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THE QUANTITATIVE ASSESSMENT OF CHROMATOGRAPHICALLY SEPARATED CHROMOGENS AND FLUOROGENS

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SUMMARY

In this review the conditions required to produce chromatograms suitable for quantitative analysis (*i.e.* scanning) are briefly described. This is followed by a description of a simple filter transmission instrument and a more complicated double beam, flying spot, ratio forming device; the scanning records and calibration curves obtained from these two machines are illustrated and compared. Finally an automated computational procedure for the analysis of digital records is described and compared with alternative techniques of analysis.

INTRODUCTION

Since its introduction in the mid-forty's thin-media chromatography has become the principal tool for the separation and analysis of a vast array of substances. It is used by nearly all branches of science and in biochemistry, chemistry, pharmacy, biology and medicine is indispensable. Because of the information contained in any thin-media separation it is not surprising that many have concentrated on the quantitative aspects (*i.e.* scanning) of analysis and as proof of this interest we have today at least fifteen to twenty commercial scanning devices. These scanning devices range from simple filter transmission instruments up to more complicated double-beam types. It might be useful in this review to summarize briefly some facets of thin-media chromatographic analysis. More detailed comments on recent advances in photodensitometry can be found in a later paper[1] presented at this symposium.

QUALITATIVE ANALYSIS

Although thin-media separations are used widely and regularly, in a qualitative sense, by all of us, it is worth noting that such analyses even when performed with numerous different solvent systems and detection reagents do not lead unambiguously to a positive identification. In order to be sure that an unknown substance is what you think it is, other physico-chemical techniques of analysis must be included; in this respect the use of mass spectrometry as an adjunct to chromatography offers much for the future. The basic steps for any thin-media separation are summarized below.

Separation. This is an empirical process and although common sense and even certain theoretical considerations (for instance the R_M theory[2,3]) can be used in a predictive manner the best solvent systems usually evolve by searching the literature and on a trial and error basis. Because individual requirements vary, the length of time of any separation can usually be arranged to take from a few minutes to overnight or longer by a careful selection of the type of support medium.

Detection. Under this heading we can consider the sensitivity and specificity of the detection procedure. Taking specificity first this may be general as would be the case for instance with ninhydrin (nitrogenous substances) or Ehrlichs (indolic substances) reagents or more specific so that only one or two like substances are stained. An example of this is the so-called 'pink spot'. This substance, originally thought to be β-3,4-dimethoxyphenylethylamine and occurring uniquely in the urine of schizophrenic patients, stains a pink colour when the constituents of a pH 9.5 urine extract are separated overnight on paper and treated sequentially with ninhydrin and Ehrlichs reagent. A more detailed examination of the constituents of the pink spot zone (R_F value about 0.6, solvent system *n*-butanol–acetic acid–water, 4:1:1) reveals that there are at least eighteen of them. Some specific and sensitive procedures have been developed for the analysis of some of these constituents. For instance β-3,4-dimethoxyphenylethylamine produces a characteristic fluorophore when treated with an acidified glycine–formaldehyde reagent[4,5]; *p*-tyramine, another constituent, yields a fairly specific fluorophore after treatment with 1-nitroso-2-naphthol[6]. Although these substances are nearly isographic following separation of a urinary pH 9.5 extract they can easily be qualitatively and quantitatively detected by these more specific procedures.

With respect to sensitivity, it is on the whole a truism that fluorimetric procedures are more sensitive than absorptiometry. Using the example described above this is demonstrated in Fig. 1. In order to be able to detect a pink spot of any of the individual or combined constituents approximately 5 μg need to be present on the paper chromatogram; although by scanning at 530 nm a linear relationship has been shown to exist between optical density and concentration this represents a very crude and non-specific analysis. Use of the fluorophores produced by glycine–formaldehyde or 1-nitroso-2-naphthol reagents increases both the specificity and sensitivity (see Fig. 1). A still more sensitive and specific procedure involves the use of Dansyl derivatives. In these cases as little as 5 ng may be detected and quantitated (see refs. 6–10 for further details).

The advantage of preparing, and then separating, derivatives of a substance, or group of substances, present in a mixture rather than attempting to separate the mixture itself is that cleaner separations with an improved sensitivity and specificity are obtained. Examples are the use of fluorodinitrobenzene and Dansyl derivatives in the elegant studies on protein structure and the recent analysis of certain cerebral constituents[7,9,10] using Dansyl derivatives.

In summary the requirements for a successful qualitative separation are:

(1) Prefractionation (in order to isolate a group of substances)—this group may then be separated directly or after conversion to suitable derivatives.

(2) Separation—on a trial and error basis the most appropriate support medium and solvent systems are selected to give the separation required in the desired time period.

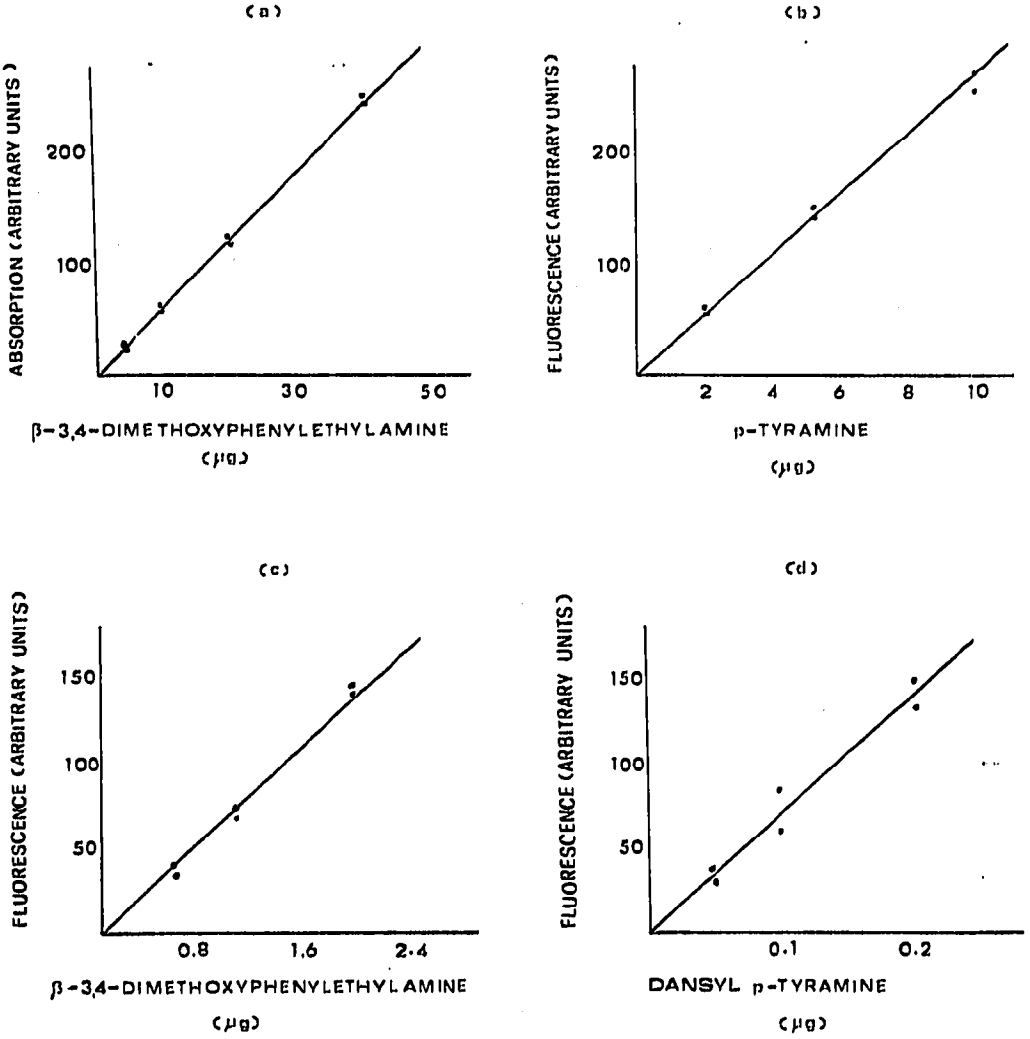

Fig. 1. (a) Calibration curve for urinary pink spot constituents. A pH 9.5 urine extract was separated on Whatman No. 2 paper strip overnight in *n*-butanol–acetic acid–water (4:1:1) and treated sequentially with ninhydrin and Ehrlichs reagent. After air drying the chromatograms were scanned at 530 nm. Range (using ß-3,4-dimethoxyphenylethylamine as reference substance), 5–50 μg; error, ± 6.4% (95% fiducial limits). (b) Calibration curve for *p*-tyramine. A urinary sample (extracted and separated as in Fig. 1a) was dipped through 1-nitroso-2-naphthol reagent (0.1 g dissolved in 90 ml ethanol and 0.5 ml aqueous 2.5% sodium nitrate added, the mixture then carefully made up to 100 ml with conc. HNO_3), heated for 15 min at 125°, allowed to stand for at least 30 min and then scanned (activation 365 nm, fluorescence 530 nm). Range, 0.5–10 μg; error, ± 5.2% (95% fiducial limits). (c) Calibration curve for β-3,4-dimethoxyphenylethylamine. Chromatograms of urinary samples (extracted and separated as in Fig. 1a) or β-3,4-dimethoxyphenylethylamine were sprayed with acidified glycine solution (5% aqueous glycine adjusted to pH 3.0 with conc. HCl), partially dried and then suspended in formaldehyde vapour for 3 h at 65°. After extensive drying in a ventilated fume hood the strips were scanned (activation 365 nm, fluorescence 470 nm). Range, 0.25–5 μg; error, ± 10.4% (95% fiducial limits). (d) Calibration curve for Dansyl *p*-tyramine. *p*-Tyramine eluted from the *p*-tyramine zone of a chromatogram was dissolved in 100 μl of 0.1 *M* $NaHCO_3$. After adding 100 μl of the Dansyl reagent (1 mg/ml in acetone), mixing, standing overnight and removing the excess $NaHCO_3$, the reaction products were separated by chromatography and the Dansyl *p*-tyramine zone was scanned (activation 365 nm, fluorescence, 510 nm). Range, 25–500 ng; error, ± 10.2% (95% fiducial limits).

(3) Detection—a general or specific detection reagent is selected to give the desired specificity and sensitivity of detection.

QUANTITATIVE ANALYSIS

In order to obtain quantitative as well as qualitative information from thin chromatographic media it is necessary to include a few precautions in the handling, preparation, detection and drying (see ref. 6 and 11 for further details) of the chromatograms.

There are essentially three ways of evaluating, in a quantitative sense, chromogens and fluorogens separated on thin-media chromatograms. The first is semiquantitative and consists quite simply of separating and treating standard substances in parallel with the unknown followed by visual comparisons. The accuracy of this method is poor but, nevertheless, quite adequate in some situations.

Elution techniques

Quantitation by spectrophotometry or spectrophotofluorimetry of chromogens and fluorogens eluted directly from chromatograms is straightforward and possesses the advantages that the separated substances are often required in solution for other reasons (radioactive counting, preparation of derivatives, recording of spectra, etc.). Disadvantages are that this procedure is much more time-consuming than direct scanning, that there is sometimes an irreproducible recovery and that irreversible changes can occur. It is, however, better to employ elution techniques than an uncritical use of a scanning device. By this I mean that for direct scanning it is important to observe the precautions listed above; in addition, in the case of absorptiometry the solutes must be applied in bands rather than spots. Finally in the quantitation of the analogue or digital scanning records an awareness of the limitations of the various procedures used is important; this is especially important when overlapping peaks are analyzed by numerical integration.

Direct scanning

As already mentioned there are available many different scanning devices both of a commercial and a laboratory prototype nature. These machines operate either in the transmission or reflection mode, or both; incorporate either filters or monochromators, or both; use either photomultipliers or photodiodes for photoelectric conversion; incorporate a wide variety of light sources from simple low-pressure mercury arc lamps (with discontinuous line spectra in the ultraviolet) up to xenon arc lamps (continuous spectrum in both the ultraviolet and visible regions); include various other sophistications such as fibre optics, flying spot systems or double beam difference or ratio systems; digital and/or analogue outputs and automated peak area evaluation ranging from straightforward numerical integration to computer-aided curve fitting procedures. A detailed survey and criticism of these various devices has been published[6,11]; in this review, therefore, I shall mention only briefly our original filter transmission instrument which incorporated automated analysis of the digital scanning record and follow this with a mention of a new double beam, flying spot, ratio-forming photodensitometer. This latter machine is described and discussed in some detail by POLLAK later in this symposium[1]. The appearance and schematic representation of a filter transmission instrument built primarily to aid in the evaluation of chromogens and fluorogens separated on paper is illustrated in Fig. 2. In the

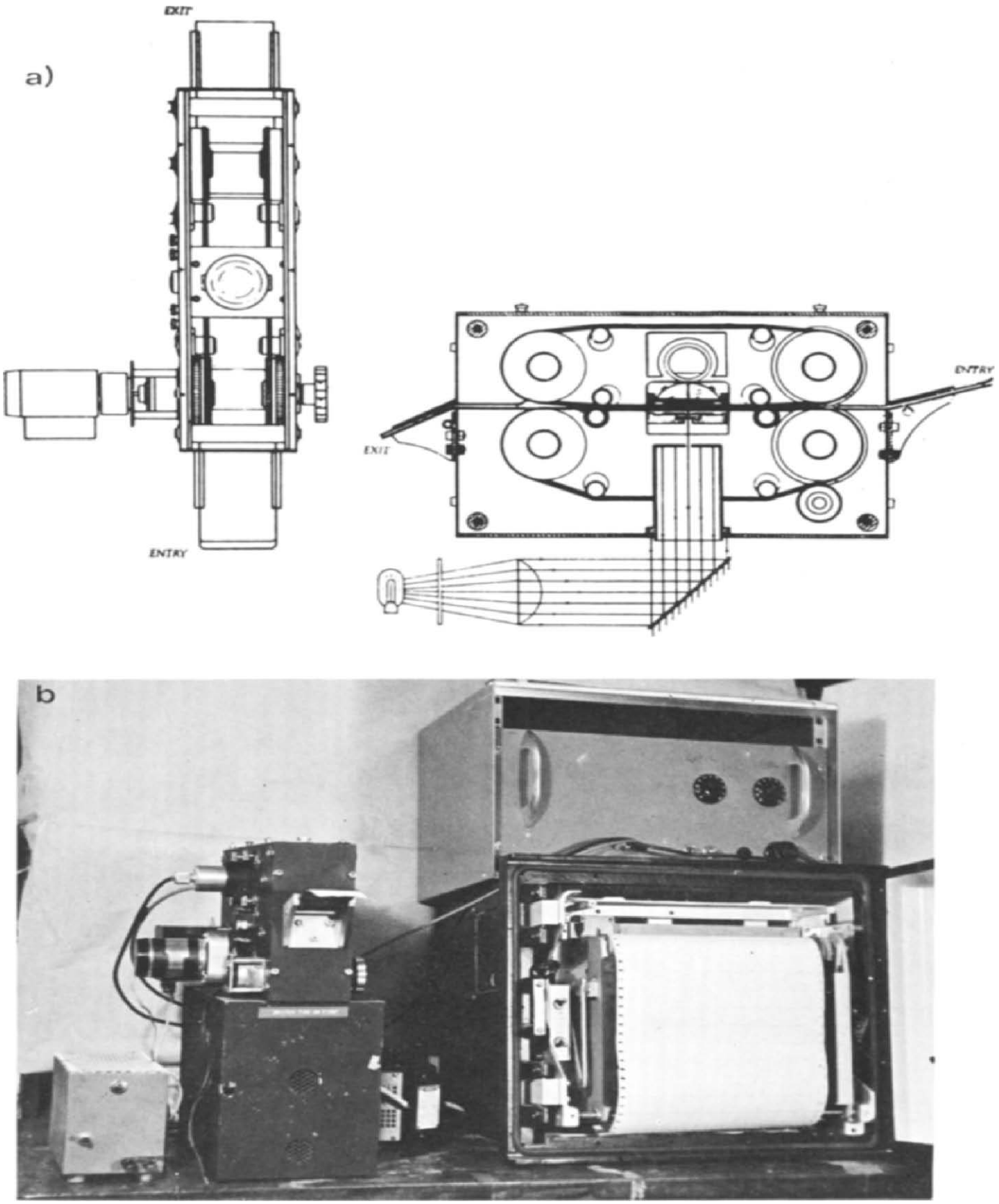

Fig. 2. (a) Schematic illustration of a single-beam filter transmission scanning device (see text for further details). (b) Scanning device arranged for absorptiometric analysis.

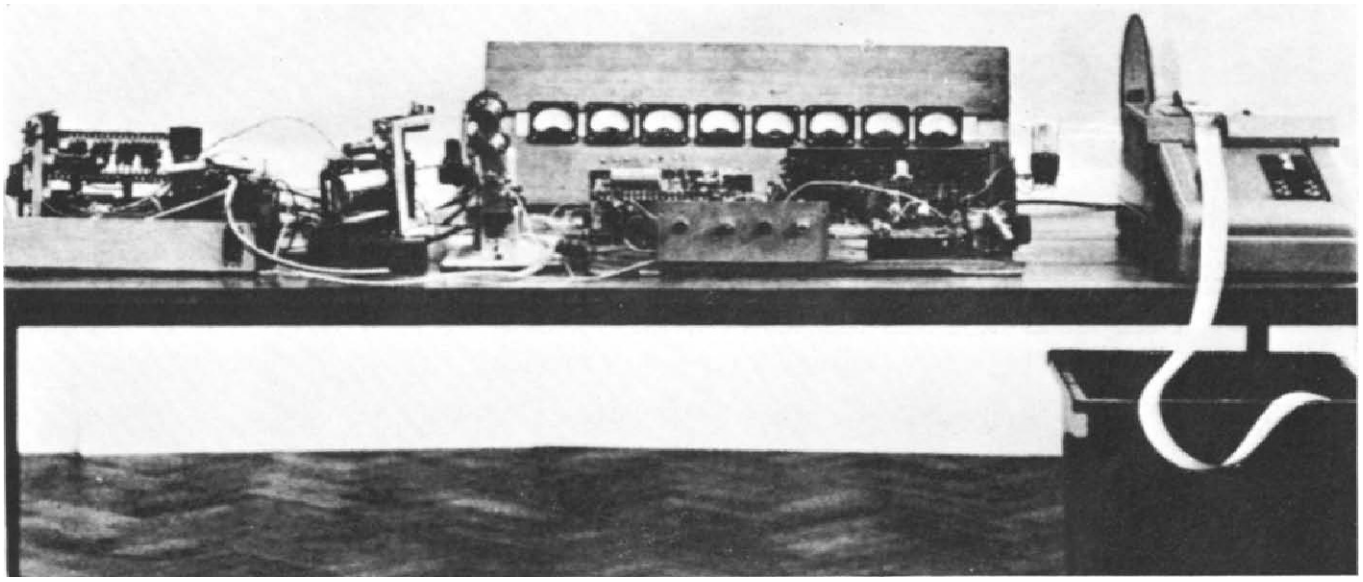

Fig. 3. Prototype apparatus for the production of digital records.

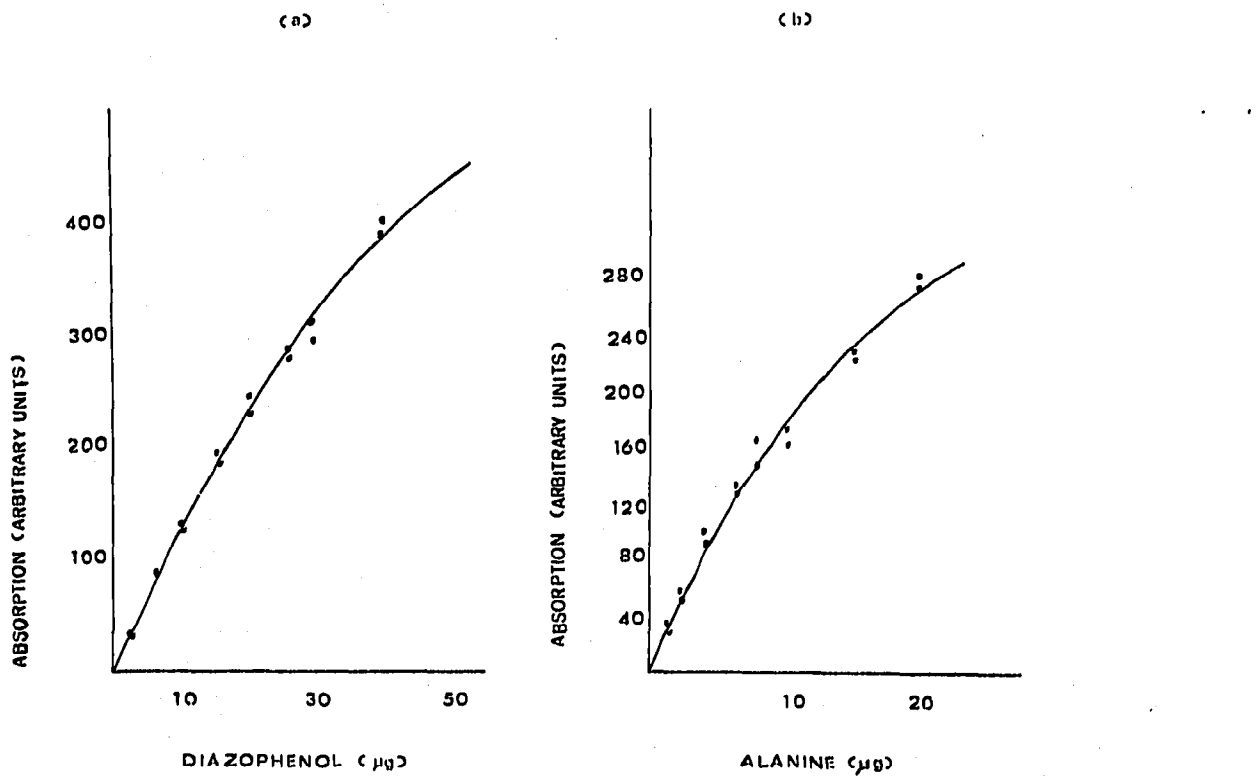

Fig. 4. (a) Calibration curve for 4-hydroxy-4'-nitroazobenzene. This derivative of phenol was separated during 4 h on Whatman No. 2 paper strip in the solvent system light petroleum (b.p. 100–120°)–toluene–acetic acid–water (133:66:170:30) and after air drying scanned at 380 nm. (b) Calibration curve for alanine. Alanine was separated on Whatman No. 3MM paper strips in *n*-butanol–acetic acid–water (4:1:1) and then dipped through a ninhydrin–cadmium acetate reagent[8,9], allowed to dry in a sealed system and then scanned at 530 nm.

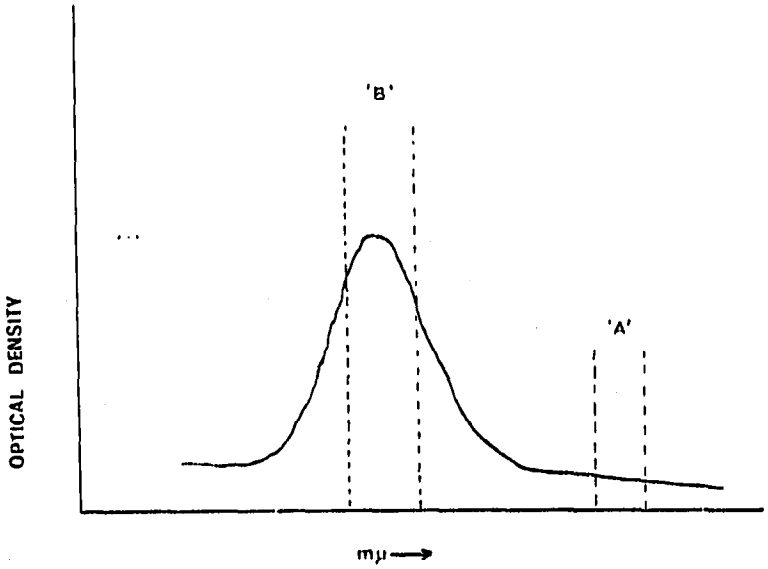

Fig. 5. Diagram illustrating the wavelengths of the two scanning beams used in double-beam photodensitometers.

case of fluorescence measurements light from a low-pressure mercury lamp is filtered on the primary side just below the entrance slit to produce the activating wavelength; on the secondary side (just above the exit slit) a second filter transmits only the principal fluorescent wavelength. After photoelectric conversion the signal is amplified, divided and fed to both the analogue recorder, and after analogue to digital conversion to the paper tape punch (Fig. 3). Typical calibration curves obtained by scanning some fluorophores are illustrated in Figs. 1b, c and d.

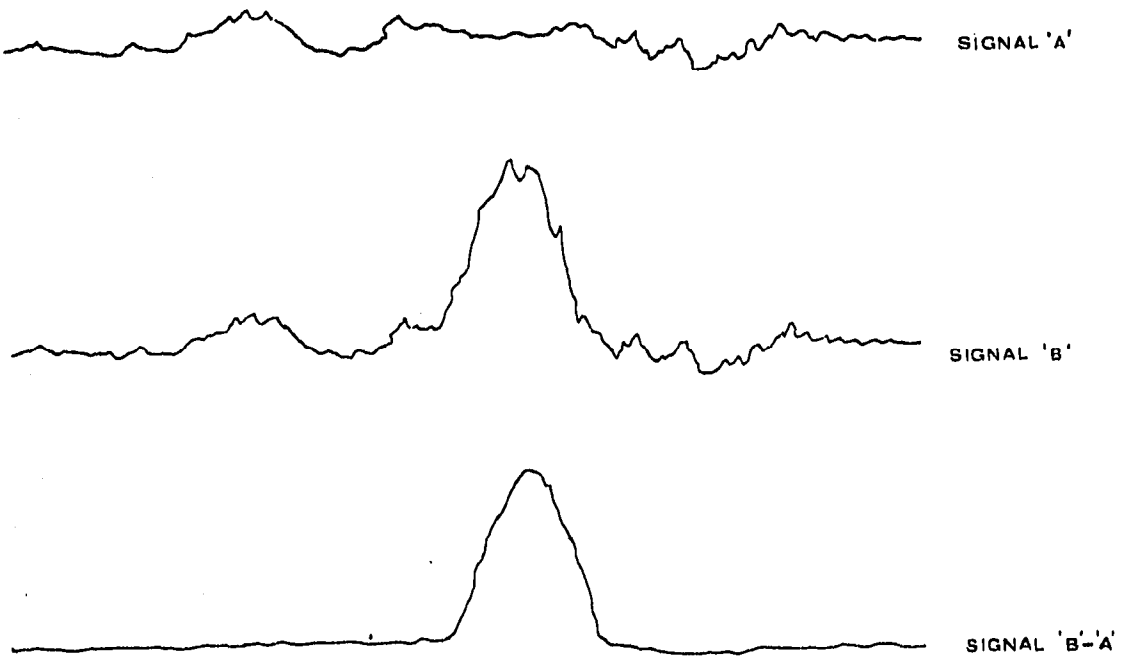

Fig. 6. Diagram illustrating the typical scanning signals obtained from a double-beam difference system.

In the case of absorptiometric analyses the apparatus (see Fig. 2b) was slightly modified so that light from a cooled quartz/halogen lamp is filtered on the primary side and after conversion through a log circuit based on that first described by SWEET[12] recorded (analogue only) to produce scanning records (typical calibration curves for which are illustrated in Figs. 1a and 4). It can be seen by inspecting the calibration curves shown in Figs. 4a and 4b that a simple logarithmic conversion does not produce a linear relationship between optical density and concentration as would be the case if Beer's law were obeyed.

The lower sensitivity level of any scanning device is limited by 'noise' of both electrical and optical origin. In single-beam devices the 'noise' is overwhelmingly optical in origin[13–15]; this limits any further improvement in performance. In a double-beam difference system[14–16] two light beams selected as shown in Fig. 5 are used alternately to illuminate the chromatogram. The separate signals from these two beams are respectively 'A' and 'B' in Fig. 6. By subtracting signal 'A' from 'B' a marked increase in sensitivity of detection is achieved with of course a considerable improvement in the state of the background. In a device recently completed in our laboratories, this concept (as described in a later paper[1]) has been taken a step further so that instead of the difference between signals 'B' and 'A' the ratio B/A is formed. In addition the chromatogram to be scanned is illuminated by a spot of light moving rapidly from left to right (*i.e.* flying spot); this flying spot mechanism in addition to negating any problems arising as a consequence of zone geometry also further reduces the noise component[1,13,14].

With the same chromogens as indicated in Fig. 4 preliminary calibration curves using this double-beam, flying spot, ratio-forming photodensitometer are illustrated

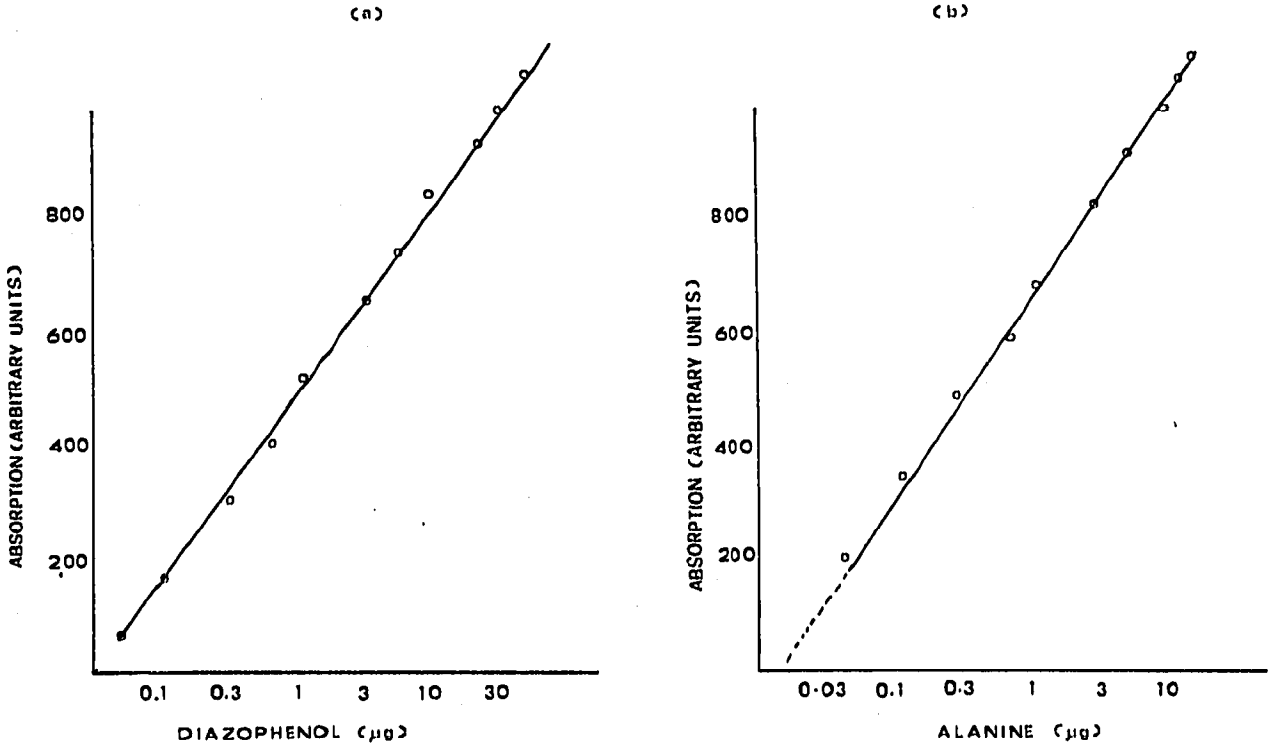

Fig. 7. (a) Calibration curve for 4-hydroxy-4'-nitroazobenzene. (b) Calibration curve for alanine.

in Fig. 7. It can be seen that the linearity between optical density and concentration and the lower level of detection has improved considerably.

ANALYSIS OF ANALOGUE AND DIGITAL SCANNING RECORDS

There are many ways in which quantitative data may be obtained manually from an analogue scanning record: (1) planimetry, (2) peak height, (3) triangulation, (4) tracing of peaks followed by cutting and weighing, and (5) excision and weighing of peaks on analogue record itself.

Although these procedures (except 2 and 3) produce acceptable results they are extremely laborious and time-consuming (see ref. 6 for precise comparisons). With certain assumptions numerical integration can produce accurate values during the actual recording of the scan.

In the filter transmission device shown in Fig. 2 the photomultiplier signal was divided to produce both an analogue and a digital record. The digital record was punched on paper tape as a binary 8-bit character arranged so that $255 \pm 1 = 2.55$ V. A typical paper tape with an arbitrary coding system is shown in Fig. 8. The com-

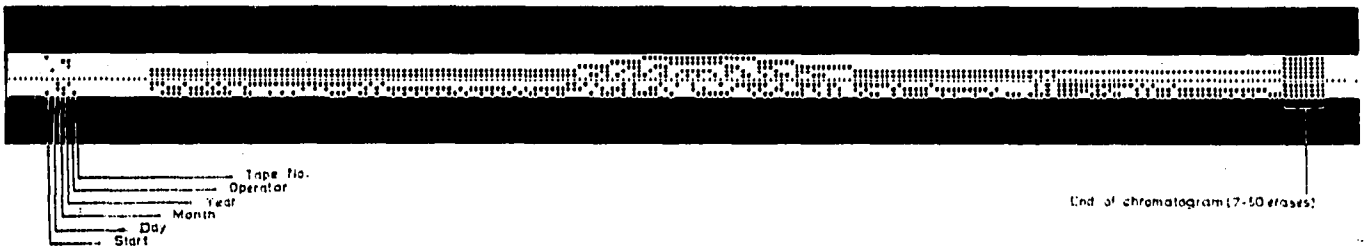

Fig. 8. Typical punched paper tape showing a part of the baseline, a peak and the code routines used to start, stop and list logistic data.

putational analytical procedure developed in collaboration with Drs. H. Ross and R. HOLDER (see refs. 6 and 11 for further details) includes an iterative statistical procedure in which the best 'Gaussian' profile is fitted to the digital data recorded during scanning. The validity of this assumption is illustrated in the block of overlapping peaks (Dansyl amino acids only partially resolved by light petroleum (b.p. 100–120°)–acetic acid–water (10:9:1) on a Whatman No. 2 paper strip) shown in Fig. 9a and some amino acids present in locust haemolymph (kindly made available by Dr. I. HARRIS) and separated by ion-exchange chromatography (Fig. 9b). Another aspect of the computer programme used to quantitate digitized scanning record was an ability to detect and correct mispunched points, correct discontinuities (such as baseline shifts), and assess very large and very small peaks on the same record. Quantitative data obtained using this computational procedure were superior to all other manual and electric methods of peak area evaluation in terms of accuracy, reproducibility and speed.

Future developments

It seems that perhaps two types of scanning device will be required for the future. The first will be a special-purpose device which, depending upon need, could

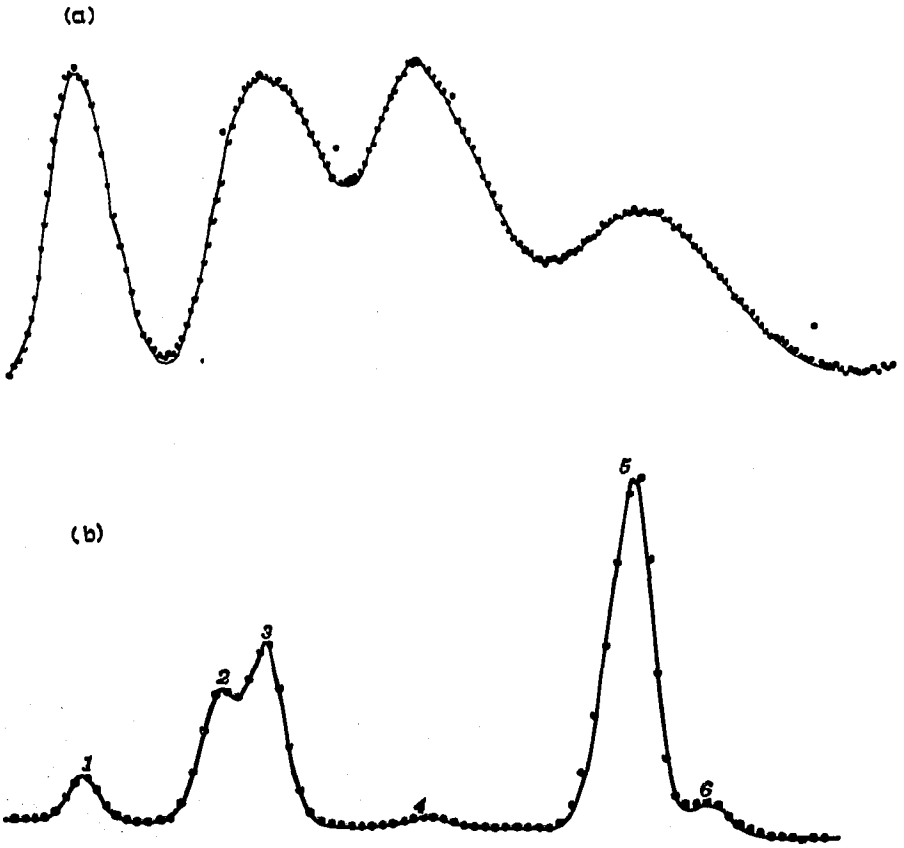

Fig. 9. Validity of the Gaussian assumption as used in the statistical curve fitting procedure. (a) Partially resolved mixture of Dansyl amino acids. (b) Amino acids separated on an ion-exchange column.

be mechanically and optically relatively simple but incorporating sophisticated automatic data assessment; the second will be a much more complicated device offering transmission and reflection modes of operation, double-beam and flying spot optics, the ability to record spectra *in situ*, capable of scanning all types of thin media, analogue and digital outputs and suitable software for computational analyses. The linitations for the future seem no longer to be instrumental; improvements and alterations in the support media toward increased reproducibility and sensitivity, however, would be useful.

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