

QUANTITATIVE ANALYSIS ON THIN LAYER CHROMATOGRAMS: A THEORY FOR LIGHT ABSORPTION METHODS WITH AN EXPERIMENTAL VERIFICATION

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INTRODUCTION

The facility of thin layer chromatography for effecting rapid separations of both organic and inorganic mixtures has not yet been followed by an equally convenient way of assaying the separated components. Various methods, which fall into two classes, elution before measurement and measurement *in situ*, have been published¹. The latter method has generally been accepted as the simpler, but has suffered from having no theoretical basis. The methods that have been employed are usually based upon empirical calibration of data obtained by a method that happened to be convenient for the type of instrument used.

We have studied the possibilities of a theoretical basis for making measurements by optical transmission and reflection in materials such as silica gel thin layers.

THEORY

The transmission and reflection of light in highly scattering media has been discussed by CHANDRASEKHAR² in his book on radiative transfer. There he gives the basic integro-differential transport equation. As this equation has no analytical solution, all useful equations have been developed as simplifications of the actual case. There are several of these equations. One of these, used extensively up to now in the paper and paints industries is that proposed by KUBELKA AND MUNK³. This theory has been the basis of the work done by us.

Summary of the Kubelka-Munk theory

Their differential equations were of the form:

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

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

where:

- i = intensity of light travelling inside the specimen towards its unilluminated surface
 j = intensity of light travelling inside the specimen towards its illuminated surface
 S = coefficient of scatter per unit depth or thickness
 K = coefficient of absorption per unit depth or thickness
 x = distance from the unilluminated surface of the specimen.

The following conditions are implicit in these simple equations:

- (1) The light beam is parallel and normal to the specimen plane.
- (2) The reflections and absorptions occur at infinitesimal distances, and are constant over: (i) the frequency range of the light, (ii) the area illuminated by the light beam, (iii) the depth of the specimen.
- (3) The light, when reflected, is turned through 180° , *i.e.* the only direction of travel of the light is perpendicular to the specimen plane.

The general solutions in i and j are:

$$i = A \sinh b' Sx + B \cosh b Sx$$

$$j = (aA - bB) \sinh b Sx + (aB - bA) \cosh b Sx$$

where:

$$a = \frac{S + K}{S}$$

$$b = \sqrt{a^2 - 1}$$

A and B are arbitrary constants

Using the following boundary conditions:

when $x = 0$; $i = I_0 T$ and $j = 0$

and when $x = X$; $i = I_0$ and $j = I_0 R$

it is found that:

$$\text{Transmittance, } T = \frac{b}{a \sinh b SX + b \cosh b SX} \quad (1)$$

and

$$\text{Reflectance, } R = \frac{\sinh b SX}{a \sinh b SX + b \cosh b SX} \quad (2)$$

X is the thickness of the specimen measured from the unilluminated side, and I_0 is the intensity of the incident light.

When the light scattering layer does not absorb light, *i.e.* when $K = 0$, then

$$T = T_0 = \frac{1}{SX + 1} \quad (3)$$

and

$$R = R_0 = \frac{SX}{SX + 1} \quad (4)$$

These equations provide a simple means of determining SX , the scattering power. It is also found that in eqns. (1) and (2) S , K and X need not be determined separately, but only SX and KX have to be determined, since

$$a = \frac{S + K}{S} = \frac{SX + KX}{SX}$$

and

$$b = \sqrt{a^2 - 1} = \frac{\sqrt{KX(2SX + KX)}}{SX}$$

This is convenient as the term KX is proportional to the weight of light absorbing compound per unit area.

Concepts from the theory

Before determining the utility of eqns. (1)–(4), for quantitative evaluation of light absorbing compounds adsorbed on thin layer (or paper) chromatograms, the conditions under which they may be valid must be considered.

Condition (1) need not be adhered to explicitly as when considered in conjunction with condition (3) it is seen that the third condition is very much an oversimplification. Thus the equations would be no less applicable if the light entering were not parallel or not perpendicular to the sample. However it would seem provident to keep the angular distribution of the incident light constant, if not parallel and normal to the sample.

Conditions under (2) are very important. The following constraints are apparent:

(i) Unless the light absorbing compound is perfectly grey, the illumination must be sufficiently monochromatic to be uniformly absorbed by the compound over its spectral band width. This may be checked by testing the absorption of the compound in solution to see if it sufficiently obeys Beer's law with the filter or monochromator used. (Beer's law is a limiting case of the Kubelka–Munk theory, obtained when $S \rightarrow 0$). Even with a perfectly grey compound it is likely that S is a function of spectral frequency, so that this in itself would necessitate monochromatic light.

(ii) A familiar concentration profile is that of a spot on a thin layer chromatographic surface, which displays concentration gradients in the two surface dimensions. In order to comply with requirement 2(ii) above, the illuminating aperture must be small, so as to cover on the steepest gradients present a limiting maximum range of absorptions. This maximum allowable range depends on the precision required, and the average absorption of the measured area. As each measurement will represent an area much smaller than the chromatographed zone, many such measurements at sufficiently small intervals in the two dimensions would be required in order to assess the zone in total. This could be accomplished readily by a flying spot scanning device, in the form of a small spot of light which scans the zone in successive segments, each the width of the light spot and slightly longer than the maximum width of the sample zone.

If the sample zone is a band with no concentration gradient in the transverse direction it need only be scanned in the other direction. However, this was not found

to be generally suitable for chromatographic analyses, as variations occurred in the transverse dimension after chromatographic development for more than a short distance.

As we had no flying-spot scanner we had to use a one dimensional scanning procedure with a slit, applied to bands which had travelled for a distance of only 0.6 cm. This was adequate for an experimental verification of the Kubelka-Munk theory.

(iii) Non uniformity of the sample spot can occur through the depth, if the adsorbent layer is thick enough for secondary chromatography to occur through this depth during the evaporation-drying stage. This effect will be greatest with compounds having high R_F values in the solvent being evaporated. This type of non-uniformity will also be likely to have a greater effect on reflection measurements than on transmission. With respect to condition (2) very little mention has been made of the scattering coefficient. This is because any variation of it over the area of illumination, through the depth of the thin layer, or over the frequency range, is likely to be much smaller than the corresponding variations in the light absorbing compound.

So far, no account has been taken of possible light absorption by the thin layer of adsorbent itself. In the visible it is reasonable to assume that most of the purified adsorbents which appear to be colourless do not in fact absorb above 400 $m\mu$. In the case of the adsorbent used by us (Merck's Kieselgel G), reflection measurement against a magnesium oxide standard confirmed that no absorption occurred. The use of the theory in the case where the adsorbent also absorbs light introduces a third independent variable (K of substrate). However this would not make the theory less valid.

APPLICATIONS

Slit and spectral band widths

To keep within the constraints required by the theory, it is important that the

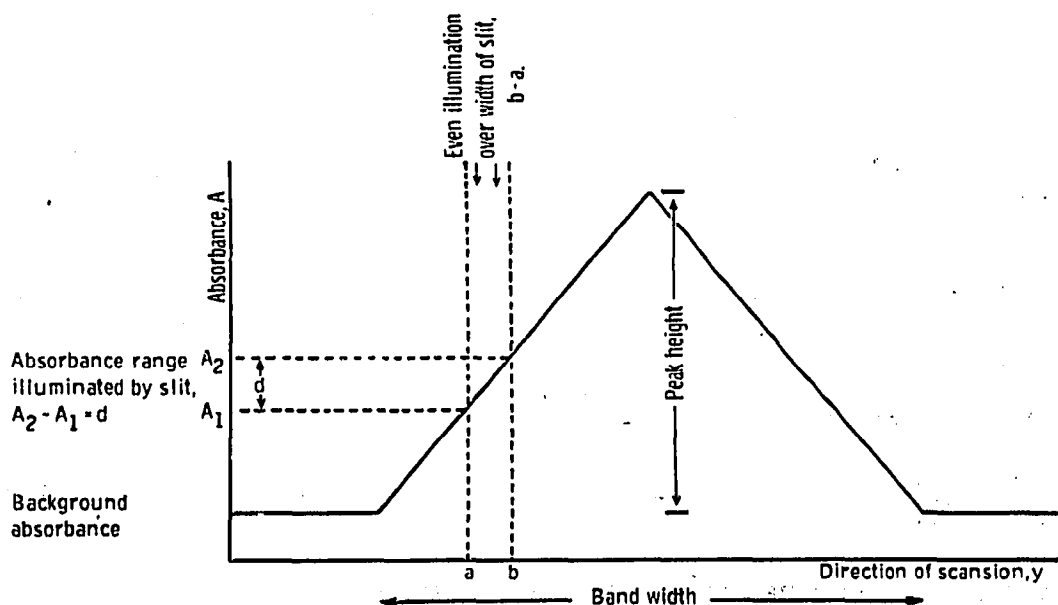


Fig. 1. Diagram of a simplified absorbance profile of a chromatographed band.

aperture should be sufficiently small and the light sufficiently monochromatic. The absorption range over which the light should be of the required monochromaticity can be shown readily by experiment, *viz.*, by checking whether Beer's law holds for solutions of the compound in question, using the same filter or monochromator as for the thin layer measurements. A good agreement for solutions in methanol covering the absorbance range used in thin layer measurements was established for all compounds used by us.

The limiting aperture size could not be demonstrated readily by experimental means. However a simple calculation is easily made to give an approximate measure of the effect of increasing aperture size.

For this purpose it is sufficient to approximate the absorbance profile of a chromatographed band in the direction of development (and scansion) to that of an isosceles triangle (see Fig. 1). Neglecting those parts of the scansion of the band during which the slit includes the start, apex, or end (these parts will be small if the slit width is much smaller than the band width) the slit will be illuminating equally the range of absorbance, d , from A_1 to A_2 given by

$$d = \frac{\text{peak height}}{\frac{1}{2} \times \text{band width}} \times \text{slit width.}$$

The actual absorbance at the mean position of the slit is

$$\frac{A_1 + A_2}{2}.$$

The measured absorbance of the illumination is:

$$-\log_{10} \left[\frac{1}{b-a} \int_a^b T \, dy \right]$$

where:

$$T = 10^{-A}$$

$$dy = \frac{\frac{1}{2} \times \text{band width}}{\text{peak height}} \cdot dA$$

and

$$\frac{1}{(b-a)} = \frac{\text{peak height}}{\frac{1}{2} \times \text{band width}} \cdot \frac{1}{A_2 - A_1}.$$

Thus, substituting the corresponding limits A_1 to A_2 for a and b , respectively,

$$-\log_{10} \left[\frac{1}{b-a} \int_a^b T \, dy \right] = -\log_{10} \left[\frac{1}{A_2 - A_1} \int_{A_1}^{A_2} 10^{-A} \, dA \right] = \log_{10} \left[\frac{2.303 (A_2 - A_1)}{10^{-A_1} - 10^{-A_2}} \right].$$

The discrepancy, θ , between the actual and measured absorbances due to the use of a slit of finite width is therefore given by

$$\theta = \frac{A_1 + A_2}{2} - \log_{10} \left[\frac{2.303 (A_2 - A_1)}{10^{-A_1} - 10^{-A_2}} \right]$$

Substituting d for $A_2 - A_1$ leads to

$$\theta = \log_{10} \left[\frac{\sinh (1.151 d)}{1.151 d} \right]$$

From this equation it is seen that the difference between the actual and observed absorbance, θ , is only dependent upon the range of absorbance, d , illuminated by the slit. Thus in the situation exemplified in the above diagram (Fig. 1) and within the simplifications stated, the measured absorbance profile would show a constant reduction in absorbance equal to θ . The consequent decrease in peak area is therefore

$$\theta \times \text{band width}$$

which as a fraction of the peak area, $1/2 \times \text{band width} \times \text{peak height}$, is

$$\frac{2 \times \theta}{\text{peak height}}$$

For a reduction in peak area of less than 1%, θ must be less than 0.5% of the peak height. The corresponding maximum value of d may be found from the graph of θ vs. d in Fig. 2. The slit width is related to d by

$$\text{slit width} = \frac{d \times \text{band width}}{2 \times \text{peak height}}$$

For example, for bands with maximum absorbance of 1.0, relative to the background, θ for 1% precision on peak area is 0.005.

From Fig. 2, this corresponds to $d = 0.23$.

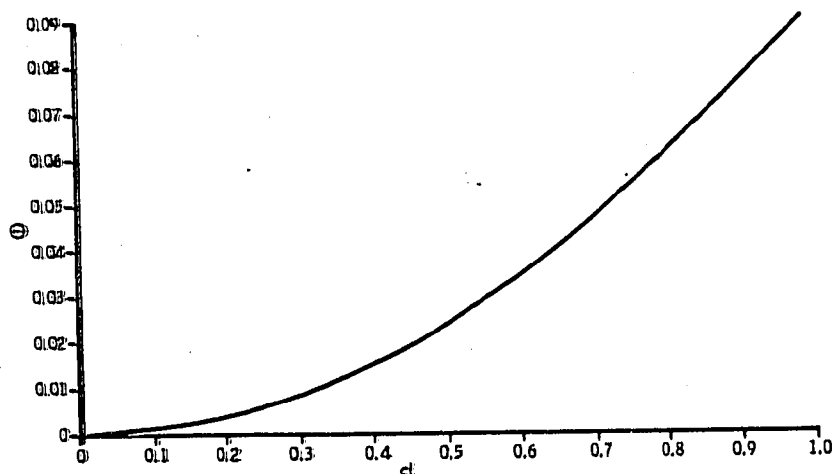


Fig. 2. Relation between linear absorbance range, d , covered by slit, and the resulting bias, θ , between the actual and measured absorbances.

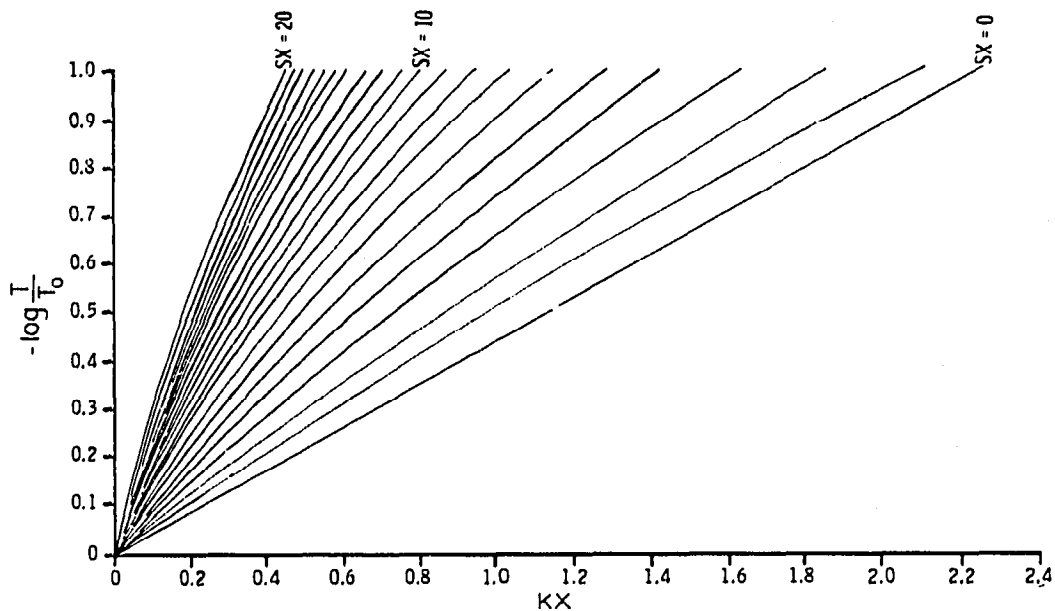


Fig. 3. Absorbance by transmission ($-\log T/T_0$) for a range of KX (\propto substance concentration) on a non-absorbing, light scattering substrate according to the Kubelka-Munk theory. Each curve corresponds to a different scattering power, SX (\propto substrate thickness). The curve when $SX = 0$ defines Beer's law.

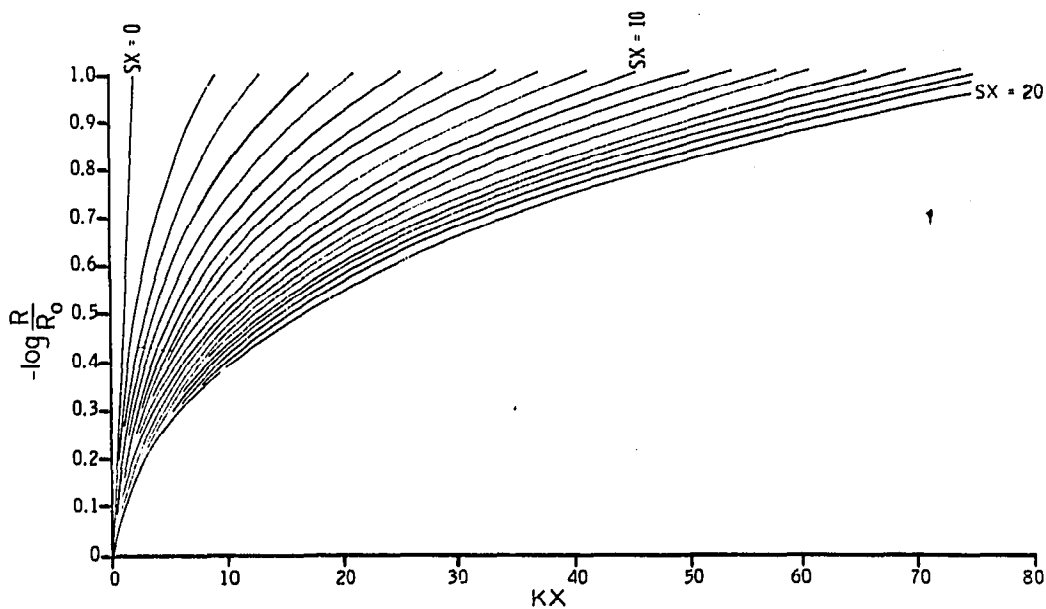


Fig. 4. Absorbance by reflection ($-\log R/R_0$) for a range of KX (\propto substance concentration) on a non-absorbing, light scattering substrate according to the Kubelka-Munk theory. Each curve corresponds to a different scattering power, SX (\propto substrate thickness). The curve when $SX = 0$ defines Beer's law.

Thus

$$\text{slit width (max.)} = \frac{0.23 \times \text{band width}}{2 \times 1.0} = 0.12 \times \text{band width.}$$

With a knowledge of the chromatographic band width and the expected maximum absorbance, the maximum slit width appropriate for any desired degree of precision can be calculated, provided that the absorbance is approximately linearly related to concentration.

Examination of the Kubelka-Munk theory

Eqns. (1)–(4) were computed to give a wide range of relationships between useful experimental variables. These were in our case: $-\log (T/T_0)$ and $-\log (R/R_0)$ vs. KX for values of SX from 1 to 20. Figs. 3 and 4 show $-\log (T/T_0)$ and $-\log (R/R_0)$ respectively over the range 0 to 1.0 for integer values of SX from 0 to 20, vs. KX . From these the following observations are apparent:

(i) The response in transmission, $-\log (T/T_0)/KX$, is much more constant as compared with that in reflection, *i.e.* $-\log (R/R_0)/KX$, which curves rapidly away from the slope, 0.4343 at $SX = 0$ (Beer's law).

(ii) The reflection response is always much less than 0.4343, whereas in transmission the response is always greater than 0.4343.

(iii) For any fixed value of KX the sample absorbance increases with SX in transmission, and decreases with SX in reflection. Thus the SX value of the thin layer must be measured to the same degree of accuracy as the sample absorption, to obtain a quantitative evaluation of KX .

We decided that the only reliable method of determining SX for thin layers would be optical, as it is an optical constant. Any mechanical method (*e.g.* measurement of layer thickness, or weight per unit area etc.) would not take into account variables such as packing density, particle size or variation of scattering coefficient with light frequency. When the thin layer does not absorb light SX may be evaluated via eqns. (3) and (4) to give:

$$SX = \frac{1 - T_0}{T_0} = \frac{R_0}{1 - R_0}$$

It is seen that R_0 is the least sensitive to changes in SX for values of SX above 1. That is, as SX becomes large $R_0 \rightarrow 1$ and $T_0 \rightarrow 1/SX$. Therefore transmission was used to determine the SX value.

For the several reasons discussed above, transmission rather than reflection measurements are more suitable for experimental work.

Calculation of semi-empirical transmission equation

For the case of transmission, the Kubelka-Munk theory gives eqn. (1), which expressed T in terms of SX and KX . In order to have more practical value, it is desirable to express KX in terms of T and SX since the latter two are related to measured variables. However, theoretically this is quite complicated and the following is an empirical attempt to overcome this difficulty and to derive a simple equation.

The experimental variables are $A_0 = \log 1/T_0$ and $A = \log 1/T - \log 1/T_0$.

Eqn. (3) gives the relationship between SX and T_0 . The suggested relationship between KX , A and A_0 is assumed to be of the form:

$$KX = f(A_0) g(A) \quad (5)$$

since it seems reasonable to suppose that, to a first approximation, A and A_0 affect KX independently. It is further assumed that $g(A)$ can be represented by a polynomial in A . Fig. 3 shows that KX and A are zero together for all A_0 and so

$$g(A) = A + \alpha A^2 + \beta A^3 + \dots \quad (6)$$

where α, β, \dots are constants to be determined. (The constant multiplying A may be taken as unity without loss of generality).

Hence:

$$\left[\frac{d(KX)}{dA} \right]_{A=0} = f(A_0).$$

Now

$$\frac{d(KX)}{dA} = \left[\frac{dA}{d(KX)} \right]^{-1} \text{ and } A = 0 \equiv KX = 0$$

and so

$$\left[\frac{dA}{d(KX)} \right]_{KX=0} = \frac{1}{f(A_0)}.$$

Now

$$A = \log \frac{1}{T} - \log \frac{1}{T_0}$$

thus

$$\frac{dA}{d(KX)} = \frac{T}{2.303} \frac{d}{d(KX)} \left[\frac{1}{T} \right].$$

Since

$$\frac{1}{T} = \frac{a}{b} \sinh (bSX) + \cosh (bSX)$$

then

$$\frac{dA}{d(KX)} = \frac{1}{2.303} \frac{(ab^2SX - 1) \sinh (bSX) + a^2bSX \cosh (bSX)}{b^2SX (a \sinh (bSX) + b \cosh (bSX))}.$$

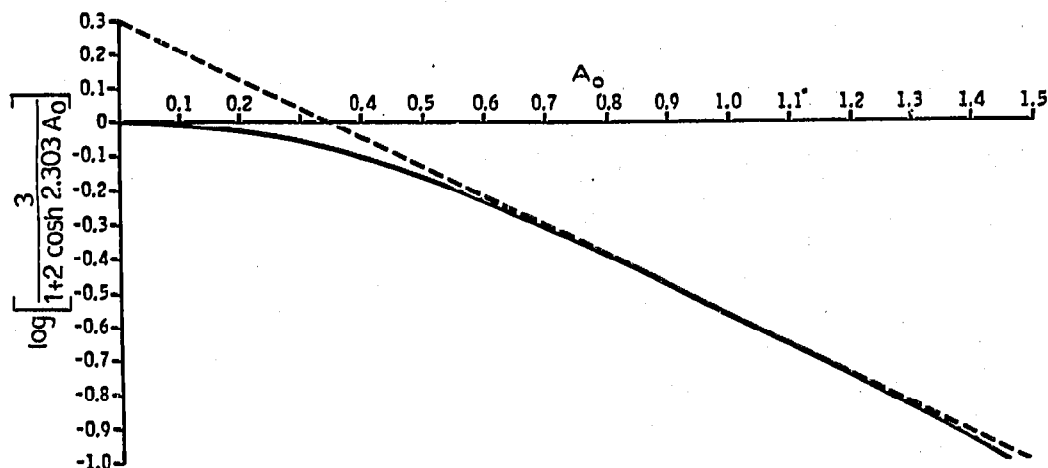


Fig. 5. Graph of $\log \left[\frac{3}{1 + 2 \cosh 2.303 A_0} \right]$ vs. A_0 (—), showing close approximation to the linear function, $-0.868 A_0 + 0.301$ (---), over the range $0.7 < A_0 < 1.3$.

As $K \rightarrow 0$, $a \rightarrow 1$ and $b \rightarrow 0$, hence

$$\left[\frac{dA}{d(KX)} \right]_{KX=0} = \frac{1}{2.303} \frac{(SX)^2 + 3SX + 3}{3SX + 3}$$

Finally

$$f(A_0) = 2.303 \frac{3}{1 + 2 \cosh (2.303 A_0)}$$

on using eqn. (3) and the definition of A_0 .

It now remains to investigate the range of values of A and A_0 for which eqns. (5) and (6) are a reasonable approximation and to determine appropriate values for the constants in eqn. (6). The relevant range of values for A is $0 < A < 1$ and, to a first approximation, $g(A)$ is assumed to be quadratic since if A is in the above range the higher-order terms in $g(A)$ will tend to become negligible compared to those retained.

Hence, the approximation takes the form

$$KX = f(A_0) (A + \alpha A^2)$$

This equation was fitted for different values of SX using the data in Fig. 3. It was found that for $4 \leq SX \leq 20$, i.e. $0.7 < A_0 < 1.3$, α varied from 0.33 ($SX = 4$) to 0.46 ($SX = 20$). In order to minimize the error, the value of α was taken to be 0.40.

A graph of A_0 vs. $\log \left[\frac{3}{1 + 2 \cosh (2.303 A_0)} \right]$ was drawn (Fig. 5) and shows that, over the range $0.7 < A_0 < 1.3$,

$$\log \frac{3}{1 + 2 \cosh (2.303 A_0)} \approx -0.868 A_0 + 0.301$$

i.e.

$$\frac{3}{1 + 2 \cosh (2.303 A_0)} \approx -2.00 \exp [-2.00 A_0].$$

Finally

$$0.434 KX \approx 2 \exp [-2 A_0] (A + 0.4 A^2) \quad (7)$$

where the decimal zeros have been omitted. The term $2 \exp [-2 A_0]$ allows for the hyperchromic effect over the range $0.7 < A_0 < 1.3$ and the term $(A + 0.4 A^2)$ corrects for the curvature of KX vs. A over the ranges $0 < A < 1.0$ and $0.7 < A_0 < 1.3$.

EXPERIMENTAL

Eqn. (7) shows two effects caused by light transmission measurement in light scattering media:

(i) The hyperchromic effect, which changes the overall response by the factor $\exp [2 A_0]/2$, for $0.7 < A_0 < 1.3$.

(ii) The curvature (from Beer's law as linear) which is accounted for by $(A + 0.4 A^2)$ over the range, $0 < A < 1.0$, and within the range for (i) to apply.

Of the two factors, (i) and (ii) above, (i) will have the more dominant effect on deviations from the simple Beer's law; $A = 0.434 KX$.

The experimental work is taken in two parts:

(i) A comparison of the theoretical "hyperchromaticity" with that found experimentally for a wide range of background absorbances.

(ii) A comparison of the theoretical "curvature of response" with that found experimentally.

In each part both effects were taken into consideration. However the experiments were designed so that the effect not being tested was of minor significance in the results.

(i) Hyperchromic effect

Procedure

Preparation of thin layers on slides. The potential simplicity and speed of operation with thin layers on microscope slides is very attractive and so we used them. Also the good optical qualities of microscope slides and the more reasonable expense if extension of quantitative analysis into the U.V. is contemplated, makes them preferable to conventional sizes.

A simple brass slurry box was made to run over microscope slides placed transversely on a brass bedplate. The depth of layer is the clearance between the lower face of the slurry box and the top of the slide. This clearance varies with the thickness of the glass slide. Usually it is about 0.25 mm. In the following experiment the slides were of various thicknesses, so that a range of adsorbent scattering powers (SX) could be tested. Silica Gel G (Merck) has been used throughout this work.

Application of zones. The photograph Fig. 6 shows a simple device which will apply a band of solution of maximum length 5.4 cm at an accurate rate of 1.85 $\mu\text{l}/\text{cm}$. It is built around a Hamilton 10 μl syringe and holder, the barrel of which runs on

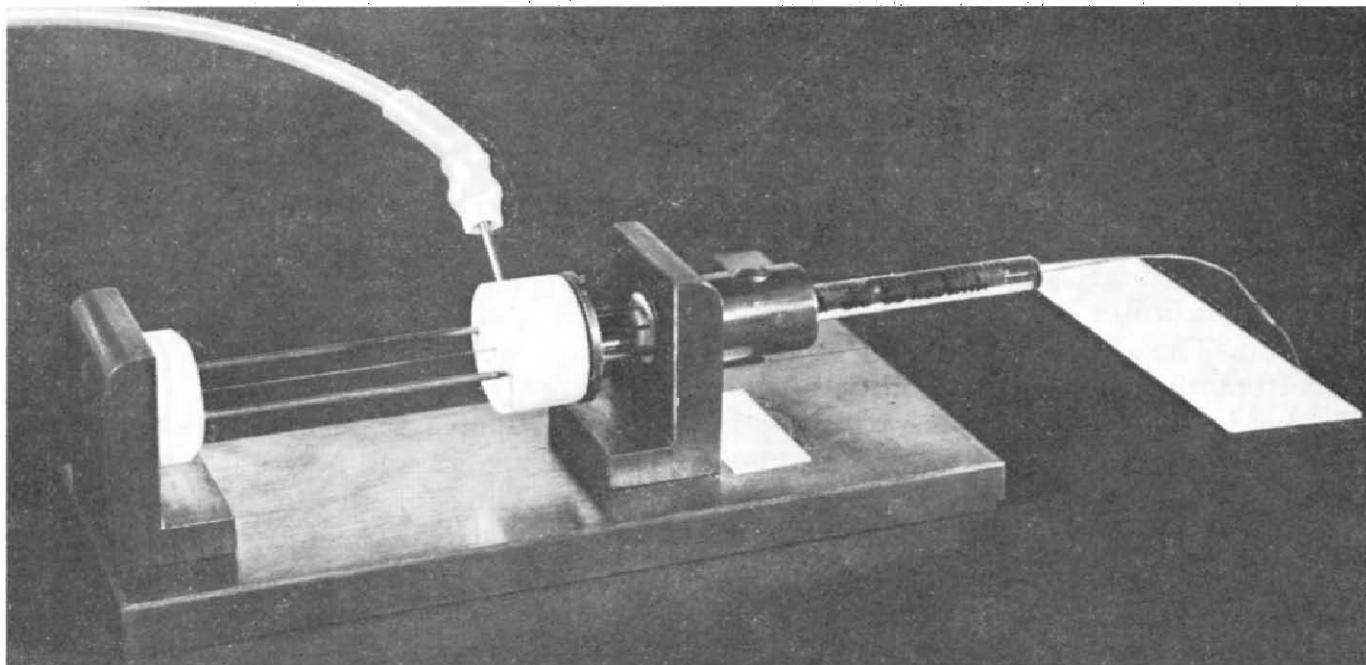


Fig. 6. Apparatus devised from a 10 μ l Hamilton syringe to apply solutions to thin layer plates as bands of constant concentration.

the guide rods supplied with the holder, and in a P.T.F.E.-bushed brass cylinder. The plunger is fixed, so that the quantity of liquid ejected depends upon the position of the barrel. The needle is bent and a tapered fine piece of polyethylene tubing is attached to the end, so that it runs on the surface of the adsorbent without scoring it.

The vacuum filling device is made from a block of P.T.F.E. which is sealed to the flange by a rubber sealing ring and a brass face-plate. The leak past the tip of the plunger is produced by erosion from dipping into 2 *N* HCl for a distance of 10–15 mm for 5–10 min. The plunger enters into the vacuum chamber via a hole of close fit, thus forming a seal. Two holes 0.8 mm in diameter are drilled at 45° to the vertical into the vacuum chamber, one of which is widened for about 6 mm to take a catheter which is connected to the vacuum line. The other hole is used as a quick seal and release by placing a finger over it.

To fill the syringe, the barrel is moved to withdraw the plunger as far as possible, the vacuum turned on, a finger placed over the air leak hole and the tip dipped into the required solution. When the liquid reaches the end of the plunger, as observed through the sight hole, the finger is taken off the air-leak. After use, the remaining small volume of liquid can similarly be removed by fully extending the barrel and resealing the air leak.

The major advantage of this device is that there is no air space in the syringe barrel. This prevents the occurrence of variable output caused by compression and decompression of such an air bubble.

Development. Sudan III (0.243 μ g/ μ l) in chloroform was applied at 1.85 μ l/cm to form a band across the full width of each micro-plate used, at a distance of about 1 cm from the bottom. Each micro-plate was then placed into a 3 oz (approx. 80 ml) amber glass bottle with a wide neck (overall dimensions 5 cm \times 9 cm) which comprises the developing chamber. A pool of toluene of depth about 5 mm had been

previously placed in each bottle. Fifteen such experiments were set up. The plates were allowed to develop with toluene till the solvent front had risen 2 cm (60 sec time). The R_F of Sudan III is 0.33 in this system. The developed plates were allowed to dry slowly at room temperature during 30 min under draught-proof conditions and well separated from each other.

Scanning details. The plates were scanned using a slit (rotatable for alignment with the bands) of 0.25×5 mm and a 5300 Å interference filter. The band widths were approximately 6 mm and the maximum peak height was approximately 0.8 so that a maximum slit width for 1% precision on the peak areas would be $0.12 \times 6 = 0.7$ mm. The slit width of 0.25 mm was therefore sufficiently small. The recording densitometer used was a modified "Chromoscan" made by Joyce Loebel, Gateshead, England, operated at gear ratio 14.88:1 and absorbance scale of 0.0596 units/cm. The "sample" photomultiplier cathode is 0.8 cm \times 2.3 cm and is situated 7 cm from, and normal to, the aperture image on the sample. Thus for all absorption measurements of thin layers, the angular distribution of the transmitted light has to be assumed constant*.

The areas under the recorded peaks, P , were determined by triangulation (*i.e.* width at half peak height \times peak height) and written in absorbance \times cm units. The background absorbances, A_0 , could only be determined relatively within the fifteen results and so is expressed as $\Delta A_0 (= A_0 - \bar{A}_0)$.

Results

The experimental data obtained were:

Peak heights, p , in absorbance units

Peak areas, P , in absorbance \times centimeter units

Relative background, ΔA_0 , in absorbance units

Weight of Sudan III per centimeter of band ($1.85 \times 0.243 = 0.450 \mu\text{g cm}^{-1}$)

Absorptivity of Sudan III in methanol solution using the 5300 Å interference filter (82.5)

To allow substitution of these terms into eqn. (7), the following operations were carried out. Integrating with respect to the direction of scanning, y , gives

$$\int_{y_1}^{y_2} 0.434 KX \cdot dy = \int_{y_1}^{y_2} z \cdot \exp[-z A_0] (A + 0.4 A^2) dy.$$

* Probably the most serious change in angular distribution would be that caused by the transmission of parallel light, *i.e.* light not scattered by the thin layer. Other causes could be the change in angular distribution of scattered light with increasing layer thickness and with increasing concentration of light absorbing compound.

It is indicated by the absence of unaccountable anomalies in the results that these effects have not been serious over the range of layer thicknesses and compound concentrations used by us. The integrating sphere and opal glass techniques have been used⁴ to compensate for such effects, should they occur. Unfortunately such methods, for averaging the contribution of each part of the hemisphere of transmitted light, seem to be technically difficult to put into operation in this case without introducing the consideration of transmission of light through "two homogeneous scattering sheets"⁵.

The limits y_1 to y_2 are from one side of the band to the other, *i.e.* $y_2 - y_1 =$ band width. Assuming the background absorption to be constant over this range, then:

$$\int_{y_1}^{y_2} 0.434 KX \cdot dy = 2 \exp [-2 A_0] \int_{y_1}^{y_2} (A + 0.4 A^2) dy.$$

Taking the absorbance profile to be triangular, as a simple approximation, with peak height p , and base width $y_2 - y_1$, then

$$\int_{y_1}^{y_2} (A + 0.4 A^2) dy = \frac{(y_2 - y_1)}{p} \int_0^p (A + 0.4 A^2) dA = \frac{p(y_2 - y_1)}{2} (1 + 0.27 p)$$

where

$$\frac{p(y_2 - y_1)}{2} = P, \text{ the peak area,}$$

thus

$$\int_{y_1}^{y_2} 0.434 \cdot KX \cdot dy = 2 \exp [-2 A_0] P(1 + 0.27 p).$$

The term

$$\int_{y_1}^{y_2} 0.434 KX \cdot dy$$

is proportional to the weight of Sudan III per centimeter, transversely, and is thus a constant, C .

Substituting P^1 for $P(1 + 0.27 p)$

$$C = 2 \exp [-2 A_0] P^1.$$

Taking \log_{10} , and substituting $(\Delta A_0 + \bar{A}_0)$ for A_0 ,

$$\log P^1 = 0.868 \Delta A_0 + \log \frac{C}{2} + 0.868 \bar{A}_0.$$

As \bar{A}_0 and C are constants, a graph of $\log P^1$ vs. ΔA_0 should be a straight line with slope 0.868, and crossing the $\log P^1$ axis ($\Delta A_0 = 0$) at,

$$(\log P^1)_0 = \log \frac{C}{2} + 0.868 \bar{A}_0. \quad (8)$$

Fifteen sets of experimental values of P , p , $\log P^1$ and ΔA_0 are given in Table I. The graph of $\log P^1$ vs. ΔA_0 is shown in Fig. 7, where the experimental points are compared to the straight line of slope 0.868 predicted by theory. Clearly there is a close correlation between the experimental points and the theoretical straight line,

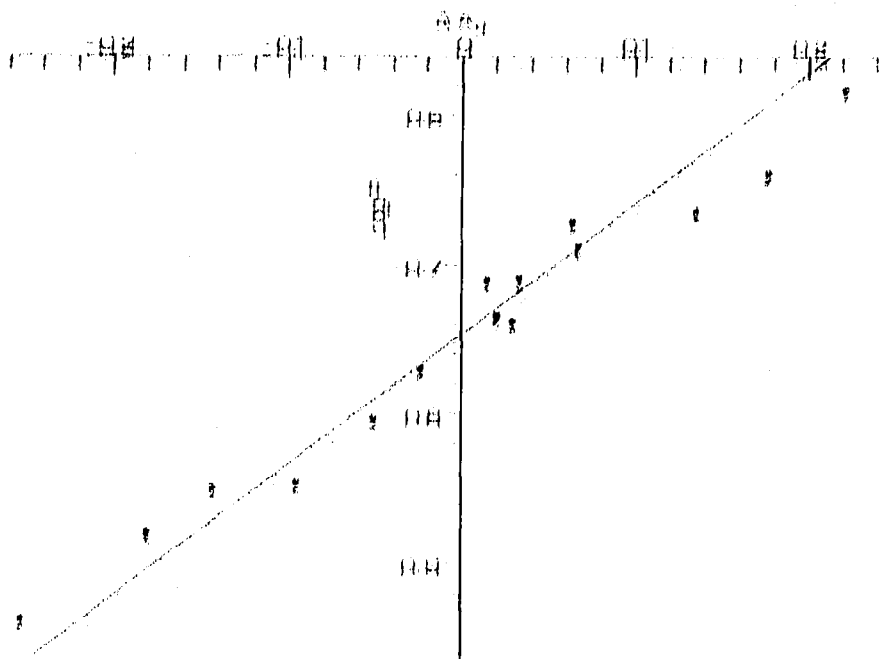


Fig. 2. Comparison of the theoretical relation between $\log I'$ and $-\log I_0$ with that found experimentally. Experimental points are shown as ●.

which confirms that a significant hyperchromic effect occurs and also that the Kautsky-Munk theory gives a close approximation to the actual hyperchromic effect.

I_0 can be determined by substitution into (8) for $(\log I')_0$ ($\epsilon = 0.250$) and $C' = (0.45 \times 10^{-4} \text{ mole/l.})$ to give:

$$I_0 = 1.13$$

This value is approximate, as it includes the assumption that the absorptivity of

TABLE I

COMPARISON OF RELATIVE HYPERCHROMIC ABUNDANCE, I_0 , WITH CONDITIONS OF CONSTANT DEBYE HÜCKEL f^{\pm} , $f^{\pm} = 0.47 f_0$

f^{\pm}	f	$\log I'$	$-\log I_0$
0.047	0.135	0.047	0.000
0.050	0.130	0.050	0.051
0.055	0.124	0.054	0.014
0.058	0.118	0.057	0.030
0.060	0.115	0.059	0.040
0.065	0.107	0.063	0.013
0.077	0.097	0.070	0.000
0.084	0.090	0.075	0.134
0.091	0.083	0.079	0.179
0.114	0.070	0.090	0.431
0.102	0.080	0.085	0.354
0.131	0.060	0.100	0.000
0.150	0.050	0.110	0.119
0.155	0.049	0.112	0.035
0.160	0.048	0.113	0.003

Sudan III on silica gel is identical to that in methanol solution. Thus the range of A_0 using this value, of from 0.88 to 1.35, is sufficiently close to the required range of 0.7 to 1.3 to justify the inclusion of the two uppermost points in Fig. 7.

(ii) *Curvature of response*

Procedure

Using our densitometer with a 420 m μ interference filter, agreement with Beer's law was shown for acetone dinitrophenylhydrazone in methanol solution over the range $0 < A < 0.9$. The absorptivity was found to be 25.9.

Solutions of the compound were made in methanol at 0, 0.27, 0.54, 0.81 and 1.08 $\mu\text{g}/\mu\text{l}$. 2 μl spots of each solution were dispensed in triplicate side by side via a capillary pipette on to a silica Gel G coated microscope slide. The slide was specially chosen to have a uniform thin layer. The purpose of this procedure was to obtain uniformly adsorbed discs of the compound, all of the same diameter. The acetone dinitrophenylhydrazone was completely unadsorbed onto silica gel from methanol and so could be expected to produce such a disc. The solution was applied as rapidly as possible, and in one operation, so as to minimise the effects of evaporation. After drying off the methanol, each of the three concentration sets was scanned in our densitometer through their diameters, using a circular aperture of 0.5 mm diameter and a gear ratio of 4.96:1 and an absorbance scale of 0.0655 units/cm.

Results

From the densitometer recordings, the uniformity of the discs of acetone dinitrophenylhydrazone on the slide was confirmed. The absorbances and diameters were

TABLE II

CURVATURE OF RESPONSE OF CORRECTED ABSORBANCE (b), WITH INCREASING KX , AND OBSERVED LINEARITY WHEN USING THE THEORETICAL CURVATURE EXPRESSION (a)

(a) $(A + 0.4 A^2) \exp [-2 \Delta A_0]$.

(b) $A \exp [-2 \Delta A_0]$.

Weight of acetone dinitrophenylhydrazone	KX	ΔA_0	A	(a)	(b)
0	0	—	0	0	0
0.54	0.194	—0.006	0.262	0.293	0.265
0.54	0.194	+0.021	0.288	0.308	0.276
0.54	0.194	+0.008	0.262	0.286	0.259
1.08	0.388	—0.006	0.459	0.550	0.465
1.08	0.388	+0.014	0.485	0.562	0.471
1.08	0.388	—0.006	0.472	0.568	0.478
1.62	0.582	—0.012	0.622	0.795	0.637
1.62	0.582	+0.008	0.675	0.843	0.664
1.62	0.582	—0.012	0.622	0.795	0.637
2.16	0.776	—0.006	0.806	1.079	0.816
2.16	0.776	+0.008	0.878	1.167	0.864
2.16	0.776	—0.012	0.812	1.101	0.831

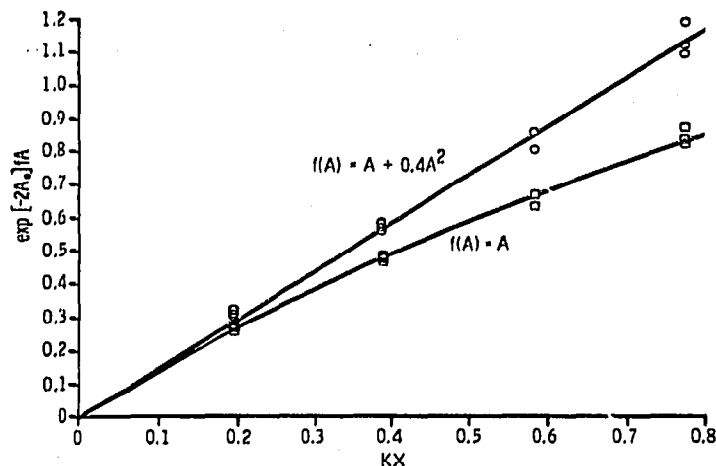


Fig. 8. Graph showing experimental curvature of response as absorbance (\square), and linearity when plotted in the theoretical expression (\circ).

measured, as well as the relative background absorbances, ΔA_0 . The average spot diameter was found to be 0.46 cm, corresponding to an area of 0.166 cm². Assuming the absorptivity of acetone dinitrophenylhydrazone in methanol solution to be the same as when adsorbed onto silica gel,

$$KX = \frac{2.303 \times 25.9 \times \mu\text{g of acetone DNPH}}{0.166 \times 1000}$$

Substituting $(\Delta A_0 + \bar{A}_0)$ for A_0 in eqn. (7) gives,

$$0.434 KX = 2 \exp[-2\bar{A}_0] \exp[-2\Delta A_0] (A + 0.4A^2)$$

As \bar{A}_0 is constant, a graph of $\exp[-2\Delta A_0] (A + 0.4A^2)$ vs. KX should yield a straight line. The slope of this straight line may be equated to $(0.434/2) \exp[2\bar{A}_0]$ to obtain an approximate value for \bar{A}_0 .

Table II shows the weight of acetone dinitrophenylhydrazone, KX , ΔA_0 , $\exp[-2\Delta A_0] (A + 0.4A^2)$ and also $\exp[-2\Delta A_0] A$. This last expression should give a curvature, when plotted against KX , similar to that for the curves in Fig. 3. This is shown to be so by the linearity of the same points when the curvature expression is used instead of A , as shown in Fig. 8.

Equating the slope of this straight line to $(0.434/2) \exp[2\bar{A}_0]$ gives:

$$\bar{A}_0 = 0.948$$

which is within the limiting A_0 range of $0.7 < A_0 < 1.3$. The small variations in A_0 correspond to an overall 8% variation in thickness of the layer. Thus the concentration of methanol which saturated the thin layer to give the spots will also have varied to a similar extent causing some of the scatter in the points in Fig. 8.

CONCLUSION

The Kubelka-Munk theory of radiative transfer has been summarised. The basic concepts have been discussed in relation to *in situ* quantitative analysis of thin layer chromatograms by light absorption methods. It is shown, on theoretical grounds, that measurement by transmission is more advantageous than by reflection. For the region of interest the Kubelka-Munk equation for transmission has been simplified to eqn. (7), expressing the absorption coefficient, $0.434 KX$, in terms of the two separated and useful variables A_0 and A :

$$0.434 KX = 2 \exp [-2 A_0] (A + 0.4 A^2) \quad (7)$$

$0.434 KX$ is proportional to the weight of light absorbing substance per unit area and equals the absorptivity \times mg \times cm⁻² of the substance.

A_0 is the background absorbance due only to scattering and not to absorption.

A is the absorbance in the presence of the light absorbing substance relative to the background.

Eqn. (7) is an approximation to the Kubelka-Munk theory over the ranges $0 < A < 1.0$ and $0.7 < A_0 < 1.3$. This spread of A_0 embraces a five-fold range of layer thickness, which includes the thicknesses of silica gel commonly used.

The two effects shown in eqn. (7), *i.e.* that of background absorbance (exp[-2 A_0]) and the curvature of response ($A + 0.4 A^2$) have been investigated experimentally. The first by scanning of bands of Sudan III on a wide range of layer thicknesses, and the second by measurement of a range of acetone dinitrophenylhydrazone concentrations on a layer of constant thickness. Both experiments show close agreement with theory. The overall expression is therefore shown to be valid.

It is hoped that the comparatively simple nature of our derived expression (7) will reduce the need for empiricism in quantitative analysis by thin layer chromatography.

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SUMMARY

A simple theoretical relationship applicable to *in situ* quantitative analysis by light absorption is proposed. The equation is based upon the Kubelka-Munk theory of radiative transfer. The conditions necessary for the application of the theory to quantitative analysis have been discussed. The simple theory is composed of two separable effects, "hyperchromaticity" and "curvature of response". These two effects have been evaluated experimentally on silica gel layers and correspond closely to that predicted. The validity of the theory is thus confirmed.

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