ASPECTS OF THE DIRECT PHOTOMETRY OF SUBSTANCES DISTRIBUTED ON TRANSLUCENT SHEETS

J. W. H. LUGG

Department of Biochemistry, University of Western Australia, Perth (Australia)

INTRODUCTION

Provided that reasonably complete resolutions of mixtures of substances can be effected on sheet material (zone-electrophoretically, chromatographically), that the individual substances can be located and identified, and that either they themselves or something with which they have reacted or the two together can be eluted satisfactorily from excised portions of the sheet, then circumstances exist for the quantitative estimation of the individual substances. And the circumstances suffice whether the resolution can or cannot be conducted in a closely reproducible way.

The current biochemical literature furnishes various examples of the use of methods of this type. However, a desire to reduce the time and equipment involved by eliminating the elution step has led to the development of many methods involving direct photometry (transmission, reflection) of the substance bands on the sheets, only a limited number of which have proved satisfactory, however.

It is the purpose of this communication to recount an analysis of the difficulties which appear, hitherto, to have militated against the achievement of success in work of this type, and of means which might be adopted to surmount these difficulties and to place the photometry on a footing more nearly comparable with that prevailing in work with solutions (LUGG AND MCEVOY-BOWE[1]). Although transmission methods will be considered primarily here the conclusions have implications for reflection methods also, and these will be indicated later.

In "one-dimensional" resolutions, with the mixture to be resolved streaked uniformly on a starting line, the bands will normally move with but little lateral spreading, a small defect which can be eliminated by carrying the starting line along the full width of a strip of sheet. Provided that the sheet material is of uniform thickness and of fine texture, and that the absorption of light by the substance is in effect in accordance with Beer's law in respect of "concentration" (density in the sheet), then any such clearly separated band may readily be submitted to scanning with a slit photometer of suitable design, with integration for the number of slit-frames required to cover the band. The resolution does not need to be conducted in a closely reproducible way if the photometry can be conducted in an ideal way. Any overlapping of bands, however, must obviously lead to some uncertainty about values.

With "two-dimensional" techniques the resolution is much higher and can usually be made much more complete. However, with sheets of uniform thickness and fine texture, and the most favourable conditions for reproducible chromatography

prevailing, the distribution of substance in a band about the position of maximum density will yet not, in general, be radially symmetrical nor will it be of the same form for all bands, even if the initial spot has been applied uniformly as a disc of definite diameter to the sheet. It can readily be shown on optical grounds that, even if the absorption of light by the substance in different parts of the band conforms in effect with Beer's law in respect of density and the photometry can be conducted in an otherwise ideal way, yet the integrated value obtained by slit-scanning such a band will vary with the orientation of the band relative to the direction of traverse of the slit.

And so, even if the resolution can be made reproducible, there remains the problem of ensuring reproducible orientation in the slit-scanning photometry, and in any event the abandonment of any hope of establishing other than an empirical relationship between the integrated photometer results and the quantity (Q) of that substance in the initial spot, irrespective of the possible existence of a formal relationship between Q and the density of substance in a specified part of its band. For these reasons the slit-scanning of such bands cannot be favoured: the so-called "maximum density" type of photometry holds far more promise.

The photometer (densitometer) must, however, have a circular aperture and the response of the photocell to light must be uniform across the exposed portion of its face, if the reading is to be independent of the orientation of the band in the instrument. We (LUGG AND MCEVOY-BOWE[1-3]) have exclusively employed instruments of this type, in which also the response has been closely proportional to light flux.

CHOICE OF CHROMATOGRAPHIC PROCEDURE: IDEALISED CONDITIONS

However regular may be the construction of an actual sheet the distribution of centres of specific attraction (Van der Waals and electrostatic type) is unlikely to exhibit the degree of regularity desirable for the purpose under consideration. That is to say, liquid–liquid partition rather than adsorption or ion-exchange should determine the R_F value. Again, it is with this type of partitioning that the distribution coefficient of the substance will usually vary least with concentration. Correspondingly, if the cross-sectional thicknesses of stationary and mobile phase do not vary over the sheet, the more likely is the relationship between Q and the maximum density of substance in its band to be linear.

The plain fact is that every substance is distributed in a two-dimensional chromatogram over the entire sheet beyond the position of first contact with each of the two mobile phases in turn, up to the limit of excursion of these phases. The confines of a band, however sharply defined they may appear to be, are merely the limits of detectability under the detection criteria in use. The maximum density, however, has a position and a value, related indeed to the sheet-wide overall distribution, but instrumentally determined by a single reading and requiring no precise information about the sheet-wide distribution

For ideal liquid–liquid partition chromatograms on sheets of fine structure and uniform gross thickness the overall distribution might be computed from MARTIN AND SYNGE'S[4] theoretical treatment of the column partition chromatogram for substances having constant coefficients of distribution between the two liquid phases, if the cross-sectional thicknesses (areas, per unit width) were known for stationary

phase and mobile phase. The computation requires that due allowance be made for diffusion (diffusion coefficients in both phases being assumed constant) which, in the direction of the "run" has the effect of increasing the "height-equivalent of the theoretical plate" and which, normal thereto, leads to the lateral spreading of the bands. The effect can be computed by analogy from the well-established equation for the loss of heat by conduction from a source (see also BRIMLEY[5]). There is also the effect of micro-scale angle-channelling of mobile phase between the textural elements of the sheet upon lateral spreading, but the channelling could reasonably be assumed independent of the (low) concentration of substance.

Under these conditions the quantity of substance through which the light is regarded as passing is directly proportional to Q, neglecting the minute contributions from the attenuated parts of other bands and the (usually) far more important contribution of the "blank" (the total being the "background"). Reasonable allowance for the "background" can be made by subjecting a portion of the sheet remote from visible limits of bands to the photometry.

IDEALISED TRANSMISSION PHOTOMETRY OF BAND AND BACKGROUND

Consider the transmission photometry of a substance in solution contained in a cuvette. Of the monochromatic incident light flux (IL) some (RL) is reflected back from the interfaces and irretrievably lost. Of the balance, which we may call the "entering" light (EL), a portion (AL) is absorbed and the rest, the transmitted light (TL), reaches the photocell and is measured:

$$EL = IL - RL = AL + TL \qquad (1)$$

If, as is usually the case, the absorption of entering light by materials in the light path is governed by Lambert's law in respect of thickness, if the absorption by the substance is governed by Beer's law in respect of density, if optical densities are additive, and if due correction can be made for the blank, then the concentration of the substance can be estimated accurately if the entering light can be maintained constant. But, whereas the constancy of IL can be assured readily enough, EL must be presumed to vary with substance concentration to some extent if IL is constant. This variation is customarily ignored, but it is of interest that, such as it may be, the variation is in the direction which would account, at least in part, for the commonly observed departure from Beer's law.

In both the strip-scanning and maximum density types of transmission photometry of bands on a sheet, RL is usually a large, not small, fraction of IL, so that even a relatively small variation with substance density can seriously affect EL when IL is constant. Any attempt, therefore, to employ the classically derived relationship:

$$Q = kD = k \log (1/T) \qquad (2)$$

where D is the optical density of the band material, T being the transmittance relative to the sheet, and k a constant, requires that the reflected-back light be restored. This can be done conveniently in one or both of two ways which will be discussed later.

WORK WITH ACTUAL SHEETS

Our work has been confined to so-called "single-phase" two-dimensional chromatography of amino acids[1-3] and of sugars[6] on Whatman No. 1 filter paper sheets, colour development being conducted under conditions designed to yield colour substances in quantities proportional to those of the parent substances. With amino acids, ninhydrin and moist chlorine gas were colour and bleaching reagents, respectively. The evidence is that these single-phase solvents behave as conventional solvent pairs in association with the fibre (see MARTIN[7]), and the preliminary assumption is, then, that the chromatograms are ideal within the limitations imposed by the deficiencies of the sheets.

These sheets are of fibrous cellulose and their structure is depicted in Figs. 1–3. This evidence and information obtained by weighing various areas of sheet indicate that individual sheets are of appreciable uniformity in respect of gross physical thickness, but that there is some variation between sheets of a batch, and that the textural irregularities are not notably fine in relation to the magnitude of the instrument aperture (3 mm diam.). Thus, photometry of a remote part of the sheet could not be assumed to yield an adequate estimate of the optical density of sheet plus background in the region of minimum transmission of the band.

A solution of this problem was sought by measuring the transmissions in the two places on excised portions of the sheet, bleaching the portions and returning them in precisely the same positions and orientation for re-measurement. A microscope mechanical stage attached to the instrument is used for the purpose. From these values the transmittance of the band material relative to the sheet could be computed much more reliably than was otherwise possible, the variance of the mean with replicate chromatograms being reduced to about one quarter of what was otherwise attainable.

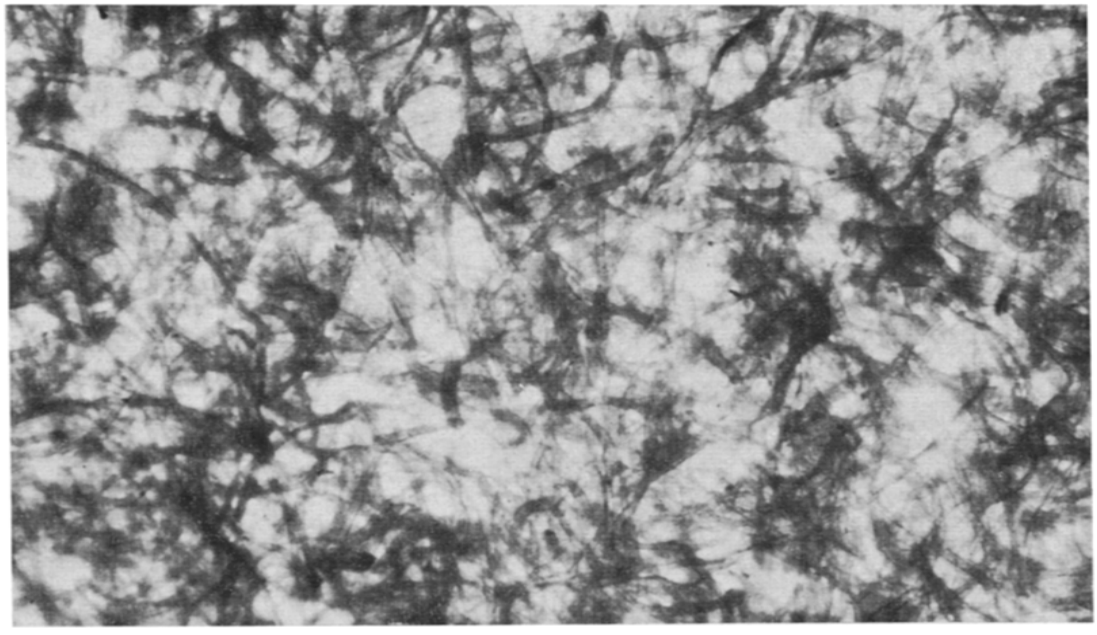

Fig. 1. View into Whatman No. 1 filter paper, with light source below. Focus half-way through the paper. Mag. (paper to photo) × 40.

Fig. 2. View into Whatman No. 1 filter paper, with light source below. Focus almost superficial. Mag. (paper to photo) × 200.

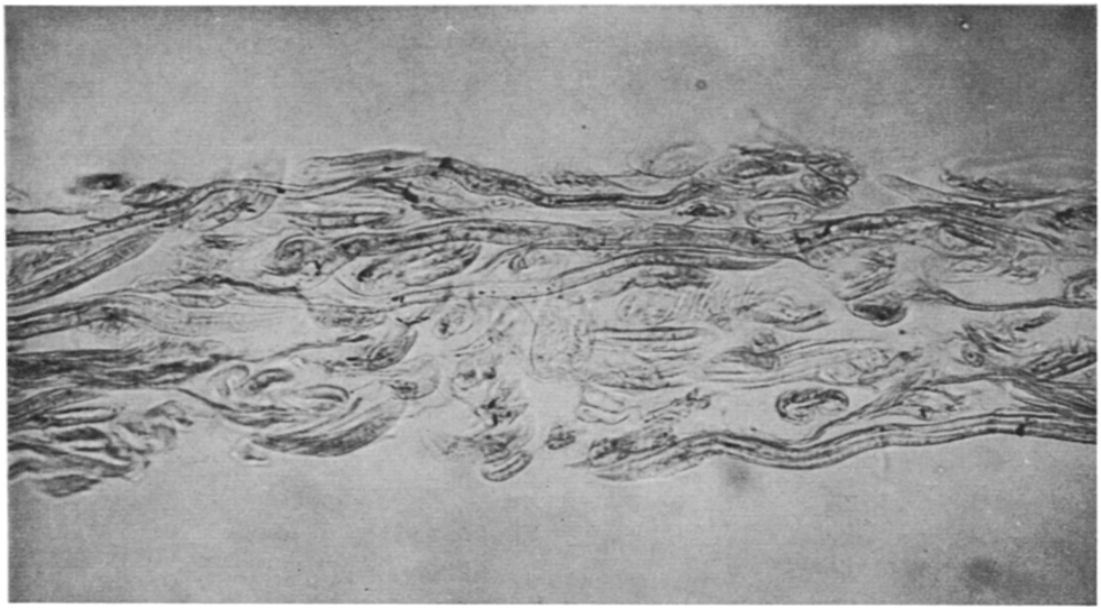

Fig. 3. Cross-section (10 μ thick, wax-embedded, wax removed) of Whatman No. 1 filter paper. Note some shrinkage of fibre due to embedding. Mag. (section to photo) × 200.

As the transmittance so computed is still in respect of constant incident light instead of constant entering light it is under-estimated to a degree which increases with substance density, and the correction for background is likewise somewhat defective. Equation (2) could therefore not be expected to hold, even if monochromatic light were used in place of the near-white light actually employed in our work. The value, however, could be used with a calibration curve and, within fairly wide limits, was found to be reasonably satisfactory also for use in a semi-empirical equation:

$$Q = k_1 [(1/T) - 1] \quad (3)$$

where k_1 is a constant.

Close control of the chromatography and of the colour development, coupled with this photometry refinement, yielded means of triplicate assays of amino acids which were as reliable[3] as the means of about one hundred replicates in the procedure recommended by BLOCK, DURRUM AND ZWEIG[8].

FURTHER CONSIDERATIONS

With the advances related above we have thus far been satisfied in applied studies, despite the appreciable empiricism still plaguing the photometry and the fact that eqn. (3) could be far less valid with other types of sheet. The challenge has been taken up, however, in further study and in the designing of instrument accessories.

A presumed (in effect) validity of Lambert's law for "thickness of sheet" in respect of entering light can be tested by measuring the transmitted light with increasing numbers of sheets in contact, incident light being kept constant and the reflected-back light being absorbed by the black enclosure. For sheets of Whatman No. 1 paper beyond the third (and so, for part but not all of the third itself) accordance with the law has been found excellent, near-white and green-filtered light being used in this work; and we conclude that the flux of reflected-back light has by then become virtually constant. In passing it should be mentioned that the term "thickness of sheet" in the above context must signify "mean density of sheet material in the region for unit thickness, times the number (including fractional number) of thicknesses considered".

The observation furnishes a simple solution of the problem of ensuring constancy of the entering light—simply interpose several thicknesses (or their optical equivalent in other material) as backing sheets between the source and the test sheet and as close to it as practicable.

An alternative solution is to collimate the incident beam and use a large almost hemi-spherical reflector with its centre of curvature at the site of incidence of the beam on the sheet—nearly all the light could be restored in summation of an infinite series of successive reflections from and restorations to the sheet. Such a reflector would make the instrument cumbersome. A smaller reflector is less efficient but, used with only a few backing sheets, achieves a satisfactory result. Interposition of the backing sheets reduces the sensitivity of the instrument, but compensation for this can be made by inserting a Perspex light conductor in the collimator tube.

The instrument and its accessories have been described fully elsewhere[1] and need not be detailed here. Figs. 4 and 5 show the main body of the densitometer with the lamp-house detached and folded back to reveal the position of the collimator-reflector

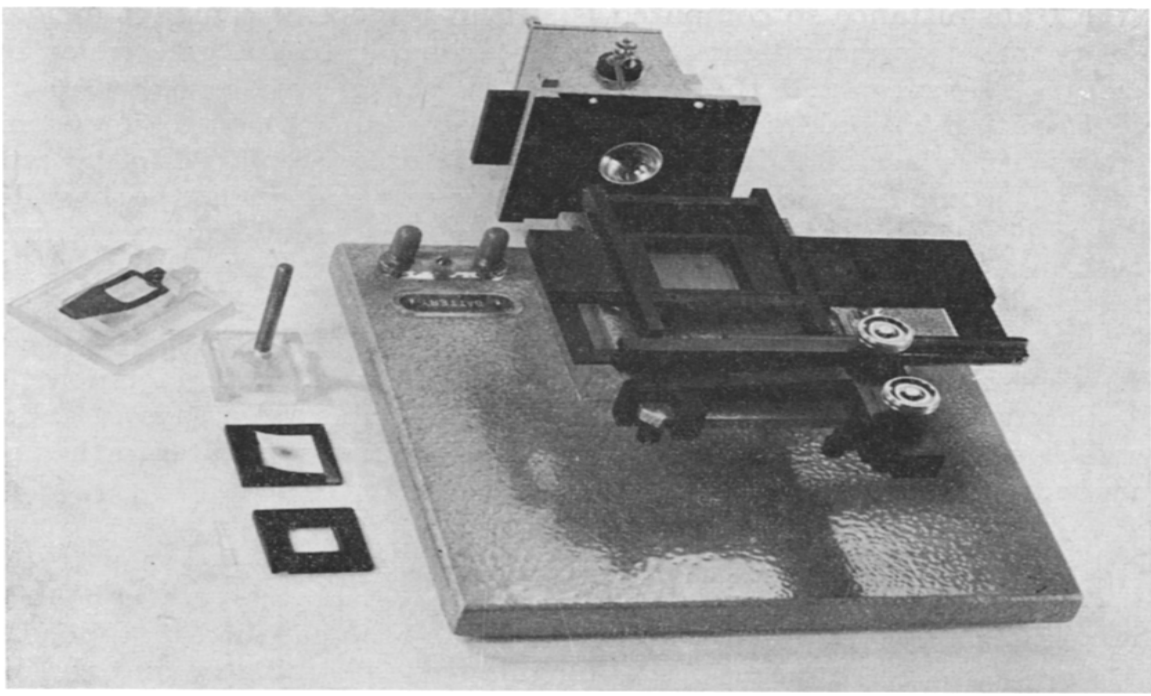

Fig. 4. The densitometer, with the lamp-house folded back to show the collimator-reflector assembly. A sheet-portion is shown on one (inverted) of the sheet-holders at the left.

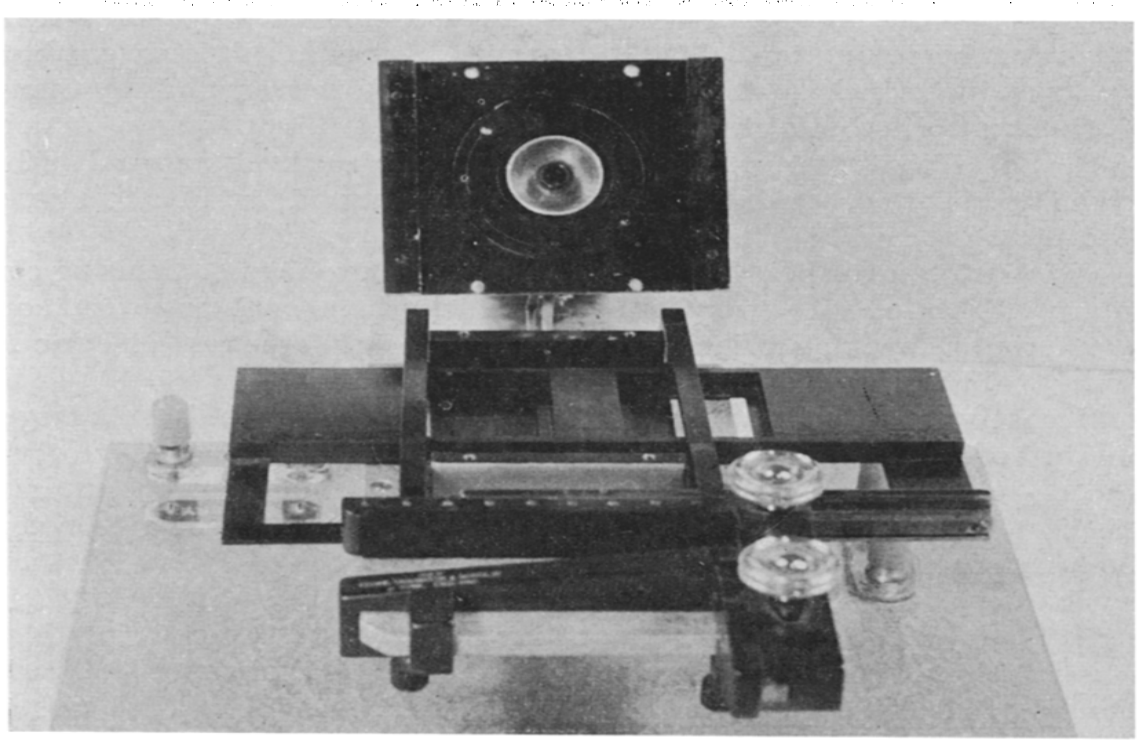

Fig. 5. Densitometer, showing microscope mechanical stage fitted to carrier, and with the lamp-house folded back to reveal the collimator-reflector assembly in its base.

assembly in the base. At the left hand side of Fig. 4 are shown the creaser and stamper (the crease ensures positional replacement of a sheet portion after bleaching), an inverted sheet-holder with sheet portion attached, and an unloaded sheet-holder with its securing wedge at its side.

This model differs from the earlier one[1] in that the slide to carry a diffuser and/or colour filter has been raised to just beneath the lamp and just above the top of the collimator, thereby obviating the need to insert diffuser and/or filter in a collimator well.

To some extent the nature of the textural irregularities might affect the density of chromatographed substance in and upon the fibre there. If, for example, the irregularities were in respect of fibre density for uniform physical thickness of sheet the cross-sectional thicknesses of mobile and stationary phase would not be in as constant ratio minutely over the entire sheet as would be expected if the fibre density were minutely uniform, the irregularities being only in local physical thickness of sheet.

In the latter of these extreme cases the amount of substance in the light path would be deemed proportional not only to Q but to the amount of fibre there. Equation (2) might be expected to apply more closely then, if the term on the right were multiplied by the ratio of the mean thickness over all the sheet to the mean thickness at the photometer aperture. An approximation to this factor could be computed from the photometer readings for sundry bleached portions of the sheet. The prospect is of potential importance in that the variability of replicates might thereby be substantially reduced.

In the former of these two extreme cases the amount of substance in an irregularity would not be proportional to the amount of fibre there. A possibility is that its distribution at the aperture might be rather uniform, with eqn. (2) applying fairly closely.

The photomicrographs of the paper we used suggest, perhaps, that the irregularities are somewhat of a mixture of the two extreme types considered above. It can readily be shown that for sheets of grossly uniform texture and physical thickness the constant, k, in eqn. (2) or its variant should be constant for small variations in physical thickness between individual sheets of a batch, if the assumptions concerning the chromatography are valid. The soundness of this important principle is not dependent upon the nature of the (small) irregularities[1].

The data with which eqn. (3) was found to accord fairly closely for a ten-fold range of Q were, in fact, fairly well represented by eqn. (2) for much smaller ranges of Q. Although systematic studies of the same type have not been made with provision for restoration of reflected-back light, existing data[1] make it plain that such provision would greatly increase the range of Q for which eqn. (2) might apply fairly closely. Indeed, with monochromatic (if not even with appropriately filtered) light, eqn. (2) could, in the author's opinion, be used to summarize data with many different types of sheet material.

Just how it comes to pass that Beer's law should show such promise of being valid (in effect at least) is a matter of conjecture. The "in effect" qualification might merely be an aspect of real validity if the substance is wholly dissolved in sorbed moisture on the sheet material. Indeed, we have recently found[9] that in drier environments eqn. (3) is reasonably applicable only up to somewhat lower levels of Q.

With the development of newer types of sheet of fine and uniform structure the

irregularity problem may be so reduced as to make the bleaching and repositioning of sheet portions unnecessary in some work. Indeed, much has been claimed for the slit-scanning of bands on granular-textured cellulose acetate strips[10] after the state of the sheet material has been changed from translucent to virtually transparent with the aid of organic solvents. However well-justified the claim may be, it is questionable that successful "maximum density" work could be done with sheets so treated: if the sheet thickness and with it the condition and/or state of aggregation of the substance were to vary capriciously, more could be lost than gained in so simple a solution of the reflection-back problem.

The problem posed by "reflection-back" in the transmission photometry of substances on translucent sheets has as its logically obvious counterpart a "transmission-forward" problem in reflection photometry. Analogous solutions are, of course, to be found in the use of backing sheets and/or reflector on the side of the test sheet distal to the light source, but a plane reflector against the test sheet (or backing sheets, if used) is required. Such reflection photometry may have advantages over the transmission type if the sheet transmission itself is very low.

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SUMMARY

In the direct photometry of substances resolved on translucent sheet electrophoretograms and chromatograms textural irregularities in the sheet may be responsible for pronounced variability of replicates, and loss of reflected-back light in transmission work or of transmitted-forward light in reflection work can result in serious departure from otherwise valid, formally derived relationships between the quantity or density of a substance and the photometer results.

These difficulties and means which have been devised to reduce their significance are discussed, primarily in relation to "maximum density" type transmission photometry of liquid–liquid partition chromatograms. A photometer and procedure suitable for such work are briefly discussed. However, the conclusions reached are considered to be of widely general validity, and the broad design of a corresponding reflection photometer is indicated.

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