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USE OF A SCANNING LASER FLUOROMETRIC DETECTOR IN HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY

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SUMMARY

A laser fluorometric detector for quantitative analysis of 5-dimethylamino-naphthalene-1-sulphonyl (Dns) amino acids has been developed. Photon counting was used for fluorescence detection and a computer was used for data processing. Different types of scanning were used to determine the amount of substance in a spot. Calibration curves for Dns-Ile and Dns-Gly down to $5 \cdot 10^{-14}$ mol per spot were obtained with an error of less than 10% for each component. Unknown quantities of Dns-Ile and Dns-Gly were determined with an accuracy of 7%.

INTRODUCTION

The investigation of structure–function relationships in proteins and peptides available only in very small quantities is currently attracting much attention. Effective investigation of these substances is impossible without apparatus for analysing compounds down to the 10^{-12}–10^{-13} M level.

High-performance thin-layer chromatography (HPTLC) is a well known method in bioorganic and medicinal chemistry. The main advantages of the method are simplicity of the analysis, good sensitivity and high resolution. Its disadvantages are a low quantitation accuracy because of the poor precision of the available detectors, and unsatisfactory sensitivity in some applications. Different detectors have been used for quantifying spots[1], of which the most sensitive are fluorometric detectors with a lamp[2] or a laser[3] as excitation sources, the corresponding detection limits being 10^{-12} and $5 \cdot 10^{-13}$ M of 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole NBD)-methylamine (signal-to-noise ratio 2).

The present paper describes an improvement in these methods using a lower wavelength laser (325 *vs.* 488 nm, previously) with higher power (15 *vs.* 8 mW). In comparison with a lamp excitation source, the laser has a higher spectral intensity and can be confined to zones of 5–10 μm in diameter (100 μm was used in the present work). These advantages in conjunction with specific types of scanning, photon counting techniques and computer data processing lead to important advances in quantitative HPTLC.

MATERIALS AND METHODS

Dns (5-dimethylaminonaphthalene-1-sulphonyl) derivatives of glycine (Gly), phenylalanine (Phe) and isoleucine (Ile) (Serva, F.R.G.) were chosen for the present investigation because of their different R_F values. Polyamide plates (Schleicher & Schüll, F.R.G.) and silica plates (grain diameter 5–7 μm) prepared as previously reported[4] were examined.

The separation of Dns-amino acids was performed by means of one-dimensional HPTLC in the solvent systems chloroform–benzyl alcohol–ethyl acetate–acetic acid (6:4:5:0.2) for silica plates, and 1.5% formic acid in water for polyamide plates. Before separation, the plates were activated for 1 h at 110°C and samples of 0.5 μl were spotted. Before detection, the plates were dried for 3 min with hot air.

Apparatus

The quantitation of Dns-amino acids is based on the detection of fluorescence intensity which is proportional to the amount of excited substance. Fluorescence was excited by helium–cadmium laser light (power output 15 mW) focused onto a small circular spot (the illuminated zone). The apparatus is shown in Fig. 1.
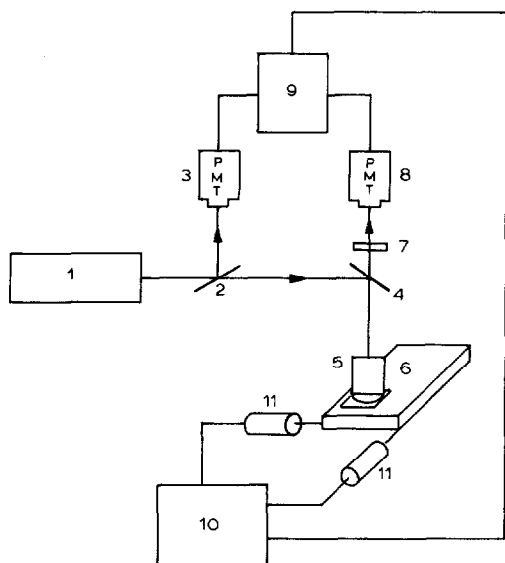

Fig. 1. Diagram of the laser fluorometric detector. 1 = Helium–cadmium laser; 2 = dividing quartz plate; 3 = reference channel PMT; 4 = selective mirror; 5 = quartz lens; 6 = scanning table; 7 = filter; 8 = operating channel PMT; 9 = preliminary data processor; 10 = computer; 11 = stepping motor.

The helium–cadmium laser (1) is most suitable for analysing Dns-amino acids because the radiation is in the UV spectrum at 325 nm, corresponding to the low frequency absorption band of the Dns-amino acids. The spectral maximum of the emitted fluorescence is in the 550–560 nm green-yellow range[5]. Therefore, filtering of scattered laser light at the photomultiplier tube (PMT) is not difficult.

Fig. 1 shows that the laser beam passes through a beam dividing plate (2) where part of the energy is directed into the reference channel PMT (3). The other part is reflected by the selective mirror (4) and focused by the UV-transparant quartz lens (5) onto the surface of the HPTLC plate fixed on the scanning table (6). The emitted fluorescence was collected by the same quartz lens, passed through the selective mirror, light filter (7) (to remove scattered 325-nm light) and directed into the operating channel PMT (8). Electric signals from the operating and reference channel PMTs (operated in the photon-counting regime) were conditioned in the preliminary data processor (9) and further by an Iskra-226 personal computer (10). The computer also controls the stepping motor (11) which moves the scanning table in 200-μm steps (stepwidth may be reduced to 10 μm) of 0.9 s per step to record the laser zone of 100 μm in diameter.

Measurements

Scanning of a chromatographic spot was performed either along mutually perpendicular directions or in a "meander-scanning" mode as shown in Fig. 2. In the first case, the integral fluorescence intensity may be calculated from[6]

$$I = K \cdot \frac{\sum_i (I_i^x - I_b) \sum_j (I_j^y - I_b)}{H - I_b} \tag{1}$$

where I_i^x and I_j^y are the fluorescence intensities for measurements i and j along two mutually perpendicular lines, passing through the centre of the chromatographic spot having a fluorescence maximum, H; K is the dimensionless constant which corrects, for the varying plate thickness, the quantum yield of the Dns derivatives, and reflects the plate temperature, sorbent moisture, etc., and I_b is the background fluorescence intensity which was determined at points A, B, C (see Fig. 2a) five times before and five times after the spot along each line. Eqn. 1 may be employed when the spot is gaussian and the position of maximum H is known. These can hardly occur in experiments with extremely small amounts of substances. An advantage of meander-scanning is that it does not assume knowledge of the spot shape or of the location of the maximum fluorescence. In this case (Fig. 2b)
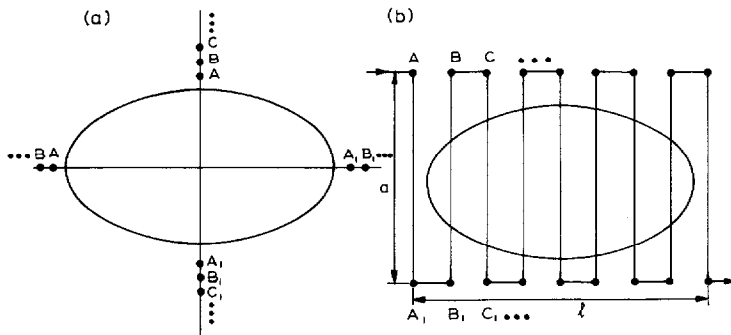

Fig. 2. (a) Perpendicular scanning along 90° directions through the centre of the spot. (b) Meander-scanning: a = track length; l = meander length; A, A$_1$, B, B$_1$, etc. = points where background was detected.

$$I = K \cdot \frac{al}{N} \left(\sum_{i=1}^{N} I_i - NI_b \right) \tag{2}$$

were a is the meander depth, l is the meander length and N is the total number of measurements (I_b was determined at A, A_1, B, B_1, etc.). There is a well known criterion for spot resolution[6]. However, if the spot is gaussian, a different criterion can be used for meander-scanning. This enables higher resolution, since chromatographic spots having elliptical shapes will have narrower peak widths, W_1 and W_2 (Fig. 3), than those determined by perpendicular scanning along the line XX; so, the spots can be considered resolved, if

$$R = \frac{2d}{W_1 + W_2} \geqslant 1 \tag{3}$$

where d is the distance between tracks having maximum intensity for the two spots. It is seen that if $W_1 + W_2$ is reduced, the value d also decreases, *i.e.*, the resolution is improved.
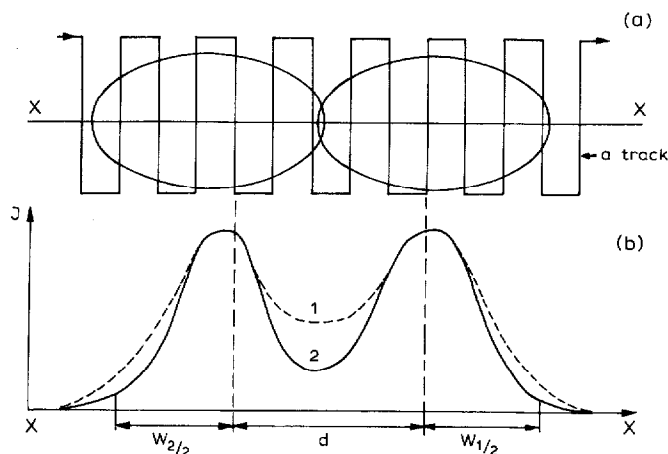

Fig. 3. Resolution of the chromatographic spots using meander-scanning. (a) Scanning direction. (b) Dependence of the fluorescence intensity in a track on its position along XX (2) and on the position of a point along XX (1). The curves are normalized.

RESULTS AND DISCUSSION

Fig. 4 shows plots of the amounts, M, of Dns-amino acids *vs.* their fluorescence intensities, I, upon separation. The plots are linear and the mean squares regression parameters are given in Table I. The variation in experimental points was less than 8% for silica plates and 10% for polyamide plates. The detection limit on silica plates (lines 1 and 2 in Fig. 4) was $5 \cdot 10^{-13}$ M, and on polyamide plates (lines 3 and 4 in Fig. 4) was $5 \cdot 10^{-14}$ M.
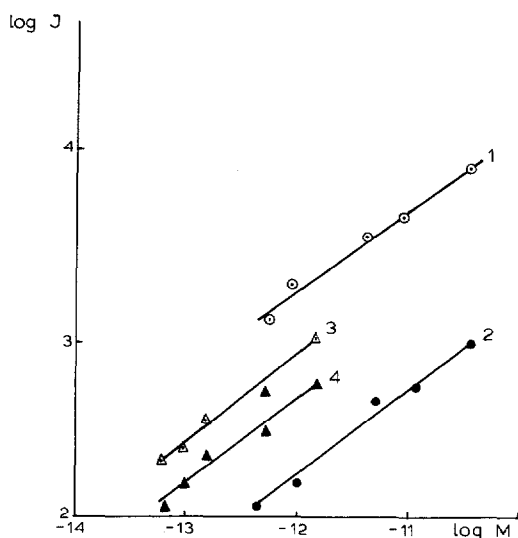
Fig. 4. Dependence of the fluorescence intensity, I, on the amount of substance, M, in the HPTLC spots: 1, Dns-Phe; 2, Dns-Ile; 3, Dns-Gly; 4, Dns-Ile.

The analysis of two unknown quantities of Dns-Gly and Dns-Ile has been carried out. The unknown quantities were determined with an accuracy of 7%.

To estimate the reproducibility of the method, the scanning of each chromatographic spot was repeated four times. The standard error of each measurement was <8%.

In the calibration plot, the slope characterizes the detector linearity and deviations from a slope of 1 reflect insufficient dynamic range of the detector. The coefficient K in eqns. 1 and 2 reflects the dependence of the results on the plate preparation method. However, non-homogeneity of the plate layer was found to have little effect when a large number of measurements were made (about 350–400 per spot) and the data were averaged.

Photodestruction of the Dns-amino acids by intense light source was evaluated. When the power density of the exciting light is 100 W/cm², the fluorescence intensity of the Dns-amino acids decreases by a factor of e in 5 ± 1 s. Thus, the detection time per step should be less than this value. The dependence of K on the time between

TABLE I

COEFFICIENTS OF THE LINEAR REGRESSION $\log I = a \cdot \log M + b$ AND THEIR DISPERSIONS

Compound	Detection limit (mol)	a	b	σ_a (%)	σ_b (%)
Dns-Phe	$5 \cdot 10^{-13}$	0.92	15.4	4	6
Dns-Ile	$5 \cdot 10^{-13}$	0.89	13.1	7	8
Dns-Gly	$5 \cdot 10^{-14}$	0.88	14.2	9	10
Dns-Ile	$5 \cdot 10^{-14}$	0.90	14.0	6	7

the separation and the detection may be taken into account with calibration samples used on the same plates[7].

CONCLUSIONS

A laser fluorometric detector for HPTLC with a sensitivity 5–10 times better than available detectors was constructed. The detection limits were $5 \cdot 10^{-14}$ M for polyamide and $5 \cdot 10^{-13}$ M for silica plates with reproducibilities below the measurement error, 10 and 8% respectively. The diameter of the illuminated zone in the experiments was 100 μm and determined the resolution of the spots.

This detector will have wide application in high-sensitivity analysis in biochemical and medical investigations.

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