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AN ANALOGUE DEVICE FOR CORRECTING DENSITOMETRIC MEASUREMENTS OF COLOURED SPOTS ON CHROMATOGRAMS FOR THE EFFECT OF LIGHT SCATTERING

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SUMMARY

A device is described which corrects absorbances measured by the Vitatron TLD 100 Densitometer for the effect of light scattering ($A + 0.4A^2$ factor). This permits quantitative densitometry of thin-layer chromatograms without the necessity of using very pale spots (low concentrations), thus increasing the accuracy and versatility of the instrument. The system produced linear calibration curves for β-carotene and iodine-stained triolein.

INTRODUCTION

The advent of thin-layer chromatography (TLC) has made possible the rapid separation of many types of substances. Its main drawback has been the lack of methods for *in situ* quantitative analysis. The slit densitometer is intended for the assay of uniform bands of colour such as those produced by electrophoresis. It has been used for the assay of charred spots on thin-layer chromatograms[1] but it is clear that results can only be of a semi-quantitative nature because the measurement is only that of the area occupied by the spot. If used to measure a coloured spot the result obtained is the average percentage transmission over the length of the slit and not the average optical density which is the parameter required[2].

This fault has been overcome by the scanning densitometer (a commercial model is the Vitatron TLD 100), which measures light transmission at a point in the chromatogram and therefore permits conversion to O.D. units (log amplifier) before an average is derived. The difficulty remains, however, that the scanning densitometer assumes that Lambert–Beer's law remains approximately valid over the range of optical densities encountered[3]. Lambert–Beer's law applies only to transparent media, the laws of light absorption in light scattering media being much more complex[4]. GOLDMAN AND GOODALL[5,6] used this basic theory to derive the following function for the absorption of light by coloured compounds in light scattering media:

$$m = f \cdot e^{-2 A_0} (A + 0.4A^2)$$

where m = mass of coloured substance present in the light scattering layer;

A = measured absorbance of the compound present in the light scattering layer;

$e^{-2 A_0}$ = a correction factor for variations in thickness of the layer.

It follows from this equation that Lambert–Beer's law is valid only at very low optical densities. However, at these low spot densities fluctuations of the background become severe in practice. At higher concentrations results for chromatographically produced spots can only be semi-quantitative.

The present paper is concerned with the design and evaluation of an analogue device, for use with the Vitatron TLD 100 densitometer, which would correct measured absorbances for the factor $A + 0.4A^2$ and thus enable quantitative measurements to be made over a range of spot densities where background effects are of minor significance.

EXPERIMENTAL

TLC was carried out on 20 × 20 cm plates precoated with 0.25-mm layers of silica gel (Merck, Darmstadt, G.F.R.). Development was performed in unlined tanks.

All densitometric measurements were made with the Vitatron TLD 100 Hg densitometer used in the transmission mode. Plates were scanned at 6 cm/min. A recorder chart speed of 20 cm/min and an integrator setting of 8 were used throughout.

Voltage measurements were made with a Model 8 Avometer.

RESULTS

Design of the correction device

When the TLD 100 is operated in the − log mode (normal transmission measurements) the signal from the photomultiplier is passed firstly through a logarithmetric amplifier, then through an amplifier of variable amplification factor. The ouput from the latter, which is proportional to the absorbance measured by the optical system, is subjected to "cosine correction" to compensate for the sinusoidal nature of the scanning motion, and finally the voltage is integrated by an RC network before
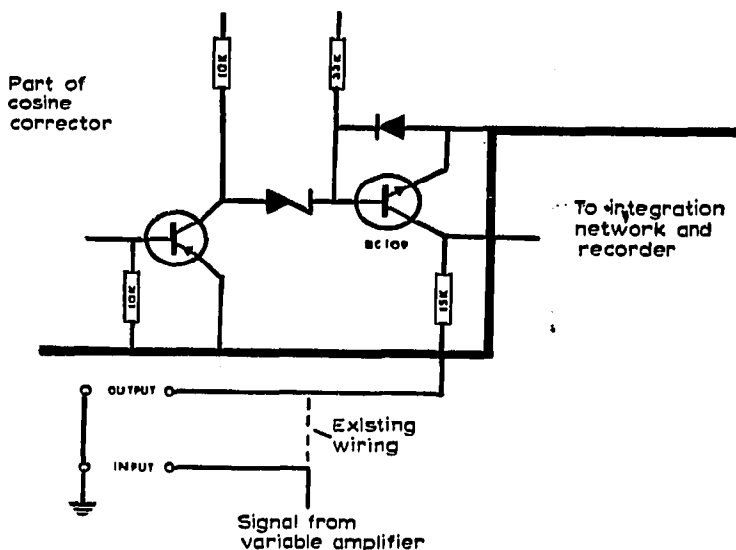

Fig. 1. Connection of correction to printed circuit board B of the Vitatron TLD 100 densitometer.

being fed into a potentiometric recorder. A logical place to apply the correction is between the variable amplifier and the cosine corrector as shown in Fig. 1. Measurement of voltages at this point showed that it was possible, by adjustment of the variable amplifier, to arrive at a situation when $1V = 1$ O.D. unit. Substitution of the voltage (V) at this point by $V + 0.4V^2$ would effect the correction required.

The schematic diagram of the device is shown in Fig. 2. It employs three operational amplifiers and a four quadrant multiplier. Operational amplifier A could be omitted if operational amplifier C were used as an inverting subtracter. The circuit diagram is given in Fig. 3. The circuit of the quadrant multiplier with operational amplifier level shift is as given by Signetics Corporation[7]. The inverter and inverting

Fig. 2. Schematic diagram of the correction device.

Fig. 3. Circuit diagram of the correction device (power supplies omitted).

adder are both basic operational amplifier circuits[8]. A 2,200 pF capacitor was added across the input to prevent parasitic oscillations. To facilitate setting up adjustments, the prototype was provided with its own power supplies (15 V monolithic voltage regulators, R. S. Components Ltd., London).

Setting up adjustments

The offset voltage of operational amplifier A was nulled by its offset adjust potentiometer (input grounded). The quadrant multiplier was adjusted as detailed by Signetics[7] so that its output voltage was $0.1 \cdot$ (input voltage)^2. Operational amplifier C was temporarily adjusted (offset adjust) to give zero output with its input grounded. The device was then checked, on the bench, for correct static performance by applying known voltages (derived from potential dividers across the power supply) to the input and measuring the output voltages obtained.

Connection to the densitometer was carried out as indicated in Fig. 1. The instrument was switched on and a blank area of plate scanned (cosine corrector functioning). By temporarily disconnecting the quadrant multiplier circuit the span control was adjusted so that at a deflection corresponding to 1 O.D. unit (TLD span calibration graph) the additional deflection obtained on reconnecting the quadrant multiplier was exactly 0.4 O.D. units. This was found to be span setting 5.28. The offset potentiometer of operational amplifier C was then reset to give a deflection of 10 recorder chart units when the input to the device was grounded.

Comparison of the performance of the densitometer with and without the compensator

β-Carotene (B.D.H. Ltd., Poole, Dorset) was chosen as the first substance for examination. It was thought desirable to know if this substance behaved as predicted by the equation derived by GOLDMAN AND GOODALL. This required a range of concentrations of β-carotene to be applied as uniform spots to a thin-layer plate. A series of concentrations of β-carotene in chloroform were prepared and spotted on to
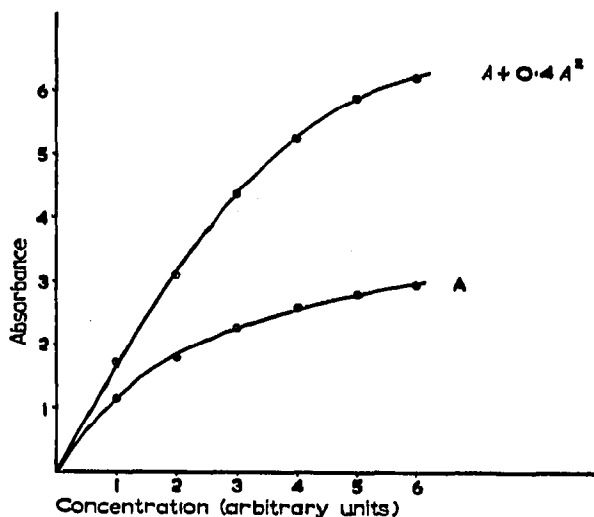

Fig. 4. Graph of measured absorbance (A) and $A + 0.4A^2$ against concentration for uniform spots of β-carotene.

a thin-layer plate with a Pasteur pipette from a height of 2 cm. Uniform spots of consistent optical density were easily prepared in this way. These were measured with the TLD 100 densitometer (uncorrected for $A + 0.4A^2$) using a U8 filter and results are shown in Fig. 4. The upper curve is corrected arithmetically for $A + 0.4A^2$, and shows that when this correction is applied an approximately linear relationship up to measured absorbances greater than 2 is obtained. Since this range of O.D.'s is beyond the range of the recorder at span setting 5.28, more dilute series of similar standard spots were prepared, and their optical densities measured with and without the $0.4A^2$ correction connected. The results are shown in Fig. 5 (average of two readings). A linear response was produced only when the $A + 0.4A^2$ correction was applied, even at these comparatively low optical densities. Note that these same recorder deflections may represent much larger momentary O.D.'s when chromatographically produced spots instead of areas of uniform density are being scanned.
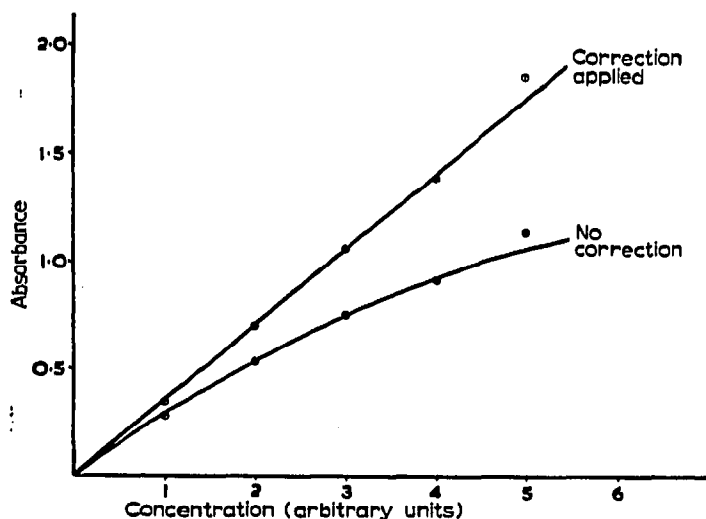

Fig. 5. Graph of corrected and uncorrected absorbances against concentration for uniform spots of β-carotene.

Having verified the properties of β-carotene and the response of the machine using uniform areas of colour, the evaluation was continued using chromatographically produced spots. 2, 4, 6, 8 and 10 μl of a solution of β-carotene in petroleum ether, b.p. 40–60° (0.12 mg/ml) were spotted (μl syringe) on to a Merck silica gel prepared plate which was then developed with petroleum ether–diethyl ether (99:1). The spots were scanned with the densitometer with and without the $A + 0.4A^2$ correction applied. Results are shown in Fig. 6. The application of the correction gives a linear response up to about 0.8 μg after which it falls off gradually. The whole of the uncorrected curve is non-linear.

The device was also tested on lipid spots stained with iodine vapour. 2, 4, 6, 8 and 10 μg of triolein (Sigma London Chemical Co. Ltd., Kingston-upon-Thames) were applied to a silica gel thin-layer plate which was then developed with petroleum ether–diethyl ether–acetic acid (90:10:1), sprayed lightly with water to reduce iodine uptake by the background and then stained with iodine for 10 min. Staining was
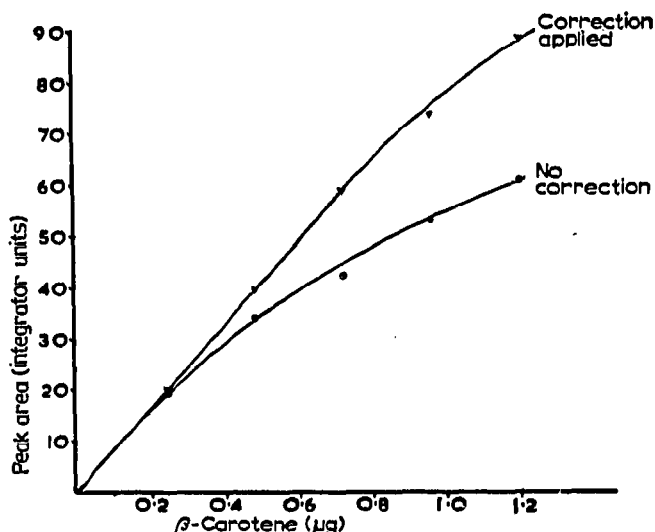
Fig. 6. Calibration graphs for chromatographed β-carotene.

carried out in a large desiccator containing solid iodine and a motor-driven glass and PTFE paddle. The stained plate was covered with a 20 × 20 cm piece of glass (uncoated TLC plate)and the edges sealed with transparent adhesive tape. Densitometry was carried out as above and results are given in Fig. 7. Again the application of the correction produced a linear calibration curve, this time up to 8 μg. The uncorrected curve has a linear portion which does not pass through the origin. This is often found when Lambert–Beer linearity is exceeded with the unmodified machine.
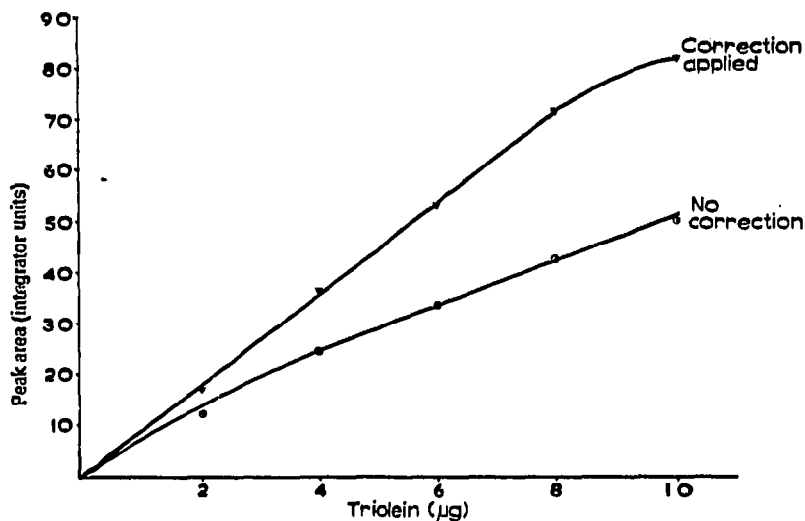

Fig. 7. Calibration graphs for chromatographed triolein stained with iodine vapour.

DISCUSSION

Clearly much improved results were obtained when the correction was applied and this should greatly extend the usefulness of the machine. There now appears no reason why any substance, which is either coloured, or for which a quantitative colour-yielding reaction can be performed, should not be assayed *in situ* on thin-layer chromatograms.

It should be possible to incorporate the device described into the existing electronics of the densitometer utilising the available power supplies. The need to use span setting 5.28 presents variation of the sensitivity of the machine, but this could be overcome by replacing the feedback resistor of operational amplifier C with a variable component.

CONCLUSIONS

A device is described which applies the $A + 0.4A^2$ correction to absorbance measurements made with the Vitatron TLD 100 flying spot densitometer. A comparison of the performance of the machine, with and without the compensator, demonstrated that the compensated machine gave a greatly superior performance, for both uniform and chromatographically produced spots.

The extended range of concentrations over which linear calibration curves may be obtained allows measurements to be made under conditions where background fluctuations are of minor importance. Quantitative densitometry is thus extended to the large number of analyses where the production of very pale spots on an essentially uniform background is not possible.

ACKNOWLEDGEMENT

REFERENCES

1 E. STAHL (Editor), *Thin-Layer Chromatography, A Laboratory Handbook*, Springer-Verlag, 1965.
2 H. KOOPMANS, *Quantitative Thin-Layer Chromatography*, Fisons Scientific Apparatus, Loughborough, Leicestershire, 1971, p. 15.
3 H. J. KOOPMANS, *Application Report TLD 100*, Vitatron Scientific Instruments, p. 2.
4 P. KUBELKA AND F. MUNK, *Z. Tech. Phys.*, 12 (1931) 593.
5 J. GOLDMAN AND R. R. GOODALL, *J. Chromatogr.*, 32 (1968) 24.
6 J. GOLDMAN AND R. R. GOODALL, *J. Chromatogr.*, 40 (1969) 235.
7 *Application Sheet Linear Four Quadrant Multiplier 5595*, Signetics Corporation, Sunnyvale, Calif., 1970.
8 G. B. CLAYTON, *Operational Amplifiers*, Butterworth, London, 1971.