CHROM. 3774

AN IMPROVED METHOD OF DENSITOMETRIC THIN LAYER CHROMATOGRAPHY AS APPLIED TO THE DETERMINATION OF SAPOGENIN IN *DIOSCOREA* TUBERS

K. R. BRAIN AND ROLAND HARDMAN
Pharmacognosy Group, School of Pharmacy, Bath University of Technology, Claverton Down, Bath, Somerset (Great Britain)
(Received August 1st, 1968)

SUMMARY

A cheap, easily made and generally applicable template and syringe assembly is described which gives a more rapid and accurate densitometric thin layer chromatographic procedure. This is illustrated by the estimation of sapogenin in *Dioscorea* tubers. Factors limiting the precision of the assay method have been investigated and a standard deviation of 1.1 % has been obtained.

INTRODUCTION

The commercial production of biologically active steroids commonly begins from steroidal sapogenins. The latter may be accurately estimated in plant material by infra-red spectrometry and the improved procedure of BRAIN *et al.*[1] is rapid and affords the biologically interesting data of the amounts of the 25α- and 25β-epimers contributing to the total sapogenin. The method has the limitations that the sample must yield about 50 mg of sapogenin and that, in certain cases, interference in the infra-red spectrum, due to absorption by non-sapogenin compounds in the solution of crude sapogenin, has been observed.

These limitations were partially overcome by BLUNDEN, HARDMAN AND MORRISON[2] when they applied densitometric TLC to the determination of diosgenin in *Dioscorea* tubers, with an error of approximately 7 %. Recently they have extended the method to include the estimation of the mono- and di-hydroxy sapogenins of species of *Yucca* and *Asparagus*[3].

Our work, on the factors controlling the sapogenin available from harvested plant material, required a more accurate means of estimating the sapogenin and we have used densitometric TLC to give a method accurate to $\pm$ 2 %.

It is well known that one of the major sources of error in quantitative TLC is the spotting procedure and we have devised a relatively simple and inexpensive application apparatus which optimises the requirements of speed and accuracy.

BLUNDEN *et al.*[2,3] found that a linear relationship held between the logarithm of the weight of sapogenin applied to the plate and the square root of the absorbance

TABLE I

ERRORS IN THE APPLICATION OF SAMPLES TO THIN LAYER CHROMATOGRAPHIC PLATES

No.	*Method*	*Number of plates*	*Number of spots on each plate*	*Total number of spots scanned*	*Mean % S.D.*	*Variation of % S.D.*
i	Hand-held disposable micropipette[a]	6	9	54	9.3	± 5.4
ii	Stand-held 10 μl microsyringe[b]. Hand set	4	8	32	10.6	± 0.4
iii	Stand-held 10 μl microsyringe[b]. Automatic setting by Chaney adaptor	5	7	35	7.9	± 3.4
iv	Stand-held Agla micrometer syringe[c]. Hand set	6	7	42	7.7	± 2.7
v	Hand-held 250 μl microsyringe with repeating dispenser[b] and special template[d]	9	7	63	2.8	± 0.3
vi	As (v) but with polythene sleeve on tip of the needle	6	7	42	6.4	± 1.9

[a] Drummond Scientific Co., Broomall, Pa., U.S.A.
[b] Hamilton Co., P.O. Box 307, Whittier, Calif., U.S.A.
[c] Burroughs, Wellcome and Co. Ltd., Dartford, England.
[d] Described in this paper.

value of the spot produced. However, under our conditions, a direct relationship was found between the weight of sapogenin applied and the integrated absorbance reading. This may well be due to the different chromatographic procedures used and hence to the different shape, both in area and profile, of the spots of sapogenin, as well as the use of the Chromoscan* rather than the Vitatron** densitometer.

EXPERIMENTAL

Isolation of crude sapogenin

The sapogenin was isolated in the manner described by BRAIN *et al.*[1] but the final residue was dissolved in sufficient chloroform to give a total sapogenin concentration of approximately 0.1 %.

Thin layer chromatography

A slurry was prepared by shaking 30 g of Silica Gel G (Merck) with 60 ml of distilled water and was applied as a 250 μ thick layer to 20 × 20 cm glass plates using a Shandon adjustable spreader and Unoplan leveller. The plates were air-dried for 15 min before activation at 110° for 30 min.

Application of the samples. When some commonly used procedures[4] for the application of samples to TLC plates were tried it became obvious that the application process was a major source of error. Therefore an evaluation of six different methods was undertaken. By each procedure a series of 5 μl spots of standard sapogenin solution, 1 μg/μl, was applied, the chromatogram developed, sprayed and scanned by

* Joyce Loebl & Co. Ltd., Gateshead, England.
** Fison's Scientific Apparatus Ltd., Loughborough, England.

transmittance. The % S.D. for each plate and the mean % S.D. for each series of plates were calculated (Table I).

Procedure v, using the template and syringe assembly described below (Fig. 1) was the best both for speed and accuracy. The template was constructed of perspex as follows: the base-plate, 21.5 × 21.5 cm, had two opposite side pieces, 17 × 0.5 × 2 cm high, cemented to it at the edges, and a third side, the end-piece, 21.5 × 0.5 × 2

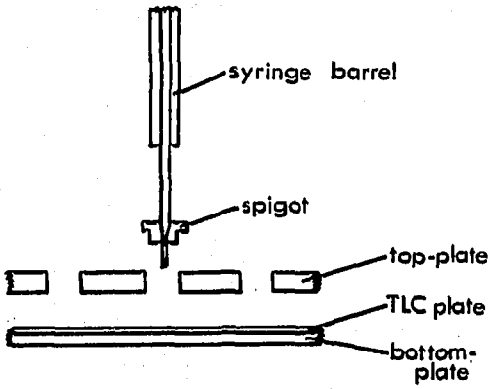

Fig. 1. Template and syringe assembly.

cm, was added. A top-plate, 21.5 × 0.5 × 2 cm, was fixed at the lower edge, across the side and end pieces, and a series of twelve holes, 0.5 cm in diameter, with 1.5 cm centres, was drilled through it, 1.5 cm from the end-piece. A 250 μl Hamilton microsyringe was fitted into a Hamilton PB 600 repeating dispenser, which, on depression of the spring-loaded button, moved the syringe piston through one-fiftieth of its total travel length, thus expelling a fixed volume of liquid, 5 μl. A perspex spigot was constructed of shaft diameter 0.49 cm with a collar of 1 cm diameter. A hole of 0.3 mm diameter was drilled through the centre of the spigot. This allowed the final centimetre of the syringe to pass through but the spigot was a tight fit at the position where the needle diameter expanded to 0.5 mm.

The syringe was filled with the test solution in the usual manner, then held vertically, in one hand, over the template. It was lowered until the spigot engaged with the first template hole and a single 'shot' of liquid ejected. This was repeated with the same solution for alternate holes and the intermediate positions spotted in the same manner with standard solution. The procedure was extremely rapid and required only about 15 sec to apply five spots of one solution. In the sapogenin assay five spots, each of 5 μl, of a standard sapogenin solution of 1 μg/μl, and another five spots, each of 5 μl, of an unknown solution of approximately equal concentration, were applied alternately to each of two plates.

Solvent system. Each plate was developed in a Desaga S-chamber for a distance of 15 cm (25–30 min), with *n*-hexane–acetone (8:1). The chromatographic separation is shown in Fig. 5.

Detection of sapogenin. Antimony trichloride, 300 g, in concentrated hydrochloric acid, 100 ml, was used to char the sapogenin spots. The plate was air-dried for 2 min before residual solvent was removed at 110° for 30 min in a mechanical convection oven. The plate was allowed to cool for 15 min before scanning.

Densitometry

A Chromoscan double-beam recording and integrating densitometer with thin layer scanner (for optical system see SHELLARD AND ALAM[5]) was used for quantitation in the transmission mode under the following conditions: wedge 2, cam C, gain 5, aperture 1005, tungsten lamp with no filter.

TABLE II

MEAN VARIATION OF THE DENSITOMETER READINGS ACROSS THE THIN LAYER CHROMATOGRAPHIC PLATE

Standard spots	*Unknown spots*	*Ratio of adjacent spots*
2.8 %	3.2 %	3.6 %

The plate was positioned with the slit 1.5 cm to higher R_F value of the first sapogenin spot, the baseline count set to one unit per 5 sec, and the spot scanned at a sample:record expansion ratio of 1:4. The area under the densitometer curve was automatically calculated and expressed as a readout on the built-in digital integrator (see Discussion). Subsequent spots were scanned similarly, working from right to left, and then each spot was scanned a second time, working from left to right.

Calibration curves

Mixtures of the C_{25} epimers, for example diosgenin with yamogenin, normally occur in nature and the relative proportions of the two components can readily be determined by infrared spectrometry[1]. A 0.1 % w/v solution in chloroform of a natural mixture, having approximately the same $25\alpha:25\beta$ ratio as the test sample, was used as the reference material to minimise any difference in response of the C_{25} isomers to the assay procedure.

Three spots, each of 5 μl, of four standard diosgenin/yamogenin solutions of concentrations ranging from 2 to 14 μg/μl were applied to a TLC plate as described above. Fig. 2 shows the relationship between functions of concentration and integrator readings.

In another experiment three spots, each of 5 μl, of five standard sapogenin solutions of concentrations ranging from 0.88 to 1.28 μg/μl were applied to each of four TLC plates and treated similarly. The linear relationship between the integrator reading and the sapogenin concentration is shown in Fig. 3.

TABLE III

DETERMINATION OF THE ACCURACY OF THE PROCEDURE

	Mean % S.D. of duplicate determinations
(a) Thirteen TLC plates each scanned twice	0.80
(b) Six sapogenin solutions each determined twice	0.95
(c) Four plant samples each assayed twice	1.10

RESULTS AND DISCUSSION

The precision of the densitometer was determined by scanning a single 5 μg spot of sapogenin 26 times without resetting the scan path. The integrator reading was noted in each case and the % S.D. of the readings was 1.1 %. The built-in digital integrator was used successfully in this work as there was negligible fluctuation observed in the baseline.

There must always be some degree of spatial separation between the standard and unknown on a TLC plate and it is impossible to spray the whole surface evenly. To minimise the effects of this each plate was spotted with five standard and five unknown spots and the mean values calculated. The variation in the densitometer readings of 40 spots, five each of standard and unknown on each of four plates is given in Table II. The variation in the values for individual spots was slightly lower than the variation in the ratio of adjacent spots.

The precision of the assay procedure was estimated in three ways (Table III). The mean % S.D. was calculated from the results of thirteen TLC plates each scanned twice. This gave a figure of 0.80 % for the error in the densitometric determination. The errors involved in the chromatography and detection stages were estimated from the means of six sapogenin solutions each determined twice. The value of 0.95 % was only slightly greater than the figure above indicating that there was little error introduced at these stages. The accuracy of the extraction procedure was determined by assaying each of four plant samples twice. Again the figure of 1.10 % was only slightly greater than the previous figure indicating complete and repeatable extraction.

The basis of the high precision of the whole assay procedure is repeated determination. The final result is the mean of ten separate spot values each determined twice. This duplication of the estimation is readily achieved with the aid of the template and syringe assembly.

The figures for the errors in the application of samples to TLC plates by various methods (Table I) show that not only does the precision of application vary from method to method but also wide variation can occur in the accuracy of a given procedure. For example method (i): a hand-held disposable micropipette, had an error range of approximately 5–15 %. This variation in the accuracy is due in part to the artifice of the particular operator.

Our finally adopted procedure (method v) has the advantage that variation caused by the operator's manipulations are minimised and controlled: location of the spigot and depression of the plunger are the only actions required. Connection of the tip of the needle to the TLC plate by means of a piece of flexible polythene tubing (method vi) failed: the accuracy was lower and damage to the surface of the plate was frequent. Method (v) was as rapid as method (i) and these procedures were considerably faster than any of the others; the time taken to spot 5 × 5 μl of two different solutions to each of two plates was approximately 3 min.

Recently FAIRBAIRN AND RELPH[6] have published the results of an investigation of the errors in spotting paper and thin layers. They quote a coefficient of variation of 9.74 % for delivery of 25 μl of a methanolic solution of morphine from a Hamilton repeating dispenser. BRIDGER AND RELPH[7] have constructed a machine for automatic application with a coefficient of variation of 2.5 % and our value of 2.8 % compares

very favourably with this, especially when the simplicity of our apparatus is noted.

Considerable variation has occurred in the results of different workers as to the relationship between the absorbance and the concentration of the compound in both densitometric PC and TLC. Several authors[8–16] have found that a direct relationship occurs under suitable conditions between the integrated area under a densitometric peak, the absorbance value of the centre of the spot, or the optical density of the whole spot, and the quantity of the compound applied. A variety of compounds have been studied including Solanaceous alkaloids[8], *Digitalis* glycosides[9], anthraquinones[10,11], cholesterol[12], cholesterol and monopalmitin[13], methyl and cholesteryl palmitates[14], sugars[15], and amphetamines and barbiturates[16].

PURDY AND TRUTER[17] found that there was a linear relationship between the logarithm of the weight of substance applied and the square root of the area of the spot produced. BLUNDEN *et al.*[2] found that this relationship also held for the logarithm of the weight of sapogenin against the square root of the integrated absorbance reading produced.

JOHNSTONE AND BRINER[18] found a linear relationship between the logarithm of the absorbance area and the logarithm of the concentration of certain purines and SHELLARD AND ALAM[5] have found a linear relationship between the curve area and the square root of the amount of compound for the *Mitragyna* oxindole alkaloids.

If the BEER–LAMBERT law were obeyed then a linear relationship would be expected between the logarithm of the integrated area and the concentration of the compound in the layer. However, this law is not obeyed as the thin layer plate is translucent and a high proportion of the light is scattered. The KUBELKA–MUNK[19] equation for the absorption of light in highly scattering media has recently been applied by GOLDMAN AND GOODALL[20] to the problem of quantitative analysis of thin layer chromatograms. The KUBELKA–MUNK equation overlaps the BEER–LAMBERT law at low concentrations and a direct relationship between the logarithm of the curve area and the compound concentration can occur over short distances[21].

In the case of the mixture of diosgenin with yamogenin under our experimental conditions, various functions of our results were plotted and Fig. 2 shows these over the range 10–70 μg of total sapogenin. All deviated to some extent from a linear relationship and no method was better than the direct one of sapogenin concentration plotted against integrator reading. Over a narrow range of 4.5 to 6.5 μg of total sapogenin (Fig. 3) a linear relationship was obtained. This relationship between integrator reading and sapogenin concentration is not direct and a correction curve (Fig. 4) was drawn up for use with a 5 μg standard. The apparent sapogenin concentration was calculated from the standard and unknown readings assuming a direct relationship and this was then correlated with the absolute concentration by means of the correction graph and expressed as a percentage of the original plant material on a moisture-free basis.

Fig. 5 shows the TLC separation and corresponding densitometer trace for the same extract from a sample of *Dioscorea deltoidea* tuber powder under (A) the conditions of BLUNDEN *et al.*[2], *n*-hexane–ethyl acetate (4:1) in an ordinary saturated TLC tank, (B) using *n*-hexane–ethyl acetate (4:1) in an S-chamber, and (C) our present conditions of *n*-hexane–acetone (8:1) in an S-chamber. Under condition (A) a diffuse sapogenin spot was produced of diameter 15 mm. Using the same solvent system but in the S-chamber the sapogenin was deposited as a circular spot of diameter 10 mm.

In the S-chamber with the *n*-hexane–acetone solvent system a narrow 2 × 8 mm band was produced.

The distribution of spots in depth in thin and thick layers has been the subject of speculation. Under conditions of perfect saturation there should be an even distribution of the compound throughout the thickness of the layer but under normal chromatographic conditions there is a concentration of the substance towards the

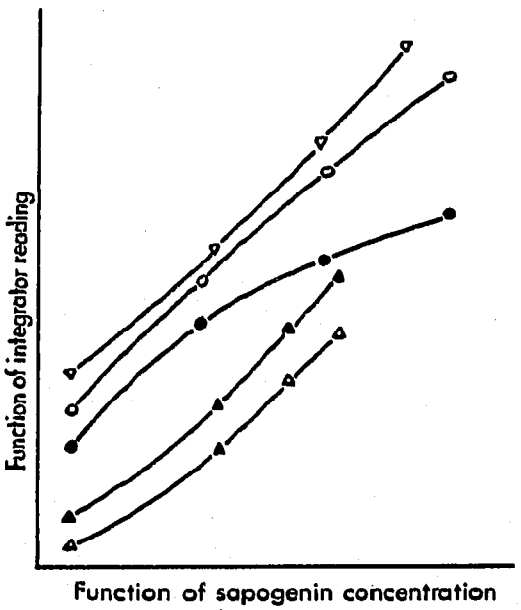

Fig. 2. Relationship between various functions of integrator reading and of sapogenin concentration over the range 10–70 μg of diosgenin and in the transmission mode. (○—○) Integrator reading *vs.* sapogenin concentration; (●—●) log integrator reading *vs.* sapogenin concentration (BEER–LAMBERT law); (▲—▲) log integrator reading *vs.* log sapogenin concentration; (△—△) √(integrator reading) *vs.* log sapogenin concentration; (▽—▽) integrator reading *vs.* √(sapogenin concentration).

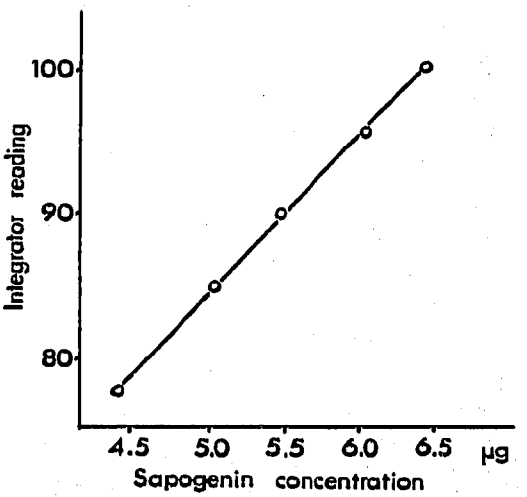

Fig. 3. Relationship between integrator reading and sapogenin concentration in the transmission mode over the range 4.5–6.5 μg of diosgenin.

upper surface of the layer. The S-chamber gives a close approximation to perfect saturation and suppresses the movement of solvent towards the surface of the layer giving a more even distribution in depth and hence a sharper spot edge and narrower band width.

Using transmission scanning (true densitometry) the same amount of light will be absorbed by the compound no matter what the thickness of the layer although the background absorbance will vary with layer thickness. A problem encountered using transmission methods has been the preparation of TLC plates of even thickness but we have found that, provided the plates were run in the direction of spreading, this gave no difficulty especially as the high contrast of the spot and background allowed the use of a low cam setting.

With reflectance scanning the distribution of the compound in the layer is of greater importance as reflectance takes place to the greatest extent at the surface of the layer. We found that the response rapidly dropped off with increasing concentration (Fig. 6) and we opted for the transmission mode.

In transmission scanning the light passes through a certain amount of the layer before it reaches the spot and according to the length of this path so the scatter will vary. As the spot becomes more compact scatter becomes relatively less important. SHELLARD AND ALAM[5] found in the case of the *Mitragyna* alkaloids that if the plate was sprayed with petroleum–liquid paraffin (1:1) to make it transparent before

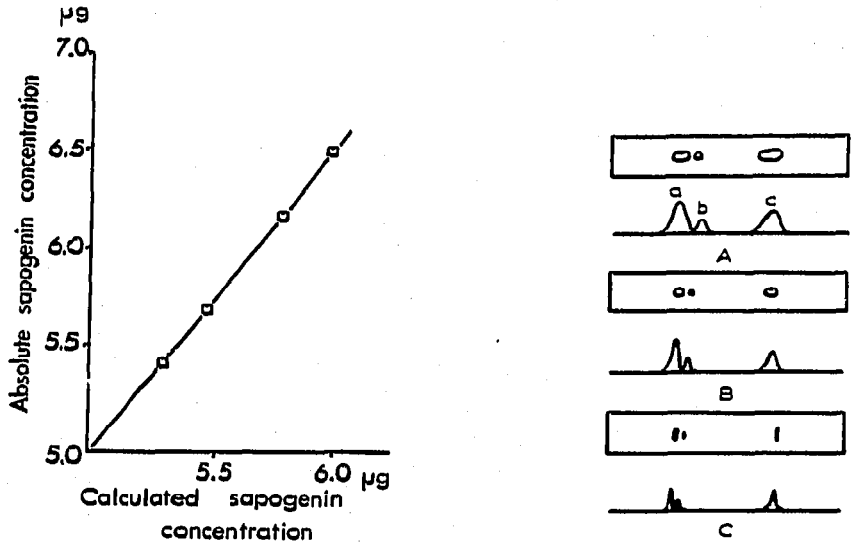

Fig. 4. Correction curve using 5 μg standard and transmission scanning.

Fig. 5. Chromatographic separations and densitometer traces from an extract of *Dioscorea deltoidea* tuber run under different conditions, *viz.* (A) *n*-hexane–ethyl acetate (4:1) in an ordinary tank; (B) *n*-hexane–ethyl acetate (4:1) in an S-chamber; (C) *n*-hexane–acetone (8:1) in an S-chamber. a = diosgenin/yamogenin; b = sterol; c = spirostan-3,5-diene.

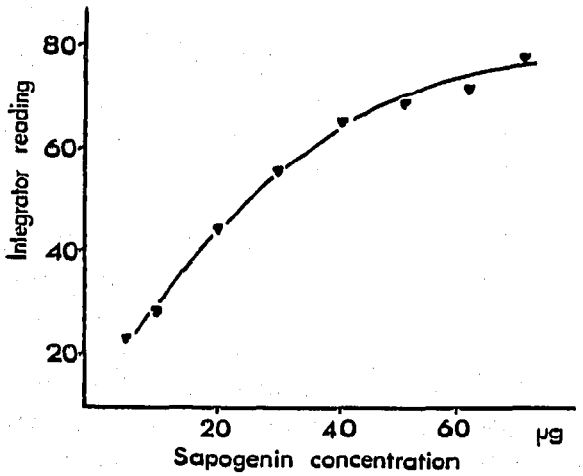

Fig. 6. Relationship between integrator reading and sapogenin concentration in the reflectance mode over the range 5–70 μg of diosgenin.

transmission scanning a linear relationship existed between peak area and the amount of alkaloid present whereas if the untreated plate was scanned a curvo-linear relationship was obtained. It is suggested that due to the even distribution in depth of the compound throughout the layer under our conditions the situation approximates to that found by SHELLARD AND ALAM[5] and the layer acts as if it were transparent.

ACKNOWLEDGEMENTS

We wish to thank the National Research Development Corporation for financial assistance and one of us (K.R.B.) thanks the Tropical Products Institute, Ministry of Overseas Development, for the award of a research studentship. Mr. A. G. KENYON and Mr. H. L. PEERMAN, of the Institute and Corporation respectively, are thanked for their assistance with supplies of plant material, and Mr. R. M. SADLER for his technical assistance.

REFERENCES

1 K. R. BRAIN, F. R. Y. FAZLI, R. HARDMAN AND A. B. WOOD, *Phytochem.*, 7 (1968) 1815.
2 G. BLUNDEN, R. HARDMAN AND J. C. MORRISON, *J. Pharm. Sci.*, 56 (1967) 948.
3 G. BLUNDEN AND R. HARDMAN, *J. Chromatog.*, 34 (1968) 507.
4 E. STAHL (Editor), *Thin Layer Chromatography*, Springer Verlag, Berlin, Heidelberg and Academic Press, New York, 1965.
5 E. J. SHELLARD AND M. Z. ALAM, *J. Chromatog.*, 33 (1968) 347.
6 J. W. FAIRBAIRN AND S. J. RELPH, *J. Chromatog.*, 33 (1968) 494.
7 Y. E. BRIDGER AND S. J. RELPH, *Brit. Pat.*, Appl. Nos. 54215/65 and 40136/66.
8 T. J. QUINN, J. G. JEFFREY AND W. C. MACAULAY, *J. Pharm. Sci.*, 46 (1957) 384.
9 A. ULUBELEN, *J. Pharm. Sci.*, 51 (1962) 62.
10 J. SCHRATZ, *Planta Med.*, 6 (1958) 44.
11 R. DEQUEKER, J. LEMLIET AND J. CUVEELE, *Planta Med.*, 12 (1964) 51.
12 J. J. PEIFER, *Mikrochim. Acta*, 34 (1962) 529.
13 J. G. LINES, in A. C. FRAZER (Editor), *Biochemical Problems of Lipids*, Elsevier, New York, 1963.
14 O. S. PRIVETT, M. L. BLANK, D. W. CODDING AND E. C. NICKELL, *J. Am. Oil Chemists' Soc.*, 42 (1965) 381.
15 J. W. MIZELLE, W. J. DUNLAP AND S. H. WENDER, *J. Chromatog.*, 28 (1967) 427.
16 J. C. MORRISON AND J. M. ORR, *J. Pharm. Sci.*, 55 (1966) 936.
17 S. J. PURDY AND E. V. TRUTER, *Analyst*, 87 (1962) 802.
18 B. M. JOHNSTONE AND G. P. BRINER, *Nature*, 183 (1959) 681.
19 P. KUBELKA AND F. MUNK, *Z. Tech. Phys.*, 12 (1931) 593; English transl. by F. A. STEELE, *Paper Trade J.*, 100 (1935) 37.
20 J. GOLDMAN AND R. R. GOODALL, *J. Chromatog.*, 32 (1968) 24.
21 G. FRANGLEN, in E. J. SHELLARD (Editor), *Quantitative Paper and Thin Layer Chromatography*, Academic Press, London, 1968.