RECENT ADVANCES IN THE PHOTODENSITOMETRIC EVALUATION OF THIN-MEDIA CHROMATOGRAMS

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SUMMARY

The article brings a survey of recent developments in theory and instrumentation for the quantitative evaluation of thin-media chromatograms by photodensitometric measurements. Optical and electrical noise as factors limiting the performance of the method are discussed and attention is called to the possible use of semiconductor photodetectors. Double-beam scanning in its various modifications is considered the best way of reducing the effect of the optical background noise and flying spot scanning is shown to remove errors due to irregular geometry and non-uniform density profiles of the zones. The paper ends with the brief description of a newly developed instrument incorporating these features.

INTRODUCTION

At present photometric methods seem to be the most convenient means for the quantitative determination of the amount of separated substance on a solid substrate. To this purpose the change in one of the optical transfer parameters of the medium caused by the presence of the investigated substance is determined and correlated with the quantity of the latter. Transmittance, reflectance, and fluorescence are all used and have their specific merits. Criteria of the method are sensitivity, reproducibility and accuracy.

The performance of a measuring system of any type is directly correlated to the amount of information about the measured magnitude, which can be collected and processed. It can be shown in a very general way that the amount of information which can be utilized is proportional to the ratio of the intensity of the total signal at the output of the measuring device to the sum of all its unwanted components. Unwanted, here, refers to components which do not carry information pertaining to the investigated parameters. Though coming from different sources the unwanted components are usually lumped together under the common term "noise". To obtain best performance the ratio of signal to noise has to be maximised.

NOISE IN PHOTODENSITOMETERS

In photodensitometric instrumentation essentially two types of noise are encountered. One is electrical in nature and originates mainly in the photodetector elements. The other one is of optical origin and caused largely by fluctuations of the optical transfer parameters of the medium. Mains pickup and radio interferences are excluded from these considerations. Their effect is similar to that of noise; by proper design (e.g. shielding, correct grounding, smoothing of the current supply, etc.) they can be kept at a negligible level.

Both types of noise mentioned are of random character and uncorrelated. Their combined effect is, therefore, determined by the square root of the sum of the respective squared amplitudes.

$$E_{n_t} = \sqrt{E_{el}^2 + E_{opt}^2} \tag{1}$$

If the two components are unequal in amplitude, the stronger one obviously prevails and determines almost exclusively the total noise signal. In general, therefore, only that component has to be considered.

The principal source of electrical noise is as mentioned above the photodetector. The most common type of detector used in photometric equipment is the photomultiplier tube. Only recently have solid-state photodetectors been developed to a degree which makes their use feasible[1].

The noise contribution of a photodetector element is conveniently expressed in optical terms as the "Noise Equivalent Power" (NEP) of the detector. This parameter determines the intensity of a light beam impinging upon the detector element, which produces an output signal with an amplitude equal to the amplitude of the noise signal generated in the photodetector itself. The amplitude of the noise signal is frequently proportional to the square root of the electrical bandwidth. Noise of this type is called "white" in analogy to white light in optics. The NEP value quoted in manufacturer's specifications is usually referred to a bandwidth of 1 Hz. It determines in a sense the lowest light intensity which can be reasonably detected with a particular type of photodetector. The electrical bandwidth determines the response time of the system; a bandwidth of 1 Hz implies a response time of the order of 1 sec. In general a faster response is required entailing the use of a larger bandwidth. The approximate optical threshold sensitivity is then obtained by multiplying the NEP value with two times the square root of the inverse value of the response time.

The NEP value of a standard photomultiplier tube is of the order of 10^{-16} W; however, tubes with NEP values down to 10^{-20} W are available. On the other hand, the best solid-state photodetectors presently available have NEP values of the order of only 10^{-13} W. Semiconductor photodetectors need, therefore, for efficient operation an intensity of illumination which is several orders of magnitude higher than that required by a photomultiplier tube. However, this intensity is not beyond that which can be achieved by a well designed illuminating system using a standard light source of several hundred Watts input power. Then they offer many practical advantages over photomultiplier tubes, which appear to outweigh the need for a more efficient and sophisticated illuminating system. There will, of course, always be applications where the photomultiplier cannot be replaced.

The signal picked up by the photodetector is, of course, proportional to the light intensity, which is available at the entrance window of the photodetector. Since the electrical noise is constant, the signal to electrical noise ratio can be made nearly arbitrarily small using a sufficiently high intensity of illumination and a photodetector with reasonably low NEP value. From eqn. (1) it can, however, be seen, that there is no point in making the signal to electrical noise ratio much smaller than the signal to optical noise ratio. With sufficient light available it is the latter which determines the performance parameters of the instrument.
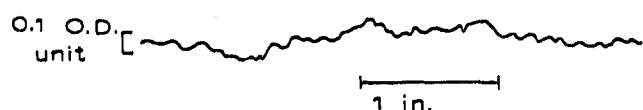

Fig. 1. Fluctuations of the transmittance of Whatman No. 2 chromatographic paper.

The optical noise is caused by fluctuations of the optical transfer of the medium (Fig. 1). Whilst the electrical noise is additive in character, the optical noise affects the illuminating light beam and consequently the instrument response in a multiplicative way[2]. For this reason the optical signal to noise ratio is independent of the illuminating light intensity and only a function of the medium.

The magnitude of the optical noise is determined by the non-homogeneity of the substrate. By improved manufacturing methods a considerable improvement over the present state should be obtainable. Besides that there are, however, very efficient means available, which permit to reduce the optical noise signal after pick-up by two orders of magnitude and possibly more.

The only practical way to reduce the electrical noise signal once it has been generated is a decrease in electrical bandwidth at the expense of the response time of the system. The final limitation is here the desired scanning speed of the instrument. The optical noise decreases also with decreasing bandwidth, though not to the same extent as the electrical noise. Its bandwidth is, however, determined not only by the electrical parameters of the system, but also by the spatial bandwidth determined by the size of the illuminated area. Increasing that size is equivalent to a decrease in bandwidth of the optical signal. There are, however, limits to what can be obtained in this way. Much more efficient in reducing optical noise is another approach, which is based on the fact that the useful information required is usually contained in only a narrow region of the optical spectrum, whilst the noise information is spread out over a much broader spectral range.

DOUBLE-BEAM SCANNING

To take advantage of this situation two scanning beams of different wavelengths can be employed[3]. One beam is centered around the wavelength, where the substance under investigation has maximum absorbance. The other beam occupies a spectral region, where the absorbance of the chromogen is negligible. Consequently the first beam carries information about both the wanted signal and the noise, whilst the second beam contains only noise information. Contrary to common belief, there is no need to employ strictly monochromatic light for either beam (Fig. 2). A spectral width of

several hundred Ångströms does in most cases not produce any significant error and eases problems of illumination[4].

The two beam signals can now be processed in such a way as to suppress a large part of the noise information whilst leaving the wanted information unaffected. To this purpose it has been proposed by SALGANICOFF et al. in 1968[5] to form the difference of the two beam signals (Fig. 3). This can be done in a very simple way by applying the two signals to the input of a conventional differential amplifier. The noise cancellation obtainable in this way is mainly dependent on the degree of equality between the two beam signals.
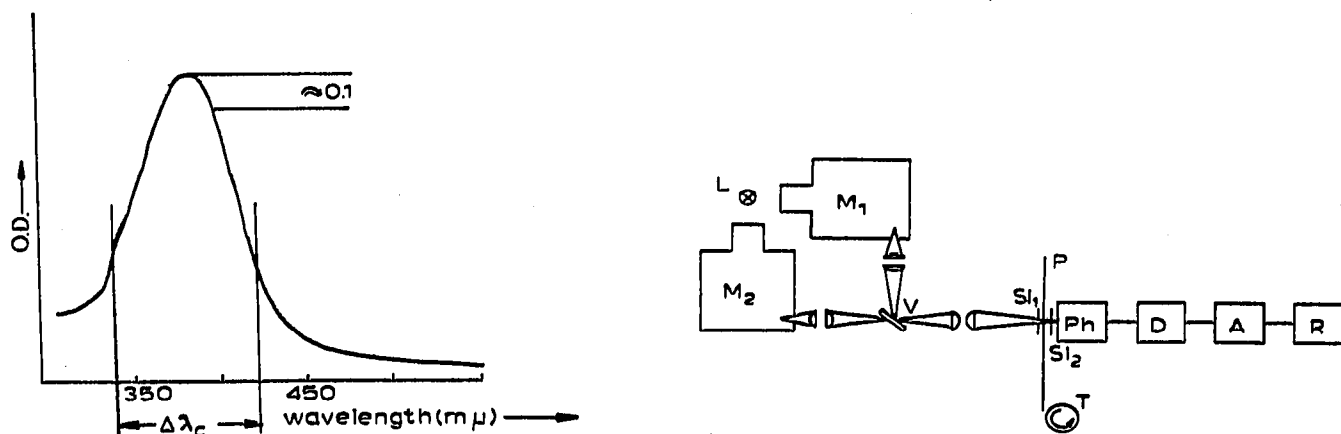

Fig. 2. Typical shape of the absorbance spectrum of a chromogen.

Fig. 3. The optical pathways of a double-beam difference forming system as described in ref. 5.

Another way to suppress the optical noise is to form not the difference but the ratio of the two beam signals (as proposed by BUSH[6]). Circuit elements to carry out this operation have become available recently at low cost. The advantage here is that small differences in the average intensity of the two beam signals do not affect the degree of noise suppression. A further important advantage is that the results of measurements obtained in this way are almost independent of fluctuations in the intensity of the illuminating light beams. Consequently much less stabilization of the light source is needed and also the uniformity of illumination over the width of the medium is not critical. Ratio forming appears, therefore, to be in all respects superior to difference forming. If the useful signal is very weak, the requirements on the accuracy of the ratio forming circuits become exceedingly high. This problem is best solved by digital processing. If analog processing is required, a combination of the difference and ratio forming procedures seems to give best results. It should be noted that neither method improves the noise of electrical origin.

For either of the two methods to provide effective noise cancellation the two beams have to carry the same noise information. To this purpose they should at any given time illuminate identical areas of the medium. Small deviations from this principle can be tolerated, as long as they are small in comparison with the spatial wavelength of the noise on the medium. No noise cancellation is achieved if the two beams illuminate essentially different areas of the chromatogram. On the contrary the optical noise is then increased by $\sqrt{2}$

TRANSMITTANCE *versus* REFLECTANCE

Most of the substrates used in chromatography have besides absorbance a relatively high coefficient of scattering. The theoretical expressions describing the optical transfer of such media are extremely complex. For technical purposes, however, a simplified theory developed in the late thirties by KUBELKA AND MUNK is adequate[7-11]. If the concentration of chromogen in the medium is not too high, it can be assumed that it affects only the absorbance, whilst the coefficient of scattering remains unchanged. If the medium does not scatter, the exponential dependence of transmittance on concentration postulated by Beer's law holds ideally. Besides specular reflection at the surface, which, of course, does not carry any information about the interior of the medium, no reflection occurs. A medium with very little scattering is, therefore, not suitable for reflectance measurements. With increasing scattering power of the medium deviations from Beer's law begin to appear. They become most pronounced in media with strong scattering and little absorbance, *e.g.* paper. The slope of the curve of transmittance plotted over absorbance decreases with increasing scatter, resulting in a loss of sensitivity and accuracy (Fig. 4). The deviations from Beer's law encountered in this region are in themselves not too troublesome. For small concentrations they can usually be neglected. At larger concentrations an individual calibration curve is commonly used. If the output signal of the photometer is available in digital form, a computer can be used for linearization. An automatic analog linearizing device, which can also be used for reflectance measurements, is presently being developed in our laboratories.

If the medium employed has strong scattering and a high transmission loss, reflectance measurements become attractive. A closer analysis shows that good sensitiv-
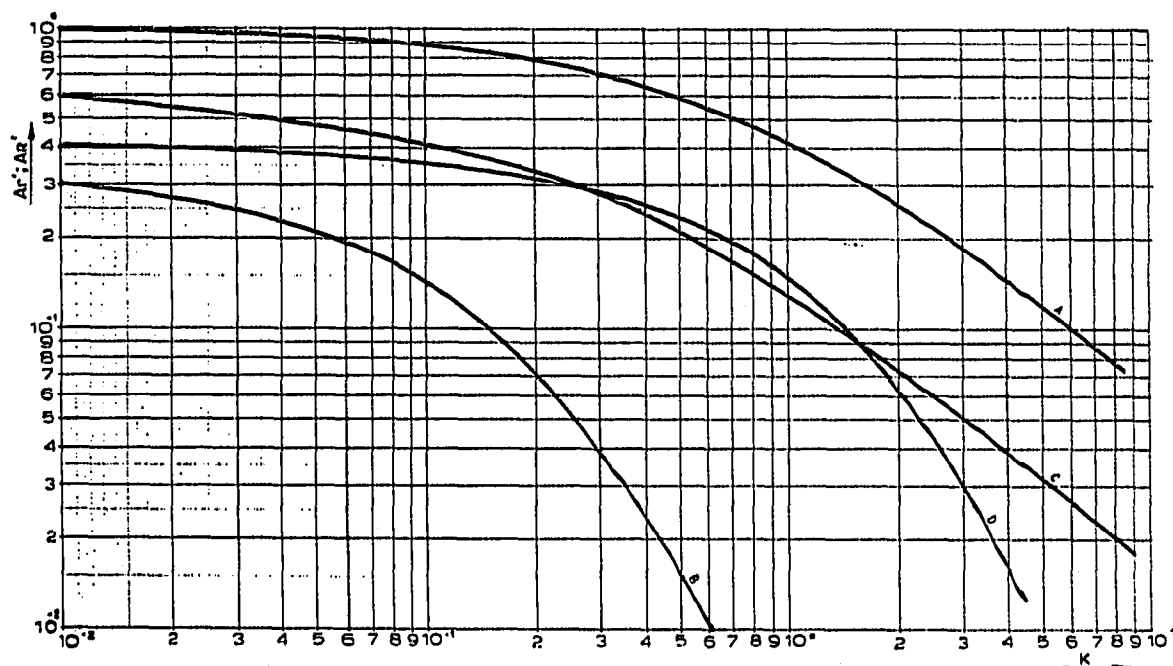

Fig. 4. The sensitivity $\partial A/\partial K$ for measurements of transmittance A_T and of reflectance A_R in dependence on the coefficient of absorbance K and for extreme values of the coefficient of scattering S of the medium. A: $\partial A_T/\partial K$ ($S = 0$); B: $\partial A_T/\partial K$ ($S = 20$); C: $\partial A_R/\partial K$ ($S = 1$); D: $\partial A_R/\partial K$ ($S = 20$).

ity and accuracy can be expected only if the absorbance of the medium is smaller than the coefficient of scattering.

Double-beam methods provide for reflectance spectroscopy the same advantages as for transmittance measurements, in addition to that they largely suppress the masking effect of the specular component of the reflected beam. The non-linearity of reflectance measurements is in the range quoted above compatible to that of transmittance measurements on the same type of medium. Linearization is possible in the same way as described before for transmittance measurements.

In both cases an additional error is encountered if the concentration of the separated substance varies considerably with distance from the surface. The magnitude of this error, which becomes noticeable only at rather high concentrations, depends on the degree to which the optical transfer function of the medium deviates from Beer's law. .

SLIT SCANNING *versus* FLYING SPOT SCANNING

It was pointed out above that the relationship between the light transfer of the medium and the spatial concentration of separated substance is non-linear. A partial exception from this general rule applies only for fluorescence measurements. The response of the photodetector itself is, however, strictly proportional to the received light intensity[4]. If the concentration of chromogen in the illuminated area is not uniform, the output signal of the photodetector corresponds to the spatial mean value of light transfer over that area. This value is, however, not proportional to the mean spatial value of concentration and there is not even a unique relationship between these two. Consequently large errors which cannot be compensated may occur, if there is an appreciable variation of concentration over the area of the scan. This limitation can be virtually removed and the error due to an irregular geometry of the zone and a non-uniform concentration profile be made vanishingly small if the illuminated area is made small enough so that variations of concentration over that area can be neglected. This approach leads to a replacement of the conventional slit-shaped illu-
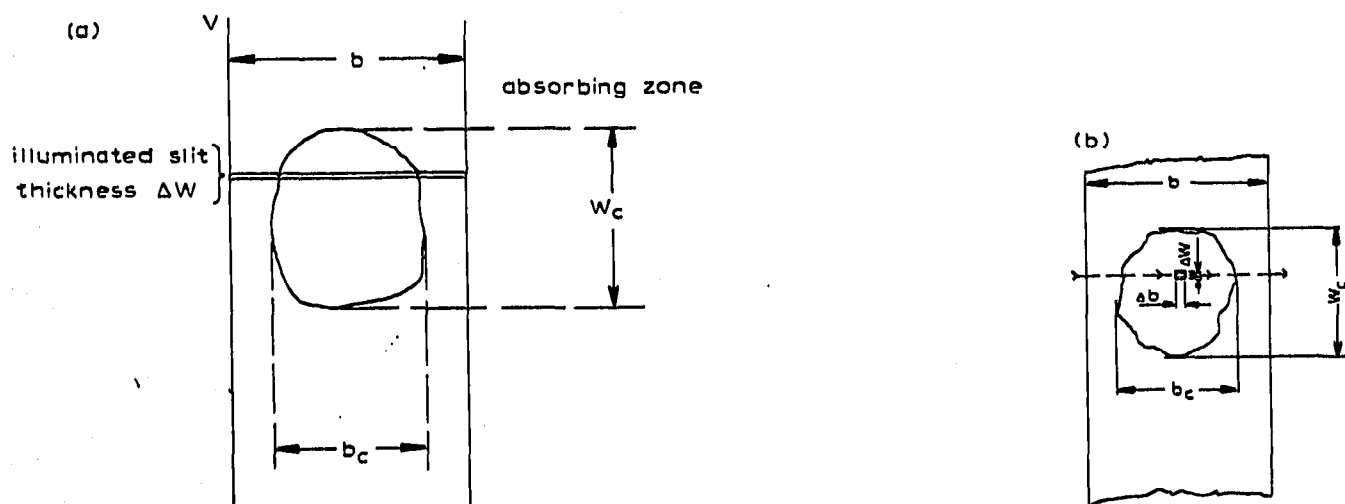

Fig. 5. Schematical representation of (a) slit scanning and (b) flying spot scanning.

minating aperture by a flying spot arrangement (Fig. 5). To obtain the total amount of substance present in a particular zone, the output of the photodetector system has to be integrated over the area of that zone. This can be done electrically using an integrating amplifier. The period of integration is conveniently made equal to the duration of a scanning line. Before integration can take place the signal has to be linearized. In cases where Beer's law is followed with reasonable accuracy, simple logarithmic conversion of the signal to be integrated is adequate. High-performance high-speed logarithmic amplifiers for this purpose are presently available at moderate cost. If Beer's law does not represent a reasonable approximation more elaborate means of linearization have to be used. Digital processing on a computer is probably the best solution, though analog methods are equally feasible.

An additional advantage gained by flying spot scanning is an improvement in optical noise. The optical noise picked up originally by a flying spot system is of course larger than that of a slit scanning system since the illuminated area is so much smaller and the spatial bandwidth consequently larger. Subsequent integration over the length of a scanning line tends to equalize this difference. However, in the flying spot system integration is carried out after linearization of the signal and therefore more efficient in combatting noise than the direct optical integration by the photodetector in the slit scanning arrangement which is affected by the non-linearity of the transfer function of the medium. The noise advantage of the flying spot becomes mainly evident on media with intense background noise, e.g. some types of paper.

A flying spot scanning device is no doubt more complex and therefore more costly than a slit type scanning device. Still we believe that the improved performance of the flying spot device more than outweighs these drawbacks.

A NEW INSTRUMENT

The theoretical considerations presented so far (and expounded in more detail in the literature quoted below) served as a basis for the development of a new type of photodensitometer for chromatographic and related applications in our laboratories. The design has reached the prototype stage and the results of some preliminary measurements seem to confirm the theoretical expectations.

The instrument can accept strip-shaped substrates of up to 25 × 500 mm. It offers a choice of two light sources: a tungsten halogen lamp for work in the longer wavelength range of the visible spectrum and a xenon-mercury short arc lamp for UV and the extreme blue range of the spectrum. Shifting a lever changes the mode of operation from transmission to reflection. Transition to fluorescence measurements from either side of the medium is equally simple. A mechanical flying spot arrangement is used with a spot diameter of approximately 1.5 mm. The light is chopped by a mechanical chopper at a frequency of 450 Hz. The processing speed is 5 mm/sec. The instrument is of the double beam type; interference filters are used to determine the spectral position of the two beams. Two PIN-Schottky barrier diodes are used as photodetectors. The output signals of the two beams are carefully balanced and then amplified, rectified and fed into two logarithmic converters. At the output of the converters the two signals are subtracted from each other and fed to an integrator and from there to a conventional line recorder, which records the ratio of the two beam signals on a logarithmic scale (Fig. 6).
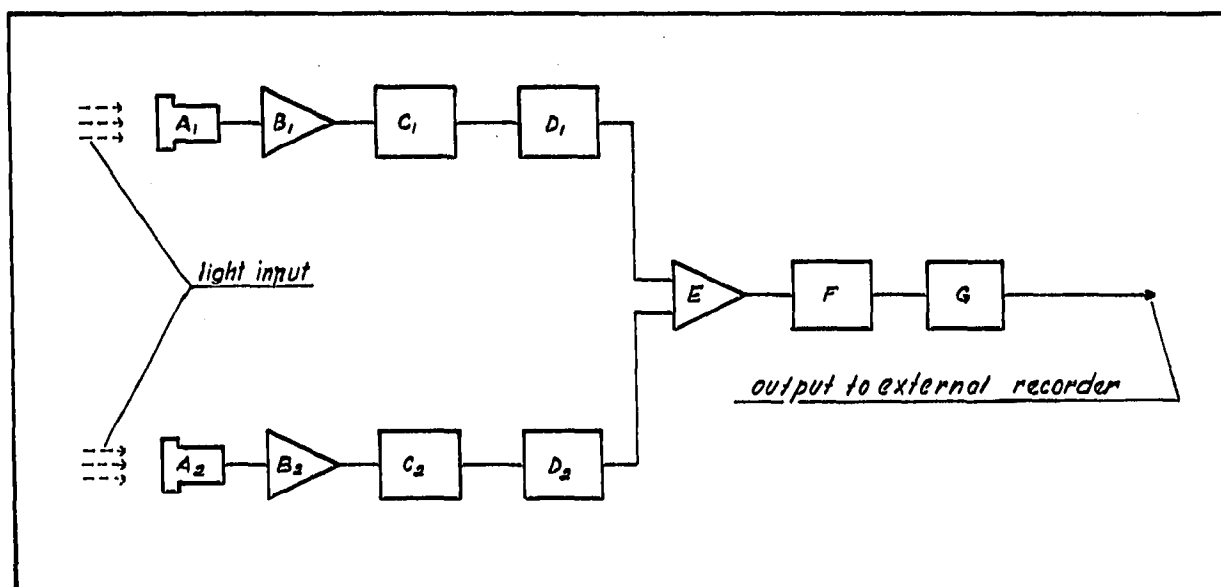
Fig. 6. Simplified block diagram of the electronics. A_1, A_2 = Photodiode; B_1, B_2 = amplifier; C_1, C_2 = rectifier-filter; D_1, D_2 = logarithmic converter; E = differential amplifier; F = integrator; G = sample and hold circuit.

Besides the analog output a digital output is provided as well. The digitized data are stored on a commercial computer compatible magnetic tape unit which serves as input medium for off-line processing on a computer. It is expected that by suitable processing it will be possible to increase the sensitivity of the instrument by another order of magnitude. Research work to establish a basis for the development of the necessary software is at present under way.

All preliminary testing was done in the transmission mode and only in the visible region. Some of the test results are shown in Figs. 7 and 8. The sensitivity limit with phenylalanine on Whatman No. 2 paper was about 5 ng. The reproducibility of the results was excellent. At present flaws are being ironed out and minor changes for improvement are made. It is expected that the final sensitivity threshold in the transmission mode will be of the order of about a nanogram of phenylalanine. Computer processing should enable us to push this limit still further into the sub-nanogram region. Performance assessments in the reflection mode remain still to be
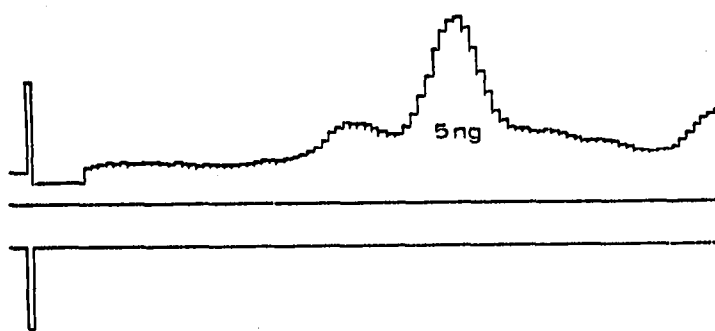

Fig. 7. A typical scan. The height of each step is proportional to the measured concentration.
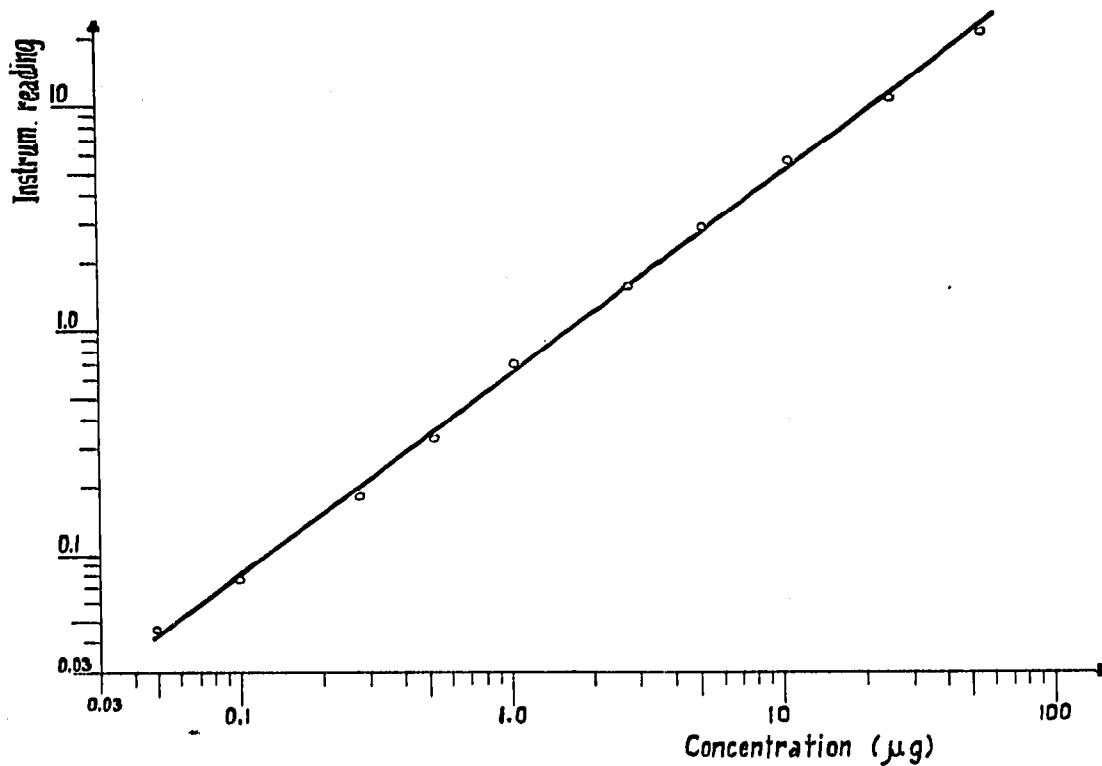
Fig. 8. Calibration curve for azobenzene chromogens (preliminary results).

carried out. The same applies to fluorescence measurements, where, however, the theoretical groundwork has still to be completed.

Altogether we believe that this new development will show superior performance and be a valuable contribution to chromatography and related disciplines.

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