# QUANTITATIVE ESTIMATION OF PAPER CHROMATOGRAMS BY DIRECT PHOTOMETRY

## I. RELATIVE MERITS OF USING TRANSMITTED LIGHT AND REFLECTED LIGHT AND OF USING DRY AND TRANSPARENT PAPER*

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(Received November 24th, 1961)

### INTRODUCTION

Direct photometric evaluation of paper chromatograms has been used increasingly in the past decade[1–28] and is now accepted as a standard method of estimation. This method is exceptionally rapid and, under suitable conditions, gives accurate results. In practice, however, it is often difficult to choose the most suitable direct photometric method from a variety of modifications. The general view that estimation by transmitted light is superior to that by reflected light requires further investigation, as does also the practice of rendering the paper transparent.

Most workers have preferred to use transmitted rather than reflected light, probably because it is easier to design a transmission photometer than a reflection photometer, especially when scanning has to be carried out. It is widely accepted that estimations based on transmission are rather more sensitive than those based on reflection. The more important question is, however, the relative accuracy of the two methods. It does not, of course, follow that the more sensitive method is necessarily the more accurate one. CROOK[29] has stated that transmission measurements are greatly to be preferred because the variations in surface texture and the difference in degree of penetration of the colour throughout the paper adversely affect the accuracy of reflection measurements. On the other hand, SAKURABA[30] has pointed out that the non-homogeneity of the paper can produce serious errors in transmission photometry.

Many workers[7,31–37] who have used transmitted light have rendered the paper transparent. This procedure may have three effects:

(i) To increase the light transmission,
(ii) To alter the nature of the function between the transmittance and the concentration and,
(iii) To alter the optical uniformity of the paper.

In the present work, the nature of these effects is studied, both using transmitted light and using reflected light, in an attempt to establish which is the more suitable method of estimation.

* Part of this paper is based on a thesis for M.Sc. submitted to Durham University, Great Britain, by R. B. INGLE.

## EXPERIMENTAL

### *Apparatus*

All photometric measurements were made in the Hilger Uvispek Model 700 Spectrophotometer. Reflection measurements were made with the aid of the Uvispek reflection attachment which was used in conjunction with the appropriate supplementary lens. This lens converts the slit of light to a beam measuring approximately 3 mm × 3 mm in cross section. The attachment, the principle of which is shown in Fig. 1, measures the reflectance of a small area of the spot relative to that of the blank paper. Unfortunately, the attachment does not permit scanning, nor does it allow

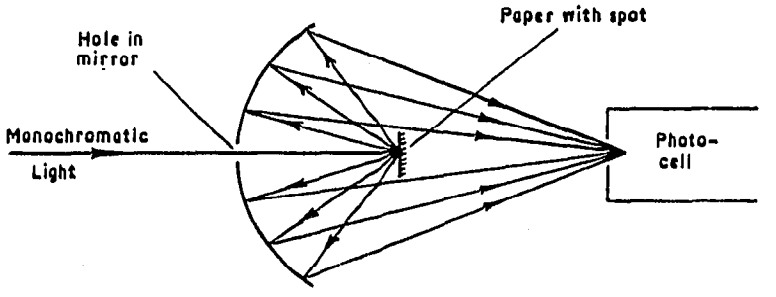


Fig. 1. Principle of reflection attachment.

adjustment of the paper while a light measurement is being taken. Transmission measurements were taken in a versatile transmission adaptor, shown in Fig. 2, which enables small sheets of paper to be scanned either by a slit or by a small square aperture. The paper can be moved vertically or horizontally. The adaptor is complicated for routine measurements but was designed to enable the paper to be scanned either dry or immersed in a liquid.

### *Application and development of spots*

The estimation of non-chromatographed spots was studied first, using a substance which could readily be developed by a suitable complexing agent. Copper, developed by rubeanic acid, was chosen for detailed study as the complex is easily developed, does not fade in the absence of light, and also because copper is present only in very minute quantities in the ordinary grades of paper.

Standard solutions were prepared by dissolving copper sulphate in 3 *N* HCl. 2.64 $\mu$l volumes of the resulting solutions were applied to Whatman No. 2 paper using a micropipette. Very uniform spots were produced. The paper containing the spots was dried for at least 10 min, and then developed by:

(i) Hanging in ammonia vapour for half a minute,
(ii) Immersing in 0.1% alcoholic rubeanic acid for 1 min with agitation,
(iii) Hanging again in ammonia vapour for half a minute, and
(iv) Washing in alcohol for half a minute.

The paper was then allowed to dry in a current of air at 20° for at least 5 min before measuring in the spectrophotometer. This method was found to give highly reproducible development of copper and was used in all subsequent work. In particular it was found that:

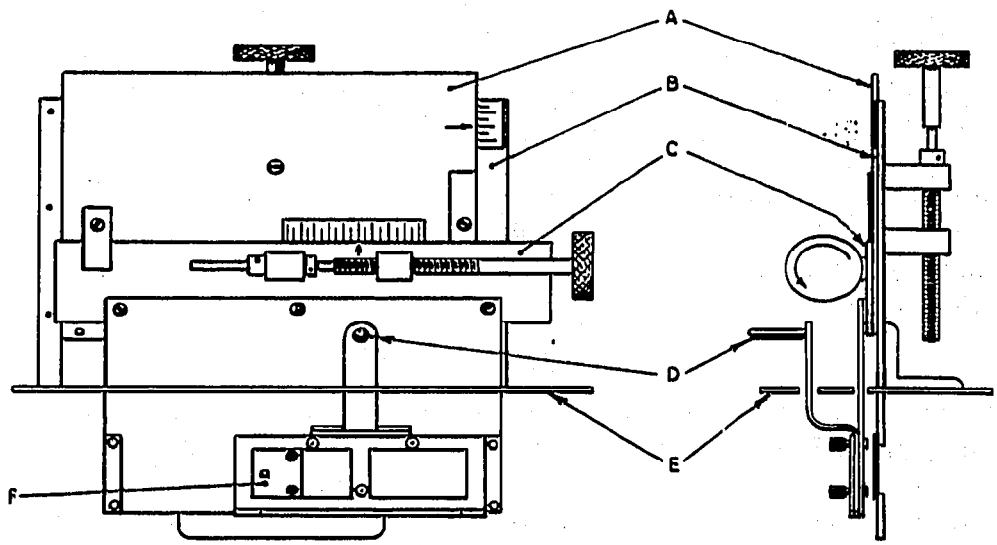


Fig. 2. Scanning transmission adaptor. The attachment is built about a horizontal plate E which fits into the top of the measurement well of the spectrophotometer. To this plate is fixed rigidly a vertical plate B which extends both above and below the horizontal plate E. A plate A may be moved up and down and another plate C may be moved from side to side by the screw mechanism shown. The plate C extends below the horizontal plate E to the paper holder, which consists of two compartments, one for the spot and the other for the blank paper. The paper may be held directly in the holder, or alternatively it may be immersed in a liquid contained in a small cell which is placed in the holder. The holder may be moved from the standard to the measure position by the handle D. The adaptor is rendered light-tight by means of black velvet. A neutral density filter, of approximately the same density as paper, can be rapidly inserted in, or removed from, the light beam, just behind the aperture F. This is controlled by a small lever on the upper side of the plate E. (This mechanism is not shown.)

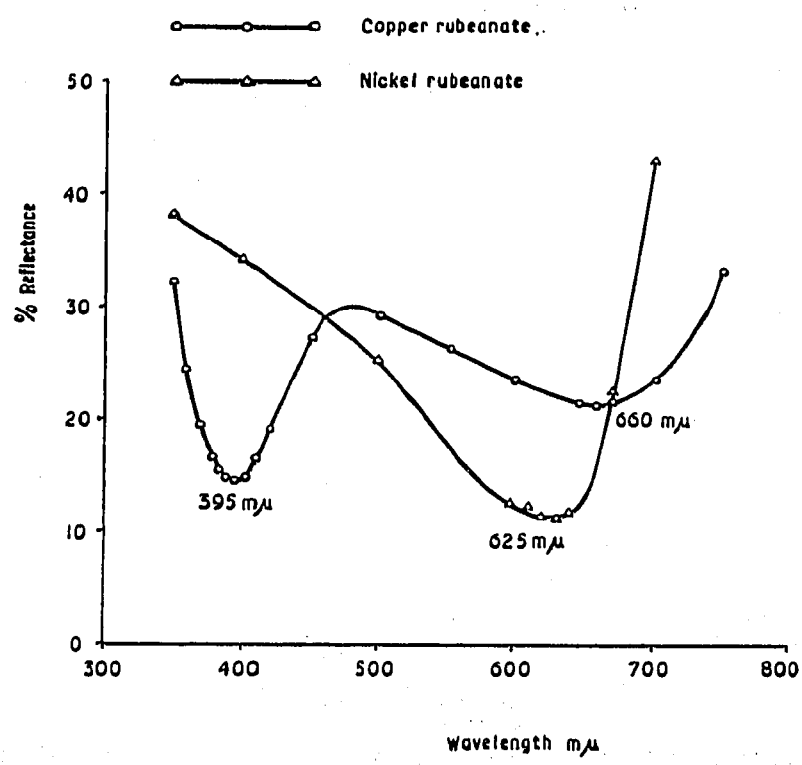


Fig. 3. Reflectance *versus* wavelength curves of copper and nickel rubeanates.

(i) Total immersion led to more reproducible development than either spraying or dipping the paper in a trough in a see-saw fashion,

(ii) Development by hanging in ammonia vapour followed by immersion in alcoholic rubeanic acid, led to much more reproducible results than development by immersion in rubeanic acid containing ammonia,

(iii) Hanging the paper in ammonia after immersion in rubeanic acid led to slightly more complete development, and

(iv) Washing in alcohol was necessary in order to obtain reproducible white areas for reference in photometry.

*Spectrophotometer wavelength and slit width*

The absorption maxima of copper rubeanate were found to occur at 395 m$\mu$ and 660 m$\mu$ as shown in Fig. 3, both by transmitted and by reflected light. Subsequent measurements were taken at 660 m$\mu$ because the spectrophotometer measurements were found to be rather more precise at this wavelength, particularly when only a small quantity of light was incident on the photo-cell. Normally the stronger absorption maximum of 395 m$\mu$ would be used. A slit width of 0.2 mm giving a band of 1.0 m$\mu$ was used for reflection measurements and for transmission measurements on transparent paper. The slit width was increased to 0.4 mm for transmission measurements on dry paper in order to admit more light to the photo-cell.

*Standards for reflection measurements*

Several sheets of white filter paper were used as backgrounds both to the spot and to the blank, rather than the black surface of the holder. The effect of varying the number of sheets in the measure compartment when nine sheets of white paper were held in the standard compartment is shown in Fig. 4. From this it is clear that six

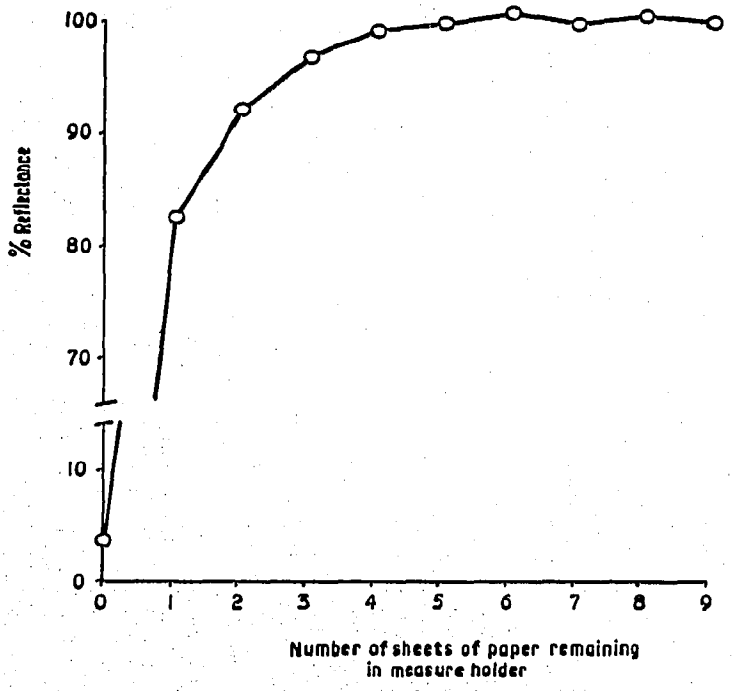


Fig. 4. Effect of varying the number of sheets of white paper used as a background (9 sheets of white paper in the standard holder).

sheets of paper are sufficient to give a reflection reading independent of the background. This number was used in all subsequent work.

Reflection readings were taken by placing the developed spot in one compartment of the reflection attachment while the blank, cut from the same sheet of paper containing the spots, was placed in the other compartment. In both cases, six sheets of white paper were used as a background. The blank paper was first placed opposite the beam of light and the spectrophotometer was set to 100% reflectance. The spot was then rapidly slid into the same position before measuring its reflectance.

*Standards for transmission measurements*

The choice of a suitable standard for transmission readings is considerably more difficult than for reflection readings because paper appears optically less uniform by transmitted light than by reflected light. The simplest method of using the scanning adaptor is to place the spot in one window, and the blank paper in the other window. This method, which will be termed the "direct standard method" is fairly satisfactory, provided that the area of the adaptor aperture is not too small. Most of the work was done, however, using rather small apertures and it was then found that the direct standard method did not give satisfactory results. For, if the instrument was set to 100% transmittance on only one position of the blank, the part of the paper chosen might be rather more or less opaque than the average. This could result in increasing or decreasing all the readings on the spot by as much as 10%. Alternatively, the instrument could be set to 100% transmittance each time a reading was taken, which would mean that a different area of the blank paper was used for each reading. This procedure would, admittedly, lead to a certain averaging out of errors, but it would still have the disadvantage that various errors, some positive and some negative would be imposed on each of the readings. This would make the interpretation of the results much more difficult, particularly when studying the uniformity of the paper.

In order to overcome this disadvantage, a method which will be termed the "indirect standard method" was employed in all transmission work. The purpose of this method was to relate the readings taken on the spot to the *mean* transmittance of the blank paper, rather than to the transmittance of a particular portion of the blank paper. The adaptor was fitted with a filter of approximately the same optical density as paper. This filter (a piece of photographic film was used) could be inserted in, or removed from, the path of the light beam by moving a small lever on the scanning adaptor. Readings were then taken on each spot with the "standard" window empty, while the paper with the spot was placed in the "measure" window. The following procedure was then carried out:

(i) The filter was placed in the beam of light, the adaptor was set to "standard", and the instrument was adjusted to 100% transmittance.

(ii) The filter was removed, the adaptor was set to "measure", and transmission readings were taken as the paper was moved across the beam of light.

The piece of paper chosen to act as a standard was then placed in the "measure" window, the "standard" window again being left empty. Readings were taken as described above, a series being obtained which scanned a representative area of the paper.

The mean transmittance of the standard paper was noted and this enabled each reading on the spot to be expressed as a percentage of the *mean* transmittance of the blank paper.

*Optical uniformity of dry paper by transmitted light*

One of the chief factors which reduced the accuracy of photometric measurements on paper is the non-homogeneity of the paper itself. This non-homogeneity gives rise to optical non-uniformity, both by reflected and by transmitted light. Dry paper was scanned using transmitted light, as shown in Fig. 5. It is clear that apertures of 2 mm × 2 mm or less should not be used for transmission measurements.

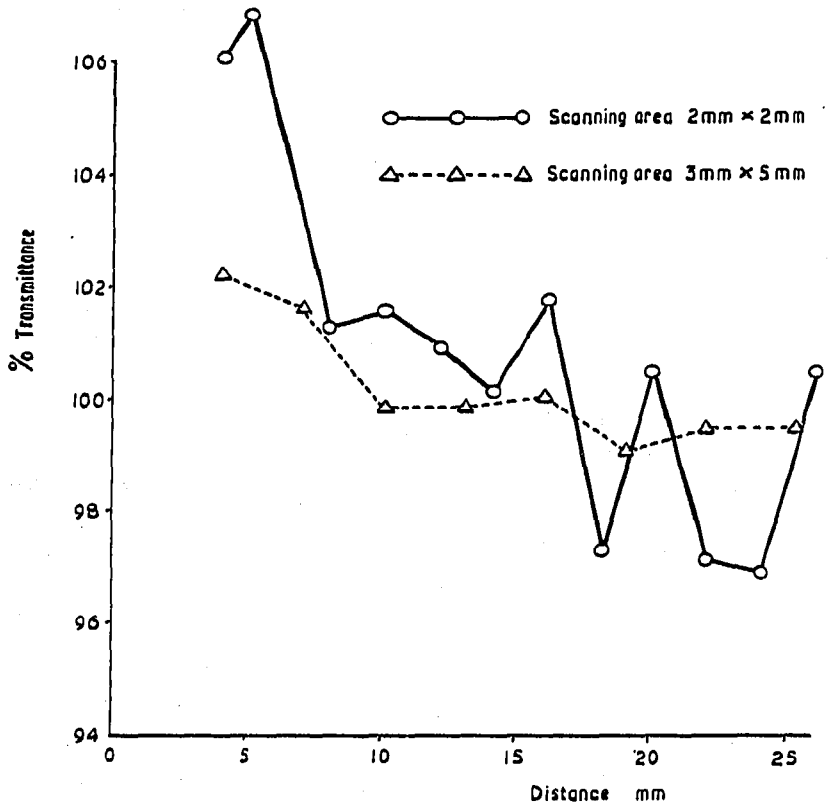


Fig. 5. Effect of scanning area on variation in transmittance of white paper.

*Methods of rendering paper transparent*

(*i*) *Using transmitted light*. Paper was first rendered transparent by total immersion in liquids contained in a small cell. The paper was scanned using an aperture of 2 mm × 2 mm, both before and after rendering transparent. Several liquids were investigated: xylene as shown in Fig. 6, water as shown in Fig. 7, paraffin, paraffin diluted with petrol ether, glycerine, and glycerine diluted with water. It was found that rendering paper transparent certainly did not improve the optical uniformity of paper by transmitted light. Furthermore, very long periods were required before the transmittance readings became steady.

A second method of rendering the paper transparent was investigated, which was similar to that described by BLOCK *et al.*[38]. The paper was dipped in paraffin for one minute, allowed to drain for a few minutes, and then blotted lightly between filter paper several times until no more paraffin could be removed. Paper was scanned both before and after it had been rendered transparent in this way; the results are shown in Fig. 8. Once again, there was no improvement in the optical uniformity,

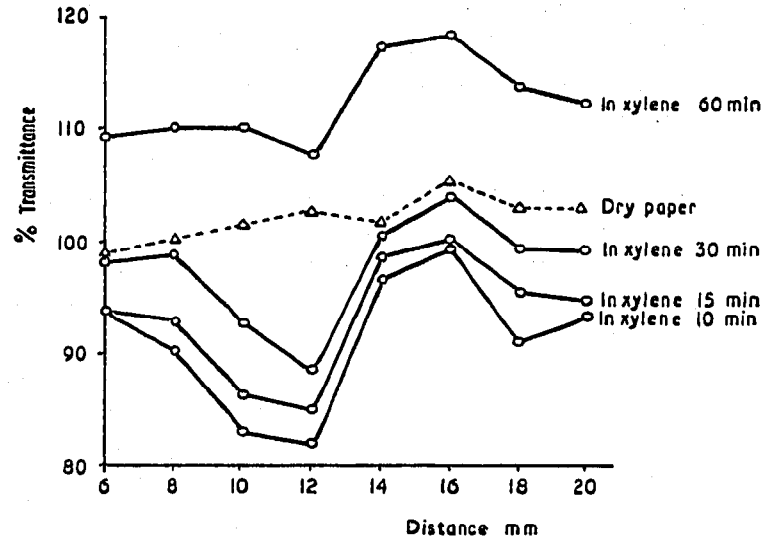


Fig. 6. Variations in transmittance of paper dry, and totally immersed in xylene. Scanning area 2 mm × 2 mm; the same standard filter was used for all readings taken on paper immersed in xylene, in order to show up the variations in transmittance with time; a different standard filter was used for readings taken on dry paper.

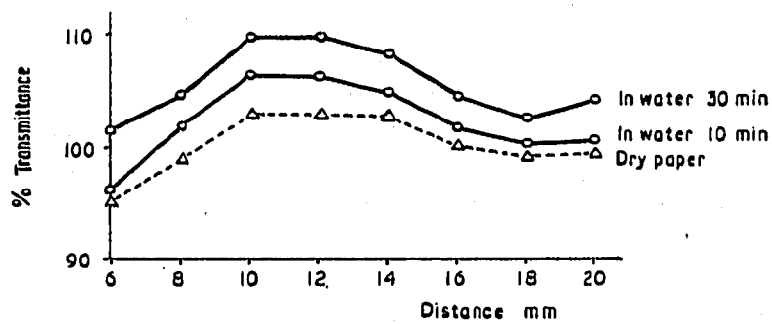


Fig. 7. Variations in transmittance of paper dry, and totally immersed in water. Scanning area 2 mm × 2 mm; the same standard filter was used for all readings taken on paper immersed in water, but a different one was used for readings taken on dry paper.

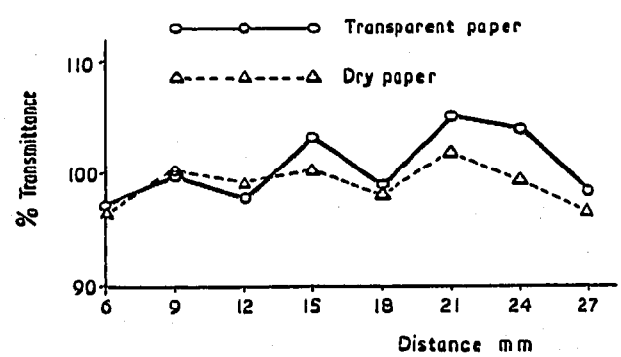


Fig. 8. Variations in transmittance of paper dry, and rendered transparent by dipping in paraffin, draining, and blotting. Scanning area 3 mm × 3 mm; a different standard filter was used for the two sets of readings.

but the method was much quicker and more convenient than that of total immersion. The readings varied very little with time, although they were dependent to a slight extent on the amount of paraffin remaining on the paper. This method of rendering paper transparent was used in subsequent work in preference to that of total immersion.

*(ii) Using reflected light.* Two methods of rendering paper transparent were investigated. In the first, the paper was backed by a layer of magnesium carbonate over which a cover slip had been placed. A drop of paraffin was placed on this cover slip and the paper was placed thereon. A further drop of paraffin was added and another cover slip was placed on top. Both the spot and the blank paper were treated in the same way. A reflection reading was then taken in the usual way. This method was found to be very inconvenient and took a long time. The readings varied rapidly with time, the quantity of paraffin used affected the readings, and no doubt errors might be caused by entrapped air. For these reasons, this method was rejected.

In the second method, the paper was dipped in paraffin, allowed to drain, and then blotted. The transparent paper was placed in the reflection holder, backed by six sheets of white paper over which a cover slip had been laid. This method was found to be much more satisfactory than the first method and was used in all subsequent work. There appeared to be little advantage in using the rather whiter surface of magnesium carbonate.

*Comparison of optical uniformity of dry and of transparent paper using both reflected and transmitted light*

Twenty pieces of dry paper were measured both by reflected light and by transmitted light. This experiment was then repeated after rendering the paper transparent. In all cases, an area of light measuring approximately 3 mm × 3 mm was incident on the paper. The standard deviations are shown in Table I.

TABLE I

STANDARD DEVIATIONS ON BLANK PAPER, MEASURED BY FOUR METHODS

(Relative to a mean reflectance or transmittance of 100)

| *Method* | *Standard deviation* |
|---|---|
| (i) Reflection on dry paper | ± 0.62 |
| (ii) Reflection on transparent paper | ± 0.33 |
| (iii) Transmission on dry paper | ± 1.43 |
| (iv) Transmission on transparent paper | ± 7.9 |

It is clear that paper appears optically more uniform by reflected light than by transmitted light. Rendering paper transparent decreases its optical uniformity to transmitted light.

*Calibration curves for non-chromatographed spots by reflection*

The theory of KUBELKA AND MUNK[39] (commented upon by STEELE[40] and JUDD[41]) makes possible a prediction of the relation between the reflectance and the concen-

tration of a coloured substance on paper. VAECK[14] applied this theory to the estimation of non-chromatographed spots on paper, and showed that, in general, a straight line is obtained on plotting the ratio of absorption coefficient to scattering coefficient ($K/S$) *versus* the concentration of the substance in the spot. The ratio $K/S$ is related to $R_\infty$ by the relation

$$K/S = (1 - R_\infty)/2R_\infty$$

where $R_\infty$ is the monochromatic reflectance of a material of such thickness that a further increase in thickness does not alter the reflection reading. In practice, the reflectance of the spot against a white background is measured. The ratio $K/S$ may be obtained from the reflectance using Table 34 on p. D22.

Copper spots, each of volume 2.64 $\mu$l, but of various concentrations, were placed on the same sheet of paper, developed, and measured by reflection (on dry paper). This experiment was repeated, but the paper was rendered transparent after development. The results are shown in Table II.

TABLE II

% REFLECTANCE (% $R$) AND $K/S$ VALUES OF COPPER RUBEANATE ON DRY AND ON TRANSPARENT PAPER AT 660 m$\mu$

| *Concentration* g $Cu^{2+}$/l | *Dry paper* | | *Transparent paper* | |
|---|---|---|---|---|
| | % $R$ | $K/S$ | % $R$ | $K/S$ |
| 0.05 | 78.8 | 0.029 | 76.9 | 0.035 |
| 0.1 | 67.0 | 0.081 | 58.9 | 0.143 |
| 0.2 | 52.0 | 0.222 | 39.1 | 0.474 |
| 0.4 | 40.3 | 0.442 | 22.5 | 1.335 |
| 0.6 | 32.2 | 0.714 | 13.1 | 2.88 |
| 0.8 | 26.4 | 1.026 | 9.80 | 4.15 |
| 1.0 | 23.4 | 1.254 | 7.70 | 5.53 |

The plot of $K/S$ *versus* concentration is shown in Fig. 9. It should be noted that the slope of the curve is much steeper when the readings are taken on transparent paper. It was found that at concentrations above 1 g $Cu^{2+}$/l, the calibration curve began to flatten out, and that at very high concentrations the readings became scattered.

*Calibration curves for non-chromatographed spots by transmission*

Developed spots of various concentrations were also measured by transmission, both on dry and on transparent paper. The results are shown in Table III.

The optical density was plotted against the concentration as shown in Fig. 10. At low concentrations the slope of the curve relating to dry paper is very much steeper than that relating to transparent paper.

The manner in which the reflectance and the transmittance vary with concentration both on dry and on transparent paper can be seen in Fig. 11.

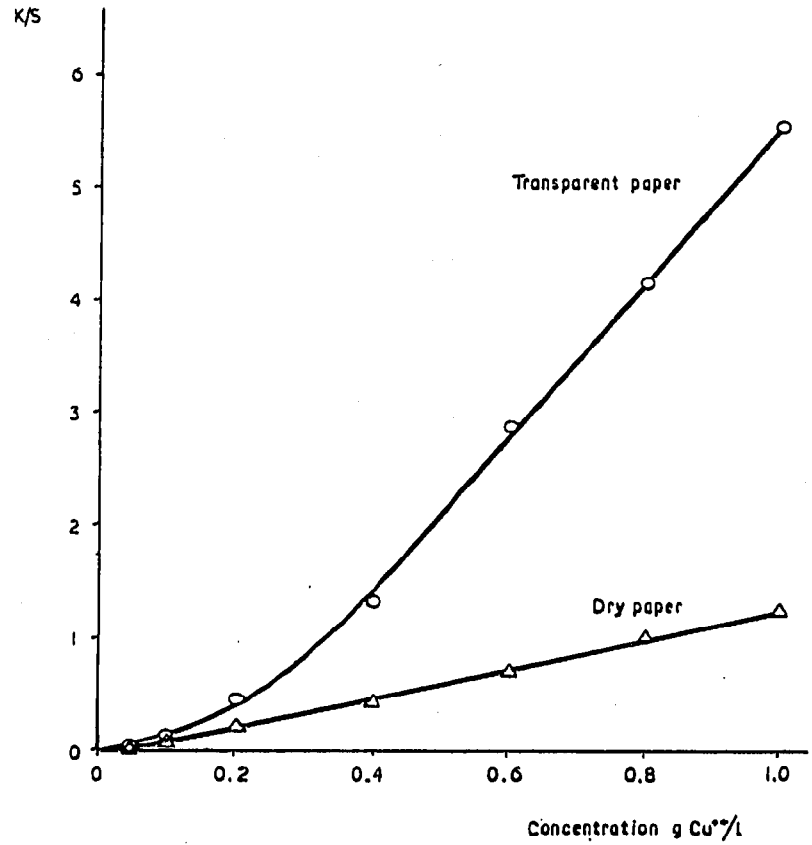


Fig. 9. Calibration curves of copper rubeanate on dry and on transparent paper by reflection.

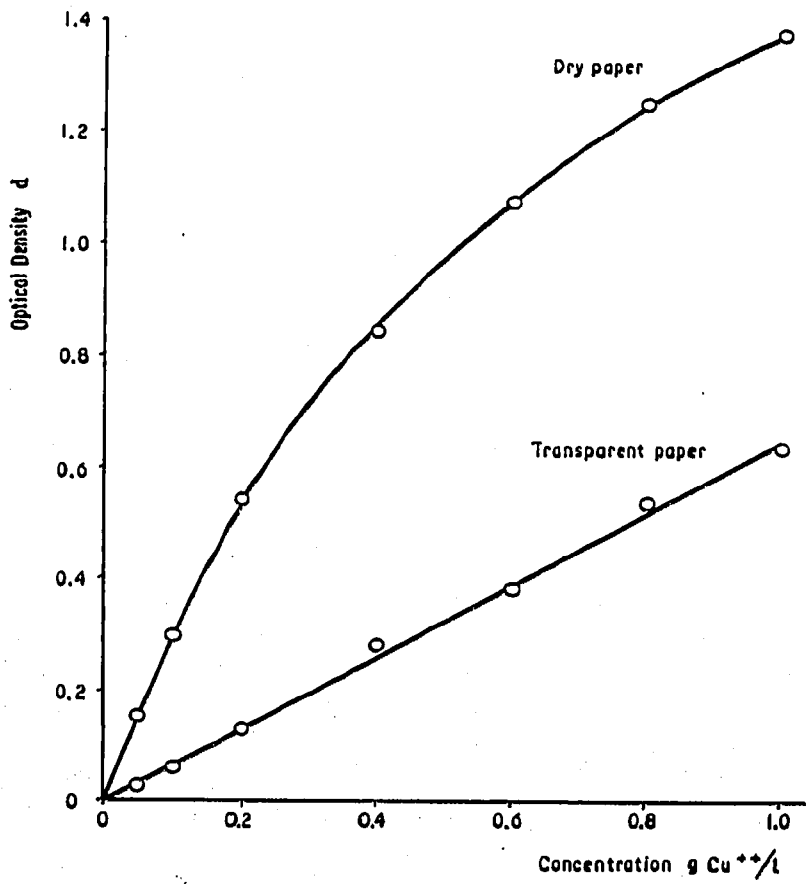


Fig. 10. Calibration curves of copper rubeanate on dry and on transparent paper by transmission.

TABLE III

% TRANSMITTANCE (% $T$) AND OPTICAL DENSITY ($d$) OF COPPER RUBEANATE ON DRY AND ON TRANSPARENT PAPER AT 660 mμ

| *Concentration* g $Cu^{2+}/l$ | *Dry paper* | | *Transparent paper* | |
|---|---|---|---|---|
| | % $T$ | $d$ | % $T$ | $d$ |
| 0.05 | 70.8 | 0.15 | 93.8 | 0.028 |
| 0.1 | 50.6 | 0.30 | 87.1 | 0.060 |
| 0.2 | 28.9 | 0.54 | 74.0 | 0.131 |
| 0.4 | 14.6 | 0.84 | 52.5 | 0.280 |
| 0.6 | 8.58 | 1.07 | 41.6 | 0.380 |
| 0.8 | 5.72 | 1.24 | 29.2 | 0.535 |
| 1.0 | 4.22 | 1.37 | 23.4 | 0.631 |

*Effect of spectrophotometer slit width on instrumental error*

Spots of concentration 0.4 g $Cu^{2+}/l$ were each measured ten times in succession at different slit widths. This procedure was carried out for each of the four methods of measurement, as shown in Table IV.

The standard deviations are less than 0.1, using a slit width of 0.2 mm (1 mμ) except in the case of transmission measurements on dry paper. These results are a measure of the instrumental error, since the spot was not disturbed in the paper holder while each series of readings was obtained. The instrumental errors are considerably less than the errors due to lack of uniformity in the paper (see Table I).

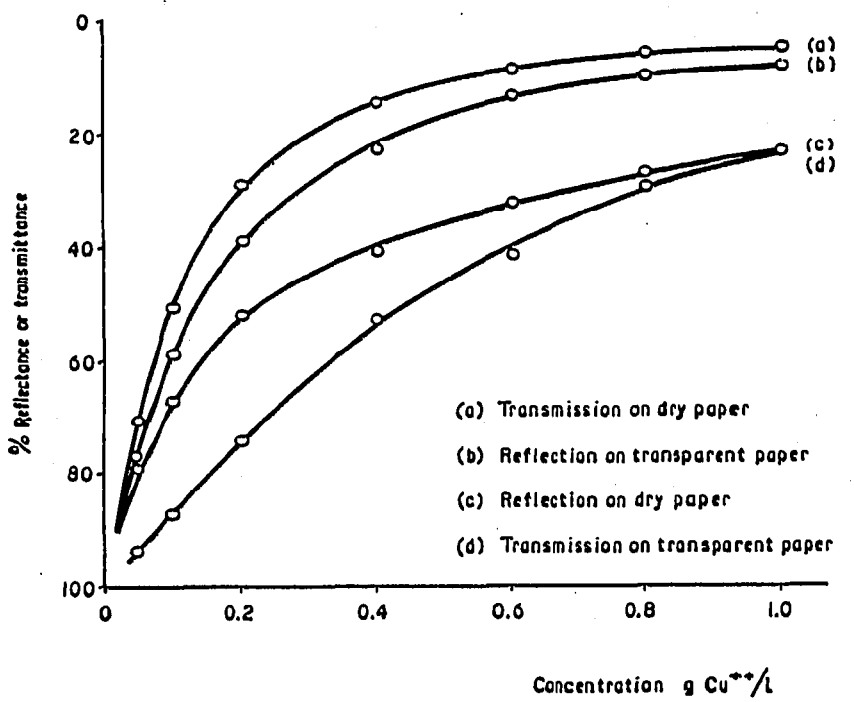


Fig. 11. Reflectance (or transmittance) *versus* concentration curves for the four methods of measurement.

TABLE IV

EFFECT OF SPECTROPHOTOMETER SLIT WIDTH ON INSTRUMENTAL ERROR

(Concentration of spots 0.4 g $Cu^{2+}$/l in each case)

| *Method* | *% Reflectance or % transmittance, with standard deviation from 10 readings* | | | | |
|---|---|---|---|---|---|
| | *Slit width mm* | | | | |
| | *0.05* | *0.10* | *0.20* | *0.40* | *1.00* |
| (i) Reflection on dry paper | 37.9 ± 0.20 | 37.5 ± 0.08 | 37.5 ± 0.05 | 37.6 ± 0.06 | — — |
| (ii) Reflection on transparent paper | 22.9 ± 0.34 | 22.6 ± 0.05 | 22.6 ± 0.05 | 22.7 ± 0.06 | — — |
| (iii) Transmission on dry paper | — — | 14.6 ± 0.66 | 14.6 ± 0.34 | 14.5 ± 0.06 | 14.4 ± 0.04 |
| (iv) Transmission on transparent paper | 52.6 ± 0.11 | 52.5 ± 0.04 | 52.5 ± 0.04 | 52.5 ± 0.05 | 52.8 ± 0.05 |

TABLE V

% REFLECTANCE READINGS ON NON-CHROMATOGRAPHED SPOTS ON DRY PAPER

(Spectrophotometer slit width 0.2 mm)

| | *Concentration g $Cu^{2+}$/l* | | | | |
|---|---|---|---|---|---|
| | *1* | *0.4* | *0.1* | *0.05* | *0** |
| Mean % reflectance of readings from 10 spots | 22.8 | 39.5 | 69.3 | 80.0 | 100 |
| Standard deviation of reflectance | ± 0.52 | ± 0.43 | ± 0.65 | ± 1.4 | ± 0.62 |
| Corresponding change in measured concentration g $Cu^{2+}$/l | ± 0.036 | ± 0.007 | ± 0.005 | ± 0.004 | — |
| Probable % relative error in concentration | ± 3.6 | ± 1.8 | ± 5.0 | ± 8.0 | — |

* From Table I.

TABLE VI

% REFLECTANCE READINGS ON NON-CHROMATOGRAPHED SPOTS ON TRANSPARENT PAPER
(Spectrophotometer slit width 0.2 mm)

| | *Concentration g $Cu^{2+}$/l* | | | | |
|---|---|---|---|---|---|
| | *1* | *0.4* | *0.1* | *0.05* | *0** |
| Mean % reflectance of readings from 10 spots | 7.8 | 22.5 | 59.0 | 77.0 | 100 |
| Standard deviation of reflectance | ± 0.46 | ± 0.63 | ± 0.78 | ± 0.80 | ± 0.33 |
| Corresponding change in measured concentration g $Cu^{2+}$/l | ± 0.050 | ± 0.0072 | ± 0.0035 | ± 0.0017 | — |
| Probable % relative error in concentration | ± 5.0 | ± 1.8 | ± 3.5 | ± 3.4 | — |

* From Table I.

TABLE VII

% TRANSMITTANCE READINGS ON NON-CHROMATOGRAPHED SPOTS ON DRY PAPER
(Spectrophotometer slit width 0.4 mm)

| | *Concentration g $Cu^{2+}$/l* | | | | |
|---|---|---|---|---|---|
| | *1* | *0.4* | *0.1* | *0.05* | *0** |
| Mean % transmittance of readings from 10 spots | 4.2 | 14.5 | 50.6 | 70.8 | 100 |
| Standard deviation of transmittance | ± 0.36 | ± 0.80 | ± 2.2 | ± 2.5 | ± 1.43 |
| Corresponding change in measured concentration g $Cu^{2+}$/l | ± 0.060 | ± 0.019 | ± 0.0063 | ± 0.0050 | — |
| Probable % relative error in concentration | ± 6.0 | ± 4.8 | ± 6.3 | ± 10 | — |

* From Table I.

TABLE VIII

% TRANSMITTANCE READINGS ON NON-CHROMATOGRAPHED SPOTS ON TRANSPARENT PAPER
(Spectrophotometer slit width 0.2 mm)

| | *Concentration g $Cu^{2+}$/l* | | | |
|---|---|---|---|---|
| | *1* | *0.4* | *0.1* | *0** |
| Mean % transmittance of readings from 10 spots | 23.8 | 52.5 | 90.2 | 100 |
| Standard deviation of transmittance | ± 1.8 | ± 4.2 | ± 5.0 | ± 7.9 |
| Corresponding change in measured concentration g $Cu^{2+}$/l | ± 0.048 | ± 0.053 | ± 0.037 | — |
| Probable % relative error in concentration | ± 5 | ± 13 | ± 37 | — |

* From Table I.

*Comparison of reflection and transmission methods*

Ten spots, each of concentration 1 g $Cu^{2+}$/l, were placed on a strip of paper. After development each spot was measured by reflection. This was repeated at different concentrations (Table V). The same experiment was carried out on spots which had been rendered transparent (Table VI).

Tables VII and VIII show the results obtained by transmission measurements on dry and on transparent paper respectively.

The standard deviation (in reflectance or transmittance) was calculated from each set of ten spots. The change in concentration corresponding to each value of this deviation was found from the appropriate calibration graph (Figs. 9 and 10). This was expressed as a probable % relative error in concentration:

Probable % relative error in concentration

$$= \frac{100 \times (\text{change in concentration})}{\text{concentration}}.$$

For convenience, the probable errors of the four methods of measurement are summarised in Table IX.

TABLE IX

PROBABLE RELATIVE ERROR OF THE FOUR METHODS OF MEASUREMENT
(Area of light incident on paper = 3 mm × 3 mm)

| *Method* | *Probable % relative error at concentrations (g $Cu^{2+}$/l) of* | | | |
|---|---|---|---|---|
| | *1.0* | *0.4* | *0.1* | *0.05* |
| (i) Reflection on dry paper | ± 3.6 | ± 1.8 | ± 5.0 | ± 8.0 |
| (ii) Reflection on transparent paper | ± 5.0 | ± 1.8 | ± 3.5 | ± 3.4 |
| (iii) Transmission on dry paper | ± 6.0 | ± 4.8 | ± 6.3 | ± 10 |
| (iv) Transmission on transparent paper | ± 5 | ± 13 | ± 37 | — |

## DISCUSSION

The relative merits of the four methods of measurement can be considered from various standpoints:

### (*i*) *Ease of calculation*

Readings on dry paper by reflected light and on transparent paper by transmitted light both have the advantage that straight line calibration graphs are obtained. Readings on transparent paper by reflected light produce a nearly straight line graph except at low concentrations. Straight line calibration graphs are very desirable, since unknowns are best estimated by comparison with standards placed on the same sheet of paper and developed under identical conditions. It is usually only practicable to place a few standards on a single chromatogram, so it is difficult to draw an accurate calibration curve unless it is a straight line.

### (*ii*) *Instrumental convenience*

It is much easier to design a spectrophotometer adaptor for transmission readings than for reflection readings, especially when scanning has to be carried out. Rendering the paper transparent greatly increases the proportion of light transmitted, but this is not in itself an important advantage when a sensitive spectrophotometer is used. The process of rendering the paper transparent is time consuming and can lead to the introduction of errors if the process is not carried out carefully. Clearly, the paper should only be rendered transparent if there is some definite advantage to be gained.

### (*iii*) *Probable error*

The results in Table I show that paper appears optically more uniform by reflected light than by transmitted light. This is not surprising in view of the fibre structure of paper. As the presence of a coloured spot cannot remove the optical non-uniformity, the precision of reflection readings would be expected to be greater than those of transmission readings. Tables V–VIII show that this is in fact the case.

The probable error in a final result depends both on the precision with which the reflectance or the transmittance is obtained and on the slope of the appropriate calibration graph. Inspection of Fig. 11 shows the close similarity in the spectrophotometer readings obtained by reflection on transparent paper and by transmission on dry paper. The slope of both curves is steep at low concentrations and the reflectance (or transmittance) reaches a much lower value at a concentration of 1 g $Cu^{2+}$/l than by either of the other two methods of measurement.

As can be seen from Table IX, the method with the least error is that of reflection on transparent paper. This is because reflection readings are very precise and because the calibration curve is steep. The errors involved in the method of reflection on dry paper are greater than those of reflection on transparent paper—although still quite small. This is because the slope of the $K/S$ *versus* concentration curve (Fig. 9) is less than that obtained on transparent paper. The process of rendering the paper transparent increases the slope of this curve because much more light now passes twice through a greater depth of the paper, thus increasing the ratio of absorbed light relative to scattered light.

The errors involved in transmission measurements are greater than those in

reflection measurements because the paper appears optically less uniform by transmitted light than by reflected light. The errors in transmission measurements on dry paper are, perhaps, not much greater than those of reflection measurements on dry paper, but the errors in transmission measurements on transparent paper are considerably greater.

## CONCLUSIONS

Of the four methods of estimation, transmission on transparent paper involves the largest errors and has therefore been rejected for further work. Reflection on transparent paper has also been rejected because, although the errors are less than in any of the other methods, there is no linear relation with concentration, and also because of the additional work involved in rendering the paper transparent.

The two most suitable methods are thus transmission on dry paper and reflection on dry paper. The only important advantage of transmission is that it is easier to design a transmission adaptor than a reflection adaptor, especially when scanning has to be carried out. Readings taken by reflection involve rather smaller errors and produce a linear relationship with concentration which renders this method more attractive. For these reasons, the method of reflection on dry paper has been chosen for further work.

## ACKNOWLEDGEMENT

The authors are indebted to the Board of Governors and Principal of the Bristol College of Science and Technology for facilities enabling this work to be undertaken.

## SUMMARY

Attention is drawn to the greater optical uniformity of filter paper by reflected light than by transmitted light. The relative merits of employing reflected light and transmitted light, both on dry and transparent paper, are considered from the standpoints of ease of calculation, instrumental convenience and error. Under the conditions described here, in which an aperture of only 3 mm × 3 mm is used, the reflection method on dry paper is recommended.

Measurements may also be taken on transparent paper. This causes an increase in error when transmission is used, but decreases the error when reflection is used.

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