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REVIEW

PROGRESS IN DENSITOMETRY FOR QUANTITATION IN PLANAR CHROMATOGRAPHY

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CONTENTS

LIST OF ABBREVIATIONS

BHT	2 6-Di-*tert* -butyl-4-methylphenol
DRIFT	Diffuse reflectance Fourier transform infrared

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EHB	Ethyl *p*-hydroxybenzoate
ERR	Emission response ratio
FAB	Fast atom bombardment
HPLC	High-performance liquid chromatography
HPTLC	High-performance thin-layer chromatography
PAS	Photoacoustic spectroscopy
PHB	Propyl *p*-hydroxybenzoate
R.S.D.	Relative standard deviation
SERS	Surface enhanced Raman spectroscopy
SIMS	Secondary ion mass spectrometry
TLC	Thin-layer chromatography

1 INTRODUCTION

Progress in planar chromatography in the last decade resulted from a synergistic interaction of developments that took place in layer technology, sample application, development methods, and quantitation by densitometry [1–9]. It is the latter aspect of planar chromatography that we will focus on here Without the commercial availability of reliable instrumentation for in situ quantitation of planar separations there is no doubt that planar chromatography would not have made the progress and attained the acceptance that it has as a major analytical tool for quantitative analysis of mixtures. Earlier methods of quantitation such as visual comparison of colored or chemically visualized zones or excising of separated zones from the layer followed by elution from the sorbent and spectrophotometric analysis are too insensitive, too imprecise, too labor-intensive, and too tedious for acceptance in a modern analytical laboratory Detection had to be as convenient and as reliable as other competing chromatographic methods for planar chromatography to survive and prosper This it has done, and numerous applications of quantitative thin-layer chromatography (TLC) to a diverse range of biomedical problems have been developed, particularly in the areas of amino acids, peptides, antibiotics, carbohydrates, lipids, pharmaceuticals, nucleic acids, steroids, toxins, and vitamins, analyses in all kinds of sample matrices. Some contemporary examples of the practical utility of quantitative TLC will be discussed in Section 8

Separations in planar chromatography occur because of differential migration velocities through the sorbent layer in a fixed separation time The sample zones are fixed in space at the completion of the separation allowing off-line monitoring. The separation step and detection process could thus be optimized separately increasing the flexibility of methods development and choice of mobile phase, etc However, at the detection step the sample and stationary phase remain unseparated. The zone matrix is optically opaque and strongly light scattering so that photometric measurements of separated substances are much more difficult than equivalent measurements in solution The Beer–Lambert

law is invalid and an alternative relationship between the sample amount and the amount of light absorbed (or emitted in fluorescence) must be sought

2 QUANTITATIVE ANALYSIS BY OPTICAL SCANNING DENSITOMETRY

2 1 Theoretical considerations

All optical methods for the quantitative evaluation of planar chromatograms are based upon measuring the difference in optical response between blank portions of the medium and regions where a separated substance is present When monochromatic light falls on an opaque medium some light may be reflected from the surface, some may be absorbed by the medium and dissipated in some way such as by conversion to heat, and the remainder will be diffusely reflected or transmitted by the medium. Specularly reflected light is important when the surface is smooth but is of little interest chromatographically as it conveys no useful information of the sample distributed within the sorbent layer It can, however, contribute to the associated noise signal in scanning densitometry as the specularly reflected component cannot be distinguished from the diffusely reflected component For quantitation it is the diffusely reflected/transmitted light which is of importance and we must assume that the specularly reflected component from the sorbent surface is very small The propagation of light within an opaque medium is a very complex process that can only be solved mathematically if certain simplifying assumptions are made [10–12] The most generally accepted theory is due to Kubelka and Munk which assumes that the transmitted and reflected components of the incident light are made up only of rays propagating inside the sorbent in a direction perpendicular to the plane of the plate surface All other directions lead to much longer pathways and, therefore, much stronger absorption Consequently they contribute only negligibly to the total amount of transmitted or reflected light The restriction to propagation in only the forward and reverse direction does not apply to light exiting the medium and at the plate air boundary light is distributed over all possible angles with the surface

In formal terms the Kubelka–Munk model can be described as follows The sorbent layer is of thickness Z and the propagation of light in an infinitesimal portion, dz, parallel to the plate surface is considered. The sorbent layer is illuminated with monochromatic light in the $-Z$ direction The radiation fluxes in the layer dz in the direction of $-Z$ and $+Z$ are I and J, respectively This approach leads to two differential equations describing the propagation of light in parallel and opposite directions normal to the plate surface, eqns 1 and 2

$$-\frac{dI}{dz} = -(K+S)I + SJ \qquad (1)$$

$$\frac{dJ}{dz} = -(K+S)J + SI \tag{2}$$

S is the coefficient of scatter per unit thickness and K the coefficient of absorption per unit thickness. In physical terms, eqn. 1 states that the intensity of light travelling in the direction of transmission is decreased by absorption, K, and scattering, S, and reinforced by scattering from light travelling in the opposite direction, J Eqn. 2 is subject to similar interpretation but this time for light moving in the opposite direction, reflection. An exponential solution to the pair of simultaneous equations was derived by Kubelka and Munk, eqn 3, that has become the fundamental law of diffuse reflectance spectroscopy

$$\frac{K}{S} = \frac{(1-R\infty)^2}{2R\infty} \tag{3}$$

where $R\infty$ is the reflectance for an infinitely thick opaque layer By assuming that the scatter coefficient of the sorbent is unchanged by the presence of the sample, eqn 3 can be written in the more generally useful form

$$\frac{(1-R\infty)^2}{2R\infty} = \frac{2.303}{S} \cdot \epsilon \cdot c \tag{4}$$

where ϵ is the extinction coefficient of the sample and c the molar concentration of the sample. Although eqn 4 relates the intensity of reflected light to sample concentration it cannot be considered ideal for chromatographic purposes as it assumes a sorbent layer of infinite thickness For a thin layer of thickness Z the explicit hyperbolic solution for reflectance and transmission, also derived by Kubelka and Munk, are more meaningful

$$R = \frac{\sinh[bSZ]}{a \cdot \sinh[bSZ] + b \cdot \cosh[bSZ]} \tag{5}$$

$$T = \frac{b}{a \cdot \sinh[bSZ] + b \cdot \cosh[bSZ]} \tag{6}$$

where R is the intensity of reflected light, T the intensity of transmitted light, $a = (SZ+KZ)/SZ$, and $b = (a^2-1)^{1/2}$. The application of eqns 5 and 6 to quantitative analysis of thin-layer chromatograms is still quite complex as will be demonstrated later

Continuum theories of absorption in opaque media, such as the Kubelka–Munk theory, provide a reasonable description of the absorbing and scattering properties of the medium One weakness of these theories is that they fail to account for the interaction of light with individual particles of the layer Multilayer models solved by using the mathematical theory of Bodo or Markov chains have been suggested [6,13–16] In general, these equations do not offer any improvement from a practical standpoint except in one respect, they can

accommodate concentration gradients in the sorbent layer that Kubelka–Munk theory cannot. In the derivation of the Kubelka–Munk theory it was assumed that the sample was homogeneously distributed throughout the sorbent sample zone. A discrete step model to simulate the transmission of light by chromatographic media by computer has been proposed [17]. Good agreement was obtained with experimental observations and it was claimed that this new model provides a more exact solution to the problem of light propagation in a thin-sorbent layer than the Kubelka–Munk theory.

Many approaches can be used to calculate the fluorescence intensity of a sample in a thin opaque layer [18–20]. The simplest phenomenological approach is based on the Beer–Lambert law If I_0 is the intensity of the incident beam and I is the intensity of light at the non-illuminated side of the plate then $(I_0 - I)$ is the light flux absorbed by the layer. The intensity of the transmitted light is given by eqn. 7 and the amount of light absorbed by eqn 8

$$I = I_0 \cdot e^{-abc} \tag{7}$$

$$(I_0 - I) = I_0(1 - e^{-abc}) \tag{8}$$

where a is the absorption coefficient, b the thickness of the TLC layer, and c the sample amount. Only a fraction of the absorbed light is re-emitted as fluorescence due to energy loss to the sorbent. This is taken into account by the quantum yield, Φ The fluorescence emission is, therefore, given by eqn 9.

$$F = \Phi I_0(1 - e^{-abc}) \tag{9}$$

where F is the fluorescence flux (emission) For low sample concentrations the simplifying assumption $e^{-abc} = 1 - abc$ can be made and eqn. 9 rearranged to give

$$F = \Phi I_0 abc \tag{10}$$

Since all terms in eqn. 10 are constant or fixed by the experiment the fluorescence emission is linearly dependent on the sample amount The fluorescence intensity is also independent of the spot shape provided that the spot is completely contained within the measuring beam Experience supports the above equation in spite of the fact that it was derived without considering the affect of absorption and scatter by the medium on both the excitation and emission wavelengths Similar results can also be derived from the Kubelka–Munk equation which incorporates absorption and scatter by the chromatographic sorbent [19].

2 2 Practical considerations

Reflectance and transmission measurements are particularly sensitive to small changes in experimental technique and materials. Variations in the layer

thickness, layer quality (particle size and particle size distribution), spot shape and spot size, and uniformity of the sample development will affect the accuracy and precision of quantitative measurements [21] To improve precision calibration standards should be run on each plate used for analysis Calibration standards should be prepared in different concentrations and spotted as a fixed constant volume [22,23]. Multiple spotting of a single standard solution to generate a calibration curve is not acceptable as this practice invariably results in the formation of a group of developed spots with different sizes It should be noted that there is no simple correlation between signal response for a constant amount of substance and spot size in scanning densitometry [24]. Thus, standards and samples must have the same spot size for accurate quantitation

Either peak-height or peak-area measurements can be used for calibration. Peak-height measurements are easier to determine with high accuracy and provide more reliable data for fused peaks. However, they are considerably influenced by chromatographic parameters that affect spot shape and position in the chromatogram [24] The shape of calibration curves for absorption measurements has caused some concern since they are generally inherently nonlinear in accordance with theoretical considerations based on the Kubelka–Munk theory. As illustrated in Fig 1, calibration curves are individual in shape, generally comprise a pseudolinear region at low sample concentration curving

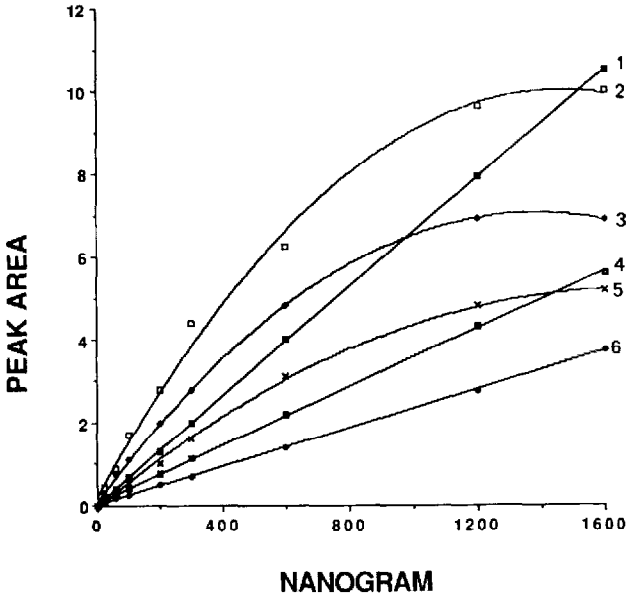

Fig 1 Some typical calibration curves for several substances measured by absorption in the reflectance mode Substance identification 1 = practolol, 2 = azobenzene, 3 = diphenylacetylene, 4 = alprenolol, 5 = estrone, 6 = pamatolol

towards the concentration axis at higher concentrations and eventually reaching some asymptotic value where signal and sample concentration are no longer correlated The extent of the individual ranges or sections of the calibration curve are frequently very different for different substances and, while in some instances the pseudolinear range may be adequate for most analytical purposes, in others no reasonable linear range may exist

Several transformation techniques have been described to linearize the normally non-linear calibration curves The simplest of these involves the conversion of the sample and/or signal into reciprocals [2], logarithms [2,21], squared terms [25,26], or use of the Michaelis-Menten function [26–31] In general, each approximation is only successful for linearizing part of the calibration curve and the region linearized is frequently different for the different methods [26] However, the principal problem with all of these linearization methods is the manner in which errors associated with the experimental measurements become propagated [27,31] Errors in the original data are also transformed in the above methods leading to inhomogeneous variances in the transformed data and unreliable regression analysis This occurrence can be detected quite simply by using the regression equation to recalculate the original experimental data By this method it has been shown that large errors can be introduced in some cases requiring that these methods should be used with due care or not at all [26,31] Alternatively, non-linear regression methods based on second-order polynomials described by eqn 11a [2,32] or eqn 11b [33,34] can be used without disturbing the error distribution

$$\ln R = A_0 + A_1 \ln M + A_2 (\ln M)^2 \tag{11a}$$

$$R = A_0 + A_1 M + A_2 M^2 \tag{11b}$$

For TLC purposes only the second- and third-order polynomials are useful Higher-order polynomials can introduce invalid maxima or minima into the approximated calibration curve De Spiegeleer et al [32] compared six calibration methods for the determination of methyl nicotinate in pharmaceutical creams They concluded that the best fit was obtained using second-order polynomials while reciprocal transformation was the best of the linear approximations

The continuing introduction and use of personal computers in the analytical laboratory should make the use of non-linear regression a more widely used technique favoring this approach for calibration in quantitative TLC However, more work is required to select the best regression models and, in all likelihood, no single model will be best for all calibration curves Several models should be tested for each data set (compound–plate type combination) using statistical tests to select the best model

The statistical validity of using least-squares linear regression in conjunction with the linearized data sets for TLC calibration has been questioned

[35] For least-squares linear regression the independent variable should be known with high accuracy and it is assumed that all the measurement errors are contained in the dependent variable Because of spotting and chromatographic errors this is not the case for TLC calibration data. For TLC data the quality coefficient for the confidence interval may represent a better statistical test of the accuracy of the linearization methods [2,27]. Despite sound theoretical considerations Ebel [27] found very little difference in the results obtained by using linear regression or linear correlation methods.

Some modern densitometers of the 'flying spot' kind using either software or electronic tuning provide for linearization of absorption measurements utilizing some mathematical solution of the Kubelka–Munk model such as those given by eqns 5 and 6 [2,36–39]. To implement this procedure the sample track is scanned by a spot or rectangular beam of small dimensions relative to the spot size in a saw tooth or square wave pattern by fixing the measuring beam in one position and mechanically moving the plate in the x and y direction to generate the desired pattern. An advantage of flying spot scanning is that the quantitative information obtained is independent of the spot shape and the sample concentration distribution within the spot

In the Shimadzu (Columbia, MA, U S A) CS-900 Series scanning densitometers linearization by the flying spot method is achieved electronically The signal produced during each swing of the measuring beam across the spot is converted to an absorbance value using eqn 12

$$D=\log(R_0/R_1) \tag{12}$$

where D is the observed response (absorbance) R_1 the reflectance determined by eqn 5, and R_0 the reflectance determined for $K=0$. It is further assumed that the sorbent layer does not adsorb light and, therefore, the value of KZ represents the absorbance due to the sample Electronic linearization of the signal is then possible by estimating the scatter component of the layer SZ.

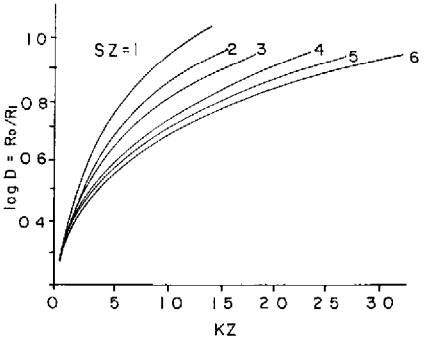

Fig 2 Relationship between the absorption signal (D) and sample amount (KZ) for different values of the plate scattering parameter (SZ) This relationship is used in conjunction with eqn 5 to perform calibration curve linearization

This can be done from plots of the kind shown in Fig. 2 in which values for the absorbance D can be converted to values of KZ as a function of the scatter component SZ. Values for SZ are determined empirically for different sorbent media Once a value of SZ is established the value of KZ is obtained from the Kubelka–Munk equation and the total absorbance by integration of the values for each swing of the measuring beam across the spot. In the absence of the scatter term the relationship between KZ and sample concentration is linear while the absorbance term, D, shows the expected curvature observed for normal calibration. To test the linearizer several standards covering a reasonable calibration range should be scanned A lack of linearity in the calibration curves indicates the wrong value for SZ, the scatter component, and a different value should be selected from the range of allowed values available.

Calibration in fluorescence rarely produces any problems. Calibration curves are usually linear over two to three orders of magnitude. At higher sample amounts self-absorption becomes a problem and the calibration lines curve over towards the weight axis An alternative method of calibration, designed for use in screening studies where several analytes are to be quantified in multiple samples has been suggested [40–42]. Known as the two-point calibration method it requires only a single standard for each substance to be determined and only a single track for all calibration standards It is based on the observation that there is a linear relationship between the fluorescence signal and slit width dimensions in fluorescence densitometry [43] and that the slope of the detector response versus slit width curve is proportional to the sample amount [40] Combining these two observations yields the calibration eqn 13

$$M_u = M_s \left(\frac{S_u}{S_s} \right) \tag{13}$$

where M_u is the unknown sample amount, M_s the known amount of sample (standard), and S_u and S_s the slope of the detector response versus slit width curve for the unknown and standard, respectively. The values for S_u and S_s are determined from two measurements of the signal response at slit widths of 0 4 and 0.8 mm Thus, in practice, calibration is performed by scanning each sample and the lane occupied by standards twice at the selected values for the optimum slit widths The two-point calibration method cannot be used to replace normal calibration when the highest degree of accuracy is required (see Table 1), but it is a very useful method as a scouting technique to determine approximate sample amounts.

On occasion it will be observed that the fluorescence response for a substance in TLC will be far less than expected from measurements made in solution, will occur at different excitation and emission wavelengths, and may diminish at varying rates over time [18,20,44]. Two phenomena, fluorescence quenching and catalytic degradation of the sample, are responsible for this

TABLE 1

COMPARISON OF CALIBRATION STANDARDS AND CALCULATED AMOUNTS AT DIFFERENT SAMPLE SIZES ($n=15$)

The reference standard for the two-point calibration was 10 ng (Reproduced with permission from ref 40)

Calibration standard (ng)	Calculated amount by two-point calibration (ng)
20 0	17 45 ± 1 30
15 0	13 97 ± 0 90
5 0	5 20 ± 0 30
2 0	2 22 ± 0 19
1 5	1 78 ± 0 14
1 0	1 14 ± 0 20
0 50	0 55 ± 0 09
0 20	0 24 ± 0 05
0 15	0 17 ± 0 05

The first is largely physical and generally reversible The most common fluorescence quenching agent is oxygen. Fortunately its effect is generally small and can be masked by displacing air with nitrogen from the measurement region of the densitometer [45]. Often a more convenient method is to impregnate the sorbent layer with an antioxidant such as 2,6-di-*tert.*-butyl-4-methylphenol (BHT) [46–48] The fluorescence signal in many cases can be enhanced by a post-chromatographic treatment of the TLC plate with a viscous solvent such as liquid paraffin [49,50], glycerol [51], cyclodextrin [52], triethanolamine [53,54], Triton X-100 (Cole-Parmer, Chicago, IL, U S.A) [23,40,44,55], or Fomblin H-Vac (Montedison, New York, NY, U S A) [23,44,47,48]. The extent of fluorescence quenching often depends on the sorbent medium and is frequently more severe for silica gel than for bonded-phase sorbents In favorable cases application of fluorescent enhancing reagent can increase the signal response as much as 10- to 200-fold. The mechanism of mechanisms of fluorescence quenching are not known with certainty, but it is generally assumed that adsorption onto silica gel provides additional non-radiative pathways for dissipation of the excitation energy that is at least partly relieved when silica gel is impregnated with a viscous liquid [18,56]. Viscous liquids are employed to minimize diffusion-induced zone broadening of the sample in the wet layer during the time required to scan all the samples on the plate. An example of fluorescence quenching and the use of fluorescence enhancing reagents is shown in Fig 3 for a sample of naphthylpiazselenole (a fluorescent derivative used to determine selenium) on a silica gel plate [53] The response of the derivative is increased by impregnating the silica gel plate with liquid paraffin (peaks b and c) and Triton X-100 (peaks d and e) In both

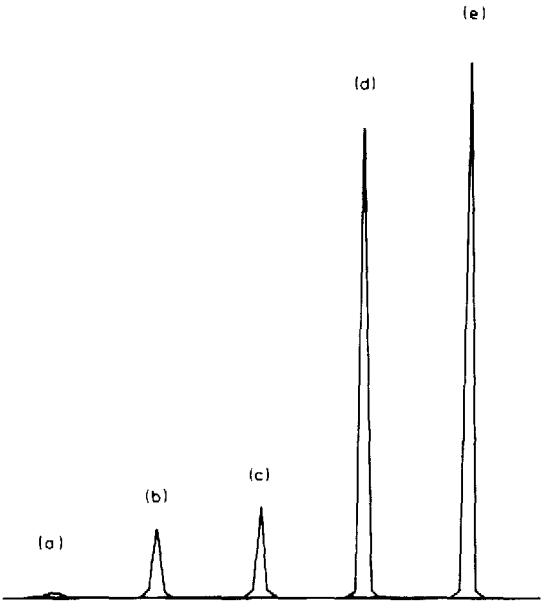

Fig 3 Fluorescence enhancement of naphthylpiazselenole on (a) silica gel HPTLC plate, (b) HPTLC plate dipped into paraffin-*n*-hexane (2 1), (c) measurement of (b) in a nitrogen atmosphere, (d) HPTLC plate dipped into Triton X-100–chloroform (1 4), and (e) measurement of (d) in a nitrogen atmosphere (Reproduced with permission from ref 53)

cases measuring the response in a nitrogen atmosphere (peaks c and e) produces a small enhancement in the response compared to the value measured in air (peaks b and d) For complex sample extracts matrix interferences may also cause either enhancement or quenching Zennie [57] observed more than 100% recoveries of aflatoxins from spiked corn samples when using TLC for their determination This was shown to be due to co-elution of free fatty acids with the aflatoxins that behaved as enhancing reagents, increasing the fluorescence response of the aflatoxins by 14–36%. This problem was solved by modifying the mobile phase so that the aflatoxins were separated from the fatty acid contaminants

The most common chemical reactions causing an unstable fluorescence response are substrate catalytic oxidations or photolytic degradation. Seifert [58] observed the oxidation of polycyclic aromatic hydrocarbons on silica gel plates to produce new products of low fluorescence yield These chemical reactions often exhibit a specific time dependence which makes quantitation difficult This was observed to be the case for the fluorescamine derivatives of cephalosporins on silica gel plates [59] and 1-aminopyrene on different sorbents [46–48] In instances of this kind impregnating the plate with an antioxidant and/or shielding the sample on the plate from exposure to room light may

substantially reduce the rate of reaction although it may fail to eliminate it entirely.

2.3 *Instrumental considerations*

Instruments for scanning densitometry using absorbance or fluorescence measurements in the reflectance, transmission, or combined reflectance–transmission mode first appeared in the middle 1960s and since then have undergone continuous change with the introduction of new technology [2,5,8,11,18,20,23,37,60,61]. The most obvious technical change has been the greater use of computers and microprocessors which have revolutionized data handling and permitted a greater degree of automation of the scanning process.

Commercial instruments for scanning densitometry share many features in common. Different lamps must be used as light sources in order to cover the entire UV–visible range from 200 to 800 nm. Halogen or tungsten lamps are used for the visible region and deuterium lamps for the UV region. High-intensity mercury or xenon arc sources are preferred for fluorescence measurements. To select the measuring wavelength either a monochromator or filter is used. Filter densitometers usually employ a mercury line source and filters to pass only light corresponding to individual wavelengths available from the source. Their advantage is their low cost, otherwise broad spectral sources and grating monochromators offer greater versatility for optimizing sample absorption wavelengths and are generally preferred. For fluorescence measurements, a monochromator or filter is used to select the excitation wavelength. A cutoff filter, which transmits the emission wavelength envelope but attenuates the excitation wavelength, is placed between the detector and the plate. Interference filters can be substituted for the cutoff filters if greater selectivity is required, albeit with some sacrifice in sensitivity. Photomultipliers or photodiodes are generally used for signal measurements. Instruments employing a laser as the primary source or as an accessory have recently appeared largely for use in scanning electrophoretic gels. Laser beams can be collimated to much smaller beam sizes (a few microns) than is possible with conventional sources and optics. Such spatial resolution may be needed for scanning two-dimensional electrophoretic separations but is generally unnecessary for TLC separations. Lasers can be used as sources for fluorescence measurements where their high power and small beams widths could be used to enhance sensitivity [62–64]. Selectivity might also be improved by exploring so-called non-linear fluorescence processes, such as two-photon excited fluorescence and sequentially excited fluorescence, which only become feasible when laser sources are used. However, to date, laser-based densitometers have not demonstrated any significant improvement in sample detectability over densitometers employing conventional arc sources. Apart from the additional equipment costs, lasers generally have a single or a limited number of useful wavelengths for detection

(particularly in the UV), which limits their use for general applications The high power of the laser source may also cause sample decomposition which can influence the reproducibility of quantitative measurements

Three optical geometries are predominantly used in contemporary scanning densitometers. The single-beam mode is the simplest optical arrangement and is capable of producing excellent quantitative results, but spurious background noise resulting from fluctuations in the source output, inhomogeneity in the distribution of extraneous adsorbed impurities, and irregularities in the plate surface can be troublesome These problems can be minimized by good analytical practices and through software correction Background disturbances can be compensated for to some extent, by double-beam operation The two beams can be either separated in time at the same point on the plate or separated in space and recorded simultaneously by two detectors

The double beam in space optical arrangement divides a single beam of monochromatic light into two beams that scan different positions on the plate One beam scans the sample lane while the other traverses the blank region between sample lanes The two beams are subsequently detected by matched photomultipliers and a difference signal is fed to the recorder, fluctuations in the source output are corrected in this way As the two beams impinge on different areas of the plate, however, small irregularities in the plate surface and undesired background contributions for impurities in the sorbent layer may still pose problems

In the single-beam dual-wavelength mode, fluctuations caused by scattering at a light absorbing wavelength (λ_1) are compensated for by subtracting the fluctuations at a different wavelength (λ_2) at which the spot exhibits no absorption but experiences the same scatter. The two beams are altered by a chopper and combined into a single beam to provide the difference signal at the detector As the scatter coefficient is to some extent wavelength-dependent the background correction is better when λ_1 and λ_2 are as nearly identical as possible This requirement is often difficult or impossible to meet as absorption spectra are usually broad and it may be impossible to find two similar wavelengths at which absorption occurs for one wavelength and not for the other In favorable circumstances background correction in this mode can be very good as illustrated by Fig. 4 [65]

For technical reasons the sample beam is fixed and the plate is scanned by mounting it on a movable stage controlled by stepping motors. The most common method of scanning is slit scanning in which the sample beam is shaped into a rectangular area on the plate surface through which the plate is transported in the direction of development (Fig 5) Each scan, therefore, represents a lane whose length is defined by the sample migration distance and whose width is determined by the slit dimensions Some instruments have a turntable-type scanning stage for peripheral and radial scanning (see Fig 5) used for chromatograms developed in the circular or anticircular mode As well

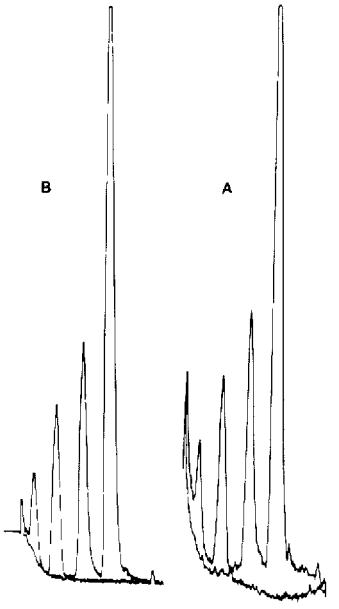

Fig 4 Use of background correction to improve baseline stability in the analysis of a mixture containing the drug metoprolol and some potential contaminants (A) Single-wavelength mode, $\lambda = 280$ nm, and (B) single-beam dual-wavelength mode, $\lambda_1 = 280$ nm and $\lambda_2 = 300$ nm The background contribution resulting from spurious absorption by plate contaminants is eliminated in (B) (Reproduced with permission from ref 65)

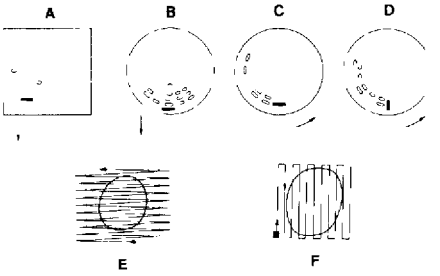

Fig 5 Methods of scanning thin-layer chromatograms A = linear slit scanning, B = radial scanning, C and D = peripheral scanning, E = zig-zag scanning of an individual spot, F = meander scanning of an individual spot The arrows indicate the direction of plate movement

as slit scanning point scanning can also be used The measuring beam is shaped into a spot or rectangle of dimensions much smaller than the chromatographic zones to be scanned By moving the scanning stage in the x and y direction a zig-zag or meander scan is possible (Fig 5) Zig-zag and meander scanning allow zones of any shape to be accurately quantified using computer algorithms to perform the integration. For very small spots errors may arise because the

scanning beam dimensions cannot be made sufficiently small to permit a sufficient number of sampling points for the spot The method employed for baseline correction is also important.

For linear slit scanning densitometers it is well established that chromatographic resolution and sample detectability can be impaired by the choice of slit dimensions defining the area of the measuring beam on the plate surface, the scan rate, and the total electronic time constant of the densitometer and recording device [20,23,43,66–68] In the absorption mode the ratio of the slit height to spot diameter has a large influence on sample detectability. Slit heights less than the diameter of the spot produce the highest sensitivity but unless position scanning (see later) is used, may lead to unacceptable errors due to incorrect alignment of the sample beam and spot centers throughout the track Generally a slit height equivalent to the diameter of the largest spot to be scanned is selected as a compromise In fluorescence the signal increases linearly with increasing slit width and shows a general increase with increasing slit height until a slit height as large as the spot diameter is reached For slit heights greater than the spot diameter there is little further change in sensitivity In both absorption and fluorescence there is a reciprocal relationship between the signal and the scan rate At high scan rates resolution can be degraded and the signal may be attenuated if the time constant of the signal processing device is inadequate

Little progress has been made towards developing effective protocols for comparing the performance of different densitometers The spatial resolving power of a densitometer can be ascertained by scanning photographic test patterns or some similar standard [67,69] As these test patterns generally consist of narrow-width, equal-density squares or lines they bear little resemblance to actual chromatographic separations Commercially available densitometers have spatial resolving powers of between 10 and 200 μm but this term is not quantitatively related to chromatographic resolution. As a practical, although imperfect measure of densitometer performance, the perceived resolution measured from a strip chart chromatogram for a pair of partially separated spots with the resolution calculated from individual stands run in different lanes can be used [4,23,67] Test plates of chosen separations can by used for side-by-side comparisons of individual densitometers [61] A standard protocol has been suggested for comparing the sensitivity of slit scanning densitometers based on the use of standard substances, chromatographic conditions, and instrument parameters [20,70,71]

The principal sources of error in scanning densitometry have been identified as the reproducibility of sample application, the reproducibility of chromatographic conditions, the reproducibility of positioning the spot in the center of the measuring beam, and the reproducibility of the measurement [72,73]. The measurement error can be determined by repeatedly scanning a single track of the TLC plate without changing any experimental variables between scans It

is composed of errors due to the optical measurement, electronic amplification, and the recording device The measurement error is dependent on the signal-to-noise ratio but for a properly adjusted instrument typical values fall into the range 0 2–0.7% [2,72]. The error of positioning the spot in the measuring beam and the sample application error should be controlled by good analytical practices The chromatographic error is generally easily the most significant error and is only reduced by minimizing the variability in the development process The data pair technique can be used to minimize errors due to migration differences as a result of edge effects, deviations in layer thickness, non-linear solvent fronts, etc [74] In this technique an internal compensation approach is used by pairing up the measurements of two spots on the same plate In modern scanning densitometry with high-performance thin-layer chromatography (HPTLC) plates the relative standard deviation from all errors can be maintained below 2% making it a very reliable quantitative tool The densitometer is not the principal source of most of the experimental error which can only be controlled or reduced further by paying adequate attention to the sample application and chromatographic development steps

First- and second-order derivatives of the analog signal can provide a rapid, direct, and simple means of verifying the homogeneity of any given spot, and in many instances, enhancing the resolving power of the chromatographic system to a point where the quantitative assay of severely overlapped components becomes a possibility [75–79] When the chromatographic resolution exceeds 1 0 there is little difficulty in quantitation, for resolution decreasing to 0 7 either peak height measurements or derivative recording should be equally reliable, for resolution values less than 0 7 only derivative recording can provide accurate results As a practical example consider the separation of ethyl (EHB) and propyl (PHB) *p*-hydroxybenzoates shown in Fig 6 [77] From the analog signal it would be difficult to extract any useful quantitative information, particularly from Fig 6B However, the two components are well resolved in the derivative recording and can be quantified from the peak heights of the satellites marked OA and OB in Fig 6 The discriminating power of the derivative spectra is such that the component of interest can be quantified in the presence of a relatively large excess of resolved interfering components as may occur in minor component analysis [76] Only first- and second-order derivatives are useful for densitometric recording as higher-order derivatives produce poor signal-to-noise characteristics [78] When using electronic differentiation optimization of the operating variables for the densitometer is critically important [76]

It should be noted that although derivative recording is not a new technique in analytical chemistry its application to densitometric measurements in TLC is still relatively novel There are two general approaches available The use of analog electrical devices containing RC circuits combined with operational amplifiers sold as accessories for spectrophotometers Alternatively, through

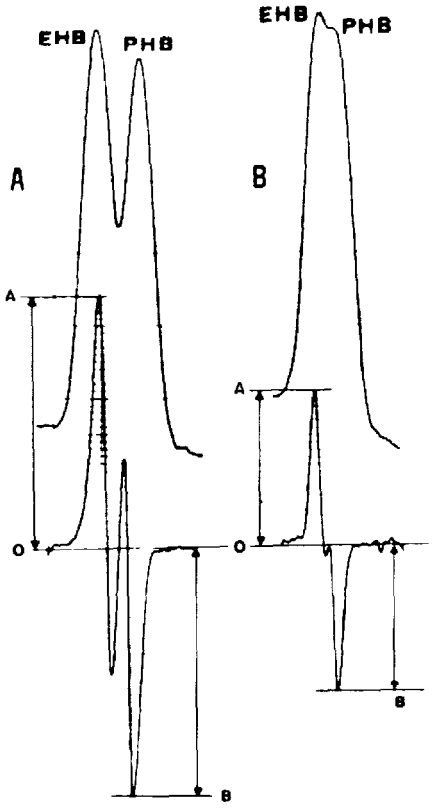

Fig 6 First-derivative measurement of unresolved ethyl *p*-hydroxybenzoate (EHB) and propyl *p*-hydroxybenzoate (PHB) at resolutions of 0 70 (A) and 0 30 (B) (Reproduced with permission from ref 77)

software differentiation is possible after first approximating the experimental data by a polynomial function [75] This method is convenient when computerized instruments are used, particularly as the raw data can be manipulated off-line without loss of the original signal information. However, both processes may lead to unreliable results if the chosen conditions are less than optimum Analog devices may cause damping and distortion of the signal due to selection of inappropriate time constants A variable time constant differentiator for chromatographic purposes was recently described which might also find use in scanning densitometry [80] Digital methods may also produce errors depending on the number of data points taken and the order of polynomial used to fit the experimental data

2 4 Automation through computer control

TLC is a data-intensive technique The operations of scanning, calibration, and quantitation can be both time-consuming and tedious when performed

manually These procedures are also prone to variable errors such as that due to positioning of the spot in the measuring beam and estimating the correct shape of the calibration curve. A further advantage of computers is that they simplify report-writing by combining graphical, statistical, and word processing capabilities and simplify the archival process of data storage Digital data stored on floppy disks can be reassessed and recalculated numerous times as the goals and interests of the analysis change without having to rerun the chromatogram These and other considerations have been reviewed in the application of computers to TLC [2,11,60,81].

The computer can be used as an intelligent integrator performing all the calculations and smoothing of the digital data received from the densitometer without communicating to the densitometer in other respects [11,81,82]. The link between the computer and the densitometer is made via an analog-to-digital converter In more advanced instruments the densitometer is a slave to the computer which controls the scan function, selection of the measuring wavelength and other optional experimental variables [7,11,24,60,83] These variables are usually established in a pre-run dialog with the operator in accordance with a fixed menu of available options. These options might include the method used for scanning, the number of tracks and their length to be scanned, whether internal or external standards, or both are to be used for calibration and, if so, in which tracks this information is to be found, which spots are to be quantified based on preselected R_F values, the calibration and calculation methods to be used, whether spectral plots or multiple-wavelength scanning is needed., and the format for the final report.

Automation of the scanning function is one of the more obvious parameters to control as when this is achieved the only operator intervention required is to position the plate in the densitometer and to remove it at some time later when all the data have been recorded. In simple instruments a microprocessor is used for this purpose Normally, the first track to be scanned is manually positioned in the measuring beam and then the values for track length, distance between tracks, and number of tracks to be scanned is entered. The densitometer then scans the plate following the established geometric pattern without further intervention by the operator The disadvantage of this method is that if the samples migrate irregularly then the spot may become misaligned with respect to the beam position and erroneous data generated. More sophisticated programs may recenter the measuring beam for the first spot of each track prior to scanning in a linear manner, execute a meander or zig-zag scan function, or optimize the beam spot co-ordinates for each spot in the chromatogram The latter option called position scanning is shown diagrammatically in Fig 7 [60] In this mode the spot is detected first by making large-search steps in the direction of chromatography, the peak maxima are then found by making smaller steps, the plate is then moved away from the established maxima in the direction perpendicular to the direction of chromatography, and the

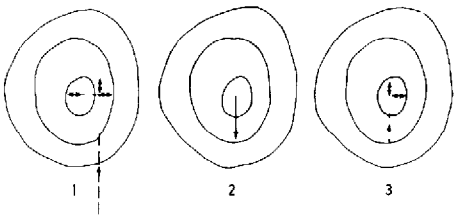

Fig 7 Example of the use of computer-controlled scanning densitometry to locate the exact center of a spot by position scanning (1) The spot center is searched for, (2) the beam returns to a prespot position, (3) a second positioning is made to confirm the spot center location (Reproduced with permission from ref 60)

positioning repeated. This process is continued until the true peak maxima are located. Each spot, in this way, is optimally located in the measuring beam independent of the irregularities in the chromatographic development

Automatic scanning routines combined with video integration allow the correct determination of the baseline under each peak. During the scan mode the background signal before and after each spot is acquired and used to compute the baseline position The baseline in TLC may vary at different positions in the chromatogram due to matrix interferents and impurity gradients in the sorbent media These can be eliminated from the signal by software control or with the intervention of the operator by video integration of the chromatographic data displayed on a monitor Some progress has been made in subtracting the entire background contribution of the plate to the analytical signal with the intent of improving detection limits [7] With complete computer control of data acquisition and manipulation the reproducibility of densitometric measurements can be lowered to below 1% relative standard deviation (R S D)

3 ELECTRONIC SCANNING DENSITOMETERS

The detection process in TLC can be considered static as the sample zones are stationary when the development process is terminated All information concerning the chromatographic experiment can be made available as a three-dimensional array in which the x and y co-ordinates define the spot position and the z direction the sample amount using image analysis techniques. Scanning takes place electronically and the plate is stationary Detectors used for this purpose are either vacuum tube sensors or solid state devices which can be subdivided into line and area (matrix) sensors [7,84–88] The equipment requirements for image analysis are a computer with video digitizer, light source and appropriate optics such as lenses, filters, and monochromators, and a vidicon tube or charged-coupled video cameras [89–95] Suitable arrangements for image analysis in the transmission and reflectance mode are shown in Fig. 8 [85] The plate is evenly illuminated with (monochromatic) light and the

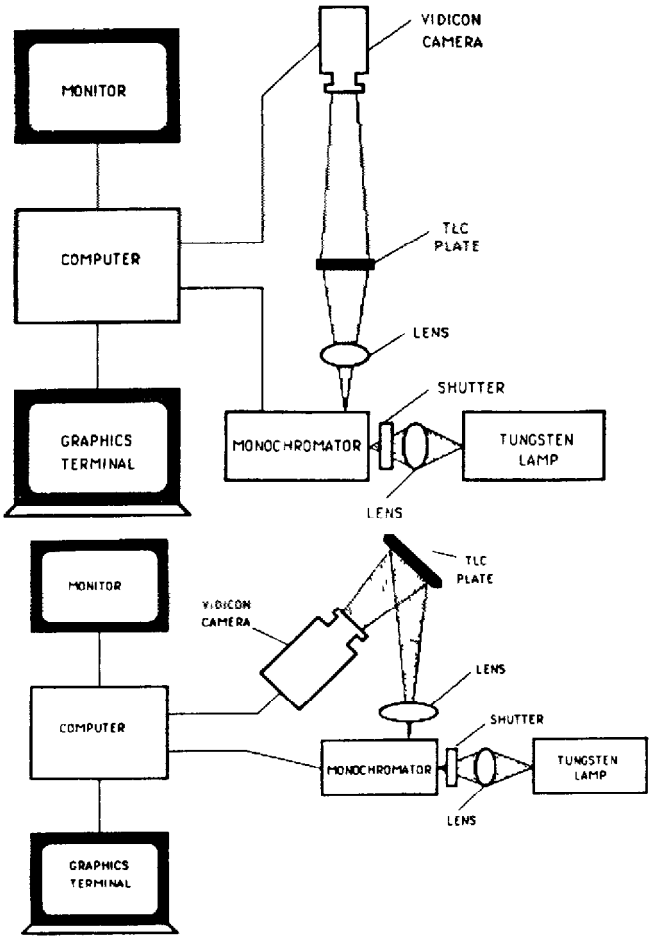

Fig 8 Component layout for electronic scanning of TLC plates in the transmission (top) and reflectance (bottom) mode (Reproduced with permission from ref 85)

reflected or transmitted light focussed as a scaled image of the plate directly onto the active element of the vidicon To obtain wavelength discrimination for spectroscopic identification or selective detection with overlapping spots a second monochromator between the plate and the camera is needed. Similarly, for fluorescence analysis either a filter or monochromator is required to shield the vidicon from light originating from the source The vidicon functions as a two-dimensional array of unit detectors continuously scanned by an electron beam These unit detectors are periodically discharged by the electron beam and the signal digitized for analysis by computer Image resolution is limited by the number of detectors, known as picture elements or pixels, forming the array This is typically 512×512 pixels for commercially available vidicon

cameras which translates to the smallest spot that can be distinguished on a 10×10 cm plate of about 0.6×0.6 mm [87] This is just about adequate for many TLC applications.

For analysis the captured images are collected, stored, and transformed by computer into chromatographic data To a large extent the number of images that can be stored, and the type of calculation algorithms used, is a function of the capacity of the computer. Image analysis is a data-intensive technique and, although many of the simpler systems described employ personal computers for data analysis, their facilities for image storage, image subtraction, filtering, thresholding, and the use of false color for image enhancement are greatly restricted. Common to all techniques of data handling is background subtraction in which the accumulated images of a blank plate are subtracted from the analytical plate on a pixel by pixel basis Thresholding is also routinely used to ensure that negative values for the plate luminance do not occur In only a few cases are exact details of the procedure used to convert images into x and y co-ordinates and optical density given [95]

The main advantages of electronic scanning are the fast acquisition of data, the absence of moving parts, simple instrument design, and compatibility with two-dimensional chromatograms which are very difficult to scan using conventional slit-scanning densitometers On the other hand with today's technology electronic scanners cannot compare with slit-scanning densitometers in terms of cost, sensitivity, available wavelength measuring range, and dynamic signal range Technology changes rapidly in the field of image analysis and it is not inconceivable that electronic scanners will be more widely used in the future, particularly for scanning two-dimensional TLC plates and electrophoretic separations.

4 RADIOISOTOPE IMAGING SYSTEMS

The principal methods for quantifying radioactive substances after separation by TLC are autoradiography, liquid scintillation counting, and direct scanning with radiation detectors [96–99] Autoradiography is the least expensive involving exposure of the chromatogram to an X-ray film which can then be converted into intensity measurements using a photodensitometer Autoradiography is generally slow and has poor accuracy and precision For scintillation counting spots or bands are removed from the plate, mixed with a scintillation fluid, and then counted in the usual way Sensitivity and quantitative accuracy are good but the process is time-consuming and spatial resolution is often compromised Direct scanning is the method of choice when many samples are to be scanned on a routine basis. Earlier instruments commercialized for this purpose used radiation-sensitive detectors such as the Geiger counter and operated in a manner similar to optical scanning densitom-

eters [100]. However, they were slow and very insensitive. Modern densitometers employ windowless gas-flow proportional counters as imaging detectors

TLC imaging systems count the amount of radiation emanating from each track simultaneously utilizing a rectangular proportional counter positioned directly over the track to be counted The imaging process is thus static for each track and the scan function is used for lane changes only The proportional counter is filled with argon–methane gas which is ionized locally by collision with β or γ rays produced by decay of the radioisotopic sample zones The proportional counter contains a high-voltage anode wire running the length of the detector. This causes the local bursts of ionized gas molecules to be accelerated and registered as a pulse upon collision with the anode. A delay line is used to indicate the position on the wire at which the pulse occurred and this information is digitized, counted, and stored in the memory location of the computer Pulses corresponding to specific locations along the wire are counted individually and stored in the computer for display in real time on a video monitor The spatial resolution is primarily limited by the relatively large volume of the detector and the energy of the emitting particle. For currently available instruments this corresponds to about 1 mm for ^{14}C sources and 0 5 mm for ^{3}H sources. The absolute sensitivity is limited primarily by self-absorption of low-energy β particles by the sorbent medium A good account of the various processes that effect calibration linearly and analytical precision is given in ref 96

5 FLAME IONIZATION DETECTION

In this technique samples are separated on thin rods and the separated zones detected by moving the rods through a flame ionization detector [101–103] The rods are 0 9 mm in diameter and 15 cm long coated with a 50–100 μm layer of powdered glass and sorbent fired onto the quartz core at 900°C [104] The sample is applied to the rod about 2 cm from one end; typical loadings are volumes of 0 1–3 μl containing 1–50 μg of sample. The rods are developed in the normal way, usually held in a support frame that also serves as the scan stage after the rods have been removed from the development chamber and excess solvent evaporated away in a drying oven. Several rods can be held in the support frame which are then automatically scanned in order. The rods are moved at a controlled speed through a hydrogen flame and the signal processed in a similar manner to the flame ionization detector used in gas chromatography The flame is a good deal larger than detectors used in gas chromatography and considerably more noisier so that detection limits are not comparable The linear working range of the detector is about 3–30 μg for most substances and the response nearly universal Recently, a thermionic detector has been introduced for the specific detection of nitrogen-containing compounds.

There are substantial differences of opinion as to the quantitative reliability of the technique Several authors have noted rod-to-rod, run-to-run, and compound-to-compound response variations [103,105] The scan rate and uniformity of the development process are critical parameters for obtaining reproducible quantitative data [103,106] It has been postulated that one source of variability is radiant heat transfer along the rod that causes evaporation of volatile samples prior to the sample zone entering the flame [103,107,108] This obviously affects volatile and thermally labile substances more so than others TLC with flame ionization detection has been used primarily for the analysis of heavy solutes lacking a chromophore for optical detection such as oils, tars, lipids, surfactants, resins, etc [103]

6 PHOTOTHERMAL DEFLECTION DENSITOMETERS

Photothermal deflection spectroscopy involves the measurement of the refractive index gradient formed in the gas phase over a solid sample heated by a laser If light is absorbed by the sample some of this radiation is converted to heat by radiationless transitions in the sample producing thermal gradients within the sample and refractive index changes in the layer of air in contact with the sample This refractive index change is detected by the deflection of a low-power laser beam propagating parallel to the plate surface Operation of the photothermal densitometer is somewhat difficult as the response is a function of several complex variables, and, for high sensitivity, a close match between the absorption maxima of the sample and the operating wavelength of the laser is required [109–112] Distribution of the laser energy over the total sample surface is necessary in order to minimize or avoid thermal damage to the sample which was a problem in early densitometer designs One solution is the use of masks (Hadamard encoding) to spatially distribute the laser beam across the imaging area and then to back transform the signal by software procedures [113–115] In favorable cases detection limits similar to conventional optical scanning densitometers in the absorption mode have been achieved

7 QUALITATIVE SAMPLE IDENTIFICATION

A prerequisite for quantitative analysis is the certain identification of the analyte to be determined and the certainty that the observed signal resulted only from the desired analyte One general weakness of TLC methods is that they provide insufficient qualitative information to remove any doubt that a substance having a specific R_F value could not be incorrectly identified This is a problem common to all chromatographic techniques that is usually solved by interfacing the chromatographic system to a detector capable of yielding structural information from the sample The techniques available in TLC are

optical spectroscopy, infrared and Raman spectroscopy, and mass spectrometry Coupling of TLC to these techniques in an on-line manner has been achieved with various degrees of success. With the exception of optical spectroscopy many of these techniques are still too insensitive to yield complete spectral features at sample loads typical of modern TLC or suffer from spectral or physical interferences resulting from the presence of the sorbent medium However, real progress has been made in the last few years to overcome or circumvent many of these problems

7 1 Optical spectroscopy

Most scanning densitometers make some provision for either manually or automatically recording the in situ spectra of any desired number of spots For automatic spectrum recording a motor-driven monochromator controlled by a computer is used For manual recording of spectra the spot is scanned repetitively while the monochromator position, or fluorescence emission filter for recording the fluorescence spectrum, is changed by fixed-wavelength increments between scans A line connecting the individual peak maxima gives the substance-characteristic absorption or fluorescence emission envelope The absorption spectra are rarely sufficiently characteristic for substance identification except by direct comparison with a standard measured on the same plate The correspondence between solution spectra and in situ absorption spectra can be quite poor in part due to the fact that the in situ spectra are usually measured under low-resolution conditions To maximize light throughput the band pass of most monochromators used in scanning densitometry is in the range 10–30 nm There may also be some contribution to the measured spectra from plate absorption or scatter which may in itself change with the measuring wavelength In the case of the fluorescence spectra, the solution and in situ spectra may show little correspondence due to the concert of energy loss and energy conversion mechanisms available to the adsorbed sample

It is less time-consuming to scan a separation sequentially at several characteristic wavelengths than to record the full spectra of each spot [21,116,117] The ratios of the response values obtained at these characteristic wavelengths can be used to confirm the similarity between samples and standards or to indicate contamination of a sample spot with other components The wavelength selected for identification should emphasize the spectral characteristics of the substance of interest, for example, wavelengths corresponding to peaks and troughs in the spectra, etc If standards are run on the same plate with the samples then the reproducibility of absorbance ratios is reasonable, R S D = 1–6% [117] Otherwise, the reproducibility of the absorbance response ratios will depend on how accurately the monochromator can be reset to a particular wavelength between measurements For the same reason, the accuracy is improved if all samples and standards are first scanned at one wavelength and

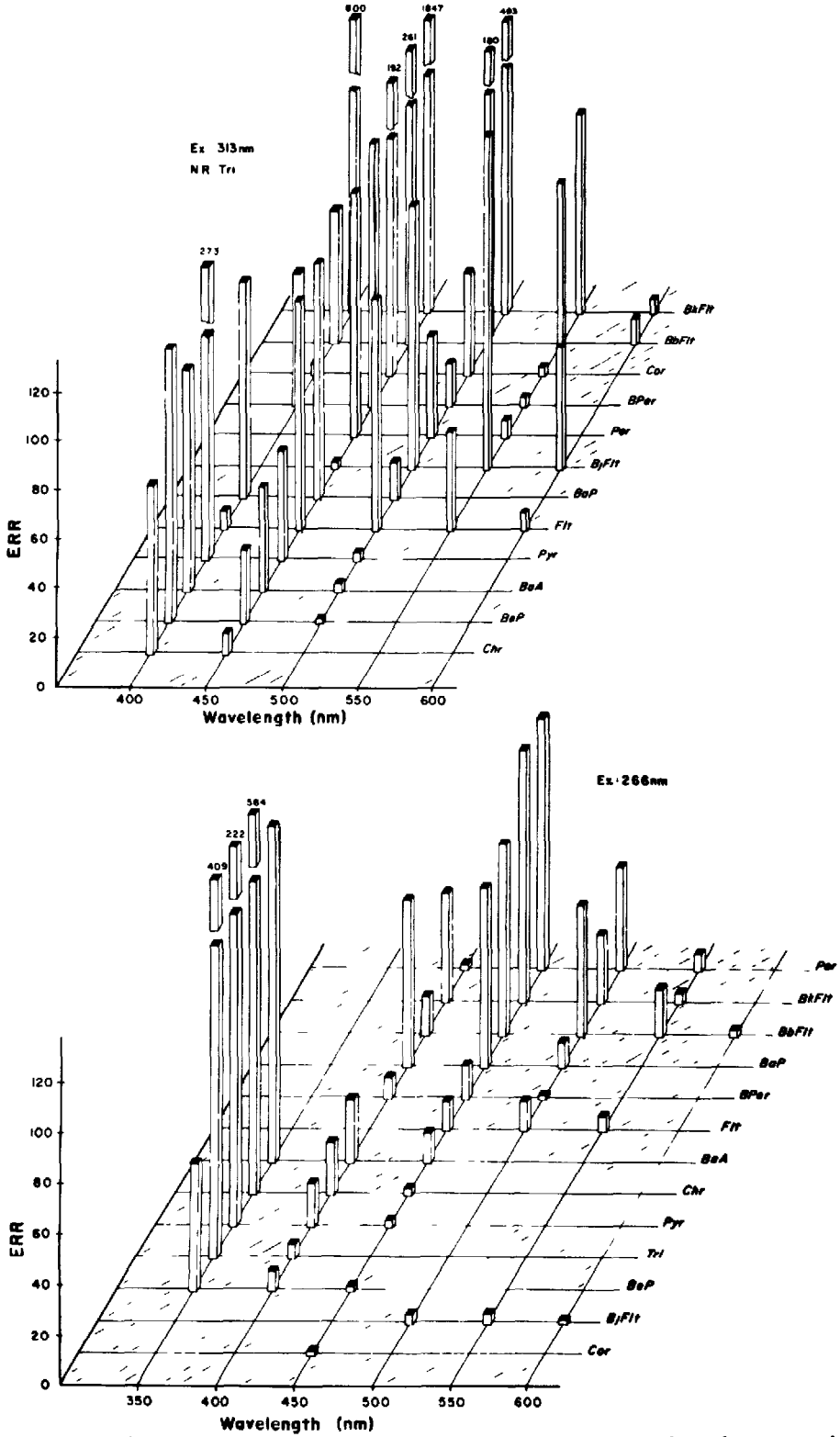

Fig 9 Plot of emission response ratios for some polycyclic aromatic hydrocarbons at a relatively non-selective excitation wavelength (313 nm) and a more selective excitation wavelength (266 nm) (Reproduced with permission from ref 118)

then the monochromator adjusted to the next position and the scanning repeated. Combining the information from coincidence of migration properties of samples and standards in the same chromatographic system and acceptable agreement between the absorbance or fluorescence emission response ratios is the most widely used technique for in situ substance identification in HPTLC

Spectroscopic selectivity is much higher in the fluorescence mode because two different wavelengths, an excitation wavelength and an emission wavelength, are used for each measurement. Additionally, the majority of organic compounds are not naturally fluorescent, but of those that are, several are of environmental or biological importance The fluorescence emission properties as a function of the excitation wavelength for polycyclic aromatic hydrocarbons can be expressed on a standardized scale using eqn. 14 [41,118].

$$\mathrm{ERR(ex,em)} = \frac{[\mathrm{PAH(ex,em)}]/ng}{[\mathrm{Per(ex,em)}]/ng^*} \times 100 \qquad (14)$$

where ERR(ex,em) is the emission response ratio at a given excitation and emission wavelength, PAH(ex,em) the peak area of a test compound at a given excitation and emission wavelength, Per(ex,em) the peak area for perylene at the given excitation and reference emission wavelength, ng the amount of PAH in nanograms, and ng* the amount of perylene in nanograms For sample amounts that remain within the linear response range of the densitometer, the emission response ratios can be reproduced with an R S.D $=3\%$ from day to day

The ERR values can be used in several ways Plotted in three dimensions as a function of compound type and emission wavelength at a fixed excitation wavelength (Fig 9) they can be used to select optimum conditions to maximize sample detectability or selectivity The product obtained by dividing any combination of ERR values by each other and normalizing the ratios provides a substance-specific ratio suitable for qualitative identification of polycyclic aromatic hydrocarbons in environmental samples [40,41] The ERR values were developed for the identification of polycyclic aromatic compounds but there is no fundamental reason why the same approach could not be applied to other fluorescent compounds

7 2 Infrared and Raman spectroscopy

Infrared spectra of separated TLC spots have been recorded by three different techniques [119] The off-line method involves the in situ elution of the spot using a commercial device, Eluchrom^{TM}, into a small amount of powdered potassium bromide followed by evaporation of the solvent The sample and potassium bromide powder are then pressed into a micro pellet that is suitable for recording spectra using a beam condenser and a standard grating infrared spectrometer [120] About 5 μg of sample is needed to obtain a useful spectrum

A commercial device is available for the in situ recording of infrared spectra of TLC spots by diffuse reflectance Fourier transform infrared (DRIFT) spectroscopy [121] For this purpose the spots are positioned manually below the probe beam of the attachment. The beam diameter is 1 0 mm. To obtain reasonable infrared spectra all solvents must be scrupulously removed prior to measurement and background correction made for absorption by the plate. The plate background correction was made by developing two identical plates simultaneously One plate contained the sample and the other acts as the blank Blank spectra are then subtracted from sample spectra at the same position on both plates. Even with background subtraction a major disadvantage of this technique is that all common TLC media have regions of strong infrared absorption which prevents reliable spectral data being measured in these regions [122]. For silica gel, for example, it is difficult to obtain any useful information in the regions 3700–3100 and 1650–800 cm^{-1}. At least 1 μg of material is required to detect individual functional groups and at least ten times this amount for partial spectral recording Also, compounds bind differently to different sorbents and this is usually reflected in changes in band positions which make spectral interpretation less reliable without reference spectra obtained on the same sorbent.

An alternative to in situ measurement is to perform the measurement after transfer of the chromatogram to an infrared transparent substrate without loss of the chromatographic resolution [123–126]. In this way detection limits can be lowered by an order of magnitude and loss of spectral information due to absorption by the TLC sorbent eliminated The Chromalect™ device contains a long metal strip with 58 sample cups, 1 mm in diameter, and 1 6 mm apart Each cup contains a wick and is filled to the top with an infrared transparent powder The metal strip is placed along one edge of the TLC plate orthogonal to the direction of chromatographic development. The separated spots are transferred to the metal strip by elution with a stronger solvent than used for the chromatography When the solvent reaches the wicks the sample is transferred into the infrared transparent powder and the solvent removed by controlled evaporation with a stream of air After the transfer is completed, the metal strip is placed in an automated diffuse reflectance sampler for measurement Spectra of the analytes are identical to published reference spectra in contrast to the case of in situ measurements and full spectra are easily obtained from about 20 μg per component

Infrared spectra have also been recorded using photoacoustic spectroscopy (PAS). PAS spectra are usually measured by enclosing the sample in a fixed volume cell The sample is then illuminated through a window with chopped monochromatic radiation The radiation energy absorbed by the sample is converted to heat which flows to the surrounding gas and generates a pressure pulse that is detected with a microphone connected to the sample chamber The output from the microphone is fed to a loc-in-amplifier referenced at the

frequency of the source radiation The PAS absorption spectra are then generated by plotting the lock-in-amplifier signal as a function of the wavelength of the incident radiation. Samples were applied to small discs of silica gel or spots cut out from plastic-backed TLC plates and mounted in a standard PAS cell [127,128]. Similarly to the DRIFT method discussed above it is difficult to extract any meaningful information in those regions of the spectra where silica gel shows strong absorption Also, it is necessary to purge the cell of vapors from the TLC plate, carbon dioxide, water, etc , that can easily swamp the PAS signal arising from the condensed phase. After background subtraction for the absorption due to silica gel a detection limit of 1 μg of caffeine (carbonyl absorption band) was obtained [127]. PAS has also been used to measure visible spectra of TLC spots In early experiments the sample spot was scrapped from the plate and sealed in the PAS cell [129] It was very difficult to obtain reproducible signals by this method as the signal generated was influenced by both the particle size and amount of silica gel sealed into the cell In later experiments 7 mm^2 portions of a flexible TLC plate were cut from the chromatogram and mounted onto the tip of a quartz rod by double-sided adhesive tape which was then positioned in the PAS cell [130]. A detection limit of 0.1 ng of cobalt and nickel and 0 4 ng of copper as their 1-(2-pyridylazo)-2-naphthol chelates was obtained. A complete visible spectrum could be obtained from 10 ng of chelate. Fishman and Bard [131] developed an open-ended cell for PAS that could be attached to different regions of the TLC plate. The illuminated area on the plate was 0 35 cm^2. With this movable cell a detection limit of 0 4 μg of rose bengal dye was obtained

Surface enhanced Raman spectroscopy (SERS) is unaffected by the problems due to moisture and background absorption that plague in situ infrared measurements [132,133] The sensitivity and spatial resolving power of the technique are compatible with the modern practice of TLC. Resonable spectra can be obtained from as little as 10–60 ng of sample per spot. Compared to solution spectra changes in band intensities and positions are sometimes observed for the sorbent adsorbed species Further work is required to define the optimum parameters for recording spectra, for example, whether a post-chromatographic treatment with a silver colloid is needed as claimed in one report [133] Not withstanding these procedural difficulties, the method holds a great deal of promise for the future.

7 3 Mass spectrometry

Four methods have been described for obtaining mass spectra of TLC spots The spot can be scrapped from the TLC layer and the sample eluted with solvent and then evaporated to a residue for insertion into the mass spectrometer or the sample plus sorbent can be inserted directly into the mass spectrometer [134–136] The direct insertion of sample plus sorbent into the ion source

generally requires relatively high temperatures for desorption and large sample amounts to give usable mass spectra Polar and high-molecular-mass samples are not easily desorbed and separation of the sample from the sorbent is generally preferred for these samples

A direct interface for obtaining chemical ionization mass spectra from spots on various types of TLC plates has also been described [137,138] A scanning device is used to move the TLC plate past a source of desorption energy, either a low-power pulsed carbon dioxide laser or tungsten filament incandescent lamp The chemical ionization gas is then used to sweep the desorbed materials through a heated inlet system into the ion source of a quadrupole mass spectrometer. The laser method provides very little bulk heating of the plate and is preferred to the incandescent filament when thermally unstable and involatile compounds are to be examined. From studies of model compounds it was shown that polar and high-molecular-mass compounds are only poorly transferred to the mass spectrometer This may be due to inefficient desorption from the plate or recondensation on colder zones within the scanner unit Compounds with molecular masses greater than 200 showed a considerable decrease in sensitivity compared to lower-molecular-mass compounds and few compounds with molecular mass greater than 300 could be detected at all. In favourable cases detection limits of about 1 0 ng were obtained by single ion monitoring and about 10 ng when a small mass range was scanned However, the precision was very poor when attempts were made to make quantitative measurements of TLC spots

Fast atom bombardment (FAB) mass spectra of thermally labile and involatile samples can be obtained directly from TLC plates [139,140]. The standard FAB probe is covered with a strip of double-sided masking tape and the tip of the probe pressed against the TLC spot to lift it from the plate Small volumes of solvent and FAB matrix solution, a few microliters, are then added to the sorbent and the probe introduced into the mass spectrometer to acquire mass spectra in the usual way Useful mass spectra were obtained from samples in the 10 ng–1 μg range

Novak and Hercules [141] have demonstrated that mass spectra can be obtained from involatile samples without removal from the TLC plate using the laser microprobe mass analyzer This instrument is designed for surface analysis and uses a laser beam for ablation and ionization of the surface species under high-vacuum conditions. Good-quality mass spectra were obtained from 10-ng samples for a number of ionic dyes on TLC plates The use of a laser provides high spatial resolution and single spots could be sampled at several positions to determine sample homogeneity and to mass-resolve incompletely separated components. Both the availability of the apparatus and the sample requirements (very low volatility) make this procedure more specialized than the others we have discussed in this section

Liquid secondary ion mass spectrometry (SIMS) has recently been dem-

onstrated as a suitable technique for obtaining mass spectra of in situ samples on TLC plates [142–147]. The mass spectra obtained by SIMS typically contain even-electron ions of the same type observed in chemical ionization mass spectra and the fragmentation processes observed are analogous in many cases In the SIMS experiment the scrupulously dry plate in a vacuum chamber is scanned through an ion beam using a motor-driven scanning stage similar in principle to optical scanning densitometers. The primary ion beam, usually argon or cesium, is accelerated to high energy and collides with the surface transferring its energy in a collision cascade to atoms and molecules in the surface region Neutral molecules, ions, electrons, and photons are ejected from the surface. The ions of interest are drawn into the mass spectrometer by an extraction potential where they are analyzed. The sensitivity of the technique can be improved by using a phase transition matrix and time averaging [143–146] Since ions are removed only from the surface the bulk of the sample is not ionized by the primary ion beam unless a mechanism can be found to extract and cycle the major portion of the sample to the surface in a continuous manner Impregnating the chromatogram with a viscous liquid or low-melting-point solid such as glycerol or 1,2,3,4-butanetetrol extracts the sample from the sorbent and allows its vertical movement to the surface when ionization occurs (the primary ion beam provides sufficient energy to melt the solid matrix in the neighborhood of its point of impact, this provides extraction without zone broadening) By using a phase transition matrix a steady signal can be obtained for several hours permitting extended data collection and lowering of the sensitivity of the technique to the 10-ng range More usual sample sizes for obtaining full mass spectra without averaging are in the microgram range.

8 BIOMEDICAL APPLICATIONS

Biomedical applications of TLC are far too numerous to survey in detail Some examples of separations using scanning densitometry will be given in this section to indicate the scope and analytical utility of quantitative TLC in the life sciences. Citations to compound type may be found in the Bibliography Section of the Journal of Chromatography, Fundamental Reviews Section of Analytical Chemistry, and in the Camag Bibliography Service (Camag, Muttenz, Switzerland) The book edited by Treiber [11] contains chapters on the quantitative analysis of steroids, vitamins, alkaloids, antibiotics, bioactive material in plant extracts, and proteins by TLC Fried and Sherma [97] have reviewed the application of TLC to the separation and analysis of lipids, amino acids, carbohydrates, natural pigments, vitamins, nucleic acids, steroids, and pharmaceuticals. More recent subject reviews published since the above compilations include the analysis of foods [147], surfactants [148], drugs in formulations and biological fluids [149–152], lipids [153–155], polyamines [156], amino acids [157], carbohydrates [158] and mycotoxins [154, 159] by TLC

Separations by high-performance liquid chromatography (HPLC) and TLC occur by essentially the same physical methods The two techniques are frequently considered as competitors when it would be more realistic to consider them as complementary to each other By HPLC it is easier to generate large numbers of theoretical plates and simpler to automate the analytical procedure than is the case for TLC. On the other hand TLC offers a much higher sample throughput due to the possibility of performing separations simultaneously and can handle cruder samples since the separation medium is used only once and then discarded. There is no possibility of column contamination which decreases the practical sample throughput of HPLC due to the delay in restoring the column to its original separation condition. The detection process in HPLC is dynamic while in TLC it can be considered to be static. The TLC plate is a storage detector which can be evaluated at intermediate times during the separation, as is commonly done in multiple development, or can be evaluated sequentially by different detection techniques at the completion of the separation It is relatively simple to apply chemical reagents to enhance detection selectivity and sensitivity in a manner unrestricted by the time constraints that must apply to a dynamic detection system. This enhances the flexibility of detection and makes possible the routine analysis of substances containing weak chromophores.

In light of the above discussion we would anticipate TLC methods being most widely used for the cost-effective analysis of simple mixtures where the sample load is large, for the rapid analysis of samples requiring minimum sample cleanup, for the analysis of samples containing matrix components that remain sorbed to the separation medium or contain suspended microparticles, and for the analysis of substances with poor detection characteristics that require post-chromatographic treatment for their determination Examples of the above points will follow. Several studies have demonstrated that when the above constraints apply TLC methods have a substantial economic advantage compared to HPLC methods [2,149,150,160–162]. There is no doubt that TLC methods could be more widely used than they are. There seem to be two primary reasons for this. Modern TLC is equipment-intensive and less likely to be available to the analyst than a liquid chromatograph. Thus analytical methods become automatically developed around HPLC even if there are substantial economic and practical reasons to use TLC. It is often overlooked that a densitometer can serve several projects with minimum reconfiguration and virtually no down time between projects while a high-performance liquid chromatograph usually has to be dedicated to a particular assay and multiple assays generally require the availability of several instruments Samples which are analyzed only occasionally become a problem using HPLC due to the time required to establish the new operating conditions for the sample. The second problem is part psychological and educational Most analysts have been trained by conventional TLC practices where low separation efficiency and semi-

quantitative analysis are accepted in return for simplicity, low cost, and speed. This establishes their horizons for TLC. Few analysts are knowledgeable about developments that have occurred in modern TLC and have no hand-on experience of the technique. Their evaluations are often made using faulty and biased information form the collectively larger HPLC marketing strategists who do not want to loose sales to a competing product line in which they have no representation

High-volume samples can be simple mixtures such as formulations of approximately known concentration or complex mixtures where it is desired to identify and quantify only a few substances or to obtain a fingerprint characteristic of the sample. Formulations may be analyzed for quality control applications, for forensic identification, or for stability evaluation In the U S.A. seven ethynyl steroids are currently permitted for use as oral contraceptives or used to control menstrual disorders. Individual ethynyl steroids in any tablet formulation can be identified with a single TLC system and the ratio of the components used to identify the make of tablet (Fig. 10) [34]. The concentration of individual active ingredients can be determined with an R.S D of between 1 and 1 5% with minimal sample cleanup. The tablets are simply dissolved by sonication in a suitable solvent and, after centrifugation to precipitate insoluble carrier substances, applied directly to the TLC plate

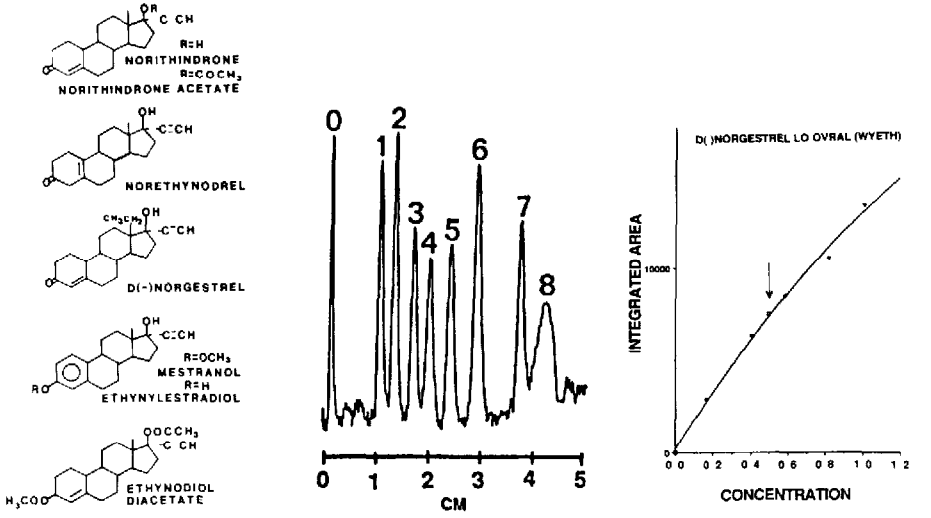

Fig 10 Separation of ethynyl steroids on silica gel 60 HPTLC plates using two 15-min developments in the solvent system hexane–chloroform–carbon tetrachloride–ethanol (7 18 22 1, v/v) and the determination of D-(−)norgestrel (arrow) in Lo-Ovral contraceptive tablets Steroid identification 0 = methyl green (lane marker), 1 = 17α-ethynylestradiol, 2 = norethindrone, 3 = norgestrel, 4 = norethynodrel, 5 = norethindrone acetate, 6 = mestranol, 7 = ethynodiol diacetate, 8 = solvent front (Reproduced with permission from ref 34)

TLC is frequently used for purity determination of pharmaceutical products in which, generally, the contaminants represent only a small fraction of the extractable sample An example is shown in Fig. 11, for the determination of D-tyrosine in L-tyrosine at the 0 1% contamination level [163]. The separation of enantiomers based on ligand exchange (largely for amino acids, α-hydroxy acids, peptides and their derivatives) [163,164] and of diverse small molecules (drugs, metallocenes, crown ethers, nicotine derivatives, etc) by host–guest complexation with soluble β-cyclodextrins [165] are now well established techniques The limit of determination for minor components will depend on the sensitivity of the contaminant to detection, the operation of the densitometer, and the selectivity, both chromatographic and spectroscopic, of the method. Fig. 11 provides an example of the determination of minor contaminants in the solvent-extractable fraction of a metoprolol tartrate tablet as part of a stability study [65] Two sample loadings were used, a 200-nl sample to determine 1-(isopropylamino)-3-[4-(2-hydroxyethyl)phenoxy]-2-propanol (II) and 1 0 μl for 4-(2-methoxyethyl)phenol (III) which is present in lower concentration At the higher sample loading component II is inadequately resolved from the major component and, therefore, both components cannot be determined in a single sample application. In the example shown the average amount of II was 0 78% (w/w) and of III 0.06% (w/w). The TLC identification of minor components in street heroin can be used to identify potential sources of the illicit drug [166] A quantitative TLC method has also been

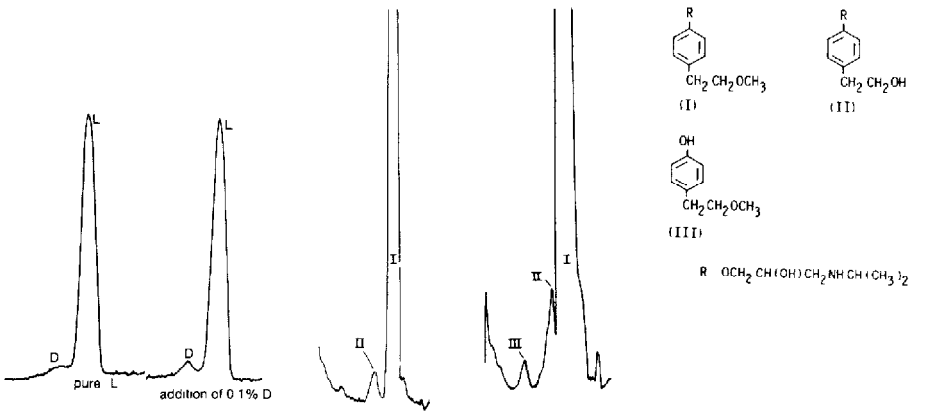

Fig 11 Determination of minor component contaminants in pure substances On the left the detection of D-tyrosine in L-tyrosine on a Chiralplate (Macherey-Nagel, Duren, F R G) after visualization with ninhydrin and right the determination of compounds II and III in aged tablet formulations of metoprolol tartrate (I) after separation on a Whatman KC_{18} reversed-phase plate for 10 min in the solvent system 10% aqueous ammonia–methanol (3 7) and detection in the dual-wavelength single-beam mode at $\lambda_1 = 280$ nm and $\lambda_2 = 300$ nm (Adapted with permission from refs 65 and 163)

described for accelerated stability studies for prediction of inherent sensitivity of drugs toward oxygen [167].

Other examples of stability and impurity analysis by quantitative TLC include the determination of nicotinic acid esters in pharmaceutical creams [32], impurities in mebeverine hydrochloride tablets [168], and impurities in the antibiotic erythromycin [169]

Quantitative TLC is ideally suited to large-scale screening programs since costs can be minimized using parallel separations and certain kinds of matrix interferences can often be tolerated to a higher level than is possible with HPLC. Recreational drug use continues to be a major national problem in the U S A as evidenced during the past several years by extensive drug-testing programs launched by the Armed Forces and industry. Most drug-testing programs contain two elements, a preliminary urine screen to target samples for further analysis, and a second, more specific confirmatory analysis. For screening purposes either immunoassays or TLC are used [151,152,170–172] Since TLC incorporates a separation step in the identification it is generally more specific than the immunoassays (cross-reactions are a problem in immunoassay) and for screening urine for unspecified drugs TLC has a greater potential for identification (many immunoassays are specific for an individual drug) Various estimates place the number of urine samples currently submitted for drug screening at about 18 million annually in the U.S A., a number that is anticipated to increase in future years [171] The principal drugs of abuse are amphetamines, barbiturates, benzodiazepines, cannabinoids, cocaine, methaqualone, opiates, and anorexicants. The detection sensitivity required for drug screening is not too stringent predicated by the desire to avoid certifying as positive passive or accidental exposure to illicit drugs

Detection limits vary substantially for individual drugs and the specifics of their metabolism, but in general, minimum detection requirements in the range 20–300 ng/ml is presently accepted as adequate For most drugs, this level is easily reached by scanning densitometry Parallels exist for drug abuse in sports for the purpose of improving athletic performance. Fig 12 shows a separation of a series of performance-enhancing drugs by forced-flow TLC [173] The average time required for a single urine analysis including extraction, sample application, development, and detection was about 8–10 min for these drugs Other large-scale screening programs are employed in pharmacognosy to identify individual plant species and their biologically active components. Some recent applications include the fingerprinting of crude gensengs from various sources [174], the characterization of tropane alkaloids [175], coumarin isomers [176], iridoid glycosides [177], and gensenosides [178]. Recent publications on the identification of individual drug groups include sulfonamides after conversion to their fluorescamine derivatives [179], adrenergic β-blockers without derivatization [180] and as their dansyl derivatives [181], diuretics [182], steroids [183], and macrolide antibiotics [184,185]

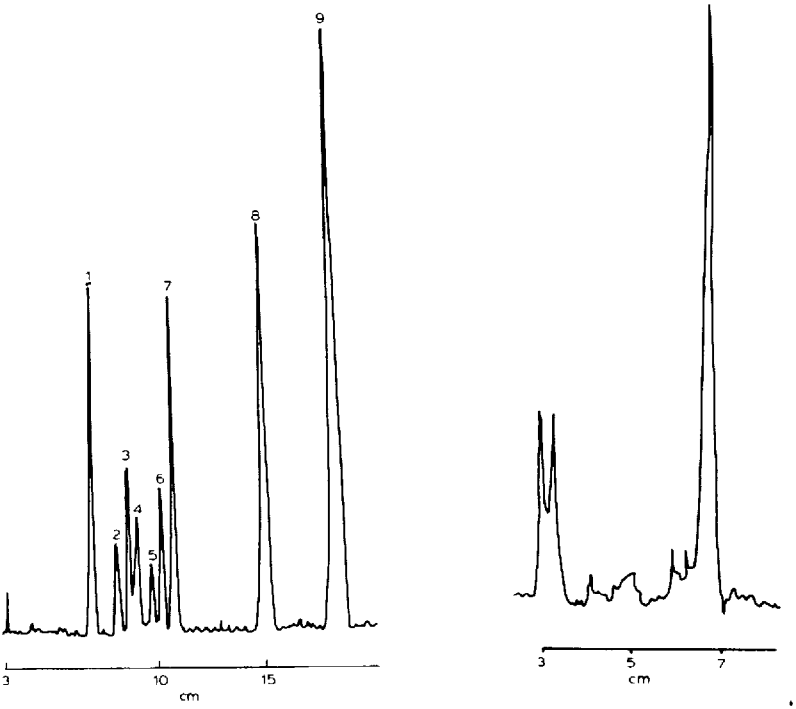

Fig 12 Separation of doping agents and a urine extract by forced-flow TLC on a silica gel 60 HPTLC plate with the mobile phase *n*-butanol–chloroform–methyl ethyl ketone–water–acetic acid (25 17 8 4 6) at a linear velocity of 0 85 cm/min Identification of standards 1 = strychnine, 2 = ephedrine, 3 = methamphetamine, 4 = phenmetrazine, 5 = methylphenidate, 6 = amphetamine, 7 = desopimon, 8 = coramin, 9 = caffeine (Reproduced with permission from ref 173)

The quantitative analysis of drugs in blood and biological fluids is complicated by the low concentrations normally encountered and by interferences from metabolic products which may closely resemble the parent drug Quantitative TLC methods have been used for bioavailability studies in man and animals, for metabolism and pharmacokinetic studies, and for therapeutic monitoring.

Chlorpromazine is a widely administered psychopharmacologic agent in mental health facilities. Estimates of the amount of chlorpromazine present in blood serum during therapy range from about 10 to 300 ng/ml Chlorpromazine and other tricyclic antidepressants can be determined at therapeutic levels after extraction of 1 0 ml of serum at high pH, back-extraction into acid, and re-extraction after adjusting the pH [186,187]. After solvent reduction the extracts can be applied directly to the TLC plate for separation and quantitation (Fig 13) The recovery and reproducibility of the assay were improved by adding internal standards, butaperazine for chlorpromazine and loxapine for the tricyclic antidepressants, as well as carrier substance, perphenazine, to

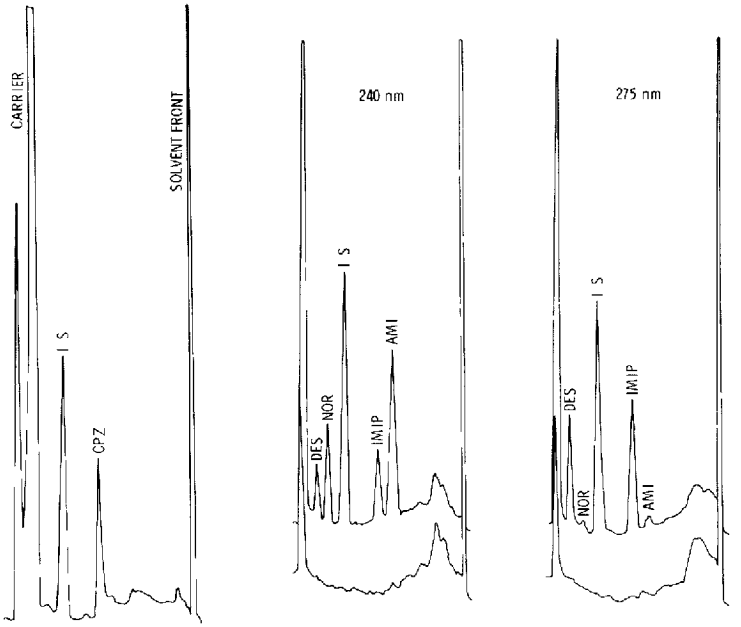

Fig 13 Separation of tricyclic antidepressants after extraction from serum on a silica gel HPTLC plate with hexane–acetone–diethylamine (80 20 3) as the mobile phase Peaks CPZ = chlorpromazine (corresponding to 45 ng/ml), AMI = amitriptyline, NOR = nortriptyline, IMIP = imipramine, DES = desipramine (Reproduced with permission from ref 186)

the serum sample Perphenazine was selected as a carrier substance because it is structurally similar to the drugs being determined but has a low R_F value in the separation system and could be used in large excess without interfering with the compounds being measured Its influence is most dramatically seen on the recovery and reproducibility of the assay for the drugs at low concentrations (10 ng/ml) but it still provides a useful improvement at higher levels (Table 2) To maximize the detectability of the tricyclic antidepressants measurements were made at two different wavelengths, 240 and 275 nm, corresponding to the different absorption maxima of the individual drugs. The lower trace in Fig. 13 is the serum blank and indicates that the analysis is free from any co-extracted interferences

A microanalytical procedure utilizing TLC has been described for the determination of the anticonvulsant drugs phenobarbital, phenytoin, primidone, and carbamazepine in 50 μl of plasma [188] The drugs were isolated in a single extraction and the extracts dried and applied to the TLC plate by contact spotting The method lends itself to the analysis of numerous samples as well as to emergency situations, where a rapid and accurate assay is required TLC with a post-chromatographic derivatization reaction was used to identify the metabolites and monitor the pharmacokinetics of nafazatrom in man and animals

TABLE 2

RECOVERY AND REPRODUCIBILITY OF THE HPTLC ASSAY FOR PSYCHOPHARMACOLOGICALLY ACTIVE DRUGS IN SERUM USING PERPHENAZINE AS A CARRIER SUBSTANCE

Drug	Concentration in serum (ng/ml)	Recovery (%)		Reproducibility (C V [a], %)	
		With carrier	Without carrier	With carrier	Without carrier
Chlorpromazine	100	96	87	3 3	2 6
	10	80	54	8 0	34 2
Amitriptyline	100	99	87	2 6	7 9
	10	95	71	7 8	11 3
Nortriptyline	100	77	73	3 1	12 4
	10	83	62	8 5	18 5
Imipramine	100	91	86	4 0	7 9
	10	86	77	4 2	11 3
Desipramine	100	70	62	6 0	13 5
	10	82	72	10 7	17 7

[a]Coefficient of variation

[189]. Nafazatrom, 3-methyl-1-[2-(naphthyloxy)ethyl]-2-pyrazolin-5-one, was extracted directly from 1 0 ml of serum or urine containing L-cysteine hydrochloride as an antioxidant and the extract applied directly to the TLC plate. After development the plate was dipped twice in a solution of 4-dimethylaminobenzaldehyde and heated for 10 min at 80°C The reagent reacts selectively with the pyrazolin-5-one ring to yield orange–red spots on a white background that are stable for several weeks (Fig. 14). The detection limit for nafazatrom was about 0 2 ng per spot corresponding to 5 ng/ml in biological fluids.

By a similar procedure the pharmacokinetics of the diuretic muzolimine, 3-amino-1-(3,4-dichloro-α-methylbenzyl)-2-pyrazoline-2-one, was studied after post-chromatographic derivatization with 4-dimethylaminocinnamaldehyde [190]. The limit of determination of muzolimine in urine or serum was 1 ng/ml for a 1-ml sample

Other notable pharmacokinetic studies using quantitative TLC have been published for salbutamol [191], 1,2,3,9-tetrahydro-9-methyl-3-(2-methyl-1H-imidazol-1-yl)-4H-carbazol-4-one [192], antipyrine [193], ampicillin [194], and digitoxin [195]

TLC has been used to determine porphyrins in biological fluids which act as biological markers for a range of diseases known as porphyrias Each of the porphyrias is characterized by a specific pattern of porphyrins and porphyrin precursors in body fluids, feces, and tissue as a result of overproduction and accumulation Separation of porphyrins by TLC (Fig 15) with prior deriva-

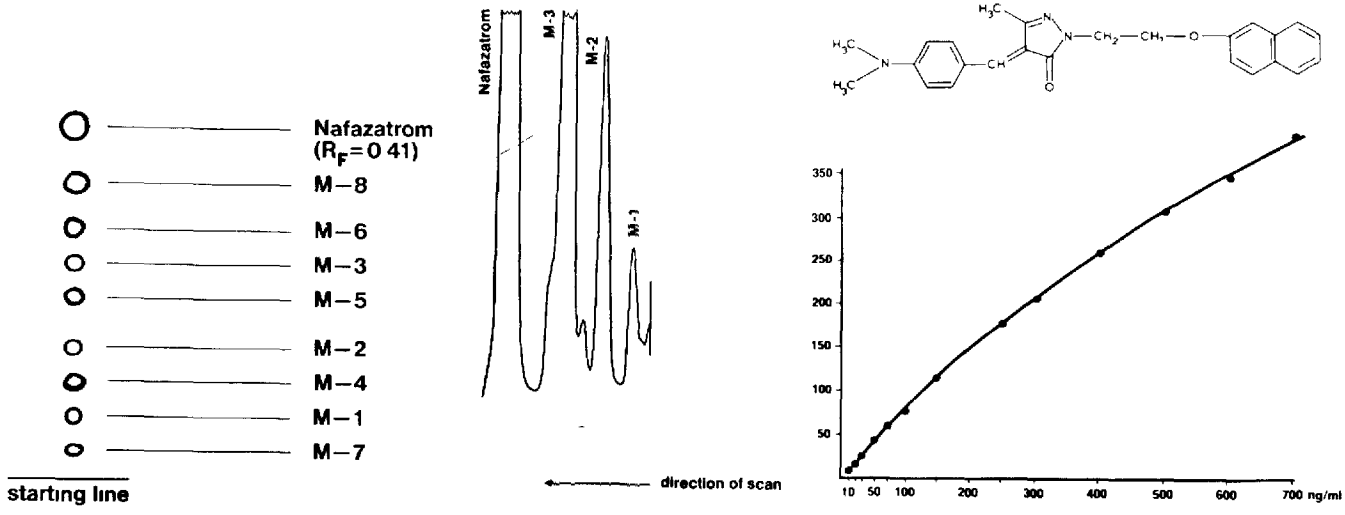

Fig 14 Separation of nafazatrom and its metabolites in human urine (solid line) and dog urine (broken line) by TLC after post-chromatographic derivatization with 4-dimethylaminobenzaldehyde for detection at $\lambda = 490$ nm The mobile phase for the separation was chloroform–ethanol–acetone (90 5 5) on silica gel HPTLC plates (Reproduced with permission from ref 189)

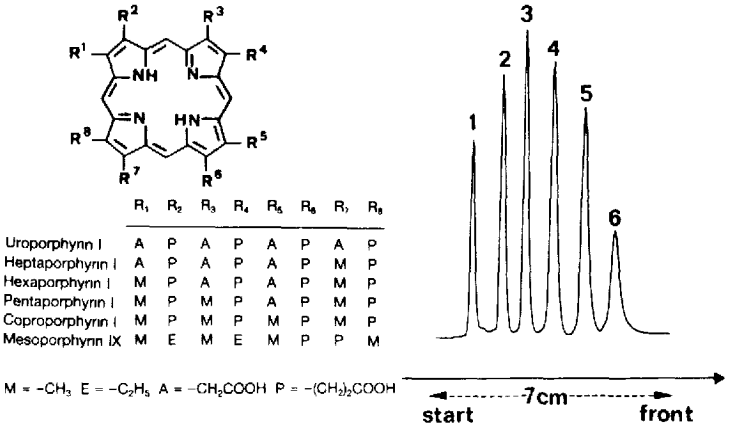

Fig 15 Separation of urinary porphyrins by reversed-phase TLC in a buffered mobile phase containing N-cetyl-N,N,N-trimethylammonium bromide as ion-pair reagent The porphyrins were detected by their fluorescence at 560 nm after excitation at 404 nm Each peak corresponds to 3 pmol of porphyrin Separation order 1 = mesoporphyrin IX, 2 = coporphyrin 1, 3 = pentaporphyrin 1, 4 = hexaporphyrin 1, 5 = heptaporphyrin 1, 6 = uroporphyrin 1 (Reproduced with permission from ref 196)

tization offers a sensitive, rapid, and reliable method of clinical diagnosis [196] The detection limit for the porphyrins was about 10 fmol and calibration curves were linear over the range 0.15–3 fmol per spot. Matrix co-extractants from urine have mainly blue fluorescence and appear in the upper part of the chromatogram and so do not interfere in the analysis

Probably the singularly most widely employed TLC method in biomedicine is the determination of lipids Lipids are critical components of the enzymatic, conduction, and transport systems of biological membranes. They comprise hundreds of distinct molecular species but, in the course of numerous chromatographic studies, it has become apparent that many organisms, tissues, cells, and subcellular components posses characteristic lipid compositions that can be recognized without complete resolution and determination of individual molecular species Quantitative estimation of a partially resolved lipid profile is frequently sufficient for establishing the origin of the total lipid extract and for assessing its relationship to normal or abnormal metabolic states. Few lipids have significant UV chromophores, limiting detection possibilities in HPLC and TLC, but post-chromatographic derivatization methods, easily applied in TLC, result in almost universal detection The displacement of conventional TLC methods by high-performance techniques has resulted in better resolution between critical lipid groups, faster separations, improved interlaboratory reproducibility, and improved detection because of the smaller, compact separated zones and the more stable densitometric plate background [197–206] Fig 16 is a representative chromatogram of a membrane lipid profile and il-

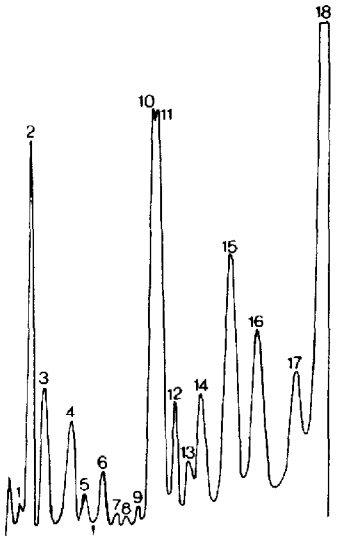

Fig 16 Separation of lipids from a mouse sciatic nerve extract fortefied with lysophosphatidylcholine, lysophosphatidylethanolamine, phosphatidic acid, phosphatidylglycerol, digalactosyldiacylglycerol, and monogalactosyldiacylglycerols Solvent system, methyl acetate–*n*-propanol–chloroform–methanol–0 25% aqueous potassium chloride (25 25 28 10 7) on a silica gel HPTLC plate Identification 1 = lysophosphatidylcholine, 2 = sphingomyelin, 3 = phosphatidylcholine, 4 = phosphatidylserine, 5 = lysophosphatidylethanolamine, 6 = phosphatidylinositol, 7 = phosphatidic acid, 8 = cardiolipin, 9 = phosphatidylglycerol, 10 = plasmalogen, 11 = plant plasmalogen, 12 = digalactosyldiacylglycerol, 13 = hydroxysulfatides, 14 = non-hydroxysulfatides, 15 = hydroxycerebrosides, 16 = non-hydroxycerebrosides, 17 = monogalactosyldiacylglycerol, 18 = neutral lipids merged with the solvent front The lipid zones were visualized by charring with copper acetate–phosphoric acid and scanning at 366 nm in the absorption mode (Reproduced with permission from ref 199)

lustrates the potential of modern TLC to obtain clinically useful characteristic profiles of complex extracts [199]

9 CONCLUSION

Optical scanning densitometry is now a mature instrumental technique Those changes which have occurred in recent years have largely been associated with the matching of conventional optical and mechanical designs to the enhanced capacity of computer control for optimization, automation, and data handling These changes have made optical scanning densitometry more convenient for the user while simultaneously reducing experimental errors. There is little evidence that the performance of modern scanning densitometers degrade the chromatographic resolution and in terms of accuracy and precision chromatographic and sampling errors seem to be more important in contemporary practice than errors associated with the operation of the densitometer.

Electronic scanning densitometers have improved rapidly in performance in recent years and may one day rival slit scanning densitometers for routine quantitative analysis. They will be most important for recording chromatographic information from two-dimensional separations that are difficult to scan mechanically Electronic scanning densitometers cannot compete with mechanical scanning densitometers at present in terms of cost or performance but these considerations can change rapidly as new technology develops and competes in the market place Improvements can also be expected in deriving structural information from separated zones on TLC layers without the need of overloading the separation layer and degrading resolution and, where appropriate, without having to separate the sample from the sorbent. Spectroscopists are starting to appreciate the unique flexibility of TLC to provide static separations simplifying interface design, to tolerate crude samples simplifying sample preparation, and to separate multiple samples simultaneously providing a high sample throughput

10 SUMMARY

The principal methods for obtaining quantitative information from separations performed by planar chromatographic techniques are reviewed. Recent advances in obtaining structural information for sample identification of separated components are also discussed Reasonable expectations concerning future developments in densitometry are made.

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