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COMPARISON OF OPTICAL TRANSMITTANCE AND REFLECTANCE MEASUREMENTS ON THIN-MEDIA CHROMATOGRAMS

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SUMMARY

This paper compares the relative merits of transmittance and reflectance procedures in the photodensitometric analysis of substances separated on thin-media chromatograms. The considerations used are based upon the equations of Kubelka and Munk, which describe in simplified terms the optical transfer properties of turbid media. The parameters used in the comparison are sensitivity, linearity and signal amplitude.

The theoretical values of these parameters were calculated using a computer and represented in graphical form. For improved linearity a logarithmic transform of the primary signal was satisfactory in the case of transmittance. For reflectance an inverse representation gave the best linearity. Both transforms promise improvement only if the primary signal-to-noise ratio is reasonably high. When very low concentrations are to be measured, the primary signal provides adequate linearity and no transform is necessary.

At larger concentrations transmittance with logarithmic conversion and reflectance with inverse representation give nearly equal performance for most media. With media of very high optical density the light intensity available in the transmittance mode may be insufficient compared to the electrical noise of the photodetector and reflectance is then the mode of choice. Reflectance may also have a slight advantage over transmittance with regard to optical noise if specularly reflected light can be kept away from the photodetectors. Any linearising transform has to be carried out before the total amount of substance is determined by integration. For full exploitation of the benefits of these transforms a flying spot scanner is essential.

A method is also described which permits the approximate determination of the coefficients of scattering and absorption of the medium from simple photodensitometric measurements. Knowledge of these parameters is required if more sophisticated methods of linearisation than logarithm forming or inversion are to be employed, *e.g.*, by using a computer.

The conclusions obtained do not apply to methods based upon fluorescence.

INTRODUCTION

Photometric methods are, at present, the most expedient means for the quantitative determination of the amount of a substance concentrated in a zone on a thin-medium chromatogram. In the main, transmittance or reflectance measurements are used. They cover a wide range of wavelengths of the optical radiation extending from the red end of the visible spectrum to the far UV.

The materials used for the active medium and partly also for the support are usually translucent, but not transparent. Expressed more accurately, they exhibit not only absorption, but also scattering of the incident radiation at optical wavelengths. The rules determining the transfer of radiant energy in such a medium are extremely complex. For practical applications, however, a simplified theory developed in 1931 by Kubelka and Munk[1] gives, in most cases, reasonably accurate results. The main simplifying assumptions of the theory apply quite adequately to thin-media chromatography. The resultant expressions are however, still much too complex to be employed in day-to-day usage in chromatography and related fields. In two recent papers[2,3] the authors presented modified expressions, which were based upon an electrical transmission line as a model. In this paper an effort has been made to use graphical representations of these expressions in order to illustrate some of the basic optical properties of turbid media and to compare the relative merits of transmittance and reflectance measurements for media with different optical parameters.

BASIC EXPRESSIONS

For the purpose of the Kubelka and Munk theory a turbid medium is characterised by two parameters, *viz.* the coefficient of scattering, S, and the coefficient of absorption, K. The theory assumes that the principal surfaces of the medium are planar and parallel to each other, as well as perpendicular to the incident radiation. The total values of S and K are proportional to the thickness of the medium. For simplicity, however, a thickness of unity will be assumed. This assumption represents only a scale factor, which does not affect the generality of the results.

The expressions for transmittance, A_T, and reflectance, A_R, as derived in ref. 2 are:

$$A_T = e^{-\gamma} \frac{1 - \varrho^2}{1 - (\varrho \cdot e^{-\gamma})^2} \tag{1}$$

$$A_R = \varrho \frac{1 - (e^{-\gamma})^2}{1 - (\varrho \cdot e^{-\gamma})^2} \tag{2}$$

The numerical value of the decimal logarithm of A_T is commonly called the (diffuse) optical density of the medium. Both A_T and A_R are obviously dependent upon the parameters ϱ and γ, which in turn are functions of the coefficients S and K.

$$\gamma = \sqrt{[K(2S + K)]} \tag{3}$$

$$\varrho = \frac{\sqrt{K} - \sqrt{(2S + K)}}{\sqrt{K} + \sqrt{(2S + K)}} = \frac{K - \gamma}{K + \gamma} \tag{4}$$

The parameter ϱ, defined by eqn. 4, has an immediate physical meaning. It

represents the reflectance of the medium if its absorption is large enough to make the transmittance A_T negligibly small. For this reason ϱ is frequently termed the remission factor. At this point, it might be useful to point out that in this context the term "back scatter" would be more accurate than the customary terms reflection or remission. It can be easily verified that the diffuse reflectance A_R of a medium without scattering ($S = 0$) is zero. As a matter of fact, reflection taking place at the surface has to be discounted from the incident illumination. It obviously does not carry information about the interior of the medium although it may be affected by substances located very near to the illuminated surface, as well as by irregularities in this surface.

Inspection of eqn. 4 shows that ϱ is independent of the thickness of the medium. Fluctuations in the thickness and density of the medium are, however, an important source of optical noise in photodensitometric measurements[4]. Since the value of the reflectance A_R is to a large extent determined by ϱ, it might be expected that reflectance measurements are less affected by optical noise than transmittance measurements.

A physical interpretation of the coefficient γ is more involved. It can be considered as a measure of the loss of incident light intensity if the amount of back scattered radiation becomes negligibly small. Contrary to ϱ, the coefficient γ varies proportionately with the thickness of the medium. Since the transmittance A_T is largely determined by the term $e^{-\gamma}$, transmittance measurements might be expected to be more affected by optical noise than are reflectance measurements. This fact, together with the larger light intensity usually available in reflectance measurements (which reduces the importance of the electrical noise originating in the measuring electronics), would appear to represent an important advantage of reflectance measurements. In practice, however, this advantage is smaller than might be expected. With reflectance methods it is all but impossible to prevent part of the surface-reflected light to reach the photodetector receiving also the diffuse reflected signal. Fluctuations of this signal produce a noise signal which may be even stronger than any improvement in noise from thickness and density variations. For this reason, when measuring in the reflectance mode, all possible steps should be taken to reduce the amount of specularly reflected light capable of affecting the photodetector (polarising filters, suitable geometry of the optical system, even electrical compensation, etc.).

The different ways in which optical noise is generated in transmittance and reflectance measurements appear to preclude the cancellation of optical noise by forming the difference or ratio of $A_T(K)$ and $A_R(K)$[5]. A similar reasoning applies to fluorescence measurements. Measurements in the transmittance and reflectance mode are influenced by fluctuations of the optical parameters of the medium in different ways and can therefore be combined only to a limited extent for the cancellation of the residual noise in fluorescence measurements[6].

RELATIONSHIP BETWEEN CHANGES IN THE OPTICAL TRANSFER PARAMETERS AND CHANGES IN THE CONCENTRATION OF SEPARATED SUBSTANCE

In general, it can be assumed that the (spatial) concentration c of separated substance, provided it is not too high, does not affect the coefficient of scattering S, but only the absorbance K.

$$K = K_0 + c \cdot \eta \tag{5}$$

K_0 is, in this case, the absorbance of the blank medium and η the absorbance (co-efficient of extinction) of the separated substance; η is, in general, the change in transmittance or reflectance due to the concentration c.

$$\Delta A_T(c) = A_T(0) - A_T(c) \tag{6}$$

$$\Delta A_R(c) = A_R(0) - A_R(c) \tag{7}$$

NON-LINEARITY ERROR AND SENSITIVITY

As in most other measuring methods, a linear dependence between the measured parameter [in our case $\Delta A(c)$] and the investigated variable (in our case the concentration c) is highly desirable. The great advantage is that the system can be calibrated in terms of the measured magnitude alone and that a change in other parameters affects, at most, only the sensitivity (the calibration constant). If the response of the measuring system was non-linear in terms of the measured variable, individual calibration curves would have to be established for any change in the parameters of the system.

In chromatographic applications linearity is still more important than in most other fields. What is really required is not the concentration, but the total amount of separated substance Q in a given zone, and this, of course, is obtained by integration over the area F of the zone. If $\Delta A(c)$ is a linear function of c, this is an easy problem:

$$c = a\Delta A(c) \tag{8}$$

$$Q = \int_F c\,df = a \int_F \Delta A(c)\,df \tag{9}$$

The absence of the index at $\Delta A(c)$ indicates that the relation refers equally to transmittance and reflectance.

Inspection of eqns. 1–5 shows, however, that in general $\Delta A(c)$ is not a linear function of K and, therefore, of c neither. Simple integration as indicated by eqn. 8 produces, therefore, an error ε, which is the larger, the more $\Delta A(c)$ deviates from linearity.

It can be shown mathematically that an arbitrary optical transfer function $\Delta A(c)$ may be expanded into a series of the following type (Taylor series):

$$\Delta A(0) = 0$$

$$\Delta A(c) = A'(0){\cdot}c + A''(0){\cdot}\frac{c^2}{2} + \ldots \tag{10}$$

In this expression $A'(0)$ and $A''(0)$ represent the first and second derivatives, respectively, of $A(c)$, with respect to c at zero concentration. If $A(c)$ is a linear function of c, the term $A''(0)$ —and all higher derivatives of $A(c)$— are obviously zero. For values of c that are not too large it is possible to terminate the series after the second-order term without incurring excessive error. For very small concentrations, therefore, a linear approximation is always feasible.

Comparing eqn. 8 and the linear part of eqn. 10 yields

$$a = \frac{1}{A'(0)} \tag{11}$$

The non-linearity error ε can now be defined as the difference.

$$\varepsilon = \int_F \left[c - \frac{\Delta A(c)}{A'(0)} \right] df \approx \frac{A''(0)}{A'(0)} \int_F \frac{c^2}{2} df \qquad (12)$$

ε is the absolute error. In general the relative error is more important.

$$\varepsilon_R \% = 100 \cdot \frac{\varepsilon}{\int_F c df} \approx 100 \cdot \frac{A''(0)}{2A'(0)} \cdot \frac{\int_F c^2 df}{\int_F c df} \qquad (13)$$

In first approximation it will be assumed that the concentration c is constant over the whole area F of the zone. This permits eqn. 13 to be simplified:

$$\int_F c^2 df \approx c^2 F$$

$$\int_F c df \approx cF$$

$$\varepsilon_R \% \approx 100 \cdot \frac{A''(0)}{A'(0)} \cdot c \qquad (14)$$

Expressed in words, eqn. 14 states that the percentage error due to the non-linear dependence of the optical signal upon concentration is proportional to concentration and to the ratio of the second derivative of the optical energy transfer to the first derivative, both taken with respect to concentration changes. The first derivative $A'(0)$ determines the change in optical signal caused by an elementary change in concentration δc:

$$\Delta A(\delta c) \approx \frac{dA(0)}{dc} \cdot \delta c = A'(0) \cdot \delta c \qquad (15)$$

The larger $A'(0)$, the larger is the change in the measured optical signal due to the same change in concentration. $A'(0)$ determines, therefore, the sensitivity of the method. $A''(0)$ can be shown to be a measure of the curvature of the graph of the transfer function $\Delta A(c)$. The percentage error ε_R is, therefore, proportional to the ratio of curvature to sensitivity.

COMPENSATION OF THE NON-LINEARITY ERROR

In many cases of practical importance the non-linearity of $\Delta A(c)$ is so large that individual calibration curves become necessary. In addition, except for very low concentrations, the integration error ε_R may reach undesirably high values if the simple linear approximation to $\Delta A(c)$ shown in eqn. 8 is used. In these cases it is necessary to determine the inverse $\Delta A^{-1}(c)$ of the function $\Delta A(c)$, which is then used in the integration:

$$c = \Delta A^{-1}(c)$$

$$\int_F c df = \int_F \Delta A^{-1}(c) df \qquad (16)$$

In the general case the expressions 1 and 2 have to be inverted, that is, solved in terms of concentration. This is not a simple operation, not even when using a computer. However, the computer inversion of a two- to three-term polynomial approximation of the type shown in eqn. 10 can easily be implemented. It is not too time consuming and results in most cases in a negligible error.

Rigid inversion of eqns. 1 and 2 by analog techniques can also be carried out, in a not too complicated manner, by using the electrical transmission line model described in refs. 2 and 3. Neither method has, however, found so far widespread application in chromatographic practice.

Fig. 1 shows the transmittance $A_T(K)$ of a turbid medium plotted as a function of K; Fig. 2 shows the curve based on log $A_T(K)$. In both cases the coefficient of scattering, S, appears as parameter. Relation 5 gives the connection between absorbance and concentration. Comparison of the two graphs shows that the logarithm of $A_T(K)$ approaches a linear dependence over a much wider range than the original function. The logarithm of $A_T(K)$ can, however, be easily produced using a logarithmic converter ahead of the integrating stage (Fig. 3). This procedure, which can
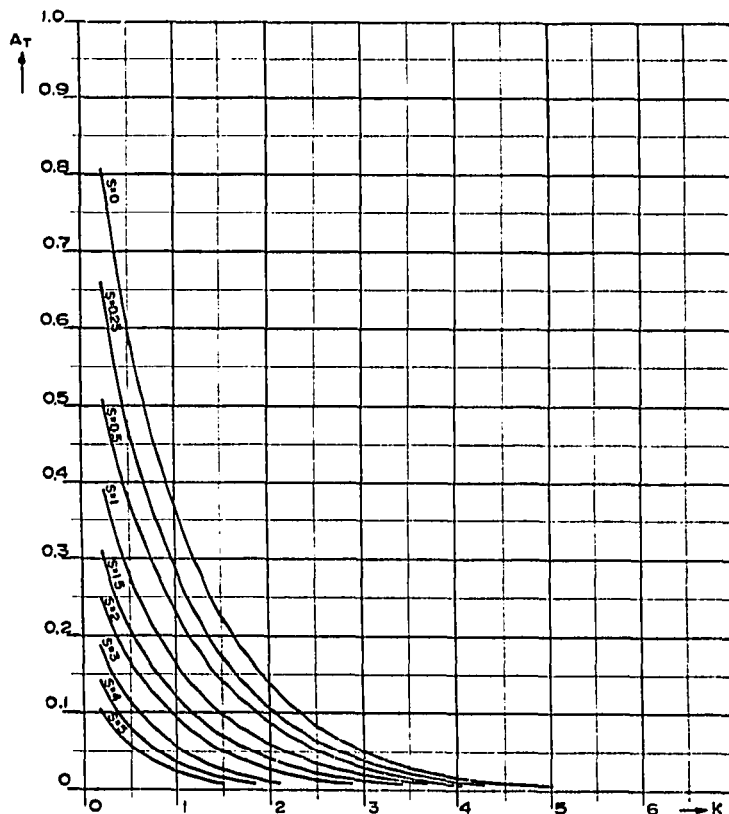
Fig. 1. The transmittance A_T (K) of a turbid medium as a function of absorption K with scattering power S as parameter (linear scale).
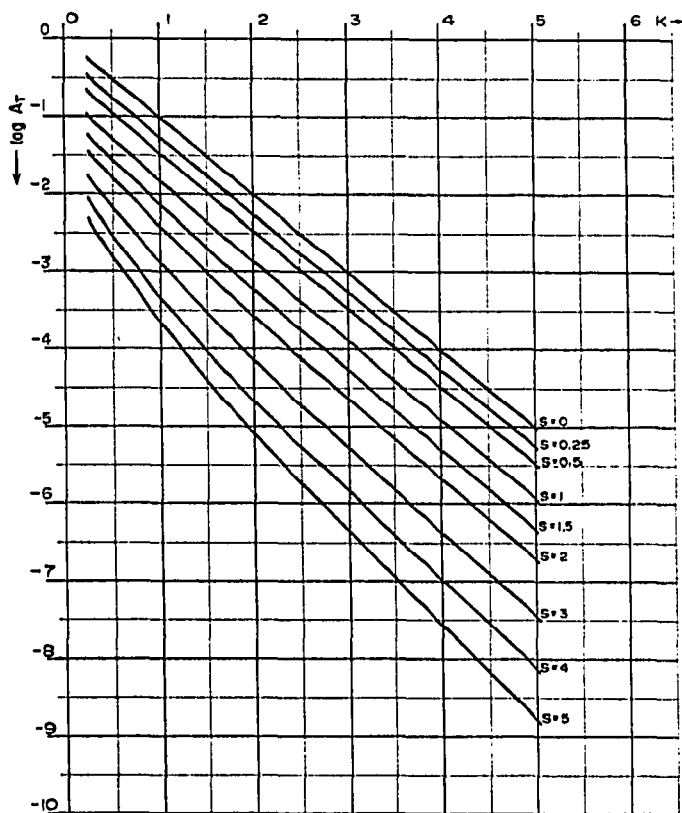
Fig. 2. The same as Fig. 1 but on a logarithmic scale. The right-hand scale is expressed in optical density units.

be carried out with reasonable ease and accuracy both digitally or by analog means, is very effective in reducing the nonlinearity error of the subsequent integration. It is less efficient in reflectance measurements (see Figs. 4 and 5). An inverse scale is here much more effective (see Fig. 6). With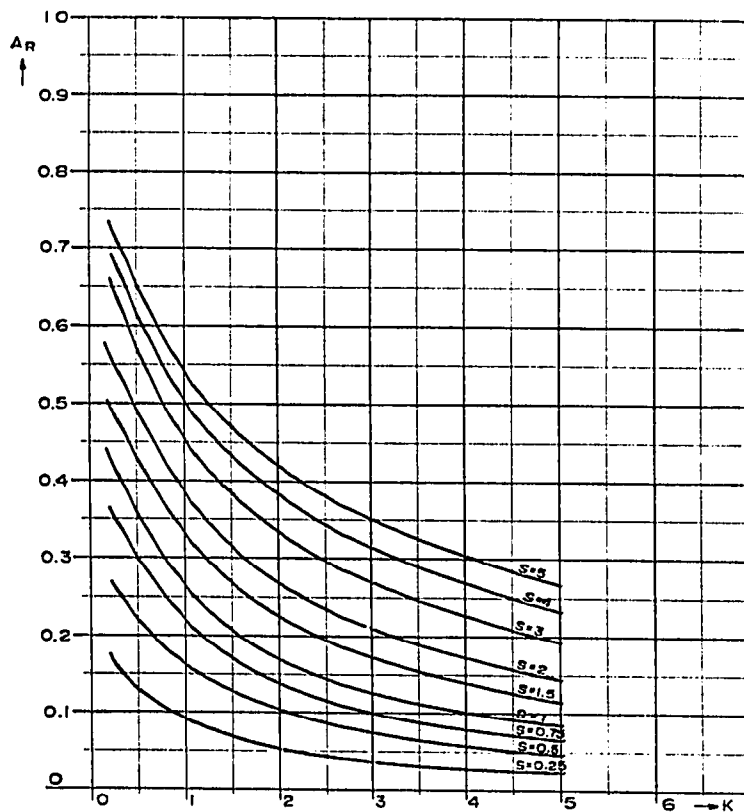 contemporary analog electronic circuitry, this scale can be easily implemented; digital implementation is equally easy.
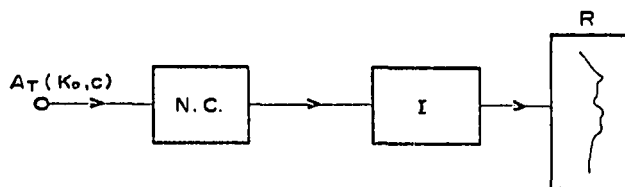
Fig. 3. Analog linearisation of the function A_T (K) using a non-linear (logarithmic, polynomial, square root or the like) converter. Block diagram (schematic). N.C. = Non-linear converter; I = integrator; R = recorder.

Fig. 4. The reflectance A_R (K) of a turbid medium as a function of absorption K with scattering S as parameter (linear scale).
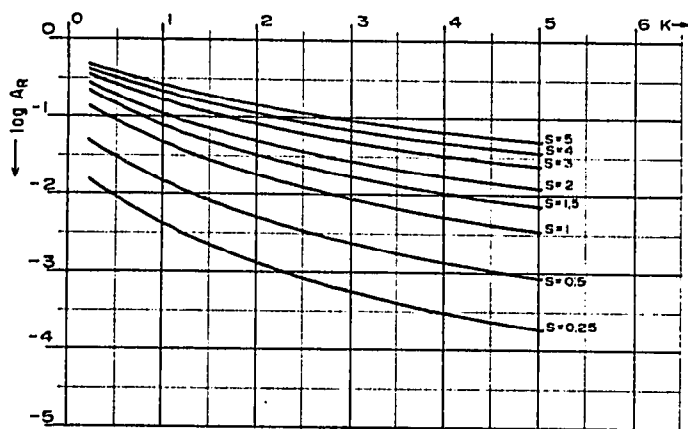
Fig. 5. The same as Fig. 4 but on a logarithmic scale.
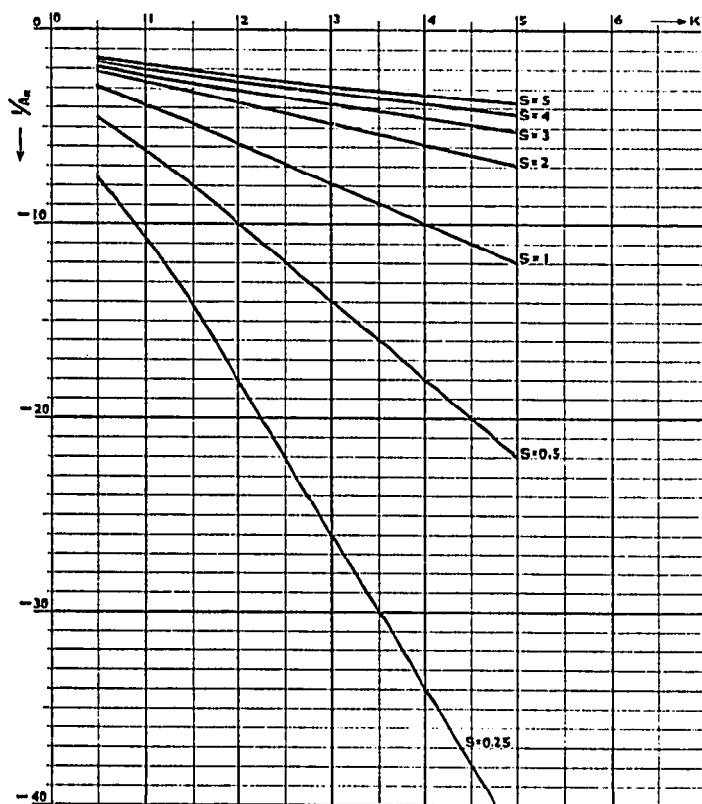
Fig. 6. The same as Fig. 4 but on an inverse scale.

FLYING SPOT SCANNING

It should be noted that, in general, the concentration c of separated substance varies both along the width and along the length of the medium. Almost any type of photodetector, however, produces a signal that is proportional to the total intensity of light impinging upon the sensitive area of the photodetector. This means that the photodetector performs a linear integration of the whole area it "sees". Applying correction procedures, such as simple logarithmic (for transmittance) or inverse (for reflectance) conversion, to the output of the photodetector is effective only if the output signal has not yet been affected by an integration error. Application of the solute in a uniform narrow band across the width of the medium improves the uniformity of the transverse concentration profile; some residual variations in concentration across the width of the medium, however, cannot be avoided. The best way to keep the integration error arising in the photodetector element small is to keep the area of the zone seen at any one instant of time by the photodetector so small that the concentration in this area can always be considered as constant. This requirement of necessity leads to the flying spot system, which in this respect is vastly superior to the common fixed slit system. The linearisation procedures mentioned above and demonstrated
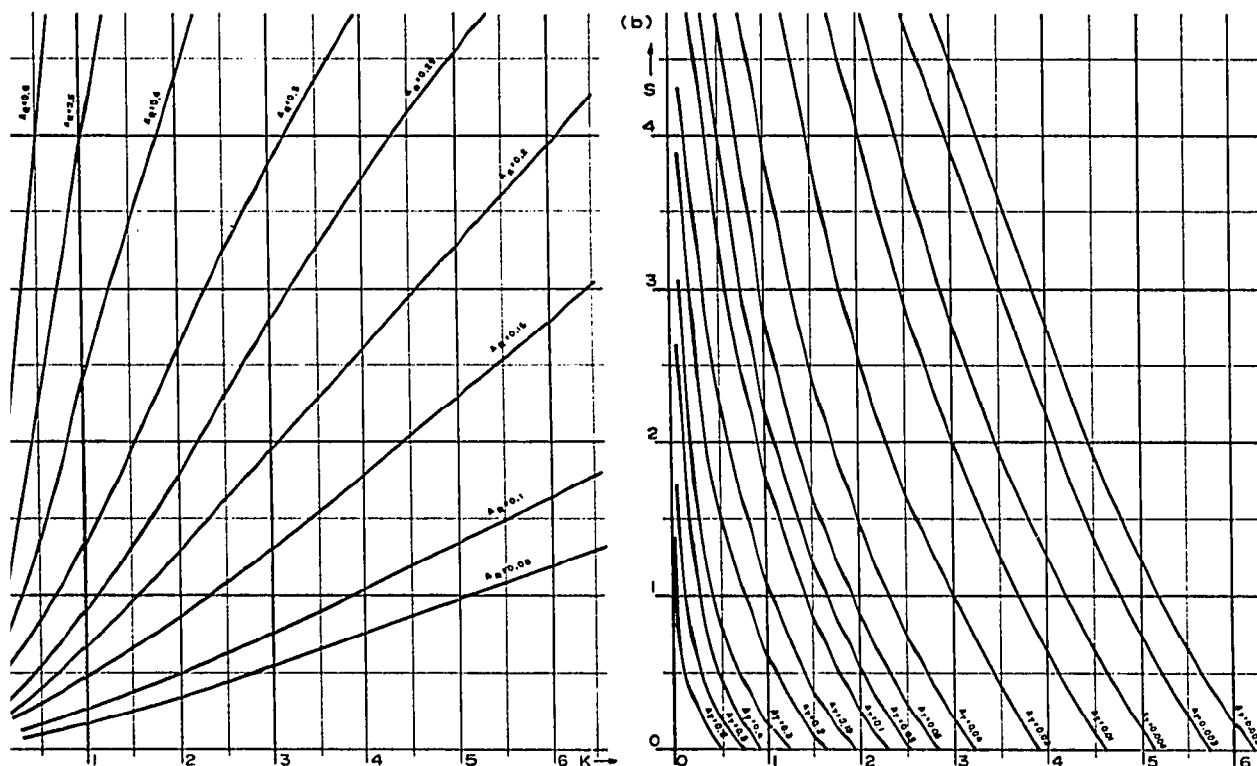
Fig. 7. Curves of (a) equal transmittance, A_T (S, K) = const., and (b) equal reflectance, A_R (S, K) = const., in dependence upon the coefficients S and K of the medium. (To be used for the determination of S and K_0 from transmittance and reflectance measurements on the blank medium.)

in the curves shown in Figs. 2, 5 and 6 are important especially in connection with flying spot scanning.

GRAPHICAL REPRESENTATIONS

What has been said above is illustrated in Figs. 1–6. The curves shown were plotted using a computer to calculate the corresponding values from eqns. 1 through 4. To apply them to any given medium the characteristic constants S and K_0 have first to be determined. This can be done in several ways. The simplest approach is to measure both the reflectance $\Delta A_R(0)$ and the transmittance $\Delta A_T(0)$ of the blank medium. These measurements can be performed on most photodensitometers used in chromatography after suitable calibration. Fig. 7 can then be used to determine the
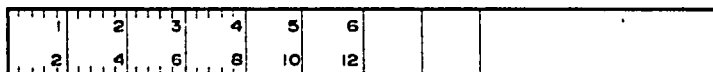

Fig. 8. Cursor with two scales for the determination of S and K_0 from measurements on media with single and double thickness.

constants S and K_0 of the medium; they are obtained as the coordinates of the point where the curves for the measured values of $\Delta A_R(0)$ and $\Delta A_T(0)$ intersect.

From Fig. 7 it can be seen that the method described gives reasonably accurate values of S and K_0 only for a limited range of parameters. Outside this range satisfactory results can be produced by a modification. In this case either the transmittance $\Delta A_T(0)$ or the reflectance $\Delta A_R(0)$ is measured twice: once on a single layer of medium and then on two layers stacked together face to face. A cursor with two scales, the step size on one scale being twice that used for the other one (see Fig. 8), is pivoted around the origin of Fig. 7 until equally marked points on both scales intersect the curves corresponding to the measured values of A_T or A_K for single and double thickness, respectively. The coordinates of the point of intersection with the curve for a medium of single thickness are then the required coefficients S and K_0. By choosing the appropriate transfer function (A_T or A_R) this method produces usually acceptable results, provided the optical density of the medium is not too high. Errors may be introduced by surface reflection at the contact surface of the two layers.

The parameter S of the medium is then used to determine the appropriate curve from the family of curves shown in the graphs. K_0 determines then the origin on this curve for the determination of changes in absorbance due to the presence of
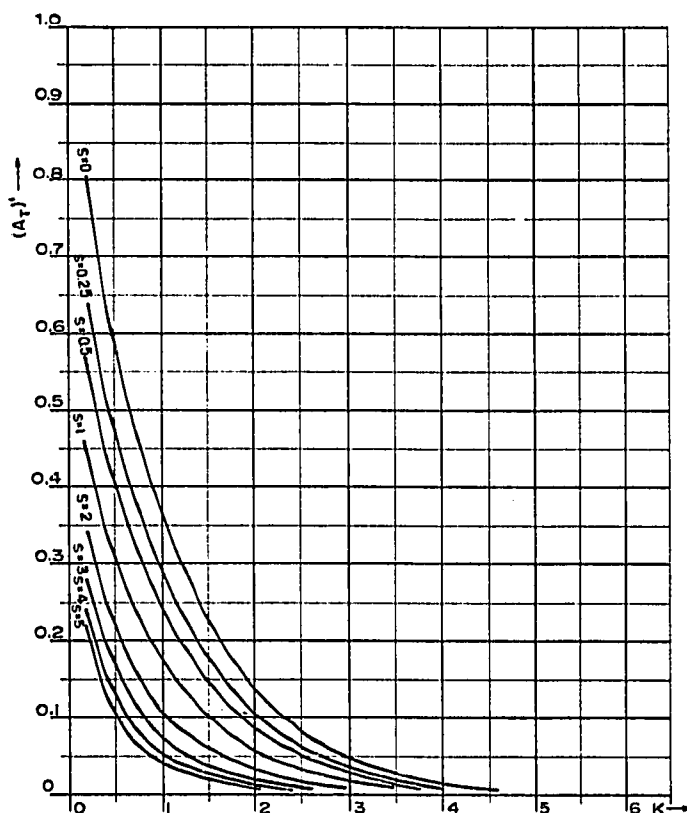

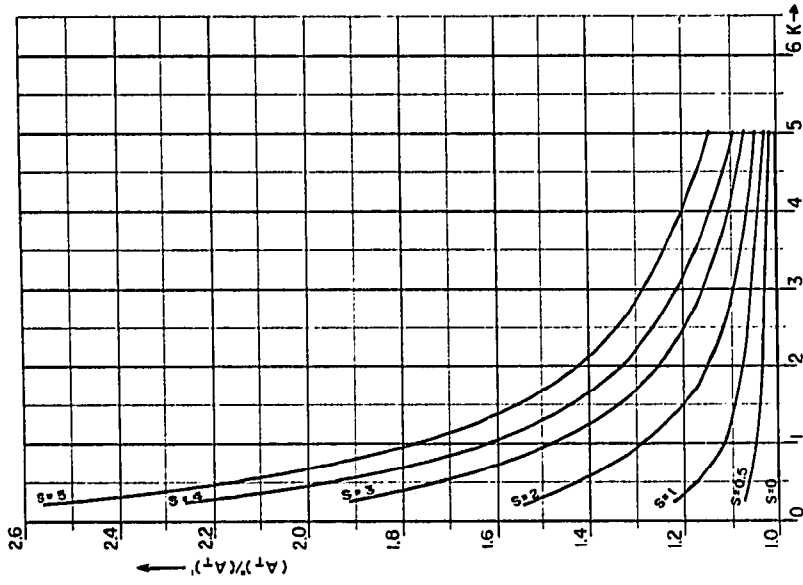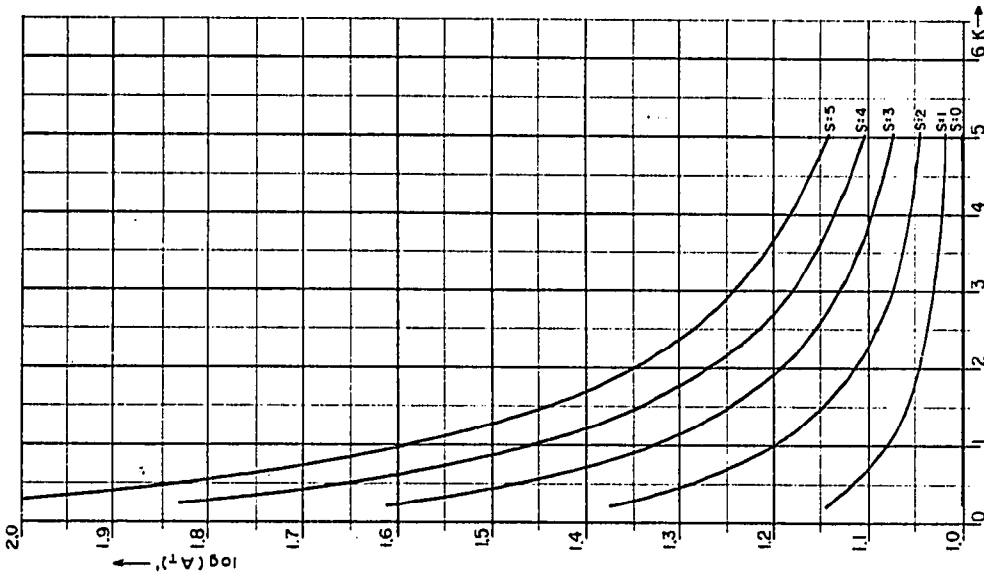Fig. 9. Graph of the sensitivity —first derivative of A_T (K)— of transmittance measurements.

Fig. 10. The same as Fig. 9 but on a logarithmic scale.

Fig. 11. Graph of the non-linearity of the transmittance characteristic defined as $A_T (K)''/A_T (K)'$.
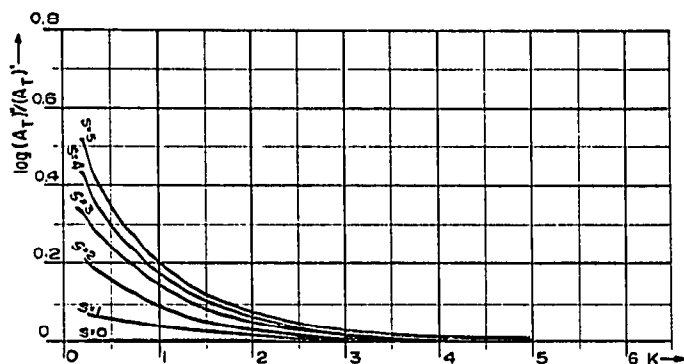
Fig. 12. The same as Fig. 11 but with $A_T (K)$ measured on a logarithmic scale.

separated substance. The same procedure applies to graphs 9–18 which indicate the sensitivity and non-linearity of the method when using a particular functional transform of $A(K)$. The relation between an increment c in concentration and the resulting change in absorbance is determined by eqn. 5.

Fig. 9 shows the sensitivity (the first derivative) of transmittance measurements. Fig. 10 indicates the non-linearity of the results obtained with respect to an increment in absorbance; the non-linearity is here defined as the ratio of second to first derivative both with respect to K. It should be remembered that it is this ratio which appears in eqn. 12 for the integration error. Fig. 11 shows the non-linearity when logarithmic conversion is used.

Fig. 12 shows the sensitivity of reflectance measurements and Fig. 13 indicates the corresponding non-linearity defined in the same way as above. Fig. 14 shows the non-linearity if an inverse transform is used. It can be seen that an inverse scale
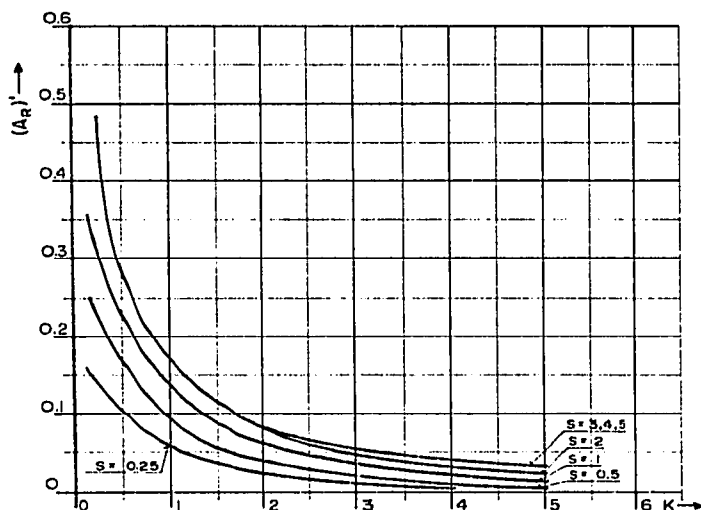

Fig. 13. Graph of the sensitivity $[A_R (K)]'$ of reflectance measurements.
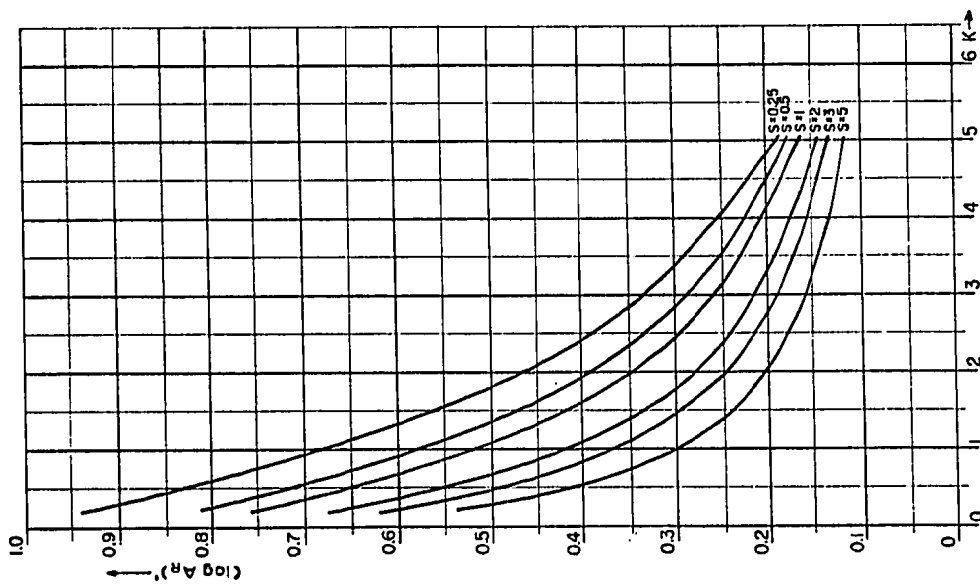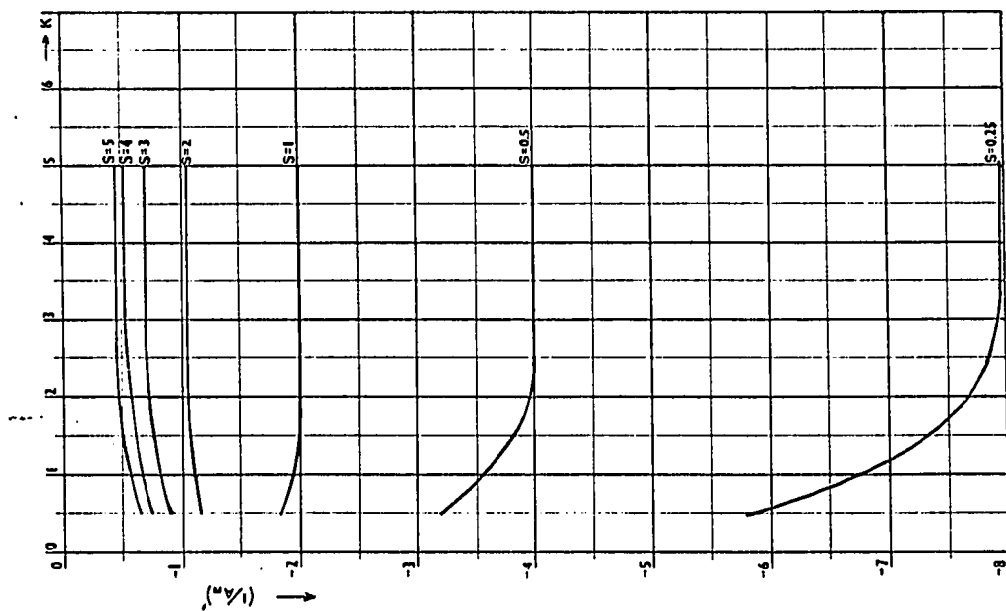
Fig. 14. The same as Fig. 13 but with $A_R(K)$ measured on a logarithmic scale.

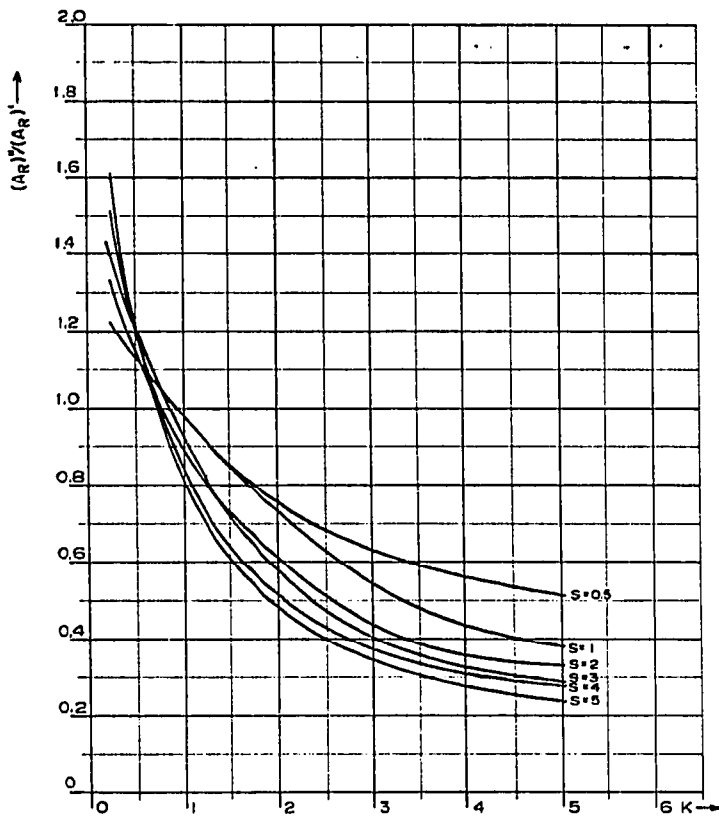Fig. 15. The same as Fig. 13 but with $A_R(K)$ measured on a reciprocal scale.

Fig. 16. Graph of the non-linearity of the reflectance characteristic defined as $A_R(K)''/A_R(K)'$.
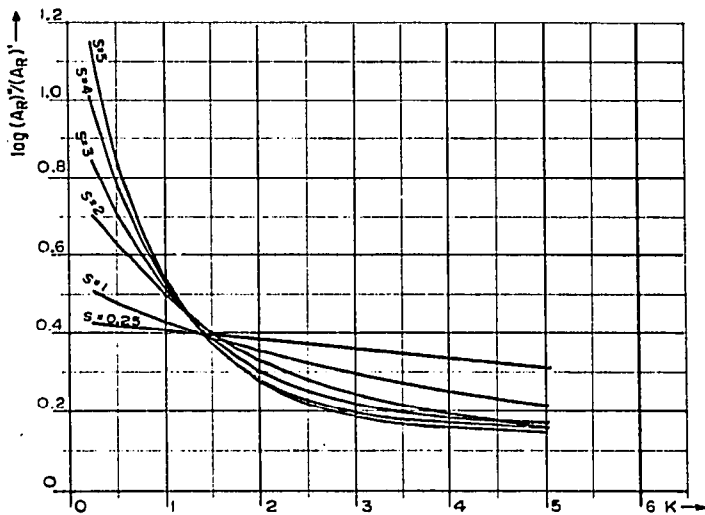

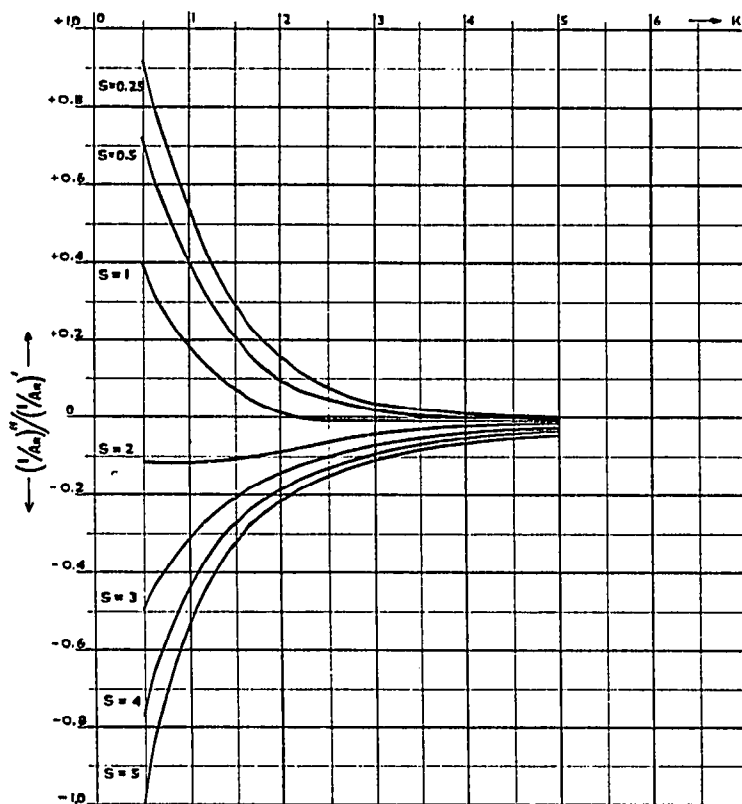Fig. 17. The same as Fig. 16 but measured on a logarithmic scale.

Fig. 18. The same as Fig. 16 but for A_R (K) measured on an inverse scale.

improves the linearity of reflectance measurements substantially making it comparable to that of transmittance measurements using logarithmic conversion.

DISCUSSION

The principal conclusions arrived at in this paper follow from a comparison of the graphs shown for transmittance and reflectance measurements.

For good accuracy and low concentration thresholds the first derivative of the measured optical transfer function (transmittance or reflectance) with respect to absorbance should be large. It determines the amplitude of the useful signal at a given level of illumination and characterises, therefore, the sensitivity of the system. The better the sensitivity, the lower the concentrations which can be measured before the electrical noise of the photodetectors comes into appearance. In a separate paper it will be shown[9] that the sensitivity is of less importance for the optical signal-to-noise ratio, mainly when the double-beam system is used. Comparison of Figs. 9 and 12 shows that media with low intrinsic absorbance and scatter provide the best sensitivity for transmittance measurements.

Reflectance measurements yield the highest sensitivity if the coefficient of

scattering is large but the intrinsic absorption small, resulting in a medium with low to moderate optical density. At high values of optical density both methods have approximately equal sensitivities. Reflectance yields, however, a stronger signal (see Fig. 4) and is, therefore, less susceptible to electrical noise. At very high optical densities, transmittance measurement may be altogether unfeasible and reflectance is then the only choice.

The linearity of both straight transmittance and straight reflectance measurements is quite poor as evident from Figs. 10 and 13.

The primary magnitude measured by the photodetector is the optical transfer function A_T or A_R. If the signal-to-noise ratio of this signal is sufficiently high, the acquired data can be subjected to a suitable transform in order to improve the linearity of the output with respect to an increment in absorbance. Of the simpler and relatively easy to implement transform operations available logarithmic conversion appears to be most suitable for transmittance measurements and the inverse transform $(1/A_R)$ for reflectance methods.

For transmittance measurements logarithmic conversion provides the best linearity for media with only little scattering (see Fig. 10). For media with appreciable scattering the best linearity is obtained if the medium has also moderate intrinsic absorbance, K_0.

Reflectance measurements using the inverse transform have nearly ideal linearity for almost any medium and, from the point of view of linearity, appear, therefore, to be superior.

Sensitivity and linearity turn out to be to some extent conflicting requirements. However, when very low concentrations are to be measured, both transmittance and reflectance can be considered to be virtually linear in terms of concentration, since the higher terms in the series expansion of eqn. 10 can be neglected. Transformation is then unnecessary and may even deteriorate the signal-to-noise ratio. The most important parameter is then the sensitivity of the method. Many of the commonly employed chromatographic media have relatively low optical density and moderate scattering; transmittance measurements should in these cases yield somewhat superior results.

At larger concentrations sensitivity becomes less important and linearity is then the most important factor. Reflectance measurements with inverse transform have then a slight advantage. An exception are separations on media with low optical density and little scattering, where transmittance with logarithmic conversion becomes equal or even superior to reflectance.

Altogether it appears that for most media used in thin-layer chromatography transmittance and reflectance measurements produce comparable results and, in the general case, neither method has a decisive superiority. It should be noted that transformation of the primary signal to improve linearity has to be performed before integration. The conclusions pointed out above apply only to straight transmittance or reflectance measurements, not however to methods based upon fluorescence, which provide results which are over a wide range intrinsically linear with respect to concentration.

In cases where the simple logarithmic or inverse transforms of the received signals are not satisfactory, there remains still the possibility of actual inversion either using an analog model[2] or by computer solutions of a series expansion of the basic

equations. The latter approach is particularly useful when deviations from the theoretic expressions are observed because it can be applied to empirically found curves. The availability of powerful low-cost computers had made this solution practical also for institutions with moderate budgets.

ACKNOWLEDGEMENTS

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