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# MODE SELECTION AND OPTIMIZATION OF PARAMETERS FOR RECORDING HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAMS BY TRANSMISSION MEASUREMENTS

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## SUMMARY

Optimum conditions to maximize the observed resolution and signal-to-noise ratio of a scanning densitometer for high-performance thin-layer chromatography are described. Transmission measurements are compared in three operating modes; two being pseudo dual-beam methods and the third a dual-wavelength single-beam mode. In all modes resolution is shown to be independent of those parameters which control the dimensions of the measuring beam within their accessible ranges. Signal-to-noise ratios depend on both the operating mode and the dimensions of the measuring beam. In particular, signal shows a marked dependence on the ratio of slit height to spot diameter. Similar values for resolution and better signal-to-noise characteristics may be obtained in the transmission mode compared to reflectance for substances absorbing in the visible range.

## INTRODUCTION

Commercial instruments for performing quantitative evaluation of thin-layer chromatograms (TLC) first appeared in about 1967 (see ref. 1). Such instruments have played an important role in the evolution of modern TLC; without such equipment the exquisite resolution obtained by high-performance thin-layer chromatography (HPTLC) would be to no avail and TLC would have remained a semi-quantitative technique. At best, inspection by eye of a TLC plate is capable of detecting about 1–10 $\mu$g of colored components with a reproducibility of not better than 10–30%. Excising the separated spots, eluting the substance from the sorbent material, and measurement by solution photometry is time-consuming and fairly insensitive. Difficulties in accurately locating the edge of the spot by eye, incomplete elution of the sample from the sorbent and non-specific background absorbance due to colloidal sorbent particles in the analytical solution add to the problem. *In situ* detection is essential for the accurate measurement of both spot size and location, for a true measure of inter-spot separation, and for rapid, accurate quantitation.

*In situ* measurements of substances on HPTLC plates can be made by a variety of methods: reflectance, transmission, simultaneous reflectance and transmission, flu-

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orescence quenching, and fluorescence[2,3]. Light striking the plate is both transmitted and diffusely scattered by the layer. Light striking a spot on the plate will undergo absorption so that the light transmitted or reflected is diminished in intensity at those wavelengths forming the absorption profile of the spot. The measurement of the signal diminution in the transmission or reflectance mode due to absorption by the spot provides the mechanism for *in situ* quantitation. Transmission measurements are limited to those wavelengths greater than 320 nm due to strong absorption by the glass backing plate and the sorbent itself at shorter wavelengths. However, for compounds absorbing in the visible region either reflectance or transmission may be used for quantitation. In this paper we will evaluate the instrument parameters which affect the observed resolution and signal-to-noise ratios in transmission scanning densitometry and compare these criteria to those established for reflectance. The approach adopted follows closely our previous studies of reflectance[4] and fluorescence[5] scanning densitometry. The instrument parameters of importance are the dimensions of the measuring beam (controlled by the slit height and slit width), scan speed, and the time constants of electronic recording devices. Although data are provided for a single instrument, the results obtained should be directly applicable to other commercially available densitometers, and the approach taken of general value for comparing the performance of different instruments.

## EXPERIMENTAL

All solvents were HPLC grade (Burdick & Jackson, Muskegon, MI, U.S.A.). Reagent-grade azobenzene and *o*-nitroaniline (Matheson, Coleman and Bell, Norwood, OH, U.S.A.) and *p*-nitroaniline (Eastman Kodak, Rochester, NY, U.S.A.) were used as received.

Separations were carried out on silica gel HPTLC plates (Whatman, Clifton, NJ, U.S.A.). Sample volumes of 200 nl were applied to the plates using fixed-volume Pt–Ir dosimeters (Applied Analytical Industries, Wilmington, NC, U.S.A.) in conjunction with a Nanomat HPTLC spotter (Camag, Muttenz, Switzerland) or a rocking applicator (W & W, Basel, Switzerland). Samples were spotted 1.0 cm apart and 1.0 cm from the bottom edge of the plate. The plates were developed in position 4 of a short-bed continuous-development chamber (SB/CD) (Regis, Morton Grove, IL, U.S.A.). Azobenzene standards were developed for 3 to 10 min with hexane–methylene chloride (1:1) as mobile phase to provide a range of spot sizes. The nitroaniline isomers were separated by development for 5 min using hexane–methylene chloride (3:2) as mobile phase.

All chromatograms were recorded using a Shimadzu CS-910 scanning densitometer (Shimadzu, Columbia, MD, U.S.A.) in the single- or dual-beam transmission mode without filtering. Azobenzene was determined at 350 or 470 nm and the nitroaniline isomers at 370 nm. The reference wavelength in the dual wavelength mode was 500 nm. The scanning stage and recorder were matched at 48 mm min$^{-1}$. Peak profiles were recorded on a Shimadzu U-135 strip chart recorder and peak areas with a Spectra Physics minigrator (Spectra Physics, Santa Clara, CA, U.S.A.). In some experiments the photomultiplier voltage was monitored using a Tektronix DM502 digital multi-meter (Beaverton, OR, U.S.A.).

## RESULTS AND DISCUSSION

Developing an effective criterion for characterizing the performance of a scanning densitometer is not a simple task as there is no absolute standard for which resolution and sensitivity information can be measured. Spatial resolving power can be measured by scanning a test strip containing a series of lines with sharply defined boundaries separated by varying distances[6]. However, this measure of resolution is completely artificial as real spots do not have sharp boundaries but a Gaussian-like sample distribution. A practical measure of instrument resolution is to compare the actual resolution measured for a partially separated pair of spots with that calculated from the profiles of individual standards run in parallel tracks[4]. It is also important that the conditions used to obtain maximum resolution do not compromise instrument sensitivity. As the same parameters which may influence resolution also affect the signal-to-noise ratio some compromise or optimum value for these parameters normally exists.

*Dependence of resolution on measuring conditions*

For slit scanning densitometers the image of the sampling beam on the plate is a rectangle defined by the slit width and the slit height. The slit width is the parameter which fixes the dimensions of the beam in the direction of scanning; the slit height defines the beam dimensions in the orthogonal direction. There are no slits on the collection side of the densitometer and the bolus of reflected light from the plate surface is collected by a hemispherical mirror and focused onto a photomultiplier above the scanning stage (reflectance mode) or the light passing through the plate falls onto a second photomultiplier located below the scanning stage (transmission mode). Within the normal range of slit settings the observed resolution is independent of the slit settings in the reflectance mode except for the largest available values[4]. Using the nitroaniline isomers to provide a series of partially separated peaks similar results were obtained in the transmission mode. For example, two spots with a mean resolution value of 1.08 were scanned at all slit width settings between 0.2 and 1.0 mm (constant slit height = 1.5 mm) and at all slit height settings between 1.0 and 10.0 mm (constant slit width = 0.5 mm). The relative standard deviation for changes in slit width was 2.4% and changes in slit height 4.2%, both within the precision with which manual measurements of peak profiles can be reproduced. Thus, signal-to-noise characteristics are more critical in defining optimum measuring conditions than resolution for spots of sizes normally encountered in HPTLC. Resolution can be considered independent of the measuring beam dimensions within its selectable range in the transmission mode.

*Mode selection for transmission measurements*

In the transmission mode the signal is defined as the diminution of light passing through the plate as the sampling beam traverses a spot compared to the value obtained as it traverses a blank area of the plate. The densitometer used in this study enables the transmission signal to be measured in three different ways. Two matched photomultipliers are used, one which measures the amount of light transmitted through the plate, and a second photomultiplier located above the stage, which views ligth from the source and reflected light from the plate. The light striking this second photomultiplier establishes the gain (negative voltage) on the transmission photomultiplier and thus directly influences instrument sensitivity and noise during measurement. A mechanical shutter can be used to block reflected light from the plate. In operating mode A, the reference photomultiplier views the source output directly. It thus provides some correction for fluctuations in the source output but not for plate inhomogeneity. With the mechanical shutter removed, the reference photomultiplier views light from both the source and reflected light from the plate (mode B). This provides some correction for fluctuations in the source output and for plate inhomogeneity. However, reflected light arises mainly from the plate surface and cannot be expected to completely allow for the vertical inhomogeneity in the separation media experienced by the transmitted beam. Both modes A and B are pseudo dual-beam single-wavelength measurement techniques. The third operating mode, mode C, is described as the dual-wavelength single-beam mode. Dual monochromators are used to select a measuring wavelength and a reference wavelength. The two beams are chopped and combined into a single beam which is transmitted by the plate and detected by the transmission photomultiplier and phase-locked amplifier circuit. In this configuration the reference photomultiplier is inoperative and the gain is established entirely by the intensity of the reference beam and is independent of the sampling beam. The dual-wavelength single-beam mode provides correction for both fluctuations in the source output and vertical inhomogeneity in the separation medium. The plate scatter coefficient is wavelength-dependent, so ideally the sample and reference wavelengths should have values close to each other. In practice, this is rarely the case, except for compounds having a sharp absorption profile, and less than ideal conditions are frequently used. To maintain high sensitivity the sample should not absorb significantly at the reference wavelength.

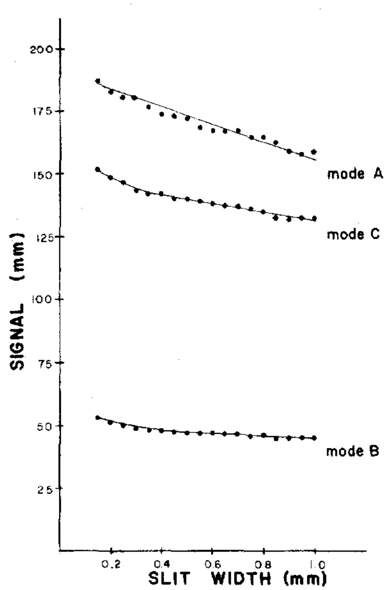



Fig. 1. Relationship between signal and slit width setting at a constant slit height (1.5 mm) for transmission measurements.

*Sensitivity-dependence on slit width*

Fig. 1 is a plot of signal (peak height) for azobenzene (1.11 mg $ml^{-1}$) measured at various slit widths and at a constant slit height of 1.5 mm. Modes A and C produce a considerably higher signal response than mode B, due to the higher gain of the photomultiplier established in modes A and C. The change in signal with increasing slit widths is similar for all three modes. The signal is greatest at small slit width settings and declines slightly as the slit width is increased. Again this follows the trend in photomultiplier gain which declines in proportion to the area of the measuring beam.

The dependence of the signal-to-noise ratio on slit width settings is illustrated in Fig. 2. For these measurements the signal is defined as the peak height of the response profile in millimeters and the noise as the absolute pen deflection on the recorder in millimeters as the sampling beam is held stationary over a blank area of the plate for 2 min[4]. As the plate is static during the noise measurements this value represents instrumental (electronic and optical) noise. However, the noise profile does not change upon scanning unless the plate surface has been damaged.

Mode C, Fig. 2, clearly provides the highest signal-to-noise ratio. The signal-to-noise ratio increases fairly uniformly with increasing slit width and reaches a maximum at the largest setting. Comparing Figs. 1 and 2, it can be seen that in mode C the noise decreases faster than the signal as the slit width is increased. A similar trend is observed for mode A, which generates the highest noise signal, and consequently a lower signal-to-noise ratio at all slit settings. The above results are characteristic

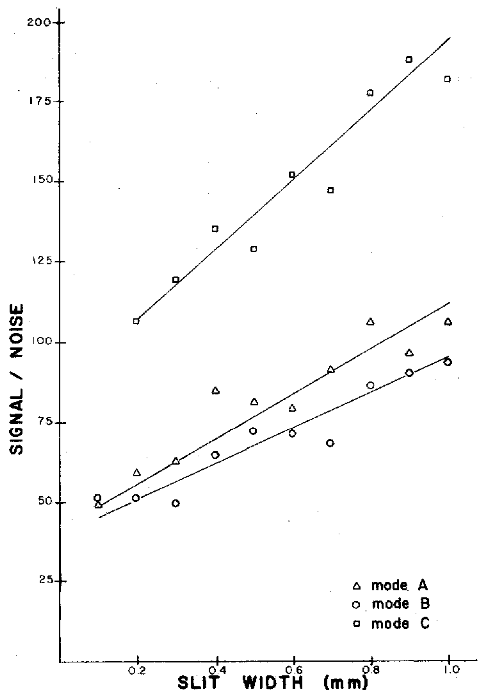
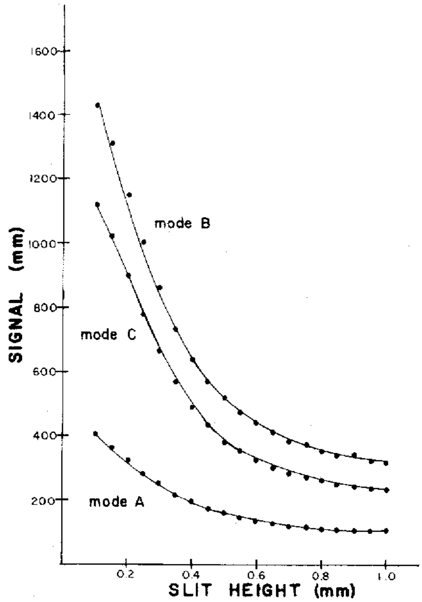



Fig. 2. Relationship between signal-to-noise ratios and slit-width setting at a constant slit height (1.5 mm) for transmission measurements.



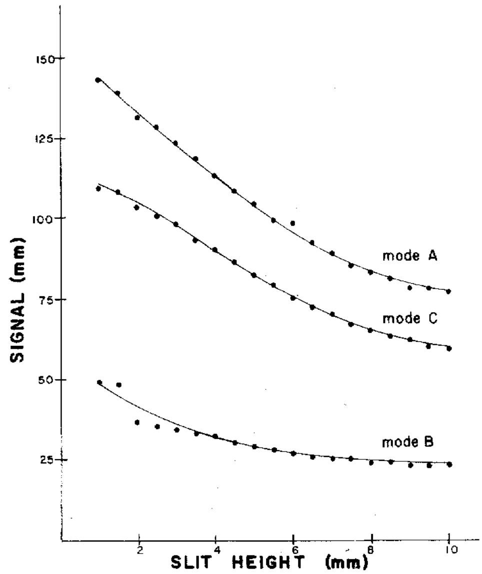



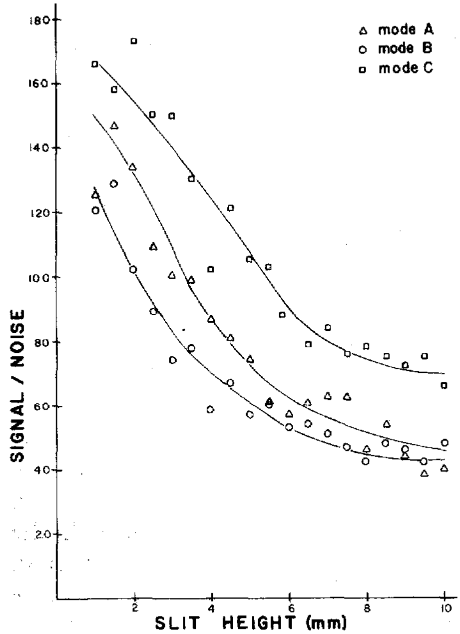

Fig. 3. Relationship between signal and slit-height setting at a constant slit width (0.5 mm) for a compact spot (3.0 mm).

Fig. 4. Relationship between signal and slit-height setting at a constant slit width (0.5 mm) for a diffuse spot (6.6 mm).

of the selected mode of measurement and the trends described independent of sample concentration and spot size. In all cases the maximum in the signal-to-noise ratio is found for the largest slit width setting available, 1.00 mm. As this does not compromise sample resolution the use of large slit width settings is recommended.

*The influence of the slit height–spot size ratio on sensitivity*

The signal (peak height or integrated area) shows a marked dependence on the slit height–spot size ratio at a constant slit width setting. The two extremes for the three measuring modes are shown in Fig. 3 for a compact spot (3 mm diameter) and a diffuse spot, Fig. 4 (6.6 mm diameter), each containing the same sample concentration. Intermediate spot sizes produce similar results lying between the two extremes. For the large spot size in which the diameter of the spot approaches the largest slit-height dimension available, the decline in signal with increasing slit height is fairly shallow. For small spots the signal changes more dramatically with slit-height dimensions. When the slit height is large, compared to the diameter of the spot, a large amount of light is transmitted from the blank area of the plate and the contribution from subtracted (absorbed) light is small. The signal is thus weak. As the slit height is reduced to values close to the spot diameter there is an almost linear increase in signal. The amount of light transmitted by the blank plate area is diminished while the amount of light absorbed by the spot remains constant under these circumstances. As the sample concentration across the diameter of the spot is not constant the signal continues to increase as the slit height is reduced to less than the spot diameter.



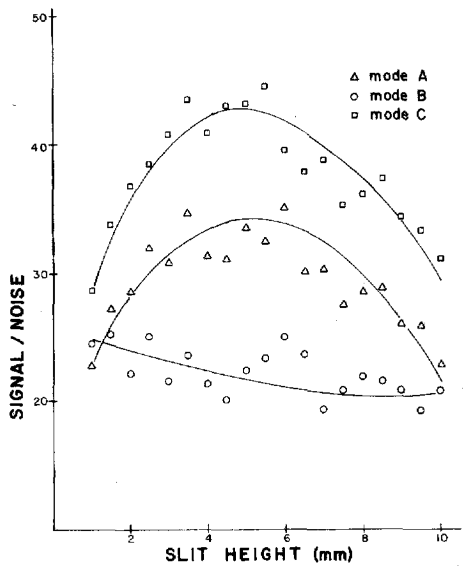




Fig. 5. Relationship between signal-to-noise and slit-height setting at a constant slit width (0.5 mm) for a compact spot (3.0 mm).

Fig. 6. Relationship between signal-to-noise and slit-height setting at a constant slit width (0.5 mm) for a diffuse spot (6.6 mm).

The dependence of the signal-to-noise ratio on slit height for a compact and a diffuse spot is shown in Figs. 5 and 6. In all cases the dual-wavelength single-beam mode, mode C, provides the highest signal-to-noise ratio followed by mode A, and finally mode B. For small spots the signal-to-noise ratio reaches a maximum at small slit-height values. At larger slit-height values the signal declines while the noise remains fairly constant producing a gradual decline in the signal-to-noise ratio driven largely by changes in the magnitude of the signal. For diffuse spots (Fig. 6) the maximum in the signal-to-noise ratio is displaced to larger slit-height values. The signal-to-noise ratio for mode B is virtually constant for all slit-height values greater than 1.5 mm. For modes A and C a plateau region with a fairly constant signal-to-noise ratio exists for slit heights between approximately 3.0 and 6.0 mm. For slit-height values less than 3.0 mm the noise is high compared to the signal while at large slit-height values ($>6.0$ mm) the signal is declining slowly while the noise remains approximately constant.

A comparison of Figs. 5 and 6 indicates that the maximum signal-to-noise ratio is obtained with the dual-wavelength single-beam transmission mode and is dependent on spot size. A slit-height value between 2.0 and 3.0 mm is a reasonable compromise for scanning separations containing spots of different sizes.

*Comparison of transmission and reflectance*

Under optimum measuring conditions the observed resolution of separated

TABLE I

COMPARISON OF SIGNAL-TO-NOISE RATIOS FOR AZOBENZENE MEASURED IN THE TRANSMISSION AND REFLECTANCE MODE

| *Method of measurement* | *Signal-to-noise ratio* | |
|---|---|---|
| | *Transmission* | *Reflectance* |
| Mode A | 30.8 | |
| Mode B | 21.3 | |
| Mode C | 40.8 | 27.8 |
| Single wavelength | – | 22.1 |

spots on HPTLC plates are identical in both the transmission and reflectance mode. It has been claimed that sensitivity is superior in the reflectance mode, for although the signal is generally greater in the transmission mode, the noise associated with the signal is also much greater[2,7,8]. In Table I the signal-to-noise ratios under optimum conditions for the different measuring modes in both reflectance and transmission are compared. Transmission measurements made by mode B clearly provide the poorest sensitivity. The dual-wavelength mode provides the highest sensitivity in both reflectance and transmission. Dual wavelength transmission measurements provide greater sensitivity than dual-wavelength reflectance measurements. This is probably due to a greater reduction in the noise signal associated with light scattered at the plate surface. A limitation of the dual-wavelength mode is the requirement that sample absorption in the reference beam should be negligible. In practice it is sometimes difficult to meet this requirement within the spectral range of the tungsten or deuterium source. This will depend on the absorption properties of the sample, and when these are unfavourable, mode A would be preferred.

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