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QUANTITATIVE ANALYSIS ON THIN-LAYER CHROMATOGRAMS

THEORY OF ABSORPTION AND FLUORESCENT DENSITOMETRY

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SUMMARY

The precision of *in situ* optical quantitative thin-layer chromatographic analysis is dependent on the following: load application, reversible chromatography, optical-quality thin-layer chromatographic plates, quantitative relation between photometry and concentration, and accurate recognition of location and extent of the chromatographed zone.

The main contribution of this paper is a new theory for fluorescence densitometry in reflection and transmission modes and its qualitative interpretation. A method for programmed recognition of the boundaries of a chromatographed zone is also described.

SPOT APPLICATION

Application by capillary and rinsing with a polar solvent as described earlier[1] is the method preferred by the author for the absolute loading of thin-layer chromatographic (TLC) plates. The use of a syringe can give rise to problems of evaporation, solvent leakage and intersample contamination. The use of an internal standard in every analysis has the disadvantages of increased data processing as well as the impracticality of finding a suitable substance with an R_F value that does not interfere with the sample zones, and which itself does not give rise to any impurity zones.

CHROMATOGRAPHY

Reversible chromatography is essential because any irreversible adsorption to the chromatogram substrate causes a loss in the zone, and also interferes with the level of light absorption of the background. Quantitation of a zone which is in the "tail" of an irreversible compound will be influenced not only by the presence of an additional amount of light-absorbing material within the zone but also by the false value for the optical thickness of the chromatographic substrate around the zone, as obtained under the assumption of freedom from contamination.

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OPTICAL QUALITY OF TLC PLATES

There are many optically interfering hazards present in TLC plates, which, for ordinary chromatographic purposes, are not usually noticed. Under the exacting conditions of absolute *in situ* quantitation, such small defects as air bubbles, specks of dust, inhomogeneous crystallization of binder (gypsum), light-absorbing (or fluorescing) impurities in the substrate or chromatographic solvent and scratches, etc., in the glass support plate, must be obviated by improved techniques together with careful scrutiny and rejection when necessary. It is feasible to use a suitable program at the data processing stage to recognize and ignore blemishes, and this has, to a limited extent, been tried, but it was found to be simpler to avoid such sophistication whenever possible.

THEORY OF DENSITOMETRIC QUANTITATION

Absorption

The densitometric application of the absorptiometry theory of Kubelka and Munk, based on a one-dimensional approximation to transfer of radiation in scattering media, has been described earlier[1-3].

The basis for the theory is summarised in the equations:

$$-\frac{di}{dx} = -(S+K)i + Sj \qquad (1)$$

$$\frac{dj}{dx} = -(S+K)j + Si \qquad (2)$$

which can be appreciated physically by saying that the light that is travelling in the transmission direction (i) loses light by absorption (K) and scattering (S) and gains from the scattering that occurs to the light travelling in the opposite direction (j).

Exactly the same occurs to light that is travelling in the reflection direction (j), the only difference being the reversal of direction.

The solution of these equations for a plane layer of thickness X is:

$$T = \frac{b}{a \sinh bSX + b \cosh bSX} \qquad (3)$$

and

$$R = \frac{\sinh bSX}{a \sinh bSX + b \cosh bSX} \qquad (4)$$

in which $a = (SX + KX)/SX$ and $b = \sqrt{(a^2 - 1)}$.

In this form, such equations convey very little meaning, although it is possible to use them for the calculation of concentrations in TLC when the data are processed by computer. However, an appreciation of the range of applicability is perhaps easy to see from a graphical representation of absorbance *vs.* sample concentration, as in Figs. 1 and 2. Figs. 1 and 2 show the difference in absorbance between the

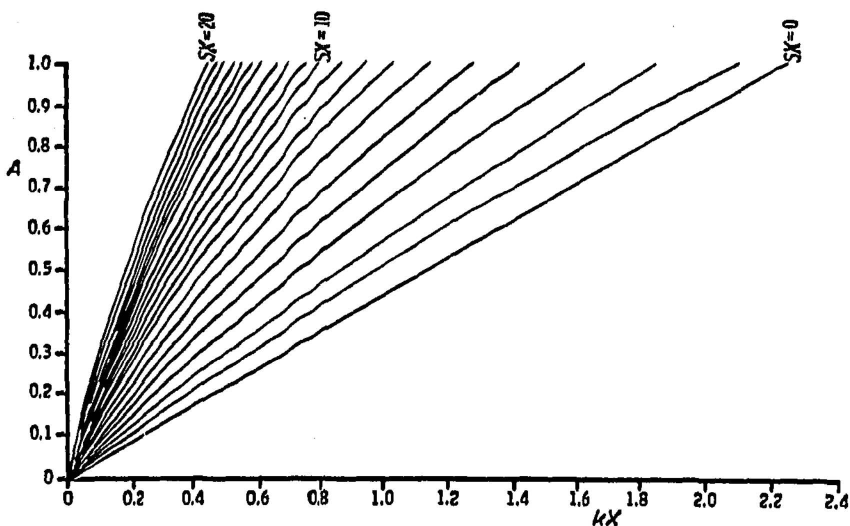

Fig. 1. Transmission. Relative absorbance A vs. KX = 0.434 × absorptivity × μg cm^{-2}, for the range of scattering powers $0 \leqq SX \leqq 20$.

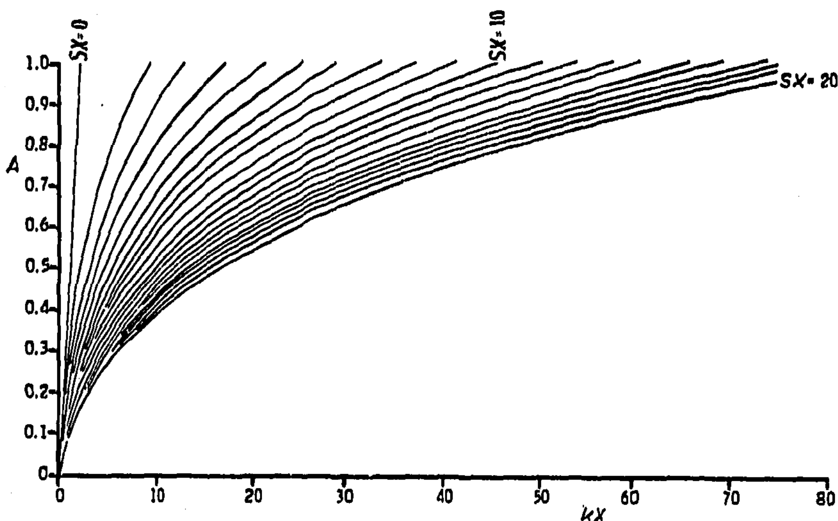

Fig. 2. Reflection. Relative absorbance A vs. KX = 0.434 × absorptivity × μg cm^{-2}, for the range of scattering powders $0 \leqq SX \leqq 20$.

non-absorbing substrate background and concentrations of the absorbing compound up to those necessary to produce a whole unit of absorbance, for layers of 0–20 units of scattering power.

The difference between Figs. 1 and 2 is that Fig. 1 is for transmitted light and Fig. 2 is for reflected light. It can clearly be seen that a much higher concentration is needed to give the same level of relative absorbance in reflection as in transmission. However, much more light is reflected than transmitted, so that the energy available for detection is greater. Hence the argument over the relative sensitivity

of transmission *vs.* reflection depends upon the technical application, namely, whether the limit to detection is caused by absolute or relative "noise" in the detection signal.

The response in transmission is curved, decreasing in slope from initially $((SX)^2 + 3SX + 3)/(2.303(3SX + 3))$ towards that of Beer's law, $1/2.303$, as the absorption increases. Consequently, when carrying out analyses in UV light on, for example, silica gel, the reduced precision in determining the scattering power of the thin layer, because of the difficulties in obtaining an accurate value for the absorption to scattering ratio, is compensated for by the decreased effect of the thickness of the adsorbent layer on response.

In reflection, the response decreases from $(2SX + 3)/(2.303(3SX + 3))$ at $KX = 0$ to zero as $KX \rightarrow \infty$. The fact that, for large $SX (> 10)$, the initial adsorbance *vs.* KX curve is independent of SX with gradient $2/(3 \times 2.303)$ cannot be used because of the short range over which it is applicable and the difficulty of measuring very small proportionate decreases in light level.

Fluorescence

For fluorescence densitometry, both illuminant and fluorescent light are present in the layer. By using a similar one-dimensional approximation as for absorption, two pairs of differential equations are obtained:

$$-\frac{di}{dx} = -(s + k)i + sj \tag{5}$$

$$\frac{dj}{dx} = -(s + k)j + si \tag{6}$$

and

$$-\frac{dI}{dx} = -SI + SJ + \tfrac{1}{2}(i + j)\alpha k \tag{7}$$

$$\frac{dJ}{dx} = -SJ + SI + \tfrac{1}{2}(i + j)\alpha k \tag{8}$$

Eqns. 5 and 6 correspond exactly to those for absorption, i and j being the illuminant light, s and k the scattering and absorption coefficients for this light. Eqns. 7 and 8 are for the fluorescent light, travelling in "transmission" (I) and "reflection" (J) directions. It is assumed that this light is not absorbed. The scattering coefficient for the fluoresced light is denoted S, and the proportion of absorbed light converted into fluorescence is α. The general solutions of eqns. 5 and 6 are:

$$i = A \sinh bsx + B \cosh bsx \tag{9}$$

$$j = (aA - bB) \sinh bsx + (aB - bA) \cosh bsx \tag{10}$$

where $a = (s + k)/s$ and $b = \sqrt{(a^2 - 1)}$.

Boundary conditions on eqns. 9 and 10 give:

$$i = i_0 \frac{(a \sinh bsx + b \cosh bsx)}{(a \sinh bsX + b \cosh bsX)} \tag{11}$$

$$j = \frac{i_0(\sinh bsx)}{(a \sinh bsX + b \cosh bsX)} \tag{12}$$

X is the thickness of the scattering media and i_0 is the initial illuminant intensity. (The boundary conditions are $i = i_0$ at $x = X$ and $j = 0$ at $x = 0$.) Substituting the illuminant solutions, eqns. 11 and 12, into the fluorescent equations, eqns. 7 and 8, and solving, provides the general solutions, whose arbitrary constants are eliminated through the boundary conditions $I = 0$ at $x = X$ and $J = 0$ at $x = 0$.

Finally, the required equations are for $I^+ = I$ at $x = 0$, and $J^+ = J$ at $x = X$, which are the fluorescences obtained in transmission and reflection, respectively:

$$I^+ = i_0\alpha \frac{[bs(a-1) + 2bS]\sinh bsX + [b^2s + 2S(a-1)]\cosh bsX - [(2SX+1)b^2s + 2S(a-1)]}{2bs(SX+1) \times (a\sinh bsX + b\cosh bsX)} \quad (13)$$

$$J^+ = i_0\alpha \frac{[(2SX+1)bs(a-1) - 2bS]\sinh bsX + [(2SX+1)b^2s - 2S(a-1)]\cosh bsX + [2S(a-1) - b^2s]}{2bs(SX+1) \times (a\sinh bsX + b\cosh bsX)} \quad (14)$$

The complexity of these expressions would not be a problem if a computer could be used to process the fluorimetric densitometry data. However, some experimental difficulty may be experienced in the determination of the parameters, i_0, α, sX and SX, which are needed so as to permit the computation of kX from the measured value of I^+ or J^+. Rough sketches of eqns. 13 and 14 are shown to three scale ranges in Figs. 3, 4 and 5. S and s are assumed to be equal in these graphs. Paper and thin layers of silica gel commonly have SX values in the range 5–20. Each graph shows both transmitted, I^+, and reflected, J^+, fluorescent light intensities.

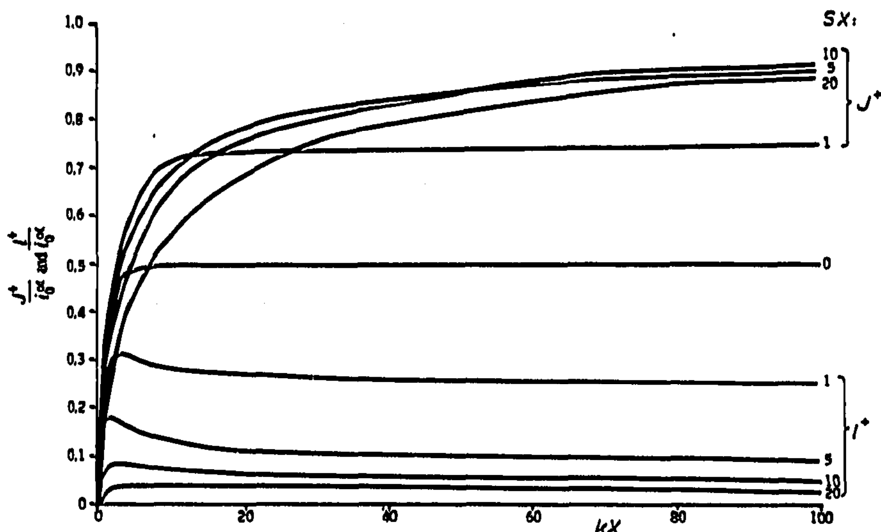

Fig. 3. Reflected, J^+, and transmitted, I^+, fluorescence for the range of absorptive powers of fluorescer, $0 < kX < 100$, at scattering powers $SX = 0, 1, 5, 10$ and 20.

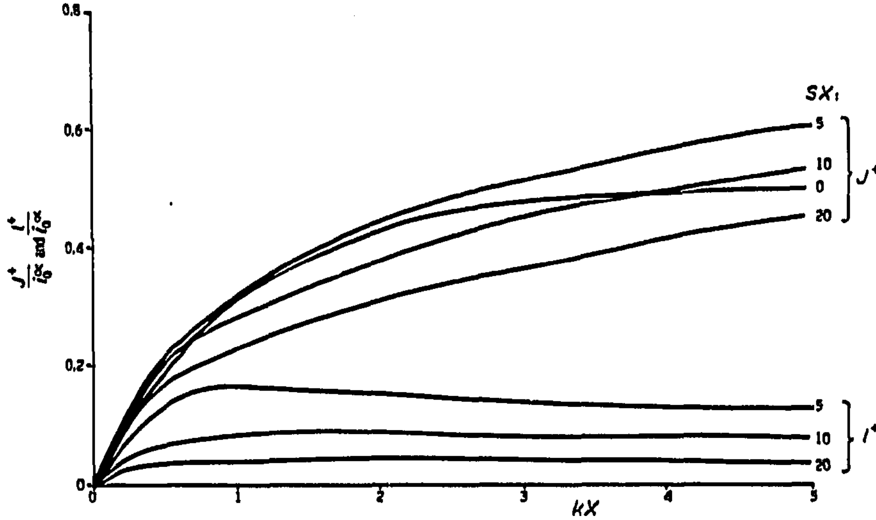

Fig. 4. Reflected, J^+, and transmitted, I^+, fluorescence for the range of absorptive powers of fluorescer, $0 < kX < 5$, at scattering powers $SX = 0, 5, 10$ and 20.

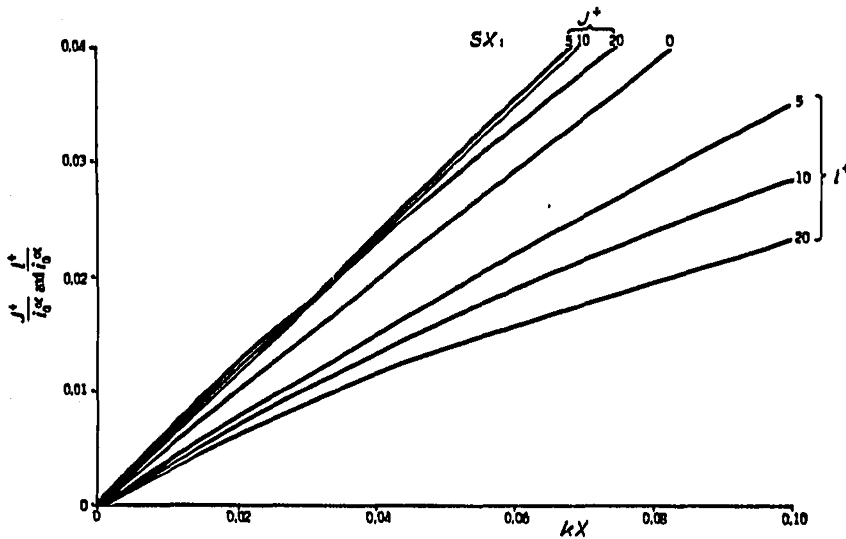

Fig. 5. Reflected, J^+, and transmitted, I^+, fluorescence for the range of absorptive powers of fluorescer, $0 < kX < 0.1$, at scattering powers $SX = 0, 5, 10$ and 20.

The curve at $SX = 0$ is the function $\frac{1}{2}(1 - e^{-kX})$ for non-scattering fluorescence, and is the same for both reflection and transmission.

The application of this theory to fluorescent emission densitometry will be considered first. A constant flux from the light source should maintain i_0 constant. α, the conversion factor from illuminant to fluorescent light, can also be assumed to be constant. Fig. 3 shows how the response becomes independent of the concen-

tration of fluorescer as the slopes of the curves in both reflection and transmission modes become zero for large kX. The asymptotic light levels are:

$$I^+ = i_0\alpha \frac{\frac{1}{2}}{SX + 1} \tag{15}$$

and

$$J^+ = i_0\alpha \frac{SX + \frac{1}{2}}{SX + 1} \tag{16}$$

for $k/s \to \infty$.

Fig. 4 shows the response over the intermediate range of fluorescence. The reflection curves are non-linear in kX and strongly dependent on SX. In transmission, the response has the interesting property of becoming negative for k/s greater than 0.2, approaching the limit in eqn. 15 slowly from above. In this region, the transmitted fluorescence is proportional to $1/(SX + 1)$, for k/s constant.

The curves in Fig. 5 show the relationships for very low concentrations of fluorescer. Reflection has some advantages over transmission in the range $5 < SX < 20$. The overall response is greater (*ca.* 2/3 kX compared with 1/3 kX) and the range of linearity is approximately twice that of transmission in terms of kX, which therefore corresponds to four times the range in fluorescence. Also, the effect of scattering power on the reflection curves is very much reduced because the opposing scattering effects of decreased sensitivity and increased collection cancel each other. The equations for small kX, up to second order (writing s and S for sX and SX) are:

$$\frac{I^+}{i_0\alpha} = \frac{kX(2Ss+3S+3s+3)}{6(S+1)(s+1)}\left[1 - kX\left(\frac{S(14s^3+69s^2+105s+60)+15(s+1)^2(s+3)}{30(s+1)(2Ss+3S+3s+3)}\right)\right] \tag{17}$$

$$\frac{J^+}{i_0\alpha} = \frac{kX(4Ss+3S+3s+3)}{6(S+1)(s+1)}\left[1 - kX\left(\frac{S(16s^3+81s^2+105s+30)+15(s+1)^2(s+3)}{30(s+1)(4Ss+3S+3s+3)}\right)\right] \tag{18}$$

which simplify for large SX and sX to:

$$\frac{I^+}{i_0\alpha} = \tfrac{1}{3}kX(1 - \tfrac{7}{30}sX \cdot kX) \tag{19}$$

and

$$\frac{J^+}{i_0\alpha} = \tfrac{2}{3}kX(1 - \tfrac{4}{30}sX \cdot kX) \tag{20}$$

to show more clearly the initial independence from sX, responses and ranges of linearity. Hence, for reflected fluorescence from non-absorbing substrates, containing kX not greater than 0.06, there is no need for either flying spot scanning or correction for substrate scattering power in the range 5–20. This ideally simple technique would involve only uniform illumination of the zone and a single estimation of the reflected fluorescent light intensity. Unfortunately, there are likely to be few

applications because of the difficulty of obtaining a chromatographic substrate with a sufficiently low absorption at the wavelength of excitation of the fluorescent zone. The necessary limit on the ratio of the substrate absorption coefficient, L, to scattering coefficient, s, for thin films with a scattering power of only 10 is 0.006.

In the presence of a moderate to large substrate absorption and with the concentration of fluorescent substance CX, the expressions for fluorescence, I^* and J^*, are obtained by the substitution of $\beta CX/(CX + LX)$ for α and $CX + LX$ for kX, where β is the efficiency for the fluorescer C.
Hence,

$$I^* = i_0\beta \frac{CX}{CX + LX}\left(\frac{I^+}{i_0\alpha}\right)_{(kX=CX+LX)} \tag{21}$$

and

$$J^* = i_0\beta \frac{CX}{CX + LX}\left(\frac{J^+}{i_0\alpha}\right)_{(kX=CX+LX)} \tag{22}$$

and when CX is small compared with LX, these equations become linear in CX:

$$I^* = CX \frac{i_0\beta}{LX}\left(\frac{I^+}{i_0\alpha}\right)_{(kX=LX)} \tag{23}$$

and

$$J^* = CX \frac{i_0\beta}{LX}\left(\frac{J^+}{i_0\alpha}\right)_{(kX=LX)} \tag{24}$$

Although reflection has the advantages of higher sensitivity and lower dependence on layer thickness (proportional to $1/X$ compared with $1/X^2$ in transmission), it would appear to be more feasible to use transmission measurement by the following technique. Add a small fixed concentration of some inert fluorescent substance to the substrate to give an additional absorption coefficient, D. Eqn. 23 then becomes:

$$I^* = i_0 \frac{\beta CX + \gamma DX}{LX + DX}\left(\frac{I^+}{i_0\alpha}\right)_{(kX=LX+DX)} \tag{25}$$

where γ is the fluorescence conversion coefficient for D.

For $(L + D)/s$ greater than 0.2, the background fluorescence I_0^*, for $CX = 0$, is proportional to $I/(SX + 1)$. The fluorescence due to the chromatographed zone is then: '

$$\begin{aligned} I^* - I_0^* &= CX \frac{i_0\beta}{LX + DX}\left(\frac{I^+}{i_0\alpha}\right)_{(kX=LX+DX)} \\ &\propto CX \frac{1}{X(SX+1)} \\ &\propto CX(I_0^*)^2 \qquad \text{(approx.)} \end{aligned}$$

$$CX = \text{const.}\left(\frac{I^* - I_0^*}{(I_0^*)^2}\right) \tag{26}$$

This expression is linear in I^* when CX is small compared with $LX + DX$, so one-dimensional scanning by an evenly illuminated slit is sufficient and the resultant peak area measurements can be corrected by dividing by the square of the background fluorescent level.

The fluorescence quenching technique involves the measurement of the reduction in fluorescence of a fixed concentration of a fluorescent additive in the presence of an absorbing non-fluorescing compound. If the absorptivity due to this compound is L and that due to the fluorescent additive is C, then kX in eqns. 13 and 14 becomes $CX + LX$, and if the efficiency of the fluorescer is β then $\alpha = \beta CX/(LX + CX)$ and so the equations are exactly the same as eqns. 21 and 22 for I^* and J^* except that now LX is the term that is proportional to the concentration of the zone and C can be considered to be essentially constant provided that sufficient precautions are taken. The measurement involved in this technique is of a reduction of light intensity from that of the background. Consequently, it is impractical to work in a limiting small linear range of LX, because small changes in light intensity would be subject to the relatively large errors that arise from source fluctuations and detector and amplifier response variations.

The simplest relationship between fluorescence and LX is obtained when the background fluorescence is approximately independent of fluorescer concentration. In reflection, this occurs when the ratio C/s is greater than 4, which is not only liable to cause interference with the chromatographic properties of the substrate, but also necessitates high concentrations in the chromatographed zones (approximately 0.5 mg/cm² for a 50% light intensity change for an absorptivity of 40 at $sX = 10$). In transmission, the fluorescence is relatively uniform for C/s greater than 0.1, thus enabling reasonable measurements to be made at zone concentrations of the order of 10 μg/cm². An additional advantage of transmission is that the background fluorescence is proportional to $1/(SX + 1)$ and so can be used to correct the data for its dependence on layer thickness.

The background fluorescence, T_0^+, from eqn. 21 is:

$$T_0^+ = i_0\beta\left(\frac{I^+}{i_0\alpha}\right)_{(kX=CX)}$$

$$\propto \frac{1}{SX+1}$$

and the relative transmitted fluorescence, T^+, in the presence of the sample zone is:

$$T^+ = \frac{CX}{CX + LX}$$

so that

$$LX \propto \left(\frac{1 - T^+}{T^+}\right) X$$

and finally, taking $(SX + 1) \sim SX$:

$$LX = \text{const.}\left(\frac{1 - T^+}{T^+}\right)\frac{1}{T_0^+} \tag{27}$$

Eqn. 27 is non-linear, so two-dimensional scanning is necessary. The measured fluorescence can be converted immediately into a signal that is proportional to sample concentration by using a linearizing amplifier, after setting the background signal level to give zero output, and the linearized data are integrated before finally correcting for the layer thickness.

If the substrate has an intrinsic absorptivity of D, say, at the exciting wavelength, then the only change corresponds to the decrease of β by the factor $C/(C + D)$ and the increase of C to $C + D$. This only decreases the overall intensities of fluoresced light, and has no effect on the calculation of LX by eqn. 27. In summary, then, the theoretically most advantageous modes of operation using the different optical techniques are as follows:

Absorption. Transmission using two-dimensional scanning and either the simplification of the Kubelka–Munk expression $e^{-2A_0}(A + 0.4A^2)$, as described earlier[1], if the substrate is non-absorbing, or the complete expression as described in a subsequent paper[3] if the chromatographic substrate does absorb.

Fluorescence. Reflection without scanning or correction for layer thickness at small levels of concentration if the substrate is non-absorbing. If the layer is not free from absorption, then reflection at low concentrations can be carried out without scanning because of the linearity, but layer-thickness controls must be applied as the fluorescence reflection is inversely proportional to the thickness of the substrate.

Fluorescence transmission on substrates with intrinsic absorption of the illuminant light together with a small substrate fluorescence has the advantage of simplicity. Two-dimensional scanning is unnecessary for small zone concentrations, and the background fluorescence level can be readily used to correct the sample readings for layer thickness variation according to the expression $(I^* - I_0^*)/(I_0^*)^2$.

Fluorescence quenching. Transmission on a layer containing a suitable concentration of fluorescent agent with corresponding zone concentrations requires two-dimensional scanning, but can be linearized and integrated before correction for layer thickness, which is inversely proportional to the background transmitted fluorescence. The technique is independent of substrate absorptivity. Sample concentration is proportional to $(1 - T^+)/T^+ \cdot T_0^+$.

TABLE I

SUMMARY OF METHODS FOR THIN-LAYER DENSITOMETRY

Technique	*Mode*	*Expression*	*Dependence on SX*	*Linearity*	*Approx. KX range*	*Substrate absorption*
Absorption	Transmission	$e^{-2A_0}(A+0.4A^2)$	Separable	Non-linear	0.5	Non-absorbing
	Transmission	$K - M$	Non-separable	Non-linear	1.0	Absorbing
Fluorescence	Reflection	J^+	Independent	Linear	0.06	Non-absorbing
	Reflection	$(J^+)sx$	Controlled	Linear	0.03	Absorbing
	Transmission	$\frac{I^* - I_0^*}{(I_0^*)^2}$	Separable	Linear	0.2	Absorbing and fluorescing
Fluorescence quenching	Transmission	$\frac{(1 - T^+)}{(T^+)}\frac{1}{T_0^+}$	Separable	Non-linear	2.0	Independent

```
THIS IS ZONE NUMBER 3

MICROGRAMS X ABSORPTIVITY = 27.643
MEAN SCATTERING POWER = 11.692 WITH MAX OF 12.38 AND MIN OF 11.13
LENGTH OF SCAN USED = 7.5 MM WITH 18.0 MM SPARE AT START AND 35.5 MM SPARE AT END
WIDTH OF SCAN = 17.0 MM
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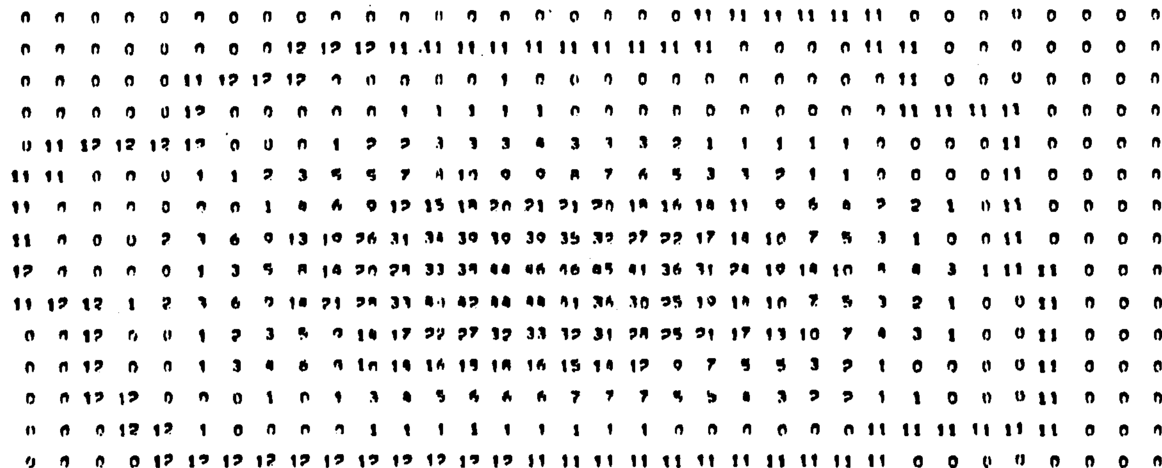

0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	11	11	11	11	11	11	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	12	12	12	11	11	11	11	11	11	11	11	11	11	0	0	0	0	11	11	0	0	0	0	0	0	0
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12	0	0	0	0	1	3	5	8	14	20	28	33	39	44	46	46	45	41	36	31	24	19	14	10	8	4	3	1	11	11	0	0	0
11	12	12	1	2	3	6	9	14	21	28	33	40	42	44	44	41	36	30	25	19	14	10	7	5	3	2	1	0	0	11	0	0	0
0	0	12	0	0	1	2	3	5	9	14	17	22	27	32	33	32	31	28	25	21	17	13	10	7	4	3	1	0	0	11	0	0	0
0	0	12	0	0	1	3	4	6	9	10	14	16	19	18	16	15	14	12	9	7	5	5	3	2	1	0	0	0	0	11	0	0	0
0	0	12	12	0	0	0	1	0	1	3	4	5	6	6	6	7	7	7	5	5	4	3	2	2	1	1	0	0	0	11	0	0	0
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0	0	0	0	12	12	12	12	12	12	12	12	12	12	12	11	11	11	11	11	11	11	11	11	11	11	0	0	0	0	0	0	0	0

Fig. 6. Two-dimensional scanning at 340 nm by transmission through silica gel ($L/S = 0.013$). The computer printout shows the value of $KX \times 100$ at 0.5-mm intervals enclosed by the zone boundary. The zone boundary is found as in the text and displayed by printing in the values of SX(11–12).

For the applications of these theories, one must remain aware of the simple first-order nature of the concept of scattering used in their derivation. The angular distribution of the light is neglected and the scattering medium is assumed to be homogeneous. However, these effects are of relative insignificance when compared with the difference between scattering and not scattering.

More important is the necessity for the illuminant light to be relatively monochromatic. This is probably unnecessary for the fluoresced light.

Zone recognition

Densitometry of one-dimensional chromatograms by techniques that do not require scanning for the purpose of data processing can be carried out by slit scanning, and the zones can be located visually before integration.

Flying spot scanning is necessary to enable the measured light flux, I, to be converted to concentration, $C(I)$, before summation when $C(I)$ is not a linear function. The reason for this is that, in general, $\Sigma C(I) \neq C(\Sigma I)$ if $C(I)$ is not a linear function (by definition of linear function).

When two-dimensional scanning is necessary, but linearization can be carried out before determination of substrate thickness, the data can be linearized and integrated transversely, and then displayed in the direction of chromatography, so that zones can be easily located before completing the integration and making final corrections for layer thickness.

The third and most general case, when the linearizing function is dependent on substrate thickness, requires concentration to be determined at each point over the zone in two dimensions before integration. This technique is most readily carried out by using a computer. The location of the zone in the two-dimensional array of data can then be found and its extent determined by an automatic procedure written into the program. A program written for densitometry by transmitted absorption in TLC has been used which locates each zone in order of decreasing maximum absorption. The extent of the zone is found by creating an array containing 2's wherever the relative transmission is less than 0.9 or the transmittance gradient is above 0.05, and 1's elsewhere. This picture is created throughout a sufficiently large region to contain the zone. Starting from near the middle of the zone, a search is conducted in a straight line until an array value of 1 is met. The value 3 is then inserted at this boundary point. The search for the next boundary point is made by testing the four perpendicular surrounding points in a clockwise direction starting from the point to the left of the direction of arrival until a number 1 is met. Thus 3's are inserted in a continuous boundary until the starting point is reached again. This is repeated for one more cycle round the first boundary to obtain a reasonable distance between the zone and its boundary curve. This procedure ensures that only one zone is enclosed and any neighbouring specks of dust or other interfering blemishes do not also receive a boundary, although it may have a sufficient contrast and gradient to be included in the creation of the initial contrast array.

The layer thickness X is then calculated at every point (x, y) of the boundary and interpolated to each interior point (a, b) by the equation:

$$X(a,b) = \sum_{x,y} \frac{X(x,y)\,[(b-y)\Delta x + (a-x)\Delta y]}{[(a-x)^2 + (b-y)^2]^2} \Bigg/ \sum_{x,y} \frac{(b-y)\Delta x + (a-x)\Delta y}{[(a-x)^2 + (b-y)^2]^2}$$

Δx and Δy are the distances between neighbouring boundary points in the two coordinate directions, and take the values -1, 0 or 1.

The determination of which points are interior to the boundary is made by testing whether

$$\sum_{x,y} \frac{(b-y)\Delta x + (a-x)\Delta y}{(a-x)^2 + (b-y)^2}$$

is greater or less than 4. For interior points, the expression is approximately 2π, and exterior points approximately zero.

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REFERENCES

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DISCUSSION

DEYL: Have you any experimental data which would support or illustrate your theories?

GOLDMAN: The new methods for TLC quantitation by fluorescence are derived mathematically and have not been tested in practice, but it is my opinion that the hypotheses implicit in the differential equations are reasonable.

GOODALL: Mr. GOLDMAN has already established his theory for light absorption methods with verification in the visible and now in the UV (*cf.* GOODALL, this issue, p. 153). The mathematical treatment of fluorescence and fluorescence quenching in flat-bed chromatograms in the present paper is an extension of the above theory to the more complicated cases arising from fluorescence and fluorescence quenching. Thus, there is reason to believe that the extension is likely to apply. Experimental tests of the derived equations will probably not be attempted, however, by Mr. GOLDMAN or myself.