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QUANTITATIVE ANALYSIS ON THIN LAYER CHROMATOGRAMS: APPLICATION OF AN OPTICAL THEORY TO *IN SITU* ANALYSIS

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SUMMARY

A method for the *in situ* quantitative analysis of thin layer chromatographic zones on microscope slides is described. It comprises the quantitative application of 1 μl of solution to the plate, two-dimensional scanning of the zone with a point of monochromatic illumination, then off-line data processing by computer so that a simplified form of the general KUBELKA–MUNK equation for transmission can be applied. The standard deviation observed on independently analysed 0.510 μg loads of acetone dinitrophenylhydrazone on silica gel thin layers using the experimental prototype apparatus is 2.7%.

INTRODUCTION

The purpose of this paper is to demonstrate the feasibility of quantitative analysis *in situ* on thin layer chromatograms by an absorptiometric method based upon the optical properties of light-scattering thin layers, and to indicate the order of precision attainable.

Quantitative analysis on paper and thin layer chromatograms by light absorption has been attempted by various workers[1–17], and several instruments are at present marketed for this purpose. The bulk of the methods published are empirical in nature.

The exact relation between incident and emergent light energy in highly scattering media is complex, so in an earlier paper[18] we have developed an approximate equation relating all the important variables that remain after applying appropriate constraints. The equation is based on a simplified theory of radiative transfer. It is hoped that the advantages of having a theoretical basis, rather than resorting to complete empiricism, will outweigh the disadvantage of increased complexity of the analytical method. The advantages expected are a wide generality of application, and an accuracy which is only dependent on the control or estimation of known variables. The disadvantage of complexity is quite serious, but the increasing use of high-speed computers for data processing has considerably reduced this problem.

THEORY

Summary

In ref. 18 the advantages of transmission rather than reflection for *in situ* quantitative analysis are defined. What is believed to be a sufficiently accurate, but more easily used, approximation to the KUBELKA–MUNK transmission equation[19,20] is derived:

$$0.434\,KX = 2 \exp\,[-2A_0]\,(A + 0.4\,A^2) \qquad (1)$$

where:

$0.434\,KX$ is proportional to the weight of light-absorbing substance per unit area and equals absorptivity × mg × cm^{-2}.

A_0 is the background absorbance due only to scattering (no absorbing substance present). A_0 is related to the scattering power (SX) of the thin layer by:

$$A_0 = \log\,[1 + SX]$$

since

$$T_0 = \frac{1}{1 + SX}$$

and

$$A_0 = \log \left[\frac{1}{T_0}\right].$$

A is the increase over the background absorbance due to the presence of a light-absorbing substance.

The equation is applicable over the range:

$$0.7 < A_0 < 1.3$$
$$0 < A < 1.0$$

Furthermore, the KUBELKA–MUNK equation, and consequently eqn. (1), can only be applied when the measurements of the absorbances are carried out within certain constraints. The more important experimental implications of these are:

(i) The light used must be sufficiently monochromatic.
(ii) The absorption must be adequately uniform over the area illuminated.
(iii) The angular distribution of incident light must be constant.
(iv) The angular orientation of the light-collecting or measuring device must not vary.

Application

From eqn. (1) it can be seen that the concentration (C) of a compound in mg × cm^{-2} at any point on a thin layer chromatogram is given by:

$$C = \frac{2 \exp\,[-2A_0]}{a}\,(A + 0.4\,A^2)$$

where a is the absorptivity of the adsorbed substance in a chromatographic zone.

If $(\Delta A_0 + A_0')$ is substituted for A_0, where A_0' is a fixed standard absorbance level, so that $\Delta A_0 = A_0 - A_0'$, then

$$C = \frac{2 \exp [-2A_0']}{a} \cdot \exp [-2\Delta A_0] (A + 0.4 A^2) \tag{2}$$

The quantity, M, in μg of a compound in a chromatographed zone is given by:

$$M = 1000 \int_{x_1}^{x_2} \int_{y_1}^{y_2} C \, \mathrm{d}y \, \mathrm{d}x$$

$$\simeq 1000 \sum_{x_1}^{x_\Delta} \sum_{y_1}^{y_\Delta} C \, \Delta y \, \Delta x \tag{3}$$

The limits y_1 to y_2 and x_1 to x_2 are taken as distances along perpendicular axes in the plane of the thin layer, sufficient to include all the zone. The variations of C over the distances Δx and Δy have to be small for the summation in eqn. (3) to approach the true value of M. Substituting for C from eqn. (2), into eqn. (3):

$$M = 1000 \sum_{x_1}^{x_\Delta} \sum_{y_1}^{y_2} \frac{2 \exp [-2A_0']}{a} \cdot \exp [-2\Delta A_0] (A + 0.4 A^2) \, \Delta y \, \Delta x$$

$$= \frac{\exp [-2A_0']}{a} \cdot 2000 \sum_{x_1}^{x_2} \sum_{y_1}^{y_\Delta} \exp [-2\Delta A_0] (A + 0.4 A^2) \, \Delta y \, \Delta x$$

The factor

$$\frac{\exp [-2A_0']}{a}$$

is unknown, but it is constant and can be determined in practice by analysis of a known weight of material. In the remaining factor

$$2000 \sum_{x_1}^{x_2} \sum_{y_1}^{y_2} \exp [-2\Delta A_0] (A + 0.4 A^2) \, \Delta y \, \Delta x \tag{4}$$

the values of ΔA_0 and A are to be determined at intervals of Δx by Δy over the whole area from y_1 to y_2 and from x_1 to x_2. Before this is carried out, the following points require consideration:

(1) The light used must be sufficiently monochromatic. This would follow by inference from a demonstration that with the selected source and interference filter, solutions of the test compound obey Beer's law over the absorbance range in question.

(2) The measurement of absorbance is usually made over a small element of area which has, to a first approximation, a uniform gradient of absorbance. Under these conditions the experimental result (—log of the mean transmittance) is less than the actual value.

It has been shown[18] that when using uniform illumination, this bias is dependent only upon the range of absorbance covered by the illuminating aperture. It will be assumed, as a simplification, that the absorbance gradient is constant over the region of the spot, *i.e.* a conical absorbance profile, and negligible elsewhere. Then the approximate percentage reduction, R, as a result of a bias, θ, on a measurement of the "volume" of the absorbance profile of a spot of peak absorbance, P and diameter, D, is given by:

$$R = 100 \cdot \frac{\theta \frac{\pi D^2}{4}}{\frac{1}{3} P \frac{\pi D^2}{4}} = \frac{3\,\theta}{P} \cdot 100\%.$$

Thus 1% reduction in spot "volume" is brought about by a bias equal to $\frac{1}{3}$% of the peak height, *i.e.* for a peak height of 0.5, this is 0.0017 absorbance.

The relation between bias, θ, and absorbance range uniformly illuminated, d

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

is derived in ref. 18, where the graph is also given. The exact derivation of bias for a circular aperture and conical zone absorbance profile is complicated, and is not warranted in this context. So, using the graph of the above relation between θ and d, when $\theta = 0.0017$, $d \doteq 0.1$. With a spot of 0.5 absorbance peak height, the ratio of the diameters of the illuminating aperture and spot must be less than 1:10 to produce an illuminated absorbance range of less than 0.1, and thus less than 1% bias on the final summated spot profile.

In the experiment below, the maximum peak absorbance was no greater than 0.5. As the spot diameters were in the range 5–8 mm the aperture of 0.5 mm, together with some lateral diffusion of light in the thin layer, should only produce approximately 1% bias in the calculated results.

(3) The absorbance values, when estimated, are the combination of background and sample absorbance, $\Delta A_0 + A$. The procedure used to separate A and ΔA_0 was to interpolate the values of ΔA_0 within the zone from measurements around the periphery of the scanned region, where the value of A is known to be zero. Subtracting the interpolated ΔA_0 values from the measurements within the zone then gives A at each point.

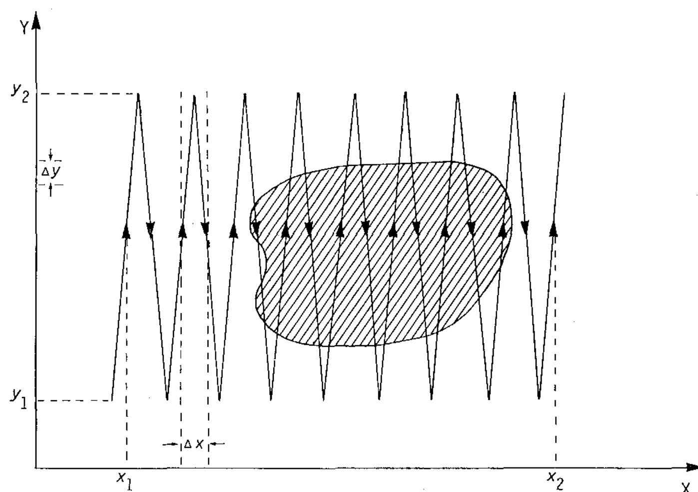

Fig. 1. Diagrammatic representation of the path of the light spot (→—) over a chromatographic zone (hatched area).

(4) Finally some means of obtaining the many absorption estimates at known spacings, Δx and Δy, in the two dimensions, x and y, is required. This can be accomplished by the use of some type of scanning motion with successively displaced scans. Two alternatives are possible. To scan the thin layer by a moving light spot, or to move the thin layer and keep the light beam stationary. We devised a mechanism to move the chromatographic slide rather than the light spot. In this way we were able to keep both the angular distribution of incident light, and the collection angle of the photomultiplier constant, as required by conditions (iii) and (iv) above.

The mechanism used for scanning (see later) gave a saw-toothed motion to the plate (Fig. 1). Δx was taken as the average spacing between scans. The actual spacing with this saw-tooth motion varies from 0 to $2\Delta x$ from one end of the scan to the other. Although this does not affect the calculations, the efficiency of this type of scanning is decreased towards the ends of the scans because of the unequal spacing. However the absorbance gradients in these regions are lower than in the central portion of the scan so that the lower efficiency can be tolerated. The transverse spacing Δy is obtained as the distance, perpendicular to the overall motion, between successive readings.

Having obtained ΔA_0, A, Δx and Δy, these values are substituted into the expression (4) and summated to give a single value which is proportional to μg of compound in the zone. The constant of proportionality is

$$\frac{\exp[-2A_0']}{a}.$$

As the expression $\exp[-2\Delta A_0](A + 0.4A^2)$ has to be evaluated $[(x_2 - x_1)(y_2 - y_1)]/(\Delta x \Delta y)$ times where Δx and Δy are of the order of 0.05 cm, and $(x_2 - x_1)$ and $(y_2 - y_1)$ are of the order of 1.5 cm, the number of times the expression is calculated is about 900 per analysis, before summation. Thus, to be useful, this method of analysis must have some rapid automatic means of computation.

EXPERIMENTAL

Instrumentation

The instrument used to apply the above concepts to quantitative analysis of thin layer chromatograms was constructed by modifications and additions to a Chromoscan densitometer (Joyce Loebl & Co. Ltd., Gateshead, England). Ideally, a new class of instrument would be preferable and more generally useful. However, for use in an exploratory experiment, of the type described below, the limitations of slow speed and poor wavelength selection imposed by adapting an existing instrument are not very serious.

The recording densitometer was fitted with a quartz iodine source, a 0.5 mm circular aperture, and a 420 nm interference filter with a half band width of 15 nm. The linear motion carriage and associated mechanism was dismantled and removed. The transmission photomultiplier stand was replaced by one occupying less space.

Inside the now empty sample compartment four legs were fitted, each with a 1 mm pitch (OB.A.) half nut soldered to the top. These support the scanning mechanism, shown in Fig. 2, by the two horizontal 1 mm pitch screwed rods. When these screwed rods rotate they impart a horizontal motion (x axis) to the mechanism, and

TABLE I

K AUTOCODE COMPUTER PROGRAM USED IN THE CALCULATION OF THE RESULTS

```
title
ici/k/17237/8  j.goldman(pharm) 2dtlc analysis

chapter0
a→1≈,b→3200≈,d→30≈,≈b→3200≈,≈c→110≈,≈d→110≈,k→110
c→110
1)newpage
read data title
read(f,a0,a1)≈,(scale and base line)
≈t=0
t=s=0
e=0
2)readword(a,p,q,r,3)
3)jump(2)a>9≈,(search for 1st scan)

(read in data)
j=0,2,100
i=1
b(30j+i)=a
4)i=i+1
readword(b(30j+i),p,q,r,5)
5)jump(7)b(30j+i)=-1≈,(end of data)
s=s+1
jump(17)q≠4≈,(mispunch)
jump(17)b(30j+i)>999
6)jump(22)i>60≈,(scan too wide)
jump(4)b(30j+i)>9
jump(4)i≈>3≈,(extra scan markers)
a=b(30j+i)
i=i-1
k(j)=ϕintpt(0.5i)≈,(k(j)=width of jth scan)
k(j+1)=i-k(j)
i=1,1,k(j+1)≈,(split double scans)
di=b(30j+k(j)+k(j+1)+1-i)
repeat
i=1,1,k(j+1)
b(30≈(j+1≈)+i)=di
repeat
≈s=s≈,(s=no of data points)
repeat≈,j=j+2
7)n=j+1
k=k(ϕmin(k0,0,n-2))
?g=≈s/j≈,(g=mean no of data per scan)

(interpolate mispunched data values)
j=1,1,n-1≈,(remove scan markers and adjacent points)
i=2,1,k-1≈,(-leave space round matrix)
≈b(jk+i)=b(30j+i-29)
repeat
repeat
jump(12)t=0≈,(no errors)
i=1,1,k
≈b(nk+i)=≈bi=10000
repeat
i=2k,k,nk
≈bi=≈b(i-k+1)=10000
repeat
j=1,1,n-1≈,(search for errors)
8)i=ϕmax(≈b0,jk+2,k≈(j+1≈)-1)
jump(11)≈bi≈>1000≈,l=0
e=0
≈q=-1,1,1
m=-1,1,1≈,(search for adjacent correct data points)
jump(9)≈b(i+≈qk+m)>1000
e=e+≈b(i+≈qk+m)
l=l+1
9)repeat
repeat
jump(10)l>0
nlcaption
too many mispunches around scan
print(j-1,2,0)
caption
point
print(i+1-kϕintpt(i/k),2,0)
jump(1)
10)≈bi=e/l≈,(corrected data value inserted)
t=t-1≈,(one less error left)
jump(12)t=0
jump(8)

(determine length of zone)
11)repeat
12)n=n-2≈,(n=no of last scan)
k=k-2≈,(k=no of points per scan)
j=0,1,n≈,(remove margin,correct-)
i=1,1,k≈,(-for drift,convert to absorbance)
b(jk+i)=f≈(≈b(≈(k+2≈)≈(j+1≈)+i+1)+≈(a0≈(j-n≈)-ja1≈)/n≈)
repeat
repeat
j=0,1,n
cj=b(ϕmax(b0,jk+1,jk+k))≈,(c=(db/dj)i=l)
repeat
j=1,1,n
≈cj=cj-c(j-1)
repeat
≈i=ϕmax(≈c0,1,n)≈,(front of spot)
≈j=ϕmin(≈c0,1,n)≈,(back of spot)
j=≈i
13)j=j-1
jump(14)≈cj≈>0.01
jump(13)j>5
14)≈i=j-5≈,(i=start of spot)
j=≈j
15)j=j+1
jump(16)≈cj>-0.01
jump(15)j≈>n-4
16)≈j=j+4≈,(j=end of spot)

(interpolate background to find sample absorbance)
j=≈i,1,≈j
≈c=≈b=0
m=j-2,1,j+2
jump(24)m≈>0
jump(25)m>n
≈b=≈b+b(mk+1)+b(mk+2)
≈c=≈c+b(mk+k)+b(mk+k-1)
jump(23)
24)≈b=≈b+b1+b2
≈c=≈c+bk+b(k-1)
jump(23)
25)≈b=≈b+b(nk+1)+b(nk+2)
≈c=≈c+b(nk+k)+b(nk+k-1)
23)repeat
```

thus to the chromatographic slide. The vertical motion (y direction) is provided by having the slide holder aligned by a tongue and groove at one end and fixed to a 1 mm pitch nut on a vertical screwed rod at the other. This vertical rod is driven via a 27:1 gear ratio from a half-crown and pinion assembly which provides alternately 13.5 cycles in one direction and 13.5 cycles in the other. Both the half-crown and the horizontal shafts are driven from the same 1.2 r.p.m. synchronous motor. The horizontal screw drive rotates 0.583 times during each half cycle of the half-crown producing 0.583 mm horizontal motion between scans. The scanning mechanism is made from F.A.C. Construction System (sold by Transitoria Trading Company A.B., Stockholm, Sweden) except for the screwed rods and microscope slide holder.

```
i=1,1,k
≈b(jk+i)=b(jk+i)-0.1≈(≈b≈(k-i≈)+≈c≈(i-1≈)≈)/≈(k-1≈)
repeat
repeat
i=3,1,k-2
≈c=≈b=0
m=i-2,1,i+2
≈b=≈b+≈b(≈ik+m)+≈b(≈ik+k+m)
≈c=≈c+≈b(≈jk+m)+≈b(≈jk-k+m)
repeat
j=≈i+2,1,≈j-2
≈b(jk+i)=≈b(jk+i)-0.1≈(≈b≈(≈j-j≈)+≈c≈(j-≈i≈)≈)/≈(≈j-≈i≈)
repeat
repeat

(apply simple k-m eqn and summate)
≈e=≈d=0
j=≈i+2,1,≈j-2
≈dj=0
i=3,1,k-2
b(jk+i)=b(jk+i)-≈b(jk+i)≈,(background)
≈dj=≈dj+≈b(jk+i)≈(1+0.4≈b(jk+i)≈)øexp(-2b(jk+i))≈,(k-m)
≈e=≈e+b(jk+i)
repeat
?≈d=≈d+≈dj≈,(integrate)
repeat


(print out results)
≈e=≈e/≈(k-4≈)≈(≈j-≈i-3≈)≈,(e=avg background)
nlcaption
micrograms x absorptivity x exp(2a0)=
print(157.5≈d/g,4,2)
nlcaption
mean background absorbance=
print(≈e,1,3)
nlcaption
with max of
print(b(ømax(b0,≈ik+1,k≈(≈j+1≈))),1,3)
caption
and min of
print(b(ømin(b0,≈ik+1,k≈(≈j+1≈))),1,3)
nlcaption
length of scan used=
print(0.5833≈(≈j-≈i+1≈),2,1)
caption
mm
nlcaption
with
print(0.5833≈i,2,1)
caption
mm spare at start
nlcaption
and
print(0.5833≈(n-≈j≈),2,1)
caption
mm spare at end
nlcaption
width of scan=
print(13.5k/g,2,1)
caption
mm
nlcaption
number of interpolated mispunches=
print(≈t,3,0)
newline

(print out zone absorbance matrix)
i=k,-1,k-1
newline
j=≈i,1,≈j
print(100b(kj+i),2,0)
repeat
repeat
i=k-2,-1,3
newline
print(100b(k≈i+i),2,0≈,100b(k≈i+k+i),2,0)
j=≈i+2,1,≈j-2
print(100≈b(kj+i),2,0)
repeat
print(100b(k≈j-k+i),2,0≈,100b(k≈j+i),2,0)
repeat
i=2,-1,1
newline
j=≈i,1,≈j
print(100b(jk+i),2,0)
repeat
repeat
jump(1)

(correct or replace mispunches)
17)jump(18)p+r≠0
jump(18)q≠3≈,(2nd digit 6 missing)
b(30j+i)=60+b(30j+i)+90øintpt(0.1b(30j+i))
jump(6)
18)jump(20)j=0
jump(21)i≈>k(j-1)+k(j-2)≈,(not end of scan)
19)b(30j+i)=0
jump(6)
20)jump(19)i>53≈,(end of 1st scan)
21)b(30j+i)=10000
t=t+1≈,(t=no of interpolable mispunches)
≈t=t
jump(6)
22)nlcaption
scan marker missing after scan
print(j,3,0)
jump(1)
close
```

The pen drive wire on the Chromoscan was extended by the manufacturer to operate a "slave" potentiometer. Approximately 10 V was applied across the two ends from 5 lead/acid accumulators via two trimming potentiometers for "scale" and "baseline" adjustment. The output from the rotating contact, adjusted to be within the range 1.00 to 9.99 V, was fed into a prototype digitiser and encoder (see Acknowledgements) to supply a digital output of 1 unit per 0.01 V in Mercury code at constant intervals of 0.926 sec. This output was punched on to 5 hole tape by a Creed 25 character/sec punch.

Connected to the motor shaft of the scanning mechanism was an arm which closed a microswitch momentarily once each cycle. This operated a short circuit in

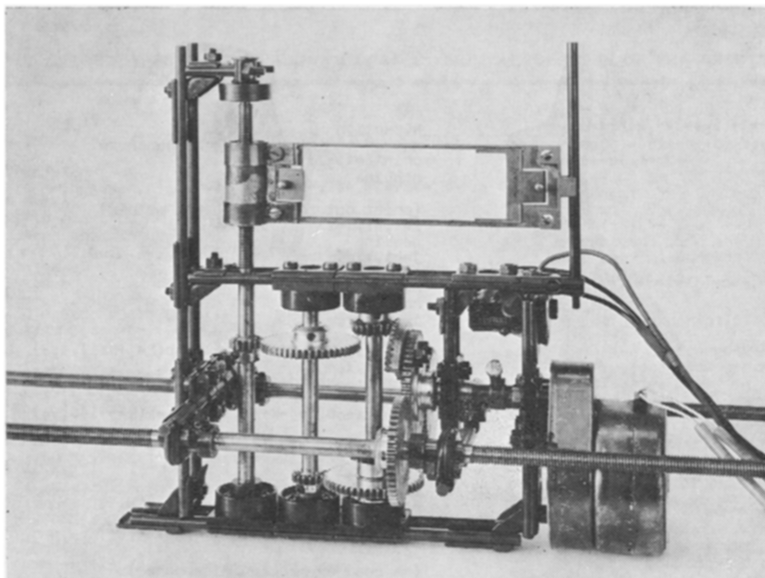

Fig. 2. Scanning mechanism, assembled from F.A.C. Construction System, so as to fit into the sample compartment of a Joyce Loebl Chromoscan. It imparts a saw-tooth motion to the microscope slide chromatogram placed in the holder.

the output voltage for about 1 sec to give one or two readings of 0.00 V. The arm was adjusted to operate when the light spot was at one extremity of the traverse across the chromatographic plate.

Program

A computer program was written in K Autocode (see Table I) to carry out the calculations on the data required for the analysis. The program aligns the data into the separate consecutive scans across the thin layer using the zero values as markers. The data which is in the form of integers between 100 and 999 is then converted into absorbance relative to the standard absorbance level and corrected for any instrumental drift during the experiment. The position and extent of the chromatographic zone is determined and the absorbance values around it are used to interpolate ΔA_0 values for each point in the region containing the zone. These are then subtracted from the absorbance values in this region to give sample absorbances A for each point. The value of the expression exp $[-2\,\Delta A_0]$ $(A + 0.4\,A^2)$ is calculated for each point and then summated over the whole region containing the zone. The result is multiplied by $2000\,\Delta x\,\Delta y$ after calculating the value of Δy (the size of Δy varies slightly as it is dependent on the time interval between successive readings).

The result, which is equal to absorptivity $\times$ exp $[2\,A_0']$ $\times$ μg of compound in the zone, is printed out, together with other data of interest, such as the mean value of ΔA_0 within the zone, the size and position of the zone etc. (see Table II). Also printed is a grid of the sample absorbance $\times$ 100, surrounded by the relative background absorbance $\times$ 100 used in the interpolation procedure.

The program also includes a correction for a specific fault which prevented the

number 6 in the tens position from being punched. There is also a general interpolation for any other type of mispunch. The data was computed on a KDF9 computer, each data tape requiring about 30 sec of computer time.

Procedure

Microscope slides, 7.5 cm × 2.5 cm, were cleaned in chromic acid, rinsed with water then methanol and dried at 100°. This seemed to reduce the probability of the gypsum binder in the adsorbent crystallising on to the glass, which would have caused optical inhomogeneities in the thin layers. Thin layers of silica gel (Merck Kieselgel G) were prepared on the microscope slides using a brass bedplate and slurry box with about 250 μ clearance above the glass surfaces. Five grams of adsorbent slurried with 10 ml of distilled water were used to prepare each batch of 12 thin layers. After drying at 90° the adsorbent was cleaned from a 1.5 cm portion at one end of each slide.

An acetone DNPH solution was prepared in methylated spirits at 0.510 mg/ml. Portions were diluted to 75, 50 and 25% of this strength. The solutions were kept in small airtight bottles with rubber lined screw caps.

One microlitre of the solution to be analysed was transferred as follows to a thin layer plate using a 1 μl capillary micropipette (Microcaps, Drummond Scientific Co., U.S.A.). The capillary was always filled from one end, and emptied from the other. It was rinsed once with clean solvent and once with the sample solution before filling. The micropipette was discharged on to the thin layer 1 cm from the bottom of the plate. Care was taken not to dislodge any adsorbent particles. It was then filled with clean solvent and again emptied on to the thin layer in the same location. The choice of the polar solvent, methylated spirits for rinsing, is to elute the spot into a ring, so that no sample is lost on adsorbent adhering to the pipette. The capillary was then cleaned by rinsing once more with clean solvent. The same pipette, which had been found to contain 1.00 μl by weighing, was used throughout.

The methylated spirits was evaporated from the spot by blowing gently for about 30 sec before development through 5 cm with toluene in a 3 oz. (approx. 80 ml) screw-topped bottle with a wide neck. The R_F value of acetone DNPH in this system is approximately 0.4. The chromatographic plate was then placed in a vacuum jar and most of the toluene evaporated under reduced pressure. However, some solvent remained due to the low temperature attained by the slide, so it was removed and warmed on a hot plate for 10 sec before returning to the vacuum jar for a further 30 sec at 0.1 mm Hg pressure.

The back of the microscope slide and the end portion with no adsorbent were carefully cleaned before placing the plate into the holder on the scanning mechanism. With the light spot falling on a background portion of the plate *i.e.* not on the spot, the pen position was adjusted to be near the lower absorbance limit on the scale. The scanning mechanism was then moved to a position where the light spot passed through a piece of diffusing glass fastened to one end of the slide holder, and the output noted. The attenuation caused by this diffuser acts as the standard absorbance level. The mechanism was then moved back to a position such that the light spot was approximately 8 mm to one side of the chromatographed zone. The chromatogram was then scanned, the output being recorded on 5 hole tape. After scanning, the standard absorbance level was again noted so as to allow for any instrument drift

TABLE II

COMPUTER RESULT CALCULATED FROM THE PRINTED DISTRIBUTION OF ABSORBANCE (× 100) OF A CHROMATOGRAPHIC ZONE

ICI/K/15586/13.26 100

MICROGRAMS X ABSORPTIVITY X EXP(2A0)= 316.52
MEAN BACKGROUND ABSORBANCE=-0.553
WITH MAX OF-0.523 AND MIN OF-0.573
LENGTH OF SCAN USED= 13.4 MM
WITH 0.0 MM SPARE AT START
AND 0.6 MM SPARE AT END
WIDTH OF SCAN= 12.1 MM
NUMBER OF INTERPOLATED MISPUNCHES= 1

-54	-55	-55	-55	-55	-55	-55	-55	-56	-56	-56	-56	-56	-56	-57	-57	-56	-56	-57	-57	-57	-57	-57
-54	-54	-55	-55	-55	-55	-55	-55	-56	-56	-56	-56	-56	-56	-56	-57	-56	-56	-57	-57	-57	-57	-57
-54	-55	-0	-0	-0	0	0	-0	-0	-0	0	0	0	0	0	-0	0	0	0	-0	-0	-57	-57
-55	-55	0	-0	-0	-0	0	-0	0	-0	1	0	0	0	0	-0	0	0	0	-0	-0	-57	-57
-55	-55	0	-0	0	0	0	-0	1	-0	5	2	2	0	0	-0	0	0	-0	-0	0	-57	-57
-55	-55	-0	-0	0	0	1	0	3	2	10	8	5	6	1	0	1	-0	0	-0	0	-57	-57
-54	-55	-0	-0	-0	-0	2	1	6	7	20	18	14	12	3	1	1	0	0	-0	0	-57	-57
-54	-55	0	0	-0	-0	2	2	12	15	28	28	22	17	7	3	1	0	0	-0	0	-57	-56
-54	-55	0	0	0	-0	2	3	13	17	30	32	27	19	9	4	2	0	1	-0	0	-57	-56
-54	-54	-0	0	0	0	2	5	14	20	29	32	24	16	8	4	1	0	0	-0	0	-57	-56
-54	-55	0	-0	-0	0	2	6	14	21	28	29	22	15	7	4	1	0	0	-1	0	-56	-56
-54	-54	-0	-0	0	0	3	6	13	22	29	27	21	14	7	3	1	0	0	-1	0	-56	-56
-54	-54	0	-0	1	1	2	6	13	23	29	27	23	14	8	3	1	0	0	-1	0	-56	-56
-54	-54	0	-0	0	1	1	6	12	23	29	29	26	16	9	3	2	-0	0	-1	0	-56	-56
-54	-54	0	-0	1	1	1	6	10	23	27	31	28	18	11	4	2	-0	0	0	0	-56	-55
-54	-54	0	-0	0	0	1	4	8	22	22	29	25	16	11	4	1	-0	0	-0	-0	-56	-55
-53	-54	0	-0	0	-0	0	2	4	16	12	18	18	9	9	3	1	-0	-0	-0	-0	-56	-55
-53	-54	0	-0	-0	-0	0	1	2	8	4	8	9	4	4	2	0	-0	0	-0	-0	-56	-55
-53	-53	0	-0	-0	-1	0	1	0	3	1	3	2	1	1	1	0	-0	0	-0	0	-56	-55
-53	-54	-0	-0	-0	-0	0	0	0	1	0	1	0	1	0	1	0	-0	0	0	0	-56	-55
-53	-54	-0	-0	-0	-0	0	-0	0	1	0	0	-0	0	-0	0	0	-0	0	0	0	-56	-55
-53	-53	1	-0	0	-1	0	0	-0	0	0	0	-0	-0	-0	0	0	-0	-0	0	-0	-55	-55
-52	-53	-52	-54	-53	-54	-54	-54	-54	-55	-54	-55	-55	-55	-55	-55	-55	-55	-55	-55	-55	-55	-55
-52	-52	-53	-54	-53	-54	-54	-54	-54	-54	-54	-55	-55	-55	-55	-55	-55	-55	-55	-55	-55	-55	-55

during the experiment. The scale was then estimated so that the output could be converted to absorbance.

The analysis of each chromatogram was completed within half an hour of development as acetone DNPH spots were not entirely stable on silica gel. A loss of about 4% after 6 h had previously been observed.

A data heading, the output scale and the first and second standard absorbance readings were punched on 5 hole tape and spliced on to the start of the data tape. The number, −1, to signify the end of data to the program, and the end of tape symbols were hand-punched on to the end of the tape. The data was computed in a manner dictated by the program, so as to provide a result which should be proportional to the weight of acetone DNPH put on to the chromatogram.

Results

The above procedure was carried out sixteen times, each on separate plates with no special layer thickness control. There were ten sample loads of 0.510 μg and two loads at 0.383, 0.255 and 0.128 μg, respectively.

TABLE III

EXPERIMENTAL RESULTS FROM ANALYSES OF KNOWN QUANTITIES OF ACETONE DINITROPHENYLHYDRAZONE

Acetone DNPH (μg)	*Result*[a]	*Results × conversion factor*[b] *(μg)*	*Error (μg)*	*Mean* ΔA_0
0.510	332.6	0.535	0.025	−0.482
0.510	328.3	0.528	0.018	−0.505
0.510	317.9	0.512	0.002	−0.581
0.510	317.5	0.511	0.001	−0.487
0.510	306.3	0.493	−0.017	−0.494
0.510	305.1	0.491	−0.019	−0.482
0.510	316.5	0.510	0.000	−0.553
0.510	318.6	0.513	0.003	−0.547
0.510	315.6	0.508	−0.002	−0.525
0.510	309.7	0.499	−0.011	−0.508
0.383	263.6	0.424	0.041	−0.550
0.383	242.5	0.390	0.007	−0.500
0.255	171.1	0.275	0.020	−0.483
0.255	165.4	0.266	0.011	−0.490
0.128	86.2	0.139	0.011	−0.501
0.128	85.9	0.138	0.010	−0.499

[a] Computed as μg × absorptivity × exp[2 A_0'].
[b] See text.

Table III shows the computed results against μg of acetone DNPH chromatographed. Dividing the mean of the ten results at 0.510 μg into 0.510 gave the conversion factor, 0.001610 μg/unit. This value was used to convert all the results to μg for comparison with the known quantities loaded on the thin layer plates. The differences between the estimated and loaded weights are given under "error". These figures were used to calculate the overall standard deviation as 0.017 μg and on the

0.510 μg level alone, 0.014 μg or 2.7%. The mean ΔA_0 is the difference between the mean background absorbance of the thin layer and the standard absorbance level, A_0'. Equating the conversion factor to

$$\frac{\exp\left[-2A_0'\right]}{a}$$

where a is the absorptivity of acetone DNPH, and rearranging gives

$$A_0' = -\tfrac{1}{2} \ln\left[0.00161\, a\right].$$

Taking the value of a as that in methanol solution, 22.4 (which will only be approximately that of acetone DNPH adsorbed on silica gel), gives A_0' as approximately 1.661. The actual background absorbance of the thin layers, A_0, may now be calculated from:

$$A_0 = A_0' + \Delta A_0$$

From the values of ΔA_0 in Table III the range of A_0 in this experiment is seen to be 1.080 to 1.179, which correspond to a range of scattering powers of 11.0 to 14.1 (using $A_0 = \log\left[1 + SX\right]$).

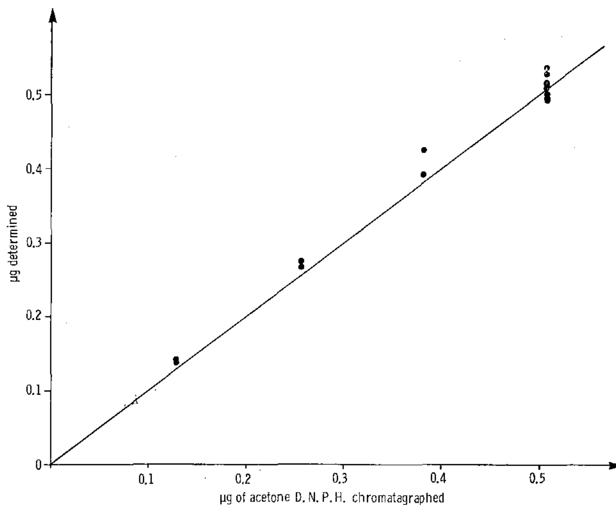

Fig. 3. Experimentally determined *vs.* actual weights of acetone DNPH in chromatographic analyses.

The graph of estimated *vs.* loaded amounts of sample, Fig. 3, may indicate a slight curvature in the experimental points as compared to the ideal straight line. This was probably caused by concentric rings of light of diminishing intensity surrounding the main point of illumination. These were due to internal reflection of diffused light back on to the chromatogram by the microscope slide surfaces. Unfortunately this phenomenon and the reason for it was not realised until after the apparatus had been dismantled. When the chromatogram is illuminated through the glass slide, as in this experiment, the diffuse light reflected back onto the thin layer

was initially reflected by the thin layer. As reflectance varies much less than transmittance these rings of light will be relatively constant in intensity. They were found by experiment to constitute about 5% of the total light energy. Improved linearity would be expected if this additional illumination were obviated. For instance, if the light were focussed onto the chromatogram from the thin layer side, then the internal reflection would be of light initially transmitted by the thin layer at the point of illumination only. Alternatively, the transmitted light could be refocussed through a small aperture before measurement.

DISCUSSION AND CONCLUSION

A theory for radiative transfer in scattering and absorbing sheets derived by KUBELKA and MUNK has been used extensively in measurements concerning the properties of paper and paints. In these fields it has become accepted as being sufficiently accurate for most purposes, although it is not an exact expression. We have reported[18] previously on the derivation and verification of the simplified form (eqn. 1) used in this paper. The advantage of this simplified equation is that the unknown, KX, which is proportional to the weight of light absorber per unit area, is explicitly related to the measured variables, A_0 and A, rather than implicitly as in the general KUBELKA–MUNK equation. This made it feasible to carry out quantification of chromatographed bands in simple cases within the described limitations, without having to use computational aids. That sufficed to obtain a reasonable verification of the simplified expression under conditions that were of limited usefulness.

The purpose of the present paper is to extend quantitative thin layer chromatography to the more general case of spots. As the absorbance of spots varies in both length and breadth, scanning in these two dimensions is necessary and inevitably large numbers of data points are accumulated. Data processing by computer is indispensable for completion of the analysis. The use of a computer brings other advantages such as corrections for instrumental faults, curve fitting and smoothing etc., which otherwise would be neglected.

The results obtained on repeat determinations of 0.510 μg of acetone DNPH on separate thin layer chromatograms showed a standard deviation of 2.7%. Improvement in the precision can be expected from developments in the instrumental design aimed at lowering mechanical and electronic tolerances. No particular control of layer thickness was attempted as there is compensation for this in the calculation of the results. Once the constant of proportionality (which is related to the absorptivity of the compound in question) is known, each analysis is an independent determination so that no calibration against adjacent standards is necessary.

The novelty of this form of analysis is not in the use of either: monochromatic light, two-dimensional scanning, a reasonably correct theory, "off-line" data handling by computer, or the accurate application of small volumes of liquid as spots. Rather it is the combination of all these aspects which provides a substantially non-empirical approach to the problem of *in situ* quantitative analysis.

To increase further the generality of application of quantitative analysis it would be necessary to use light in the ultra-violet region so that direct analysis of many more substances could be made. However, silica gel (as an example of a com-

monly used adsorbent), absorbs light itself in the region 200–275 nm, which includes the region of absorption by many of the common chromophores. Thus to work in this region on silica gel adsorbent introduces two more difficulties: a third independent variable *i.e.* the absorptivity of the substrate, and the low transmittance of the layer itself.

The first case necessitates the use of the general form of the KUBELKA–MUNK transmission equation, as the range of application of our simplified version will not be sufficient to encompass the high overall KX term, obtained by adding that of the substrate to the sample. This difficulty may be ameliorated by computational methods, although this would inevitably incur increased computing time. The KX value of the substrate in the 200–275 nm region could be determined readily by using the simple expression, derived by KUBELKA[20], for K/S in terms of the reflectance of an "infinitely thick" layer.

The magnitude of the second difficulty, that of lower light energy, can only be assessed by further experimental work.

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