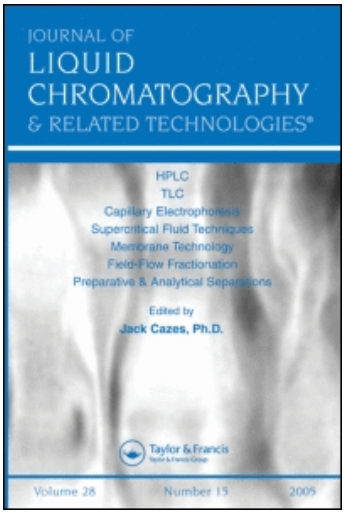

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Some Quantitative Aspects of Scanning Densitometry in High-Performance Liquid Chromatography

C. F. Poole[a]; M. E. Coddens[a]; H. T. Butler[a]; S. A. Schuette[a]; S. S. J. Ho[a]; S. Khatib[a]; L. Piet[a]; K. K. Brown[a]
[a] Department of Chemistry, Wayne State University, Detroit, Michigan

To cite this Article Poole, C. F. , Coddens, M. E. , Butler, H. T. , Schuette, S. A. , Ho, S. S. J. , Khatib, S. , Piet, L. and Brown, K. K.(1985) 'Some Quantitative Aspects of Scanning Densitometry in High-Performance Liquid Chromatography', Journal of Liquid Chromatography & Related Technologies, 8: 16, 2875 — 2926
To link to this Article: DOI: 10.1080/01483918508076609
URL: http://dx.doi.org/10.1080/01483918508076609

JOURNAL OF LIQUID CHROMATOGRAPHY, 8(16), 2875-2926 (1985)

SOME QUANTITATIVE ASPECTS OF SCANNING DENSITOMETRY IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

C. F. Poole*, M. E. Coddens, H. T. Butler,
S. A. Schuette, S. S. J. Ho, S. Khatib,
L. Piet, and K. K. Brown

Department of Chemistry
Wayne State University
Detroit, Michigan 48202

ABSTRACT

The optimization of instrumental parameters that influence resolution and signal-to-noise ratios in the recording of thin-layer chromatograms by scanning densitometry are discussed. A standardized method of determining sensitivity and detectability is proposed and used to evaluate the performance of a Shimadzu CS-910 scanning densitometer. Fluorescence enhancement reagents are discussed as a method of improving sample detectability in the fluorescence mode. The influence of the sorbent medium on both the position and intensity of the fluorescence emission signal is discussed.

INTRODUCTION

Commercial instruments for performing quantitative evaluation of thin-layer chromatograms first appeared in about 1967. Derivatives of those instruments are still in use today; details of their operating principles are reviewed elsewhere

 0148-3919/85/0816-2875$3.50/0

(1-7). 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 have been 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 μg of colored components with a reproducibility rarely 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, fluorescence quenching, and fluorescence (3,5,8,9). 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. The transmission of light by an optically dense medium is a complex process described by the Kubelka-Munk equation. As there is no exact solution to this equation a series of approximations are employed to relate sample amount on the plate to the intensity of the light transmitted and reflected. In absolute terms the results obtained are no more accurate than the approximations used in forming the model (2,10). Because absolute determinations are plagued by the above problem, calibration with standards is the method most frequently used for

quantitation. It should also be noted that the Kubelka-Munk equation predicts a non-linear relationship between reflectance and sample absorption. This is in agreement with experimental observations. Transmission measurements are limited to those wavelengths longer 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.

Absorption measurements can also be made by fluorescence quenching. In this case a special sorbent layer incorporating a fluorescent indicator is used. When such a plate is exposed to UV light of short wavelength, the UV absorbing spots appear dark against a brightly fluorescing background of lighter color. In this instance the sample spots are acting as filters: the spots absorb some of the excitation radiation thus diminishing the intensity of the fluorescence emission eminating from that area of the plate. Hence their darker color against a lighter background. Only those substances whose absorption spectra overlap the excitation spectra of the fluorescent indicator will be visualized in this way. Fluorescence quenching measurements are generally less specific and less sensitive than absorption measurements. Severe background fluctuations resulting from the inhomogeneous distribution of fluorescent indicator in the sorbent layer is the principal reason for lower sensitivity. Fluorescence quenching should primarily be considered as a visualization technique. If a scanning densitometer is available then the measurement of absorption is preferred to fluorescence quenching.

The recording of a fluorescence chromatogram differs both in principle and method to absorption. The absorption signal represents the difference in intensity of reflected or transmitted light between a blank area of the plate and the sample spot. In the fluorescence mode, light striking the plate represents a source of excitation, and the sample spot can be considered as a point source of light defined by the spot boundary and the concentration profile of the emitting molecules throughout the

spot. Fluorescence emission occurs at a longer wavelength than excitation, and is easily separated from it by isolating the emission signal with an optical filter or monochromator. In this case the densitometer views a dark background (in the optical sense) superimposed upon which are the sample components as emission sources. Compared to absorption measurements, fluorescence provides a much greater linear response range with a concentration profile independent of the spot shape. For compounds which fluoresce strongly, detection limits of 10- to 100-fold lower than for absorption measurements are obtained.

An important consideration when assessing the performance of a scanning densitometer is how faithfully it transforms the separation on the plate into a strip chart chromatogram. The main parameters of interest are resolution, dynamic signal range, and sample detectability; the last parameter is measured by the signal-to-noise ratio. The most important experimental variables affecting these parameters are the size of the measuring beam at the plate surface, the scan rate, and the total electronic time constants of the instrument and recording device* (5,7). Optimum values for recording thin-layer chromatograms in the reflectance mode (11,12), transmission mode (12), and fluorescence mode (13) have been described. No absolute measure or standard for determining resolution is available, and this makes a comparison of the performance of individual scanning densitometers difficult except for side-by-side comparisons of an identical separation recorded with both instruments set to their optimum measuring conditions. Sample detectability is more readily determined and a protocol has been suggested to standardize this parameter for TLC measurements (14,15). In this paper the above principles are applied to both optimize and characterize the performance of a commercially available scanning densitometer for TLC measurements in the absorption and fluorescence modes.

DENSITOMETER OPERATION

A brief description of the operating principle of the Shimadzu CS-910 scanning densitometer is given here to indicate

the interdependence of certain experimental parameters (1,3,4,7, 16). It will also enable the reader to discern differences between the CS-910 and other commercially available densitometers.

The CS-910 scanning densitometer can be operated in the dual-beam single-wavelength and dual-wavelength single-beam modes represented by the solid line and broken line, respectively, in Figure 1. A switching mirror is used to select between tungsten and deuterium lamps for absorption measurements and a low pressure mercury discharge lamp for fluorescence. The source output is focused onto the entrance slits of a dual monochromator. In the dual-beam single-wavelength mode a shutter is used to block the light from one monochromator while the beam exiting the other is chopped by a twin lobed mirror, rotating at 60 Hz, yielding two beams modulated at 120 Hz. These beams are referred to as the measuring beam and the reference beam. The measuring beam passes through an adjustable slit which defines the beam shape focused onto the scanning stage. The reflected light from the TLC plate on the scanning stage is collected through a 5 cm orifice in the stage lid by a 7 x 7 cm spherical mirror focused 18 degrees off axis from the measuring beam. The collected light is then directed to the reflectance PMT. The reference beam is passed directly to the reflactance PMT and is responsible for establishing the gain of the instrument. An optical attenuator, scatter plate (not shown in Figure 1), and an additional absorption zero adjust control are provided to equalize the intensity of the reference and measuring beams when the measuring beam is located over a blank area of the TLC plate. Exactly matching the intensity of the reference beam to the measuring beam using the optical attenuator (a V-shaped shutter positioned by a screw thread) is difficult and the function of the absorption zero control is to provide an additional fine-tuning mechanism. The zero adjust control directly increases or decreases the PMT voltage. It has a range of about 140 volts corresponding to approximately 20% of the usable range of the PMT.

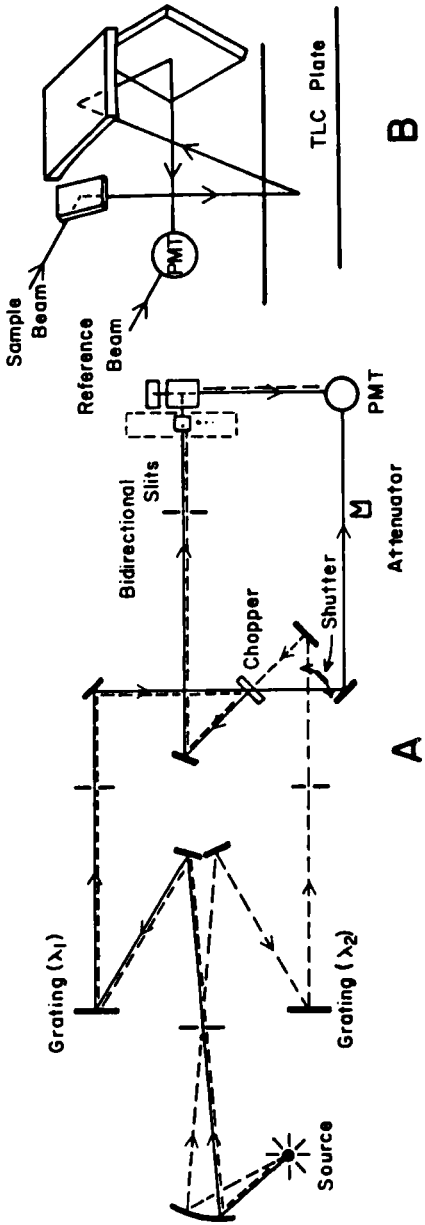

Figure 1 Simplified optical diagram for the Shimadzu CS-910 Scanning Densitometer. A = top view and B = perspective of collection optics. Solid line = dual-beam single-wavelength mode and broken line = dual-wavelength single-beam mode.

In the dual-wavelength single-beam mode, a measuring beam and a reference beam of different wavelengths are selected by the dual monochromator. The two beams are chopped and combined into a single beam which is reflected by the TLC plate and detected by the reflectance photomultiplier and a phase-locked amplifier circuit. In this mode the reference beam and the measuring beam traverse the same optical path.

For transmission measurements two matched photomultipliers are used. One photomultiplier, located beneath the scanning stage, measures the amount of light transmitted through the plate, and the second photomultiplier, located above the stage (the reflectance photomultiplier), views light from the source and reflected light from the plate. The light striking the reflectance photomultiplier is used to establishe the gain on the transmission photomultiplier and thus influences both the signal and noise during measurement. This densitometer allows transmission measurements to be made by three methods; two pseudo dual-beam single-wavelength modes and by the dual-wavelength single-beam mode. In the pseudo dual-beam single-wavelength modes a mechanical shutter can be used to block reflected light from the plate. Thus, if the shutter is in position the reflectance photomultiplier views light from the source alone. 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. 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 medium experienced by the transmitted beam. In an extensive evaluation of the two pseudo dual-beam single-wavelength modes, operation with the shutter in position provides improved signal-to-noise ratio values and was the method used in the studies reported here (12). For dual-wavelength single-beam transmission measurements the reflectance photo-

multiplier is inoperative. The measuring beam and the reference beam traverse identical optical paths and the gain on the PMT is established by the intensity of the reference beam, the later of course, is of a different wavelength to the measuring beam.

It is important to note that the overall gain of the densitometer is determined by the PMT voltage. No additional gain or offset adjustments are available on the output amplifier. The PMT voltage is in turn controlled by a feedback circuit gated to the reference beam, Figure 2. As a consequence of this design each time the wavelength, slit dimensions, position of the optical attenuator, or the absorption zero adjust control are altered the gain of the instrument will also change. Changes in the slit dimensions cause an exponential-like change in the gain, as illustrated in Figure 3. The output from the PMT amplifier is filtered by an RC electronic filter of undisclosed time constant. This filter has two settings labeled fast and slow on the instrument console. We were unable to establish the absolute value of the time constant for the filter but the difference in time constant between the fast and slow settings is about an order of magnitude. The output signal is always filtered, the extent depending on the position of the filter selection switch. As the time constant of the filter can effect peak shape, signal intensity, and electronic noise its influence on the recorded chromatogram is discussed in the optimization section.

The optical path for the operation of the densitometer in the fluorescence mode is similar to that described for the dual-beam single-wavelength reflectance mode. The principal differences are that an emission filter is inserted into the optical path between the plate and the PMT and that the absorption zero adjust control is no longer operative. A secondary zero adjust control is provided to offset background fluorescence from the TLC plate. Unlike the absorption zero control, the fluorescence zero control does not directly affect the gain on the PMT, instead, it sums an offset voltage directly to the output amplifier from the PMT. For the studies reported here we have modified the instrument slightly

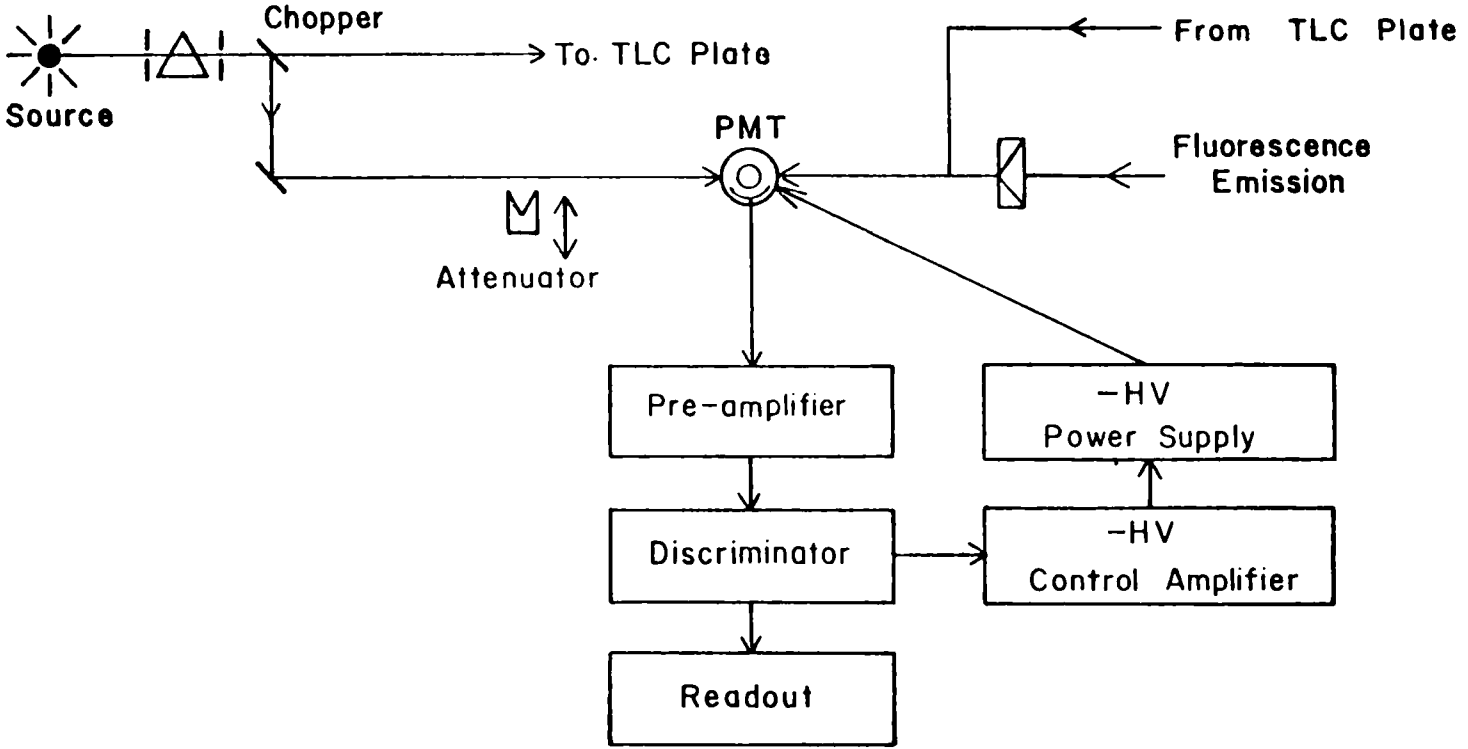

Figure 2 Block diagram of the PMT control circuit.

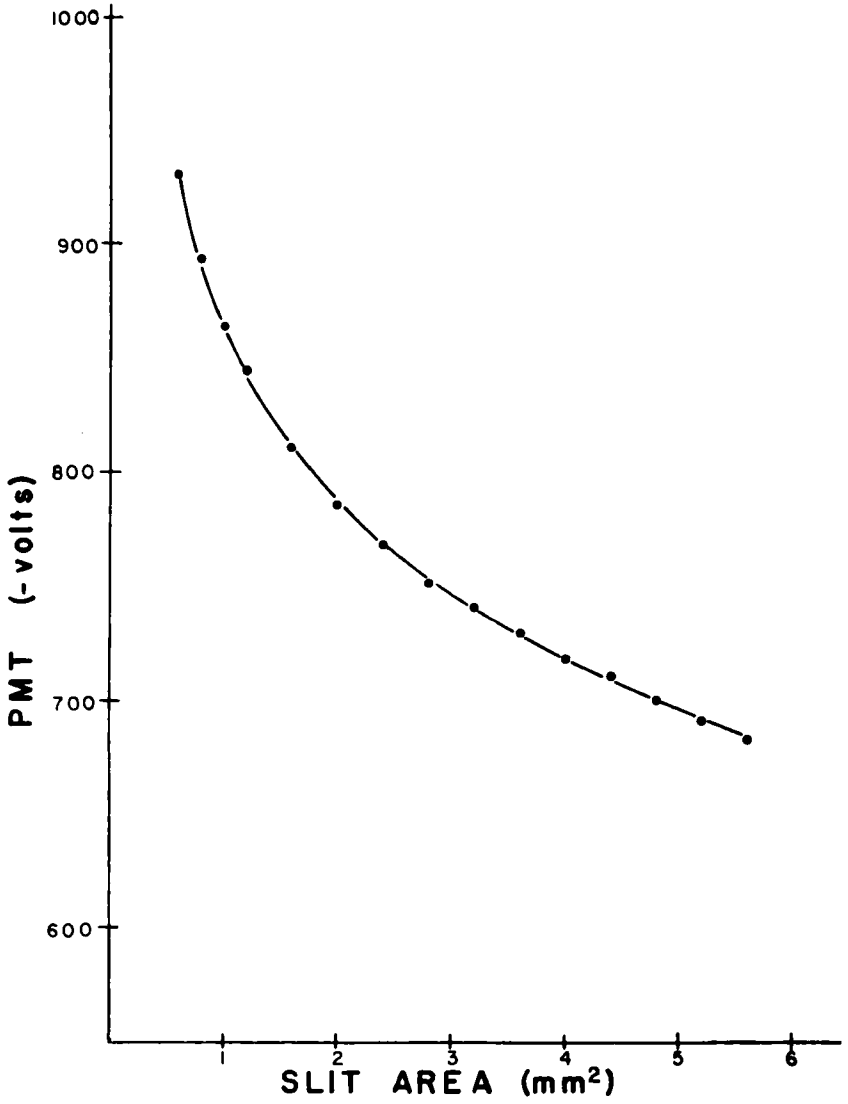

Figure 3 Relationship between PMT voltage (gain) and the area of the measuring beam (controlled by the slit dimensions).

to improve the repeatability of fluorescence measurements on a day-to-day basis (17,18). The auto feedback control, provided to adjust the gain of the PMT for fluctuations in the source output, was disabled and the PMT voltage set directly using the high voltage control for the PMT. A digital multimeter was connected via a shielded cable to a diagnostic socket providing a tap into the PMT voltage. In this way the PMT voltage could be reproducibly set to any desired value. Since the feedback mechanism is by-passed, fluctuations in the source output are not compensated for. Provided that sufficient warm up time is allowed for the lamp (> 20 min), this is not a problem.

FACTORS AFFECTING RESOLUTION IN THE REFLECTANCE AND TRANSMISSION MODES

Ideally, a densitometer should be able to faithfully reproduce the actual resolution of the components on a TLC plate into the perceived resolution of the separation in the form of a strip chart chromatogram. Unfortunately, there is no absolute method of establishing whether the above criterion is met in practice. The spatial resolving power of a densitometer can be ascertained by scanning photographic test patterns or some similar standard (11,19). As these test patterns generally consist of narrow width equal-density squares or lines with discrete boundaries they bear little resemblance to actual chromatographic separations. Commercially available densitometers have spatial resolving powers between 10 and 200 μm. The measurement of spatial resolving power provides information about the quality of optical components and the extent of their mis-alignments, if any, but no information about distortion of peak profiles pertaining to Gaussian profiles.

As a practical, although imperfect measure of densitometer performance, we have compared the perceived resolution measured from the strip chart chromatogram for a pair of partially separated spots under different densitometer operating conditions. Resolution values were calculated using standard chromatographic

TABLE 1

THE AFFECT OF MEASURING BEAM DIMENSIONS ON PERCEIVED RESOLUTION IN THE TRANSMISSION MODE FOR ORTHO- AND PARA-NITROANILINE

SLIT HEIGHT = 1.5 mm		SLIT WIDTH = 0.5 mm	
Slit Width (mm)	Resolution	Slit Height (mm)	Resolution
0.20	1.10	1.0	1.10
0.25	1.10	1.5	1.05
0.30	1.10	2.0	1.07
0.35	1.04	2.5	0.96
0.40	1.05	2.0	1.03
0.45	1.10	3.5	1.08
0.50	1.04	4.0	1.04
0.55	1.09	4.5	1.03
0.60	1.10	5.0	1.02
0.65	1.05	5.5	1.01
0.70	1.08	6.0	0.97
0.75	1.07	6.5	1.02
0.80	1.10	7.0	1.01
0.85	1.05	7.5	1.05
0.90	1.03	8.0	0.94
0.95	1.09	8.5	0.95
1.00	1.09	9.0	1.01
		9.5	1.03
		10.0	1.03
X	1.08	X	1.02
% RSD	2.42	% RSD	4.18

formulas (6) In this way, experimental variables that cause a change in the perceived resolution can be discerned. This does not prove that the chromatographic resolution and that measured by the densitometer are identical but does enable those operating conditions that result in a degradation of the chromatographic resolution to be identified. The slit width and slit height, which control the size of the measuring beam, were expected to have the greatest influence on the resolution recorded. Table 1 summarizes the observed resolution of a partially separated pair of spots. As can be seen neither slit height nor slit width has a significant affect on resolution within the range of values used for the measurements. Figure 4 illustrates a separation of three PTH-amino acid derivatives scanned repetitively with slit width

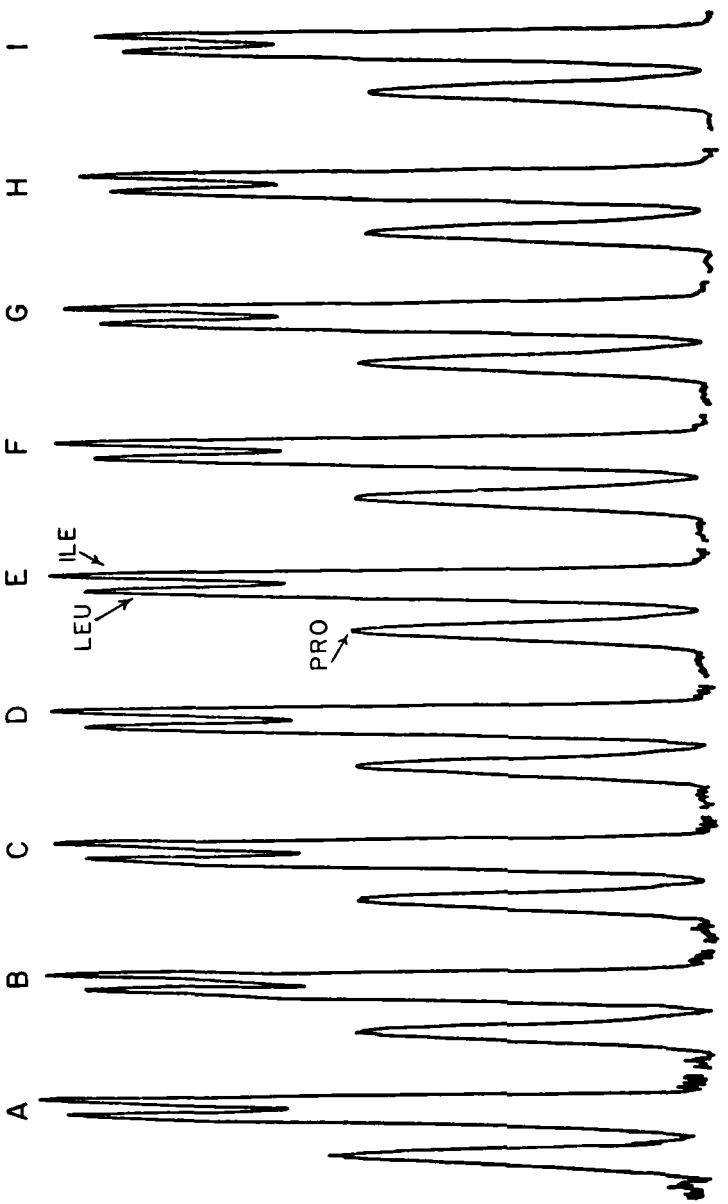

Figure 4 Observed resolution of PTH-derivatives of proline, leucine, and isoleucine measured at a constant slit height of 1.5 mm and scan rate of 24 mm/min. Slit width: A = 0.05 mm; B = 0.10 mm; C = 0.20 mm; D = 0.40 mm; E = 0.60 mm; F = 0.80 mm; G = 1.00 mm; H = 1.20 mm; and I = 1.4 mm.

settings between 0.05 andl.4 mm in the reflectance mode (11,20). Varying the slit width setting over its complete range has only a minor effect on the recorded resolution; only at the extreme of the slit width range (1.0-1.4 mm) is there any perceptible loss in resolution. Figure 5 illustrates a separation of a lipophilic dye mixture recorded in the reflectance and transmission mode. Resolution is not influenced by the choice of measuring mode when the same instrument parameters are used. For normal scan rates the observed resolution is not affected by the choice of electronic noise filter. Figure 5 also illustrates that the signal is much greater in the transmission mode; this point will be addressed later.

In both the transmission and reflectance mode larger slit widths provide better signal-to-noise characteristics. Thus an optimum value of about 0.8 mm for the slit width provides an acceptable compromise between sensitivity and resolution for this densitometer.

FACTORS AFFECTING SENSITIVITY IN THE TRANSMISSION AND REFLECTANCE MODE

The sensitivity of a scanning densitometer is a complex term that depends on the quality of the electronic and optical components of the instrument. The principal experimental variable that influences sensitivity is the measuring beam dimensions. The measuring beam is a rectangle defined by the selected values for the slit width and slit height. The slit width is continuously adjustable from 0 to 1.40 mm and defines the dimension of the measuring beam in the direction of scanning. The slit height is continuously adjustable from 0 to 10 mm and defines the measuring beam dimensions in the direction orthogonal to the direction of scanning. The change in signal with slit width is relatively small, as indicated in Figure 6. Slit height has a much more dramatic effect, as shown in Figure 7. When the slit height is large compared to the diameter of the spot, a large amount of light is transmitted/ reflected from the blank area of the plate

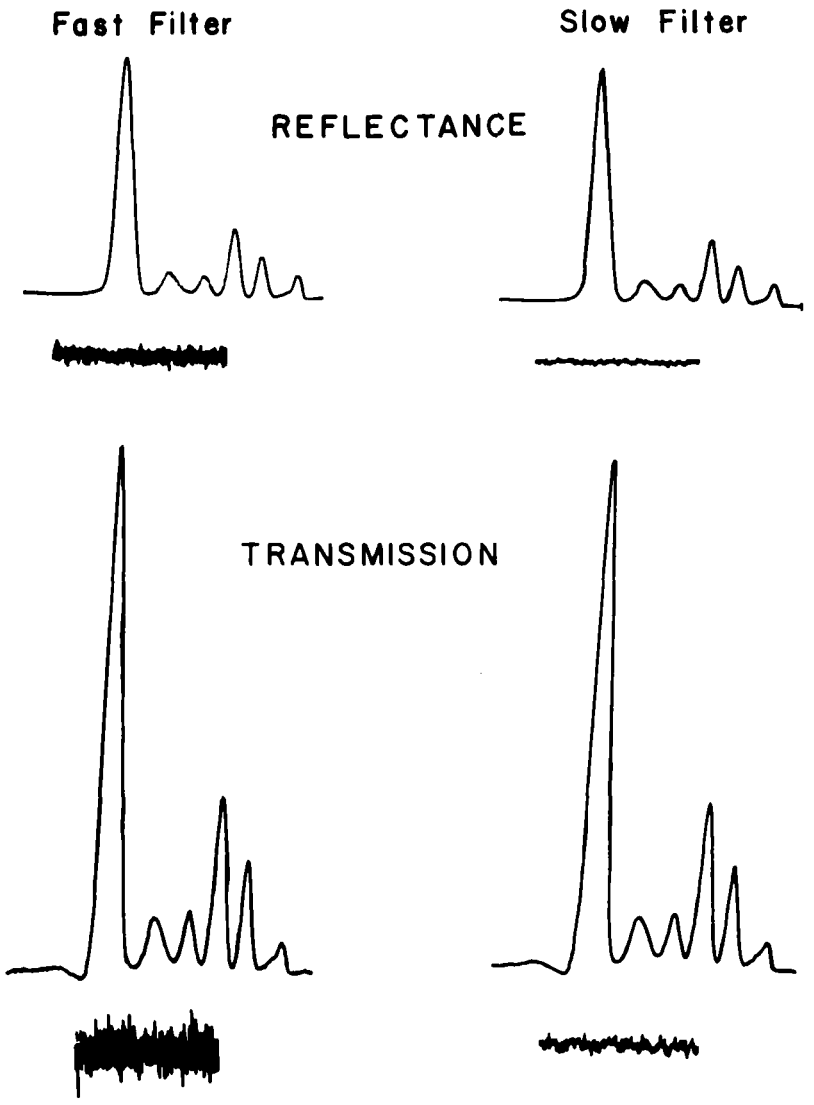

Figure 5 Comparison of Resolution in the reflectance and transmission mode for a Lipophilic dye mixture. Slit Height = 3.0 mm, width = 0.8 mm, scan rate = 24 mm/min, and measuring wavelength = 400 nm.

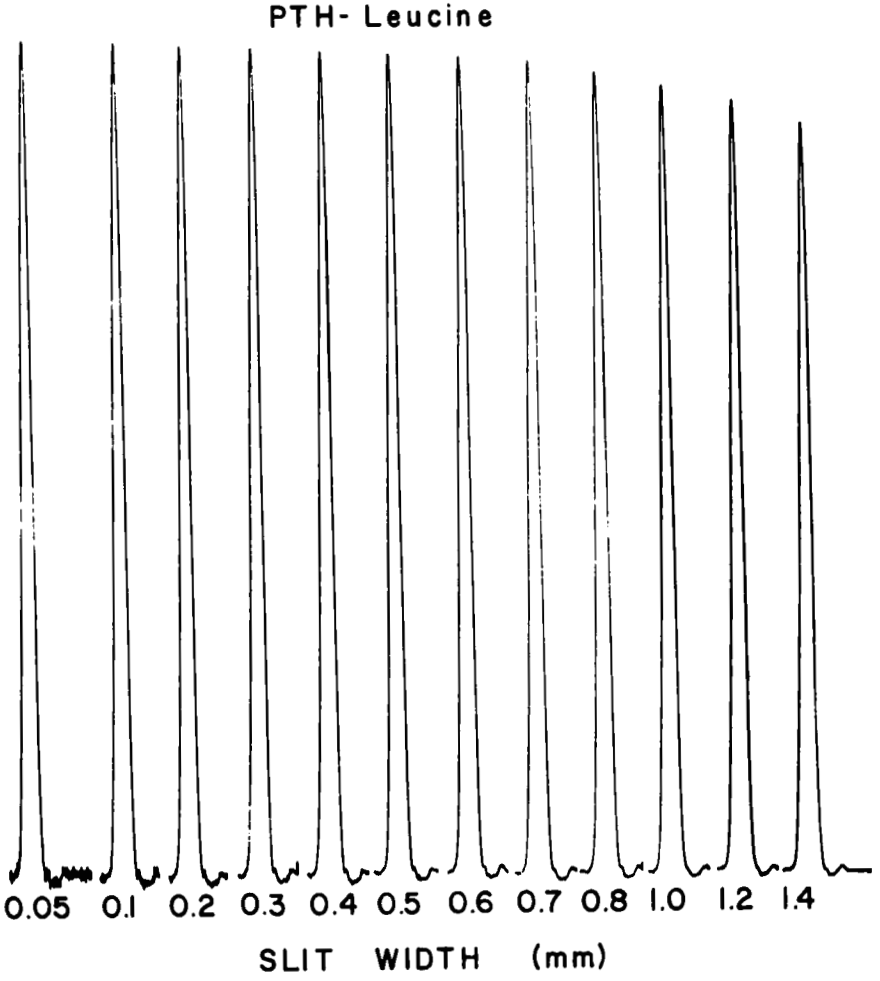

Figure 6 Variation of signal with slit width. Sample = PTH-leucine and slit height = 2.0 mm.

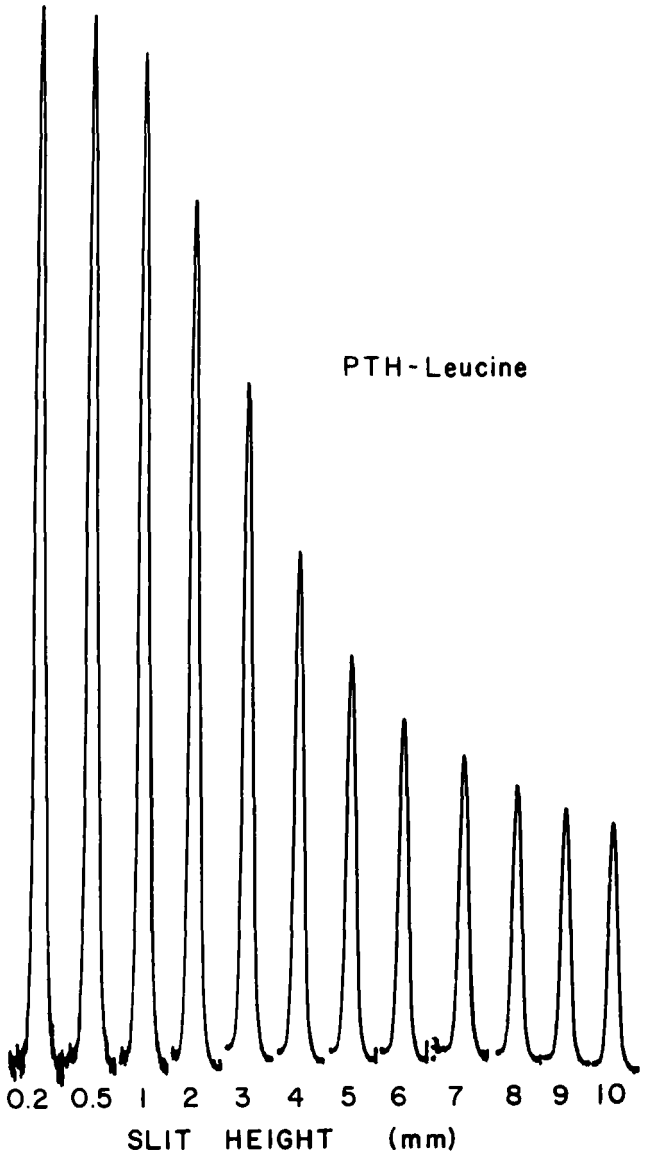

Figure 7 Variation of signal with slit height. Sample = PTH-leucine and slit width = 0.5 mm.

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/reflected by the blank area of the plate 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.

It is the signal-to-noise ratio, rather than signal, that is the more important performance characteristic of a densitometer. The signal-to-noise ratio does not necessarily follow the same trend discussed for the signal. The signal-to-noise ratio increases fairly uniformly with increasing slit width up to the maximum slit width setting. However, its influence is comparatively small compared to changes caused by varying the slit height, or more correctly, the slit height to spot diameter ratio. Figure 8 illustrates the change in signal-to-noise ratio as a function of slit height for two spots of different diameter. Both spots contain the same sample amounts. The maximum in the signal-to-noise ratio occurs at a different value of the slit height for the compact and the diffuse sample spot. As in a normal separation spots of different sizes need to be scanned, it will not be possible to scan the complete separation under optimum sensitivity conditions. The maximum sensitivity is obtained when the slit height is smaller than the spot diameter. However, for practical reasons the use of small slit heights is not recommended, since if the sample track is not perfectly linear, then different zones of the spots will be scanned producing erroneous information. A reasonable compromise value for the slit height is 3.0 mm when high sensitivity is called for. When sensitivity is not the overriding consideration than a slit height value 10% greater than the diameter of the largest spot is a reasonable value.

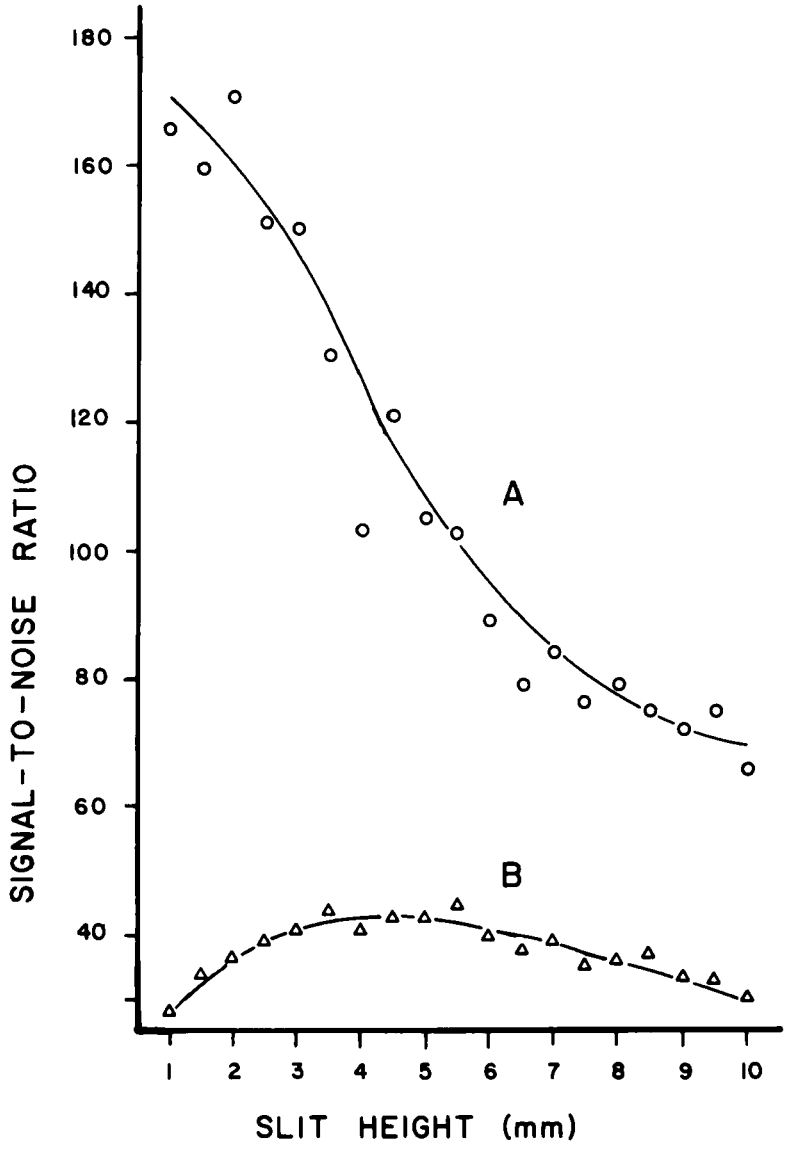

Figure 8 Relationship between signal-to-noise ratio and slit height at a constant slit width (0.5 mm) for (A) a compact spot, 3.0 mm, and (B) a diffuse spot, 6.6 mm.

OPTIMUM CONDITIONS FOR DENSITOMETER OPERATION IN THE TRANSMISSION AND REFLECTANCE MODE

Resolution is not affected by the choice of operating mode. The signal-to-noise ratios under optimum conditions for the different operating modes are summarized in Table 2. The single-beam dual-wavelength mode provides the highest sensitivity in both the reflectance and the transmission mode; the signal-to-noise ratio being greater for the transmission mode than the reflectance mode. A limitation of the single-beam dual-wavelength mode is the requirement that sample absorption in the reference beam should be negligible. This cannot always be arranged within the spectral range of the tungsten or deuterium source, in which case, the dual-beam single-wavelength mode is preferred. Again, the signal-to-noise ratio is more favorable in the transmission mode than the reflectance mode, Table 2 and Figure 5. At wavelengths shorter than 320 nm reflectance measurements are mandated by the absorption of light by the glass backing plate and the sorbent medium. Recommended operating parameters for the CS-910 scanning densitometer are summarized in Table 3 for transmission and reflectance and Table 4 for fluorescence.

Scan rates of 3, 6, 12, 24, 48, and 96 mm/min are possible with the densitometer at hand. Only at the highest scan rate of 96 mm/min was any distortion of the signal observed. Provided that fast response recorders (0.33 s for 99% full-scale deflection) or integrators of reasonable sampling frequency (e.g., 60 Hz) are used then additional signal distortion is not a problem.

TABLE 2

COMPARISON OF SIGNAL-TO-NOISE RATIOS FOR AZOBENZENE

Methods of Measurement	Signal-to-Noise Ratio	
	Transmission	Reflectance
Dual-Beam Single-Wavelength	30.8	22.1
Single-Beam Dual-Wavelength	40.8	27.8

TABLE 3

RECOMMENDED OPERATING CONDITIONS IN THE TRANSMISSION AND REFLECTANCE MODE FOR THE SHIMADZU CS-910 SCANNING DENSITOMETER

Parameter	Transmission and Reflectance
Measuring Wavelength	Single-wavelength dual-beam mode choose the absorption maximum. Dual-wavelength single-beam mode choose the absorption maximum for measuring beam and the closest wavelength at which no significant absorption occurs for the reference beam.
Slit Width	For spots of normal size a value of 0.8 mm is a reasonable compromise between resolution and sensitivity. For small spots, tightly spaced close to the origin, a smaller value should be used, 0.5 mm. For simple separations wider slits can be used to maximize sensitivity.
Slit Height	For spots of various sizes a reasonable value is 3.0 mm. When sensitivity is the overriding consideration a value 10% greater than the largest spot is recommended. For maximum sensitivity a slit height value about half the spot diameter is recommended.
Scan Rate	Scan speeds of 24 or 48 mm/min are a reasonable compromise between densitometer performance and analysis time.
RC Filter	Fast filter is generally used. If the baseline is particularly noisy then the slow filter can be used without introducing significant chromatographic distortion at normal scan rates.

TABLE 4

RECOMMENDED OPERATING CONDITIONS IN THE FLUORESCENCE MODE FOR THE SHIMADZU CS-910 SCANNING DENSITOMETER

Parameter	Comments
Measuring Wavelength	Choose excitation and emission wavelengths to maximize either sensitivity or selectivity
Slit Width	For spots of normal size a value of 1.0 mm is a resonable compromise between resolution and sensitivity. Other conditions are the same as for absorption.
Slit Height	The slit height should be slightly greater than the largest spot. For spots of 2-6 mm in diameter a value of 8 mm is adequate for most purposes.
Scan Rate	As for absorption measurements (Table 3)
RC Filter	Slow filter is generally used. No adverse effects at scan rates < 12 mm/min. Between 12 and 48 mm/min there is a small loss in signal (<5%). Slow filter should not be used at 96 mm/min.
PMT Voltage	Normal operation range should be confined to -400 to -750 V. Optimum range -500 to -600 V. Greater voltages (-ve) increase noise faster than signal and induce baseline drift.

DETERMINATION OF DENSITOMETER SENSITIVITY IN THE ABSORPTION MODE

No simple equation exists that relates all the chromatographic and instrumental parameters that influence sample detectability in scanning densitometry. Chromatographic parameters that affect the response include spot shape, spot size, and sample amount. Instrumental parameters of importance are the measuring wavelength, the dimensions of the measuring beam, scan rate, recorder and amplifier time constants, and the operating mode (e.g., reflectance or transmission). The change in integrated

area for spots of different size and sample amount is shown in Figure 9. Provided that the sample concentration lies within the pseudolinear response region of the calibration curve the variation in integrated area with spot size is generally small, showing a slight increase for larger spots. Outside the pseudolinear range variations in the integrated area are more marked and less predictable. Furthermore, the absolute value of the integrated area varies with the slit height used for the measurement. For small slit-height values larger integrated areas are obtained at all concentrations.

The existence of a pseudolinear calibration range is demonstrated in Figure 10. For any given migration distance an individual calibration curve exists; or in other words, the response to a fixed sample concentration will only be constant for spots of identical size. The shape of the calibration curve is independent of the slit-height value, only the absolute integrated area is changed. Peak height measurements show similar variations to those observed for integrated area, Figures 11 and 12. This could be anticipated from the linear relationship between spot width, migration distance, and integrated area, Figure 13. The observed trend in peak height is the opposite to that for integrated area being larger in value for spots with small diameters (provided that all measurements are made within the pseudolinear region of the calibration curves).

The use of peak height measurements in any protocol designed to establish the sensitivity of a scanning densitometer simplifies the calculation of units. The noise signal can then be measured as the average peak-to-peak pen displacement at some blank portion of the plate, in the same units used to measure sample peak heights. Detectability can then be expressed in nanograms.

The product of the reciprocal of the scan rate and integrated area (or peak height) is a constant, Figure 14. This holds true provided that the signal is not attenuated by the time constant of the amplifier and recording device.

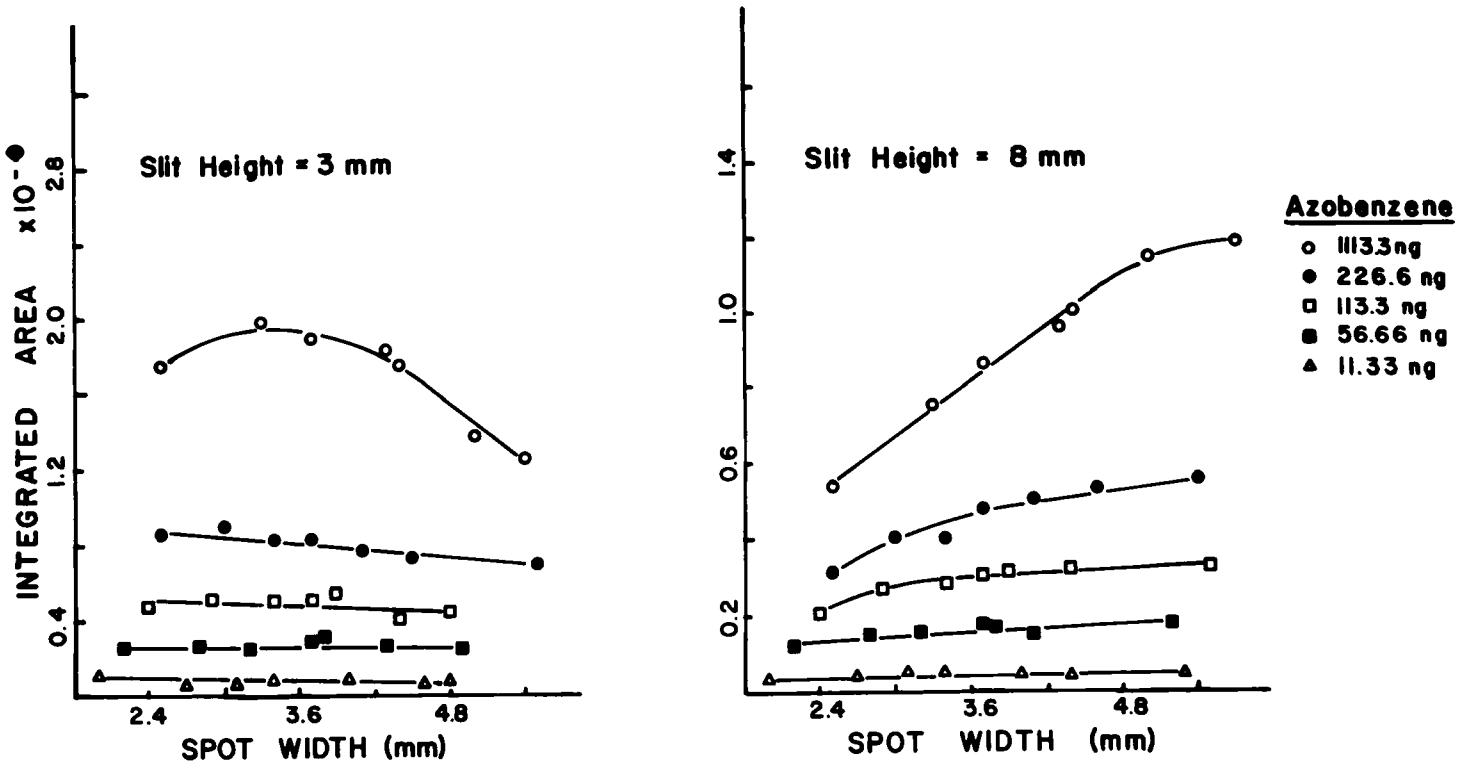

Figure 9 Signal as a function of spot width for different concentrations of azobenzene.

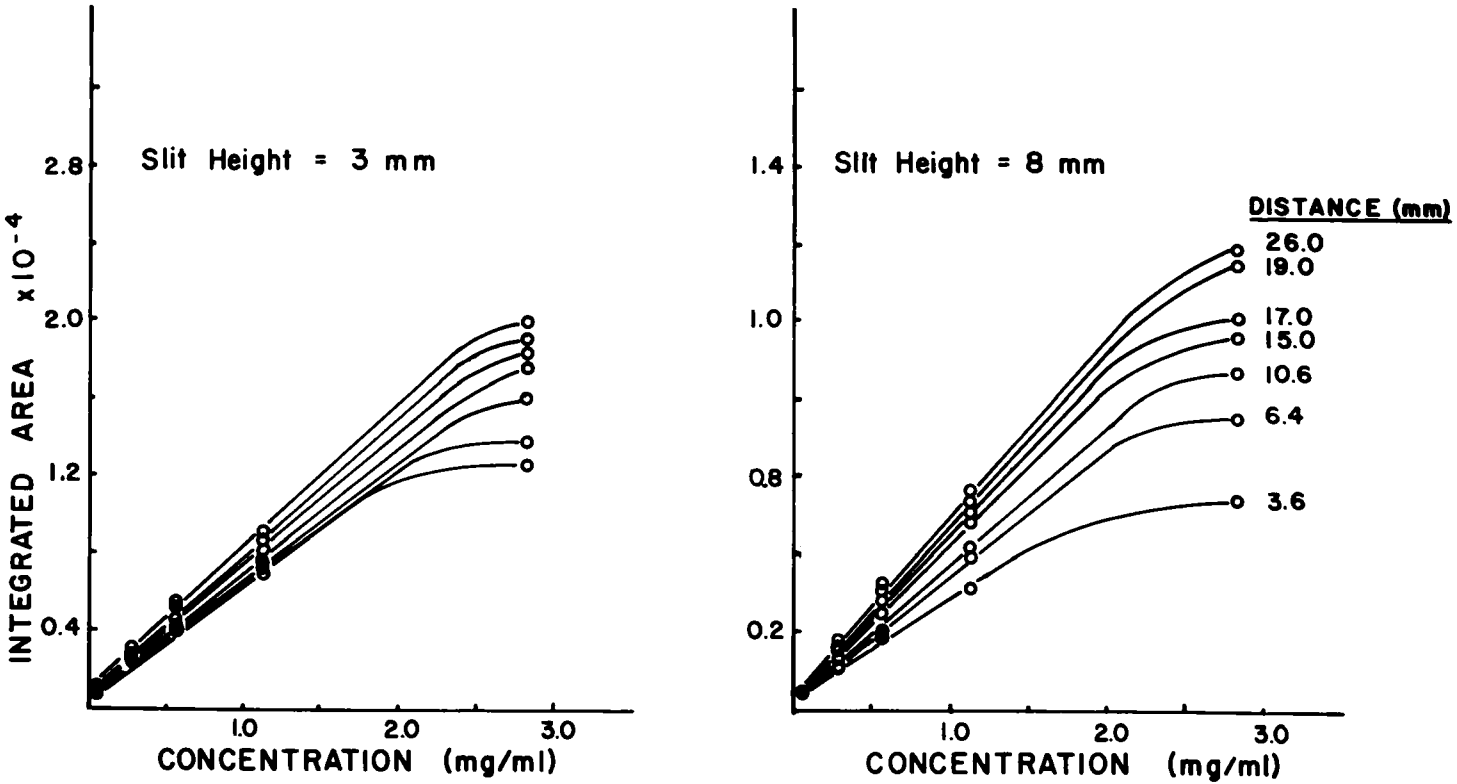

Figure 10 Signal as a function of azobenzene concentration for spots of different size.

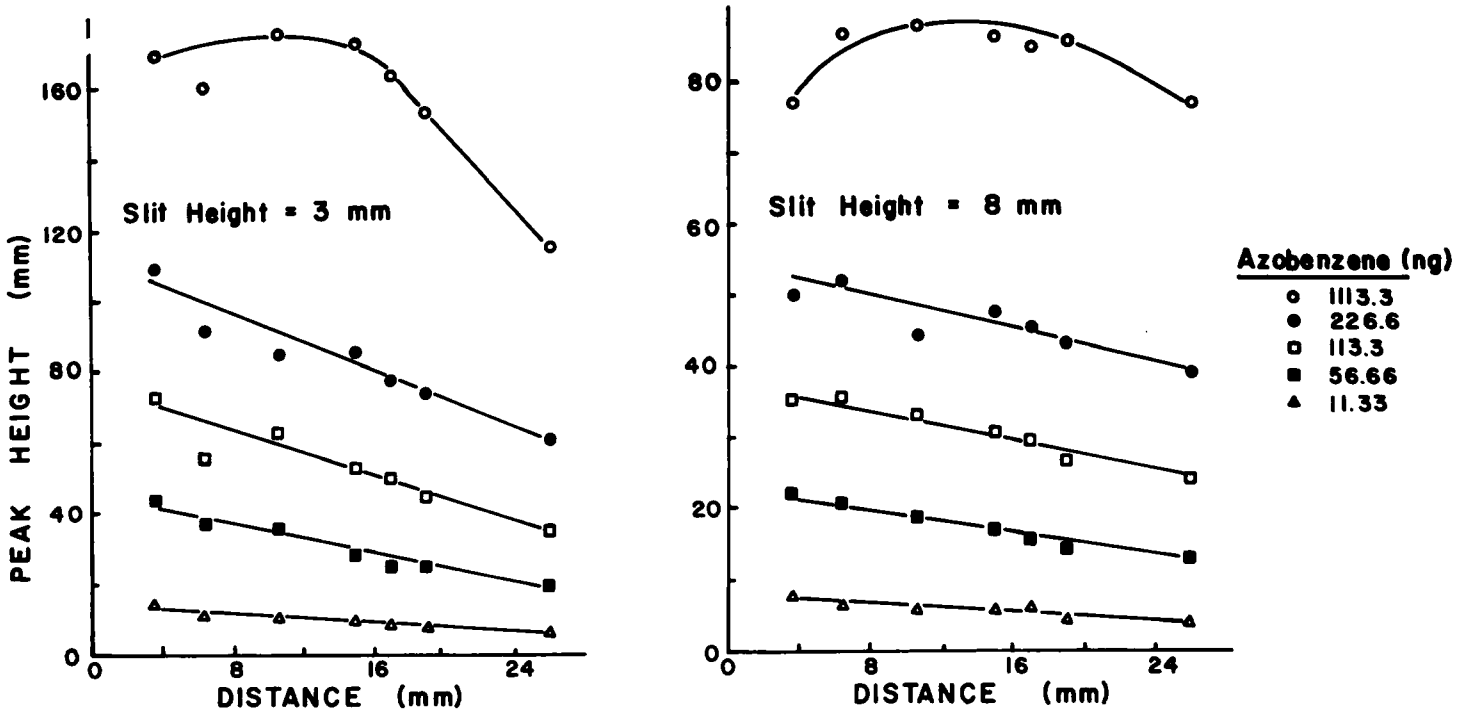

Figure 11 Peak height as a function of migration distance for different concentrations of azobenzene.

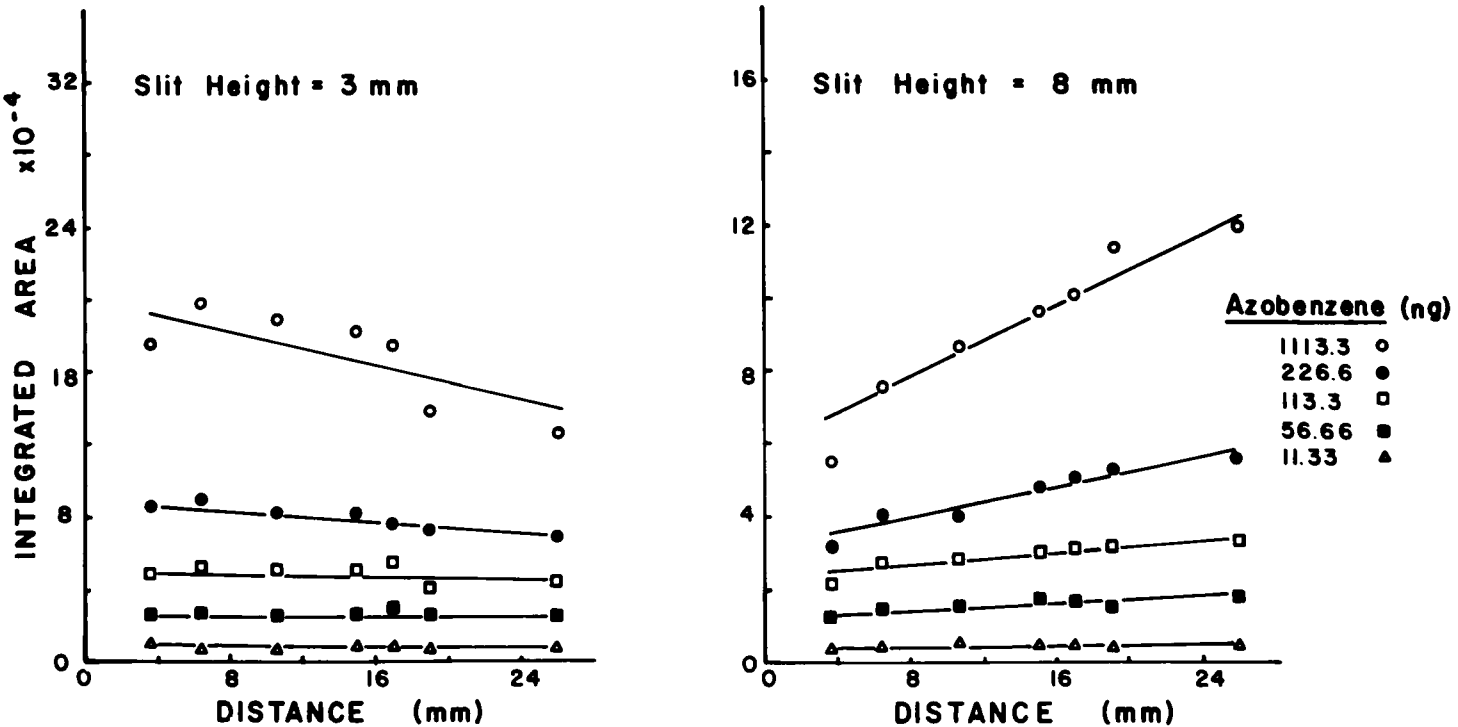

Figure 12 Signal as a function of migration distance for different concentrations of azobenzene.

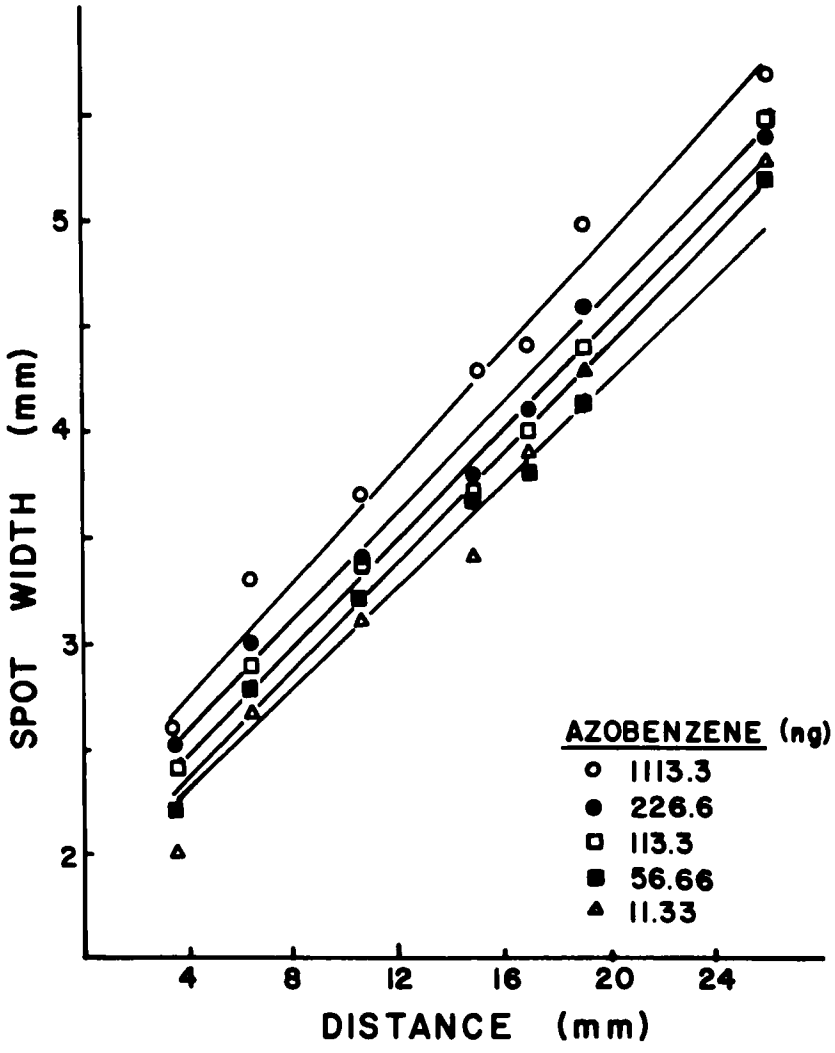

Figure 13 Relationship between spot width and migration distance for spots containing different amounts of azobenzene.

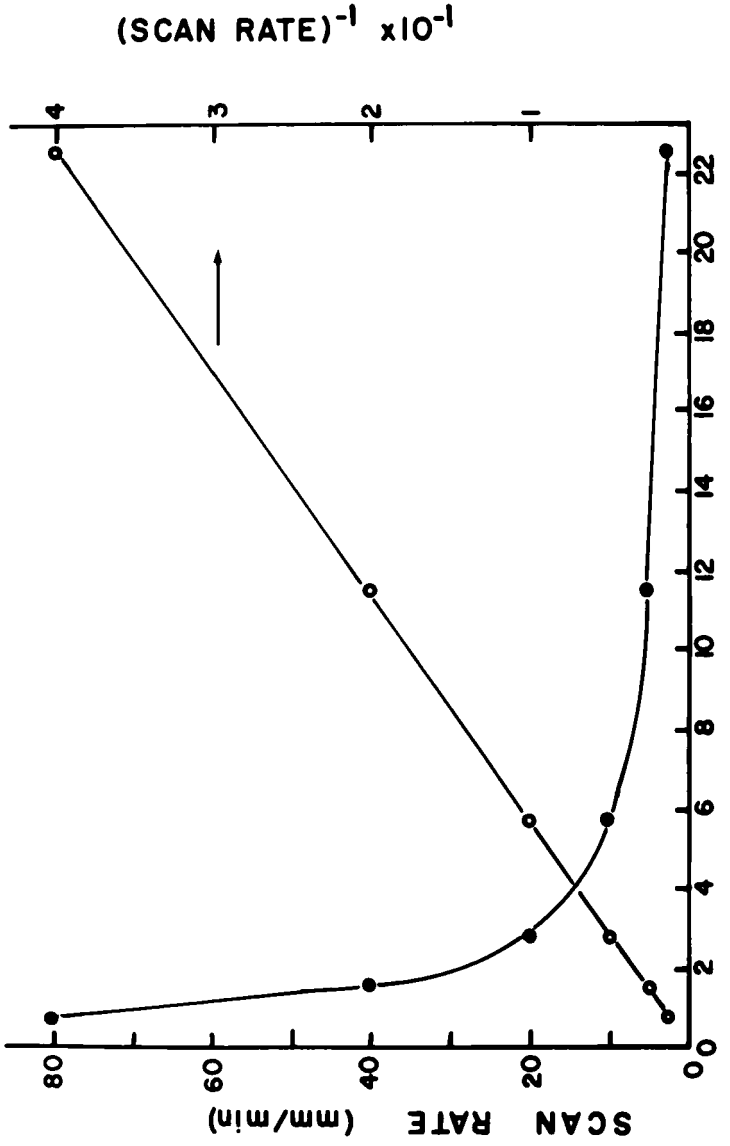

Figure 14 Relationship between signal and scan rate.

To develop a protocol suitable for determining the sensitivity of a scanning densitometer it is necessary to define a standard (or standards) to be used for all measurements, to specify the sample amounts to be used, to specify the chromatographic parameters for the development of standards, and to specify the operating conditions of the densitometer. In this way a protocol can be devised that provides a practical and consistent method for comparing the performance of different densitometers and a method that enables detection limits and other sensitivity-dependent parameters to be determined under conditions of known sensitivity.

Azobenzene and diphenylacetylene are suggested for use as standards. Both compounds are stable and readily available in a pure form or can be easily purified. Azobenzene absorbes throughout the UV and visible regions with two convenient absorption maxima for making measurements with either deuterium (200-400 nm) or tungsten (340-800 nm) sources, Figure 15. It is light yellow in color, which facilitates the alignment of the spot on the plate with the measuring beam. Diphenylacetylene is used as a secondary standard for making measurements in the dual-wavelength single-beam mode. For this purpose a standard with a sharp UV cutoff is required. The monochromators used in most scanning densitometers have fairly wide pass bands and cannot be reset precisely. For this reason standards with relatively flat-topped peak maxima were selected to minimize the importance of wavelength selection on the accuracy of measurements.

Chromatographic and instrumental parameters for determining sensitivity and detectability values of a scanning densitometer are given in Table 5. The sample concentration was selected to provide a signal-to-noise ratio between 10 and 100 at the defined spot size. This provides easily measured peaks while remaining within the pseudolinear portion of the calibration curve. A spot size of 4.0 mm was selected as this is in the middle of the range of spot sizes, 2.0 to 6.0 mm, commonly encountered in HPTLC. There is no need to accurately define the mobile phase composi-

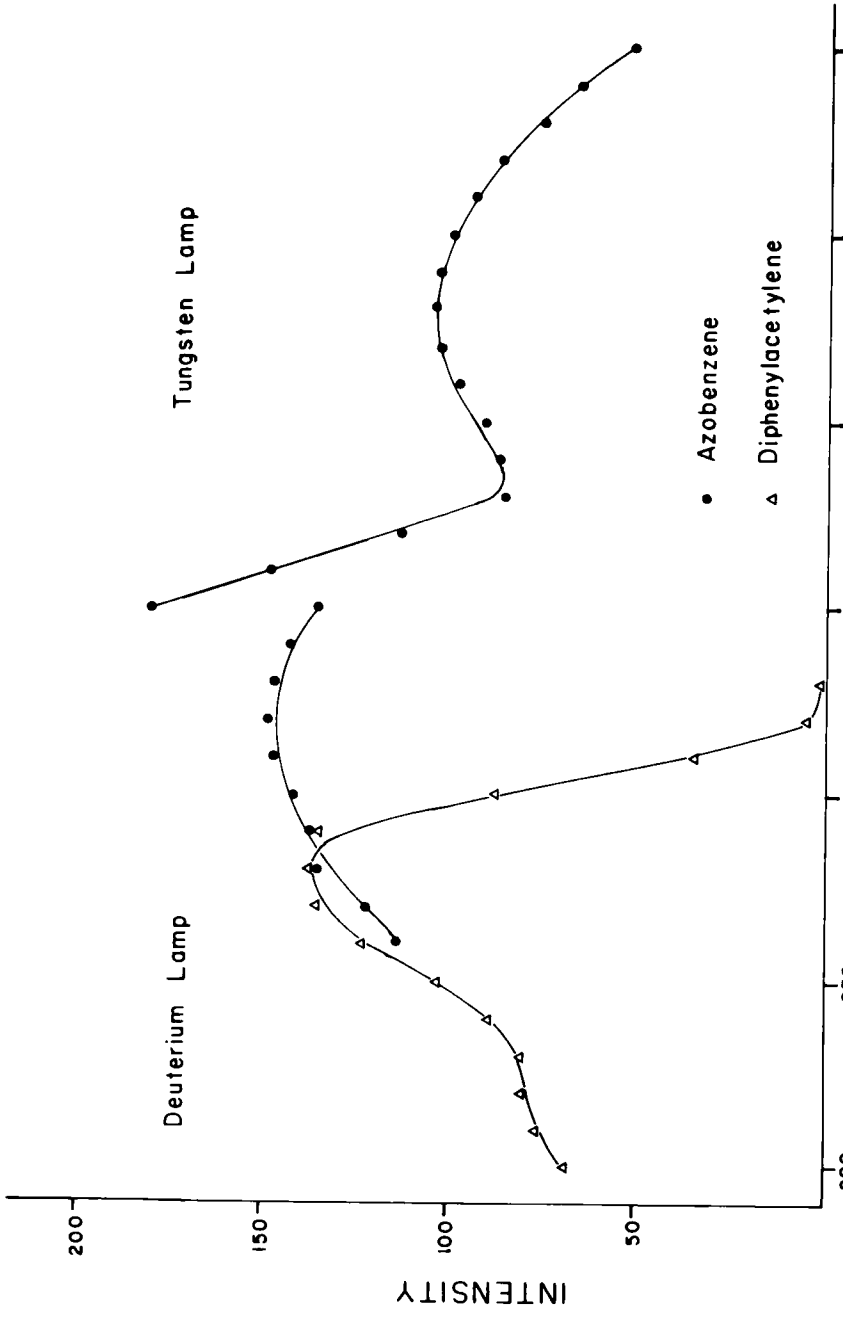

Figure 15 In situ UV/Vis spectra of azobenzene and diphenylacetylene.

TABLE 5

VALUES OF CHROMATOGRAPHIC AND INSTRUMENTAL PARAMETERS FOR DETERMINING SENSITIVITY AND DETECTABILITY VALUES OF A SCANNING DENSITOMETER

Parameter	Diphenylacetylene	Azobenzene
Sample concentration (mg/ml)	0.1	1.0
Sample size (nl)	200	200
Developed spot width (mm)	4.0	4.0
Mobile phase (dichloromethane/hexane)	1:3	1:1
Measuring wavelength (nm)	290	320 (UV) 430 (visible)
Reference wavelength (Single beam dual wavelength mode)	350	---
Scan mode	Linear	Linear
Scan rate (mm/min)	24	24
Slit width (mm)	0.8	0.8
Slit height (mm)	4.4	4.4

tion, plate activation, etc., since the time required for a spot to migrate a certain distance and, consequently achieve a certain size, is easily predicted from a few preliminary experiments.

The choice of measuring beam dimensions, as discussed earlier, is a critical decision. A slit width of 0.8 mm was selected as this value provides an acceptable signal-to-noise ratio without compromising resolution. For the slit height, a value 10% greater than the spot diameter was chosen. Smaller slit height values will produce higher sensitivity, Figure 16. However, small variations in the position of the measuring beam with respect to the spot diameter produce relatively large variations in the signal. Thus, the larger slit height value was selected for practical convenience and to improve the precision of measurements made over a long period of time.

Typical values obtained for sensitivity and detectability of the CS-910 scanning densitometer are given in Table 6. The detectability determined with azobenzene indicates a lower limit of detection when transmission as opposed to reflectance measurements are made in the dual-beam single-wavelength mode.

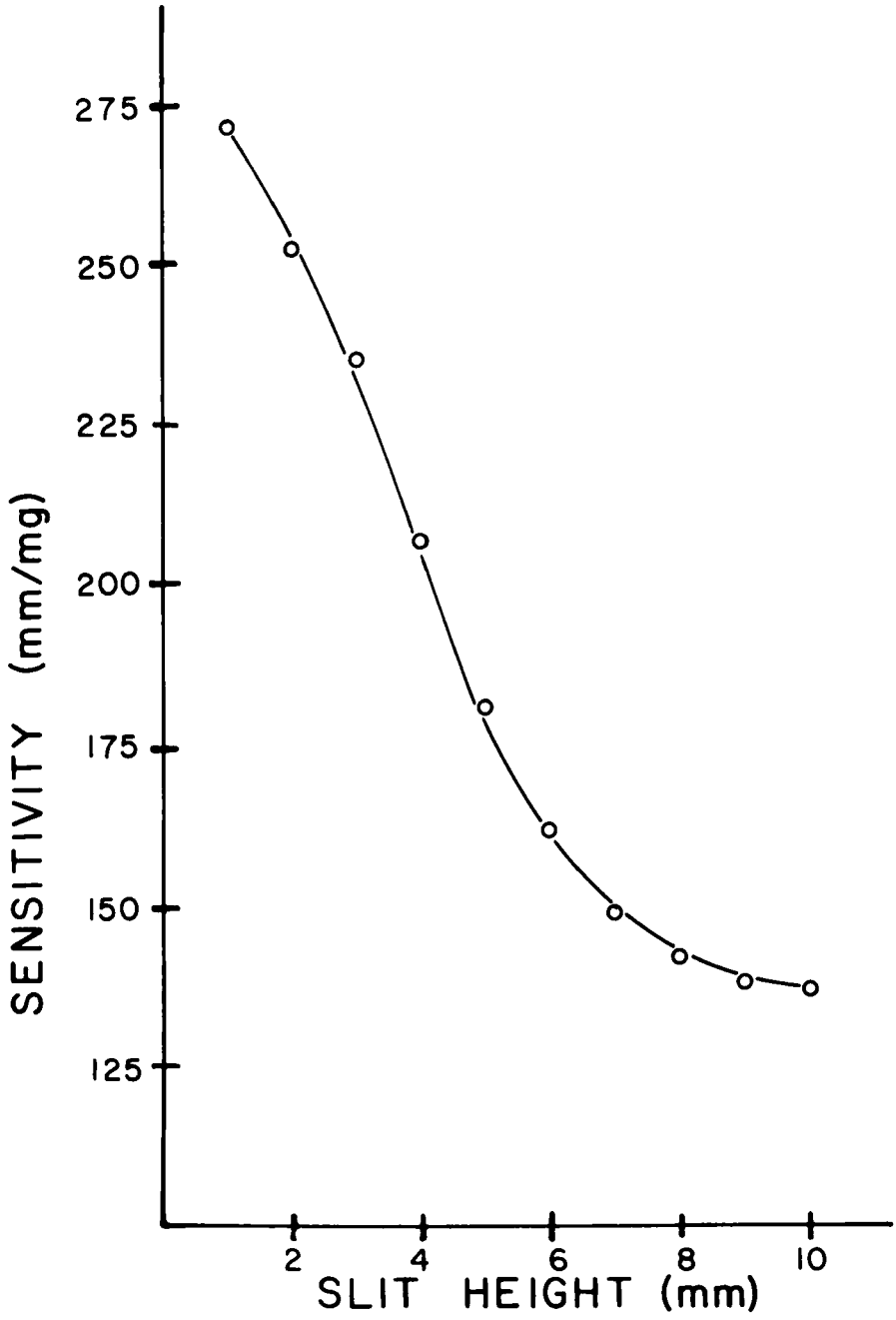

Figure 16 Relationship between densitometer sensitivity and slit height for azobenzene. Spot diameter = 4.0 mm.

Table 6

SUMMARY OF SENSITIVITY AND DETECTABILITY DATA FOR THE CS-910 SCANNING DENSITOMETER

Standard	Mode	Wavelength (nm)	Signal (mm)	Noise (mm)	Sensitivity (mm/ng)	Detectability (ng)
Azobenzene	Transmission (single wavelength)	430	56.14±1.81%	0.38	0.28±1.8%	3.6±1.85%
	Reflectance (single wavelength)	430	51.52±1.58%	0.45	0.258±1.58%	3.49±1.61%
Diphenylacetylene	Reflectance (single wavelength)	290	66.0±4.9%	3.0	3.27±4.9%	1.84±5.5%
	Reflectance (dual wavelength)	290	56.0±7.2%	2.5	2.79±7.2%	1.80±8.1%

Sensitivity (S) = peak height/sample weight; detectability = 2 x noise/S. The noise signal and peak height are measured in mm from the baseline and scaled to the same recorder response response setting. Sample weight is in ng.

The data for diphenylacetylene indicates a lower limit of detection using the dual-wavelength single-beam mode compared to dual-beam single-wavelength operation. In both cases the differences are quite small.

FACTORS AFFECTING RESOLUTION AND SENSITIVITY IN THE FLUORESCENCE MODE

Resolution is not significantly affected by changes in the measuring beam dimensions for spots of normal size. Only at the extremes of the slit width setting are any changes observed and, as was discussed for absorption measurements, these changes are very small. Signal intensity, however, is adversely affected by the use of small slit widths. Figure 17 shows a sample of benzo[g,h,i]perylene scanned at several slit widths with a constant slit height. There is a regular decrease in signal in going from large to small slit-width values. The reason being that as the slit width is decreased, the amount of sample excited during the time the spot is being scanned, is reduced. Consequently, fewer molecules are fluorescing and the signal decreases. Figure 18 shows the relationship between signal intensity and slit width for various concentrations of fluoranthene. There is a linear relationship between slit width and detector response, and furthermore, the slopes of the peak area versus slit width relationships are proportional to sample amount. This relationship is sufficiently reproducible to be used as a method of calibration (18).

The signal intensity is also influenced by the choice of slit height, Figure 19. For slit-height values smaller than the spot diameter the signal intensity increases linearly with increasing slit height until the slit height reaches the same value as the spot diameter. At this point the signal intensity levels off and only increases slightly with larger slit-height values.

Increasing the area of the measuring beam from its smallest to largest setting results in an increase in the noise level of about 10%. The source of this noise is an increase in the

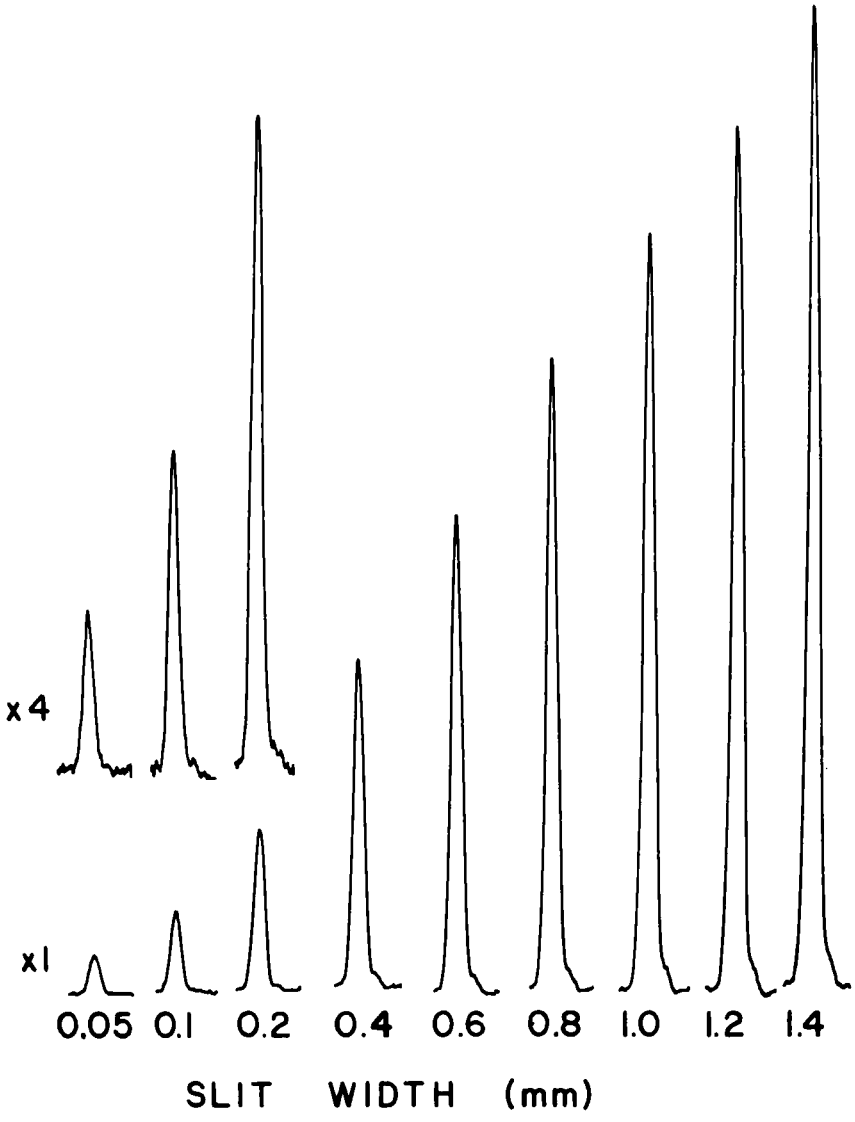

Figure 17 Variation of fluorescence intensity with slit width for benzo[g,h,i]perylene (26 ng) slit height = 6.0 mm, ex = 365 nm, and em = 500 nm.

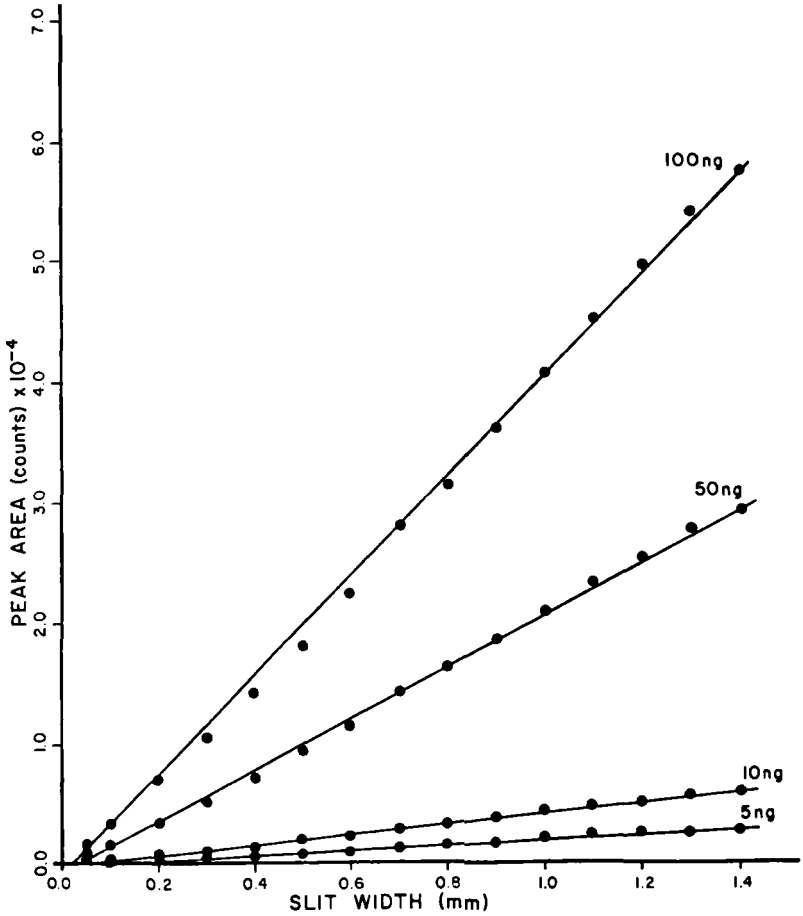

Figure 18 Relationship between fluorescence response and slit width for different amounts of fluoranthene.

background fluorescence from the plate surface. This contribution to the total noise signal is very small compared to the electronic noise associated with increasing the gain of the PMT. The effect of the PMT voltage (gain) on the signal-to-noise ratio in the fluorescence mode is shown in Figure 20. The signal increases with increasing gain but not as fast as the noise signal. Due to

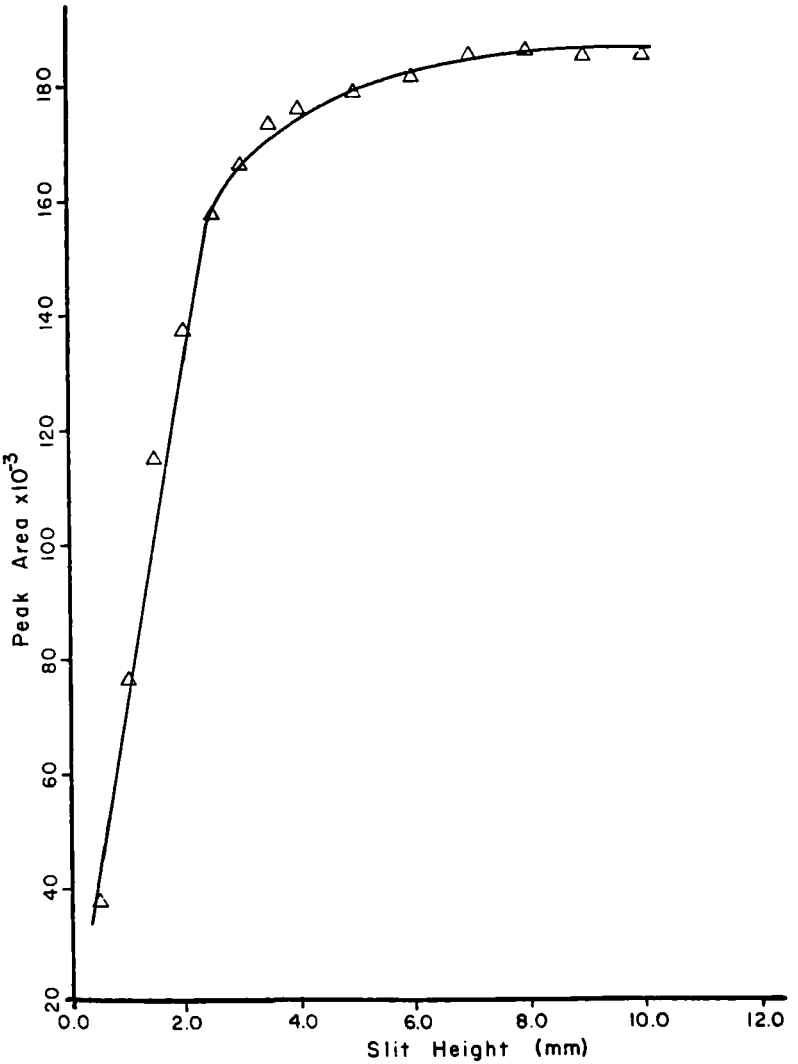

Figure 19 Relationship between fluorescence intensity and slit height for fluoranthene (50 ng). Spot width ≈ 4.0 mm.

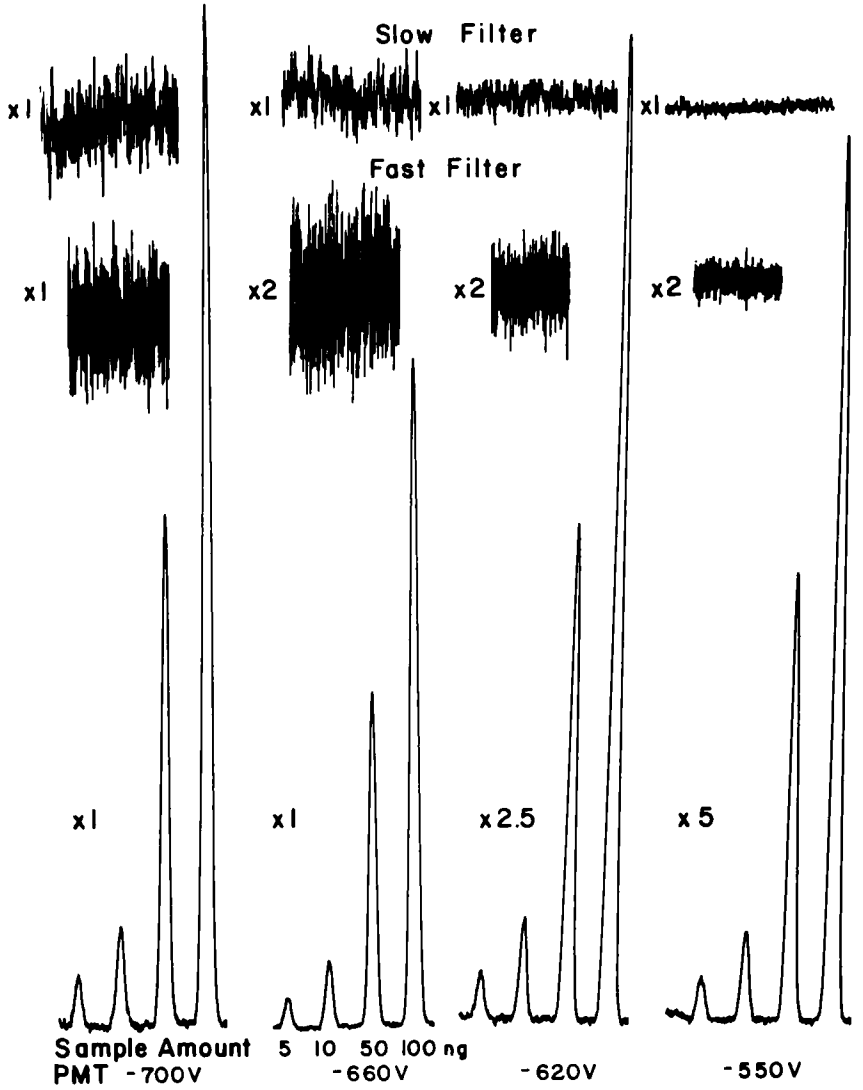

Figure 20 Relationship between signal and noise as a function of PMT voltage. Sample = fluoranthene, slit width = 0.5 mm, slit height = 6.0 mm, em = 254 nm, ex = 500 nm, and scan rate = 24 mm/min.

TABLE 7

SIGNAL-TO-NOISE RATIO IN THE FLUORESCENCE MODE AS A FUNCTION OF PMT VOLTAGE

	Fluoranthene Amount (ng)							
PMT	Fast Filter				Slow Filter			
Voltage	100	50	10	5	100	50	10	5
-500	48	25	5.6	2.5	190	100	23	10
-550	49	25	5.0	2.4	137	70	14	6.7
-620	48	23	5.0	2.4	123	60	13	6.2
-660	42	21	4.2	1.9	98	49	10	4.3
-700	44	22	4.4	2.2	100	50	10	5.0
maximum (=-950 volts)	34	17	3.7	1.9	84	42	9	4.7

fluctuations in the output of the mercury lamp additional electronic noise filtering is invariably required. This is provided for on the CS-910 densitometer by a two position RC filter of undisclosed time constant. The difference in time constant between the slow and fast settings is about an order of magnitude. The influence of the noise filter on the signal-to-noise ratio at different PMT voltages is given in Table 7. Sample detectability is favored by use of the slow filter and PMT voltages between -500 to -600 volts. It should be noted that no difference in the signal-to-noise ratio was observed whether the feedback system was used or by-passed. Apparently, the noise associated with the source and electronics at the higher gain used in the fluorescence mode is not compensated for by the feedback circuit.

FLUORESCENCE ENHANCEMENT FOR IMPROVED SAMPLE DETECTABILITY

Fluorescence scanning densitometry, compared to absorption measurements, provides greater sensitivity and selectivity, linear calibration plots, and a signal that is virtually independent of spot size. When the sample, or an easily prepared derivative of it, are naturally fluorescent, it is the detection method of choice. Occasionally, however, there is a large disparity between the detectability observed for a fluorescent compound in solution and that measured from an HPTLC plate. Two general causes of this

phenomena have been identified, namely, catalytic decomposition and fluorescence quenching.

The extent of fluorescence quenching often depends on the sorbent medium and is frequently more severe for silica gel than bonded-phase sorbents. Figure 21 illustrates the fluorescence response for a 100 ng sample of 2-aminofluoranthene on silica gel and an octadecylsilanized silica gel HPTLC plate. The response on the silica gel sorbent is only a fraction of that observed on the bonded-phase sorbent. Fluorescence quenching may also occur with a change in the emission wavelength maximum. Figure 22 illustrates the emission response as a function of wavelength for a 200 ng sample of indeno[1,2,3-c,d]pyrene on silica gel and on an octadecylsilanized silica gel HPTLC plate. The signals are measured in 50 nm increments using narrow band interference filters. The shift in emission maximum between the silica and bonded-phase sorbent is thus quite large. The mechanism of fluorescence quenching is not completely understood but it is generally assumed that adsorption onto silica gel provides additional nonradiative pathways for loss of the fluorescence excitation energy which is diminished when the silica gel is at least partially covered with bonded organic groups. It has also been observed that spraying a developed silica gel plate with a viscous solvent such as liquid paraffin (21-24), glycerol (24-26), triethanolamine (22,27,28), Triton X-100, (22,23,25,26,29) or Fomblin H-Vac (29-31) prior to detection can enhance the fluorescence response of the sample, in favorable cases, by as much as 10- to 200-fold. The mechanism of this process remains speculative (29).

Fluorescence enhancement depends on a number of factors, of which, the most important are the sample studied, the reagent and its concentration used for enhancement, and the time between impregnating the plate with reagent and making measurements. In practice other considerations are equally important such as the extent of spot broadening due to maintaining the sample in a wet layer and shifts in the emission maximum induced by interaction between the sample and the fluorescence enhancing reagent.

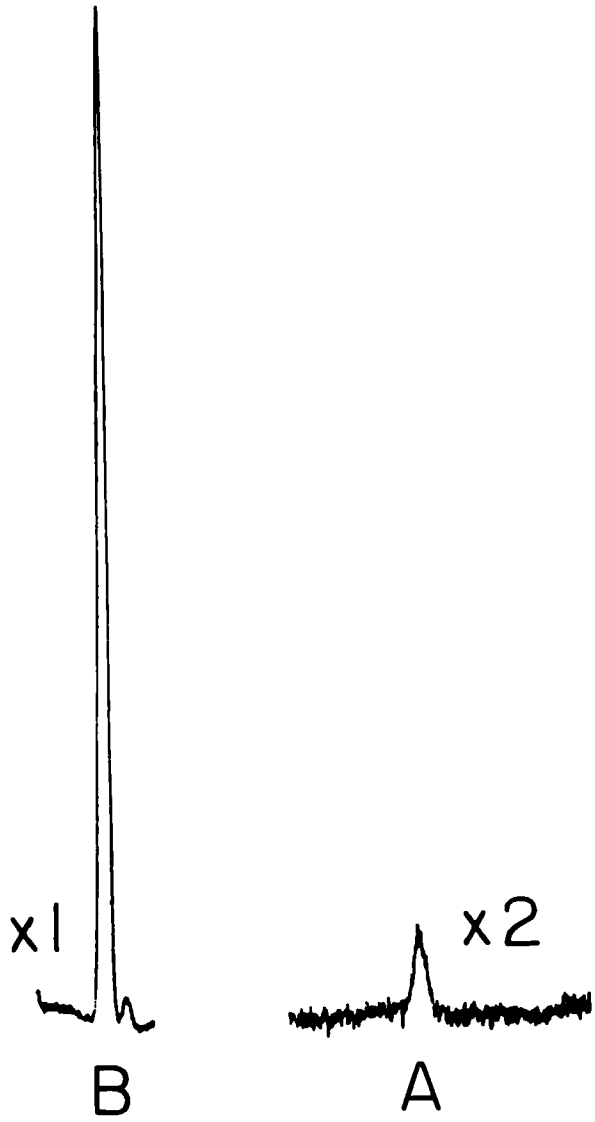

Figure 21 Fluorescence response of 2-aminofluoranthene (100 ng) on (A) silica gel and (B) octadecylsilanized silica gel.

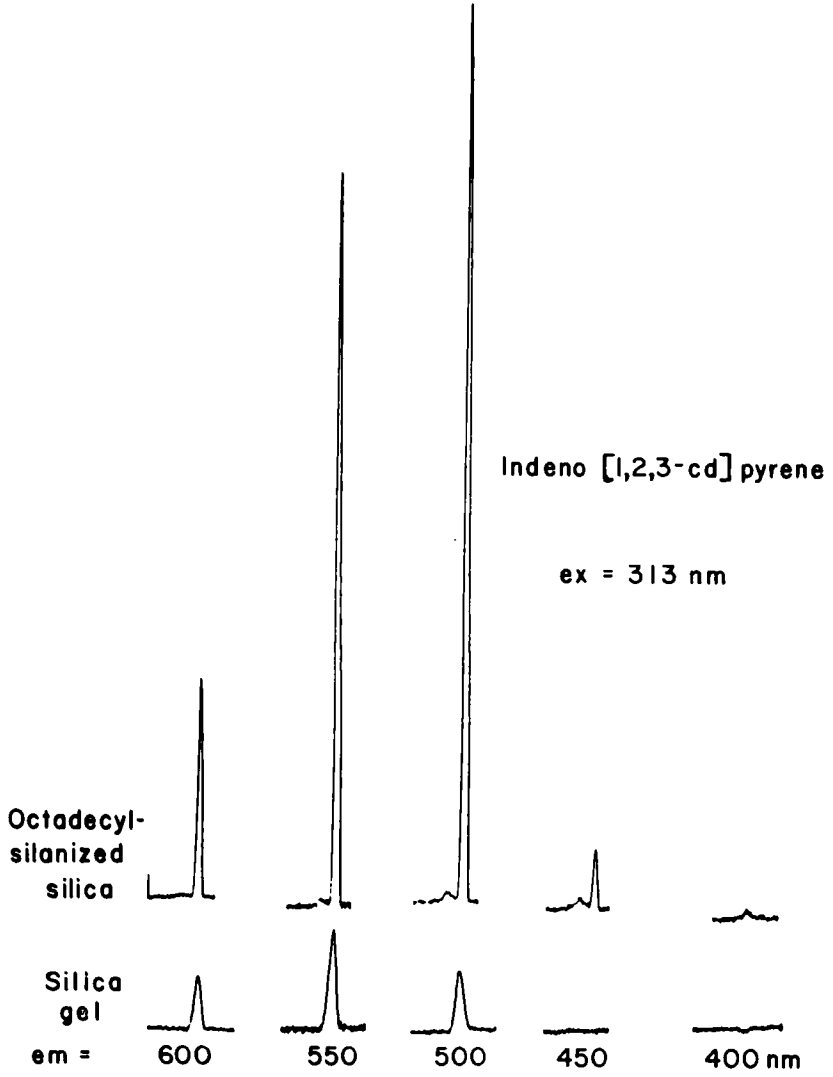

Figure 22 Change in fluorescence response for indeno[1,2,3-c,d]-pyrene on silica gel and octadecylsilanized silica gel.

TABLE 8

DIFFUSION-INDUCED BAND BROADENING OF FLUORANTHENE ON A SILICA GEL PLATE IMPREGNATED WITH DODECANE

Time After Impregnating the Plate (min)	Change in Peak Width At Half-Height (%)
10	6.5
20	11.0
30	15.5
40	21.5
50	26.5
60	31.5
70	35.5
80	38.0
90	41.0
100	43.5

Diffusion-induced band broadening of the sample on plates impregnated with reagents of low viscosity leads to a reduction in sample resolution. The extent of band broadening can be substantial as indicated in Table 8. For this reason fluorescence enhancing reagents of high viscosity are preferred.

A striking time dependence of the fluorescence enhancement ratio after impregnating silica gel plates with Triton X-100 is often observed. Consider, for example, the change in fluorescence enhancement of fluoranthene, Figure 23, as a function of time. More extensive data is summarized in Table 9, including measurements of band broadening. In this case the maximum in the fluorescence enhancement ratio was not reached until about 60 minutes after applying the reagent.

The poly(perfluoroalkyl ether), Fomblin H-Vac has some unique properties that make it very useful as a fluorescence enhancing reagent. For polycyclic aromatic hydrocarbons on silica gel plates it provides the highest fluorescence enhancement ratios in general, no significant spot broadening after the plate is impregnated, and permits measurement throughout the full fluorescence emission region of the samples. The stability of the fluorescence enhancement ratio and the degree of band broadening

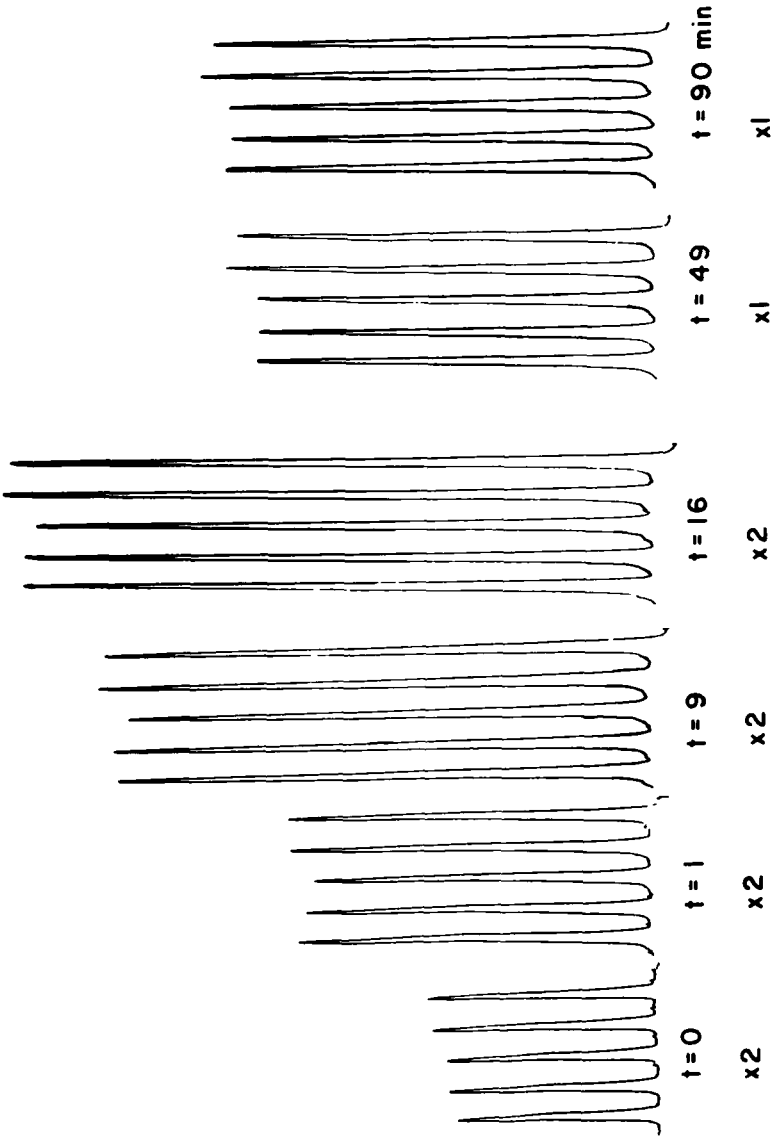

Figure 23 Fluorescence enhancement of fluoranthene (40 ng) on a silica gel HPTLC plate impregnated with Triton X-100 by dipping into a hexane saturated solution. Ex = 254 nm and em = 500 nm.

TABLE 9

TIME DEPENDENCE OF THE FLUORESCENCE ENHANCEMENT RATIO FOR A SAMPLE OF FLUORANTHENE (40 ng) ON A SILICA GEL HPTLC PLATE DIPPED IN A SOLUTION OF HEXANE SATURATED WITH TRITON X-100

Time After Impregnating the Plate (min)	Change in Peak Width at Half-Height (%)	Peak Area	Fluorescence Enhancement Ratio
0	3.9	6336	3.7
1	3.4	6756	4.0
2	4.4	7274	4.3
9	7.3	10522	6.2
12	5.1	11164	6.5
16	8.3	12662	7.4
28	8.3	15126	8.9
30	11.2	15219	8.9
32	11.2	15524	9.1
34	10.2	15656	9.2
49	10.3	16940	9.9
60	11.2	17616	10.3
70	11.2	17835	10.4
77	10.2	17705	10.4
91	10.2	17781	10.4

TABLE 10

TIME DEPENDENCE OF THE FLUORESCENCE ENHANCEMENT RATIO AND SPOT BROADENING FOR FLUORANTHENE (40 ng) ON A SILICA GEL PLATE IMPREGNATED WITH FOMBLIN H-VAC

Time After Impregnating The Plate (min)	Change in Peak Width at Half-Height (%)	Peak Area	Fluorescence Enhancement Ratio
Before	---	3118	
After (t =o)	2.5	9928	3.2
13	2.4	9240	3.0
29	2.0	9195	3.0
52	2.0	9163	2.9
91	1.5	8940	2.9

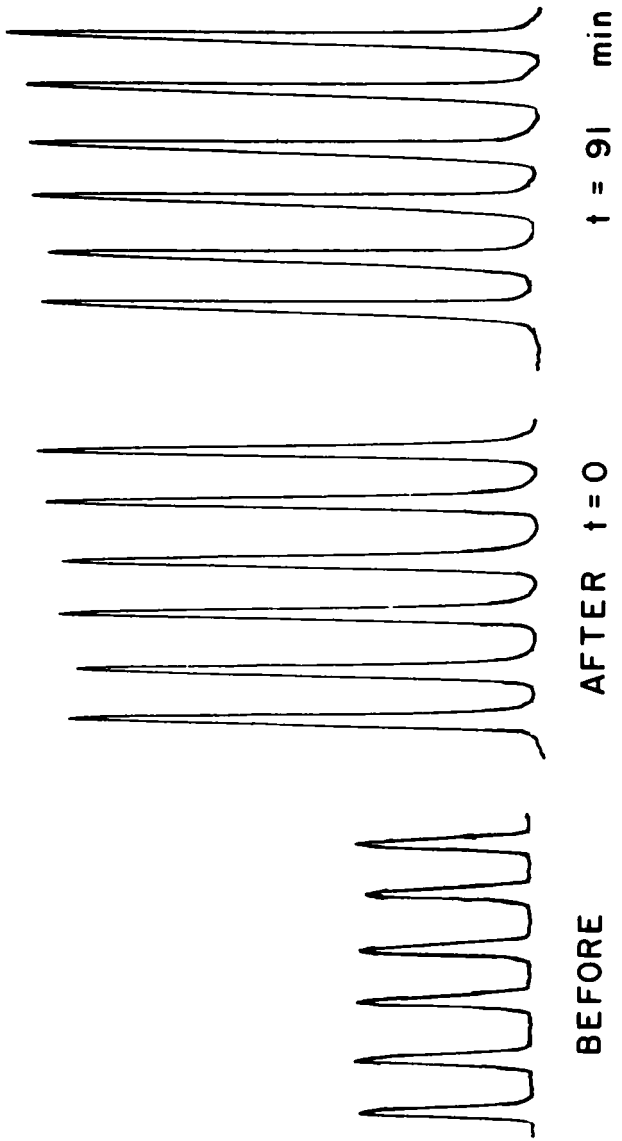

Figure 24 Fluorescence enhancement of fluoranthene (40 ng) on a silica gel HPTLC plate impregnated with Fomblin H-Vac by dipping into a 30% (v/v) solution in 1,1,2-trichlorotrifluoroethane. Ex = 254 nm and em = 500 nm.

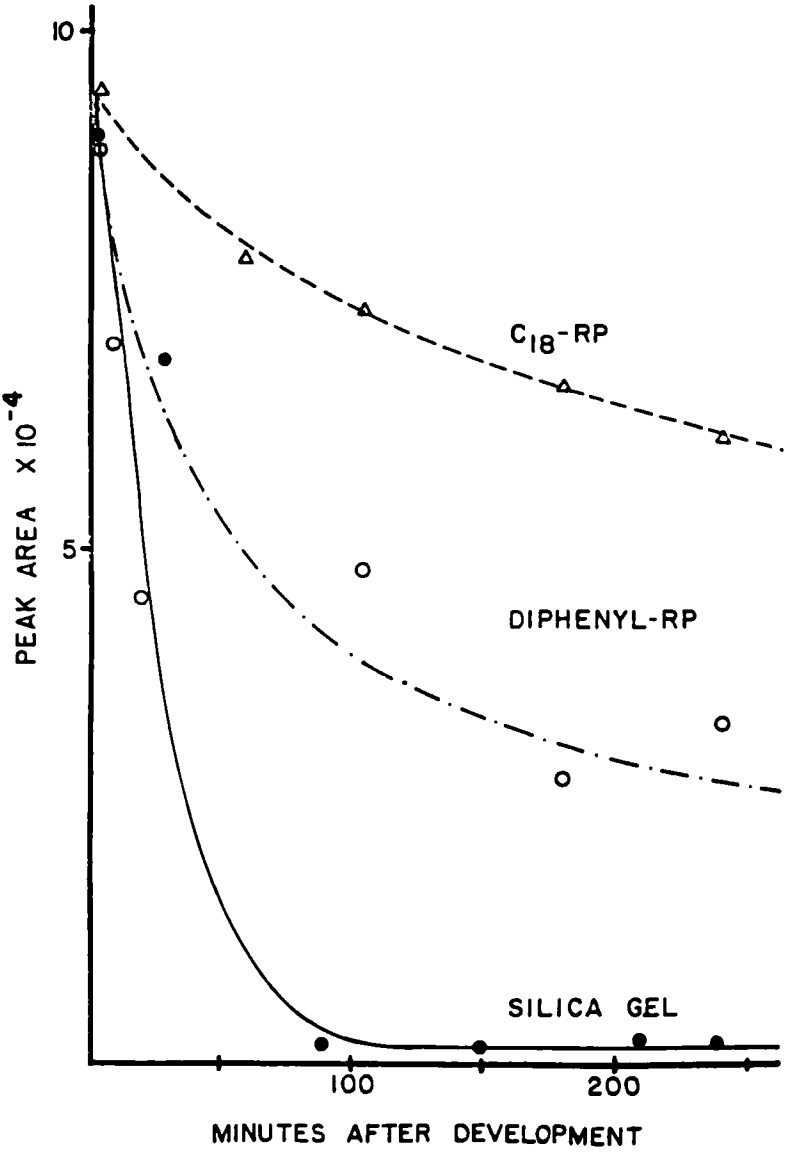

Figure 25 Rate of fluorescence decay of 1-aminopyrene (100 ng) on HPTLC plates coated with different sorbents.

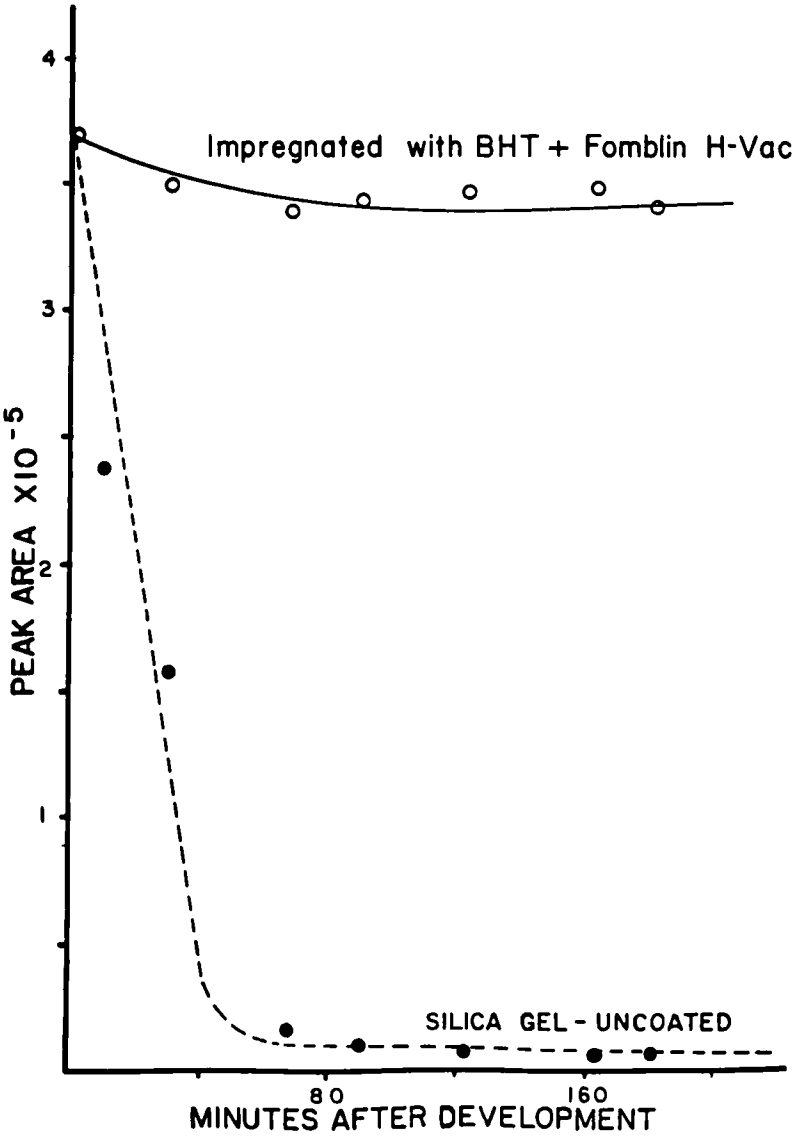

Figure 26 Inhibiton of fluorescence decay of 1-aminopyrene on silica gel HPTLC plates.

for fluoranthene on a silica gel HPTLC plate impregnated with a solution of 30% Fomblin H-Vac in 1,1,2-trichlorotrifluoroethane is illustrated in Table 10 and Figure 24.

Fluorescence quenching can also occur through chemical reaction while the sample is absorbed on the sorbent. One of the most frequently observed sorbent-catalyzed reactions, or presumed new products have been identified, in others only a physical interaction seems to be involved. In many cases these reactions show a time dependence that makes accurate quantitation difficult. Consider, for example, the fluorescence decay of 1-aminopyrene on different sorbents shown in Figure 25. The rate of fluorescence decay depends on the sorbent medium and is greatest for silica gel and lower for bonded-phase sorbents. In instances of this kind, impregnating the plate with an antioxidant, such as 2,6-di-tert-butyl-4-methylphenol (BHT), will diminish the rate of reaction substantially, although it may fail to eliminate it entirely (21,30,31). In general, the antioxidant is applied as a postchromatographic treatment in combination with a fluorescence enhancing reagent. For 1-aminopyrene, impregnating the plate after development with a solution of BHT and Fomblin H-Vac provides a stable signal suitable for quantitative analysis, Figure 26. Under these conditions some fluorescence decay occurred during the first hour following impregnation, but afterwards, the fluorescence signal was stable for at least 2 h. and changed less than 0.5% during that time.

ACKNOWLEDGMENT

Work in the authors' laboratory is supported by the United States Environmental Protection Agency. Although this research was funded wholly by the U.S. EPA under assistance agreement number R-808854-01-0 to C. F. Poole, it has not been subjected to the Agency's required peer and administrative review and, therefore, does not necessarily reflect the view of the Agency and no official endorsement should be inferred.

REFERENCES

(1) Touchstone, J. C. and Sherma, J. (Eds.), Densitometry in Thin-Layer Chromatography. Practice and Application, Wiley, New York, 1979.

(2) Pollak, V., Adv. Chromatogr., 17, 1 (1979).

(3) Bertsch, W., Hara, S., Kaiser, R. E. and Zlatkis, A., (Eds.), Instrumental HPTLC, Huthig, Heidelberg, 1980.

(4) Hurtubise, R. J., Solid Surface Luminescence Analysis, Dekker, New York, 1981.

(5) Coddens, M. E., Butler, H. T., Schuette, S. A. and Poole, C. F., LC Magzn., 1, 282 (1983).

(6) Poole, C. F. and Schuette, S. A., Contemporary Practice of Chromatography, Elsevier, Amsterdam, 1984.

(7) Poole, C. F., Butler, H. T., Coddens, M. E. and Schuette, S. A. Analytical and Chromatographic Techniques in Radiopharmaceutical Chemistry, Wieland, D. M., Manger, T. J. and Tobes, M. C., (Eds.), Springer-Verlag, New York, 1985 (in press).

(8) Zlatkis, A. and Kaiser, R. E., (Eds.), High Performance Thin-Layer Chromatography, Elsevier, Amsterdam, 1977.

(9) Fenimore, D. C. and Davis, C. M., Anal. Chem., 53, 252A (1981).

(10) Bush, I. E. and Greeley, H. P., Anal. Chem., 56, 91 (1984).

(11) Butler, H. T., Schuette, S. A., Pacholec, F. and Poole, C. F., J. Chromatogr., 261, 55 (1983).

(12) Coddens, M. E., Khatib, S., Butler, H. T. and Poole, C. F., J. Chromatogr., 280, 15 (1983).

(13) Butler, H. T. and Poole, C. F., HRC & CC, 6, 77 (1983).

(14) Coddens, M. E. and Poole, C. F., Anal. Chem., 55, 2429 (1983).

(15) Coddens, M. E. and Poole, C. F., LC Magzn., 2, 34 (1984).

(16) Yamamoto, H., Kurita, T., Suzuri, J., Hira, R., Nakano, K., Makabe, H. and Shibata, K., J. Chromatogr., 116, 29 (1976).

(17) Butler, H. T., Pacholec, F. and Poole, C. F., HRC & CC 5, 580 (1982).

(18) Butler, H. T. and Poole, C. F., J. Chromatogr. Sci., 21, 385 (1983).

(19) Apotheker, R., Advances in Thin-Layer Chromatography: Clinical and Environmental Applications, Touchstone, J. C., (Ed.), Wiley, New York, 1982, p. 149.

(20) Schuette, S. A. and Poole, C. F., J. Chromatogr., 239, 251 (1982).

(21) Woolbeck, D., Kleist, E. V., Elmadfa, I. and Funk, W., HRC & CC, 7, 473 (1984).

(22) Funk, W., Kerler, R., Schiller, J. Th., Dammann, V. and Arndt, F., HR., HRC & CC, 5, 534 (1982).

(23) Funk, W., Kerler, R., Boll, E. and Dammann, V., J. Chromatogr., 217, 349 (1981).

(24) Uchiyama, S. and Uchiyama, M., J. Chromatogr., 153 135 (1978).

(25) Uchiyama, S. and Uchiyama, M. Liq. Chromatogr., 3, 681 (1980).

(26) Uchiyama, S. and Uchiyama, M., J. Chromatogr., 262, 240 (1983).

(27) Seiler, N. and Weichman, M., Z. Anal. Chem., 220, 109 (1966).

(28) Oztunc, A., Analyst, 107, 585 (1982).

(29) Ho, S. S. J., Butler, H. T., and Poole, C. F., J. Chromatogr., 281, 330 (1983).

(30) Brown, K. K. and Poole, C. F., LC Magzn., 2, 526 (1984).

(31) Brown, K. K. and Poole, C. F., HRC & CC, 7, 520 (1984).

(32) Futoma, D. J., Smith, S. R., Tanaka, J. and Smith, T. E., CRC Crit. Revs. Anal. Chem., 11, 69 (1981).

(33) Seiferet, B., J. Chromataogr., 131, 417 (1977).

(34) Issaq, H. J., Advances in Thin-Layer Chromatography: Clinical and Environmental Applications, Touchstone, J. C., (Ed.), Wiley, New York, 1982, p. 457.