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AN EXPERIMENTAL HIGH-PERFORMANCE PHOTODENSITOMETER FOR QUANTITATIVE CHROMATOGRAPHY

II. PRELIMINARY ASSESSMENT (PAPER)

A. A. BOULTON, W. GIETZ
Psychiatric Research Division, University Hospital, Saskatoon, Saskatchewan S7N OW8 (Canada)
and
V. POLLAK
Biomedical Engineering Division, University, of Saskatchewan, Sascatoon Saskatchewan (Canada)
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SUMMARY

Paper chromatograms on which different concentrations of various chromogens and fluorogens were separated were assessed in the reflectance and transmittance modes of operation in a double beam, flying spot, ratio forming prototype scanning photodensitometer. Linear calibration curves were obtained in most instances with the lower level of detection in the case of colored substances being about 6.2×10^{-11} moles and in the case of fluorescent substances being about 5.0×10^{-12} moles.

The scanning device exhibited a stability and reproducibility as high as $\pm 0.7\%$ (fluorescence) and $\pm 1.3\%$ (absorption–transmission) when moderate quantities (0.5 μg) of fluorogens and chromogens were separated. These values decreased to $\pm 6.4\%$ (fluorescence) and $\pm 8.5\%$ (absorption–transmission) respectively when much smaller quantities (25–100 ng) were separated. The assessment of separated chromogens by reflectance appeared in the main to be less satisfactory than the transmittance procedure.

INTRODUCTION

Among the various chromatogram scanning devices built in research laboratories or available commercially, the dual beam, flying spot, ratio forming one described in the preceding paper[1] was the first to incorporate all of the features necessary substantially to suppress noise (optical or electrical) and produce greater sensitivity, accuracy and reproducibility (for arguments and reasons see the references quoted in the preceding paper). The performance of this machine with respect to paper strips has been assessed using two chromogens, one natural (phenosafranine) and the other produced by the reaction of the amino acid alanine with ninhydrin and with a fluorophore (dansyl ethylamine). The colored substances were assessed

(scanned) in both the transmission and relection modes of operation of the photometer while the fluorescent substance was scanned only in the transmission mode.

The most suitable paper quality chosen following an investigation of their spectra in the range 300–800 nm were those exhibiting a low absorbance and flat spectrum (Whatman No. 2 and No. 4 quality). In order that the chromatograms selected for assessment might be considered representative the natural chromogen (safranine) represented a "clean" chromatogram (*i.e.* stained zone on unstained background) and the ninhydrin–alanine chromogen because it represented a colored zone on a differently colored background. The results of scanning of fluorescent substances are, for a variety of reasons, usually superior to those obtained in the case of colored compounds. Dansyl phenylethylamine was therefore assessed for comparative purposes. In this preliminary paper a computational analysis of a digital output was not attempted, and all analyses were effected by a planimetric evaluation of an analogue output. The assessment of chromogens and fluorogens separated on other forms of chromatographic media (*i.e.* silica gel on glass and other solid supports, electrophoretograms, gels, etc.) will be reported later.

MATERIALS AND METHODS

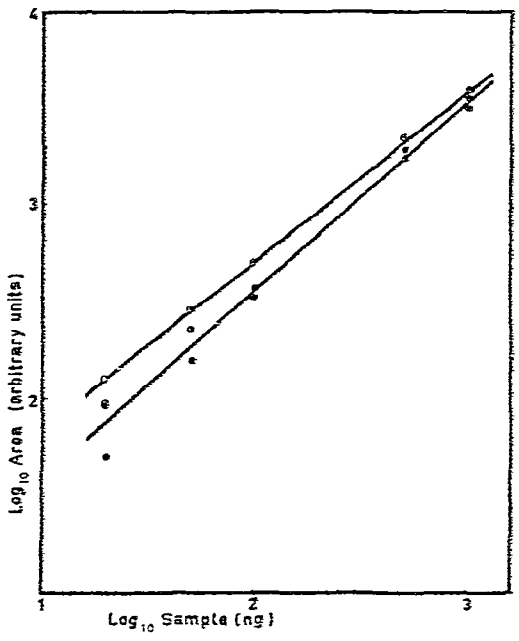
Phenosafranine (3,7-diamino-5-phenylphenazinium chloride) was purchased from BDH (Dorval, Quebec, Canada) and dansyl ethylamine was a gift from Dr. Nikolaus Seiler (Frankfurt am Main, G.F.R.). The paper strips, quality No. 2 and No. 4 (5 × 57 cm) were obtained from Canadian Laboratories (Winnipeg, Manitoba, Canada). Interference filters (9 altogether) with a bandwidth of 18 nm at half the maximum peak height were obtained from Optics Technology, Redwood City, Calif., U.S.A. They covered at equal intervals the spectral range 408–708 nm. The solutes dissolved in water or ethanol were diluted to provide a range of concentrations and then applied carefully (no-touch technique, see ref. 2) as a small band (dimensions 24 × 4 mm) in duplicate to the chromatogram origin in 10-μl volumes using disposable micropipettes. After air drying, the strips were placed on stainless-steel frames, transferred to lined and insulated glass tanks (internal dimensions 30 × 20 × 57 cm) and after equilibration at 25° for 2 h the solvent was added and the chromatograms developed by the descending technique. Alanine was separated during 15 h in solvent system A [*n*-butanol–acetic acid–water, 4:1:1 (v/v)], phenosafranine during 2 and 4 h in systems A and B [diisopropyl ether–methyl ethyl ketone–acetic acid–water, 17:3:10:10 (v/v)] and dansyl ethylamine during 2 h in system C [light petroleum (b.p. 100–120°)–toluene–acetic acid–water, 200:100:255:45 (v/v)]. After development the dansyl ethylamine and phenosafranine chromatograms were removed, dried in air for 30 min and then scanned. In the case of dansyl ethylamine the machine was operated in the transmission mode, single measuring beam 533 nm; phenosafranine was assessed in the transmission and reflection modes, reference beam 600 nm, measuring beam 533 nm. The alanine chromatograms were air dried during 30 min, treated with the ninhydrin–cadmium acetate reagent[2,3] and scanned in the transmission and reflection modes (reference beam 600 nm, measuring beam 500 nm) as soon as possible after drying.

Several types of analyses were performed; in the first, chromatograms on which the chromogen or fluorogen in the concentration range 0.1 ng–1 μg had been

separated, were scanned in order to establish calibration curves and the lower limits of sensitivity. Later experiments included (a) the repetitive scanning of chromatograms on which the chromogens and fluorogen had been separated at both a high and a low concentration in order to evaluate machine stability and reproducibility and (b) the assessment of a series of chromatograms on which equal amounts of the various solutes at both high and low concentrations had been separated in order to establish the reproducibility of the overall preparative, separative and quantitative procedure. Quantitation was achieved by planimetric tracing around the peaks contained on the analogue output from the scanning device. In the main the quality of the scanning records in the non-absorbing areas of the chromatogram were acceptably flat so that drawing a baseline "by-eye" to delineate the base of the peak was relatively easy.

RESULTS AND DISCUSSION

Typical calibration curves are shown in Figs. 1–4. In the case of phenosafranine (Figs. 1 and 2) it can be seen that a linear relationship obtains between photometer response and concentration in the concentration range 20–1000 ng in both the transmission and reflection modes of operation and on both qualities of paper. It might have been expected that Whatman No. 4 paper would have produced larger signals and hence greater sensitivity because of its greater light transmission properties. The fact that the sensitivity on Whatman No. 2 paper was similar, however, can probably be explained by assuming an increased signal due to the greater compactness of the



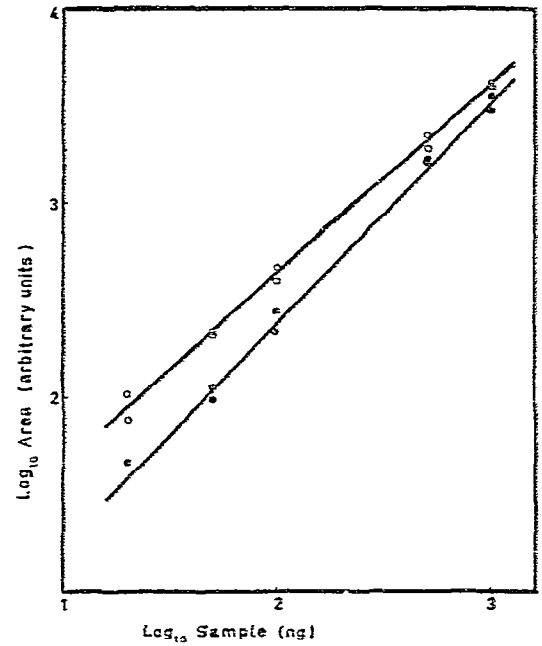


Fig. 1. Calibration curves for phenosafranine separated on Whatman No. 4 paper. ○, Transmittance mode of operation; ●, reflectance mode.

Fig. 2. Calibration curves for phenosafranine separated on Whatman No. 2 paper. ○, Transmittance mode; ●, reflectance mode.

separated zones. It is also apparent from an examination of Figs. 1 and 2 that there is a lower spread between duplicate analyses when the device is arranged to measure in the transmittance mode than is the case for reflectance. The lower level of detection was determined to be 20 ng.

In the case of alanine (Fig. 3) it is apparent that with Whatman No. 4 quality paper a non-linear relationship between concentration and photometer response was obtained. It is also apparent that an improved sensitivity was obtained when the machine was operated in the transmission as opposed to reflectance mode. In the former case 25 ng was the minimum detectable level and it is clear that the machine response at any particular concentration was greater and that closer agreement between duplicate values was obtained. It is possible that the observed lack of linearity was caused by a non-stoichiometric reaction between the reagent and the amino acid; this could arise if an insufficient amount of reagent were available within the reactive zone at the higher concentration levels. It is readily apparent, however, that in the transmission mode within the range 25–1000 ng an acceptable linear relationship exists between photometer response and the alanine–ninhydrin chromogen.

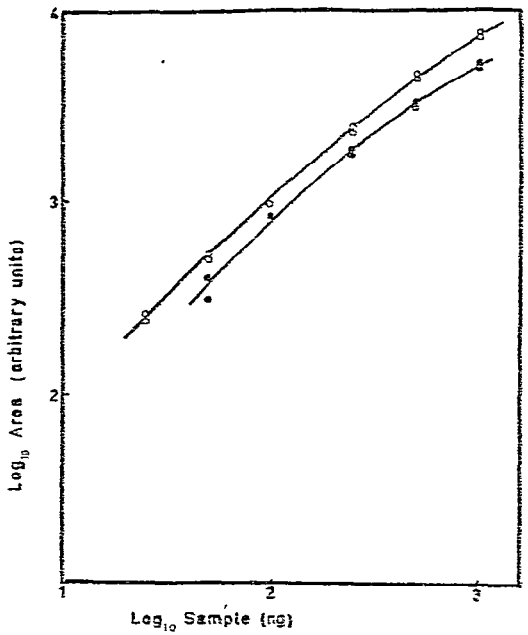


Fig. 3. Calibration curves for alanine. After separation of the amino acid on Whatman No. 4 quality paper the chromogen was formed by reaction with ninhydrin (see text for details). ○, Transmittance; ●, reflectance.

As might be expected a straight-line relationship was obtained when dansyl ethylamine was assessed (see Fig. 4) and a lower level of detection was reached.

The results listed above demonstrate an improvement of about two orders of magnitude over the data obtained by Salganicoff *et al.*[4] who used a time sharing dual wavelength (difference) machine; they in turn claimed an improvement of about two orders of magnitude over single-beam instruments.

The reproducibility of the photometer in its various operative modes with

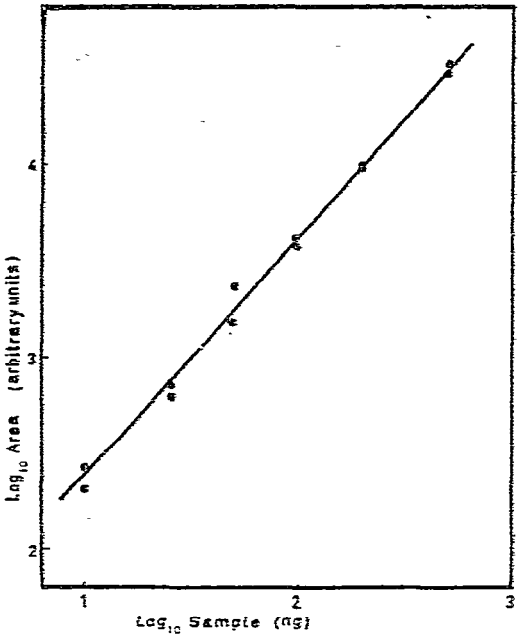


Fig. 4. Calibration curve for dansyl ethylamine. The fluorophore was separated on Whatman No. 4 quality paper and assessed in the transmittance mode.

respect to the different chromogens and fluorogens at both a high and a low concentration level was assessed by scanning (usually 16 times) the same chromatogram. The results are listed in Table I. It is apparent that at a high concentration (note that high in this context is a relative term, 0.5 μg is in reality a tiny amount) the scanning device is stable and highly reproducible. In the transmission mode the deviation from the mean was only $\pm 1.3\%$ in the case of colored substances and $\pm 0.7\%$ in the case of the dansyl fluorophore. In the reflectance mode, deviation from the mean was in the range $\pm 3.0\%$ to $\pm 4.2\%$. When smaller quantities (near the limit of detection sensitivity) were assessed, the reproducibility in the transmission mode decreased. In view of the fact that the amounts assessed were of the order of 25–100 ng the values obtained ($\pm 6.4\%$ to $\pm 8.5\%$) seem quite acceptable. Inspection of Table I indicates that at these low concentration levels the reflectance mode of operation is only marginally less acceptable than is the case for transmission measurements. It is highly probable that all of these values will be much improved when the scanning signal, converted to digital form, is assessed by a computer whether it be a small on-line dedicated machine or a large installation assessing data that have been pre-recorded either on digital tape or paper.

The assessment of the overall analytical procedure was accomplished by preparing several chromatograms (usually 16) and subjecting them to the separative, and where necessary, derivatisation procedures, followed by scanning. The results obtained are listed in Table II. As would be expected the standard deviation increased in all cases. Parts of this increase can be accounted for by variations in the chromatogram thickness and homogeneity, the accuracy of the various pipettes, handling the chromatograms, variations in equilibration and during solvent development, derivati-

TABLE I

CHARACTERISTICS OF MACHINE REPRODUCIBILITY

All separations were performed using Whatman No. 4 quality paper.

Sample	*Amount separated (ng)*	*Transmission mode (% standard deviation)*	*Reflectance mode (% standard deviation)*
Phenosafranine*	500	1.3	4.2
	50	8.5	10.7
Alanine*	500	1.3	3.0
	100	—	6.1
	50	6.5	—
Dansyl ethylamine**	500	0.7	—
	25	6.4	—
	10	12.2	—

* Solvent system A (see text).
** Solvent system C (see text).

sation, chemical changes (photodecomposition for example), and tracking of the different chromatograms through the light beam. The use of an automatic apparatus to apply the solute and an automated digital computer process incorporated in the quantitation procedure would produce significant improvements. Despite these increases, however, it can be seen that at relatively high concentrations the percentage error in both the reflectance and transmission modes and for all the substrates used was only in the range $\pm 5.4\%$ to $\pm 8.8\%$. At the very low concentration levels (10–50 ng) it is likely than an error of $\pm 11.4\%$ to $\pm 17.8\%$ is quite acceptable.

TABLE II

CHARACTERISTICS OF THE OVERALL ANALYTICAL PROCEDURE

All separations were performed using Whatman No. 4 quality paper.

Sample	*Amount separated (ng)*	*Transmission mode (% standard diviation)*	*Reflectance mode (% standard deviation)*
Phenosafranine*	500	5.4	5.7
	50	15.8***	14.0
Phenylalanine*	500	7.8	6.3
	100	—	10.7
	50	11.4	—
Dansyl ethylamine**	200	8.8	—
	10	17.8	—

* Solvent system A (see text).
** Solvent system C (see text).
*** This series of experiments was performed using solvent system B.

It is recognized of course that chromatographic separations employing thin layers of absorbants on supports such as glass, polyethylene and aluminum, are now more extensively used than paper. The machine described in the previous paper is quite capable of analyzing these types of thin media provided their width is 5 cm or less. Preliminary results using such media have proved to be superior to those obtained for paper as described in this article. The explanation for this can probably be

attributed to increased light transmission and hence less optical noise, better homogeneity of the medium and improved characteristics of the separated zone. These more recent data will be presented in a subsequent publication.

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