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DIRECT QUANTIFICATION OF THIN-LAYER CHROMATOGRAMS BY EMPIRICAL METHODS

MATHEMATICAL ANALYSIS

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SUMMARY

Ideally, the material in a spot on a two-dimensional chromatogram forms a bivariate normal distribution in which the base of the figure corresponds to the dimensions of the circular spot and the height of the curve is a function of the maximum absorbance. Using a paraboloid as an approximation of the Gaussian figure, it has been possible to construct a mathematical model of a series of chromatographic standards over a five hundred-fold range of values and to define mathematically deviant "spots" which were more compact or more diffuse than the standard series. The model has been used to evaluate the various empirical techniques of direct chromatographic analysis including: spot length, area, maximum absorbance, (area) × (maximum absorbance), total absorbance and slit scanning with fixed and fixed-ratio slit length. It was found that slit scanning where the length of the slit is a constant fraction of the spot diameter is probably the best technique for mono-dimensional chromatograms while for two-dimensional chromatograms the product of (spot area) × (maximum absorbance) appears to be the best method.

INTRODUCTION

In chromatography a mixture of substances in solution is applied to the stationary phase as a sharply defined zone. The mobile phase is then introduced and to a greater or lesser extent resolves the mixture into its separate components. As the solute molecules migrate and interact between the two phases, diffusion occurs. The substances move as zones, most concentrated at the center and decreasing towards the periphery. In practice many other factors can and do interfere but ideally, the profile of the molecular distribution in the direction of migration approximates a Gaussian curve[1,2]. This is most evident in the recorder output of gas or high-pressure liquid chromatograms and paper chromatography (PC) or thin-layer chromatography (TLC) scanners.

In the special case of a two-dimensional paper or thin-layer chromatogram, a plot of concentration *vs.* distance along any diameter of a spot ideally resembles

a bell curve and the concentration within a TLC or PC spot can be expressed as a bivariate distribution.

In a set of chromatographic standards in which all conditions are identical except for the quantity of the substance (Q), the series of spots can be represented by a corresponding series of geometrically similar three-dimensional graphs whose volumes are equivalent to the respective Q values. The area of each figure in the x, y plane is equal to the area of the corresponding chromatographic spot while the height is a function of the maximum absorbance. Spots which are more compact or more diffuse are represented, respectively, by graphs of more or less peakedness (kurtosis) than the norm. Distortions due to heading or tailing are reflected in the skewedness of the graphic figure.

This mathematical description can be used to construct a theoretical chromatogram free of human error and variation. Such a model can be used for a critical comparison of the different —often contradictory— techniques of direct chromatographic quantification that have been used empirically[3–5]. The theoretical validity of the various methods can be examined and their effectiveness determined when factors such as sample range or spot shape are varied mathematically.

MATHEMATICAL METHODS

The concentration of material per unit area of a chromatographic spot can be described by a circular paraboloid approximating a bivariate normal distribution. Mathematically it is easier to manipulate a parabola than the normal probability curve since by definition the area under any normal curve equals one. The integrations are simpler and the relationship between the volume, radius and height (eqn. 5 below) is analogous to that of a cone, hemisphere or cylinder. Moreover, the use of the paraboloid is no less accurate since a bell-shaped distribution of material may not actually be Gaussian.

The paraboloid

$$z = c(r^2 - x^2 - y^2) \tag{1}$$

(Fig. 1) has a circular base of radius r and a maximum height

$$z = h = cr^2 \tag{2}$$

when $x = 0$, $y = 0$. The multiplier c is a constant for all geometrically similar paraboloids. When $y = 0$,

$$z = c(r^2 - x^2) \tag{3}$$

which is a parabola. Rotating this curve about the z axis permits the calculation of the volume of the paraboloid, V, by the method of cylinders,

$$V = 2\pi c \int_0^r x(r^2 - x^2)\,dx \tag{4}$$

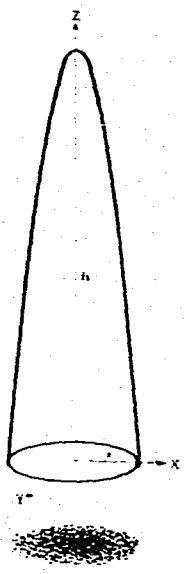


Fig. 1. Paraboloid of the form $z = c(r^2 - x^2 - y^2)$ with a maximum height of h and a maximum radius of r.

Letting the volume of the figure represent the quantity of material Q and solving the integration, we have

$$Q = \tfrac{1}{2}\pi r^2 h \tag{5}$$

where πr^2 is A, the area of the base equivalent to a circular chromatographic spot.

The maximum absorbance of a spot is related to the cylindrical volume obtained by setting the limits of integration (in eqn. 4) from 0 to a where a is the radius of the circular aperture. This quantity, M, is defined in eqn. 6

$$M = \pi a^2 h - \frac{\pi a^4}{2} \tag{6}$$

and simplifies to

$$M = \pi a^2 h \tag{7}$$

when $a \leqslant 0.25$.

In slit scanning of a spot, the quantity measured is equivalent to

$$S = c \int_{-b}^{b} dx \int_{-\sqrt{r^2-x^2}}^{\sqrt{r^2-x^2}} (r^2 - x^2 - y^2)\, dy \tag{8}$$

where the length of the slit is equal to $2b$ (Fig. 2) and is centered on the spot. Integrating eqn. 8 we have

$$S = c\left[r^2 b\sqrt{(r^2 - b^2)} + r^4 \sin^{-1}\left(\frac{b}{r}\right) + \frac{2b}{3}(r^2 - b^2)^{3/2}\right] \tag{9}$$

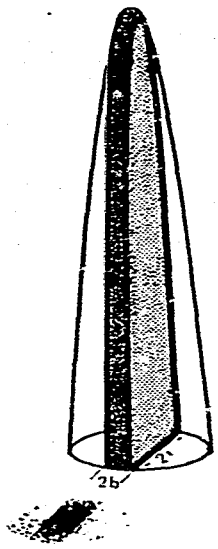


Fig. 2. Mathematical model of slit scanning of a spot with a paraboloid distribution of material: $2b$ is the slit length and $2r$ is the spot diameter.

If the quantity of material, Q, and the "similarity constant", c, are defined, then from eqns. 5 and 2 one can determine r and h.

$$r = \left(\frac{2Q}{c\pi}\right)^{\frac{1}{4}} \tag{10}$$

If the distribution of material in a chromatographic spot is unlike that in a series of standards, then c has a different value. For two dissimilar distributions containing the same quantity of material, if $c_1 = 1$ then

$$r_2 = \frac{r_1}{\sqrt[4]{c_2}} \tag{11}$$

and

$$A_2 = \frac{A_1}{\sqrt{c_2}} \tag{12}$$

A series of paraboloids was constructed by defining the quantitative relationship of a set of theoretical standards. Letting $c = 1$, "spot" radii were calculated from eqn. 10. In addition deviant values were chosen such that the spot areas were either larger or smaller than predicted by the standard series by setting $c = {}^1/_2, {}^2/_3, {}^3/_2$ and 2 (eqn. 12). These mathematical models were then used to test the effectiveness of a number of optical techniques used empirically for the direct quantification of paper and thin-layer chromatograms (Table I).

The number of methods is actually greater than that listed since linear, logarithmic and exponential relationships have been expressed for the same measurement in different studies[3-5]. These empirical variations were examined with the mathematical model. Since the model is intended to describe the actual distribution of ma-

TABLE I

BASIC METHODS USED FOR *IN SITU* QUANTIFICATION OF PAPER AND THIN-LAYER CHROMATOGRAMS

Method	*References*
Maximum spot absorbance	3, 6
Total spot absorbance	7, 8
Spot area	9, 10
(Spot area) × (maximum absorbance)	11, 12
Slit scanning	13, 14
Spot diameter	15, 16

terial in a chromatographic spot without regard to the instrumentation used to measure it, no direct consideration was given to differences between transmission and reflectance techniques or the non-linearity of Lambert–Beer's law[5,17,18]. It is assumed that laboratory determinations analogous to the theoretical values employed here are directly proportional to the actual distribution of material. Absorption measurements, whether employing the Kubelka–Munk equation[17,19] or Lambert–Beer's law, under conditions where it is applicable[5,12], are equivalent to the solution of eqn. 7.

The effect on absorbance readings of the inclusion of adjacent background in the area scanned was determined by substitution in Lambert's equation[20] (see Appendix) to obtain eqn. 13

$$D = \log \frac{1}{1 + p\,(k - 1)} \tag{13}$$

where D is the absorbance, k is the ratio of the (average) intensity of light transmitted through the spot as compared to the background and p is the ratio of the area of the spot to the total area. When $p = 1$, $D = \log 1/k$. Substituting h in the simplified form of the Kubelka–Munk equation[17] for the corrected value of the absorbance, one is able to solve for D and k. By assigning values to p one is then able to calculate D', h' and Q': the apparent values of these measurements.

RESULTS

Since a method may be essentially linear for a limited span of values but not over an extended range, the techniques were tested under both conditions. Selecting the arbitrary Q values 1–5, 10–50 and 100–500 and a similarity constant, $c = 1$, values were obtained for the parameters of a hypothetical series of standard spots (Table II). Measurements for spots more diffuse or more compact than the standard series were also calculated. For each of the quantitative relationships examined linear regression analysis was performed over the range 1–5, 1–50 and 1–500 (ref. 21). A Pearson correlation coefficient (R) of 1.000 indicates perfect linearity over the entire range; an R value decreasing as Q increases is characteristic of a curvilinear relationship (Table III, Fig. 3).

Over a short range of values, all the measurements are essentially linear with the quantity of material (Table III, Fig. 3). In fact over the range $Q = 1.00$ to 1.50 there is acceptable linearity with the radii of the hypothetical spots ($R = 0.9990$).

TABLE II

EXPERIMENTAL VALUES CORRESPONDING TO THE MATHEMATICAL MODEL
The Q and c values were defined; all others were derived from them.

c	Q	r	h	A	M^*	MA	S^{**}	S^{***}	$Q'^{\S}$
1	1	0.893	0.798	2.507	0.151	0.377	0.747	0.747	0.889
1	2	1.062	1.128	3.545	0.215	0.764	1.304	1.494	1.771
1	3	1.176	1.382	4.342	0.265	1.152	1.798	2.241	2.640
1	4	1.263	1.596	5.013	0.307	1.540	2.252	2.987	3.517
1	5	1.336	1.784	5.605	0.344	1.929	2.680	3.734	4.371
1	10	1.588	2.523	7.927	0.489	3.878	4.587	7.468	8.639
1	20	1.889	3.568	11.210	0.695	7.785	7.802	14.937	16.925
1	30	2.091	4.370	13.729	0.852	11.697	10.634	22.405	25.003
1	40	2.246	5.046	15.853	0.985	15.611	13.249	29.874	32.890
1	50	2.375	5.642	17.725	1.102	19.526	15.679	37.343	40.619
1	100	2.825	7.979	25.066	1.561	39.116	26.585	74.683	77.188
1	200	3.359	11.284	35.449	2.209	78.323	44.798	149.363	142.531
1	300	3.718	13.820	43.416	2.707	117.543	60.529	224.048	200.115
1	400	3.995	15.958	50.133	3.127	156.773	75.052	298.731	251.769
1	500	4.224	17.841	56.050	3.497	196.006	88.857	373.416	298.792
1/2	3	1.398	0.977	6.140	0.186	1.140	1.546	2.241	2.672
2/3	3	1.301	1.129	5.318	0.215	1.146	1.645	2.241	2.656
3/2	3	1.062	1.693	3.545	0.326	1.157	1.956	2.241	2.625
2	3	0.989	1.954	3.070	0.378	1.159	2.072	2.240	2.622
1/2	30	2.486	3.090	19.416	0.601	11.662	8.998	22.404	25.617
2/3	30	2.314	3.568	16.811	0.695	11.675	9.662	22.405	25.381
3/2	30	1.889	5.352	11.210	1.045	11.712	11.704	22.406	24.522
2	30	1.758	6.180	9.708	1.207	11.721	12.509	22.405	24.104
1/2	300	4.421	9.772	61.400	1.913	117.433	51.304	224.051	221.980
2/3	300	4.114	11.284	53.172	2.210	117.484	54.737	224.046	214.194
3/2	300	3.359	16.926	35.449	3.317	117.593	67.199	224.044	183.867
2	300	3.126	19.544	30.700	3.811	117.622	71.854	224.045	170.930

* The radius of the aperture is fixed at 0.25.
** The slit length ($2b$) is fixed at $\frac{1}{2}r_1 = 0.4466$.
*** The slit length is defined as $\frac{1}{2}r$.
§ $p = 0.9$.

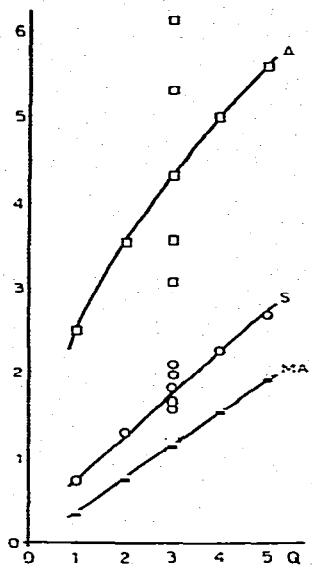
Neither the area, A, nor the "maximum absorbance", M, is linear with Q except over a limited range of values. According to the model, both have a perfectly linear relationship with the square root of Q. The model also predicts perfect linearity between MA and Q (Table III, Fig. 4).

The mathematical model of quantification by slit scanning (eqn. 9) indicates that if the length of the slit has a fixed relationship to the spot diameter, S (the quantity scanned) will be a constant fraction of Q (the total quantity). If the slit is one half the spot diameter

$$S = S' = 1.1731r^2h \tag{14}$$

or

$$S' \approx 0.75\,Q \tag{15}$$



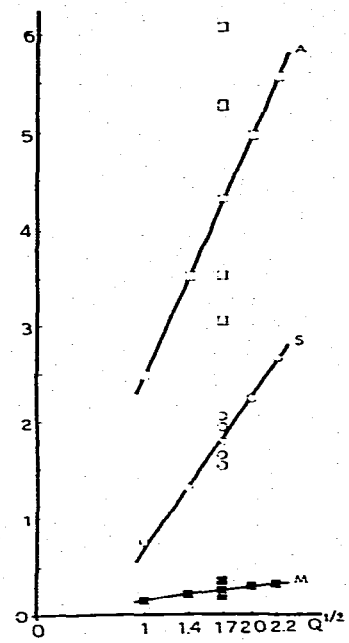


Fig. 3. Correlation of area (A), slit scan with fixed slit length (S) and ("maximum absorbance" × area) ($M \cdot A$) with the quantity of material (Q) with superimposed deviant values.

Fig. 4. Correlation of area (A), slit scan with fixed slit length (S) and "maximum absorbance" (M) with the square root of the quantity of material ($Q^{\frac{1}{2}}$) with superimposed deviant values.

If, on the other hand, the length of the scanning slit remains constant and therefore a decreasing fraction of the spot diameter, S, is not linear with either Q or $\sqrt{Q}$ except over a limited range of values. The curvature of $S \propto Q$ appears to mirror that of $S \propto \sqrt{Q}$ suggesting that $S \propto Q^{3/4}$ might be linear. In fact this relationship is observed and there is almost perfect linearity over the entire 500-fold range (Fig. 5).

Comparison of the deviant values with the standard series reveals that measurements of area or "maximum absorbance" alone cannot correct for a deviation in spot compactness–diffusiveness. The product of these two determinations can achieve this to a considerable degree (Fig. 3). Similarly, slit scanning can compensate for an error of this type if the slit length is a constant fraction of the spot diameter, otherwise the error can be considerable even when the 0.75 power is used.

Measurement of the total spot absorbance by means of a circular aperture larger than the spot gives excellent linearity even with the most deviant spots (Table III). It is evident, however, that an error of only about 11% in the aperture size (*i.e.*, about 5.4% in the radius of the aperture) can result in a sizable error in the estimated value of Q (Fig. 6). This corresponds to an inaccuracy of only about 0.5 mm in the setting of a 10-mm circular aperture. A similar error would be expected in slit scanning if the slit length was greater than the spot diameter or the radius of curvature of the spot was small.

TABLE III

RELATIONSHIPS BETWEEN VARIOUS DERIVED MEASUREMENTS AND THE QUANTITY OF MATERIAL

Range		*M/Q*	*M/Q*	*MA/Q*	*MA Q*	*M/A Q*	*S/Q*	*SA Q*	*S/Q²*	*S'/Q*	*Q'/Q*	*M/log Q*	*MA/log Q*
1–5	Slope	1.288	20.600	2.577	0.399	6.381	2.071	0.637	1.212	1.339	1.148	0.993	0.892
	Intercept	2.412	2.284	0.030	0.000	0.040	0.637	0.557	0.098	0.000	0.027	0.225	1.396
	R*	0.994	0.994				0.999	0.998			1.000	0.993	0.998
	r**	5.98%	5.98%				2.80%	1.76%			0.27%	7.83%	3.93%
1–50	R	0.984	0.984				0.997	0.995			1.000	0.973	0.992
	r	19.24%	19.24%				8.79%	6.21%			1.71%	14.89%	7.92%
1–500	R	0.972	0.972	1.000	1.000	1.000	0.995	0.991	1.000	1.000	0.997	0.933	0.980
	r	34.19%	34.19%	0.06%	0.00%	0.00%	15.28%	12.42%	0.25%	0.00%	10.72%	22.49%	12.44%

* R correlation coefficient.

** r coefficient of variation.

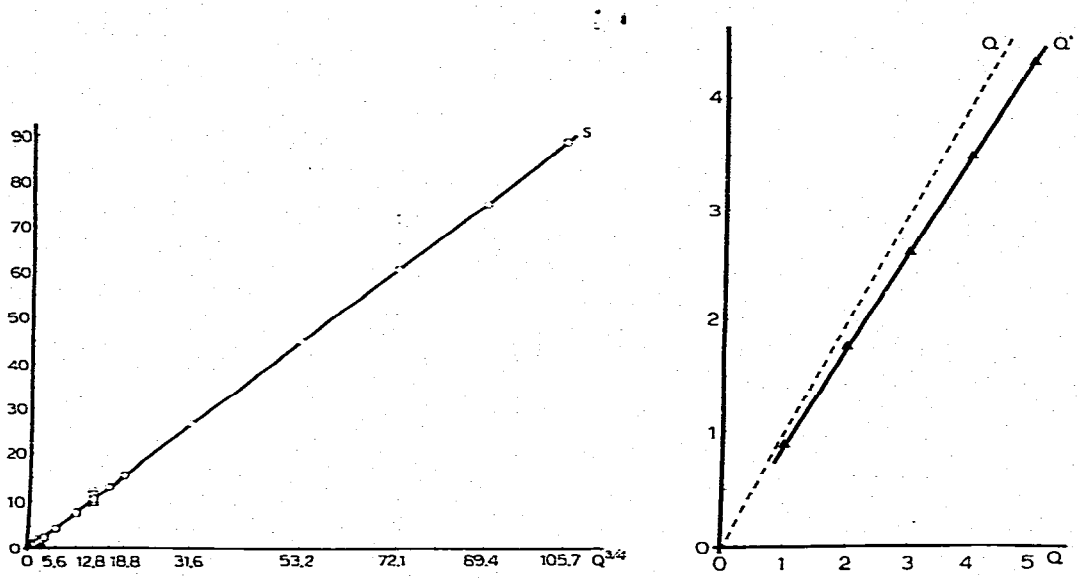


Fig. 5. Correlation of slit scan with fixed slit length (S) with the three-fourths power of the quantity of material ($Q^{\frac{3}{4}}$) with superimposed deviant values.

Fig. 6. The effect of adjacent background on total spot absorbance (Q') where the spot area is 0.9 that of the aperture.

DISCUSSION

Ideally the distribution of material in a series of standard and unknown spots on a chromatogram should be geometrically similar. The range of values should be limited and the unknowns should be bracketed physically and numerically by standards so prepared that their mobility and spot shape are exactly the same as the unknown. Under these conditions, almost any method of quantification is quite satisfactory. Curvilinear relationships approximate straight lines if the range is short enough. Certainly, if the precision is sufficiently good any shape curve is usable. Chromatograms of this quality are attainable but only at the sacrifice of speed, convenience and economy. For analytical TLC to be a practical, routine, technique, the method of quantification should be able to compensate for a resonable degree of human variation.

Spot area is undoubtedly the least expensive approach to TLC or PC quantification since one employs planimetry[3] or simply the product of the major and minor diameters[12,22]. In practice, however, area measurements are not simple. Since the spot size is critically dependent on the size of the origin spot, the precise distance migrated and the migration time —all of which increase diffusion[23]— painstaking reproducibility is most important. Furthermore, the determination of the spot boundary is difficult and somewhat arbitrary. This is a factor in a number of methods but is most serious when it is the only measurement that is taken[5].

Mathematically, the maximum absorbance of a spot appears to be no better a means of quantification than the area method. In practice, however, it can be measured more reliably[3-5]. Nevertheless, the absorbance is a function of the spot size and therefore equally dependent on factors affecting the area. If the precise reproducibility of chromatographic conditions is maintained, maximum absorbance is a simple, useful means of quantification[3,6]. The technique cannot cope with deviant spots, however. The model indicates that this measure correlates with acceptable linearity with the quantity of material over a short range of values. Actually, both maximum absorbance[24] and spot area are linearly related to the square root of the quantity of material, and over any extended range that relationship must be employed.

The method does not predict a linear relationship between the logarithm of the quantity of material and either the spot area[9] or the square root of the area[10]. Over a short range both methods are satisfactory. Diffusion is a function of spot size[23-25] but has been treated here as a constant. When diffusion is considered, spot size is reported to be related to the logarithm of the quantity of material[25]. Empirically it is difficult to establish which relationship is more correct since spot area measurements are not the most accurate[5]. Giddings and Keller[25] predict a linear relationship between the spot diameter and the square root of the logarithm of the quantity of material. The present model predicts linearity between the logarithm of the diameter and the logarithm of the quantity. Fowler[15] reported a constant relationship between the logarithm of spot content and logarithm of spot length for sucrose over a hundred-fold range. Miyaki *et al.*[16] observed a similar relationship.

According to the model, the product of the spot area and maximum density gives a far better correlation with the quantity of substance than either of these measures individually. This is especially true in the case of spots which deviate from the standards and where either the absorbance or area values alone are most unsatisfactory. This has been observed empirically[12] many times. The double measurement is able to compensate for considerable variation in the chromatography. Somewhat less compulsive attention to detail is permissible but at the price of two determinations instead of one[6].

At first glance it would appear that the easiest way to measure the total amount of colored reaction product in a spot would be with an aperture large enough to encompass all the material. This technique has been used successfully[7,8] though it has not gained wide popularity. Examination of the mathematical model reveals that if the aperture size is so controlled that the spot is a constant function of the scan area then excellent results are obtained even with very deviant spots. On the other hand, if the contribution of adjacent background area varies by as little as $\pm 11\%$, the accuracy is substantially affected. Optimally, the aperture should be exactly equal to the size of the spot but unless the spot is uniform and circular this is difficult to achieve.

Slit scanning[13,14] is particularly suitable for mono-dimensional chromatograms. The model indicates that there are better reasons for this than the mechanical convenience of automatically scanning a line of spots. If the chromatography is performed in "channels" on the plate and the origin shape is a line rather than a circle[13,14], the spots are elliptical bands and the major (transverse) diameter is constant, determined by the width of the channel. The length of the scanning slit is also fixed and, as

in the mathematical model, a constant fraction of the spot is scanned. Quantification is excellent in theory and in practice[14] even with deviant spots provided the ratio of slit length to spot "diameter" is constant. When the spots are essentially linear bands and the slit is smaller than the diameter, the error due to the inclusion of adjacent background is minimal. Scanning two-dimensional chromatograms is more difficult. The spots vary in size and unless the slit length is constantly readjusted a variable error is introduced. From the model, a linear relationship between the value obtained by scanning with a slit of fixed length and the quantity of material raised to the 0.75 power has been observed. The ability to compensate for deviant spots is rather limited, however, though considerably better than either the spot area or maximum density techniques. The background effect adjacent to the curved leading and trailing edges of a round spot may be significant, however, and would hinder the value of slit scanning for two-dimensional chromatograms.

Slit scanning in the direction of chromatography with slit length to spot diameter in a fixed ratio does appear to be the best approach to mono-dimensional TLC and PC. Modern instrumentation with stabilized electronics, reference beam background subtraction and automatic integration has permitted the widespread use of this procedure [13,14].

No comparable solution for two-dimensional chromatography is available as yet. The use of the product of the spot area and maximum absorbance can compensate for a rather severe degree of spot variation. Two manual determinations are required, however, placing great stress on the skill, patience and experience of the investigator[9-12]. Still, the procedure has significant advantages: other than a standard laboratory spectrophotometer, instrumentation costs are very low[12]. Also, the technique can be used to measure very distorted polygonal spots provided that all the members of a series are geometrically similar[26].

Measurement of total absorbance with an aperture matching the spot in size and shape is most accurate, theoretically, even for seriously distorted spots. The mechanical problem of implementing this for anything other than circular or elliptical spots[7] seems prohibitive, however. Since this technique should be equally effective with one- or two-dimensional separations, achieving this capability would be quite advantageous. Existing instrumentation is not suitable. A flying spot scanner[27] or image analysing computer[28] is very expensive and not really designed for measuring chromatograms of different sizes with spots that bleed into the background. Perhaps a special-purpose instrument could be developed.

The different techniques have been compared with respect to a mathematical model representing uniform, similar spots and others differing only in their compactness–diffusiveness. Under these optimal conditions the strengths and weaknesses of the various methods are evident. In the laboratory, heading, tailing, salt interference, medium overloading and incompleteness of the detection reagent may cause the chromatography to be far from ideal and impair any of the techniques[5]. If the background color is dark and/or irregular, errors in determining the limits of the spot and the correct value of the spot absorbance may be large and variable. None of these methods can really compensate for faulty technique.

APPENDIX

According to Lambert's law,

$$D = \log \frac{I_0}{I_i} \tag{1}$$

where D is the absorbance, I_0 is the total incident light and I_i is the total transmitted light. This can be rewritten as

$$D = \log \frac{i_0 a_0}{i_s a_s + i_b a_b} \tag{2}$$

where i is the illumination per unit area and a is the total area. The subscripts s and b denote the spot and the background, respectively. When $a_s = 0$ (*i.e.*, there is no spot),

$$D = \log \frac{i_0 a_0}{i_b a_b} = 0 \tag{3}$$

by definition. Therefore, the instrument readings are such that $i_0 = i_b$. Substituting in eqn. 2 and letting $k = i_s/i_b$, we have

$$D = \log \frac{a_0}{k a_s + a_b} \tag{4}$$

If $p = a_s/a_0$, then $1 - p = a_b/a_0$ and

$$D = \log \frac{1}{kp + 1 - p} \tag{5}$$

or

$$D = \log \frac{1}{1 + p(k - 1)}. \tag{6}$$

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