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QUANTITATIVE ANALYSIS ON THIN-LAYER CHROMATOGRAMS

AN INSTRUMENT AND METHOD FOR *IN SITU* ANALYSIS USING ULTRAVIOLET LIGHT

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SUMMARY

The construction and use of an instrument designed to facilitate research into analysis by quantitation of chromatographed zones is described. The instrument determines and records on computer tape the UV transmittance at 0.5 × 0.5 mm intervals in the two dimensions of the thin layer. The application of the general KUBELKA–MUNK transmission equation to the interpretation of the data is described. The flexibility of the method lies with the analyst who may write computer programmes suited to his analytical problems.

INTRODUCTION

A physical theory for absorptiometry on thin-layer chromatograms and its practical application have been described in two earlier papers[1,2]. The intention of this paper is to indicate how the theory and its application may be extended to suit the UV scansion of zones on silica gel adsorbent. We believe that no suitable instrument is at present commercially available, which would obtain and record the relevant data. Thus we had to design and construct an instrument to our own specifications. The principles and operation of this novel instrument are reported here.

There are many important factors to be considered in this class of analysis. Previous workers (see ref. 2, refs. 1–17) have recognised some, but not all, in their treatments. By describing the theory and principles of absorptiometric analysis on thin-layer chromatograms it is to be hoped that analysts will be discouraged from using an *ad hoc* approach and that those persons concerned with the validity of such analyses will find this paper of help in assessing experimental results.

THEORY

The general KUBELKA–MUNK theory for light transmission and reflection in highly scattering media was considered in detail in ref. 1. A simple inverted form was

calculated which approximated to the general equation for transmission over certain useful but limited ranges. In ref. 2 the adaptation of this simple equation was described for use in quantitative analysis of chromatographed zones on adsorbents which have no intrinsic absorption, that is they only scatter light. As an example the analysis of acetone dinitrophenylhydrazone on silica gel, using light of 420 nm wavelength, was described in technical detail using an adapted instrument. In the present paper it is intended to show how the general KUBELKA–MUNK transmission equation can replace our simple version, thus enabling optical analyses to be carried out on adsorbents which do have an intrinsic absorption at the chosen wavelength.

The main extension to the work in ref. 2 is in the method of calculation of C, the concentration of absorbing substance in mg $\times$ cm^{-2} on the thin layer, in the expression

$$M = 1000 \sum_{x_1}^{x_2} \sum_{y_1}^{y_2} C \Delta y \Delta x \qquad (1)$$

where:

M = micrograms of compound in the whole chromatographed zone in the region x_1 to x_2 and y_1 to y_2; Δx and Δy are the intervals in the two dimensions at which estimations of C are made. C is related to the physical parameters K and X through the absorptivity, a, by

$$C = \frac{KX}{2 \cdot 303 \cdot a} \qquad (2)$$

where:

K = coefficient of absorption per unit depth due only to the adsorbed substance.

X = thickness of adsorbent layer.

These two parameters can be related to transmittance, T, in the general KUBELKA–MUNK equation as follows:

$$T = \frac{b}{a \sinh bSX + b \cosh bSX} \qquad (3)$$

where:

$$a = \frac{SX + KX + LX}{SX}$$

$$b = \sqrt{a^2 - 1}$$

and the additional parameters are defined:

S = coefficient of scatter per unit depth;

L = coefficient of absorption per unit depth due only to the substrate.

The introduction of L involves a slight adaptation from the usual KUBELKA–MUNK expression, obtained by replacing K by the term $(K + L)$. The four parameters S, K, L and X may be reduced to three by noting that X only occurs in the

terms SX, KX and LX and never independently. Transmittance in regions of the thin layer that contain no zone may be classed as background transmittance, T_0, which is only related to two independent parameters, SX and LX, as $KX = 0$.

The calculation of KX in eqn. 3 requires a knowledge of the values of T, SX and LX. T is measurable optically. SX and LX require evaluation. By interpolating T_0 from regions of the chromatogram where $KX = 0$ to all other regions, it is possible to remove the independence of SX with respect to LX. Thus, finally, by having previously determined the ratio LX/SX $(= L/S)$, see below, both SX and LX can be calculated. It is reasonable to expect that the ratio L/S at a selected wavelength is constant throughout the life of a large batch of adsorbent, provided suitable precautions are taken. Possible causes of variation include, for example, excessive mechanical treatment which would be expected to change S independently of L, or adsorption of impurities from the air or developing solvents etc., which could change L independently of S. The ratio L/S is simply related to the reflectance of an "infinitely thick" layer, R_∞, through a limiting case of the KUBELKA–MUNK reflection equation:

$$\frac{L}{S} = \frac{(1 - R_\infty)^2}{2R_\infty} \tag{4}$$

Thus from a measurement of R_∞ at the specific wavelength using suitable equipment, L/S is readily obtained via eqn. 4. This ratio can then be used to eliminate L from eqn. 3. Equating K to zero and using the interpolated T_0 value for the data point, it is possible to calculate a sufficiently accurate value for SX by iterative approximation. With the known value of SX in eqn. 3, the only unknown is KX, which is then similarly iteratively determined for the measured T value.

The values of KX for all the points over a region chosen to include the chromatographed zone are summated in accordance with eqns. 1 and 2 which show that

$$M \cdot a = \frac{1000}{2 \cdot 303} \Delta x \Delta y \sum_{x_1}^{x_2} \sum_{y_1}^{y_2} KX \tag{5}$$

The above calculations and summation can be carried out automatically by computer in a manner similar to that described in ref. 2, the result for each chromatographed zone being printed out as micrograms × absorptivity. The conditions necessitated by the application of the theory are unchanged and are fully stated in both refs. 1 and 2.

INSTRUMENTATION

An instrument (Fig. 1) was built specifically to enable the transmittance of silica gel thin layers to be estimated in the ultraviolet at wavelengths down to 240 nm. It had previously been ascertained, using reflectance, that silica gel shows a significant absorption below 280 nm. The effect of this absorption was calculated to decrease the transmittance of a typical silica gel thin layer to 1/1000th of that usually encountered above 280 nm. Consequently the system requires a powerful UV source and a highly sensitive detecting system, with a wide range of response times. A speed appropriate to the conditions can then be selected.

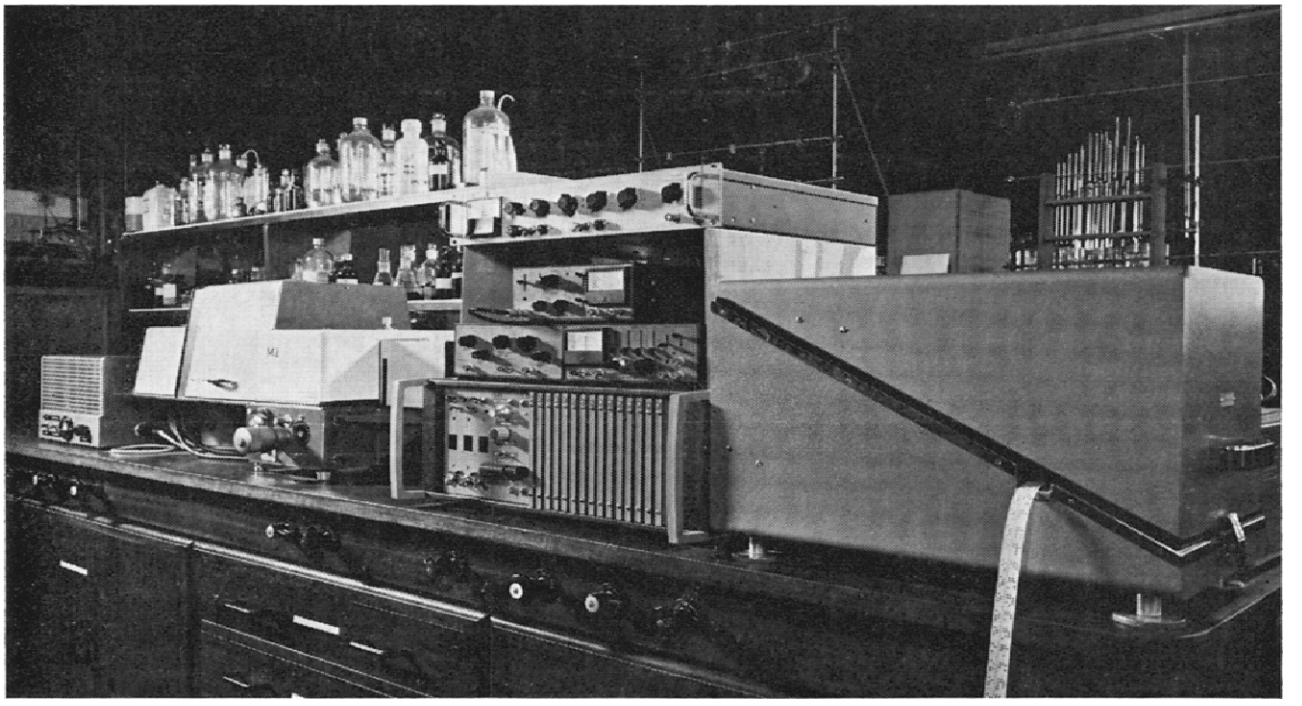

Fig. 1. General view of the complete instrument.

The structure of the instrument is indicated diagrammatically in Fig. 2. The components were obtained in modular form as far as possible, with the exception of the scanning mechanism, lamp housing and chopper. These were manufactured and mounted on the Grubb–Parsons extension plate by R.N. Saxby Ltd. (Bridge Road, Mossley Hill, Liverpool 18, Great Britain). The scanning mechanism (Fig. 3) is the main novel device in the instrument. In addition, the analogue to digital converter/encoder was specially constructed to our requirements by I.C.I. Central Instruments Research Laboratories, Wilton Works, Middlesbrough, Great Britain.

The scanning device drives the chromatogram in 0.5 mm steps in a square-wave motion (Fig. 4) of adjustable amplitude through a small point of illumination. The

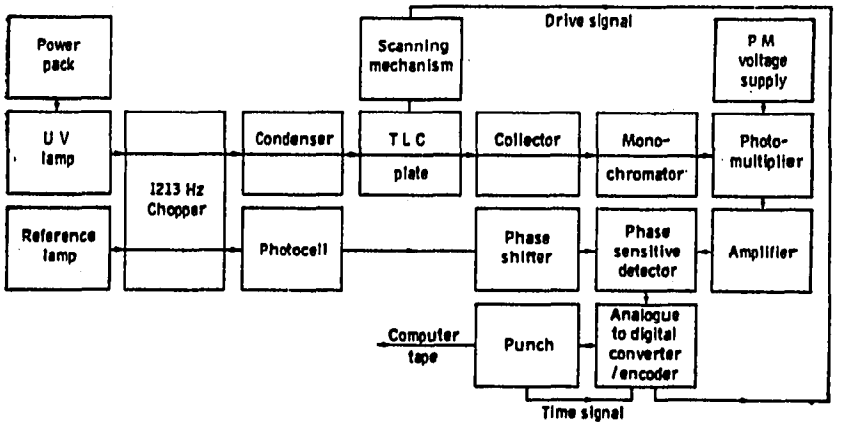

Fig. 2. Diagrammatic representation of the instrument showing the interrelation of the functional components.

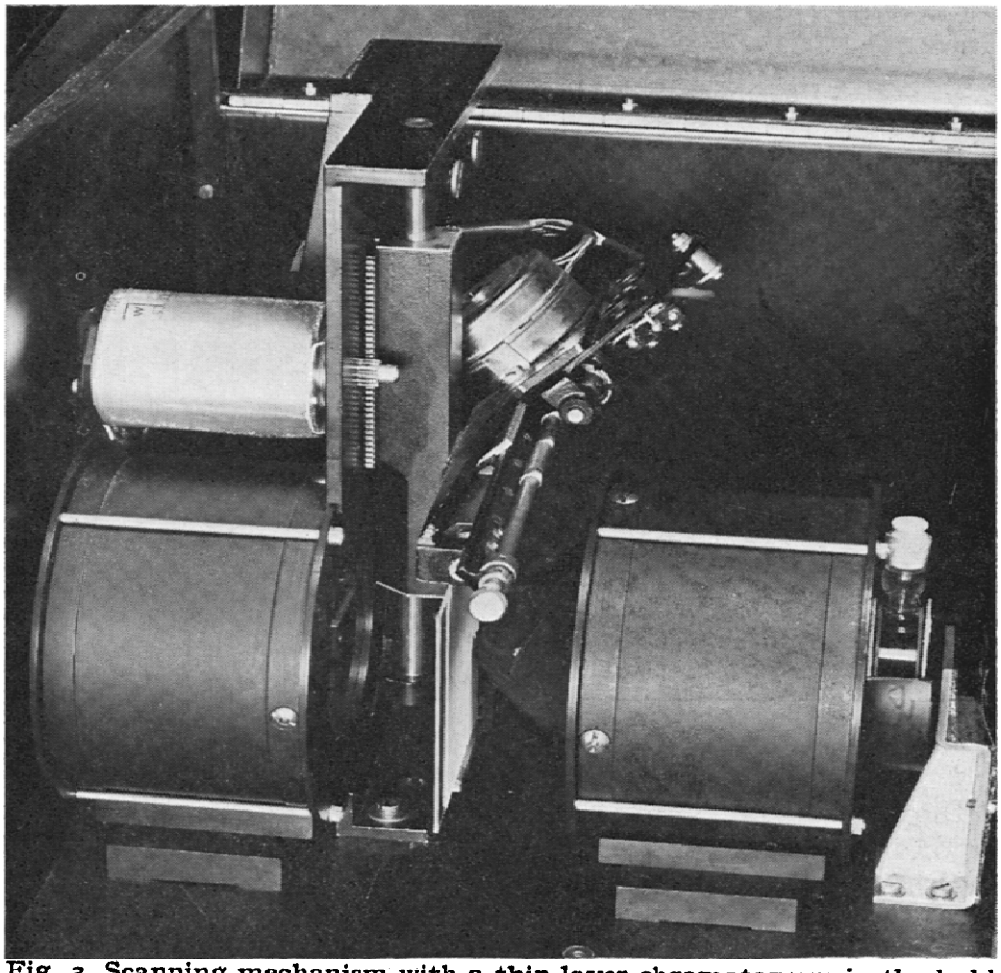

Fig. 3. Scanning mechanism with a thin-layer chromatogram in the holder.

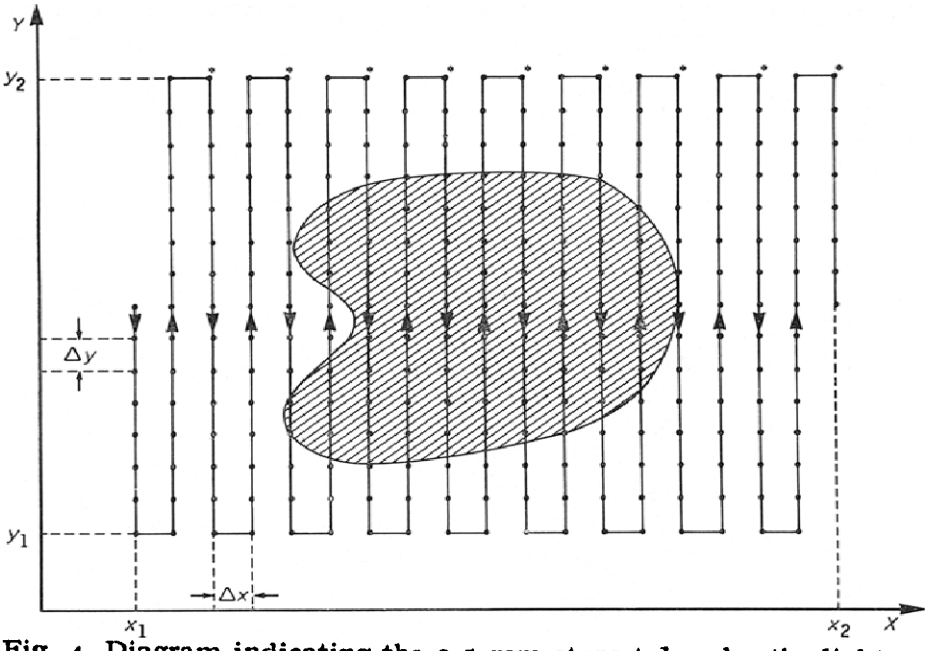

Fig. 4. Diagram indicating the 0.5 mm steps taken by the light spot over the chromatographic zone. End of scan marker occurs at the data points marked • .

time interval between steps is synchronised with the computer tape output, one reading being punched per step. The output data can be realigned into a two dimensional array through an end of scan symbol (a number nine preceding the usual three digit number) occurring at one end only of the chromatogram traverse.

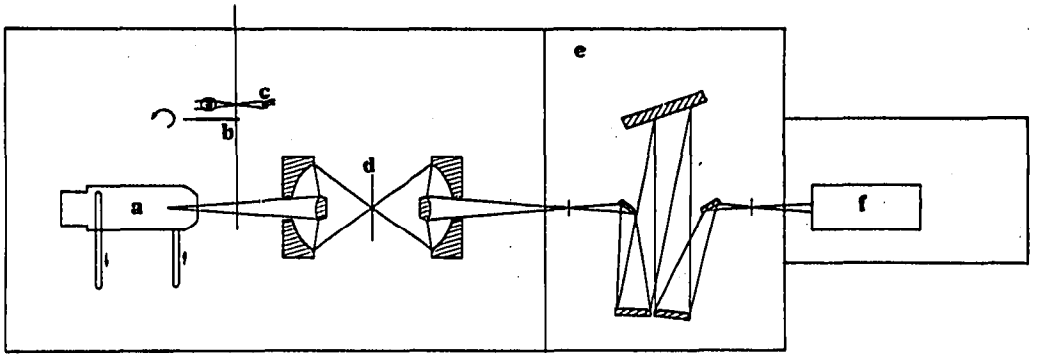

Fig. 5. Diagram of the optical components and light path: a, UV source with cooling water supply indicated; b, chopper disc; c, reference lamp and photocell; d, thin-layer chromatogram; e, grating monochromator; f, photomultiplier.

The light path and optical components are shown in greater detail in Fig. 5. The source is the 1 mm-diameter aperture of a water-cooled deuterium lamp (D150W, Quarzlampen Gesellschaft M.P.H., Hanau, G.F.R.). After passing through the 1213 Hz (26 windows, 2800 r.p.m.) chopper, the light is focussed by the Cassegrain condenser as a 1/5th size image of the source onto the chromatogram, through the Suprasil supporting plate. A proportion of the light transmitted through the thin layer is collected by a twin Cassegrain collector, which focusses a $\times 5$ magnified image of the chromatogram onto the entrance slit of the Grubb–Parsons M2 monochromator. The entrance and exit slits are masked to a height of 2.5 mm and are not opened to wider than 2.5 mm so that the equivalent of no more than a 0.5 $\times$ 0.5 mm region of the chromatogram is observed at any instant. After wavelength selection in the monochromator, the light falls onto the 10-mm-diameter end-window cathode of the 13-stage venetian blind photomultiplier, E.M.I. 6256S. A high voltage d.c. power supply (Model 412B, John Fluke Mfg. Co. Inc., Seattle, Wash., U.S.A.) which will provide over 2000 V, enables the photomultiplier to be used at up to maximum sensitivity. The a.c. output from the photomultiplier is taken into a 3 module low-noise amplifier/phase shifter/phase sensitive detector system (models 450, 421, 411, Brookdeal Electronics Ltd., Myron Place, London, Great Britain) where it is gated with the reference signal to provide a low noise d.c. output of 10 V maximum with variable time constant. The reference signal is obtained from a silicon photovoltaic cell (NSL-703P, National Semiconductors Ltd., 2150 Ward St., Montreal 9, Canada) illuminated by a small tungsten filament lamp through the chopper. The signal voltage in the range 9.99 to 0 V is converted to a 3 digit number (0.01 V per unit) to be punched in Mercury code onto 5 hole computer tape at up to 110 characters/sec on the high speed tape punch (Teletype Corporation, 5555 Touky Avenue, Shakie, Ill., U.S.A.).

The data on the computer tape are proportional to transmittance. They are converted to absolute transmittance by measurement of a reference standard of known transmittance, the two scales being coincident at zero. Autoclaved, dilute india ink is at present used as a neutral reference standard.

PROGRAM

The complexity of the data processing has increased over that described in ref. 2. The computer program now has to carry out two iterative procedures at each data point, as well as align the data, determine the length of each zone and interpolate the background transmittance into that region. Furthermore, the program has been extended to search for a pre-stated number of zones, although these need to be well separated because of the simple nature of the recognition procedure. If necessary, the analysis of less well separated and even overlapping zones could be accomplished by suitable programming. The present program uses data input proportional to transmittance, rather than absorbance as was the case in ref. 2.

The computing time per zone is approximately 45 sec on a KDF 9 computer, only a small increase over the time necessary for the simpler version previously used.

METHOD

The chromatographic procedure is similar to that described in ref. 2, except that 7.5 × 7.5 cm Suprasil plates are used instead of microscope slides. Up to 6 samples may be spotted side by side on each plate. The chromatographed zones may then be scanned transversely if all the analyses are similar and only the same zone is to be quantitated in each case. Alternatively each chromatogram may be scanned in the direction of development if more than one zone is of interest. It is possible to scan the whole thin-layer plate if for instance a two-dimensional chromatogram has been run, or several components in several side by side chromatograms are to be measured. A data tape so obtained would occupy almost the whole of an ordinary sized roll of computer tape of 300 yd. length. The present programme would not be able to organise the data so obtained, although such a program could readily be written. The main novel extension in such a program would be the recognition of the boundaries of each zone in the two dimensions.

In addition to the automatically punched data readings, each data tape has to have the following information punched at the beginning: number of zones scanned; L/S ratio of adsorbent; effective transmittance of a suitable reference standard; measured reading obtained with the reference standard in the light beam with the light also passing through a cleaned portion of the Suprasil plate. This last measurement is repeated immediately after scansion of the chromatogram and is recorded on the end of the data tape.

The L/S ratio, although initially determined by reflectance had to be altered after replication of analyses, using a range of thin-layer scattering powers, because of obvious correlations between variation in results and scattering power. The amount and direction of adjustment to L/S is arrived at by consideration of the relationship displayed in Fig. 3 in ref. 1. For the Merck's Kieselgel G used by us, the graph of L/S *vs.* wavelength (Fig. 6) was determined from the curve calculated from reflectance data, by rescaling below 270 nm to agree with experiment at 238 nm, and similarly adjusted at 298, 335 and 365 nm. The absolute value of L/S becomes more critical as it approaches zero. When using a developing solvent containing a component absorbing light at the wavelength of the analysis, such a component must be com-

pletely removed from the adsorbent, otherwise it would increase the L value independently, and cause erroneous results.

Each reference standard has to be of similar transmittance to the silica gel layer at the operative wavelength. It must also be fairly neutral as otherwise too great a proportion of stray light will be measured particularly at shorter wavelengths where the transmittance is approximately 0.000001. The transmittance value for use by the computer program has to include a factor for the increased collection efficiency when having a non-scattering as distinct from a scattering light path. This factor is difficult to determine experimentally, but geometrically it is estimated to be approximately $\times$ 3 (equivalent to 33 % collection efficiency of scattered light).

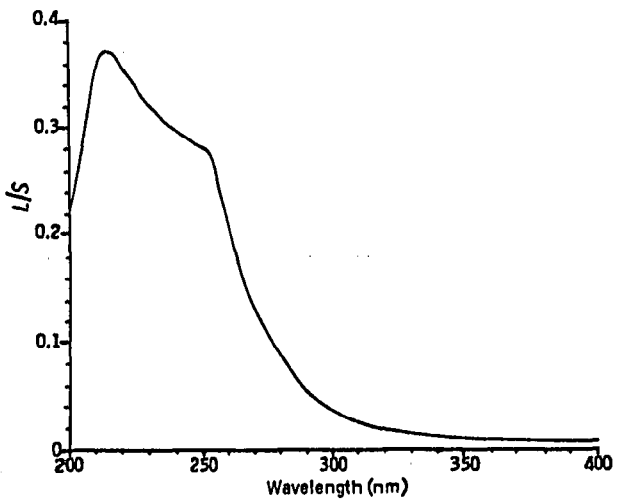

Fig. 6. Spectrum of absorption to scattering ratio of Merck's Kieselgel G.

Because of the relatively high transmittance by silica gel above 280 nm as compared with shorter wavelengths, the stray light passed by the monochromator when working below 260 nm is too great. It is reduced to an acceptable level by having a nickel and cobalt sulphate solution band pass filter (transmits between 230 nm and 350 nm), and a 240 nm interference filter in the light beam for certain portions of the short wavelength UV spectrum.

The scanning rate for analyses above 270 nm is dictated by the maximum rate of the computer tape punch. With 7 characters per data reading this is 63 msec per reading, which is synchronised with each step of the scanning device. As the stepping motors move the chromatogram through 0.5 mm to the next 0.25 mm^2 position, the maximum scanning rate is 2.4 cm^2/min. A detector system time constant of 30 msec is sufficiently short to enable the changes in signal level to be followed at this scanning rate. At lower wavelengths the light level is decreased so that a longer time constant becomes necessary in order to maintain precision. At 238 nm, the attenuation is such that a time constant of 300 msec per reading is required, equivalent to a rate of 0.25 cm^2/min, 10 times slower than maximum. The time to scan six zones side by side typically included in a 1.5 $\times$ 6 cm strip of chromatogram is under 4 min at the maximum rate.

Because of the high scanning rate the extent of photodecomposition by the

high intensity point of UV light focussed on to the sample has not so far been sufficient to cause any concern, although decomposition has been observed with some substances when left stationary for several seconds in the light beam.

So far experimental results have been obtained at various wavelengths down to 238 nm. As yet the problems involved in obtaining exact values of L/S and maintaining a stable standard reference solution have not been solved to the extent of justifying the publication of limits of precision. Also the refinement of the computer program to give a more exact delineation of the zones and the interpolation of background within these regions may still bring about some further improvements in precision.

DISCUSSION AND CONCLUSION

Quantitative analysis *in situ* of chromatographed zones may make possible analyses not previously considered. It can also be used to replace some slower methods. However, it is apparent that *in situ* absorptiometry of thin-layer chromatograms is not an especially simple analytical technique. Preparation of the adsorbent layers has to be done with care so as to prevent the occurrence of defects such as inhomogeneous crystallisation of the gypsum binder, or small holes caused by bubbles. It is essential to establish the absorption to scattering ratio of the adsorbent and to ensure that the latter receives no treatment that may alter this value. Developing solvents, if they absorb at the operative wavelength must be removable easily by gentle warming or vacuum evaporation. Other factors of influence are the quality of the separation required and the capability of the computer program to recognise zones. Thus all aspects of the method from chromatography to data processing, contribute to the overall precision.

We hope that the instrumentation and experimental conditions described here and in refs. 1 and 2, and the theoretical conditions which they were designed to fulfil, will provide analysts with a clearer view of the problems associated with absorptiometry on thin-layer chromatograms and the solutions to those problems. They should make possible a more critical assessment of devices manufactured to carry out densitometry of chromatograms and the methods of analysis in which they are used.

Because this instrument is designed specifically to collect all the necessary data precisely and rapidly, it is extremely flexible in its use. That is, the apparatus should enable a variety of types of analysis to be developed, each treating the data in a manner described by its particular program. In this sense it may be more correctly described as an instrument for research rather than for routine applications.

ACKNOWLEDGEMENT

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REFERENCES

1 J. GOLDMAN AND R. R. GOODALL, *J. Chromatog.*, 32 (1968) 24.
2 J. GOLDMAN AND R. R. GOODALL, *J. Chromatog.*, 40 (1969) 345.