QUANTITATIVE SCANNING OF FLUORESCENT OR LIGHT-ABSORBING SPOTS ON THIN-LAYER CHROMATOGRAMS

H. T. GORDON
Division of Entomology and Acarology, University of California, Berkeley, Calif. (U.S.A.)
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Many different instruments have been used for the direct measurement of light absorbance, fluorescence, or radioactivity on chromatograms, although this is generally considered to be less accurate than elution of spots for quantitative analysis[1-3]. During current research on thin-layer chromatography (TLC) of fluorescent urethanes of sterols and other higher alcohols it became clear that a fluorescence scanning apparatus was essential to provide permanent and at least roughly quantitative records of the chromatograms. Few scanners are available commercially, and their performance is not usually evaluated thoroughly. Our laboratory uses the relatively new Aminco Fluoro-Microphotometer (American Instrument Company, Silver Spring, Md.) for fluorimetry of solutions. This instrument is sensitive and stable but does not yet have an accessory for scanning thin-layer plates. Such an accessory has therefore been designed and constructed, and its performance is being reported in this paper.

DESCRIPTIONS OF SCANNING ACCESSORY

The instrument was designed to fit the Aminco Fluoro-Microphotometer with a minimum of modification and to be quickly insertable and removable to allow normal use as a solution fluorimeter or colorimeter. The sample chamber of the Fluoro-Microphotometer is a metal well 25 mm², with a depth easily extendable to 25 cm. Light (from interchangeable ultraviolet or visible lamps) enters through a 32 × 18 mm first aperture into a primary filter compartment that allows use of interchangeable light filters 50 mm² and then passes through a 32 × 18 mm second aperture into the sample chamber. Fluoresced, scattered, or reflected light passes through a 32 × 18 mm exit aperture at a right angle to the entry aperture, through a secondary filter compartment to a photomultiplier tube.

The mechanical and electrical systems of the scanner were designed and constructed by J. DIMICK (Dimick Enterprises, El Cerrito, Calif.) to perform according to the specifications of the author. The metal frame slides in vertically to the full depth of the Aminco sample chamber and is attached by a set screw. The frame carries a vertical metal movable plate, 250 × 28 mm, which is positioned diagonally in the sample chamber and can support glass plates having maximum thickness of about 3.2 mm, width of 26 mm, and length of about 200 mm. Such plates are routinely used in our laboratory for one-dimensional TLC. To the back of the metal slide is affixed a helical rack, which is driven by a helical pinion attached to a 1 r.p.m.

reversible synchronous motor, swivel-mounted so that the pinion can be quickly disengaged from the rack. The vertical motion is at a rate of 18.7 mm/min. There are upper and lower limit switches that restrict the travel to a maximum of 183 mm, starting about 3 mm from one end. A full scan takes 9.8 min. A reversing switch allows immediate shifting from downwards to upward motion. A tubular metal cover protects the light chamber from interference by room light.

The optical systems were designed by the author. For fluorescence scanning it is desirable to project on the thin-layer surface a horizontal band of light, as intense and uniform as possible. This is approximated by using a cylindrical lens (5.5 mm wide, focal length 5.6 mm) mounted on an interchangeable metal slide within the sample chamber and directly in front of the second entry aperture. The lens projects a widened image of a 1 × 3 mm rectangular slit in an interchangeable plastic slide mounted in front of the first entry aperture near the fluorescent lamp. Since the image is projected on a surface set at a 45° angle to the lens and the slit is illuminated by the very diffuse light from a GE F4T4/BL fluorescent lamp the band of light on the thin-layer surface is about 16 mm wide and not perfectly uniform in thickness or intensity.

The nature of the light band produced by this optical system was analysed by applying to a thin-layer plate a series of 1 μl spots of a fluorescent solution, positioned vertically 5 or 10 mm apart and horizontally at distances from 0 to 5 mm to the right or left of the center line. The spots had a radius of about 2 mm. When this test plate was scanned, the peak fluoresced light intensity proved to be the same for spots positioned 0 to 3 mm on either side of the center, but it decreased to 90 % of the maximum value at 4 mm and 80 % at 5 mm. Resolving power was measured by the intensity of fluoresced light in the center of the 1-mm wide dark region between spots separated by a vertical distance of 5 mm. This intensity was about 7 % of the maximum in the region from 0 to 5 mm to the left of center where the distance from lens to surface varies from 3 to 8 mm. To the right of center resolving power decreased, minimum light intensity being 25 % of maximum at 5 mm to the right. While resolving power is somewhat increased by using a 0.5 × 3 mm light slit, the major factor lowering resolution seems to be broadening of the effective light band by internal reflections within the thin layer.

The light system for fluorescence scanning can be used for reflectance scanning of light-absorbing spots, but the reflected blank light is so intense that a 0.1 × 3 mm slit is preferable; it may even be necessary to introduce a light-reducing aperture in the secondary filter compartment. The extremely wide band has the advantage that the full width of the absorbing spot is scanned, *i.e.*, all the material in the spot contributes to absorption of light. However, sensitivity (as measured by peak reduction in light intensity) is relatively low since a large fraction of the band of light never encounters any absorbing material. If a pinpoint light source is used instead of a slit the cylindrical lens still spreads it out into a relatively wide but non-uniform band of light. Therefore a second lens system, a double-convex lens (5 mm in diameter, focal length 7 mm) mounted on an interchangeable metal slide, is used to replace the cylindrical lens. The circular lens, used with a pinpoint source, can project a very small spot of light on the center of the thin-layer plate. Such a system, however, is very sensitive to small changes in shape and centering of the spot; even if the substance being chromatographed is applied as a wide band, perfect uniformity is diffi-

cult to achieve. Use of a pinpoint source has therefore been discontinued. For high-sensitivity scanning, the combination of the circular lens with a 0.1 × 3 mm slit was adopted; this projects a thin uniform band of light about 4 mm wide on the plate. This is wide enough to cover most spots or at least to have an averaging effect over the greater part of the spot width, and yet not so wide that a large fraction of the light fails to encounter the spot. It is affected by serious off-centering of the spot; however, this can be minimized by spotting a fairly wide band of material initially. Even if the initial band is not homogeneous, the averaging effect of scanning with a band of light tends to correct for this.

OPERATION, CONTROLS AND RECORDING

The recorder output of the Aminco photometer is 50 mV and any potentiometric recorder of 100 mV or higher sensitivity, 1 sec or faster response, and chart speed of the order of 20 mm/min will give satisfactory scans. We have used a Bausch and Lomb VOM-6 recorder at 25 mV sensitivity and chart speed of 25 mm/min; this amplifies the photometer output by 2, and its zero-setting control is sometimes useful to keep scans on-scale.

Except for occassional use of the zero-setting control of the recorder (and changes of lens and slit systems) all operating adjustments are made with the Fluoro-Microphotometer controls. The zero-adjust control allows setting the recorder pen to zero or 100 in the blank region of a chromatogram at the start of a scan. The meter-multiplier control allows switching of photometer sensitivity over a 1000-fold range in steps of 1.0, 0.3, 0.1, etc. The light systems of the scanner are designed so that the maximal sensitivity required is usually not greater than 0.01, where zero stability of the photometer is very high. Even the most intense spots can then be scanned at a sensitivity of 1.0.

The first scan is normally in the downward direction at maximum sensitivity. If an intense spot gives an off-scale scan the chromatogram can be re-scanned in the upward direction at a lower sensitivity by using the scanner reversing switch. Time can often be saved by reversing the scanner as soon as an off-scale spot has been scanned, simultaneously decreasing sensitivity 10-fold and readjusting the zero setting. If the reverse-scan is still off-scale or now gives too small a peak, the scanner is again reversed and sensitivity readjusted. This allows satisfactory scanning of the intense spot in a few minutes. While the area of a scanned spot can be measured with a planimeter, it is more convenient to approximate it to an isosceles triangle and measure peak height and base width in mm. The product of height × base × photometer sensitivity is the "relative area", which measures the light intensity change caused by the spot. If a spot is so spread out that it does not scan as a triangle, it is probably best to spot a more dilute solution and chromatograph it.

Scanner performance with fluorescent spots using adsorption chromatography on silica gel G

In all tests the scanning system has always given identical repetitive scans of any one thin-layer plate. The performance tests indicate the sensitivity and range normally attainable, but the variability found is entirely in the chromatographic operation.

The fluorescent test substance used was a crystalline preparation of the fluoranthenyl urethane of cholesterol, m.p. 184–186°, prepared from recrystallized cholesterol (m.p. 144–145°) and fluoranthenyl-3-isocyanate synthesized from commercial 3-aminofluoranthene and phosgene. A stock solution in toluene at 0.163 μg/μl (equivalent to 0.1 μg/μl of cholesterol), and a 1:8 dilution in toluene (equivalent to 0.0125 μg/μl of cholesterol) were prepared. Test spots were applied to the silica gel G plates (thickness of layer about 850 μ wet, 625 μ dry) in the center 10 mm from one end. A Hamilton PB-600-1 repeating dispenser with a Hamilton 705 50-μl syringe (Hamilton Company, Inc., Whittier, Calif.) was used to deliver 1-μl droplets. Quantities of 1, 2, 4, 8 and 16 μl were applied, each droplet being allowed to dry before applying the next. Chromatograms were developed in a 1:1 mixture of cyclohexane and benzene, completed in less than an hour, then dried for 10–15 min before scanning. When a plate was re-scanned several hours later the scan was identical, but after 24 h there was slight loss of fluorescence intensity.

The use of unusually thick layers made the plates even more fragile than the usual 200–300 μ layers. The intention was to increase the capacity, but the greater fragility is an inconvenience during chromatography and scanning of thinner layers would normally be preferable.

Table I lists the data for triplicate runs. Scanning used the 1 × 3 mm slit and cylindrical lens, with a primary filter of peak transmittance at 365 mμ (Corning 7-60) and a secondary filter cutting off wavelengths below 485 mμ (Wratten 8). The relative areas of triplicates show standard deviations of the order of 20%, caused by large deviations in peak heights or base widths. The mean relative area per μg of cholesterol, however, is remarkably constant over a wide range, although it declines above 0.4 μg of cholesterol. Spotting of a large volume of a dilute solution or a small volume of more concentrated solution does not seem to affect the scans. Base width of peaks is increased less than 2-fold by a 16-fold increase in volume spotted and a 100-fold increase in quantity spotted. R_F values vary; but there is no striking change with increasing load, nor any clear correlation between R_F and relative area in replicates. It seems probable that most of the fluoresced light is emitted from the upper 100 μ of the thin layer. The variability may be caused by variations in the quantity of fluorescent substance migrating upward into this surface layer as the chromatogram dries. It would probably be less if a 200 or 300 μ thick layer were used, and might also be reduced by making drying conditions uniform.

Scanner performance with fluorescent spots using adsorption and partition chromatography on Avicel–silica

Avicel–silica forms extremely tough layers on glass, and is used routinely in our laboratory for partition chromatography, in which the layer must be pre-coated with a stationary phase liquid.

The microcrystalline cellulose, Avicel (American Viscose Division of FMC Corporation, Newark, Del., U.S.A.) was introduced for TLC by WOLFROM *et al.*[4]; in our experience, layers of pure Avicel have been less satisfactory than 1:1 mixtures of Avicel and silica (without binder). The 1:1 Avicel is prepared by mixing 20 g of Avicel and 20 g of silica with 200 ml of distilled water in a Waring blendor at high speed for 15 min. The warm, creamy mixture is briefly subjected to a water-pump vacuum while gently shaking, to remove air bubbles. It is then spread on the glass

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TABLE I

SPOT SCAN DATA FOR ABSORPTION CHROMATOGRAMS OF FLUORANTHENYL URETHANE OF CHOLESTEROL ON SILICA GEL G

μl spotted	Solution*	μg cholesterol	Photometer sensitivity	Height × base (mm × mm)	Relative area**	Mean rel. area	Std. dev. (%)	Rel. area per μg cholesterol	R_F × 100
a				44 × 14	1.848				44
1 b	A	0.0125	0.003	47 × 14	1.974	2.002	0.175	160	45
c				52 × 14	2.184		(8.7%)		41
a				33 × 12	3.96				47
2 b	A	0.025	0.01	62 × 10	6.2	4.59	1.41	184	43
c				30 × 12	3.6		(31%)		39
a				62 × 18	11.16				44
4 b	A	0.05	0.01	58 × 18	10.44	9.35	2.53	187	42
c				37 × 18	6.46		(27%)		42
a				39 × 14	15.38				49
8 b	A	0.10	0.03	50 × 11	16.5	18.62	4.76	186	50
c				47 × 17	23.97		(25.6%)		46
a				36 × 21	26.68				47
16 b	A	0.20	0.03	76 × 19	43.32	31.32	9.21	157	42
c				56 × 20	33.6		(29.6%)		48
a				37 × 17	18.87				50
1 b	B	0.10	0.03	40 × 17	15.3	17.69	2.07	177	51
c				35 × 18	18.9		(11.7%)		46
a				59 × 17	30.09				43
2 b	B	0.20	0.03	109 × 10	32.7	35.33	6.81	177	45
c				90 × 16	43.2		(19.3%)		42
a			0.03	109 × 21	68.67				41
4 b	B	0.40	0.1	63 × 15	94.5	73.39	22.0	183	53
c			0.1	30 × 17	51.0		(30%)		42
a				32 × 21	67.2				
8 b	B	0.80	0.1	47 × 22	103.4	82.1	19.0	103	47
c				36 × 21	75.6		(23%)		
a			0.1	67 × 24	160.8				51
16 b	B	1.60	0.1	95 × 24	228	185.5	23.5	116	
c			0.3	43 × 13	167.7		(12.7%)		57

* Solution A contains cholesterol (as the fluoranthenyl urethane) at 0.0125 μg/μl and solution B at 0.10 μg/μl.

** Relative area is peak height × base × photometer sensitivity.

TABLE II

SPOT SCAN DATA FOR 3 TYPES OF CHROMATOGRAMS OF FLUORANTHENYL URETHANE OF CHOLESTEROL ON AVICEL–SILICA

µl spotted	*Type of chromatogram**	*Solution***	*µg cholesterol*	*Photometer sensitivity*	*Height × base (mm × mm)*	*Relative area****	*Mean rel. area*	*Std. dev. (%)*	*Rel. area per µg cholesterol*	R_F
					15 × 13	1.95				0.35
1 b	A	A	0.0125	0.01	21 × 13	2.73	2.44	0.43	195	0.39
c					24 × 11	2.64		(17.6%)		0.45
					43 × 12	5.16				0.23
1 b	P-1	A	0.0125	0.01	32 × 12	3.84	4.60	0.69	368	0.17
c					40 × 12	4.80		(15.0%)		0.27
					18 × 18	3.24				0.47
1 b	P-2	A	0.0125	0.01	27 × 12	3.24	3.12	0.21	249	0.57
c					24 × 12	2.88		(6.7%)		0.49
					34 × 12	12.24				0.44
1 b	A	B	0.10	0.03	28 × 15	12.6	12.36	0.21	123.6	0.45
c					34 × 12	12.24		(1.85%)		0.50
					52 × 15	23.4				0.23
1 b	P-1	B	0.10	0.03	47 × 17	23.97	24.61	1.64	246.1	0.38
c					63 × 14	26.46		(6.66%)		0.15
					36 × 18	19.44				0.59
1 b	P-2	B	0.10	0.03	37 × 21	23.31	20.71	2.27	207.1	0.58
c					38 × 17	19.38		(11.0%)		0.58
					26 × 21	163.8				0.57
16 b	A	B	1.60	0.3	26 × 19	148.2	148.8	14.8	93	0.54
c					28 × 16	134.4		(9.94%)		0.66
					56 × 16	268.8				0.36
16 b	P-1	B	1.60	0.3	38 × 28	319.2	287.2	28.0	180	0.24
c					38 × 24	273.6		(9.75%)		0.24
					53 × 23	365.7				0.82
16 b	P-2	B	1.60	0.3	43 × 22	283.8	314.5	45.1	197	0.55
c					49 × 20	294.0		(13.9%)		0.68

* A signifies adsorption chromatograms developed in cyclohexane–benzene (1:1); P-1 signifies partition chromatograms using 7.5% phenylacetonitrile–12.5% β-methoxypropionitrile in acetone to form the stationary phase, with development in isooctane; P-2 signifies partition chromatograms using 25% β-methoxypropionitrile in acetone to form the stationary phase, with development in isooctane.

** Solution A contains cholesterol (as the fluoranthenyl urethane) at 0.0125 µg/µl and solution B at 0.10 µg/µl.

*** Relative area is peak height × base × photometer sensitivity.

TABLE III

BAND SCAN DATA FOR PARTITION CHROMATOGRAMS OF *n*-OCTANOYLAMIDE OF 1-PHENYLAZO-2-AMINONAPHTHALENE ON AVICEL–SILICA

Partition chromatograms using 25% β-methoxypropionitrile in acetone to form the stationary phase, followed by development in isooctane. Most of the chromatograms showed some tailing, especially at higher loads, but only the main roughly triangular peak area was measured. Tailing may be caused by irreversible adsorption of part of the load, and contributes to the variance in relative area of replicates. The 12 chromatograms (3a to 24c) were scanned 3 times. The first set of scans (run at times from 24 to 62 min after chromatography) and the second set (run at 72 $\pm$ 3 h after chromatography) used the 0.1 × 3 mm slit and cylindrical lens system. The third set (run at 74 $\pm$ 2 h after chromatography) used the 0.1 × 3 mm slit and circular lens system. The higher variance of the scanning, especially with 3a and 6c, is caused by off-centering of the band.

µl spotted	*Time interval**	*µg*	*Height × base (mm × mm)*	*Relative area ***	*Mean rel. area*	*Std. dev. (%)*	*Rel. area per µg*	*K****	*R_F*
a	50 min		12 × 14	5.04					0.72
3 b	44 min	3	13 × 15	5.85	4.93	1.2	1.64	44	0.67
c	58 min		10 × 13	3.90		(24.2%)			0.70
a	40 min		34 × 15	15.3					0.70
6 b	46 min	6	19 × 14	7.98	10.32	3.49	1.69	52	0.67
c	43 min		16 × 16	7.68		(33.8%)			0.67
a	50 min		30 × 18	16.2					0.67
12 b	56 min	12	41 × 15	18.4	17.3	0.9	1.44	52	0.76
c	62 min		44 × 13	17.2		(5.2%)			0.57
a	40 min		42 × 18	22.7					0.68
24 b	24 min	24	46 × 16	22.1	25.5	4.44	1.06	51	0.77
c	30 min		59 × 18	31.8		(17.4%)			0.64
a			19 × 16	9.10					
3 b	72 h	3	18 × 16	8.63	7.71	2.01	2.57	69	
c			12 × 15	5.40		(26.1%)			

a			38 × 18	10.2				
6 b	72 h	6	23 × 16	11.0	12.9	4.62 (35.8%)	2.15	65
c			20 × 16	9.6				
a			37 × 19	21.1				
12 b	72 h	12	54 × 15	24.3	21.8	2.27 (10.4%)	1.82	65
c			51 × 13	19.9				
a			47 × 18	25.4				
24 b	72 h	24	52 × 18	28.1	28.6	3.42 (12.0%)	1.19	57
c			63 × 17	32.2				
a			20 × 14	8.40				
3 b	74 h	3	47 × 17	23.97	16.40	7.79 (47.5%)	5.47	147.5
c			33 × 17	16.83				
a			66 × 16	31.68				
6 b	74 h	6	70 × 19	39.90	26.73	16.2 (61.2%)	4.45	134
c			16 × 18	8.62				
a			53 × 20	31.2				
12 b	74 h	12	92 × 15	41.4	37.3	5.4 (14.5%)	3.11	112
c			94 × 14	39.4				
a			101 × 21	63.8				
24 b	74 h	24	82 × 16	39.3	56.6	15.1 (26.8%)	2.36	113
c			117 × 19	66.8				

* The time interval in min or h between removal of a plate from the developing solvent and beginning of the scan. No notable changes were noted during the first hour or two. Intervals marked 72 h in fact varied from 68 to 74 h.

** All scans were run at a photometer sensitivity of 0.03. Relative area is peak height × base × photometer sensitivity.

*** The hyperbolic constant, K, is (relative area) × (24 + load in μg)/(load in μg).

plates, at wet thicknesses from 400 to 800 μ (adequate to cover one or two 20 × 20 cm plates), which dry to layers of about half this thickness after standing overnight at room temperature. The layers (like those of pure Avicel) are so firm that plates can be stacked together without damaging the layer, and also can be written on with pencil. They give excellent results both in adsorption and partition chromatography, even with solvents containing 20 % water. For partition chromatography, spots are applied and allowed to dry and then the plates are coated with a solution of a stationary phase liquid in acetone, using a 1-ml pipet. The solution is first applied to the region below the spot, then directly above the spot, moving away from the spot to the upper end. Since the solution flows into the spotted area, the spots are compressed into fairly narrow bands. After air-drying for a few minutes to allow evaporation of the acetone, the plates are dipped (to a depth of 4 or 5 mm) in the mobile phase solvent for development, in a chamber saturated with stationary phase solvent vapor.

Only 3 levels of the fluoranthenyl urethane of cholesterol were tried, equivalent to 0.0125, 0.1 and 1.6 μg cholesterol. However, 3 different chromatographic systems were used: (1) adsorption chromatography using cyclohexane–benzene (1:1), as with silica gel G, (2) partition chromatography using a pre-coat of 7.5 % phenylacetonitrile and 12.5 % β-methoxypropionitrile in acetone, followed by development in isooctane, and (3) partition chromatography using a precoat of 25 % β-methoxypropionitrile in acetone, followed by development in isooctane. In partition chromatograms some shifting of the moving spot to the right or left of the center frequently occurs, since perfectly uniform pre-coating is technically difficult. Fortunately, the width of the scanning band of light is usually sufficient to encompass such deviations from the true center.

The data are listed in Table II. Scanning used the same conditions as in the work with silica gel G. There is less variation among triplicates than in the tests with silica gel G, possibly because the layers were thinner. The partition chromatograms show less variation than the adsorption chromatograms. As with silica gel G there is relatively little change in base width with increasing load. The relative area per μg of cholesterol, however, shows a consistent tendency to decrease as load increases, *i.e.*, calibration curves would be less linear than those for silica gel G. Highest sensitivity at low loads is shown by the phenylacetonitrile–β-methoxypropionitrile chromatograms. The phenylacetonitrile is of low volatility and has high solvent power; during drying it would tend to rise to the surface of the thin layer, tending to retain small loads of the fluorescent compound in solution. With large loads, however, a smaller fraction of the load can thus be carried into the upper surface. Chromatograms coated with β-methoxypropionitrile, which is a moderately volatile liquid, seem to carry a more constant fraction to the surface during drying at all loads, giving relatively better linearity. Adsorption chromatograms using the volatile benzene–cyclohexane solvent show a less efficient and constant surface-transfer effect, with lower sensitivity and less linearity. Avicel–silica layers are less translucent than silica gel G layers of equal thickness, so that less efficient light penetration is to be expected; this may enhance the effect of variation in concentration into the surface layer during drying. Nevertheless, sensitivity is of the same order of magnitude for all types of chromatography; spots barely detectable by the human eye can be quantitatively scanned. Sensitivity is also of the same order of magnitude

attained by fluorimetry in 2 ml volumes of chloroform. The major limiting factor is the apparent background fluorescence; as pointed out by UDENFRIEND[5] this includes Raman scatter, and adsorption and quenching effects may also lower sensitivity.

Scanner performance with light-absorbing spots

Only Avicel–silica layers, about 350 μ thick, were used. The test substance was a crystalline sample of the *n*-octanoylamide of 1-phenylazo-2-aminonaphthalene (m.p. 91–92°), prepared by the author, as a 1 μg/μl solution in toluene. This yellow azo dye absorbs 360 mμ light strongly, and chromatograms were scanned with the long-wave U.V. source and primary filter used for the fluorescence work but without a secondary filter. A light-reducing slit 2 mm wide was used in the secondary filter compartment to lower the light intensity on the photomultiplier tube. The optical system consisted of the 0.1 × 3 mm slit and cylindrical lens.

Three 1-μl spots (total 3 μg) centered 3 mm apart were applied to the center region of a starting line, 10 mm from one end of the plate. These generate a band 6 to 12 mm wide when chromatographed. Loads were increased by overspotting the initial spots to give 6, 12 and 24 μg. If protected from light (which decomposes azo dyes) spots do not fade with time; in fact, there is an increase in intensity after 1 or 2 days.

The scanning data are presented in Table III. Relative areas (over the limited 8-fold range tested) are neither a linear nor a logarithmic function of the load; the relation seems to be hyperbolic, the factor K [(mean rel. area) × (24 + load in μg)/(load in μg)] being nearly constant. The reason seems to be that at loads of the order of 10 μg the TLC system becomes saturated so that peak density no longer increases much with load; differences in scans are then largely caused by increases in spot size. The width of the 12 and 24 μg spots extends beyond the linear range of the light band formed by the cylindrical lens. Table III also presents data from the re-scanning of the same plates after storage in the dark for 2 days. All peak heights are increased, suggesting continued migration of dye into the upper surface of the thin layer. Variability among replicates remains high. The approximately hyperbolic relation between relative area and load still holds.

Absorbance scanning therefore seems useful over a no more than 10-fold range in load, unlike fluorescence scanning which has a 100-fold range (and 100-fold greater sensitivity). The optimal conditions for absorbance measurement are probably spotting of an initial band at least 10 mm wide (*e.g.*, four 1-μl spots 3 mm apart) and scanning with the 4-mm wide light band formed by the circular lens. Although only a fraction of the absorbing band will be scanned, it will be a relatively constant fraction and relatively unaffected by off-center migration. This technique should be satisfactory for a 2 to 20 μg range (depending on the molecular extinction coefficient, this may be higher or lower). Quantities greater than 20 μg usually exceed the capacity of TLC, so that compactness of bands and resolving power are diminished.

One can conclude that the limiting factor in quantitative scanning is the reproducibility of replicate chromatograms. Presumably this can be improved by extreme care in controlling the nature of the thin layer and the chromatography and drying of the plate. Even so, quantitative data should be based on triplicate runs to allow estimation of the variance, and on calibration curves of standards run under identical conditions.

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SUMMARY

The construction and operation of a scanning accessory for 25 × 200 mm TLC plates adapted to the Aminco Fluoro-Microphotometer are described. Fluorescent compounds can be quantitatively scanned in the range from 0.005 to 5 μg, but calibration curves are not linear. Repeated scans of any one plate are identical, but the standard deviation of triplicates is high (10 to 20 %) because of variations in dispersion of the fluorescent compound in the thin layer during chromatography and drying. Light-absorbing compounds of high molecular extinction coefficient can be quantitatively scanned by reflectance in the range from 1 to 20 μg; calibration curves are not linear and triplicates also show high variance caused by chromatography and drying.

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