PRECISION IN THE DIRECT DENSITOMETRY OF COLOURED COMPOUNDS ON THIN LAYER CHROMATOGRAMS

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SUMMARY

An experimental study has been made of several factors that can affect precision in the densitometry of coloured compounds on thin layer chromatograms. Data are given concerning methods of peak area measurement, the centering of the scanning light beam over the chromatogram, the spotting on of the solute sample and the effect of the distance of development, of the position of the origin, of the time of the run, of the solvent flow rate, of the layer thickness and of the moisture content of the adsorbent.

The most important factors are seen to be layer thickness, time of development and positioning of the sample in the densitometer. The moisture content of the adsorbent can be a significant variable and the measurement of small peak areas can lead to appreciable loss of precision. Overall, however, the loss of precision is likely in practice to be small in comparison with that occurring in the detection stage in the case of colourless compounds.

Those familiar with the technique of thin layer chromatography (TLC) will appreciate that there are cases, especially when compounds of low volatility are involved, where quantitative TLC is a good alternative to gas chromatography, if it is not the only alternative. The accuracy of gas chromatography is perhaps not always as great as is claimed; but, in a given amount of time, the precision of gas chromatography can rarely be matched by quantitative TLC.

Of the several methods of making TLC quantitative the method of direct densitometry has been chosen for study here, since it is potentially the most rapid. Attention has been focussed on the several factors that can lead to loss of precision or to lack of reproducibility in densitometry especially of coloured compounds.

These studies have been carried out with certain coloured organic compounds, chromatographed on thin layers of silica gel.

Early experience with the TLC of non-polar lipids indicated that physical factors affecting the reproducibility of R_F values needed further examination. These factors were therefore studied before the densitometry itself was examined in detail. The results of these studies have been reported previously[1] and the general conclusions simultaneously confirmed by GEISS AND SCHLITT[2,3]. The work of BLANK *et al.*[4] and of THOMAS *et al.*[5] has shown that R_F values can affect densitometry readings in many cases. Consequently considerable care has been taken over the conditions of the

chromatography in this work. The general question of R_F values in densitometry will be referred to again later in this paper.

Several details in the chromatographic procedure have been standardised as described in the experimental part. The densitometry in all cases has been performed using an automatic recording densitometer equipped for both transmission and reflectance densitometry.

EXPERIMENTAL

Chromatography

The glass plates (1.2–1.4 mm thick) were coated with Silica Gel G (Merck) with the apparatus and the procedure described by STAHL[6]. Unless otherwise stated the layer thickness was nominally 0.25 mm. The plates were activated at 110° for 30 min and stored over anhydrous calcium chloride until required.

The compound used in nearly all cases was Sudan Red G (Desaga). This substance was tested and found quite stable on a chromatoplate; it was used as solution of suitable strength in xylene.

The solutions were always applied to the layers by means of small disposable capillary pipettes (Microcap pipettes)* made in several sizes to deliver 1, 2 or 5 μl. Care was taken not to damage the surface of the layer at all.

Vertical channels of about 1 mm width were always cut in the layer in order to prevent any sideways movement of the spots during development of the chromatograms. In addition horizontal channels were cut in the layer at the precise point from the origin, to which it was desired that the solvent should move. The chromatogram was normally removed from the solvent chamber 15 min after the solvent front had reached this point.

Unless it is otherwise stated the chromatograms were developed in fresh alcohol-free chloroform (dried over calcium chloride) in an S-tank.

Densitometry

The densitometer used throughout was a Joyce Loebl 'Chromoscan' densitometer**. The basic design of the instrument was first described by LATNER *et al.*[7] The most important features of the instrument used are these:

(i) The light source is a tungsten lamp with stabilised power source.

(ii) The instrument operates on the double beam principle.

(iii) The parallel focussed beam can be adjusted to any shape inside a 1 cm diameter circle. Incident angle = 90°.

(iv) The detector is a high sensitivity photomultiplier.

(v) Direct optical density measurement is achieved by reference to an 'optical density wedge'. This wedge is in the path of the reference beam, is moved mechanically by a servo motor to the position of balance and is linked directly to the recording pen.

(vi) In reflection measurements only light scattered at an angle of 45° to the incident light beam passes, via a mirror, to the photomultiplier.

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* From Shandon Scientific Co., London and U.S.A.

** From Joyce, Loebl & Co., Gateshead-on-Tyne, England.

(vii) In transmission measurements a separate photomultiplier, situated several cm behind the specimen, is used. Thus scattered light is not received.

The spectral characteristic of the light was adjusted by broad band light filters that could be placed in the path of the incident light beam.

In all reflectance measurements a piece of thick white non-fluorescent filter paper was placed behind the sample.

In nearly all cases the peaks were clearly resolved and the ratio of recorder chart speed to sample speed was 3:1.

Gradient thickness plates

These special plates were used to study the effect of layer thickness. The method described by HONEGGER[8] for preparing TLC plates with a uniformly graded layer thickness was employed. The method does not require any special equipment other than that required to coat plates with a layer of about 1 mm thickness. The prepared plate was stored at constant humidity for three days to ensure uniform activity, spotted quickly and then developed in chloroform using an S-tank.

The actual layer thickness was measured afterwards by means of a micrometer screw gauge.

RESULTS

Measurement of peak dimensions

The densitometer has a built in integrator, which was tested and found to be satisfactory (standard deviation on repeat readings on the same sample was less than 1%), but it was not normally used, since it is efficient only if the base line is perfectly straight.

The two main alternatives, planimetry and geometrical measurement, were compared.

In the planimeter measurements a good mechanical instrument was used. Each of ten peaks of similar shape but different area were measured ten times. The results (Table I) suggest that the relative standard deviation varies in a simple manner with the area measured. From these and other results it is confirmed that the chart speed should be high, where planimetry is to be employed.

TABLE I

PRECISION IN PLANIMETRY

Mean area (cm^2)	σ (%)[a]	*Area* × σ (%)	$\frac{15.9}{Area}$
30.39	0.597	18.1	0.52
19.71	0.66	13.0	0.81
14.90	1.70	25.3	1.07
10.54	1.54	16.2	1.51
9.62	1.64	15.8	1.65
6.26	2.05	12.8	2.53
4.82	3.32	16.0	3.30
2.78	3.52	9.8	5.72
	Mean	15.9	

[a] Relative standard deviation.

Two alternatives were compared in examining the geometrical methods (triangulation methods):

(A) Area = width at half the observed peak height × observed peak height.

(B) Area = half the width at base of nearest equivalent triangle × height to apex of this triangle.

Method (A) was found to give results most consistent with the planimeter readings, method (B) usually giving results several per cent high. In general, the planimeter readings were found the most accurate but also the most time consuming.

In this investigation method (A) was used, except where it is stated otherwise.

Effect of material behind the specimen in reflectance measurements

All the reflectance measurements in this investigation were carried out with a piece of thick white non-fluorescent filter paper immediately behind the specimens. This practice was adopted since it led to straighter base lines. Scanning of the same chromatographic spot on a 0.35 mm thick layer gave peak area readings ranging from 12.3 cm^2 (black cotton) to 15.5 cm^2 (two thicknesses of Whatman 3 mm) or to 15.2 cm^2 (one thickness of Whatman 3 mm). Thus the peak area increases as the light reflected from the material behind the specimen increases.

Precision in repeated scanning of the same spot (densitometry error)

In this test a chromatostrip with a single chromatographic spot was scanned by reflectance five times. Between each scanning the sample was removed and replaced and the light beam (1 mm diameter) re-positioned to pass through the centre of the spot (the re-positioning of the light beam is the principle source of error here). Each recorded peak area was then measured ten times by planimetry. The mean planimeter readings are shown in Table II.

TABLE II

PRECISION IN POSITIONING OF SAMPLE

Scan	*Mean area*[a] *(cm^2)*	*Relative standard deviation from mean*[a] *(%)*
1	9.80	1.77
2	9.36	1.67
3	9.57	1.75
4	9.89	1.47
5	9.48	1.55
Mean	9.62	1.64

[a] Mean of ten planimeter readings.

The estimated standard deviation for the 50 readings was 1.73%.

The standard deviation of the five mean area readings (*i.e.* the scan to scan deviation) was 2.29% (or 2.37% estimated from the range[9]); hence it was calculated that the standard deviation for the scanning alone in this particular case was 2.27%. That this figure is rather high is with little doubt due to the difficulty of positioning a small beam of light in the centre of the chromatographic spot. This figure is much higher than would be expected from the results in Table III and the reason for this

must lie in the fact that the chromatographic spot in this case was very much smaller and positioning of the beam much more critical. It must therefore be close to the upper limit for the error in the actual densitometry.

Precision in spotting of substances onto the chromatoplate

In the first test the same solution of coloured material was applied with the same (2 μl) capillary pipette as ten separate spots along the origin line of a plate. After development of the chromatogram each of the ten spots was scanned in several ways. Each recorded peak was then measured by planimetry (mean of four readings).

TABLE III

PRECISION IN APPLYING SAMPLE TO CHROMATOPLATE

	Conditions of scanning			
	Test 1		*Test 2*	
	1 mm spot by reflectance	*9 × 1 mm slit by reflectance*	*1 mm spot by transmission*	*1 mm spot by reflectance*
Mean area (cm²)	21.57	9.21	14.24	22.30
Relative range (%)	3.25	5.43	9.83	8.52
Relative standard deviation (%)	1.10	1.66	3.27	2.47

The high standard deviation for scanning by transmitted light was due to the fact that a much higher noise level was obtained, which led to an increased base line error.

After taking into account the planimetry error (Table I) the standard deviation reduced to 1.03% for the 1 mm spot and to 1.38% for the 9 × 1 mm slit scanning. If the difference between these two figures is significant, then the results are the reverse to what might be expected and the explanation is difficult to find.

In a further test, directly comparable with the previous one, ten different capillary pipettes, each nominally of 2 μl capacity, were used in applying the coloured solution to the chromatoplate. In this case, after development, the ten spots were scanned only by reflectance using a 1 mm spot. The results (Table III, last column) suggest that this can be a significant source of error. By taking into account the variance due to the planimetry and to the densitometry it may be calculated that the standard deviation in the volume of different 2 μl capillaries is 2.2%. Therefore, for a peak area of the order of 20 cm², this error is greater than that due to the chromatography, the densitometry and the planimetry combined.

TABLE IV

EFFECT OF MULTIPLE SPOTTING

Spotting method	*Volume applied (µl)*	*Mean peak height (cm)*	*Mean width at half height (cm)*	*Mean peak area (cm²)*	*σ (%)*
a	2	7.08	2.03	14.36	1.85
b	5 × 2	6.57	2.06	13.53	2.14

In a third test a comparison was made on the same plate between (a) spotting once with 2 μl of solution of concentration $5x$ and (b) spotting five times with 2 μl of a similar solution of concentration x. After development of the ten individual chromatograms on the plate each was scanned once by reflectance with a 1 mm spot, the peak heights and widths being measured in each case (method A). Spotting by both methods (a) and (b) showed a relative standard deviation of approximately 2% in the areas of the peaks (Table IV).

Effect of distance of development and position of origin

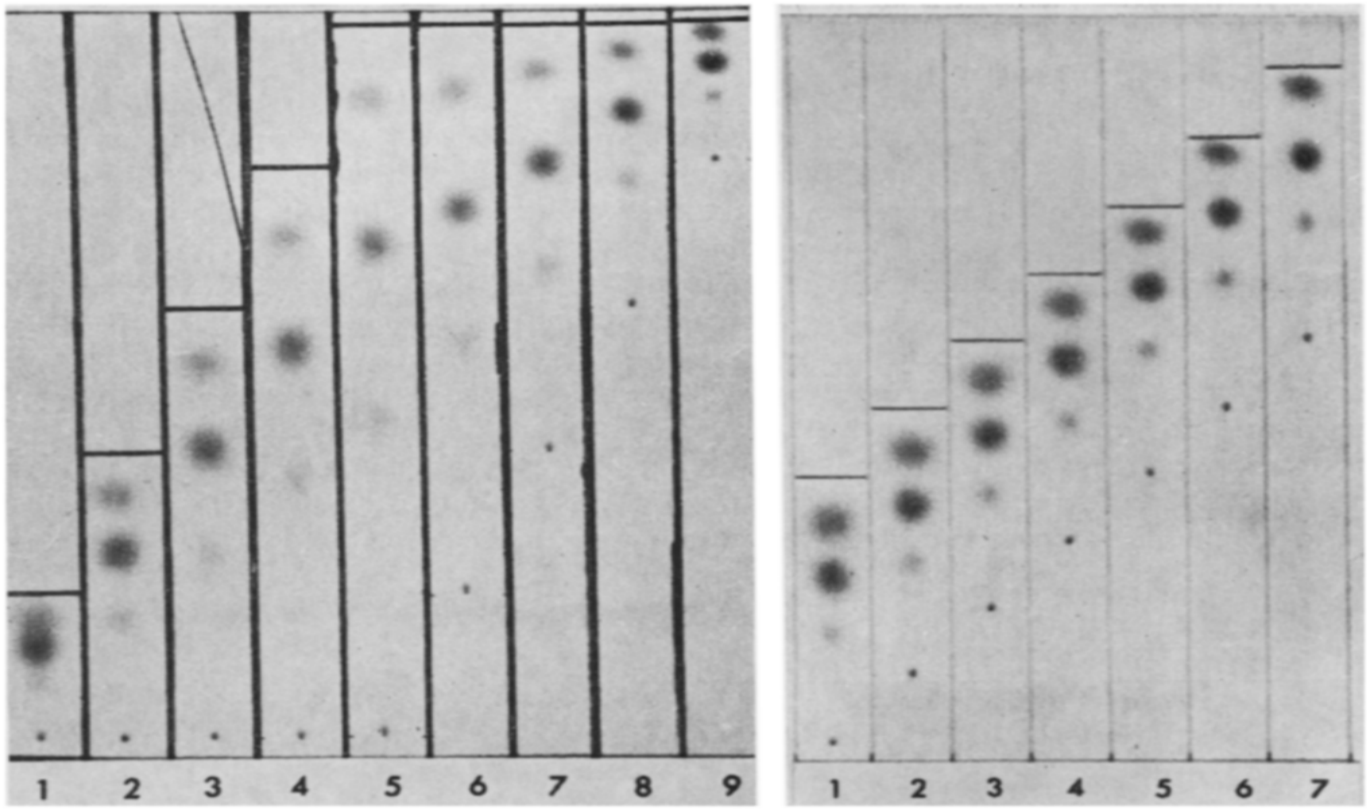
The special chromatograms shown in Figs. 1 and 2 were designed to enable several related factors to be studied.

The same amount of material was carefully applied at each origin (marked with a dot). Each of the strips was then scanned by reflectance with both a small spot and then a slit-shaped incident light beam (strip No. 1, Fig. 1, was not sufficiently well resolved for densitometry).

Several points emerge from the results given in Table V (relating to Fig. 1) and Table VI (relating to Fig. 2).

(a) The R_F values in this single solvent system increase to a small extent as the development distance, d, or the distance of the origin from the solvent surface increases. But this increase would be negligible in normal practice.

(b) Small variations in the position of the origin may be neglected. Similarly,



Figs. 1–2. Chromatograms designed to test the effect of distance of development and position of origin at constant R_F value. Only the dark middle spot, Sudan Red G, on each strip was measured (see Tables V and VI).

TABLE V

EFFECT OF TIME AND DISTANCE OF DEVELOPMENT

Adsorbent: Merck Silica Gel G; solvent: alcohol free chloroform; developed in S-tank; 22.5°; humidity 58%; layer thickness 0.24 mm. t = time of contact of solute with mobile phase; d = distance from origin to solvent front (see Fig. 1).

Strip No.	*Peak[a] height*	*Peak[a] area*	*Peak[b] area*	*t (min)*	*d (cm)*	R_F
2	8.7	18.7	6.9	62	6.0	0.65
3	8.2	18.9	6.9	62	9.0	0.67
4	8.05	18.9	7.1	62	12.0	0.68
5	7.8	19.1	7.0	62	15.0	0.69
6	8.85	19.0	7.2	59	12.0	0.68
7	9.65	18.8	7.0	54	9.0	0.68
8	11.10	17.8	5.9	45.5	6.0	0.68
9	13.75	18.6	5.9	32	3.0	0.67

[a] For scanning with 1 mm spot.
[b] For scanning with 10 × 1 mm slit.

small variations in the distance of the run are unlikely to have a significant effect on densitometry accuracy. These conclusions may not apply, where there is frontal analysis of a mixed solvent[10] or where the R_F value is much nearer to either 0 or 1.

(c) Scanning with a 1 mm diameter spot of light gives more nearly constant peak areas than scanning with a slit, which is a rather surprising result.

(d) The peak concentration in the centre of a spot varies much less with distance of development, d, than with the time of contact, t, of the solute with the mobile phase (see Table V). In practice, therefore, it seems necessary to standardise the time, t, where two chromatograms are to be compared by densitometry.

It should not be overlooked that the flow rate of the solvent varies with the position of the origin and is thus also a variable in the above tests. To gain an idea of the effect of flow rate two chromatograms were run, where R_F values, the time t and the distance of development d were kept constant. With each of the three com-

TABLE VI

EFFECT OF TIME AND FLOW RATE

Adsorbent: Merck Silica Gel G; solvent: alcohol free chloroform; developed in S-tank; 22.5°; humidity 58%; layer thickness 0.24 mm. t = time of contact of solute with mobile phase (see Fig. 2).

Strip No.	R_F *value*	*Peak[a] height*	*Peak[a] width*	*Peak[a] area*	*Peak[b] area*	*t (min)*
1	0.62	10.20	2.20	22.4	8.9	48.2
2	0.63	10.25	2.10	21.5	8.6	47.1
3	0.65	10.50	2.05	21.5	8.7	45.1
4	0.68	11.10	1.85	20.5	8.2	43.3
5	0.69	11.25	1.70	19.1	7.7	40.3
6	0.73	12.35	1.60	19.7	7.3	36.8
7	0.68	12.60	1.55	19.5	7.1	32.1

[a] For scanning with 1 mm spot.
[b] For scanning with 10 × 1 mm slit.

pounds used in this test peak areas were slightly larger where there was a much higher flow rate, the influence being greater the greater the R_F value. From these results it is concluded that, in normal practice, the variation of flow rate is likely to have a negligible effect on peak areas.

It may be added here that there was appreciably increased resolution at the low flow rate, as measured by the peak width.

Effect of layer thickness

Great care had to be taken in order to obtain both a uniformly graded thickness and a uniform activity of the silica gel before development. Using an S-tank for development of the plate is essential in order to obtain the ideal conditions, under which R_F value is independent of layer thickness[8].

TABLE VII

EFFECT OF LAYER THICKNESS

Adsorbent: Merck Silica Gel G; solvent: chloroform; developed 15 cm in S-tank; 66 min; 23°; humidity 40%. Densitometry with 3 mm diameter light beam, no filter. Solute: Sudan Red G.

Strip No.	*Layer thickness (mm)*	R_F	*Transmission*			*Reflectance*		
			Peak height	*Peak width*	*Peak area*	*Peak height*	*Peak width*	*Peak area*
1	0.52	0.31	9.70	2.30	22.31	4.20	2.30	9.66
2	0.47	0.31	9.80	2.25	22.05	4.55	2.20	10.01
3	0.39	0.31	9.55	2.15	20.53	4.90	2.15	10.54
4	0.30	0.31	9.25	2.00	18.50	5.55	2.05	11.38
5	0.13	0.31	8.15	1.85	15.08	6.25	2.10	13.13

The following conclusions were drawn from these results:

(a) Peak height, width and area all depend on layer thickness to a considerable extent.

(b) With reflected light both peak height and peak area increase as the layer thickness decreases.

(c) With transmitted light both peak height and peak area decrease as the layer thickness decreases. Extrapolation of the values in Table VII leads to an area of 12.4 at zero thickness. It is probable that this is the figure one would obtain if the layer were perfectly translucent and that the higher peak areas arise from multiple absorption effects.

(d) With transmitted light no loss of peak resolution was observed as the layer thickness increased, but with reflected light there was appreciable loss of resolution.

It is clear from these results and others that the thickness of the layer is a critical factor in densitometry. Thus from Table VII above it may be calculated that a 0.01 mm change in layer thickness led to a change of about 0.18 cm^2 in peak area (transmission).

Effect of moisture content of the adsorbent

It has been observed that a layer of silica gel on a chromatoplate can take up or lose moisture from the surrounding atmosphere quite rapidly[1,3], the final moisture

content depending on the relative humidity and the temperature. A few tests have therefore been carried out to see if there was any appreciable effect of the moisture content of silica gel on the densitometry. The results obtained so far (Table VIII) show that the water content of the gel cannot be neglected, there being a change of the order of 1% in peak area with a 3% change in the relative humidity.

TABLE VIII

EFFECT OF RELATIVE HUMIDITY ON DENSITOMETRY

Strip No.	*Original area*[a]	*Storage conditions*	*Area after storage*
1	20.8	48 h at 30% RH	21.6
2	19.6	48 h at 30% RH	20.5
3	20.3	48 h at 60% RH	19.1
4	20.5	48 h at 60% RH	19.4
1		1.5 h at 30% RH	21.3
2		1.5 h at 100% RH	16.2

[a] Relative humidity (RH) = approx. 40%. Spots scanned with 1 mm diameter spot; no filter; 3:1 gear. Areas in cm^2.

It is not clear yet to what extent this effect is due to a change in the translucence of the silica gel or to a change in the absorption spectrum due to a change in the strength and nature of the adsorption forces. Further tests using black spots obtained by charring of glycerides with acid showed that after storage at 100% humidity for 1.5 h there was, in the five cases observed, a small increase in peak area. This trend (increasing area with increasing humidity or translucence) is opposite to that observed with the red dye, but is in keeping with the general effect of layer thickness. It is quite possible, therefore, that two different mechanisms can operate simultaneously to determine the effect of humidity on the densitometry. In support of this it was noticed that the colour of the Sudan Red spot changed appreciably with humidity.

Error in detection of colourless compounds

It has been shown that a very useful degree of precision can be achieved in the densitometry of coloured compounds on chromatoplates. However, it has been found that the error in the detection stage, where colourless compounds are chromatographed, is considerably greater than that at any other stage.

Experiments are still being undertaken to assess the sources of error in the detection stage so that some attempt may be made to reduce the error. It has been found difficult to obtain a relative standard deviation, for a single reading on an average sized peak, of less than 6%; the average in practice has been observed to be nearer 10% (using the method of PRIVETT AND BLANK[11] for the detection of glycerides). With a standard error of around 10% the contribution from the densitometry itself is very minor in importance.

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