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ULTRA-MICRO THIN-LAYER CHROMATOGRAPHY ON A CYLINDRICAL SUPPORT

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SUMMARY

By eliminating "edge effects" during chromatographic separation on a cylindrical chromatogram it has proved possible to increase the sensitivity of detection of certain fluorescent 5-dimethylaminonaphthalene-1-sulphonyl derivatives by 100–1000 times. This was achieved by coating glass rods (minimum diameter 0.6 mm) with silica gel and after applying a mixture of fluorophores to one end of the rod separating them in a test tube containing a suitable solvent mixture by the ascending technique. As little as 10 pg of the derivatives, separated as compact circular zones, may be seen by the naked eye. This amount was reduced further to 2.3×10^{-14} moles in the case of benzylamine by a photographic technique of assessment. The possibility of further refinement to, and increased sensitivity of the procedure is discussed.

INTRODUCTION

Attempts to miniaturize the chromatographic process and thus improve sensitivity at the lower levels of detection by reducing the size of conventional two-dimensional media have been partially successful. It is now quite common to be able to separate, visualize and even quantitate a few nanograms of a variety of coloured or fluorescent (usually the latter) substances on thin media with dimensions of 25 × 75 mm. The miniaturization process is limited by both chemical and physical phenomena. In the former case much progress has been made in recent years by converting substances either before chromatography or else *in situ* to highly fluorescent (less frequently to coloured) derivatives. It is clear, however, that improvements in terms of chemistry (*i.e.*, nature and type of derivative and their extinction and/or fluorescent properties) cannot proceed indefinitely. With respect to the physical dimensions of the medium it is an easy matter to discover their lower limits. Reduction beyond a certain point, however, is not possible and there seem to be several reasons for this. Most of these reasons are technical, such as application of sample volume, properties of the layer, ease of equilibration and/or solvent development, handling, etc. With some ingenuity many of these difficulties can probably be overcome. Unfortunately,

however, there is a much more serious limitation, namely, "edge effects" (for a discussion of this and a theoretical derivation please refer to ref. 1).

After attempting without success conventional chromatography on very narrow bands of silica gel deposited either on a flat surface or in channels, and descending separations on cotton threads, we abandoned these types of procedures in favour of a cylindrical chromatogram (*i.e.*, silica gel deposited on glass rods).

EXPERIMENTAL

Materials

Samples of Dns*-ethylamine, Dns-dimethylamine and Dns-benzylamine were provided by Dr. N. Seiler (Frankfurt, B.R.D.), methyl red indicator (sodium salt) was purchased from Mann Research Labs. (New York, N.Y., U.S.A.); silica gel containing 5% calcium sulphate binder suitable for thin-layer chromatography was purchased from Mondray (Montreal, Canada) and cellulose powder 300 G (particle size $< 10\,\mu m$) containing 10% calcium sulphate binder from Brinkman Instruments (Toronto, Canada). Glass rods, 1.0, 0.8, 0.6 and 0.4 mm (tolerance $\pm$ 0.01 mm), respectively, in diameter, were purchased from Drummond Scientific (Broomall, Pa., U.S.A.) and disposable 10-μl micropipettes (diameter approximately 1.5 mm) from DADE (Miami, Fla., U.S.A.). An Asahi Pentax Model SL camera was used for photography using either Kodak high-speed Ektachrome (daylight type, ASA 160) or Kodak Tri-X Pan (ASA 400) film. These films were specially processed (Commercial Lab., Regina, Sask., Canada) or Medical Photography Services, University of Saskatchewan, Sask., Canada) in order to increase their effective ASA ratings from 160 to 2560 and 400 to 1600, respectively. Filters (Wratten No. 9 and No. 55) were obtained from Eastman-Kodak (Rochester, N.Y., U.S.A.).

Preparation of the cylindrical chromatograms

Rods 125 mm long (diameters 0.4, 0.6, 0.8, 1.0 and 1.5 mm; the latter size produced by sealing the ends of micropipettes) inserted in corks were dipped into a suspension of silica gel (produced by vigorous shaking of the gel in acetone and water, 5:12:2, w/v/v) or cellulose (cellulose–water, 1:6, w/v) in 10-ml cylinders. They were withdrawn slowly, inverted, placed on the bench (in the cork) and allowed to dry at room temperature. Various concentrations of a solute or mixture of solutes including the marker substance (methyl red indicator, 2–5 ng) dissolved in water were applied (gentle contact) from a 1-μl pipette to the end of the coated rod as shown in Fig. 1. The pipette was re-filled with acetone–water (80:20, v/v) and re-applied to the end of the chromatogram. This latter application served to rinse the pipette and to move the solutes and marker substance off the end of the rod. After drying in air for 30 sec the chromatogram was inverted and the solute end touched to the surface of a mixture of acetone–glacial acetic acid (3:2, v/v). After a solvent rise of 10–20 mm the rod was removed for 30 sec and the procedure then repeated until the visible marker dye was concentrated in a narrow band 5–15 mm from the end of the rod as shown in Fig. 2. The preparation of three chromatograms in this manner took about 3 min.

* Dns = 5-Dimethylaminonaphthalene-1-sulphonyl.

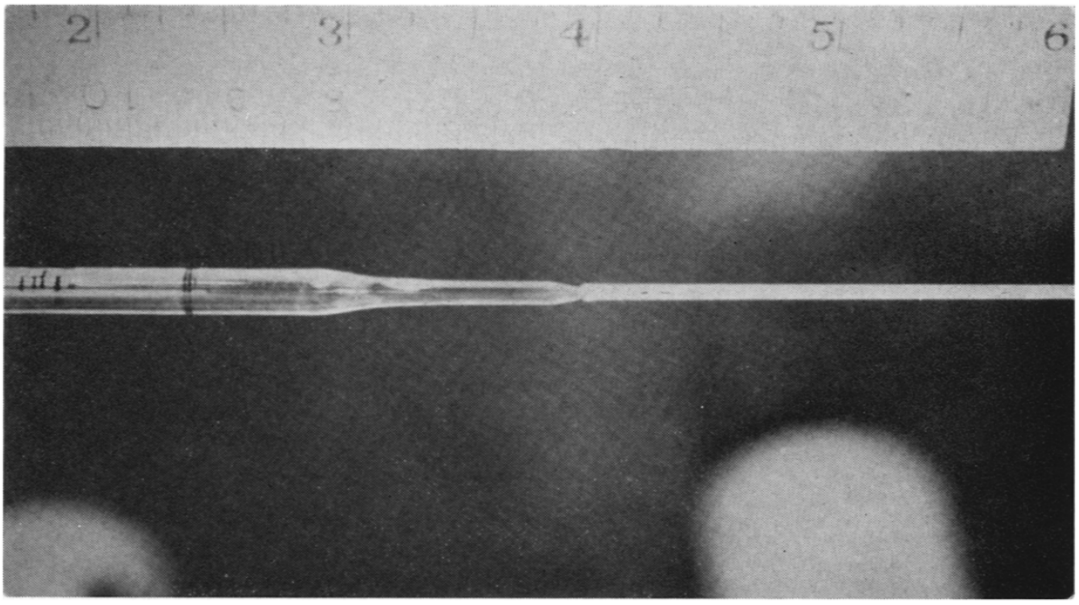

Fig. 1. Application of solute to the tip of a cylindrical chromatogram.

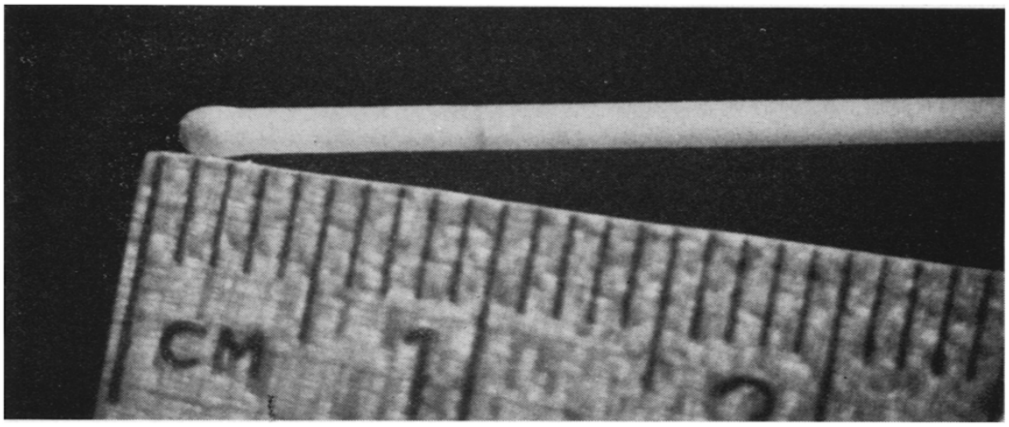

Fig. 2. A cylindrical chromatogram after concentration of sample.

Chromatogram development

The cork holding the chromatogram was placed in a test tube (20 × 150 mm) as shown in Fig. 3. The depth of solvent (benzene–acetic acid–water, 10:5:5, v/v) in the tube was arranged so as to be in contact with the silica gel coated rod but at a level lower than the solute origin. Development at room temperature varied between 12 and 40 min for an ascent of 60–90 mm after which the cork and chromatogram were removed, inverted and allowed to stand at room temperature for 5–10 min. The fluorescence of the separated derivatives was then stabilized by rotating the chromatogram in a light mist spray of triethanolamine–isopropanol (1:4, v/v). When dry, the chromatograms were stored by placing the cork in empty clean tubes.

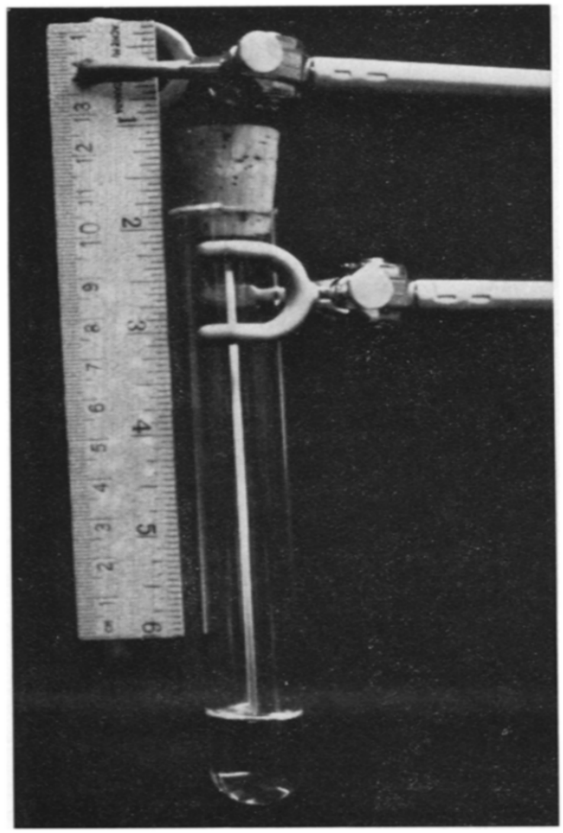

Fig. 3. Arrangement for solvent development during chromatography.

Visualization and assessment of chromatograms

The chromatograms were assessed visually by viewing in ultraviolet light (365 nm) and by photography. Photographic assessments were made as follows: The rod was clamped about 15–20 cm below an illuminating ultraviolet light source (Blak Ray, Kensington Sci. Corp., Oakland, Calif., U.S.A.), emitting at 365 nm. Two Wratten filters excluding light below 430 nm and above 620 nm (the fluorescence maxima of the Dns derivatives on the cylindrical chromatograms were in the range 510–530 nm) were placed over the camera lens system (Wratten No. 9 nearest the lens) which was situated a convenient distance (20.8 cm) from the rod. The whole arrangement was enclosed in a light-protected box painted black on the inside. The shutter was activated from outside the box by a cable release mechanism, the aperture was fixed at *f*/4 in all cases and exposure times were measured by a stopwatch.

RESULTS

The methyl red marker substance was useful as an indicator of the chromatogram origin since in the highly polar "running-up" solvent system it exhibited the same R_F value as the fluorescent derivatives; after separation, however, it possessed a lower

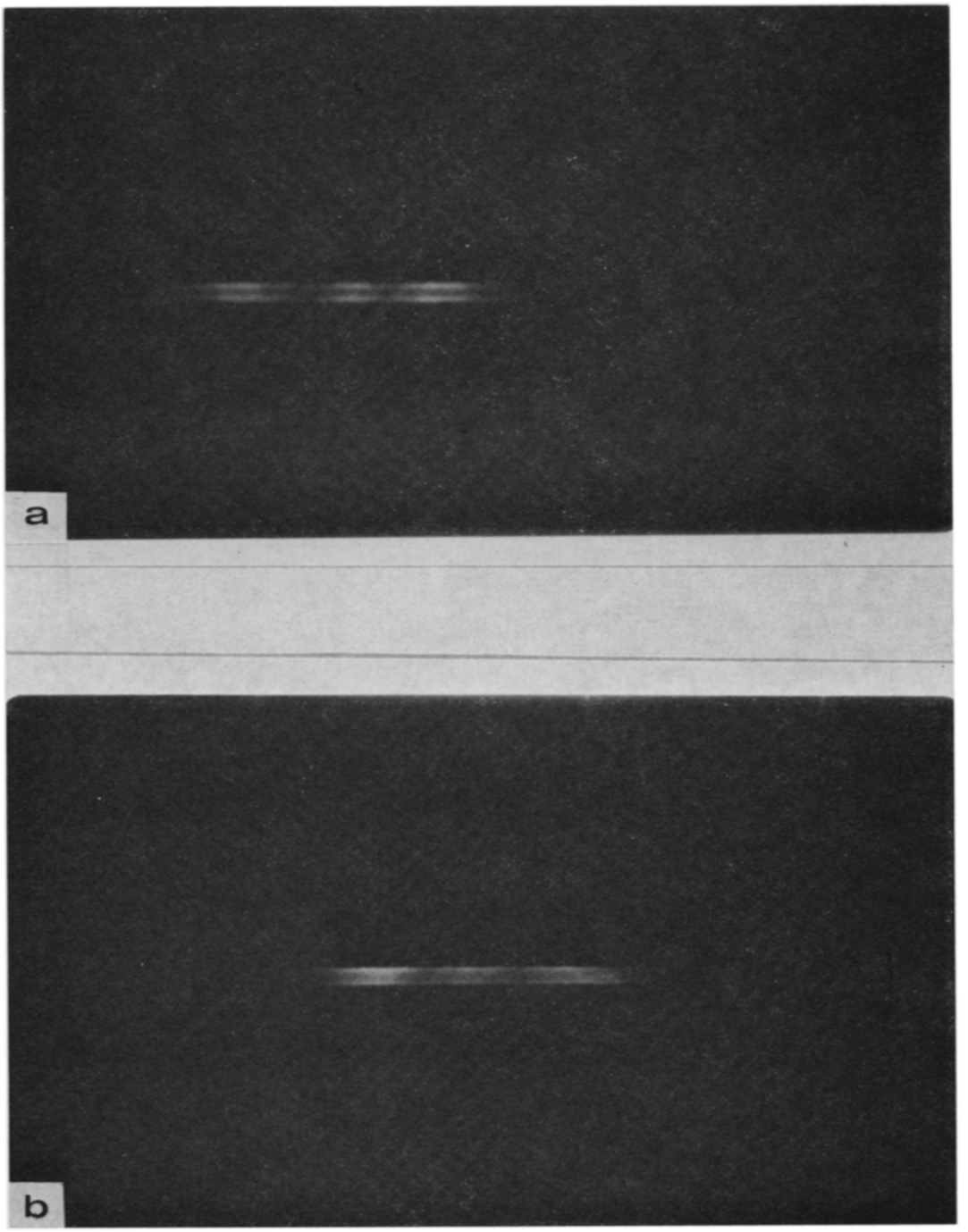

Fig. 4.

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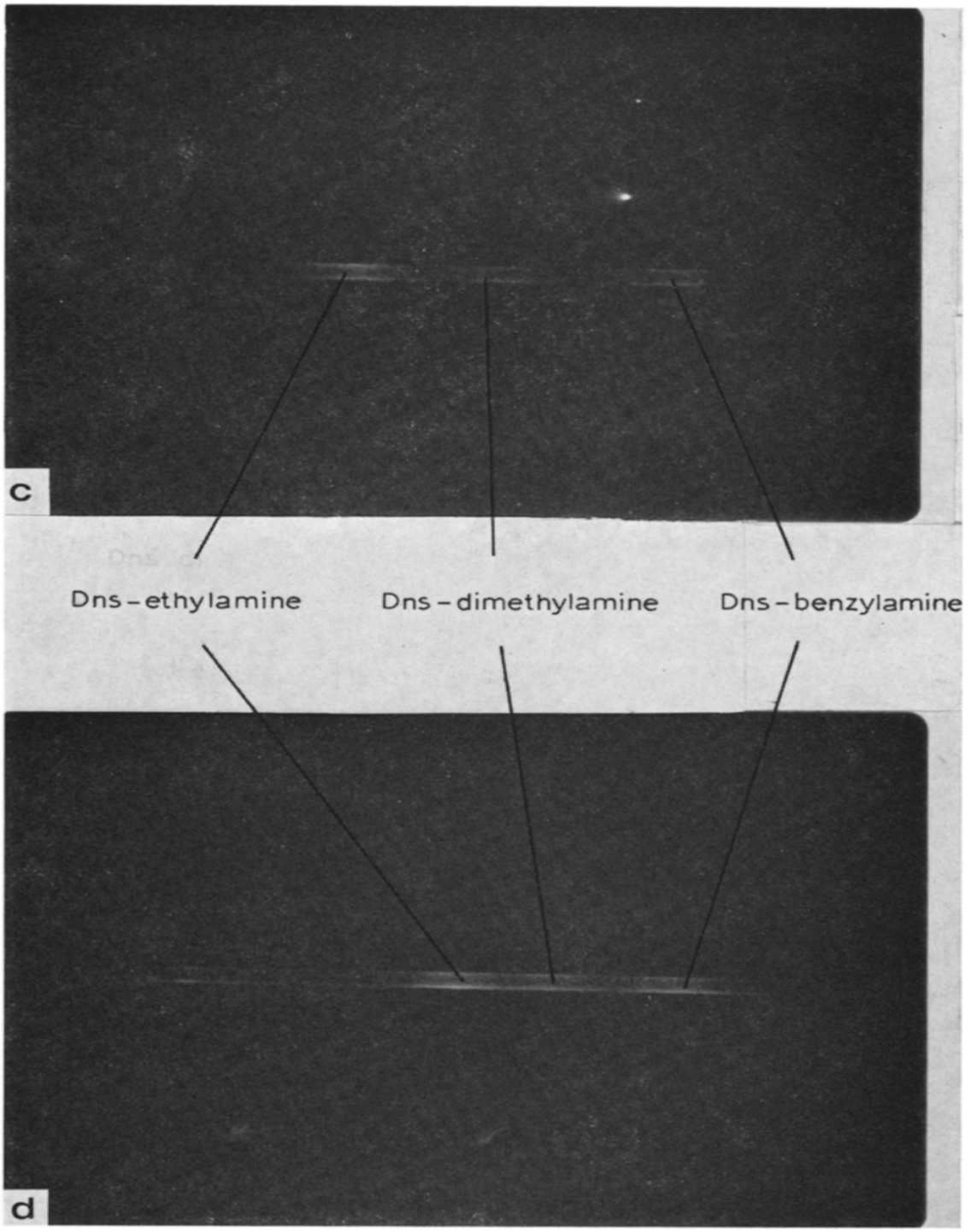

Fig. 4.

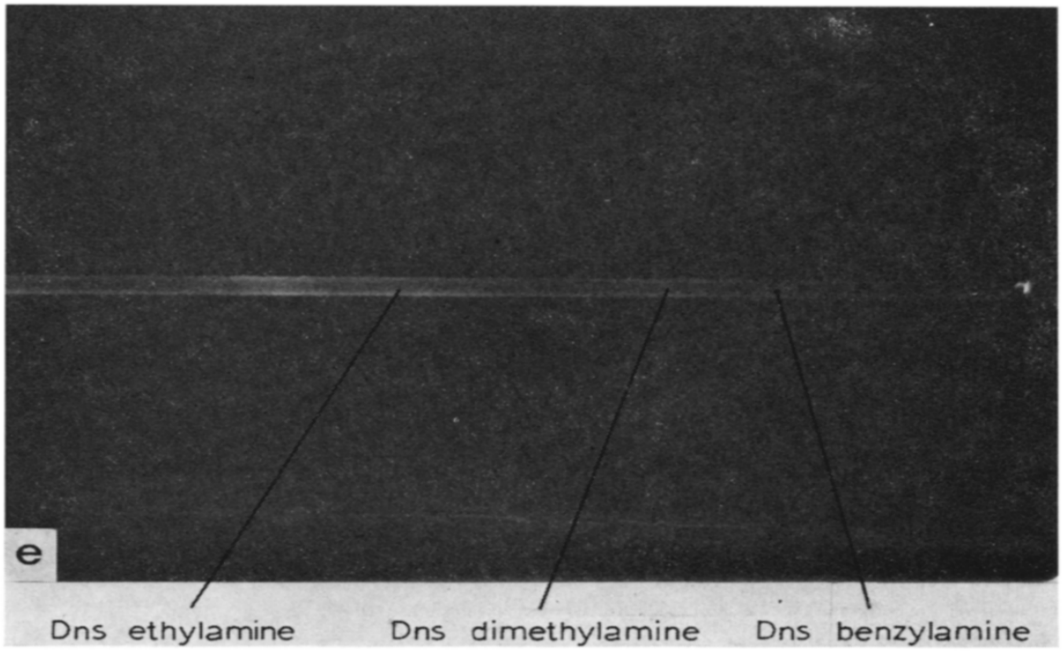

Fig. 4. Photograph of fluorescent derivatives separated by cylindrical chromatography. (a) 250 pg, exposure 0.25 sec; (b) 100 pg, exposure 0.5 sec; (c) 25 pg, exposure 2 sec; (d) 10 pg, exposure 4 sec; (e) 2.5 pg, exposure 8 sec. Direction of solvent flow is from left to right; rod diameter, 0.6 mm; high-speed Ektachrome film; distance between camera and chromatogram, 22.4 cm. Fluorescence from the methyl red marker is visible at the left in the 10- and 2.5-pg samples.

R_F value and did not overlap with, or interfere in, the assessment of fluorescence. A concentration of between 2 and 5 ng was easily visible (see Fig. 2); in the photographic assessment, however, this substance was slightly fluorescent (equivalent to about 10 pg of Dns-ethylamine) (see Figs. 4d and 4e).

The quality of the layers produced as described in Experimental were quite uniform in most cases; the thickness was about 200 μ. In the event that the rods were withdrawn from the suspension too quickly, droplets formed, which on running down the rod, led to channeling.

It was difficult to retain acceptable layers on the 0.4-mm rods but good and reproducible chromatograms were produced with diameters of 0.6 mm and higher. With experience it was possible to avoid breakage during solute application and so 0.6-mm rods were selected for a more detailed investigation. Silica gel as opposed to cellulose was selected as absorbent on the basis of (1) its lower background fluorescence as revealed both by photography and from assessment in the Aminco Bowman spectrophotofluorometer, (2) the ease of preparing better and more homogenous layers, and (3) the reproducibility and superiority of the chromatogram.

Photographs of cylindrical chromatograms on which 250, 100, 25, 10 and 2.5 pg, respectively, of Dns-ethylamine, Dns-dimethylamine and Dns-benzylamine have been separated are shown in Figs. 4a–e. These particular results are quite typical of all the results obtained to date. The minimum amount of derivative

that could be detected by eye was about 10 pg (20×10^{-14} moles); the lowest levels detected by photography using high-speed Ektachrome film with an exposure time of 8 sec were: Dns-ethylamine 5.5×10^{-14} moles, Dns-dimethylamine 5.5×10^{-14} moles, and Dns-benzylamine 2.3×10^{-14} moles.

DISCUSSION

Although the experiments reported here were performed as a feasibility study using the stable, highly fluorescent Dns derivatives about which much is already known, it is possible to claim that the technique has confirmed theoretical predictions[1] and is probably capable of considerable refinement and improvement. The advantages of the method may be summarized as:

(1) The apparatus required is cheap and simple, and exists in all types of laboratories. The camera may be considered as an optional extra since extremely tiny concentrations can be seen by eye.

(2) Tiny quantities of the substances to be separated dissolved in small, or even relatively large volumes of solvent, can be applied to the chromatograms.

(3) Clean and effective separations to produce compact circular zones are obtained.

(4) The amounts of absorbent, solvents, spray reagents, etc. are much less than is required in conventional chromatography.

(5) The minimum level of detection has been reduced by 100–1000 times.

(6) The flexibility of the procedure is considerable since by changing the rod diameter (*i.e.*, from 0.6 mm up to test tube size) solutes in the range picograms to micrograms and even milligrams may be processed.

(7) The technique may be considered as the ultimate or penultimate step in the identification and quantitation of any substance. It may also, however, be used in conjunction with other more conventional preliminary separative procedures and in conjunction with other physico-chemical analytical procedures such as mass spectrometry.

Disadvantages of a chromatographic separation on a cylindrical support in comparison with other more conventional thin-media separative procedures are:

(1) More care is required in the preparation and handling of the chromatogram.

(2) Separation in a second dimension is not possible.

For the future it is quite conceivable that by using specially prepared absorbents, modified coating techniques and smaller rod diameters greater sensitivities will be obtained. It is even possible that the layer could be deposited onto fine threads by evaporation sputtering techniques. If these threads possessed significant mechanical strength (*i.e.*, glass fibres or metal wires) and flexibility, then fragility and breakage would not be a problem. The difficulty of solute application to very fine rods could perhaps be circumvented by arranging for the chromatogram to possess a bulbous end. Quantitation of separated substances exhibiting fluorescent (either naturally fluorescent or rendered so on the chromatogram by chemical modification) or chromogenic properties could be achieved by direct photodensitometry utilising repeated photometric scanning with memory storage and computation analysis or else by scanning photographs provided that a constant relationship can be ob-

tained between the quantity of separated fluorogen or chromogen and optical density on the photograph. It seems reasonable to conclude that by utilising a more powerful light source, a better camera, more sensitive films, improved photographic conditions, and arranging for the chromatogram to revolve during filming, a further considerable improvement in sensitivity could be achieved.

ACKNOWLEDGEMENTS

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REFERENCE

1 V. Pollak and A. A. Boulton, *J. Chromatogr.*, 76 (1973) 393.