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QUANTITATIVE THIN-LAYER CHROMATOGRAPHY

VII*. FURTHER INVESTIGATIONS OF DIRECT FLUOROMETRIC SCANNING OF AMINO ACID DERIVATIVES**

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SUMMARY

Direct estimation of DANS-, DNP- and PTH-amino acids separated on silica gel and polyamide layers, by means of fluorescence and fluorescence quenching techniques, is discussed. The reproducibility of different methods of peak measurement is compared. Special reference is made to the scanning of closely neighbouring spots.

Although thin-layer chromatography (TLC) has been well established in amino acid and peptide chemistry (for a review *cf.* refs. 3–6), there is very little known about the quantitative estimation of these compounds by means of *in situ* techniques. As far as we are aware, SEILER in Germany[7] was the first to use direct fluorometry for scanning of fluorescent spots of dimethylamino-naphthalenesulphonyl-(DANS)-amino acids. Independently and simultaneously, one of us described the direct fluorometric estimation of DANS-amino acids, as well as the application of the quenching technique in quantitative evaluation of dinitrophenyl (DNP)- and phenylthiohydantoin-(PTH)-amino acids[8]. In the meantime further investigations have been carried out, and beside silica gel layers, the use of instant polyamide layers according to WANG[9] has been studied. Our recent results are described in the present communication.

METHODS

Chromatography

TLC of *DANS-amino acids* was carried out on Silica Gel G layers (containing starch)[8] with benzene–pyridine–acetic acid (80:20:2, v/v)[10] or chloroform–methanol–

* For Part VI *cf.* ref. 1.
** 8th communication on "Application of thin-layer chromatography in sequence analysis of peptides." 7th communication *cf.* ref. 2.

acetic acid (75:20:5, v/v)[11]. Alternatively, instant polyamide layers[9*] and *e.g.*, heptane–*n*-butanol–acetic acid (3:3:1, v/v)[12] were used.

DNP-amino acids were chromatographed either on Silica Gel G layers (containing starch)[8] with chloroform–benzyl alcohol–acetic acid (70:30:3 v/v)[10] or with *n*-propanol–25 % ammonia (7:3, v/v)[10], or on polyamide sheets[9*] with benzene–acetic acid (4:1, v/v)[13].

Chromatograms of *PTH-amino acids* were carried out on Silica Gel G–zinc silicate layers (containing starch)[3,4,8] with chloroform–formic acid (100:5, v/v)[10] and chloroform–methanol(9:1, v/v)[10] or on polyamide layers[9*] with formic acid 90 %–water (1:1, v/v)[14].

Quantitative estimation

The spots were scanned, after drying the chromatoplates in a stream of cold air for exactly 30 min, by fluorometry using a Turner-Fluorometer 111 fitted with a door for thin-layer plates[**]. In some experiments the improved "de-luxe" door has been used[15**]. Speed of scan: 20 mm/min.

Conditions for scanning are given in Table I. The fluorometer units were recorded, generally at a voltage of 10 mV, with a Hi-Speed Recorder (W+W model 401)[***] normally using a speed of 8 cm/min.

TABLE I

CONDITIONS OF SCANNING[a]

Compound	*Layer*	*Excitation (nm)*	*1° filter*[b]	*2° filter*[b]	*Neutral density filter in addition to the 2° filter (%)*	*Sensitivity of fluorometer*[c]
DANS-amino acids	Silica gel	366	110–811	110–816	40 + 20	10 ×
DANS-amino acids	Polyamide	366	110–811	110–816	40 + 10	1 ×
DNP-amino acids	Silica gel	254	110–810	110–816	40	5 ×
DNP-amino acids	Polyamide	254	110–810	110–816	10	5 ×
PTH-amino acids	Silica gel with zinc silicate	254	110–810	110–816	20	1 ×
PTH-amino acids	Polyamide	254	110–810	110–816	10	5 ×

[a] Aperture to door in all experiments fully open (~ 3.4 mm). The samples were applied as spots, using 2 μl microcaps.
[b] Camag catalog nos.
[c] The blends 110–842 were used.

The scan was made perpendicular to the direction of chromatography. The layer was covered, except for a small strip, 12–14 mm, containing the spot under investigation, with a plain silica gel or polyamide sheet, respectively.

RESULTS

Our previous experiments on direct fluorometry[1,8,16–18] have been carried out with a prototype of the Camag/Turner Scanner. In the meantime a number of im-

* Cheng-Chin Trading Co. Ltd., No. 75 Sec. I. Hankow St., Taipei, Taiwan.
** Camag, Muttenz, BL, Switzerland.
*** Kontron AG, Zürich, Switzerland.

provements have been made[15,19] and therefore the working conditions had to be changed to some extent. In particular, the sensitivity could be kept considerably lower, as illustrated in Fig. 1. On the other hand, it was also found that the fluorescence peak area is dependent on the *sensitivity* of the fluorometer. The effect of using different fluorometers in scanning of spots is shown in Fig. 2. Fluorescence peaks of DANS-amino acids (Fig. 3) and quenching peaks of DNP-amino acids (Fig. 4) on silica gel and on polyamide layers show very clearly that such compounds can be measured with higher sensitivities on polyamide layers. The superiority of polyamide layers with respect to the sensitivity of scanning is partly compensated by the fact that the separation of amino acid derivatives is highly dependent upon the quality of polyamide used. Whilst the chromatographic resolution of amino acids

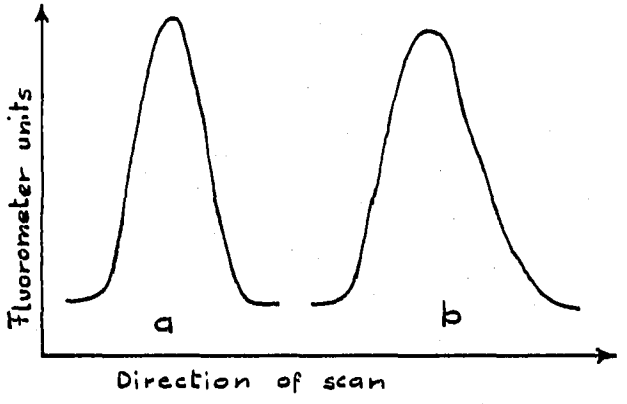

Fig. 1. Fluorescence peaks of DANS-proline on Silica Gel G. (a) 5 μg DANS-proline measured with the prototype (sensitivity: 10 ×, no neutral density filter was used). Peak area = 1420 mm^2. (b) 2 μg DANS-proline measured with the improved model (sensitivity: 10 ×, neutral density filters: 20 + 40 %). Peak area = 1840 mm^2.

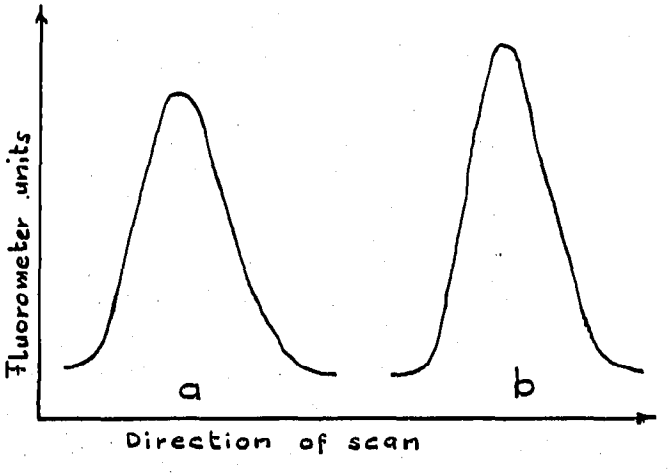

Fig. 2. Fluorescence peaks of DANS-proline on Silica Gel G. (a) 2 μg DANS-proline, measured with fluorometer I (sensitivity: 10 × ; neutral density filters: 20 + 40 %). Peak area = 1840 mm^2. (b) 2 μg DANS-proline, measured with fluorometer II (sensitivity: 10 × ; neutral density filters: 20 + 40 %). Peak area = 2040 mm^2.

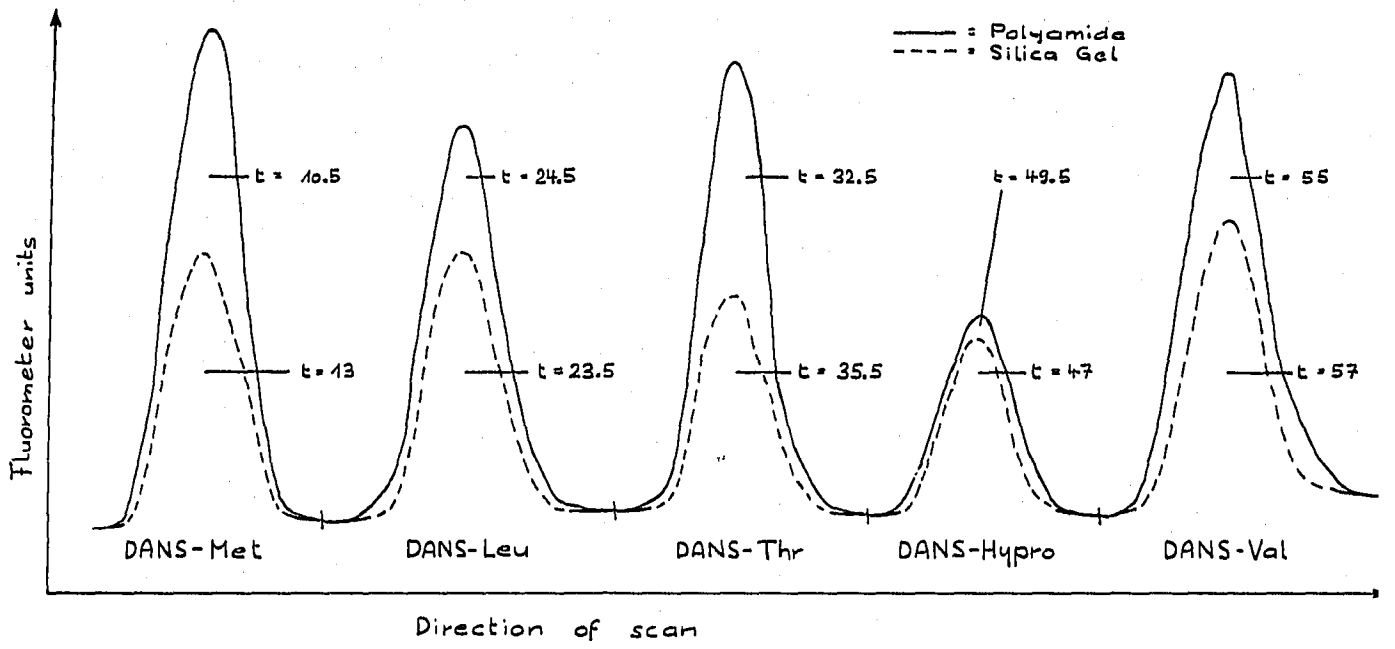

Fig. 3. Fluorescence peaks of DANS-amino acids (2 μg each) on Silica Gel G and polyamide, conditions are given in Table I. The higher sensitivity on polyamide layers is especially worthy of note. The "t-values" indicate the time (min) between plate drying and scanning (see METHODS).

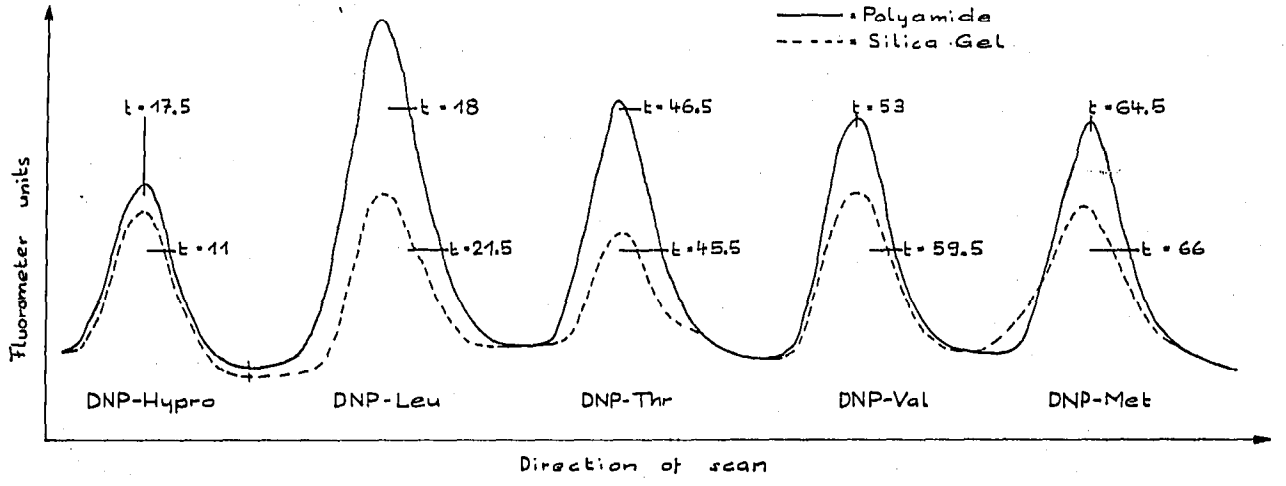

Fig. 4. Fluorescence quenching peaks of DNP-amino acids (2 μg each) on Silica Gel G and polyamide. The conditions are given in Table I. The higher sensitivity on polyamide layers is especially worthy of note. The "t-values" indicate the time (min) between plate drying and scanning (see METHODS).

on silica gel layers from different commercial suppliers seems to be hardly influenced by the quality of sorbent[20], the separation of DANS-, DNP- and PTH-derivatives is inferior using other types of polyamide. The sensitivity of scanning of PTH-amino acids on silica gel and polyamide is approximately of the same order of magnitude Fig. 5). The fluorescence peak areas of DANS-derivatives are given in Table II, while in Tables III and IV fluorescence quenching peak areas of DNP- and PTH-derivatives are given. These data may change with variation of the experimental conditions, or from one laboratory to another, but their relative values will serve as a guide line in

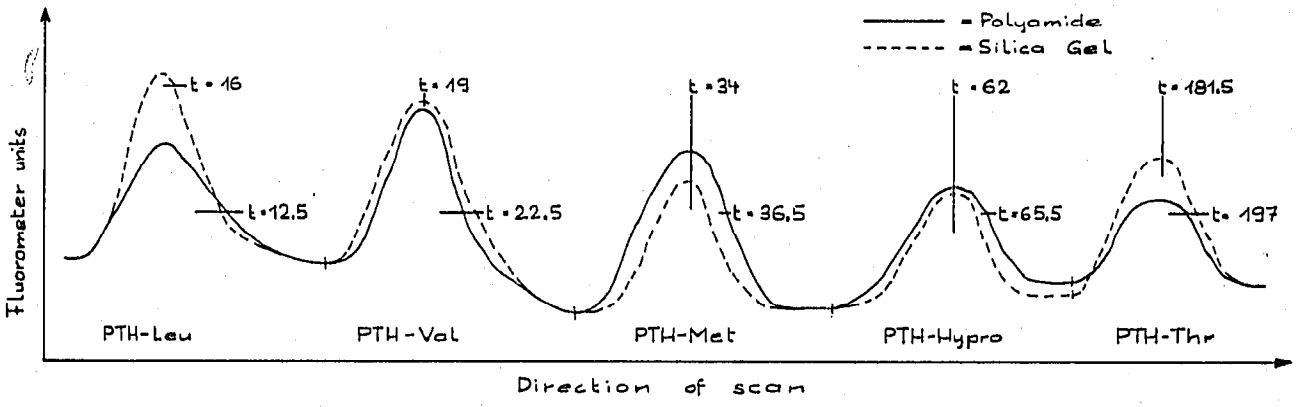

Fig. 5. Fluorescence quenching peaks of PTH-amino acids (2 μg each) on Silica Gel G (containing zinc silicate) and polyamide. The conditions are given in Table I. The "t-values" indicate the time (min) between plate drying and scanning (see METHODS).

practical work. It should be pointed out that in all cases of fluorescence quenching measurements, polyamide layers were capable of furnishing more constant baselines than silica gel layers.

In order to obtain reasonable reproducibility, the scanning must be made with care. Important influencing factors, some of which have been investigated previously[1,8,17] are: (a) positioning of the scanner; (b) standardisation of time between end of chromatography and start of scanning; (c) loading volume; (d) developing distance; (e) layer thickness. The first two (a and b) have been found very important

TABLE II

AREAS OF FLUORESCENCE PEAKS OF DANS-AMINO ACIDS (2 μg EACH) ON POLYAMIDE AND SILICA GEL LAYERS (*cf.* TEXT AND TABLE I)

DANS-	*Polyamide*		*Silica Gel*	
	Peak area[a] (mm^2)	t[b] (*min*)	*Peak area*[a] (mm^2)	t[b] (*min*)
Glutamic acid	2230	1	1500	6
Di-cystine	1870	1.5	2000	1.5
Methionine[c]	3860	10.5	2110	13
Tryptophane[c]	1850	13	740	9.5
Glycine	3530	19.5	2050	15
Serine[c]	2510	20.5	1460	22.5
Leucine[d]	3430	24.5	2010	23.5
Alanine[c]	4060	26.5	1910	28.5
Phenylalanine	3620	32.5	2300	38.5
Threonine[c]	3380	32.5	1520	35.5
Aspartic acid[d]	1570	35.5	960	32
Proline[c]	3600	44.5	1840	46.5
Hydroxyproline[d]	1590	49.5	1330	47
Valine[d]	4020	55	2190	57
Isoleucine[d]	4090	69.5	2290	68.5
Asparagine[d]	1890	79	1630	77.5

[a] Planimetry (5 times).
[b] Time (min) between plate drying and scanning (see METHODS).
[c] Cyclohexylamine salt.
[d] Piperidine salt.

TABLE III

AREAS OF FLUORESCENCE QUENCHING PEAKS OF DNP-AMINO ACIDS (2 μg EACH) ON POLYAMIDE AND SILICA GEL LAYERS (*cf.* TEXT AND TABLES I AND II)

DNP-	*Polyamide*		*Silica gel*	
	Peak area (mm^2)	*t* (*min*)	*Peak area* (mm^2)	*t* (*min*)
Di-Lysine	3160	0	2480	1
Cystine	2260	1	1460	1
Taurine	540	1	2190	4
Glycine	2800	1	1560	2.5
Cysteine	4340	2.5	2440	5
Glutamic acid	2150	3	1840	4.5
Arginine	3060	3	1020	1.5
Aspartic acid	2270	4	1020	7.5
Di-Homocystine	1610	12.5	1410	11.5
Alanine	3720	13	3290	20.5
Di-Cystine	1420	13.5	1160	15
Di-Homocysteine	3170	17.5	2100	14
Hydroxyproline	2070	17.5	1850	11
Leucine	4870	18	2570	21.5
Glutamine	1830	22	800	25.5
ε-Lysine	3540	24	3890	20
Methioninsulfone	3800	25.5	2100	24.5
Methioninsulfoxide	2220	26	1000	31.5
Isoleucine	4660	26	2210	29.5
Citrulline	4090	27	1690	14.5
β-Alanine	3990	29	2920	26
Di-Histidine[a]	2640	31.5	690	37
Phenylalanine	3260	34	2540	36
Homocitrulline	3840	35	1950	33
γ-Amino butyric acid	3780	38	1750	35.5
Tryptophane	2930	38.5	1850	41
Di-Tyrosine	2580	38.5	1910	35
α-Amino isobutyric acid	3640	40	2240	42
Proline	3930	44.5	3390	44.5
Sarcosine	5550	45	3510	50.5
Threonine	3050	46.5	1110	45.5
Serine	1750	49	1300	58
Hydroxylysine	1470	50.5	900	53
α-Amino butyric acid	4750	51.5	2710	58
Valine	3190	53	1990	59.5
Cysteic acid	3290	59.5	1760	51
im-Histidine	3010	60	2200	65
α-Amino adipic acid	3030	63	1770	78
Asparagine	1300	63	420	64
Methionine	3060	64.5	1530	66
Mono-O-Tyrosine	1880	65	420	81

[a] Impure, gives two spots on polyamide layers.

with respect to obtaining accurate results[8,15,17]. SEILER[7] reported on the influence of the moisture content of the layer on fluorescence. This effect, which has also been confirmed by us[8], can be suppressed by spraying the chromatogram with triethanolamine–isopropanol (1:4)[7,15]. Moreover the spray-technique of SEILER[7] increases the intensity of fluorescence. In addition, it has been noticed that heating the chromatograms prior to scanning in many cases caused a considerable increase in the amount of fluorescence[21,22]. The extent to which fluorescence quenching peaks are influenced

TABLE IV

AREAS OF FLUORESCENCE QUENCHING PEAKS OF PTH-AMINO ACIDS (2 μg) ON POLYAMIDE AND SILICA GEL ZINC SILICATE LAYERS (*cf.* TEXT AND TABLES I AND II)

PTH-	*Polyamide*		*Silica gel*	
	Peak area (mm^2)	*t* (*min*)	*Peak area* (mm^2)	*t* (*min*)
Δ-Serine	560	1	440	7
α-Amino butyric acid	1710	1.5	2310	1.5
Cystine	750	2	750	8
Alanine	2230	3	2300	2
Histidine			740	3
Glutamic acid	2060	9	1380	16
Homocitrulline	1520	11.5	1130	16.5
Leucine	1950	12.5	2540	16
Glycine	2120	16	1820	20
Valine	2670	22.5	2250	19
Phenylalanine	2490	27.5	1270	30.5
Methionine	2630	36.5	1800	34
Proline	2220	40	2660	40.5
Citrulline	1180	40	1150	44
Glutamine	2180	49	2160	52
Tryptophane	970	60.5	1570	60.5
Hyproline	1590	65.5	1390	62
α-Amino isobutyric acid	1320	84.5	1310	84
Methioninesulfone	2260	91.5	1240	78
Cysteic acid	1020	112.5	1210	105.5
Arginine	3140	124.5	1190	123
Asparagine[a]	1430	137.5	850	140
Aspartic acid	1710	142	1780	132
Lysine	1620	156	1560	147
Tyrosine	1340	171	1490	158
Isoleucine	1730	180	1300	168
Δ-Threonine	820	191	1250	187.5
Threonine	1270	197	1780	181.5

[a] Impure, gives a secondary spot.

by the time between drying and scanning has also been investigated[1,8,15]. Since the quenching peaks of some U.V.-absorbing substances have also shown time-dependence[1,8,15], it is a prerequisite in all cases of *in situ* scanning-fluorescence as well as quenching—to check whether the influence of time on the results has to be taken into account. The remaining influencing factors were found to be of less importance, however, their standardisation is also advisable.

Different techniques for the measurement of peak areas have been investigated. In the case of symmetrical peaks, with a uniform baseline, simple geometric measurement, planimetry and the use of a disc integrator have about the same accuracy. However, if the peaks are nonsymmetrical, which often occurs in the case of fluorescence quenching, planimetry gave the best reproducibility (Table V). The relative standard deviations* amount to 3.5–5 % in the case of fluorescence, and 5–7 % in the case of fluorescence quenching (measurement of 6 spots on the same chromatogram by planimetry). If spots from different chromatograms are to be compared,

* In all experiments the "*t*-values" (see *e.g.* Fig. 3) were kept constant. The "spray-technique" of SEILER[7] has been found less reproducible, particularly if spots from one chromatogram to another had to be compared[15] (*cf.* also ref. 23).

TABLE V

REPRODUCIBILITY OF FLUORESCENCE QUENCHING USING DIFFERENT MEASUREMENT TECHNIQUES (VALUES ON SIX DIFFERENT CHROMATOGRAMS: DNP-PROLINE, 2 μg)

Chromatogram	*Peak area (mm²)*		
	Planimetry[a]	*Geometry*[b]	*Integrator units*[c]
1	2460	3105	870
2	2350	2625	730
3	2570	3055	790
4	2650	3420	900
5	2250	2635	745
6	2100	2510	665
Mean value	2400	2890	785
Relative standard deviation	8.6%	12.3%	11.3%

[a] Measurement 5 times.
[b] Height·width at half-height.
[c] A disc integrator is used. The baseline has to be adjusted to the *same* value, before measurement.

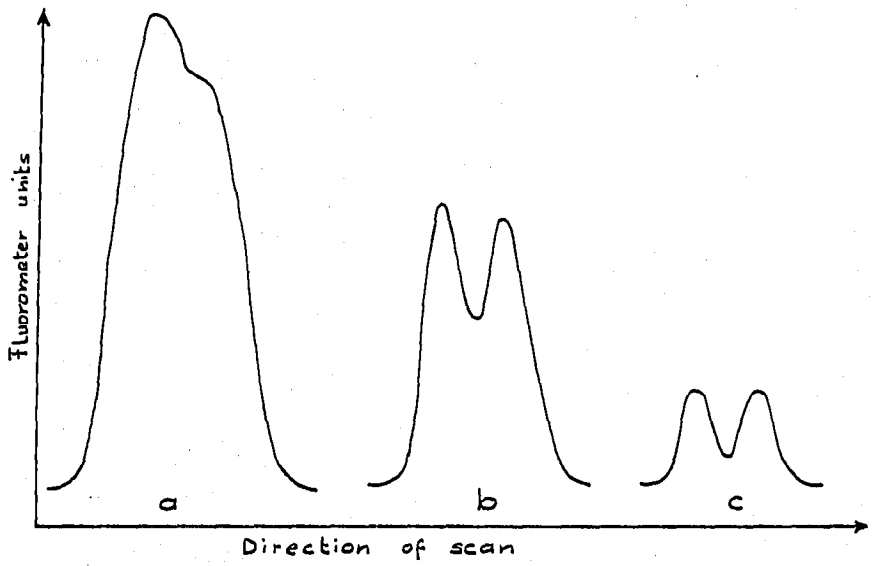

Fig. 6. Resolution of peaks of two DANS-derivatives (2 μg each). Measuring of fluorescence according to the conditions given in Table I; (a) aperture to door 3.4 mm; (b) aperture to door 1.7 mm; (c) aperture to door 0.85 mm.

relative standard deviations of about 10–15% can be obtained. An example is shown in Table V. Finally, the resolution of closely adjacent spots has been investigated. Fig. 6 shows an experiment where there is almost no resolution of two fluorescence peaks of DANS-derivatives, using the standard procedure as given in Table I. If, however, the aperture of the door is adjusted to only $^1/_2$ or even $^1/_4$ (*i.e.* 1.7 and 0.85 mm) the resolution of peaks increased considerably. The sensitivity, which is of course lower in this case, can be increased as much as is convenient by the use of other neutral density filters[23]. Nevertheless, the scanning of closely adjacent spots is sometimes difficult. The scanning of spots on two-dimensional chromatograms is troublesome and not free from error. This problem is now being investigated.

REFERENCES

1 G. PATAKI AND A. NIEDERWIESER, *J. Chromatog.*, 29 (1967) 133.
2 G. PATAKI, *Helv. Chim. Acta*, 50 (1967) 1069.
3 G. PATAKI, *Dünnschichtchromatographie in der Aminosäure und Peptidchemie*, W. de Gruyter, Berlin, 1966.
4 G. PATAKI, *Techniques of Thin-Layer Chromatography in Amino Acid and Peptide Chemistry*, Ann Arbor Science Publ., Ann Arbor, Mich., 1968.
5 G. PATAKI, *Chromatog. Rev.*, 9 (1967) 23.
6 G. PATAKI, J. BORKO, H. CH. CURTIUS AND F. TANCREDI, *Chromatographia*, in press.
7 N. SEILER AND M. WIECHMANN, *Z. Anal. Chem.*, 220 (1966) 109.
8 G. PATAKI AND E. STRASKY, *Chimia (Aarau)*, 20 (1966) 361.
9 K.-T. WANG, I. S. Y. WANG AND A. L. LIN, *J. Chinese Chem. Soc., Ser. II*, 13 (1966) 77.
10 M. BRENNER, A. NIEDERWIESER AND G. PATAKI, *Experientia*, 17 (1961) 145.
11 N. SEILER AND H. WIECHMANN, *Experientia*, 20 (1964) 559.
12 K. R. WOODS AND K.-T. WANG, *Biochim. Biophys. Acta*, 133 (1967) 369.
13 K.-T. WANG AND I. S. Y. WANG, *J. Chromatog.*, 27 (1967) 318.
14 K.-T. WANG, I. S. Y. WANG, A. L. LIN AND C.-S. WANG, *J. Chromatog.*, 26 (1967) 323.
15 D. JÄNCHEN AND G. PATAKI, *J. Chromatog.*, 33 (1968) 391.
16 G. PATAKI, *3rd Symposium on Chromatography and Electrophoresis, Brussels, 1966*, p. 305.
17 G. PATAKI AND E. STRASKY, *3rd Symposium on Chromatography and Electrophoresis, Brussels, 1966*, p. 317.
18 G. PATAKI AND A. KUNZ, *J. Chromatog.*, 23 (1966) 465.
19 *Fluorometrie*, FT-67, Camag, Muttenz (BL), Switzerland.
20 G. PATAKI, *J. Chromatog.*, 17 (1965) 580.
21 A. A. BOULTON, *Federation Europ. Biochem. Soc., 2nd Meeting, Vienna, 1962*, A 265.
22 A. A. BOULTON, in D. GLICK (Editor), *Methods of Biochemical Analysis*, Vol. 16, 1968, in press.
23 G. PATAKI, *Chromatographia*, in press.