Chapter 11

Thin Layer Chromatography Applications

Quantitative analysis by TLC is most frequently carried out by the visual comparison of the intensity of the spot of interest to that of a standard. This, for example, would allow the sample concentration to be compared with that of a legal standard to ensure compliance with regulatory limits. For more accurate work, spot scanning techniques must be used. In general, it is the optical properties of the separated material that are used for the quantitative assessment of a thin layer spot. If the spot adsorbs in the visible range of wavelengths, then both reflected and transmitted light can be measured. If, however, the substance in the spot only adsorbs in the UV range of wavelengths, then only reflected light can be measured, as the silica base of the plate itself will adsorb UV light. If the substance fluoresces, then the fluorescent light can be directly measured as emitted light, or, alternatively, if a fluorescent plate is used, the quenching due to presence of the spot can be measured. Unfortunately, there is no simple linear relationship between spot transmittance or reflectance, and calibration curves must be obtained.

The relationship (with the exception of fluorescence) between the measured property and the mass of solute in the spot is obtained by arbitrarily fitting an appropriate function (usually a second or third order polynomial) to a set of calibration data. The calibration curve is
then used for subsequent analysis. The calibration data are usually fitted to a polynomial function. However, care must be taken not to use too high an order of polynomial as the improved correlation will usually include fine structure in the curve fit which does not exist in practice and which will often lead to serious errors. To avoid this situation the order of the polynomial should never be greater than one-fifth of the number of data points. For example, to fit a second order polynomial there should be at least 10 calibration points that scan the total range of concentrations that are likely to be analyzed. If a third order polynomial is needed, then there should be at least 15 data points spanning the concentration range.

![Figure 1 Peak Height Measurements on a TLC Plate](image)

Fluorescence measurements have a number of advantages over transmittance and reflectance as the relationship between emitted light and solute mass is closely linear. In addition, the noise level is much smaller and thus the measurements more sensitive (the detection limit can be one to two orders of magnitude smaller than that obtained from transmittance or reflectance measurements) and as a result the measurements have a wider dynamic range. It is usually essential to insert a cut-off filter between the plate and the fluorescence sensor to remove all excitation light. The presence of excitation light provides a background signal which increases the noise and thus reduces both the sensitivity and the linear dynamic range of measurement.
Measurement Methods

There are two common methods of spot assessment: peak height and peak area measurements (including sliced area measurements), which have been discussed in earlier chapters. The measurement of peak heights is depicted in figure 1. The advantages of peak height measurement over peak area measurement have also been discussed in earlier chapters, but it is clear that the possibilities of error by peak overlap are much reduced if height measurements are employed. Examples of two types of peak integration are shown in figure 2.

![Total Integration and Sliced Integration Diagram](image)

**Figure 2 Different Forms of Peak Integration**

It is clear that for accurate total area integration, adequate resolution must be achieved, or there will be significant errors introduced due to
peak overlapping. However, complete resolution is far more difficult to achieve in thin layer chromatography than in other forms of chromatography and thus a modified form of peak integration is sometimes used, called sliced area integration. This procedure is depicted in the lower chromatogram in figure 2. Sliced area integration is a procedure that attempts to maintain the accuracy of area integration and maintain some of the advantages of peak height measurement. It is clear that slice integration does eliminate some of the errors caused by peak overlap. This procedure requires very careful selection of the limits used in the computer integration protocol, but if carefully carried out, can be a useful compromise between peak height and peak area measurements when using scanned data from a TLC plate.

There are a large number of applications employing TLC for quantitative analysis described in the literature, many of which, although applied to different materials, are from the point of view of the technique very similar in nature. The following examples are taken from reports mostly published either in the Analyst, Analytical Chemistry or the Journal of Planar Chromatography and these journals, together with the Journal of Liquid Chromatography are recommended as reliable sources of application information for those starting to use TLC for quantitative assays. The versatility of the technique is highlighted by the widely different application areas from which the examples are taken.

The Assay of Sulfamethazine in Pork Carcasses

Sulfonamides in general are widely used in veterinary practice as antibacterial agents, and sulfamethazine (4-amino-\(N\)-(4-6-dimethylpyrimdin-2-yl) benzene-sulfonamide) is one that is commonly used in pig husbandry.

Unfortunately, sulfamethazine can be potentially misused; for example, if there is an inadequate withdrawal period prior to slaughter, there may be a level of the drug in the meat that exceeds the minimum allowed by law. Consequently, a sensitive, rapid, on-site
analytical procedure is required to ensure regulatory compliance. Shearan and O'Keeffe [1] developed a rapid technique with a TLC 'finish' that allowed the level of sulfamethazine to be determined as below, above or coincident with the statutory limit, at a rate of 20 samples per day.

The extraction procedure was similar to that developed by Shearan et al. [2]. 0.5 g of tissue was blended with 2 g C18 reversed phase adsorbent in a mortar and pestle for 30-45 s. The blend was then packed into the syringe barrel of a solid phase extractor and the contents washed with 8 ml of hexane. The blend was then extracted with 8 ml of dichloromethane and the extract evaporated to dryness in a stream of nitrogen and the residue taken up in 100 μl of dichloromethane.

The silica plates were 10 x 10 cm with a pre-absorbent strip for spot focusing. 40 μl aliquots of extracts and standards were applied to the plate using micro capillary pipettes. The plate was dried and placed in a chromatographic tank containing methanol. The solvent was allowed to advance to the top of the pre-absorbent strip and was then removed and dried. The plate was then placed in another tank filled with ethyl acetate and the solvent allowed to advance 2 cm beyond the pre-absorbent strip; the plate was then removed and sprayed with fluorescamine solution. After 15 min the plate was viewed under UV light (366 nm) to locate the fluorescent bands. Where it was necessary to resolve, sulfadiazine, sulfathiozole and sulfurmerazine from sulfamethazine, the ethyl acetate was allowed to advance 3.5 cm beyond the pre-absorbent strip.

The technique recovered about 80% of the antibacterial agents at levels between 50-100 ppb in porcine tissue, with a minimum level of detection of about 30 ppb. The standard used contained 0.2 μg per ml; less dense spots were reported as negative, containing the antibacterial agent at a level below that permissible; more dense spots were reported as positive and indicated that the antibacterial agent was present at a level above that permissible. This is a typical example of the more common type of quantitative analysis by TLC. The analysis
determines whether a component is above or below the level of a standard and employs the eye as a comparator (for which it is most efficient) to give a pass or fail result.

The Determination of Aflatoxins in Palm Kernels

Aflatoxins are highly toxic metabolites produced by the molds Aspergillus flavus and Aspergillus parasiticus. These molds thrive on substrates rich in lipids and carbohydrates and particularly under the damp conditions resulting from the high relative humidity and elevated ambient temperatures common in tropical countries. The oil palm Elaies guineensis Jacquin occurs both wild and cultivated in the equatorial areas of Africa, South East Asia and South America. Palm oil is obtained from both the fruit and the nut, the latter containing as much as 50% of its mass as oil. The oil finds commercial use in the food and detergent industries and the residual pressed 'cake' provides a protein source for animal food. The aflatoxins are generated both before and after harvesting and their production is aggravated by cracking, insect activity and poor storage conditions. Due to the high toxicity of the aflatoxins, maximum levels of contamination have been set by most user-countries and consequently, efficient accurate and precise methods for their measurement had to be developed. Nawaz et al. [3] devised a practical analytical procedure that involved an efficient extraction procedure followed by a TLC separation. The plate was finally quantitatively assessed by automatically scanning the aflatoxin fluorescence.

Five hundred gram of finely ground palm kernel (appropriately sampled) were slurried with 750 ml of water in a blender at high speed for 3 min. A 100 g portion was then extracted by blending with 240 ml of acetone and filtered through a Whatman No. 1 filter. 5 ml of the crude extract was mixed with 5 ml of methanol, 1 ml of 20 %w/v lead acetate solution, 1 g of Celite and 63 ml of water. The lead salt was used to precipitate colloidal contaminants from the sample. The mixture was then passed through a solvated PH bonded phase cartridge at about 10 ml/min. (PH cartridge 608303 was solvated by treating with 10 ml of methanol followed by 10 ml of water). The
cartridge was then washed with 10 ml of water and the aflatoxins eluted from the cartridge with 3 ml of chloroform.

The extract was dried by passing it through a tube packed with anhydrous sodium sulfate and collected in an 8 ml vial. The solid aflatoxins were obtained by evaporation at 45°C in a stream of nitrogen. The HPTLC plates (aluminum backed, 20 x 20 cm, Merk 5547) were immersed in methanol for 1 hr to remove any adsorbed material, dried for 5 min at 100°C and stored in a dessicator. The sample extract was dissolved in 300 µl of a benzene:acetonitrile mixture (98:2). 5 µl aliquots, occasionally interspersed with standards, were applied to the plate in the absence of light with an auto sampler. Sample spots were 5 mm apart along the top edge of the plate and one 10 x 20 cm plate could accommodate 30 sample spots and three standards along the 20 cm edge. Edge effects were eliminated by removing a strip of silica gel, 3 mm wide, along the plate edge parallel to the direction of development.

Before the analytical development was commenced, a further clean up was carried out on the plate by developing the plate in 20 ml of diethyl ether for 17 min in a continuous horizontal tank (the eluent reached the top of the plate in about 3 min). Development was continued for about 14 min., and, as a consequence, there was no solvent front. The plate was dried in a darkened chamber for 3 min in a stream of nitrogen. The interfering substances were contained at the plate edge and were removed by scraping 2 cm of silica from the edge. The plate was then rotated and developed in a normal tank and developed for 20 min with 20 ml of chloroform-xylene-acetone mixture (6:3:1). The plate was again dried for 5 min and the bottom portion (1 cm) of the silica was removed to decrease the development time and increase the resolution. The plate development was then continued with the same solvent mixture for 16 min. The plate was finally dried in a fan assisted oven at 100°C and scanned for fluorescence by the reflectance mode using the TLC II scanner (Camag 76610) and the SP 4270 integrator (Camag 76650). Scanning techniques are discussed in the previous chapter.
The procedure appears complicated and is certainly lengthy for this type of analysis, but recoveries in excess of 90% were readily obtained and detection limits for the aflatoxins AFB₁, AFB₂, AFG₁ and AFG₂ were found to be 3.7, 2.5, 3.0, and 1.3 μg/kg respectively. Compared with the British Standard Method, the procedure was found to be both more efficient and more precise.

**Determination of the Platelet-Activating Factor and other Phospholipids in Human Tears**

Phospholipids are important components of biological systems and the platelet-activating factor (PAF), 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine, is particularly significant as it is an agent of immunization for a range of different diseases. PAF is difficult to determine in serum and other biological fluids as, following its secretion, it is immediately hydrolyzed by acetylhydrolase to form 1-hexade-canoyl-sn-glycero-3-phosphocholine (LysoPAF). In tears, however, acetylhydrolase is possibly absent or at least present at very low levels as in saliva, but there are also very small amounts of phospholipids present that offer the possibility of determining PAF in such samples. Nevertheless, due to the very low concentrations present, a concentrating technique is necessary. Ohyama et al. [4] developed a satisfactory analytical method using an extraction procedure followed by a TLC separation and a plate scanning 'finish'. The inorganic phosphate was liberated from the phospholipid molecules by an enzyme reaction which was followed by a high sensitivity color reaction that produced an ammonium molybdate-malachite green aggregate.

Tear samples were obtained by introducing 40 μl of physiological saline solution into the inferior conjunctival sac. After three gentle eye blinks, a 2 cm diameter circle of filter paper was placed near the inner canthus for tear collection. The filter paper was then soaked in 500 μl of chloroform-methanol-water mixture (6:69:50 v/v). The liquid was then passed through a diatomite column to remove proteins, sugar and polar inorganic substances and the lipids were then eluted with 6 ml of
a chloroform-methanol mixture (95:5). The eluate was evaporated to
dryness under vacuum and the residue dissolved in 50 μl of
chloroform, 20 μl samples of the solution were used to spot the TLC plate.

Figure 3 Typical Chromatogram of the Phospholipids in
Human Tears

A high performance silica gel plate was employed (HP-K, Whatman)
which was soaked overnight in a methanol water mixture (1:1 v/v) and
then dried for 1 hr at 120° prior to use. The plate was developed in
two dimensions; first with a hexane-diethyl ether mixture (4:1 v/v) for
the removal of lipids other than phospholipids; second the plate was
then developed at right angles with a chloroform-methanol-water
mixture (65:35:7 v/v) to separate the phospholipids. After drying, the plate was sprayed with phospholipase C (PL-C from Bacillus cereus) and then alkaline phosphatase (Al-P from human placenta) to hydrolyze the phospholipids.

The volume of each enzyme solution used was equivalent to 2 ml/100 cm² of plate. The two enzymatic reactions were allowed to take place for 10 and 15 min respectively at 45°C. The plates were then sprayed with the Mo-MG reagent (200 ml of 38 mmol/l ammonium molybdate, 100 ml of 8.7 mol/l sulfuric acid and 200 ml of 3.2 mmol/l of malachite green and 500 ml of water) at a coverage of 2 ml/100 cm² of plate. The phosphates from the phospholipids reacted with the reagent to produce the intense blue-green spot of molybdophosphate malachite green. After 5 min. the plates were scanned at 620 nm with a CS-9000 two-dimensional densitometer (Schimadzu, Kyoto, Japan). A trace of the absorbance profiles (the chromatogram) is shown in figure 3. The quantitative assessment was carried out using peak area measurements in conjunction with calibration curves from standard phospholipid solutions. The peak area was found to be linearly related to concentrations over the range of 2-100 pmol per spot (RSD was 2%, n=7) About 86% of the PAF was recovered. The proportion of the individual phospholipids appeared to be sensibly constant for healthy humans and PAF, LysoPC, PC and PE were present at 26.2, 42.3, 10 and 19.7% respectively.


A hybrid TLC plate system has been employed as a transport medium to monitor a thin layer separation by means of a mass spectrometer. The combination has been used to determine substances of biological origin at extremely low levels of concentration. The essential transport medium was a thin layer plate, half of which was coated in the normal manner and on which the separation was developed.
other half consisted of a special coating that formed the base of the so-called matrix assisted laser desorption ionization source for the mass spectrometer. A diagram of the hybrid plate is shown in figure 4. The plate was a modified form of the aluminum-backed silica gel plate manufactured by Merck (Cat. No. 1.0555). Silica was scraped from one side of the plate in such a way that resulted in a taper over a horizontal distance of 1 cm so that the thickness gradually decreased from the original 250 μm to zero over a distance of 1 cm, thus providing a gentle change from one coating to the other. About 5 g of the desorption medium containing 2,4,6 trihydroxyacetophenone or 2,5-dihydroxy benzoic acid was dispersed in a mixture of toluene and ethyl ether (60:40 for 2,4,6 trihydroxyacetophenone and 80:20 for 2,5-dihydroxy benzoic acid) and was sprayed onto the exposed plate backing.

The silica gel layer was appropriately shielded from the spray by a microscope slide. The coating was carried out after the separation had been developed. The samples used to test the device were Valinomycin, cyclosporin, and the protected peptide BOC-Phe-Lue-Phe-Lue--Phe. The TLC separation was carried out in two stages, first using chloroform which only moved the BOC-Phe-Lue-Phe-Lue--Phe

![Figure 4 The Hybrid Thin Layer Plate that Acts as a Transport Interface to a Matrix Assisted Laser Desorption Ionization Source for a Mass Spectrometer](image-url)
and then ethyl ether-toluene which only moved Valinomycin, which
provided three separate spots suitably separated. The other side of the
plate was then coated with the desorption material as described above
and the plate rotated through 90°. The solute spots were eluted into the
desorption zone by the solvent mixture chloroform-acetic acid-
methanol containing 0.1% trifluoro-acetic acid, (1.5:1:1). After
transfer of the spots by elution each spot area was treated with
methanol-water mixture (1:1) to improve analyte-desorbing agent co-
crystallization. After separation, the plate (now acting as a transport
system) was transferred to the laser desorption mass spectrometer
source and spectra taken in the usual manner. The spectrum obtained
form the Valinomycin spot is shown in figure 5, where the results
from the hybrid plate are compared with the more conventional
contact pressing transfer procedure.

Figure 5  Spectra Obtained for 4.5 pmol of Valinomycin
from the Hybrid Plate and by Contact Pressing Transfer

It is seen from figure 5 that the use of the hybrid plate increases the
signal to noise ratio significantly and consequently increases the
sensitivity of the overall system by an order of magnitude or more. For quantitative work it is clear that an external standard will be necessary and the precision of quantitative assays is not reported. However, the technique offers the possibility of very high sensitivity, specific identification and a reasonably rapid assay.

(a) 90 fmol of Valinomycin
(b) 0.83 pmol of Cyclosporin
(c) 6.3 pmol of BOC-Phe-Lue-Phe-Lue--Phe
(d) 2,4,6 trihydroxyacetophenone used as the matrix.

Figure 6 Spectra of Valinomycin, Cyclosporin, and the Protected Peptide BOC-Phe-Lue-Phe-Lue-Phe Obtained from the Hybrid Plates

The spectra obtained for the four test substances are shown in figure 6. It is seen that the sensitivity of the system ranges from about 90 fmol to about 6 pmol.
Determination of Caffeine Using Both Densitometry Measurements and an Image Analyzing System

A relatively recent method for scanning TLC plates that has been reported is by the use of an image analyzing system. This employs a digital camera to photograph the plate, the image is then stored in digital form in a computer and then processed by appropriate software. Vovk et al. [6] compared the relative precision obtainable from the image analyzing system to those obtained by normal scanning densitometry. The authors chose caffeine as the model substance and the separation was carried out on a 10 cm x 20 cm glass backed plate carrying a coated layer of silica gel 0.2 mm thick (Merck, Darmstadt, Germany). The samples were place on the plate with a Linomat IV applicator (Camag, Muttenz, Switzerland) which was equipped with a 100 μl syringe. The band was 10 mm wide, and 5 μl of sample was applied at a rate of 4 μl/s. Ten or twelve bands were applied per plate 15 mm apart and 15 mm from the bottom edge. The plates were developed with a solvent mixture consisting of dichloromethane:methanol (90:10 v/v) in an unsaturated glass twin trough chamber. The caffeine solute migrated 7.5 cm and the migration time was approximately 10 min. The plates were subsequently dried in a stream of warm air for about 5 min. A chromatogram of a separation monitored by densitometric scanning is shown in figure 7.

Figure 7 Chromatogram Obtained by Densitometer Measurements at 270 nm of a Caffeine Standard
The scanner was operated in the reflectance mode with slit widths of 0.8 mm and 6 mm respectively, the monochromator band width was 30 nm (wavelength of maximum absorbance $\lambda=270$ nm). The densitometry processing was carried out with a Camag TLC scanner II, equipped with a built-in twelve bit analog-to-digital converter and controlled by an external computer with a RS32 interface. The software used was the Q TLC-pack (KIBK-IFC, 1990).

![Calibration Curve from Densitometer Scanning](image1)

![Calibration Curve from Digital Camera](image2)

Figure 8 Calibration Curves Obtained by Densitometer Measurements at 270 nm of a Caffeine Standard and the Image Analyzing System at 259 nm

The image analyzing procedure commenced with the spots being captured on a high sensitivity CCD video camera (Hitachi Denshi Ltd.)
model HV-C20). All images were obtained by exposure to direct UV light (λ=254 nm). The data were processed using the Camag Video Documentation system in conjunction with the Reprostar 3 and the Camag Video Scan 1.16 program for the evaluation of thin layer chromatograms. Calibration curves are shown for both data processing methods in figure 8. It is seen that the calibration curve for the image analyzing procedure is linear whereas the curve for densitometry measurements is not. However, despite this, the two procedures gave very similar precision and showed no significant difference in evaluation parameters.

**The Use of Digital Auto-Radiography for the Analysis of Biological Samples and for Studying Drug Metabolism**

Digital auto radiography is another technique that is used for quantitatively assessing thin layer plates when, for example, radioactive tracers are employed to follow the metabolic pathway of certain drugs. There are two basic methods of radioactive monitoring, both of which are extremely sensitive as, by the use of extended periods of measurement, they can identify very low levels of radioactivity. The first is contact radiography where, after separation and drying, a photosensitive film is placed in contact with the plate and exposed to radiation for an appropriate time. Obviously the longer the time of exposure, the lower the radioactivity that can be detected. The film is then optically scanned to obtain density measurements which will be proportional to the number of emission particles passing through the film. The second method is to use an automatic radioactive counter-scanner that will scan the plate, in much the same way as a optical scanner, but measure the frequency of particle emission across the plate directly. Again the sensitivity can be increased by slowing the rate of scanning, making a wide range of sensitivities available, while trading in time for sensitivity. Due to the first method requiring exposure time and scanning time, in general, the later is the faster method of evaluation.

Kjebovich *et al.* [7] demonstrated the successful use of auto-radiography to follow the metabolism of deramciclane (a new...
anxiolytic drug) in dogs, from urine samples analyzed by thin layer chromatography. An example of the radio chromatograms obtained are shown in figure 9.

Urine Sample Taken 0-12 hrs after Treatment

Urine Sample Taken 12-24 hrs after Treatment

Urine Sample Taken 24-48 hrs after Treatment

Distance Migrated Along the Plate

Courtesy of J. Planar Chromatogr [ref. 7]

Figure 9 Digital Auto-Radiograms of Dog Urine Samples Taken 6 hr, 18 hr and 36 hr After Treatment with $[{}^3H]dexametacine$

The dogs were treated both orally and intravenously with $[{}^3H]dexametacine$ at doses of 3 and 10 mg per kilo respectively. Urine was collected between 10 and 12 hours, 12 and 24 hours and between
24 and 48 hours respectively. Samples were applied to the plate in 10 mm bands with a precision syringe and developed in a normal chamber to a distance of 16-18 cm. After development the plates were dried and scanned with a LB 287 type Berthold Digital Auto Radiograph in conjunction with Hewlett-Packard Vectra VL24/66 computer controlled with WinDAR software V.1.09. The instrument could scan an area of 20 x 20 cm which contained a 600 x 600 wire grid, two-dimensional, position-sensitive multiwire proportional chamber. A 9 to 1 mixture of argon and methane bubbled at 5 ml/min through methylal at 2.8°C was used as the counting gas and the potentials employed for $^3$H- and $^{14}$C-labeled compounds were 2040 and 1200 volts respectively. Signal analysis was performed by measuring 5 x 360000 detector cells per sec. The separation took about 6 hr and the detection took only 2.5 hr (compared with the two days required by contact auto radiography). Unfortunately, accuracy and precision were not reported but it was implied that both were or better or at least as good as that obtained from contact auto-radiography. The advantage that was emphasized was the significantly reduced analysis time.

**Purity Measurements of Phthaloyl-Amiodipine Using Over-Pressurized Thin Layer Chromatography**

Pressurized thin layer chromatography involves the application of a plastic membrane over the thin layer plate which is held there by hydraulic pressure applied to the opposite side of the membrane. By applying an appropriate pressure to the edge of the plate, the mobile phase is forced, in a lamina flow profile, through the adsorbent layer between the plate and the membrane. In practice, this procedure really constitutes normal liquid chromatography carried out in a column having a very thin rectangular cross section. The advantages of this procedure over the normal thin layer method appear to be improved resolution and shorter analysis time. The advantages of pressurized thin layer chromatography over a similar liquid chromatography system using conventional cylindrical columns, however, is not clear.

Phthaloyl-amiodipine (3-(ethoxycarbonyl)-2-[(2-phthalimidoethoxy)-methyl]-4-(2-chlorophenyl)-6-methyl-5-(methoxycarbonyl)-1, 4-dihy-
dropyriddyne) is the final intermediate in the synthesis of amiodipine benzylate, a highly important anti-ischemic and antihypertensive drug. The purity of the phthaloyl-amiodipine has a significant effect on the final product. Szikszay et al. [8] used both thin layer and pressurized thin layer chromatography to analyze phthaloylamidipine for impurities to assess the relative value of each technique for accurate and precise results. Separation by pressurized thin layer chromatography was carried out on fine particle silica gel plates (Merk 5548) and a mixture of n-hexane:butyl acetate:ethyl acetate:chloroform (60:15:15:20) was used as the mobile phase. An external pressure of 5 MPa was applied using 8.5 ml of mobile phase equivalent to a migration distance of 36 cm. The plate was scanned at 233 nm using a TLC Scanner 3 (Camag) in reflectance mode.

Figure 9 Comparative Scanned Chromatograms Demonstrating the Improved Performance of Over-Pressurized Chromatography Relative to Normal Thin Layer Chromatography

Copyright © 2001 by Taylor & Francis Group, LLC
The conventional thin layer separation was carried out on silica plates (Merck 5554) and a mixture of \( n\text{-hexane:butyl acetate:ethyl acetate:chloroform} \) (30:15:15:20) was used as the mobile phase. A normal chamber was employed with a saturation time of 30 min and a development distance of 15 cm. The plate was scanned in a similar manner to those obtained by normal thin layer chromatography. An example of some of the results obtained is shown in figure 9. The advantages of the pressurized system are clearly shown; the impurity is half masked by a major peak in the chromatogram obtained by normal thin layer chromatography. Out of seven replicates, the mean level of the impurity was determined as 0.847\% and 1.111\% by the pressurized and normal thin layer chromatographic procedures respectively. The precision, however, as measured by the coefficient of variation was 2.9\% and 6.1\% respectively. It is clear that the two methods differ both in accuracy and precision but the pressurized system provides the best precision.

Significant advances in thin layer chromatography have been few and far between over the last decade but progress continues, albeit at a reduced rate. To end this chapter on a positive note, a particularly interesting application was very recently described which involved a procedure that combines synthesis, separation, quantitative analysis and biological screening, consecutively carried out on a thin layer plate. This technique is particularly applicable to combinatorial chemistry and has been reported by Freemantle [9]. Details of the method have been described by Williams [10], an example of which is given as follows. Parallel syntheses were carried out in a series of spots on the baseline of a TLC plate coated with silica; the reaction was accelerated by microwave irradiation. Different synthetic conditions were used on each spot to identify the optimum conditions of the synthesis. The products were then separated by developing the plate with an appropriate solvent and a quantitative assessment carried out on each pertinent spot by suitable scanning techniques or visual comparison of the spots with a standard. Each spot was then screened \textit{in situ} for microbial growth inhibition or other biological activity by standard agar-overlay methods directly on the plate. The value of this
approach in combinatorial synthesis is clearly obvious as many different synthetic conditions can be investigated simultaneously and the biological activity of the products concurrently ascertained. This procedure is quite unique and is an excellent illustration of the extension of an ongoing and established technique to a challenging modern chemical problem.

Synopsis

The most common and inexpensive method of evaluating a thin layer plate quantitatively is by simple spot matching with the naked eye. The sample is separated with a standard (or set of standards) separated alongside. After appropriate processing, the intensity of the spot of interest is compared with that of the standard spot(s). A more accurate method is to scan the spot(s) by reflectance, employing light of appropriate wavelength or by fluorescence if naturally fluorescent or if fluorescent derivatives can be formed. Scanned chromatograms can be evaluated by using peak heights, peak areas or sliced areas. Sulfamethazine has been determined in animal tissue using the visual comparative technique at concentration levels in the original sample as low as 50 ppb. Thin layer chromatography has also been employed to determine aflatoxins in palm oils and kernels at levels of 1-2 mg/kg. Although a somewhat lengthy procedure, an extraction efficiency in excess of 90% was easily achieved. In a similar analysis which involved the determination of phospholipids in human tears. The extracted phospholipids were hydrolyzed by acetylhydrolase to form 1-hexade-canoyl-sn-glycero-3-phosphocholine and then sprayed with the Mo-MG reagent to produce the intense blue-green spot of molybdo-phosphate malachite green. Scanning at an appropriate wavelength, concentrations of 2-100 pmol were measurable with an RSD of about 2%. A hybrid thin layer plate (half covered with silica gel and half with a laser desorption matrix) has been used as a transport medium for a laser desorption sampling system of a mass spectrometer. After separation in one direction, the spots on the plate are transferred to the section coated with the desorption matrix by transverse elution with another solvent The plate is then dried and
then inserted into the mass spectrometer sampling system. The sensitivity that was realized ranged from 90 fmol to 6 pmol. Thin layer plates can also be scanned by a digital imaging camera and the resulting image passed to a computer and the analysis processed by suitable software. Calibration curves obtained by this procedure are close to linear but there appears to be little difference between the precision and repeatability obtained by this method and that obtained by conventional scanning.

The use of radioactive tracers also opens up alternative methods of scanning a thin layer plate. One method is by contact radiography where, after development and drying, the plate is placed in contact with a photographic plate or film for a day or two. The film is developed and then scanned in the usual manner. Alternatively, the plate can be scanned with a radioactive counter-scanner and the radio chromatogram produced directly. This technique can be highly sensitive and at the same time provide good precision and accuracy. Pressurized thin layer chromatography, where the flow is energy-driven instead of by surface tension forces can provide better resolution and shorter analysis times. However, the accuracy and precision obtained form such techniques appear little better than those obtained from conventional thin layer chromatography. An interesting development of the thin layer technique is to first use the plate as a synthesis medium to produce the products of interest. The plate is then used to separate the products in the conventional manner. Finally the individual spots on the plate are used to test the biological activity of each separated component.

References