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CHARACTERIZATION OF A SCANNING DENSITOMETER FOR HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY

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SUMMARY

Optimum conditions to maximize the observed resolution and signal-to-noise ratio of the Shimadzu CS-910 scanning densitometer for high-performance thin-layer chromatography (HPTLC) are described. Resolution is shown to depend only on the slit width and is constant for slit widths in the range 0.05–0.80 mm. Signal-to-noise ratios depend on the size of the sampling beam which is fixed by the dimensions of the slit. At small slit dimensions the noise component dominates the signal-to-noise ratio. For scanning HPTLC plates dual-beam single-wavelength operation, linear scanning, scan speeds of 24 or 48 mm min^{-1}, slit widths of 0.5–0.8 mm and slit height $\approx$3.0 mm are recommended for general use.

INTRODUCTION

The performance breakthrough in thin-layer chromatography (TLC) was not a result of any specific advance in instrumentation or materials. It was rather a culmination of improvements in practically all aspects of the TLC process. This has resulted in an increase in separation efficiency and sample detectability approaching an order of magnitude while simultaneously reducing analysis times by a similar amount[1–5]. Generally known as high-performance thin-layer chromatography (HPTLC), it provides separation capabilities somewhat similar to those expected from isocratic conventional high-performance liquid chromatography (HPLC) (25-cm column with a 10-μm packing). This is illustrated by the HPTLC separation of a mixture of phenylthiohydantoin (PTH)-amino acid derivatives in Fig. 1. Multiple wavelength detection, simultaneous sample analysis and freedom from column contamination problems enhance the attractiveness of HPTLC as an alternative to HPLC for many routine analytical problems.

Visual observations and evaluation of separations on an HPTLC plate will not reveal the true separation obtained. This is because the eye functions as a logarithmic integrator having poor detection sensitivity, low reproducibility and poor perception of the exact position of spot boundaries[6]. *In situ* measurements of HPTLC separations are routinely made by scanning densitometry. This work was undertaken to

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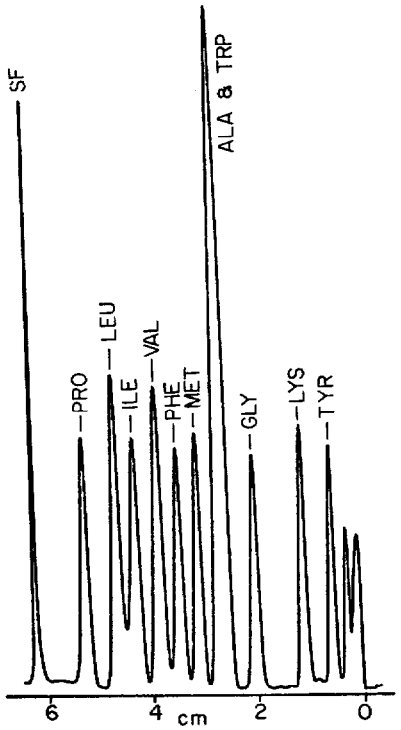

Fig. 1. Separation of a thirteen-component mixture of PTH-amino acid derivatives by continuous multiple development using the spot reconcentration technique. First development: hexane, 5 min, position 2. Second development: methylene chloride, 5 min, position 2. Third development: 2-propanol–methylene chloride (1:99), 15 min, position 4. Fourth development: 2-propanol–methylene chloride (1:99), 10 min, position 4. SF = Solvent front.

ascertain, in at least a semi-quantitative way, the experimental parameters that influence the measurement of spot profiles by scanning densitometry.

EXPERIMENTAL

Reagents

All solvents were HPLC grade (Burdick & Jackson Labs., Muskegon, MI, U.S.A.). Reagent grade azobenzene (Matheson, Coleman & Bell, Norword, OH, U.S.A.) were used as received. The separations were carried out on 10 × 10 cm HP-K precoated silica gel plates (Whatman, Clifton, NJ, U.S.A.).

Standards and conditions

Azobenzene standards in hexane were prepared by successive dilution yielding concentrations of 11.60, 5.82, 2.33, 1.16, 0.582, 0.116 and 0.0582 mg ml^{-1}. For development of silica gel HPTLC plates the mobile phase was hexane–methylene chloride (1:1) and the time 5 min in position 4 of the short bed continuous development chamber (SB/CD).

The PTH-derivatives of leucine, isoleucine and proline were dissolved in hexane at a concentration of approximately 4.0 mg ml^{-1}. For separation on silica gel HPTLC plates the mobile phase was methylene chloride–isopropanol (99:1) and the time 10 min in position 4 of the SB/CD chamber. The conditions for the multiple development separation in Fig. 1 are given in the legend.

For scanning densitometry the single wavelength reflectance mode without filtering was used throughout this study unless otherwise noted. Azobenzene was determined at $\lambda = 350$ nm and the PTH-amino acid derivatives at $\lambda = 270$ nm. The scanning stage and recorder speeds were matched at 24 mm min^{-1} except as noted.

Apparatus

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). Samples were spotted 1.0 cm apart and 1.0 cm from the bottom of the plate. The HPTLC plates were developed in a SB/CD (Regis, Morton Grove, IL, U.S.A.). *In situ* scanning of the HPTLC plates was performed with a Shimadzu CS-910 scanning densitometer (Shimadzu, Columbia, MA, U.S.A.). Peak profiles were recorded on a Shimadzu U-135 strip chart recorder and peak areas with either a Spectra Physics minigrator or SP 4100 computing integrator (Spectra Physics, Santa Clara, CA, U.S.A.).

RESULTS AND DISCUSSION

The Shimadzu CS-910 scanning densitometer is a dual-beam instrument which can measure absorption or fluorescence by reflectance or transmission, and absorption by fluorescence quenching[7,8]. Reflectance provides higher signal-to-noise ratios, and unlike transmission measurements, is not limited to wavelengths greater than $\lambda = 320$ nm due to absorption by the glass backing plate. For the purpose of this discussion only the reflectance mode is considered.

The plate can be scanned in the reflectance mode using single-wavelength or dual-wavelength separated in time modes. In the single-wavelength mode, the beam exiting the monochromator is chopped and divided to provide the sampling beam and the reference beam. The reference beam and a variable mechanical beam attenuator are used to set the gain on the photomultiplier, and to eliminate instability in the detector baseline caused by fluctuations in the source output. The sampling beam is used to scan the plate. In the dual-wavelength mode, two monochromatic light beams of different wavelengths are chopped and combined into a single beam forming the sampling beam. The reference wavelength is used to correct the measuring wavelength for changes in scatter at the plate surface. The surface homogeneity of HPTLC plates is fairly good and large changes in background scattering are not common. Also, it is often difficult to select a reference wavelength close to the measuring wavelength at which none of the sample spots absorbs. For experimental convenience single-wavelength operation is preferred and this discussion will be limited to this mode.

The plate may be scanned linearly or in a zigzag fashion. In the zigzag mode the sample beam is fixed and the scanning stage oscillated perpendicular to the di-

rection of scanning. The net result is that the spot is repeatedly sampled along its profile as the spot is scanned. Since the sample beam is small, 1.25 × 1.25 mm for conventional TLC applications, concentration variations within the beam are negligible. In conventional TLC where spots of irregular shape containing an uneven sample distribution are frequently obtained, zigzag scanning has been show to be more accurate for determining sample concentration than linear scanning[7]. For the compact spots observed in HPTLC only a few passages of the beam through the spot are possible due to the close similarity in dimensions of the spot to the forward step in the zigzag motion. For zigzag scanning of HPTLC separations the beam size is reduced to 0.6 × 0.6 mm and the scan rate to 12 mm min^{-1} increasing the analysis time compared to linear scanning. Also, the signal output in the zigzag mode comprises a series of spikes corresponding to the individual slices of the spot recorded each time the spot is moved through the beam. The envelope enclosing these spikes represents the peak profile. General laboratory chromatographic integrators do not handle this signal well. Computing integrators may be used after additional programming, but less sophisticated instruments are usually unsuitable. Linear scanning is more convenient, less time consuming and is quantitatively accurate for symmetrical spots. Distorted spots in HPTLC should be corrected by modifying the separation system rather than resorting to zigzag scanning.

The above parameters can be termed "selectable" as they do not, in general, influence the observed chromatographic resolution, although they may affect the signal-to-noise ratio. The operation of the instrument is also controlled by a second group of parameters, termed "adjustable". These include the slit width, slit height, scanning speed and the detector and recorder time constants. These parameters may change resolution directly and are considered in more detal below.

Slit width

The image of the sampling beam is a rectangle defined by the slit width and slit height. The slit width is the parameter which fixes the dimensions of the beam in the direction of scanning. The cone of reflected light from the plate surface is collected by a 7-cm concave mirror. There are no slits on the collection side of the instrument.

The first experiment performed was non-chromatographic and designed to test the spatial resolving power of the instrument. As a test system, colored slide coding dots were placed on an HPTLC plate and their boundary separation carefully measured with a calibrated ocular device. At a slit width greater than the edge-to-edge separation of the spots some fusing of the edge profiles was observed at high detector sensitivity. At normal detector settings for HPTLC measurements, it was not possible to detect any edge-to-edge fusing of the spot boundaries even in the worst case situation investigated, the spot boundaries separated by 0.2 mm and the slitm width set at 1.40 mm. Thus, boundary fusion was observed only for detection conditions uncharacteristically sensitive for practical work, were minor in extent, and were not observed for slit widths less than the edge-to-edge separation. At detection conditions closer to those used in practice, no dependence of spatial resolution with slit width setting was observed.

To investigate the effect of slit width on observed resolution a mixture of three PTH-amino acids was separated under conditions providing resolution almost to baseline between proline and leucine, and partial resolution between leucine and

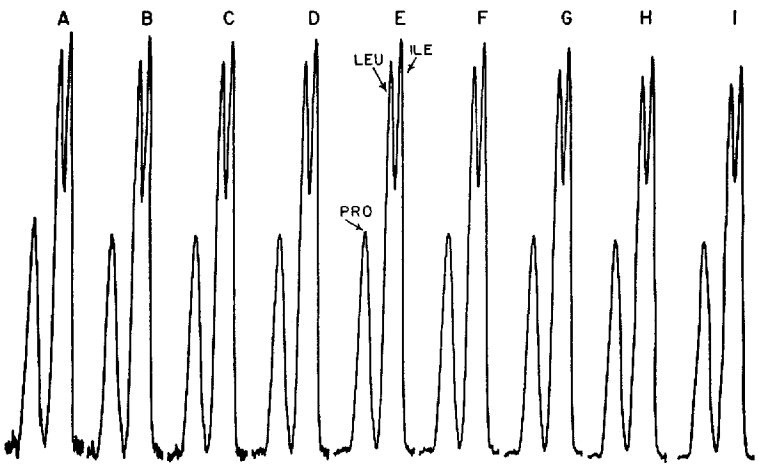

Fig. 2. Resolution of PTH-derivatives of proline, leucine and isoleucine measured at a constant slit height 1.50 mm and scan speed 24 mm min^{-1}. Slit width: A, 0.05 mm; B, 0.10 mm; C, 0.20 mm; D, 0.40 mm; E, 0.60 mm; F, 0.80 mm; G, 1.00 mm; H, 1.20 mm; I, 1.40 mm.

isoleucine. As well as the mixture, individual standards were run for the independent calculation of peak widths. The chromatograms were scanned with a constant slit height of 1.50 mm and at slit widths covering the range 1.40–0.05 mm, Fig. 2. The calculated resolution values are tabulated in Table I. Within experimental error, the observed resolution is essentially constant for slit widths of 0.05–0.80 mm. For larger slit widths a small degradation in resolution is observed (compare Fig. 2E and I).

Fig. 3 shows the effect of varying the slit width on the signal-to-noise ratio for an azobenzene standard (1.16 mg ml^{-1}). The noise level was defined as the dimensions of the peak to peak recorder pen deflections in mm as a 5-cm blank region of the plate was scanned. The origins of the noise signal and its magnitude is discussed

TABLE I

DEPENDENCE OF RESOLUTION ON SLIT WIDTH

W = Peak width at base; $W_{\frac{1}{2}}$ = peak width at half-height.

Slit width (mm)	*Leu–Pro*		*Leu–Ile*		*Ile–Pro*	
	W	$W_{\frac{1}{2}}$	W	$W_{\frac{1}{2}}$	W	$W_{\frac{1}{2}}$
1.40	1.71	1.61	0.75	0.73	2.34	2.28
1.20	1.80	1.64	0.79	0.73	2.35	2.30
1.00	1.76	1.66	0.79	0.74	2.42	2.30
0.80	1.86	1.70	0.81	0.76	2.43	2.34
0.60	1.86	1.72	0.83	0.78	2.47	2.36
0.40	1.87	1.70	0.81	0.77	2.43	2.36
0.20	1.87	1.70	0.81	0.78	2.49	2.38
0.10	1.82	1.70	0.82	0.78	2.46	2.38
0.05	1.86	1.72	0.84	0.78	2.51	2.40

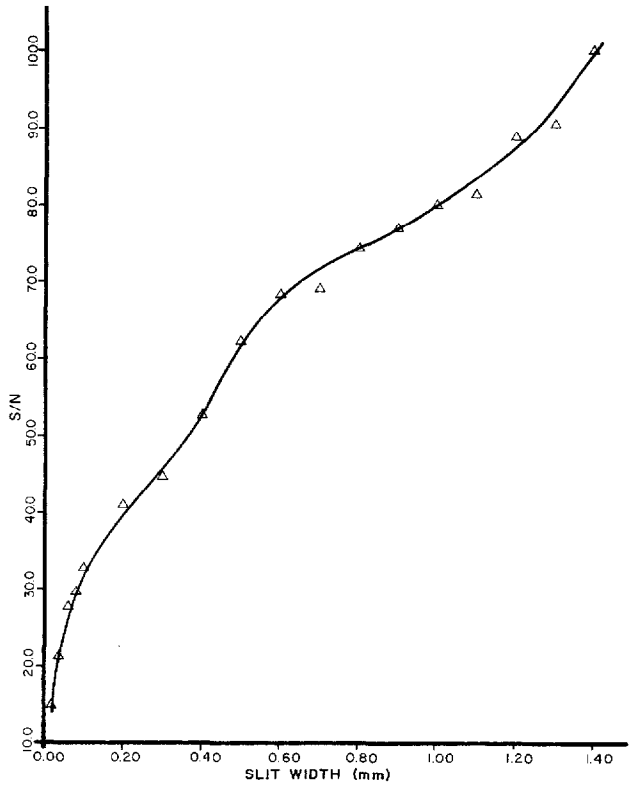

Fig. 3. Influence of slit width on the signal-to-noise ratio, S/N, for azobenzene. Total sample: 0.232 μg. Slit height: 1.50 mm.

under slit height. The noise signal is always greatest for small slit settings and is observed in the chromatograms of Fig. 2. The general shape of the curve shown in Fig. 3 was independent of concentration over the 200-fold concentration range investigated. The "knee" in the curve at a slit width of approximately 0.60 mm was apparent in all plots and was independent of spot diameter, slit height and spot concentration. There is no obvious reason for the above break in the curves and we can only speculate that it is probably a function of the collection optics of the instrument.

In summary, to maintain resolution at maximal signal-to-noise ratios, a slit width of 0.5–0.8 mm is optimum. When resolution is not the primary concern, *i.e.*, resolution > 1.5, then wider slit widths should be used to maximize the signal-to-noise ratio.

Slit height

While maintaining the slit width constant at 0.50 mm a series of azobenzene standards were scanned at slit heights in the range 10.0–0.02 mm. Fig. 4, representing the 1.16 mg ml^{-1} standard, is typical of the results obtained. In the reflectance mode, the signal is defined as the diminution of reflected light compared to a blank area of

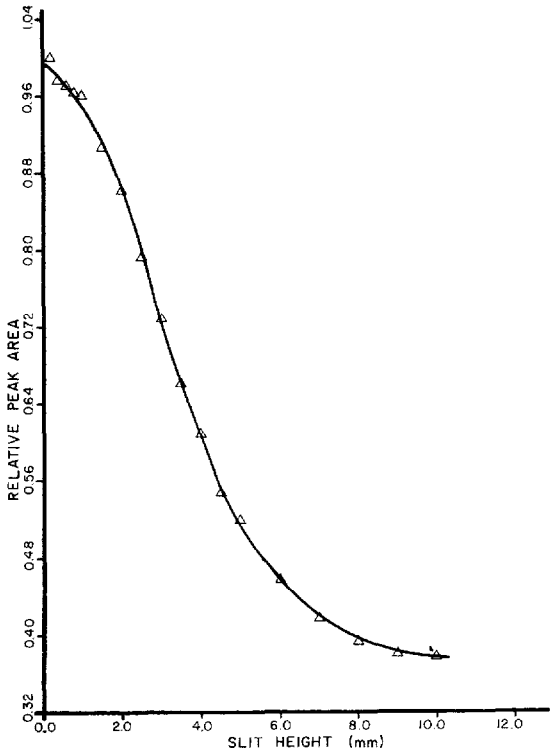

Fig. 4. Influence of slit height on signal for azobenzene. Total sample: 0.232 μg. Slit width: 0.50 mm. Peak area values are normalized.

the plate as the sampling beam traverses the spot. When the slit height is large compared to the diameter of the spot a large amount of light reflected from the plate is collected and the contribution from subtracted (adsorbed) 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 reflected from the plate background 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. The contribution of absorbing molecules at the fringe of the spot is small compared to those at the center. At very small slit heights the signal levels off as the slit height approaches the diameter of the core of the spot where sample concentration is approximately constant. The above features are clearly seen when the colored slide coding dots are scanned; the signal becomes constant once the largest dimension of the slit corresponds to the slide dot diameter.

Decreasing the slit height below the diameter of the spot resulted in an increase in signal but also a concomitant increase in the noise level. At small slit heights the observed noise is a maximum and decreases in a smooth curve as the slit height is increased. The signal-to-noise ratio, Fig. 5, passes through a maximum at a slit height setting of approximately 3.0 mm, and is independent of spot diameters in the range 3.9–5.2 mm. At slit heights less than 3.0 mm the signal-to-noise ratio is dominated

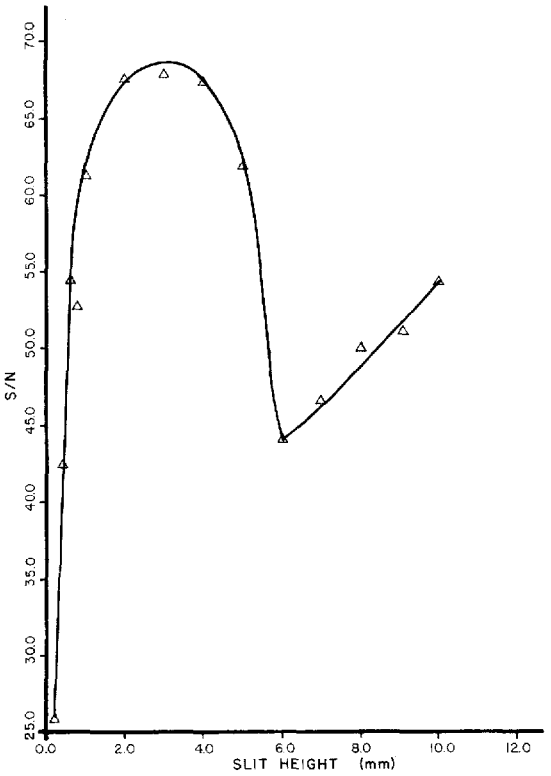

Fig. 5. Influence of slit height on the signal-to-noise ratio, S/N, for azobenzene.

by the noise component, which increases at a rate in excess of the improvement in signal. At slit heights greater than 6.0 mm a small increase in the signal-to-noise ratio is observed, as here the noise component is decreasing at a faster rate than the signal.

In the CS-910 the photomultiplier detector is operated in a feedback reference network which increases the absolute voltage to the photomultiplier tube (PTM) as the intensity of the sampling beam is diminished. Therefore as the slit dimensions are decreased the overall gain of the detection system is increased. There are no gain or offset adjustments in the output circuitry. The gain is fixed once the background level for the reflected light from a blank area of the plate has been established. The absolute PMT voltage increases in an exponential-like fashion as the slit dimensions are decreased. This partially explains why the signal rises so sharply with decreasing slit height, Fig. 4. As the slit height was reduced from 10 mm to 5 mm the PMT voltage changed $\approx 5\%$ and the signal increased $\approx 35\%$. As stated previously, the increase in signal dominates the shape of the curve in this region. Between 5 mm and 0.5 mm the PMT voltage increased an additional 35% and the signal $\approx 80\%$. In this region the large increase in the gain of the instrument appears to be the dominant factor (overall gain increases exponentially with PMT voltage). The rise in PMT voltage with decreasing slit dimensions also leads to the greater noise levels observed at the small slit dimensions. At wide slit settings baseline stability increases, and here the

principal source of noise is probably inhomogeneity in the light scattered at the plate surface.

Resolution was found to be independent of slit height.

Scan rate

A chromatogram in scanning densitometry is obtained by movement of the HPTLC plate on a motorized stage through the sampling beam while simultaneously monitoring the intensity of the light reflected from the plate surface. Scan rates of 3, 6, 12, 24, 48 and 96 mm min^{-1} are possible with the instrument at hand. At scan rates of 96 mm min^{-1} an approximate decrease of 5% in signal was observed compared to slower scan rates. Fast pen response recorders and integrators with high sampling frequency are required to avoid distortion of the peak profiles. The strip chart recorder used in these studies had a pen response of 0.33 sec for 99% full scale deflection. This was adequate for recording chromatograms up to, and including, scan rates of 48 mm min^{-1}. The minigrator, which has a sampling frequency of 10 Hz, often failed to detect small peaks and treated closely spaced but baseline resolved peaks as fused at scan rates of 48 mm min^{-1} and above. At slow scan rates, less than 12 mm min^{-1}, small or broad peaks were often treated as a shifting baseline. The Spectra Physics SP 4100 (sampling frequency 60 Hz) is not beset by these problems at scan rates of 24 and 48 mm min^{-1}. At slower scan rates small peaks and closely spaced baseline resolved peaks were often misinterpreted. The above observations were found to hold true for a wide range of integration parameters.

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