified.

Gradient layers have been discussed by Stahl (44). Recently pH gradient layers were used to separate a mixture of fluorescent dyes (45). These layers are also recommended for the separation of mixtures of compounds of widely differing polarities. Gradient

layers with levels of increasing impregnation with silver nitrate have been reported (46,47).

Complexing agents are added to the adsorbent not only for separation purposes but for visualization and identification. Some chromagenic reagents based on the formation of complexes between Fe^{+3} ion and organic acids have been reported (43,49). The formation of blue color complexes between Cu^{+2} ions and some polybasic acids has been used for the densitometric quantification of the resulting compounds (50).

A note of caution is appropriate here. When impregnation is used for adsorbent modification, care should be taken that the solvent selected does not wash away or interact with the modifier.

COATERS

The production of a uniform coat of adsorbent is important in TLC, not only for reproducible R_f values, but for accurate quantification. Today there are various types of coaters, manual or automatic, which can be selected to meet research and quantity requirements. Precoated plates, commercially available for almost all types of adsorbents, are suitable for laboratories which use only a small number. Where research dictates the use of mixed adsorbents on the same plate, a manual applicator will suffice. An automatic applicator is relatively expensive. Other considerations are the price of glass plates and the adsorbent and time necessary to prepare the plates.

SOLVENTS

Solvents used in developing TLC plates may be selected from the elutropic series depending on the type of sample to be analyzed and the adsorbent selected. A solvent may be polar or nonpolar, one component, or a mixture of two or more. Polar and nonpolar solvents are used with normal phases and polar solvents with reversed phases. The more viscous the solvent system, the slower the development, the less diffused the spots and the better the resolution. Changing the strength (polarity) of the solvent system affects not only the separation but also the R_f values. For example, in reversed phase TLC changing the percentage of water in the system may move a spot upward or downward depending on whether the water level is decreased or increased. In certain cases, changing from one alcohol to another affects the separation and R_f values. For example, changing from methanol to ethanol to n-propanol to isopropanol affected the separation and the R_f values of alkylated adenines and uracils (51). The use of mixed mobile phases, although it has its advantages, as mentioned earlier, also has some disadvantages. It is not always easy to predict the R_f values as compared with those in pure solvent. R_f values may not be reproducible due to the evaporation of one of the components. Demixing (52) of the solvent components may take place which will affect the quality of the separation (53). Methods for the elimination of demixing have been discussed (53).

Solvent modifications in TLC are not new; they are probably as old as the technique itself. Whenever more than one component sol-

vent system is used, it means that the researcher for one reason or another (separation, streaking, resolution, solubility, diffusion, visualization and identification) decided to add another solvent to aid in the chromatographic process. For example, in the separation of amino acids and their derivatives if alcohols are replaced by low-polarity liquids in the solvent system which contains water, a solubilizer such as methanol, pyridine or acetic acid is added to restore miscibility with water (54). Rasmussen (55) added fluoroscein to the solvent to aid in the UV detection of some acids on TLC plates. Aringer and Eneroth (9) and later Issaq et al. (56), added HMDS to their solvent systems to achieve the separation of the oxidation products of cholesterol.

Addition of Acids and Bases: Acids (54) or bases (51) are added to the solvent systems to prevent streaking and to produce more compact spots, when the separation of acids or bases is required, respectively. The addition of buffers and salts to control pH and ionic strength of water based phases is common in ion exchange chromatography (57).

Micellar Solutions: As was mentioned earlier, in soap TLC detergents are mixed with the adsorbent during slurry formation. In micellar solvent systems surfactants are added to the aqueous mobile phase (58,59). A review of micelle forming surfactants has been published (60). The principle of using micelle solutions is that partitioning does not occur in the bulk of the solvent but rather to highly selective species dissolved in the solvent. A simple micelle offers a variety of environments (60) from its

organic core, to the more viscous polar region toward the edge of the hydrophobic core to the highly polar and charged (in the case of micelles formed from ionic surfactants) Stern layer. The apparent polarity of a micelle mobile phase is altered by changing the concentration of surfactant in the solutions. The surfactants used were sodium dodecylsulfate, acetyltrimethylammonium bromide and Ipagal CO-710, a nonionic surfactant. The concentration of the surfactant in aqueous solutions ranged from 0.1-0.02M. They proved effective in the separation of pesticides, nucleosides and other compounds on polyamide, and alumina but not on silica gel plates.

SAMPLE PREPARATIONS

The sample mixture to be separated is dissolved, after preliminary clean-up, in an appropriate solvent then applied to the plate as a spot or band, with no chemical treatment (oxidation, reduction, etc...). However, when the mixture components are closely related separation may be difficult or impossible without chemical pretreatment. This section will discuss sample modifications, that allow the separation of such mixtures. They are carried out, either (a) in solution before spotting, or (b) on the plate before or during development.

a) In Solution: Derivatization of the sample mixture to separate closely related components has long been used in gas liquid chromatography (GLC) and recently in HPLC. However, the use of derivatization in TLC, as an aid in the separation of a mixture, has not been fully exploited. Unlike GLC, where derivatization is

used mainly to form volatile, thermally stable compounds, and HPLC where derivatization is used to introduce a chromophore, in TLC it is used to accentuate structural difference by introducing "bulky" groups into the sample mixture; make separation possible. Use is also made of steric hindrances.

Separation of the two isomers of 3-hydroxy-N-nitrosopiperidine was achieved after derivatization in solution with TSIM (29). Also, a mixture of oxidation products of cholesterols were separated on silica gel plates after derivatization with TSIM, where separation was not possible without derivatization (56).

(b) On the Plate: Hwang et al (61) combined the Schiff's base reaction characteristics with TLC to determine primary amines. The sample was spotted on the plate followed by the addition of an equal volume of 1-pyrene aldehyde in ethanol, after which the plate was sprayed with 1-butanol acidified with dry hydrochloric acid and placed in an oven for 1 hour at 70°C, before being developed. Schutz (62) used reaction on the plate to separate and identify nitrazepam and its metabolites. After separation in the first dimension of two-dimensional thin-layer chromatography, the substances were derivatized by treatment with an acidified aqueous solution of $TiCl_3$. By means of combined hydrolysis and reduction nitrazepam and its metabolites were derivatized on the plate. The subsequent separation in the second dimension permitted exact identification from the R_f values and Bratton-Marshall detection.

In another study (63), Soyasapogenols A, B, C, D and E were separated on silica gel plates after derivatization with acetic

anhydride:pyridine (1:1) to form acetyl derivatives. Nakamura and Pisano (64) derivatized the sample mixture (peptides, amino acids or amines) after spotting, by either dipping or spraying the plate with fluorescamine.

Note: When dipping or spraying is used, the reagent is dissolved in a solvent with which the sample is not miscible; otherwise runs or diffusion of the spots may occur which will affect resolution and quantification.

Post development sample modification is mainly used for visualization and identification purposes. The sample after development is sprayed, dipped or placed in a gaseous atmosphere. Some spray reagents are specific. For example $Co(SCN)_2$ aqueous solution gives a different color with nitrite, nitrate, and nitrosamines however, the reagent gives the same color with all nitrosamines tested (65).

In certain cases more than one reagent is needed. For example, nanogram amounts of adenine, guanine, uracil, cytosine and their alkylated bases, nucleotides and nucleosides were detected on TLC plate by placing the plate, after development and drying, in chlorine gas. When the excess chlorine gas was removed, the plate was sprayed with tolidine reagent to give colored spots (66). Kirchner (67) discussed reactions on the plate including oxidation, reduction, halogenation, hydrolysis, nitration and dehydration.

Many other examples can be found in the literature (67,57,68) and need not be discussed here.

DEVELOPING CHAMBERS

The plate is developed after the sample has been spotted and dried in either a stream of air or in an inert atmosphere, depending on the reactivity of the sample. Development refers to the separation of the mixture when the liquid phase moves across the plate adsorbent by capillary action. The plate, depending on the mixture to be separated, may be developed in a saturated or unsaturated atmosphere.

Any size container, depending on the size of the plate, may be used for plate development. The container may be a closed jar or a specially manufactured cylindrical or rectangular tank. The tanks most widely used for 8 X 8 cm plates measures 4 X 12 X 9 in. and are made of glass.

Generally, the tanks are not flushed with nitrogen before the plate is developed. The plate can also be developed in a refrigerated tank. Development under nitrogen and refrigeration is recommended for samples which are easily oxidized and heat sensitive.

Many significant advances in plate development have been introduced in the last few years. They include radial, anticircular, compressed, gradient, and continuous (both at room temperature and at - 77°C using a cryogenic apparatus) modes.

In radial or circular development, which has been used for years in paper chromatography, the mixture to be separated is spotted at the center of a sheet and the solvent flows from a pipette onto the plate and elutes the components in circles.

Recently, an accurate and relatively simple apparatus, the U-chamber (16), was introduced for use with high performance TLC (HPTLC). In this method the solvent is delivered to the plate at a predetermined flow rate. The sample is either spotted on a 5 X 5 cm plate or injected into the stream of the solvent. If the sample mixture is spotted at the center or injected into the stream, the components separate as circles. However, if the samples are spotted around the center, the sample components separate in arcs. The most accurate system in terms of experimental conditions is the U-chamber by Camag, which uses a micro-syringe attached to a pump to feed the solvent at constant rate. Development time is about 4 minutes, the amount of solvent needed is less than 1 ml, and the experimental conditions are reproducible.

Two other units are available for circular development; the SelectaSol by Scheicher and Schuell and the High Performance Radial Chromatography Chamber by Fotodyne Inc. Whereas the U-chamber uses a pump to deliver the solvent at a pre-determined flow rate, the Fotodyne unit works by gravity and the SelectaSol by capillary action. Both units are fast and aid in the selection of a solvent system, but are not as accurate nor as reproducible as the U-chamber, but they cost much less.

Horizontal circular centripetal TLC was introduced by van Dyk in 1969 (69). In this method the sample is applied at the circumference of a circle, and elution proceeds toward the center. The advantage of this is that the concentration per unit area is increased with decreasing diameter. Also, the separated components

can be withdrawn from the center. Later, the apparatus was simplified by Kyne & Vetters (70). Their apparatus consisted of a turntable on which the TLC plate was positioned while the solvent was introduced from a stationary syringe to a felt ribbon surrounding the plate. The system needs to be protected from drafts; otherwise, irregular circles are produced. The main problem encountered in centripetal TLC is the transfer of the eluting solvent to the plate. In 1978 a modified U-chamber was introduced by Kaiser for anticircular high performance TLC (71). The solvent is fed into the plate by capillary action from a narrow round channel. A simpler, and far cheaper anticircular TLC developing device, was reported by Kariko and Tomasz (72). The device consists of a petri dish into which the solvent is placed. Contact between the round plate and the solvent is accomplished through a cylindrical filter paper strip with feathered edges. The device is not as reproducible or consistent as the anticircular U-chamber.

Issaq, (73) combined the advantage of both the U-chamber and the petri dish in developing a simple, economical and fast apparatus. The system was built from two glass dishes arranged concentrically. A paper wick sits between the two dishes and transfers the solvent from the outer dish to the adsorbent layer. To ensure an even flow of solvent, the dishes are situated on a platform which has adjustable legs. The inner dish is used for saturation or plate conditioning by a reagent.

The advantage of anticircular TLC over circular TLC is that a large number of samples can be spotted, up to 40 samples on a 10

spot. The resolution by anticircular and triangular TLC was comparable to that of circular TLC. Also, the resulting spots are more concentrated (molecules/unit area) because the available area decreases quadratically with the solvent front. The higher the R_f, the more elongated the spots, which affects the resolution of the mixture. However, the resolution at R_f values above 0.5 was improved by developing the plate, after drying, a second time in the same solvent system, which takes only a few minutes (73). Densitometric studies showed not only that better resolution of the spots had been obtained but more uniform and higher peaks (73), which are the result of a greater concentration of the spots i.e. molecules/unit area.

Better resolution of a sample mixture was obtained when the sample solution was applied to the plate as a streak rather than a with that of circular development (74).

Soczewinski and Wawrzynowicz (75) developed a sandwich tank in which the solvent is delivered to the TLC plate from a small reservoir by a capillary siphon using a device which distributes the solvent at right angles to the direction of development. The advantages of such a system are that only one tenth as much solvent is used compared with saturated tanks, and the sample is also preconcentrated on the plate (76).

A new system using a pressurized ultra-micro chamber has been introduced (77,78). The adsorbent layer is covered by a membrane under external pressure. The solvent is introduced by means of a pump. The advantages of such a system are shorter development

time, less solvent is used, and performance is equal to that of HPLC, since there is no vapor pressure as in standard TLC. It was concluded by Kalaz (79) that more compact spots are obtained in the absence of a vapor phase.

Camag has introduce a linear-sandwich type HPTLC chamber which uses 10 X 10 cm plates. Samples are spotted on both sides of the plate which is then developed in the special chamber. The advantages of this chamber over linear TLC in a tank are the smaller volume of solvent needed and the ability to develop twice as many samples per plate, since samples are spotted on opposite sides of the plate. This results in a 50% saving in plate use. Again, one has to assume that a short development meets the needs of that particular analysis. Another advantage (80) of this system is in the two-dimensional development of four samples simultaneously, which cannot be done easily using any other TLC mode. The samples are spotted at the four corners of a 10 X 10 cm plate and developed. Then the plate is removed, dried, rotated 90° and developed again in a different solvent system. The result is the rapid two-dimensional development of four samples on the same plate. In classical TLC this development would require four separate developments - two in each solvent system.

An apparatus for continuous development (CD) TLC at room temperature (75,81) and at -77°C (82) has been reported. The theory and advantages of CD were discussed by Perry (81). The main advantage is the ability to separate closely related compounds using solvents of very low polarity. The lower the strength of the

solvent the higher its selectivity. A point of caution may be appropriate here: CD using binary (tertiary, etc.) solvent mixtures of varying polarities may lead to solvent demixing. Two approaches which have been reported (53) to minimize this effect are, (a) conditioning of plate in saturated tanks, and (b) construction of flat tanks of the sandwich type which have a minimum volume.

Continuous development at -77°C enables the separation of conformational isomers which would equilibrate at room temperature. Also more compact spots are obtained (82) which results in improved resolution.

One of the strengths of HPLC is the ability to use solvent gradient elution to separate a mixture of different polarities. In HPLC, it is (mechanically) easier to achieve such an elution than in TLC because (a) the solvent is continuously discharged from the column; (b) no vapor equilibrium is required as in TLC; and (c) the size and design of the TLC developing tank is a limiting factor. The last point merits discussion. In order to achieve reproducible R_f values in TLC, vapor saturation and equilibration in the developing tank must be achieved before plate development. The classical commercial tanks (10 X 25 X 25 cm) are too large to allow vapor equilibrium to be reached in a short time, and they require approximately 100 ml of solvent. Also, if gradient elution is used with the classical tank the design of the tank does not allow easy discharge of the solvent.

Recently, a few attempts have been made to use gradient elution in TLC. Blome (83) used gradient elution with the U-chamber, which requires a small volume of solvent (less than 1 ml). Soczowinski and Czapinska (84) used stepwise gradient development in conjunction with sandwich tanks. A capillary siphon delivered the solvent at right angles to the direction of development which permitted the composition of the solvent to be changed in a simple manner by substitution of containers. Gradient elution on reverse phase plates has been reported (85). Two pumps and a solvent programmer were used to generate the required mobile phase gradient. Three thicknesses of 100 mesh steel were used to disperse the solvent (evenly)in the developing trough, wet the adsorbent, and allow plate development. Excess solvent flowed out of the trough into a waste solvent container. A four component mixture was separated using the above system, which seems to be the best TLC - gradient elution system reported to date.

Simultaneous Plate Development. In classical TLC, more than one plate can be developed in the same tank and solvent. It is also advantageous to be able to develop the same plate in more than one solvent system or under different conditions simultaneously. The advantages of such a system would be selection of the most suitable solvent system and development conditions. This may be achieved by using one of two units (a) the Vario-KS chamber (Camag), or (b) the SelectaSol solvent selector system (Schleicher and Schuell). The Vario-KS-Chamber has three glass conditioning trays (5, 10, and 25 compartments), two stainless steel slides,

two glass solvent troughs, and a temperature control unit. It is very versatile, allowing the researcher to study the effect of relative humidity and saturation on TLC separations, select the most suitable solvent (up to five solvents can be used), develop 10 samples (up to five different solvents on one 20 X 20 cm plate), and develop continuously at a predetermined temperature, which is most advantageous when reaction chromatography is used.

The SelectaSol system can be used to select the optimum solvent system in minutes. Up to 16 different solvent systems can be run simultaneously on a 20 X 20 cm plate. Development requires less than 6 ml of solvent and is complete in 3-6 minutes. The unit is very simple and easy to operate. It consists of a base which has different size wells (0.5 in to 1.5 in internal diameter). The sample is either spotted on the plate or on a wick (the easier of the two) covered by the plate and then developed. The components of the mixture separate in concentric circles.

PLATE SHAPE

In TLC, separations are generally obtained by linear development on rectangular plates. Recently, the circular mode of development (including anticircular) is gaining popularity (U-chamber, Selecta Sol, pressurized TLC and other variations). The advantages and disadvantages of linear, circular and anticircular TLC have been discussed by Kaiser (71), who compared separation number, sensitivity, analytical separation power, R_f data, consumption of plate material and consumption of mobile phase per sample. These comparisons showed the anticircular mode to be superior to

the other two in terms of relative sensitivity, the number of samples per plate, speed of analysis, and amount of mobile phase required per sample. The linear mode was ranked second, and the circular mode last. However, the circular mode did give the best separation number. The separation number can be improved in the anticircular mode by developing the plate twice in the same solvent system (73). The initial cost of each was not compared, but the linear mode is the cheapest since it does not require the special developing apparatus, the U-chamber, needed for the circular and anticircular modes. In the long run, this initial cost will be offset if a sufficient number of analyses are needed. For the anticircular mode a simple and inexpensive apparatus has been developed (73). Another important aspect not mentioned is that in circular and anticircular TLC, advantage cannot be taken of two dimensional development. The use of two different mobile phases, one polar and one nonpolar, may reveal spots not resolved by a single development. Also, inefficient and incomplete separation of a multi-component mixture may result if a 4 cm run is made on a 10 X 10 cm plate. In classical TLC, using a 10 X 10 cm plate and two dimensional development it is theoretically possible to separate a mixture of at least 50 components which is not possible using circular, or anticircular methods. Nor is the analyst limited by the size of the plate which can be used; anticircular and circular methods use only 5 X 5 cm or 10 X 10 cm plates.

Recently, Issaq (86) combined the advantages of both the anti-circular and linear modes by using a triangular plate. In tri-

angular TLC the samples to be separated are spotted at the base of the plate and then developed in a regular tank, which may be rectangular or cylindrical in shape. The sample after development, is more concentrated (molecules/unit area) than in the linear or circular modes. In triangular TLC the plate is developed twice in the same solvent system which results in compact spots, especially at high R_f values. The advantages of triangular TLC are a 50% savings in plate use compared with conventional TLC, and restricted diffusion of the spots after development. Since the sample is spotted at the base of the plate the movement of the solvent front is from a wide to a narrow area. As a result, the spots are compact and concentrated, (molecules/unit area) which means increased sensitivity (lower detection limits). Preparative separations on triangular plates are preferred over square or circular plates because the sample is streaked at the base of the triangle. After development, the streaks are shorter and less solvent is used for eluting the samples from the plate, and no special developing apparatus is required. Plates of any size can be used, from 5 X 10 to 20 X 20 cm, without the need for special equipment or attachments. Unlike circular or anticircular methods, trianglar TLC allows two dimensional development when an equilateral triangle is used.

DETECTION OF SPOTS

Colored and fluorescent spots on plates can be easily located by white and UV light, respectively. Other spots or inorganic ions can be detected by "ripening", a process developed by Mein-

hardt and Hall (2). The major points of this process include applying gaseous reagents to the plate (after developing, the plate is placed in a tank containing gaseous reagents such as ammonia, iodine, or bromine); spraying or dipping the plate in a reagent. Hundreds of reagents are available for the detection of almost any group of compounds (57,68, 87). Fluorescent quenching of fluorescent plates is another fast technique for detecting spots. Indirect detection methods utilizing combinations of the above techniques may also be used (66).

SPOT CHARACTERIZATION AND INDENTIFICATION

Characterization of the spot is an important aspect of TLC. Separated compounds may be characterized by R_f values (useful with certain solvent systems and adsorbents); nuclear properties of ^{14}C and ^{3}H; chemical formation of colored or fluorescent compounds (spots may be sprayed in situ with a reagent, giving a colored or fluorescent spot characteristic of the material being analyzed); biological aspects (growth stimulation or inhibition of microorganisms and mammalian cell lines); UV or fluorescent measurements (spots are scanned in situ to give characteristic spectra; in certain cases where spots are nonfluorescent, spots are sprayed with a fluorescent reagent and then scanned); and elution and subsequent analysis by spectral methods such as uv-vis, fluorescence, infrared, NMR, mass spectrometry, and atomic adsorption (89,90).

Elution of the spots may be achieved by scraping the spots and extracting the compound from the adsorbent or by in situ elution of

the spots. Manual scraping has its disadvantages which include the use of large amount of solvent compared with the compound, losses due to the powdery nature of the adsorbent, and particles (adsorbent) get into the solution and may interfere in further spectrophotometric analyses. It is not recommended when working with toxic or carcinogenic compounds, which requires special care and handling. The ideal method would minimize sample losses due to scraping, require less care, use a minimum amount of solvent, and elute more than one sample simultaneously and quantitatively. The Eluchrom Automatic Elution System by Camag is used in our laboratory. This unit is capable of eluting six spots simultaneously and quantitatively and requires less than 0.2 ml of solvent per spot (91,92).

QUANTIFICATION IN TLC

Quantification in TLC may be carried out in two different ways, (a) *in situ* on the plate, or (b) after elution. The advantages and disadvantages of each will be discussed.

(a) *in-situ* quantification: Quantification of *in situ* spots on TLC plates is an established technique. The methods used are visual or spectrometric.

The visual technique is carried out by spotting the unknow sample either alongside a standard or between two standards and then comparing the intensity of the spots by fluorescence, fluorescence quenching, or color intensity. The error in this method is ±15-50%, but it is fast and can be used when semiquantitative measurements are acceptable.

The spectrophotometric technique may be divided into three different modes:

(i) Reflectance or Transmittance: Colorless compounds are made visible by charring or by treating with a reagent to produce color.

(ii) Fluorescence: Certain compounds, such as aflatoxins, fluoresce under UV radiation. The area under the peak is proportional to concentration [caution: some compounds deteriorate under UV radiation (93)]. Compounds which do not possess natural fluorescence may be made to fluoresce by spraying with a reagent. For example primary amines are sprayed with fluorescamine.

(iii) Fluorescence Quenching: Spots apear as dark circles on the fluorescent plate. This method usually give a nonlinear relationship between integrated area and sample concentration.

Fluorescence is more sensitive than absorption; also, the measured signal is a linear function of the concentration. Errors in spectrophotometic measurements are between 2-10%. The sources of error are sample application, variation in spot size, uniformity of layer thickness, and variations from plate to plate. Errors can be minimized by using plates with uniform layers. Our experience indicates that commercially precoated plates are reproducible to within ±5% (89%). Errors can also be attributed to the method used for measuring the area under the peak which can be measured manually or automatically. Manual methods, such as triangulation, cutting and weighing, or using a planimeter, are relatively slow and subject to human error. The automatic method

eliminates these disadvantages by electronically integrating the area under the peak. The source of error is the scanning speed of the TLC plate, combined with the counting/second. Our experience shows that a 2.1% error is introduced if the scanning is fast (cm/min) and the counting is slow, whereas a 0.7% error is introduced if the scan is slow and the counting is fast (94).

In our laboratory, the SD 3000 and MPF-3 densitometer with scanning attachments are connected to a Hewlett-Packard Lab Data System Model 3354A which types the integrated area, percentages of each spot from the total area, and the R_f values.

(b) Quantification after elution: Quantification through elution, which involves several steps, is of limited value in routine analysis. Limiting factors include incomplete elution of the compounds and the possibility that interfering materials be carried over with the eluate. Gravimetric determinations are often very precise, but the amounts required for an analysis are very high. Spectrophotometric (UV-vis, fluorescence) determinations of eluted fractions are feasible only when the compounds adsorb UV-vis radiation or fluoresce in solution.

Recent Advances in quantification: Few advances in quantitative TLC have been reported. Camag introduced the HPTLC Scanner for photodensitometric measurements of linear, circular and anticircular plates. This scanner has been of great help with these popular techniques.

Another advance, which has gained acceptance, is the combination of TLC/FID detection and quantification. In this procedure

the sample is spotted on a rod with a sintered silica gel or alumina layer (11). After development the rod is passed through a flame ionization detector (95). The system, Iatroscan TH-10, is manufactured by Iatroscan Labs, Inc. Tokyo, Japan. This TLC/FID combination seems to work well and give reasonable results. Since the chromarod is of the sintered type it can be reused. Background values, which can affect the quantitative measurements, can be controlled by prewashing the chromarod.

Modified radioscanners can be used to measure the activity of a 1 X 20 cm channel on the plates. The new scanners are connected to a strip-chart recorder or a video screen, which gives the analyst a quick evaluation of the chromatogram. Two new models are available from Berthold, Switzerland, and from Bioscan, Washington, D.C. Both systems are sensitive, efficient and require no sample preparation such as scraping or cutting. Measurements are made in situ on the plate. They both offer good resolution, sensitivity and are quick and easy to operate.

Another densitometer which uses a video screen is the Telechrom videodensitometer, (Chinion - Budapest, Hungary) developed by Devenye (96). It will be produced in this country in the near future. The unit has a vidicontube as the detector and scans the spots in three dimensions. The X and Y axes correspond to the shape of the spot and the Z axis correspond to its density. The size of the spot may vary from 2 X 2 cm to 2.3 X 3.6 cm. Small spots are optically magnified by the instrument.

Microprocessors are used in TLC in conjunction with fluorimeters and densitometers for quantification and for data storage and handling. Foss et al (97) described an elegant system for the acquisition and reduction of TLC data. The system consisted of a microcomputer, a cassette recorder for storage of data and programs, a television screen for visual monitoring of the chromatogram and X-Y recorder which serves as a printer/plotter. The microcomputer interfaced with a Schoeffel SD 3000 Spectrodensitometer. The data may be gathered in either absorbance or fluorescence modes. Plates may be scanned either parallel or perpedicular to the direction of developpment for best results. The system is versatile and gives the analyst a quick evaluation. Pollak (98) wrote a comprehensive review the use of microprocessors in quantitative TLC. The review discussed data acquisition, reduction and retrieval and the use of different spectrophotometers (dual and single wavelength), and scanning modes including densitometry (reflectometry and transmission), and fluorimetry. The effect of coefficients of adsorption and scatter on quantification were also discussed.

SAMPLE APPLICATION

The sample solution is applied to the plate as a spot or a thin linear band with a micropipette, microsyringe, a capillary tube or a mechanical applicator. These devices dispense from 1 µl to 25 µl of sample with a precision of ±1%. It is important that when reproducible quantitative results are sought, the spots or bands applied be reproducible in volume dispensed,

size and shape. The advantage of an automatic spotter is reproducibility of the amount spotted, the size and shape of the spot. A disadvantage is that the unit has to be cleaned after each sample; with disposable micropipettes this step is unnecessary.

The newest sample applicators for both classical and high performance TLC are the Linomatt III, and the nanoapplicator, both by Camag. The Linomat III uses an interchangeable micro syringe. The sample is applied to the plate in narrrow bands up to 20 cm in length. With the nanoapplicator the samples are applied as spots ranging in volume from 10-230 nl. The sample size to be spotted is adjusted with a micrometer to ensure both reproducibility and accuracy. Preparative TLC requires that a larger sample be used. The sample is applied as a band (not as a spot because of overloading) and should be uniform, narrow, reproducible and, when needed, quantitative. When plates with preadsorbent layers are used, the uniformity and thickness of the applied bands are not prerequisites. The device is selected on the basis of need, size of sample, accuracy, reproducibility and ease of operation. Many devices are available on the market which have various controls that aid the operator in selecting and adjusting conditions to suit requirements such as length of sample application band (20-370 mm) width of sample (0.5-5 mm), speed of sample discharge, volume of sample to be applied (2-5000 µl), and interval between sweeps (5-360 s) depending on volatility of the solvent. Some of these streakers have an electrostatic dispensing system that eliminated drop formation during or after sample application. Others have a

jet spray gun and no syringes are used, which eliminates undesirable drop formation. These streakers provide quantitative sample application. The glass reservoirs used have a capacity of 0.1-5 ml. Narrow width streakers produce bands which give better resolution.

The application of a large sample, 1-3 ml, to a preparative plate, 0.5-1 mm thick, is not an easy task. Application as a spot leads to a series of microcircular chromatograms, while application with an automatic applicator as a streak produces two opposite frontal zones with a central zone containing the most strongly retained component (99). Soczewinski and Maciejewicz (100) solved the problem by using a sandwich tank with a glass distributor. The sample is applied to the edge of the plate with the glass distributor which forms a horizontal flat pipette in contact with the edge of the adsorbent. Partial separation takes place during the application which is then completely separated after plate development in an appropriate solvent.

THEORETICAL CONSIDERATIONS

Recently, there has been much progress in our understanding of the theory of separation in both partition and adsorption chromatography, and the effects of solvent-solvent and solvent-solute interaction. A few selected works are mentioned here. It is recommended that the reader refer to these for a more detailed discussion. Solute-solvent interactions on the surface of silica gel and the effect of using polar and non-polar solvents on the solute, as well as their behavior on the silica gel layer and competition

for the available sites have been studied (101, 102). It was found that a solvent bilayer formed on the surface of silica gel when using hydrogen bonding polar solvents. It was concluded that when a low concentration of a polar modifier was employed, the solutes interacted with a primary layer of polar solvents without displacing the solvent. However, at higher concentrations, the solutes interacted with the primary layer, displacing solvents in the second layer, but not reacting with the silica gel surface itself. For solutes with a polarity comparable with that of the modifying solvent, competition with the primary layer can take place, and the solute interacts directly with the silica gel surface (103).

Perry (81) studied the relation between solvent strength, selectivity and continuous development. He concluded that selectivity (the center-to-center separation ability) increased exponentially with decrease in solvent strength. Also, at the high selectivities which become available with decreased solvent strengths, the number of theoretical plates required for resolution became negligible. Spots were then resolved after very short migrations (81).

Soczewinski (103) studied the relation between eluent composition and retention. Equations were presented for the optimization of TLC and HPLC system. It was concluded that, depending on the system type, the RM (log K') values are often a linear function of the modifiers' concentrations. Martire and Boehn (24) presented a molecular theory of liquid adsorption chromatography. The theory

addressed the molecular mechanism, based on lattice models, of retention and selectivity in both normal phase and reverse phase liquid adsorption chromatography. Solvent-solvent and solvent-solute interactions were discussed and practical applications of the theory were presented. Chen and Horvath (104) evaluated the substituent contributions to chromatographic retention for quantitative structure-retention relationship. They present data obtained with different C_{18} silica gel stationary phases at various temperatures which suggest that quantitative structure-retention relationships can be transformed from one reverse phase to another as long as the eluent composition is the same. Guiochon et al (105-109) wrote a series of five articles dealing with TLC. In their articles, they discussed particle size and its effect on spot shape, size, band broadening and plate height; effect of mobile phase flow velocity in which it was shown that adsorption of solvent vapor from the gas phase on the dry layer can affect the solvent front velocity; optimization of experimental conditions such as particle size, flow rate, analysis time, and temperature effects, in reverse phase TLC. Guiochon and his co-workers present the TLC chromatographer with clear answers and an easy-to-understand discussion.

Finally, although Snyder's book (52) on adsorption chromatography was published in 1968 it is still a useful reference work for chromatographers using TLC and HPLC. Another good work is "Introduction to Modern Liquid Chromatography" (110) by Snyder and Kirkland, especially chapters 6, 8 and 9.

The optimum composition of a binary solvent mixture in TLC is normally determined by successive trials. A theoretical estimation of the optimum separation may be obtained from the relationship between Rm values of substances 1 and 2 and the composition of the mobile phase. The most favorable Rm value can then be measured using an equation which relates the above parameters. For details see Ref. (111).

COMPARISON OF TLC WITH HPLC AND GLC

Although GLC and HPLC are more accurate and afford superior resolution, TLC offers some advantages when compared with the above techniques. TLC can handle up to 40 different samples simultaneously on a 10 X 10 cm anticircular plate, whereas GLC and HPLC are limited to one sample at a time. TLC like HPLC can handle all types of compounds (except gaseous), but GLC is limited to compounds having an appreciable vapor pressure or that can be derivatized to products that have an appreciable vapor pressure without thermal decomposition. TLC offers the analyst the advantages of *in situ* spectroscopy and bioautography (88-90). Using HPTLC plates the chromatographer can analyze from 1-40 samples in less than 5 minutes.

Furthermore, classification of a large number of compounds can be achieved faster and easier by TLC than by GLC or HPLC. Different adsorbents and various solvent systems can be used (88). Generally, presample cleanup is not a must for TLC, while GLC and HPLC require reasonably clean samples befor using injected on to the column.

The Eluchrom makes simple, fast, and quantitative elution of samples from the plate and subsequent analysis by spectroscopic methods. Recently, TLC-AAS (91), TLC/IR (89) have been achieved by use of the Eluchrom for sample elution. The coupling of the Eluchrom to mass spectrometry has also been reported (112).

Overall, TLC is cheaper and faster than the other chromatographic techniques. Also, the use of the Eluchrom and other modern instruments makes TLC a very competitive and useful analytical tool.

CONCLUSION

This review clearly demonstrates that, in recent years, research into the basic aspects of TLC has been on the rise. These included, (1) modifications of the adsorbent (reverse phase C_2, C_8, C_{18}), the sample (derivatization) and the solvent (micellar and soap chromatography); (2) scaling down the size of the adsorbent particles and controlling their size (HPTLC); (3) decreasing the size of the plate (Triangular and HPTLC) and changing its shape ((circular and triangular); (4) the introduction of controlled development modes (U-Chamber for circular and anticircular, continuous development at room temperature and in the cold, pressurized ultra micro chamber, and a new sandwich tank); (5) the used of more than one adsorbent type side-by-side on the same plate and the use of a solvent programmer for gradient elution.

Theoretical studies of the basic aspects of TLC have been published which improved our understanding of the underlying TLC processes.

The challenge to TLC from HPLC has been met with new and useful advances, which makes TLC as modern a technique and as helpful and valuable an analytical tool as ever.

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