SENSITIVE DUAL WAVELENGTH DENSITOMETER WITH SUPPRESSION OF THE SUPPORT INTRODUCED BACKGROUND OPTICAL DENSITY

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INTRODUCTION

The irregular and high background optical density of the support has been one of the major obstacles in attaining the precision and sensitivity expected from the direct quantitation of zones in paper or any thin layer chromatography or electrophoresis. Up to the present time, it has not been possible to find an adequate solution to this problem using the classical single wavelength spectrophotometric techniques.

Based on the CHANCE time sharing dual wavelength spectrophotometer, a densitometer is described that shows as its main characteristic an inherent suppression of the irregular background optical density of the support and the cancellation of the total background signal, while maintaining full spectrophotometric specificity and sensitivity. A high efficiency light gathering system, an electronic circuit with an overall noise equivalent to $4 \cdot 10^{-4}$ optical density units, and a.c. amplification permit additional advantages. These advantages include: (1) the use of narrow slits which increase the resolution of close peaks, (2) the use of unoiled supports, (3) an expanded sensitivity adjustable up to 0.05 optical density units full scale without changes in the base line. These characteristics allow an increase in precision or sensitivity of nearly two orders of magnitude in relation to any previously designed single wavelength instrument. Thus, the direct detection and quantitation of zones is limited only by the sensitivity and reproducibility of the chemical methods involved.

DESCRIPTION OF THE INSTRUMENT

The instrument is an adaptation of the dual wavelength spectrophotometer described by CHANCE[1,2] (Fig. 1). Two beams of monochromatic light are focused upon a vibrating mirror in such a way that they are alternatively flashed through the paper and then to the photoelectric detector. The mirror is mounted on the vibrating element of a Brown Company "Converter" which operates at the line frequency. Thus, each beam passes through the paper 60 times per sec. The photomultiplier reproduces the square wave resulting from light modulation. When the intensity of the monochromatic beams are equalized, no alternating component is observed. If the light absorption at either wavelength changes, the a.c. amplitude is proportional to the

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difference in absorption. A square wave of amplitude equal to this difference results. This square wave is amplified, demodulated and recorded. For economy, interference filters can be used instead of the monochromators, with a sacrifice in flexibility.

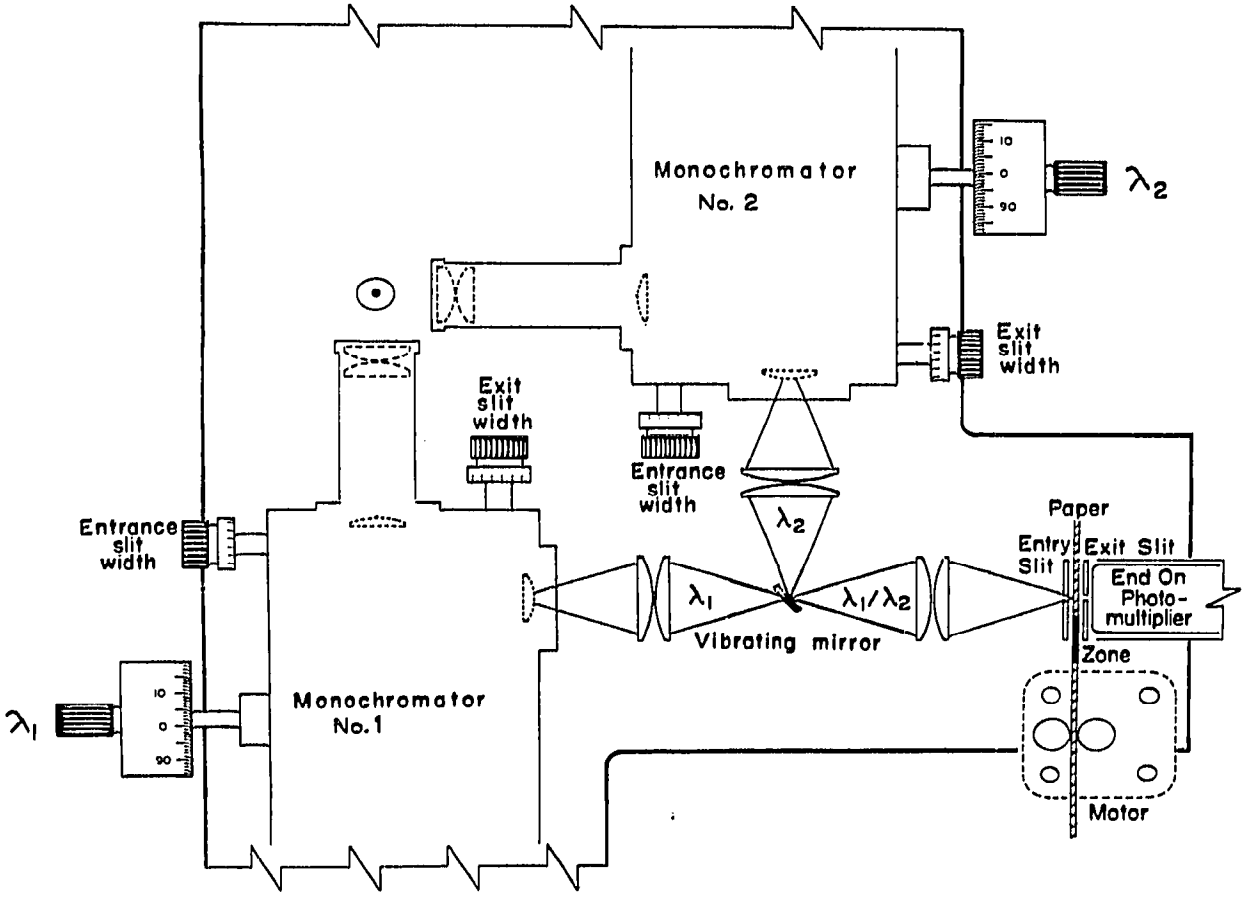

Fig. 1. Block diagram of the dual wavelength densitometer (MD 34LS).

SCANNER FOR PAPER STRIPS

A 2 r.p.m. motor having a grooved roller mounted on its shaft moves the paper strip through a channel at 1 mm/sec speed. A spring loaded counter roller on the other side of the paper insures the effectiveness of the movement. A safety switch insures that no high voltage can be applied on the photomultiplier unless the strip covers the slit apertures.

The channel has a rectangular cross section of 25 mm × 0.5 mm for the strip admission. The scanning aperture is formed by two aligned slits (12 mm long × 0.5 mm wide × 0.1 mm thickness) mounted on each side of the channel. The filament of the lamp is focused on the entry slit and the end-on photomultiplier is mounted at 2 mm from the exit slit. This type of optics allows the highest recovery of scattered light[1], while the slit system reduces to a minimum the interaction among stained and unstained zones due to rescattering[3,4].

ELECTRONIC DESIGN

The electronic circuitry associated with the time sharing dual wavelength spectrophotometer has been described by CHANCE[1,2] (Fig. 2). The transistorized electronics used in the present system follow the same philosophy but differ somewhat in layout. They are only described here for the sake of completeness.

A GE BXJ 8.5 V 4A tungsten lamp obtaining its supply voltage from a Sorensen Nobatron QB 6–8 power supply regulated to 0.01 % is used as a light source. An EMI 9524B photomultiplier obtains its dynode supply voltage from a Kepco ABC 1500 V with 10 mA maximum output current which is continuously adjustable and has an overall regulation of 0.001 %. The total high voltage is divided by a string of resistors which are directly attached to the photomultiplier socket. The photomultiplier anode is connected through a shielded cable to the load resistors at the input of the amplifier chassis. Different values assure optimum performance. The arrangement of the Zener diode IN 751A and the diode FD 300 is provided to keep the negative excursion at test point 1 between 0 and -5.1 V in order to avoid excessive input signals to the operational amplifier. The amplifier is capacitor-coupled to eliminate the unnecessary d.c. background signal. This amplifier is set up at the gain of 1 and has sufficiently low output impedance to drive the transformer of the phase sensitive detector. The variable resistor at the secondary of the transformer allows upgrading the frequency response. The actual detector consists of a single pole, double throw contact on the Brown Converter used as the light switch. In this way a locked-in-phase sequence is automatically assured. A three stage filter consisting of a box car and variable two stages allows election of bandwidths which are given in terms of the 10 to 90 % rise time from 0.05 to 5 sec. The operational amplifier at the output of the circuit employs a calibrated gain switch and supplies sufficiently low output impedance to drive a 1 mA recorder.

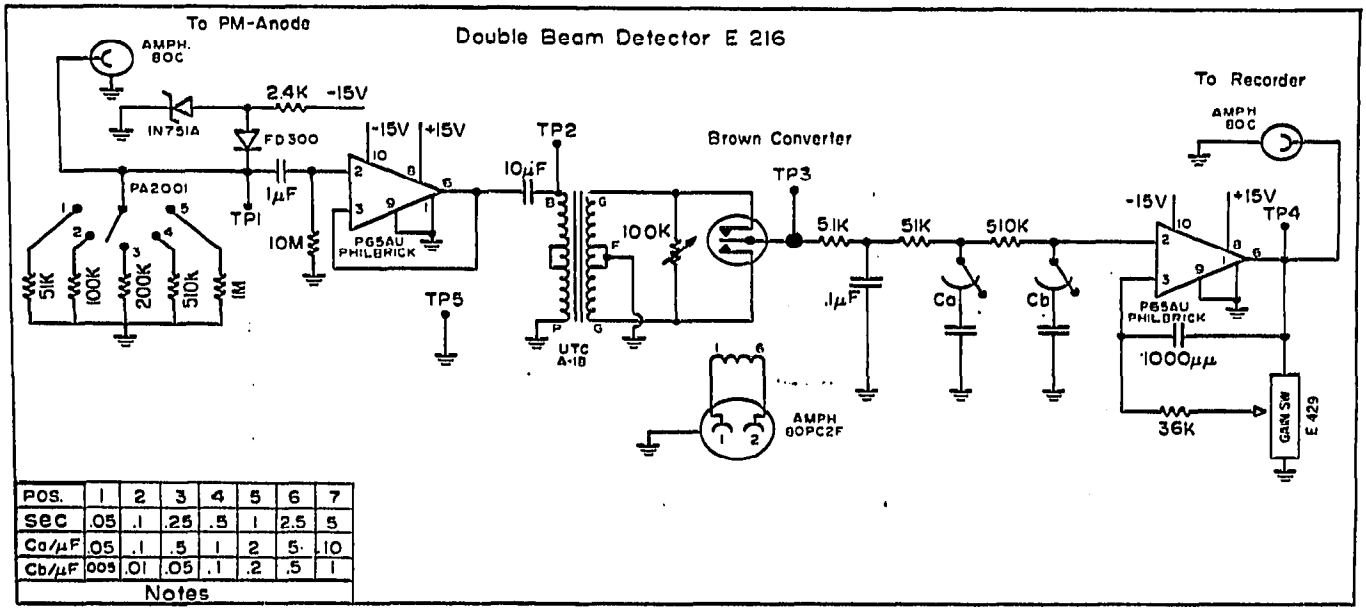

Fig. 2. Electronic design of the detection and amplification system used in the dual wavelength densitometer.

PERFORMANCE

The automatic suppression of the irregular background noise is shown in Fig. 3. In this experiment a clean strip of Whatman No. 3 filter paper with an average optical density of 3.4 is scanned with the purpose of comparing the classical single wavelength with the dual wavelength technique.

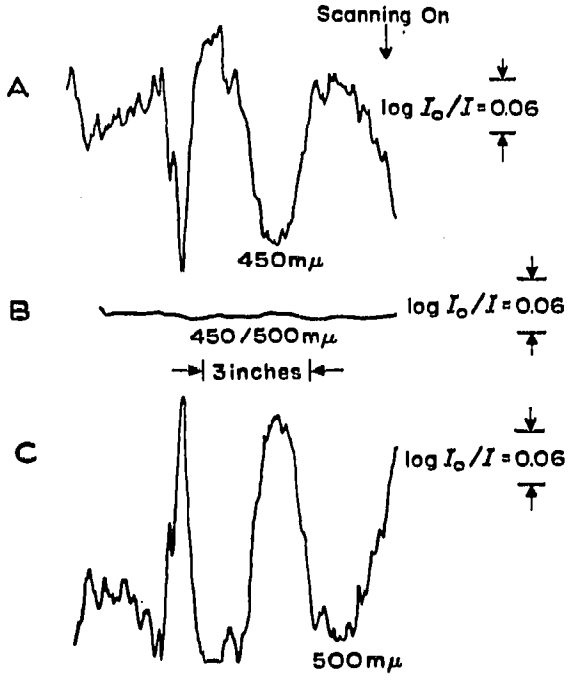

Fig. 3. Suppression of background noise by the dual wavelength technique. (A and C) Trace of a single wavelength scanning; (B) dual wavelength scanning.

For this purpose the monochromator or filter giving λ_1 is blocked and the paper is scanned with the λ_2 light (Trace A). Then the strip is returned to its original position and the operation is repeated blocking λ_2 and recording with λ_1 (Trace C). The same operation is repeated again, but using both beams (Trace B). In Traces A and C the upper part of the signal is shown, that is, the one corresponding to the irregular optical density of the paper. The rest of the signal, corresponding to the remaining optical density of the paper, has been bucked out electrically. The quasi-specular relationship of the signals is due to the 180° phase shift inherent in the fullwave detector used in the electronic circuit. The cancellation of the two signals when the dual wavelength system is used results in the signal that appears in Trace B. In the particular experiment described using a 0.5 mm slit and 450 mμ and 500 mμ as the scanning wavelengths, the maximum signal observed scanning with a single wavelength is equivalent to 0.250 optical density units. At the differential dual wavelength it is equivalent to 0.005 optical density units, thus showing a fifty-fold rejection of the undesirable background changes. The value of the last mentioned signal is due to the difference in the light scattering properties of the paper at the different wavelengths used[1,2]. As a general rule, the longer the wavelengths used and the smaller their difference, the higher the rejection of the background. In this way it is possible to attain up to a hundred-fold or more suppression of the irregular background of the paper.

If zone measurements of a signal to noise ratio of 10 are required in order to maintain a $\pm 5\,\%$ error, it can be easily seen that for equal intensity signals, the precision of the dual wavelength technique is intrinsically increased fifty to a hundred times more, thus making the background noise irrelevant to the measurements. If an increase in sensitivity, while maintaining an acceptable error is desirable, the background noise rejection value is equivalent to the increase in sensitivity obtained. To illustrate this increase, a 1 cm wide zone containing $6 \cdot 10^{-10}$ moles of alanine gives a full scale deflection with a ten-fold signal to background noise. This value is comparable to the most sensitive fluorometric methods using pyridine nucleotide coupled enzymes[5].

Fig. 4 shows a comparison between the electronic circuit and paper background noise at different sensitivities of the expanded scale. The scanned zone is the same for the three traces. It is possible to see that while the electronic noise is of an essentially statistical origin, the paper signal is completely reproducible at the different amplifications shown. Trace C shows the signal at the maximum practical amplification attainable and it is in this condition that it is possible to obtain the full scale deflection with $6 \cdot 10^{-10}$ moles of alanine as noted previously.

Fig. 5 shows a calibration curve done with alanine. Zones containing different amounts of the amino acid in the same final volume were applied at equidistant points in a strip. They were subjected to electrophoresis at 50 V/cm for 30 min, dried and stained according to the method of ATFIELD AND MORRIS[6]. Scanning was done using 450 mμ as the reference and 500 mμ as the measuring wavelength. In this special case, in which the zones have the same width, the plot of $1/C$ against $1/T$, in which C = concentration of the amino acid and T = the maximum difference in transmission in the densitometric curve gives a straight line. This discrepancy with Beer's Law is now under study, but our results are in agreement with those obtained by CROOK[4].

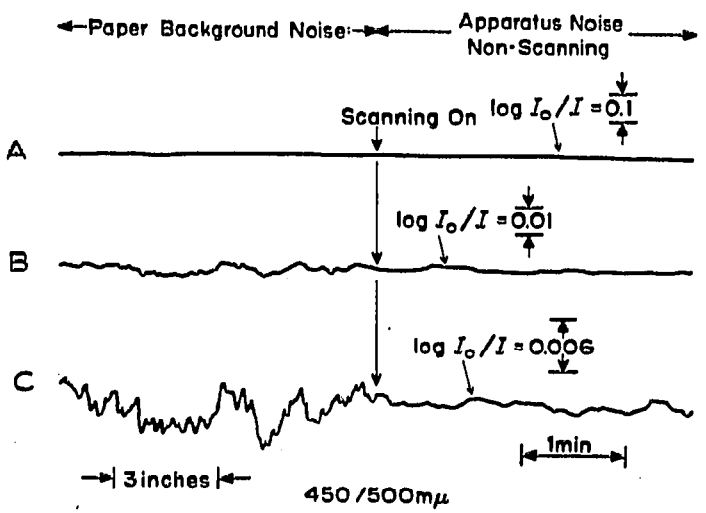

Fig. 4. Trace of electronic noise and background scanning noise at different sensitivities of the expanded scale amplification. Time constant: 1 sec.

Fig. 6 shows the trace obtained in a high voltage electrophoretic separation. A mixture of amino acids with close mobilities was studied. Separation at 30 V/cm per

2 h was done and the strip dried and stained as in Fig. 5. The trace demonstrates the background noise, the resolution of close peaks, and the low cross talk between slightly and highly stained zones.

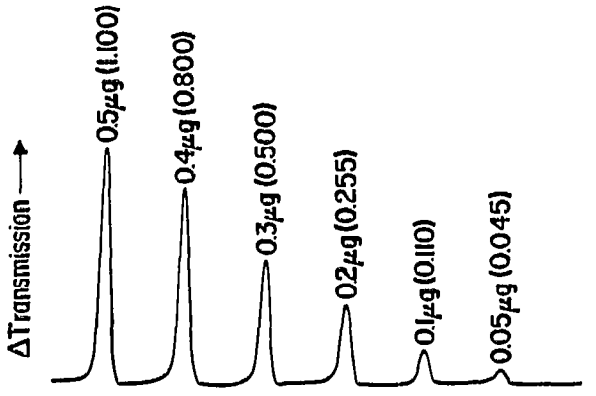

Fig. 5. Densitometry of a calibration curve of alanine (conditions described in text). Numbers in parentheses are the equivalent difference in optical density units at the peaks. Full scale sensitivity: 2.0 optical density units. Time constant: 1 sec.

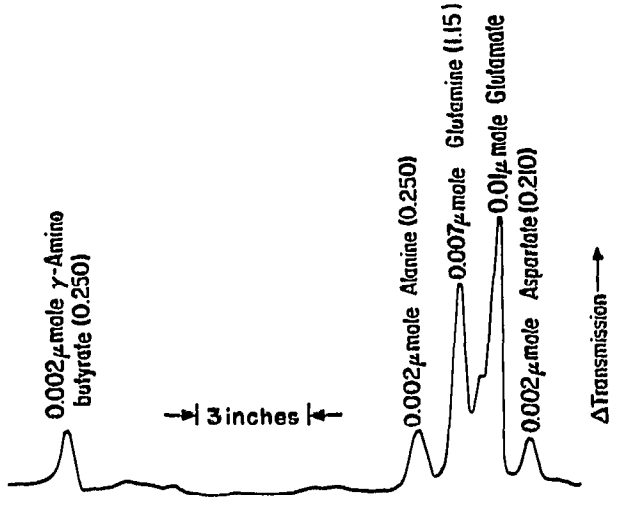

Fig. 6. High voltage electrophoretic separation of a mixture of amino acids. Full scale sensitivity: 2.0 optical density units. Time constant: 1 sec.

DISCUSSION

Differential dual wavelength colorimetry was actually suggested by TYNDALL in 1852, but was applied for the first time by MILLIKAN[7] in a technique used to avoid the influence of the physical changes (shortening, thickening, changes in blood content, etc.) on the measurement of the rate of desoxygenation of hemo- and myoglobin during the contraction of a muscle. In 1951, the principles involved in this technique were reanalyzed and perfected by CHANCE[1] for the study of cytochrome oxidoreduction changes *in vivo* and *in vitro*. These studies were done in highly turbid suspensions of particles or slices of tissue whose scattering properties changed along time or in the

course of the experiment. The success of this technique in measuring with high precision small changes in optical density in the presence of highly turbid materials is due to the combination of sophisticated electronic circuitry allowing low drift and noise, and to the technique of measurement in which the absorption bands of the cytochrome are recorded in terms of the difference of absorption at two closely spaced wavelengths, one at the peak and the other at the isobestic point of the pigment. These wavelengths are sufficiently close together that non-specific changes which vary only slowly with wavelength give very little absorbancy changes. A full discussion of the possibilities and limitations of this technique has appeared in publications by CHANCE[1,2], RIKMENSPOEL[8,9], and COWLES[10]. Although this technique has been used successfully for more than 20 years, workers in the field of quantitation of electrophoretic and chromatographic zones seem to have disregarded the possibility of applying the dual wavelength principles to the design of densitometers.

The problem of the irregular background absorption of the support remained one of the major obstacles against sensitivity and precision in direct photometry at a single wavelength. Some efforts to solve this problem have been directed toward use of a smoother base line using either low amplification or high time constants in the response of the electronic circuits. Other attempts have been made to average the base line statistically by scanning the same or another portion of the paper, or by using wider slits. The most accepted way to solve the problem, at least partially, has been to submerge the support in a solvent of a refractive index similar to the cellulose. A suggestion as to the possible use of split beam techniques was given by BEAVEN[11] in 1955, based on the "opal glass" of SHIBATA[12]. In 1963, BUSH[13] reanalyzed the problem in an excellent article and proposed for the first time a densitometer using differential dual wavelength measurements. The apparatus was described as being in the experimental stage and quantitative data on its performance were not provided.

The instrument described here shows that the technique of flashing two beams on the same part of the sample, as proposed first by CHANCE[1], is until now the best rational method of rejecting background unspecific changes in optical density due to the irregularity of the support. This technique also has the advantage of maintaining full specificity and sensitivity to the stained zones. The differential method also allows easy switching from one sensitivity to another without any calibration change in the position of the base line, thus providing flexibility for the measurement of low or highly stained zones.

The increase in sensitivity and precision allowed are by far in excess of the practical capabilities of chromatographic or electrophoretic techniques. An unsolved problem is the decision to use either difference detection as in the present apparatus, or ratio detection as proposed by BUSH[13], RIKMENSPOEL[9], and COWLES[10]. Ratio detection has the advantage of solving the basic limitations of difference detection which are automatic corrections of large changes in transmittance common to both wavelengths, and the straight line logarithmic conversion to an absorbancy scale. Although both are useful in the present problem, both assume obedience to Beer's Law. This assumption has been challenged by CROOK and others[3] in direct photometry of strips and has been confirmed with different amino acids by our preliminary experience. This problem is under study, and for the moment, we prefer to run standards of the substances to be separated together with the unknown sample. This allows internal controls and an extended range of calibration.

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SUMMARY

An instrument for the direct photometric scanning of zones is described. The method involves the alternative flashing of two beams of light through the support. The wavelengths used are the peak of absorption and a selected reference at one of the sides of the absorption curve of the colored compound in the zone. This technique allows an inherent suppression of the background optical density of the support while maintaining full sensitivity and specificity. This results in a performance increase of about two orders of magnitude when sensitivity or precision are compared with single wavelength operating instruments.

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