PITFALLS AND AIDS IN QUANTITATIVE THIN-LAYER CHROMATOGRAPHY

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Chromatography is a separation method based on the principle that components of a mixture can be separated from one another and concentrated into zones by passing the mixture through a two-phase system. One of the phases, the mobile phase, acts as carrier for the mixture and the other, the stationary phase, exerts a differential restraining effect on the components.

Chromatographic separations may be classified according to the nature of the phases, gas, liquid, solid (GC, LLC, LSC, GLC), according to the mechanism of retention (Adsorption, Partition), or according to some special feature of the technique (Ion-Exchange, Paper, Thin-Layer, Column).

Chromatography can be said to have begun from the classical work of Tswett. Developed, modified and improved by later investigators, it has become an important tool to chemists.

The principle of thin layer chromatography (TLC) was first described by the two Russian authors, Ismailov and Shraiber about the early part of this century. Meinhard and Hall, Kirchner, Miller and Keller later developed the method which then found use almost exclusively in the separation of terpenes. E. Stahl standardized the procedure in 1958 and showed chemists its wide applicability.

TLC at present has found good use for separating not only liphophilic substances but also hydrophilic compounds. Due to its sensitivity, simplicity, speed, and selectivity, it has found increasing use in Medicine, Biology, and Pharmacy.

Quantitative layer chromatography (paper and thin-layer) should in principle be accurate inasmuch as under identical conditions the quantity of substance present in the final spot should be in a fixed ratio to the quantity applied in the initial or starting spot. Also the pattern of distribution in the final spot should be constant so that the ultimate measurement should accurately represent the amount of substance originally applied. But in practice this is not so. Studies show that there are three phases in layer chromatography where errors can arise. These are 1) in the production of initial spots, 2) in the translocation of the substance from the initial to the final spot, and 3) in the treatment of the final spot and subsequent measurement.

A. PRODUCTION OF THE INITIAL SPOTS

Information gathered through studies of this phase in layer chromatography with the use of radioactive substances (radioactive glucose, tyrosine, morphine, etc.) showed errors ranging from + 6% to + 20% for individual measurements.

Formation of initial sports are commonly done in volume of 5, 2, or 1 ul using syringes and micropipettes and recently, automated devices which measure and inject the samples. Examination of the process of delivering the required volumes indicated possible sources of variation from "creep back", capillation and "operator effect".

The "operator effect" in the application of samples for quantitative work was investigated with a group of students using microcap disposable micropipette and the Hamilton syringe. A great deal of variations among individual operators was noted. With a repeating dispenser such as the Hamilton syringe, the mean coefficient of variation was 5.6% for unskilled and 3.5% for skilled operators. For the single application of samples by the microcap disposable micropipette the individual mean coefficients of variation ranged from 1.2% to as high as 53.4%. Using operators experienced in applying samples with the various instruments, errors as high as + 25% were registered.

The "creep back" effect was studied by observing with a lens the delivery of drops from the needle of an Agla syringe by free fall. It was noted that from time to time an accumulating drop suddenly slipped up slightly from the point and when the drop finally fell a certain amount remained on the stem. This "creep back" effect was cumulative and sometimes a sizeable volume remained on the stem for some time and unpredictably disappeared with the succeeding drop. This varied with solvent used but was particularly noticeable with methanol. It was observed that when delivering methanolic morphine, crystals of morphine would form a mark on the stem. Creep back would sometimes increase this mark appreciably but again unexpectedly a creep back would reach the mark and redissolve the crystals and carry it with the drop.

Another source of error arises from the fact that a measured drop may not always fall freely from the end of a needle as its weight may not be sufficient to overcome surface tension. Touching the drop on the surface of the adsorbent was found to cause a withdrawal of fluid from the lumen of the needle by capillarity which then would sometimes require 0.6—0.8 ul of solution from the barrel of the syringe into the needle before the succeeding drop appears. This effect varied according to the bore of the needle and time of contact with the adsorbent.

To minimize errors ensuing from the formation of the initial spots, it is suggested that (a) needle tips be coated with silicon, (b) the tip of the dispensing needle should be as fine as possible, (c) the tapered ends are bent till they are at right angles to the long axis and (d) an automatic device, which delivers small volumes by rapid ejection or by throwing, thereby forcing small drops to be forced onto the adsorbent without touching the surface, be used. The use of automatic injecting devices were found to give a coefficient of variation of ± 2.5%.

B. TRANSLOCATION OF THE SUBSTANCES FROM THE INITIAL TO THE FINAL SPOT

Errors during this phase of layer chromatography have been attributed to (a) lateral diffusion of solutes during the run, (b) variation in the structure of different sheets or layers, (c) variation from tank to tank and (d) retention on the starting line.

Variation from tank to tank can be eliminated by always using the same tank in identical conditions such as keeping it in a constant temperature bath and a critical solvent volume of 1%.

Lateral diffusion effect is the lateral spreading as solute molecules dissolve in the continuous sheet of flowing solvent. This effect depends on the relative rate diffusion of the solute and that of the ascending or descending mobile phase. Errors occur when varying amounts of solute diffuse out of its normal pathway during the run. This was found pronounced when running spots of different amounts side by side but cancelled out when running identical samples side by side inasmuch as the import-export of molecules between the spots balances out in a uniform surface layer. Studies with glucose showed that no significant error was found when similar quantities of glucose were chromatographed even with varying distances between spots of 4 to 30 cm. Coefficient of variation was only about 1.26% and no greater than 2.0% using a machine designed for initial spot application. However, running dissimilar samples side by side (20 ug and 200 ug) showed an increase in glucose in the glucose poor spots and a decrease in the glucose rich spots showing diffusion from glucose rich to glucose poor spots.

The solvent was found to contribute to diffusion. Single component solvent with Rf value between 0.5 and 0.75 was found to be desirable but higher Rf values were found to run into interference from adsorbent impurities carried by the solvent front and also found to enhance diffusion. It was also pointed out that in the case of mixed solvents, if demixing occurs and the compound under investigation travels with the B-front, lateral diffusion of the spots occurred.

Studies on variation between sheets or layers have been found more pronounced in TLC than in paper chromatography. Morrison and Orr. Fairbairn and Suwal and Fairbairn and Wassel, have reported that the slope of the regression line relating to the measurement of the final spot with the quantitates applied to the initial sports were consistent within one sheet or layer but were not identical with those from others even when operated under similar conditions. It was also noted that deviations between spots measure on the same plate was less than the deviation between spots on different plates.

Regarding retention on the starting line, it was observed that if the initial spots are dried vigorously some of the solutes are irreversibly attached to the layer. This could be avoided by drying the initial spots in a current of cold air.

The degree to which the type of adsorbent and the specific surface area affects quantitative measurements was studied by separating a series of mixtures of different adsorption energies and different abilities to form hydrogen bonds on di-

ferent adsorbents with different specific surface areas. The results obtained showed that: 1) The same substances on different types of adsorbents gave different spot areas and different peak areas. 2) The size of the spots decreased with an increase in the specific surface area of adsorbent. Changes in spot areas were more pronounced with adsorbent of specific surface areas less than 200 m2/g, attributed to the absence of high adsorption interactions which cause the mobile phase to induce greater diffusion. 3) The spot size depends on the specific surface area of adsorbent, in agreement with the ability of solvents to form hydrogen bonds. 4) The Peak Area depends to a great extent on the adsorbent used as the staionary phase—highest for relatively small specific surface area. 5) The Peak Area values of isomers depend on their structure.

C. TREATMENT AND EVALUATION OF THE FINAL SPOTS

Several methods had been used for the quantitative analysis of the sample resolved in the thin layers. The earlier method was to recover the substance from the adsorbent before quantitation whereas the present practice is to relate the sizes or some function of the intensities of the spots to the weights of material contained in them.

Elution Technique

The most accurate results in quantitative TLC were obtained by eluting the components analyzed from the adsorbent with a suitable solvent followed by spectrophotometric or colorimetric measurement.

Elution technique requires that components are separated into clearly defined areas which can be removed quantitatively from the plates, eluted, and estimated.

The areas in which the required adsorbed compound is located is carefully scraped and transferred to suitable flasks or beakers for further treatment. The elution may be done through simple agitation with a solvent in which the solute is soluble and the adsorbent removed by filtration or by soxhlet extraction. The eluting solvent depends on the nature of the adsorbed substance andt the adsorbent.

The quantitation of the eluate may be done by gravimetric estimation, spectrophotometry, colorimetry, flourimetry, polarography, or radiometry.

The gravimetric method in which the residue is weighed after evaporation of the eluting solvent is found to yield poor results and tends to give high results. Possible sources of error are: (a) extraction of other substances from the adsorbent, (b) the amount is too little for accurate measurement in balances hence needs a special microbalance and (c) the substance may not all be extracted.

Quantitation by spectrophotometric methods may be undertaken by direct measurements in the ultraviolet or visible wavelength range for absorbing species or by indirect measurements of non-absorbing specie by first reacting the substance with a suitable chromogenic reagent before measurement is taken.

Densitometry

Densitometry is a method whereby the intensity of color of a substance is measured directly on the chromatrogram. This method was first used for measuring amino acid concentrations in bands separated by electrophoresis and then on paper chromatograms of amino acids, sugars and steroids. It is faster than elution methods but is affected by a number of factors which are not encountered in solution densitometry for colorimetry.

Densitometric TLC may be performed indirectly. The chromatogram is photographed or photocopied and the photograph scanned.

In both direct or indirect densitometry the parameters that one must consider in relation to precision and reproducibility are those relating to the instrument and those relating to the chromatogram.

The basis of the determination is that of measuring the light absorbed when a beam of light falls on the spot. This is usually done by taking the difference of the incident light beam and the light that passed through. The difference which is the absorbed light is related to the intensity of color of the spot which is related to the amount of substance present in the spot. For precision and reproducibility the following instrumental parameters are made constant:

a) Filters — The filters are used to control the wavelengths of the incident light especially for continuum source. It allows only a certain wavelength change to pass through.

To achieve optimum results the colored substance must absorb as much of the incident light as possible and the background as little as possible. The choice of the incident light depends on the material scanned.

b) Aperture — The shape and size of the spots are the main factors to be considered when selecting the most suitable aperture. Some investigators have found a circular aperture of 1 mm diameter best, but others have found that a slit aperture gave better results and that a lower coefficient of variation was obtained when the slit length was smaller than the width of the spot.

c) Position of Slit in Relation to Spot — It is recommended that scans be run at the real peak maximum. To do this it is suggested that an initial scan be made and then scans run 1 mm shifted to each side of the optimum point be done to make sure that the scan is at the real peak maximum.

d) Method of Scanning — Scanning the spot may be done by reflectance or by transmittance method. In transmittance method attempts must be made to reduce light scattering by making the adsorbent as transparent as possible.

e) Director of Scanning — The spots can be scanned along the line of development or perpendicular to it. The choice depends on the nature of the chromatogram. In scanning parallel to the direction of development, the baseline may not return to the same level. This happens if the solvent leaves a narrow tail in the center or the spots are not completely separated or impurities are deposited between the separated spots. Generally, it is advantageous to scan in both directions and

average the two results.

It should be noted that when scanning a spot with a recorder fitted with an integrator which automatically gives a count proportional to the area of the curve, an error is incurred when the recorder does not return to the original baseline. Furthermore, it should be remembered that when scanning vertically or horizontally, care must be taken that the beam of light passes through the center of the spot.

f) Speed of Scanning — It has been noted that for any given spot, the slower the rate of scan the larger will be the integrated reading or curve area so that there is a necessity of maintaining the same gear ratio or rate of movement of TLC plate carrier for all observations of a particular determination.

The above pertains to instrumental parameters which must be fixed or set constant. But what about the parameters related to the chromatogram? These factors associated with the colored substance at the time the color instensity is taken is very important. None can be made constant so that it is essential to keep them at a minimum. With the instrumental parameters kept constant, results can only be reproduced if the colored spots are identical in shape, size, profile in depth, distribution, and the quantity of substance on the absorbent and its relationship to the background adsorbent. If the substance is colored and the background is white the measured color intensity will show a clear relationship with the quantity of substance present. If however, the substance has to be treated with a reagent to produce a colored compound the following conditions have to be observed; (a) the colored product must not diffuse out into the surrounding area, (b) the amount of color produced must be proportional to the amount of sample, (c) the colored product must be reproducible and stable and (d) the reagent should leave the background white or at least be as contrasting as possible with the spot. There might be also a time factor involved as noted in the reflectance measurements of DNP derivatives of amino acids which showed a steady drop in reflectance during the first 100 minutes and then became fairly constant for a period of time. This indicates that when doing the measurement, a definite time schedule had to be followed and measurement should be taken when change had leveled off or where the rate of change had become insignificant.

Other factors involved are (a) the variation of peak areas with shape, size, and load of the initial spot, (b) variation of peak area with the Rf value, (c) variation of peak area with the nature of the stationary and mobile phases in the separation process, (d) variation of peak area with the thickness of adsorbent layer, (e) variation of peak area with moisture content of the adsorbent layer. A change of 3% in relative humidity leads to a change in the order of 1% in the peak area irrespective of the variation due to Rf value, and (f) variation of peak area due to the presence of substances in the mixture other than those being examined. In an artificial mixture of 4 alkaloids, the coefficient of variation was about 5% but examining plant material containing 2, 3, 5, 7, and 11 alkaloids found that with 2 or 3 alkaloids the coefficient of variation was less than 4% but with 7 or 11 it was up to 10% or higher.

Densitometry is a quick and reliable method for determining the amount of substance directly from the chromatogram after its separation from a mixture. But

care should be taken in the preparation of the chromatogram and in developing the colored complex since the reproducibility and precision depends almost entirely upon the actual nature and distribution of the colored complex at the time it is scanned. Better results are obtained by using the mean of a number of determinations on several plates rather than from a calibration curve or data from one plate.

Fluorimetry

Some compounds fluorescewhen exposed to light of a given wavelength. The amount of light emitted is directly proportional to the amount of the fluorescing compound. Quantitative TLC by fluorimetry may be done by:

1) Measuring the native fluorescence after chromatrographic separation (CF).
2) Non-fluorescent compound is chromatographed then rendered fluorescent with fluorescent spray, then the fluorescence of the compound is measured (CRF).
3) The compound is rendered fluorescent, chromatographed, then the fluorescence measured (RCF).
4) The compound is chromatographed then fluorescence quenching measurement is taken (CQ).
5) The compound is reacted, chromatographed then fluorescence quenching measurement (RCQ) is made.
6) The compound is chromatographed, reacted then quenching measurement (CRQ) is taken. Measurement of fluorescence is done with a fluorimeter.

Factors to be considered in fluorescence measurements are:
a) Wavelength of exciting light. This should give a linear relationship between area of peak and amount of fluorescent compound.
b) Effect of adsorbent on the amount of fluorescence.
c) The moisture content of the layer.
d) The size of spots in fluorescence quenching measurements inasmuch as only a fraction of the depth of the spot is used in quenching, hence, it is imperative that size and loading volume of spots be consistent.

Fluorescence measurements are sensitive and measurements can be made in the nanogram range in contrast to the microgram range in density or reflectance measurements. Also relative standard deviation of 4% to 6% on the same plate and 8.6% 12% on different plates when conditions and techniques which are carefully standardized are obtainable.

Reflectance Measurements

In reflectance measurements light of preselected wavelength is made to strike the adsorbent layer vertically with the slit sharply focused onto the spot. The incident beam which strikes the adsorbent layer penetrates to a certain depth and then that which is not absorbed is reflected or scattered and the reflected light which reaches the measuring system (photomultiplier) is measured.

The ways of carrying out reflectance measurements are:
1) remove the spot and together with sufficient additional adsorbent to give a constant weight; intimately ground and pack uniformly in a cup or cell and measure. A blank consisting of adsorbent treated in the same manner is also measured. This method is more time-consuming but is more precise.
2) measure directly the spot. This method is not greatly influenced by layer thickness but needs standardized coating procedure for uniform and reproducible plates. It also needs a white background beneath the layer during measurements. This method was found to give standard deviation of 1%–5.3% on the same chromatogram and 4%–6% on different chromatograms under carefully standardized conditions. A modified method which tends to reduce systematic errors due to chromatographic parameters that influence reflectance value as a function of varying Rf values, is the in situ reflectance spectroscopy. This uses the principle of data-pair wherein the reference and standards are spotted in such a way as to give a pair for each concentration with spots 1/2 plate width apart. An average of 4 readings are taken per spot (2 forward and 2 backward scans in the direction of chromatography).

Spot Area Measurements

Measuring spot area is another means of TLC quantitation. This is usually done by (1) taking the area with a planimeter, 2) photographing and then cutting out the spot and weighing, 3) transferring to a square millimeter paper and counting the squares. Among these, the planimeter gives the best results.

The relationship between area and the amount of compound has been the subject of considerable discussions. A straight-line relationship between the area of the spot and the quantity of substance was found by Petrowitz in his studies of some insecticides and Seiler in the determination of inorganic ions. Aurenge, et. al. in their work with phenols obtained a straight-line relationship by plotting the square of the surface area against the weight of the sample. Purdy and Truter obtained a linear relationship between the square root of the area and the log of the weight of the compound in their examination of sixteen different compounds. Others not obtaining strictly linear curves had to take a compromise by using only those short sections of curves which obeyed a linear relationship.

Nybom, who investigated the relationship found that different relationships existed between area and weight of material with a different layer thickness. A thin layer gave a linear relationship between log weight and area and a thick layer between weight and square root of the area. He also discovered that visualizing agent also affects the area-weight relationship as revealed in his study of alamine.

Spot area measurements wherein the area and weight relationship is used for quantitation is not the most accurate way for TLC plates but can provide some answers in the absence of more elaborate equipment.

Radioactive Methods

With radioactive substances or radioactive labelled compounds separated by TLC, quantitation may be done by Autoradiography, Liquid Scintillation Counting, or scanning of radioactive chromatogram.

In autoradiography, an X-ray film is exposed to the developed chromatogram and the developed film is scanned with a densitometer.

In Liquid Scintillation Counting, sections of the adsorbent is transferred from the chromatogram into vials and added with scintillator and counted.

The third method uses a radiation device (Thin-window Geiger-Muller tube, gas flow Geiger-Muller) producing a signal which is recorded to give a peak or peaks corresponding to the separated compounds.

Factors to be considered when doing radioactive TLC are: a) Scan Speed — to minimize error, scan speed should be such that a high count is obtained. This, therefore, necessitates slow scanning, b) Detector Voltage — the gas flow detector requires a high voltage. The magnitude of the response from the detector and hence, detector efficiency, varies with the applied voltage. The optimum voltage can only be determined experimentally by plotting the voltage/response curve when used with a radioactive source. Response increases with voltage until a plateau is reached, then with further increase, instability occurs due to the arching of the detector. For maximum efficiency and instability the voltage should correspond to the center of the plateau. The voltage of instability depends on the level of radioactivity (lower for samples of high activity). c) *Detector Height* — Detector height affects peak height, peak width, and peak area, hence, must be maintained at constant. d) *Detector Efficiency* — The efficiency of a detector is the ratio of the number of counts recorded per unit time to the number of B-particles entering the sensitive volume of the detector per unit time.

In the scanning of radioactive TLC some of the requirements to produce optimum results are (1) a low scan speed, to enable the maximum number of counts to be recorded (2) a narrow slit for maximum peak separation, and (3) the minimum distance between chromatogram and detector, to improve resolution and detector efficiency.

There are other, newer methods/techniques which are modifications of the thin-layer scanners and detectors but the basic principles of quantitative thin-layer chromatography remains the same and hence, awareness of the pitfalls would contribute greatly in the reduction of error.

CONCLUSION

From the number of published articles in this subject, one can visualize the wide applicability and popularity of this technique. But in undertaking such analysis one must always take into consideration the nature of the substance to be analyzed, the manipulative ability and technique of the operators, the equipment available, and the time factor. Taking all these factors into account he must assess the merits of alternative methods to obtain the desired accuracy.

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