

THE REPRODUCIBILITY OF R_F VALUES USING PRECOATED TUBES AND FOILS

GEORGINA H. JOLLIFFE AND E. J. SHELLARD

Pharmacognosy Research Laboratories, Department of Pharmacy, Chelsea College of Science and Technology (University of London), London, S.W.3 (Great Britain)

SUMMARY

A statistical analysis has been made of the results obtained for the movement of simple dye mixtures on coated glass plates and commercially available factory-coated tubes and foils. The results show that the serious shortcoming of variability of the adsorbent layer on glass which rendered consistent, standardised results impractical may be partially overcome with the precoated tubes and foils.

INTRODUCTION

Thin layer chromatography (TLC) is now established as a valuable separation technique used by scientists of many disciplines. For some purposes it is essential to make use of R_F values and where this is necessary for purposes of identification considerable reliance must be placed on the reproducibility of R_F values. Factors affecting the reproducibility of R_F values in TLC have been discussed by BRENNER *et al.*¹, DALLAS², SHELLARD³ and others.

One of the most important factors is the active condition of the thin layer at the time of use and this has been found most difficult to standardise or control for the reasons discussed by SHELLARD³.

Within recent years precoated plates, tubes and foils have been described⁴⁻⁸, with the claim that they give better reproducibility of R_F values than is obtainable with the conventional layers spread on glass plates. This communication is concerned with results obtained by chromatographing simple dye mixtures on a number of the commercially available precoated tubes and foils. A statistical analysis of the results has been made and the results have been compared with those obtained using conventional layers.

EXPERIMENTAL

Conventional layers

Materials. Silica Gel G (Merck) and Alumina G (Merck).

Thickness of layer. 250 μ .

Method of spreading. Mechanical spreader (a) Desaga, (b) Shandon, or (c) Quick-fit and Quartz.

Activation. Air drying for 15 min followed by heating in an oven at 110° for 30 min.

Method of development. By ascending solvent technique.

Solvent system. Benzene.

Distance. 10 cm.

Type of tank. Large volume tank lined with paper for equilibration.

Precoated tubes and foils

Materials

Desaga Chromatotubes (Silica Gel G): (a) activated as supplied; (b) non-activated as supplied; (c) activated by heating at 110° for 30 min; (d) used tubes, eluted with methanol and reactivated as stated in (c).

Precoated foils (Macherey, Nagel and Co., Düren): Silica Gel N-HR, N-HR/UV254, S, S/UV254, and S-HR/UV254. Aluminium Oxide N and N/UV254. Cellulose MN300 and MN300 F254. Polyamide MN and MN/UV254.

Precoated foils (Eastman Kodak Ltd.): Silica Gel 6060 (formerly known as Type K301R) and Polyamide K541 V.

Thickness of layer. Eastman-Kodak silica gel stated to be 100 μ . There is no information regarding thickness of other materials used but probably 100 μ .

Activation. All silica gel and aluminium oxide foils were activated by heating at 100° for 15 min. The others were not activated. There is a warning on the box, not to activate cellulose layers.

Method of development. Ascending solvent technique.

Solvent systems. Benzene (with silica gel, alumina and chromatubes), methanol (with alumina), ammonia 880-methanol-water, 1:15:84 (with polyamide), and 25% ammonium hydroxide-2.5% sodium citrate, 2:8 (with cellulose).

Distance. 6 or 8 cm.

Type of tank. 'S' chamber (Kodak 'S' chamber Model 104, for Kodak foils).

Dyes

For the chromatotubes and silica gel layers: a Desaga dye mixture consisting of *p*-dimethylaminoazobenzene, Sudan Red G, and indophenol.

For the alumina layers: neutral red and eosin.

For the cellulose layers: amaranth and tartrazine.

For the polyamide layers: fluorescein and Orange G.

Application: in quantities of 0.06 μ l, by means of Hamilton syringe.

RESULTS

The results obtained are given in Tables I-VI. hR_F ($R_F \times 100$) values are given, having been calculated to the nearest whole number from the distance (in cm) measured from the starting line to the centre of the spot.

DISCUSSION

Although it has been shown by DALLAS² and others that the relative humidity of the atmosphere in which the adsorbent layers are maintained prior to use affects the moisture content of the layer at the time of use and that this is one of

TABLE I

hR_F VALUES OBTAINED ON CONVENTIONAL LAYERS

A. Silica gel/benzene

(a) Results obtained on plates obtained from same spread

Colour of dye	No. of plates	No. of spots per plate	Range of <i>hR_F</i> values	Mean	Mean % coefficient of variation	Variance ratio
Yellow	5	10	36-46	42.0	0.75	195
Red	5	10	14-19	16.4	0.48	104
Blue	5	10	5-8	6.7	0.40	35.6

(b) Results obtained on plates obtained from different spreads

Colour of dye	No. of plates	No. of spots per plate	Range of <i>hR_F</i> values	Mean	Mean % coefficient of variation	Variance ratio
Yellow	5	10	40-50	44.1	0.92	55
Red	5	10	15-19	16.9	0.38	67
Blue	5	10	4-7	6.1	0.42	48

B. Alumina/benzene

(a) Results obtained on plates obtained from same spread

Colour of dye	No. of plates	No. of spots per plate	Range of <i>hR_F</i> values	Mean	Mean % coefficient of variation	Variance ratio
Yellow	5	10	85-91	88.2	0.88	18
Red	5	10	60-70	65.7	0.86	50
Blue	5	10	47-56	51.8	0.91	44

(b) Results obtained on plates obtained from different spreads

Colour of dye	No. of plates	No. of spots per plate	Range of <i>hR_F</i> values	Mean	Mean % coefficient of variation	Variance ratio
Yellow	5	10	75-93	84.9	0.80	409
Red	5	10	58-77	68.3	0.94	324
Blue	5	10	47-64	55.2	0.96	224

the main factors in obtaining reproducible results, no attempt was made, in this work, to control the moisture content of the layers. This was because the literature accompanying the tubes and foils implies that no special precautions need be taken and that the materials can be used directly from the packages. Furthermore those precautions are not usually undertaken in the majority of laboratories where TLC is used as a routine method for purposes of separating mixtures of substances and it was the intention to compare the precoated layers with the conventional layers as they would normally be used.

TABLE II

 hR_F VALUES OBTAINED ON CHROMATOTUBES

A(1) = Activated Silica Gel G/benzene. Distance travelled by solvent: 8 cm.
 B(1) = Inactivated Silica Gel G/benzene.
 C(1) = Inactivated Silica Gel G activated by heating at 110° for 30 min/benzene.
 A(2) = A(1) tubes eluted with methanol and reactivated by heating at 110° for 30 min.
 B(2) = B(1) treated as A(2).
 C(2) = C(1) treated as A(2).

	Colour of dye	No. of tubes	No. of spots per tube	Range of hR_F values	Mean	Mean % coefficient of variation	Variance ratio
A(1)	Yellow	8	8	46-54	50.84	1.72	15.35
	Red	8	8	16-19	18.41	0.89	5.74
	Blue	8	8	4-6	5.61	0.68	22.1
B(1)	Yellow	8	6	75-96	87.83	7.44	777
	Red	8	6	41-91	65.65	16.02	1883
	Blue	8	6	29-86	52.75	19.52	3982
C(1)	Yellow	8	8	15-26	20.55	2.77	20.7
	Red	8	8	4-9	5.66	1.58	79.8
	Blue	8	8	0-3	1.11	0.54	19.14
A(2)	Yellow	(4)* 3	8	18-23	20.17	1.93	20.3
	Red	(4)* 3	8	3-8	5.58	1.61	36.6
	Blue	(4)* 3	8	0-1	0.67	0.48	∞
B(2)	Yellow	4	8	16-21	19.06	2.11	2.7
	Red	4	8	3-8	5.44	0.80	5.3
	Blue	4	8	0-3	0.5	0.67	1.8
C(2)	Yellow	4	8	18-28	21.77	2.84	13.0
	Red	4	8	4-8	5.47	1.31	3.4
	Blue	4	8	0-3	0.53	0.94	6.1

* One tube gave streaks *not* spots.

Chromatotubes. The reproducibility of R_F values on each of the eight activated silica gel tubes was good but the values obtained varied from tube to tube, particularly for the fast moving dye *p*-dimethylaminoazobenzene, the computed variance ratios indicating that there was a significant difference between the tubes (Table II, A(1).)

The manufacturer's leaflet *Desaga Tube Thin-layer Chromatography* includes a graph showing the relationship between R_F values and the drying temperature of the silica gel on the tubes. The expected hR_F values for *p*-dimethylaminoazobenzene, Sudan Red G and indophenol on silica gel activated at 110° for 30 min (the temperature and time given by the manufacturers for the activated tubes) is given as 14, 5 and 1 respectively. The mean hR_F values obtained were 50.8, 18.4 and 5.6 (Table II, A(1)) and it was thought that, by accident, some of the tubes giving high hR_F values had not been activated. Some inactivated tubes were, therefore, examined without activation and as shown in Table II B(1) there was a wide variation in results but with the hR_F values being much higher than those given by the activated tubes. However, on heating the inactivated tubes in a hot air oven at 110° for 30 min just before use the mean values obtained were 20.55, 5.66 and 1.1 (Table II, C(1)). These values, together with

TABLE III

 hR_F VALUES OBTAINED FROM SILICA GEL PRECOATED FOILS

A = Silica Gel N-HR, Macherey, Nagel and Co.

B = Silica Gel N-HR/UV254, Macherey, Nagel and Co.

C = Silica Gel S, Macherey, Nagel and Co.

D = Silica Gel S/UV254, Macherey, Nagel and Co.

E = Silica Gel S-HR/UV254, Macherey, Nagel and Co.

F = Silica Gel 6060, Eastman Kodak Ltd.

Solvent system: benzene.

(1) Distance travelled by solvent: 6 cm

	Colour of dye	No. of foils	No. of spots per foil	Range of hR_F values	Mean	Mean % coefficient of variation	Variance ratio
A	Yellow	8	3	23-28	24.46	2.13	20.0
	Red	8	3	8-10	8.5	0.88	5.43
	Blue	8	3	1-2	1.17	0.38	1.5
B	Yellow	8	3	27-37	32.17	3.28	5.22
	Red	8	3	8-15	11.21	1.67	6.89
	Blue	8	3	1-2	1.67	0.48	16.0
C	Yellow	8	3	20-27	22.46	1.47	3.1
	Red	8	3	7-8	7.08	0.28	4.0
	Blue	8	3	1-2	1.13	0.34	∞
D	Yellow	8	3	40-43	41.58	0.97	2.12
	Red	8	3	15-18	16.42	1.06	2.92
	Blue	8	3	5-7	5.92	1.02	7.93
E	Yellow	8	3	17-23	18.29	1.85	268
	Red	8	3	5-8	5.58	1.06	86.3
	Blue	8	3	1	1	0	0
F	Yellow	8	3	60-88	79.29	7.79	37.2
	Red	8	3	30-50	40.50	5.58	31.6
	Blue	8	3	15-38	22.04	5.61	18.5

(2) Distance travelled by solvent: 8 cm

	Colour of dye	No. of foils	No. of spots per foil	Range of hR_F values	Mean	Mean % coefficient of variation	Variance ratio
A	Yellow	8	3	20-26	21.83	1.93	8.55
	Red	8	3	6-10	7.88	1.12	9.98
	Blue	8	3	1	1	0	0
B	Yellow	8	3	20-36	27.54	5.85	31.48
	Red	8	3	6-13	9.25	2.29	1.20
	Blue	8	3	0-3	1.13	0.54	9.07
C	Yellow	8	3	20-25	22.96	1.43	1.84
	Red	8	3	5-8	7.13	1.08	1.09
	Blue	8	3	1-2	1.13	0.34	∞
D	Yellow	8	3	35-40	38.5	1.32	5.33
	Red	8	3	13-15	14.21	0.83	8.65
	Blue	8	3	4-5	4.33	0.48	2.28
E	Yellow	8	3	14-25	18.75	4.21	21.0
	Red	8	3	4-8	5.75	1.45	6.03
	Blue	8	3	1	1	0	0
F	Yellow	8	3	68-78	77.25	3.17	35.3
	Red	8	3	28-38	32.79	3.35	56.7
	Blue	8	3	10-19	13.96	3.69	131.84

TABLE IV

hR_F VALUES OBTAINED FROM ALUMINA PRECOATED FOILS

A = Aluminium Oxide N, Macherey, Nagel and Co.

B = Aluminium Oxide N/UV254, Macherey, Nagel and Co.

Solvent system: methanol.

(1) Distance travelled by solvent: 6 cm

Dyes	No. of foils	No. of spots per foil	Range of <i>hR_F</i> values	Mean	Mean % coefficient of variation	Variance ratio
A Neutral red	8	3	56-63	59.37	2.16	28.86
Eosin	8	3	30-33	31.29	1.27	5.17
B Neutral red	8	3	48-56	53.96	2.29	25.36
Eosin	8	3	25-30	27.13	1.92	13.83

(2) Distance travelled by solvent: 8 cm

Dyes	No. of foils	No. of spots per foil	Range of <i>hR_F</i> values	Mean	Mean % coefficient of variation	Variance ratio
A Neutral red	8	3	64-68	65.42	0.97	7.1
Eosin	8	3	31-38	35.08	53	4.8
B Neutral red	8	3	55-68	63.12	2.89	13.44
Eosin	8	3	24-34	29.33	2.84	6.30

TABLE V

hR_F VALUES OBTAINED FROM CELLULOSE PRECOATED LAYERS

A = Cellulose MN300, Macherey, Nagel and Co.

B = Cellulose MN300 F254, Macherey, Nagel and Co.

Solvent system: 25% ammonium hydroxide-2.5% sodium citrate (2:8).

(1) Distance travelled by solvent: 6 cm

Dyes	No. of foils	No. of spots per foil	Range of <i>hR_F</i> values	Mean	Mean % coefficient of variation	Variance ratio
A Tartrazine yellow	8	3	73-81	78.25	2.27	5.84
Amaranth	8	3	35-43	37.79	2.11	1.69
B Tartrazine yellow	8	3	66-76	69.88	3.14	8.07
Amaranth	8	3	23-30	26.46	2.32	30.4

(2) Distance travelled by solvent: 8 cm

Dyes	No. of foils	No. of spots per foil	Range of <i>hR_F</i> values	Mean	Mean % coefficient of variation	Variance ratio
A Tartrazine yellow	8	3	81-88	84.71	2.05	2.75
Amaranth	8	3	44-51	47.87	2.09	1.03
B Tartrazine yellow	8	3	66-73	69.08	2.10	1.92
Amaranth	8	3	25-33	28.37	2.24	7.62

TABLE VI

 hR_F VALUES OBTAINED FROM POLYAMIDE LAYERS

A = Polyamide MN, Macherey, Nagel and Co.

B = Polyamide MN/UV254, Macherey, Nagel and Co.

C = Polyamide K541V, Eastman Kodak Ltd.

Solvent system: ammonia 880-methanol-water (1:15:84).

(1) Distance travelled by solvent: 6 cm

Dyes	No. of layers	No. of spots on foil	Range of hR_F values	Mean	Mean % coefficient of variation	Variance ratio
A Fluorescein	8	3	78-93	85.07	5.27	70.78
Orange G	8	3	61-83	70.58	7.99	53.0
B Fluorescein	8	3	76-93	85.08	4.51	74.1
Orange G	8	3	61-85	75.04	7.17	98.9
C Fluorescein	8	3	83-93	88.83	2.84	16.97
Orange G	8	3	37-47	42.08	3.49	51.0

(2) Distance travelled by solvent: 8 cm

Dyes	No. of layers	No. of spots per layer	Range of hR_F values	Mean	Mean % coefficient of variation	Variance ratio
A Fluorescein	8	3	70-89	80.67	4.92	30.1
Orange G	8	3	53-78	66.29	6.51	30.8
B Fluorescein	8	3	81-93	87.25	4.20	24.0
Orange G	8	3	68-86	78.50	6.92	45.5
C Fluorescein	8	3	86-93	89.87	1.87	12.26
Orange G	8	3	54-61	57.42	2.08	6.26

those obtained (Table II, A(2), B(2) and C(2)) after elution of the used tubes (A(1), B(1) and C(1) in Table II) with methanol and reactivation at 110° for 30 min, were closer to the expected hR_F values than from the commercially activated tubes. The hR_F values obtained in Table II, A(1) would indicate an activation temperature of about 33° or, alternatively; and this is more likely, that the tubes had picked up moisture after activation, in spite of the fact that they are "effectively sealed against moisture".

Tubes which had been eluted with methanol and re-activated were found to be somewhat friable and in some cases after only two elutions the layer was far too broken for further use.

Precoated foils. Comparison of the variance ratio figures in Table I (A = silica gel; B = alumina—hand spread layers) with those in Tables III and IV (Table III, 1 and 2 = silica gel; Table IV, 1 and 2 = alumina—precoated under controlled conditions by the manufacturers) shows that there is less variation in R_F values on the precoated foils than on conventional layers whether obtained from the same or different spreads. However, the computed variance ratios frequently indicate a considerable

difference between different foils from the same pack so that no reproducibility of R_F values can be claimed. The variance ratio for the 6060 Eastman Kodak silica gel foils was considerably greater than those obtained for the Macherey Nagel and Co. silica gel range of foils. This may have been due to the fact that all the Macherey Nagel and Co. silica gel was of constant pH value (4.0) whilst that of the Eastman Kodak silica gel foils varied from 6.5 to 4.0. It was also noticeable that the dyes all moved towards the centre of the Eastman Kodak foils but that this could be prevented by cutting the bottom corners of the foil before placing in the tank. Even then it was necessary to cover the Kodak tank with a beaker.

In the case of the polyamide layers, the Eastman Kodak K541V had lower variance ratio than the corresponding foils in the Macherey, Nagel and Co. range.

It might be mentioned that the silica gel bound with starch was extremely friable and flaked away from the plastic backing very easily so that it was not easy to handle. The aluminium oxide foils also presented problems since the solvent frequently moved very irregularly with the result that R_F values could not be measured.

Reference to Tables III (1) and (2) shows that the mean hR_F values on the silica gel foils for the three dyes in the Desaga test mixture were, in general, lower the greater the distance moved by the solvent front. However, with one exception, polyamide MN, the reverse was true for the dyes separated on alumina (Tables IV (1) and (2)), cellulose (Tables V, (1) and (2)) and polyamide (Tables VI (1 B, C) and (2 B, C)) though not on polyamide MN (Table VI). Nevertheless, comparison of the variance ratios (Tables III-VI) for a development distance of 6 and 8 cm shows there is a general tendency for lower variance ratios with increase in the distance moved by the solvent front. Hence, for purposes of correlation of R_F values on precoated foils even with control of humidity the use of a standard distance of development would be necessary. The dependence of the R_F values upon development distance when multi-component solvents are used even when the atmosphere of the tank is saturated has been reported¹. However, in this work the silica gel and alumina foils were developed in a single solvent system so that this type of dependence cannot be attributed to "frontal analysis".

Lack of reproducibility in R_F values in TLC has led to various suggestions being made for reporting R_F values: (a) to report the average R_F values of the substances as obtained with the specified experimental conditions, (b) to report R_X values¹⁰⁻¹², and (c) to report the range of R_F values as obtained with the specified experimental conditions⁹.

If, with the increased use of commercially precoated foils, more consistent R_F values are obtained, even in the absence of preconditioning in an atmosphere of controlled relative humidity, it will be necessary to give some consideration to the definition of reproducibility and perhaps to state the coefficient of variation permitted in order to consider a single R_F value as characteristic of the substance concerned. Within this context it will be necessary to consider the precision of a quoted R_F value. Visual observation of the separated dyes on the precoated foils used in this work suggested very good reproducibility so that the computed results were extremely surprising. However, considerable variation can arise in the actual determination of R_F values since many variables are involved, e.g. selection of the centre of the spot, the actual measurement of the distances involved, correction of the figure obtained (hR_F to nearest whole number) etc. so that different workers may report different R_F values after examining the same chromatogram.

ACKNOWLEDGEMENTS

We are grateful to Mr. R. P. HIRSCH, Camlab (Glass) Ltd., Cambridge, for a generous supply of Chromatotubes (Desaga Ltd) and precoated foils (Macherey, Nagel & Co.)

REFERENCES

- 1 M. BRENNER, A. NIEDERWIESER, G. PATAKI AND H. R. FAHMY, *Experientia*, 18 (1962) 101.
- 2 M. J. J. DALLAS, *J. Chromatog.*, 17 (1965) 267.
- 3 E. J. SHELLARD, *Lab. Pract.*, 13 (1964) 293.
- 4 A. LESTIENNE, E. P. PRZYBYLOWICZ, W. J. STAUDENMAYER, E. S. PERRY, A. D. BAITSHOLTS AND T. N. TISCHER, *TLC Conf. Brussels, Sept. 1964; J. Pharm. Belg.*, 5-6 (1965) 213, 224.
- 5 E. P. PRZYBYLOWICZ, W. T. STAUDENMAYER, E. S. PERRY, A. D. BAITSHOLTS AND T. N. TISCHER, *J. Chromatog.*, 20 (1965) 506.
- 6 Advertising literature by Macherey Nagel & Co., Duren.
- 7 Advertising literature by C. Desaga Ltd., Heidelberg.
- 8 Advertising literature by Eastman Kodak, Paris.
- 9 E. STAHL, *Arch. Pharm.*, 292 (1959) 411.
- 10 J. H. DHART AND C. DE ROOY, *Analyst*, 86 (1961) 78 and 527.
- 11 J. W. C. PEEREBOOM, *J. Chromatog.*, 3 (1960) 323.
- 12 B. P. LISBON AND E. DICZFALUSY, *Acta Endocrinol.*, 40 (1962) 60.

DISCUSSION

GEISS: Do you have an air-conditioned laboratory?

SHELLARD: No.

GEISS: I believe that there is a good deal of chance variations in the results. Conditioning of Desaga Tubes at 110° does not eliminate the influence of humidity. It is only somewhat decreased in an irregular fashion. Do you believe that your comparisons are truly reliable under these conditions?

SHELLARD: As I have already stated no attempt was made to control the moisture content of the adsorbent layer on the chromatotubes because the literature stated quite definitely that no special precaution need be taken. Since the results obtained with the tubes which the manufacturers stated had been activated at 110° for 30 min corresponded with the results which would be expected if the tubes were activated at 30° it was thought that perhaps these particular chromatotubes had not been activated. That is why we compared them with chromatotubes which were labelled "Unactivated" and obtained the results already described. There is no doubt that the activated chromatotubes had picked up varying amounts of moisture after activation and for this reason no reliability can be placed upon the R_F values obtained with activated chromatotubes.

PRISTOUPIL: I should like to make a brief remark on the use of membrane filters as a suitable medium for small-scale chromatography. The membranes made of nitrocellulose, acetylcellulose, nylon, teflon etc. are usually available in uniform thin (0.1-0.05 mm) sheets, having a fine foam-like microporous structure with a relatively narrow distribution of pore sizes. Different types of membranes with mean pore sizes from 0.01 μm up to 10 μm are being produced.

The strips used for chromatography (or electrophoresis, resp. cf. J. KOHN, *Aerzt. Lab.*, 10 (1964) 233) are "self-carriers", i.e. they need no mechanical supports,

as do powdered or gel materials in common thin-layer operations, and they are easy to manipulate; since they differ also in many respects from fibrous chromatographic strips (*e.g.* paper), they seem to represent a special type of chromatography which we call "membrane chromatography".

In recent papers (*J. Chromatog.*, 28 (1967) 89 and 124) we were able to show some examples of the applicability of membrane chromatography on nitrocellulose membranes Synpor (Chemapol, Prague), either normal or wedge-compressed, both intact or impregnated with detergents or proteins, in the analysis of very small samples of proteins. Marked differences in the chromatographic behaviour of some dyes interacting with the stationary protein layer on impregnated Synpor strips were also observed. At present, a more detailed investigation of the method is under way in our laboratory, using different types of membranes, solutes and solvent systems.

J. Chromatog., 33 (1968) 165-174