QUANTITATIVE *IN SITU* FLUOROMETRY OF THIN-LAYER CHROMATOGRAMS

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SUMMARY

Two methods of fluorometric *in situ* scanning of TLC plates, *viz.* measuring the fluorescence and fluorescence quenching, are discussed. Design and operation of a suitable TLC scanner are described. The various scanning methods are demonstrated with pyrene, pregnanediol, amino acid derivatives and nucleotides. Reproducibilities of 3–5% for the fluorescence method and 5–8% for the quenching technique were observed. If results from different plates have to be compared a reproducibility of 10–15% can be reached.

Fluorometry is a particularly advantageous method among the procedures available for direct quantitative scanning of thin-layer chromatograms. A brief explanation of the terminology will be given before the special features of fluorometry as a scanning method in TLC are discussed.

The term "fluorometry" includes two different methods which have to be evaluated individually:

(A) "Fluorescence" measurements are applicable to such compounds which fluoresce on their own or which can be rendered fluorescent by suitable reagents. The method is based on the ability of a molecule to emit light of a longer wave length when it is excited by ultraviolet light which is absorbed. The background of the layer does not emit light.

(B) The "quenching" method, *i.e.* fluorescence quenching, is applicable to compounds absorbing ultraviolet light without simultaneously emitting secondary light. In order to make use of the U.V.-absorbance it is, in general, necessary to make the background of the plate fluorescent. Suitable "fluorescence indicators" are well known since the early days of TLC in 1952[1]. In some cases, and with a highly sensitive instrument, the small amount of natural fluorescence of the adsorbent layer is sufficient.

Method (A), the actual measurement of the fluorescence of a compound, is the most advantageous of all optical scanning techniques[2,3].

(1) Measuring the fluorescence is a direct method, the higher the concentration, the greater the intensity of emitted light. Therefore, within the limits of the instrument used, it is possible to adjust the sensitivity of the measurement according to the com-

pound to be scanned. All other optical techniques are based on indirect measurements, whereby the background of the plate determines the sensitivity.

(2) As the background appears dark to the instrument, most of the sources of error which are typical for transmission and remission methods are eliminated. Such interfering factors have been discussed in detail in the literature[2–5].

(3) The fluorescence method is particularly versatile. There is no doubt that there is a larger number of compounds showing native fluorescence than there are substances which absorb light of wave lengths above 400 nm, as is preferred for photometric measurements. At least this is so with scanning techniques not requiring a spray reagent, which always incorporates an additional source of error.

The versatility of the fluorometric technique is still further increased when the fluorescence quenching (method B) is included. However, in this case the advantages mentioned under 1 and 2 are no longer valid. Here the same interfering factors apply which are typical for the reflectance technique. If a compound can be scanned either by fluorescence or by fluorescence quenching, the first method should always be preferred. Basically the following procedures for fluorometric *in situ* scanning of TLC plates can be applied:

CF = chromatography, followed by fluorescence measurement (the compound shows native fluorescence)

CRF = chromatography, reaction, fluorescence measurement (the separated, nonfluorescent compound must be rendered fluorescent by a spray reagent)

RCF = reaction, chromatography, fluorescence measurement (the compound is rendered fluorescent prior to chromatography, whereby the reaction can be carried out before or after sample application)

CQ = chromatography, quenching method

CRQ = chromatography, reaction, quenching method

RCQ = reaction, chromatography, quenching method

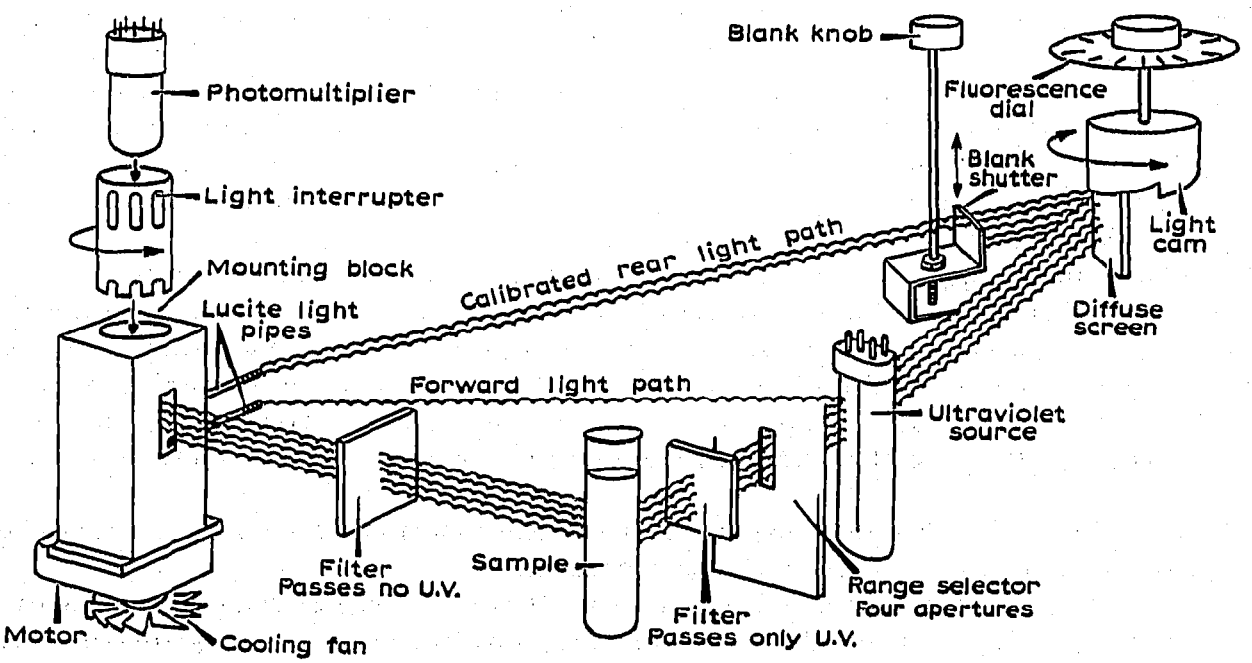


Fig. 1. Optical bridge system of the Turner fluorometer.

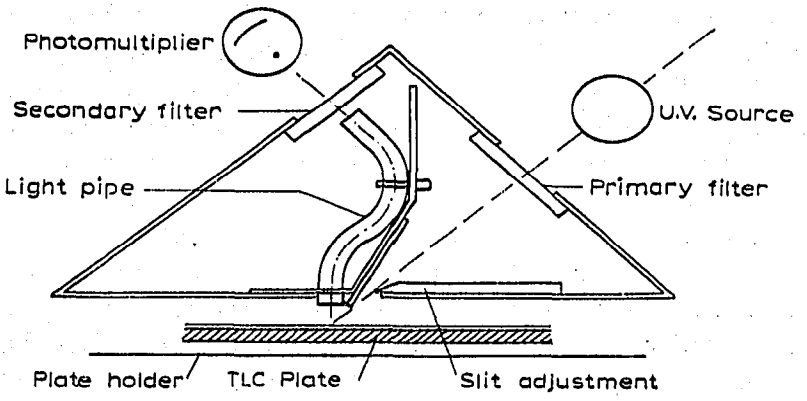


Fig. 2. Light path changed for TLC scanning.

Direct TLC scanning by fluorescence[5–9] and by fluorescence quenching[5,9–11] has been described. In this paper, examples of all the scanning methods, as mentioned, will be given with the exception of method CRQ. Although we do not have sufficient experience with this particular application, it seems to be well suited in lipid chromatography.

The measurements were carried out with a Turner model 111 filter fluorometer modified for TLC scanning[3,*]. The Turner fluorometer is a double beam instrument based on an optical bridge system. Details can be seen from Fig. 1. The choice to base the TLC scanner on this instrument was made because of its high sensitivity combined with excellent stability. As the original measuring compartment of the fluorometer was far too small to accommodate a standard size TLC plate, the optical light path had to be changed according to Fig. 2. The primary (excitation) light passes a range selector, then the primary filter. An aperture plate very close to the surface of the layer allows the adjustment of the excitation slit width between zero and 3.5 mm. The height of the slit is 15 mm and fixed. The secondary light emitted from either the spot or the layer is fed into the photomultiplier after passing the secondary filter. An

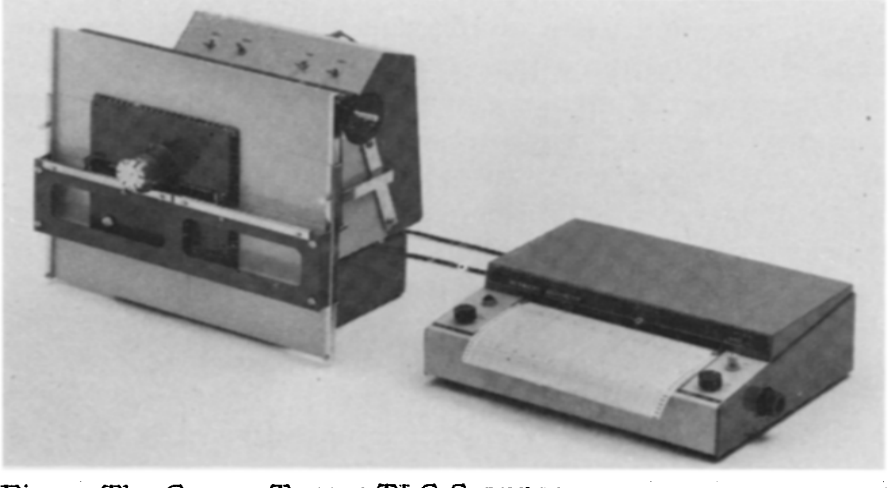


Fig. 3. The Camag-Turner TLC-Scanner.

* Commercially available from Camag, Muttenz, Switzerland.

S-shaped light pipe serves to feed maximum light intensity into the photomultiplier.

The TLC plate accomodated in the motor driven plate holder is slid at a constant speed past the scanning window. The vertical position of the plate is adjusted by the rack on which the plate holder rides. The direction of scan, either parallel or perpendicular to chromatography is determined by the way the plate is inserted in the plate holder. The scanner is depicted in Fig. 3.

A low pressure mercury lamp with maximum emission at 366 nm served for all fluorescence measurements. A Corning glass filter 7-60 (Turner 110-812) transmittant from 310 to 390 nm was used on the primary side. For the quenching technique a low pressure mercury far-U.V.-lamp with maximum emission at 254 nm was installed in combination with the primary filter Corning 7-54 (Turner 110-811) with transmittance from 220 to 400 nm. In all cases a sharp cut filter transmittant above 405 nm (Kodak-Wratten 2A - Turner 110-816) served as a secondary filter. It should be mentioned that, if necessary, the selectivity can be increased by appropriate selection of the secondary filter.

The sample quantities in the following examples were all in the microgram range, and the sensitivity of the fluorometer was still too high in the lowest range. Therefore, neutral density filters (Kodak-Wratten 96A, Turner 110-823) with transmittances ranging from 1 to 50% were inserted in addition to the secondary filter.

If the chromatographic patterns permit, the scanning direction should be perpendicular to chromatography. This will give a better base line, particularly with the quenching technique. If, in this case, scanning in the direction of chromatography cannot be avoided, it is sometimes necessary to obtain a true base line by a scan parallel to the track of the spots. Such non-uniform backgrounds occur more often with multi-component solvents. Sometimes they are noticeable even by the eye when the plate is exposed to short-wave U.V.-light. If the spots on the plate are at sufficient distance from each other the slit aperture at the instrument should be set to fully open, which contributes to a uniform base line. Resolution of the scan, however, is increased when the slit is adjusted to only 1 mm.

The instrument used for the following examples had a fixed scanning speed of 20 mm/min. The TLC scanner shown in Fig. 3 is equipped for dual speed, 10 and 20 mm/min. At low speed the resolution of the spots is increased in a similar manner to adjusting the slit to a smaller width, however, without affecting the uniform base line.

For sample application 2 μl self-filling micropipettes were used. The pipetting error plus the error in the actual fluorometric measurement, *i.e.* the total error attributed to factors other than chromatography and scanning, was estimated in the following way: The pipettes were filled with a pyrene solution containing 10 μg/2 μl and then the content delivered on small pieces of filter paper. These filter papers were eluted with benzene in 25 ml measuring flasks and the fluorescence of the solution was measured in the same fluorometer (with the appropriate cuvette sample holder). These tests showed a standard deviation of 1.7%, which of course is contained in the total variations stated below.

Table I gives a survey of the chromatographic details, the last column referring to publications. Table II contains data on the fluorometric procedures. The data on reproducibility (standard deviations) are relevant to 6 values from the same plate.

From Table II it is apparent that the reproducibility of the fluorescence measurements is better than that of the quenching scans. Even a spray reagent applied in

ABLE I

NDITIONS OF CHROMATOGRAPHIC SEPARATIONS

o.	*Compounds*	*Layer*	*Solvent system*	*Chamber type*	*Symbol for quantitation method*	*References*
	Pyrene	Silica gel	Petrol ether	Sandwich unsat.	CF	
	Pyrene	Silica gel F	Petrol ether	Sandwich unsat.	CQ	
	Pregnanediol	Silica gel	Chloroform–acetone (9:1); spray: sulphuric acid	Sandwich sat.	CRF	
	DNS-amino acids	Silica gel	Benzene–pyridine–acetic acid (80:20:2)	Tank sat.	RCF	9, 12
	DNS-amino acids	Polyamide	Heptane–butanol–acetic acid (3:3:1)	Tank sat.	RCF	9, 12
	DNP-amino acids	Silica gel	Chloroform–benzyl alcohol–acetic acid (70:30:3)	Tank sat.	RCQ	9, 12
	DNP-amino acids	Polyamide	Benzene–acetic acid (4:1)	Tank sat.	RCQ	9, 12
	PTH-amino acids	Silica gel F	Chloroform–formic acid (100:5)	Tank sat.	RCQ	9, 12
	PTH-amino acids	Polyamide	Formic acid–water (45:55)	Tank sat.	RCQ	9, 12
	Nucleotides	PEI-cellulose	0.1 → 2 *M* LiCl (gradient)	BN unsat.	CQ	13

combination with the fluorescence method (example 3) hardly affects the standard deviation. However, the fluorogenic reaction of dehydrating steroids with conc. sulphuric acid–ethanol (1:1) and heating, seems to be rather non-critical. An excess of sulphuric acid apparently has no influence on the fluorescence.

It can also be seen from Table II that the sensitivity of the fluorometer could be kept considerably lower with polyamide layers (examples 5, 7, 9) than with the corresponding silica plates. On the other hand, the better sensitivity of the polyamide layers is compensated by the better chromatographic resolution of the amino acid

ABLE II

TAILS OF FLUOROMETRIC SCANNING

o.	*Compounds*	*Quantity (µg)*	*Symbol for quantitation*	*Excitation wave length (nm)*	*Sensitivity of fluorometer*		*Standard deviation (%)*
					Range	*Transmittance of neutral density filter (%)*	
	Pyrene	0.5–10	CF	366	3 ×	1	4
	Pyrene	0.5–10	CQ	254	1 ×	10	7–8
	Pregnanediol	0.5–5	CRF	366			5
	DNS-amino acids	1–5	RCF	366	10 ×	8	3.5–5
	DNS-amino acids	1–5	RCF	366	1 ×	4	3.5–5
	DNP-amino acids	1–5	RCQ	254	5 ×	40	5–7
	DNP-amino acids	1–5	RCQ	254	5 ×	10	5–7
	PTH-amino acids	1–5	RCQ	254	1 ×	20	5–7
	PTH-amino acids	1–5	RCQ	254	5 ×	10	5–7
	Nucleotides	1–5	CQ	254	10 ×	40	5–7

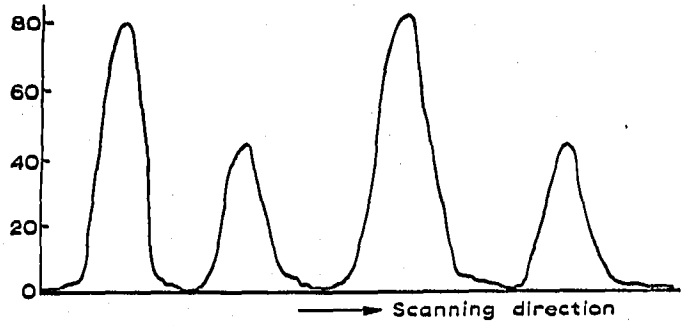


Fig. 4. Scanning chart of pregnanediol after spraying with H_2SO_4 and heating, measurement of fluorescence (CRF).

derivatives on silica gel. It should be noted that the layers of examples 6, 7 and 9 did not contain a fluorescence indicator.

Fig. 4 shows a typical scanning curve of a chromatogram of pregnanediol. For calibration actual samples must be run on the same chromatogram. Samples only applied and not run are not suitable for fluorometric scanning, since the speed of the pipette discharge becomes a factor. To ensure reliable scanning results the compounds should migrate to R_F's between 0.2 and 0.8. The area under the peak is proportional to the sample quantity. Table III shows typical values obtained from a pyrene chromatogram with well formed, nearly symmetrical peaks. In such a case planimetry and simple geometric measurement have about the same accuracy. If the peaks are non-symmetrical, due to spot deformation, area measurements by planimeter are preferable. An integrating recorder also gives equally reliable results.

The scanning peaks of a series of DNS-derivatives of amino acids are shown in Fig. 5. Fig. 6 gives a comparison between different derivatives of the same amino acid on different layers. It should be noted that the various peaks are scanned with different sensitivities.

Peak areas *versus* sample quantities are plotted in Fig. 7 and 8. A linear relationship was found for the fluorescence of the DNS-amino acids, whilst the areas of the quenching peaks of the PTH-amino acids are linear with respect to the logarithm of

TABLE III

FLUORESCENCE SCANNING OF PYRENE SPOTS (2 μg)

No.	*Peak areas (mm^2)*	
	By planimetry	*By geometry*
1	2880	2800
2	3080	3050
3	3080	3240
4	3150	3000
5	3070	3160
6	3220	3010
Mean value	3080	3040
Relative standard deviation	3.8%	5%

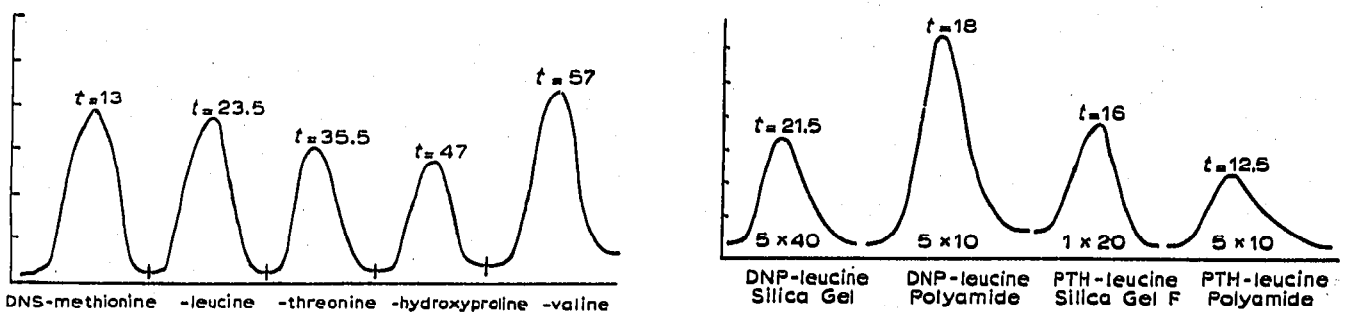


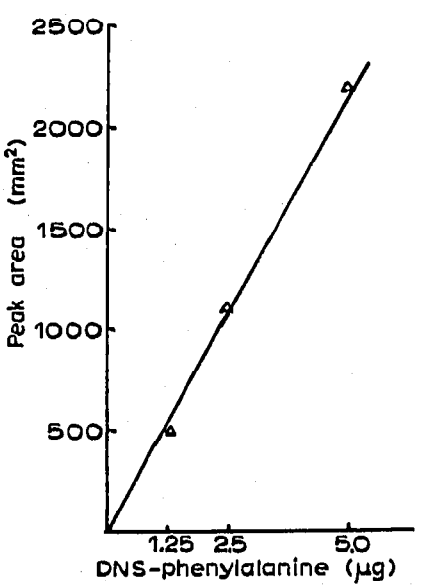
Fig. 5. Scanning peaks of DNS-amino acids (fluorescence), *t* values indicate time (min) between plate drying and scanning. Instrument sensitivity in all cases 10 × 5.

Fig. 6. Peaks of DNP- and PTC-leucine by fluorescence quenching. The figures in the peaks indicate instrument sensitivity.

the sample quantity. However, these findings should not be generalized since the results of all surface scanning methods depend on the vapour phase saturation of the chromatographic system.

The extent to which fluorometric scanning can be made reproducible if data obtained from different plates have to be compared was also investigated. This is necessary for instance in two-dimensional TLC.

The rather poor reproducibility initially observed with amino acid derivatives could be attributed to the influence of time on the fluorescence, or better, to the influence of the moisture content of the layer[6,9]. This effect can be suppressed by spraying with triethanolamine–isopropanol (1:4)[6]. Fig. 9 shows fluorescence peak areas of DNS-methionine *versus* time without and with application of the spray reagent. In Fig. 10 the influence of time on the quenching peaks of DNP-proline and PTH-proline is plotted; these, by the way, are practically not influenced by the spray reagent mentioned.



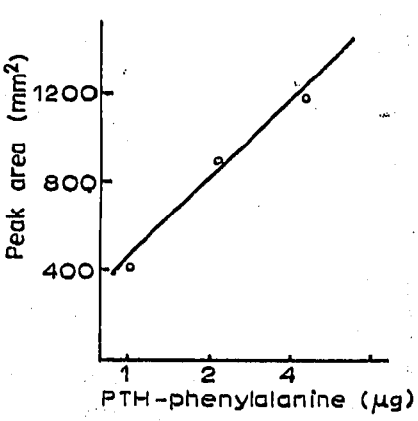


Fig. 7. Peak area *versus* quantity of DNS-phenylalanine, scanning by fluorescence.

Fig. 8. Peak area *versus* quantity of PTH-phenylalanine, scanning by fluorescence quenching.

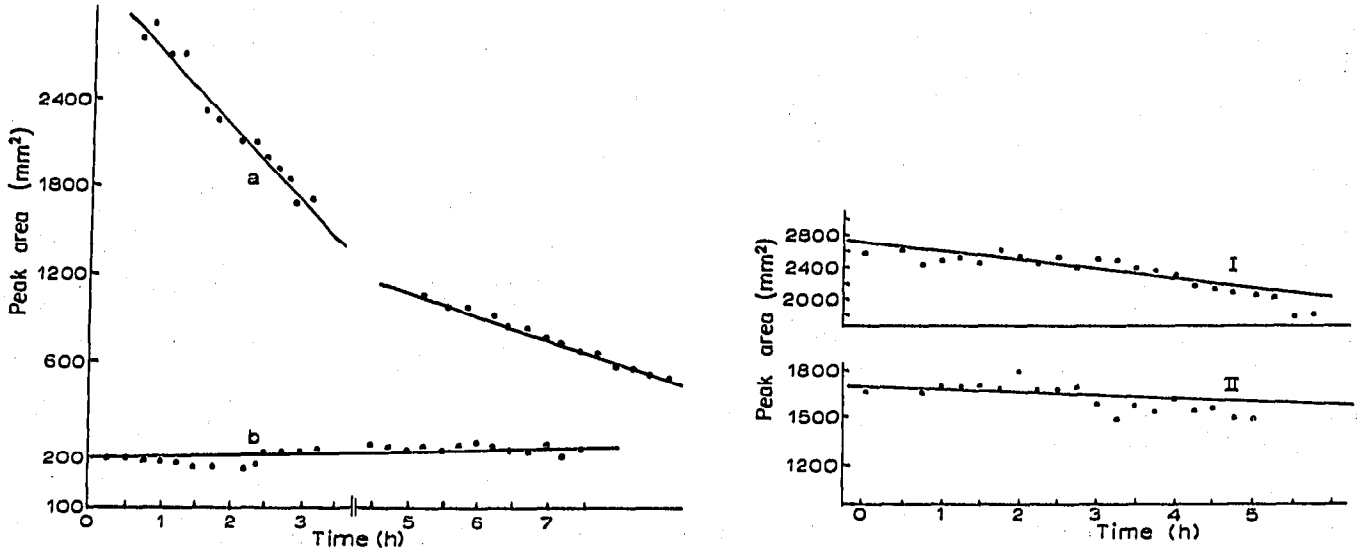


Fig. 9. Influence of time on the fluorescence of DNS-methionine, (a) unsprayed, (b) sprayed with triethanolamine reagent, plotted at different scale of ordinate; the reagent increases fluorescence.

Fig. 10. Influence of time on the fluorescence quenching of (I) DNP-proline and (II) PTH-proline.

These results indicate that such amino acid derivatives can be scanned by fluorometry with fair reproducibility, provided the time influence is suppressed by the spray reagent or that the time delay between drying the plate after the chromatographic run and scanning is standardized. It can be seen from the data of Table IV that the standardization of time is the more accurate method. Presumably a non-uniform distribution of the spray reagent is responsible for the less favourable results of that method. This particular example shows also that it is advisable in all cases of *in situ* scanning of TLC plates to check whether a time influence on the results has to be taken into account.

TABLE IV

REPRODUCIBILITY OF DNS-PROLINE SCANNING BY FLUORESCENCE

Measurement on different plates, silica gel, 2 μg DNS-proline.

No.	*Peak area (mm²)*	
	Plate sprayed with triethanolamine–isopropanol (1:4)	*Time standardized, no spray*
1	3200	1790
2	2950	1820
3	2680	1480
4	2200	1620
5	2480	1660
6	2340	1910
Mean value	2640	1710
Relative standard deviation	14.4%	9.1%

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