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SIGNIFICANT FACTORS IN THE PREPARATION AND SCANNING OF THIN-LAYER CHROMATOGRAMS, PARTICULARLY BY TRANSMISSION IN THE ULTRAVIOLET

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SUMMARY

Criteria for the selection of adsorbents, for layer preparation, for loading, and for the choice of developing solvents are discussed. From a knowledge of the absorption/scattering (*i.e.*, L/S) ratios of common adsorbents in the UV, of the emission spectrum of the source, and of the absorption spectrum of the test substance in solution, a rational choice of scanning wavelength is possible.

A favourable signal-to-noise ratio when scanning below 280 nm depends upon the adjustment of optical factors so as to maximise the light flux, in this case the source, condenser geometry, and a reduction of layer thickness. It is well established that the most significant noise arises from the particulate nature of thin layers and the irregularities associated with them. An amplifier offering a variable signal-averaging period has some advantage.

Recordings should indicate variation in layer thickness so that a correction of the Kubelka–Munk form may be applied to the results.

INTRODUCTION

The preparation of small chromatograms on plates of 75 mm × 75 mm, scanning and processing the data obtained by a "flying spot" technique, has been described in previous papers[1–3]. Having established a technique for scanning in the visible by transmitted light, the use of synthetic fused silica support plates in conjunction with a deuterium source, beam condensers, and a suitable monochromator, enabled the scope of the work to be extended into the UV down to 230 nm, and beyond in unpublished experiments. In spite of additional complications as indicated below, the measurement of UV absorbance on thin-media chromatograms is a desirable end, for example in the assay of pharmaceutically important substances.

The present communication is a review of factors which the authors have found to be significant and some of these can now be considered in more detail than in previous papers. Many of the conclusions are relevant to other procedures in this field, *e.g.*, on the normal size of thin-layer plates, or when scanning by reflection.

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ADSORBENT LAYER AND EFFECT OF VARYING THICKNESS

Before use, a prepared layer of any adsorbent should be scrutinised carefully in a strong diffuse light. The appearance by reflected light can vary from dull grey as in Kieselguhr to brilliant white as in magnesium carbonate. Cellulose and silica gel G layers are translucent. Local variations in thickness or "pin holes" where bubbles have persisted in the slurry are more easily observed as changes in transmission when looking through the plate. Plates in which such defects are observed are not suitable for scanning. The transmissions of cellulose and silica gel G layers are relatively much greater than alumina, magnesium carbonate, etc. in both the visible and UV regions.

Many theoretical and experimental difficulties connected with the adsorbent arise in the quantitative assessment of thin-layer chromatograms by optical methods and these become more acute when the UV region is considered. In solution densitometry the contribution from the background can be defined in a single experimental variable, but in thin layers the background effect arises from two non-separable variables, scattering (S) and absorbtion (L, previously named as K, but K is now assigned solely to the solute absorbance, *cf.* ref. 1.) The ratio L/S can be calculated from experimental determinations of diffuse infinite reflectance

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

The ratio is assumed to remain unchanged in a particular batch of adsorbent at a particular wavelength, but will show a marked increase as it is plotted into the UV region. Kortüm *et al.*[4] published reflection spectra of various white standards and showed that in alumina and silica gel a decline in reflection started below 300 nm. Later Goodall[2] published approximate spectra of some common adsorbents in the UV, the ordinate being L/S and alternatively $-\log T$. The spectrum of silica gel G is of particular interest and shows that the level of the ordinate increases rapidly below 280 nm, presumably due to an increase in L. In spite of this the observed transmission of the gel is much higher than that of other adsorbents.

Local variations in layer thickness across the plate will affect quantitative evaluation by direct densitometry, whether in the transmission or the reflection mode. A simple exponential term to allow for observed changes in thickness was proposed by Goldman and Goodall[5] when interpreting data obtained by transmission in the visible region. As far as the author is aware, published differential scanning methods are not designed to take account of such local variations. The effect becomes increasingly significant when operating in the UV.

OPTIMUM LOAD OF SOLUTE

It is usually possible to control the initial diameter of the spot by applying the load from a solvent of appropriate polarity. For effective separations the initial diameter should be small, but subject to the following limit. Where there is to be densitometric measurement of the developed zone of interest, the range of absorbance across this zone should not exceed 1 absorbance unit above the background. Dose–response relationships tend to flatten off above this range, and when the background absorbance already exceeds 1, as in the UV, errors of 10^{-4} in the determination of transmission become significant. If the intrinsic absorptivity of the test substance in solution is small, it can be recalled that one of the few advantages of densitometry in scattering layers is that the absorbance will be increased by a factor of 3 or more due to the hyperchromic effect in the substrate layer. It is the author's experience that photoelectric methods of detection coupled with recording are more sensitive and, of course, less subjective than corresponding colour-forming detection procedures viewed by reflection.

In practice, the analyst is frequently required to separate and determine minor proportions in admixture with an analogous major constituent. In such cases an attempt will probably be made to separate the mixture chromatographically without preliminary fractionation. The limit to the amount in the load may then be dictated by the ability of the developing solvent to pass through the high concentration on the starting line and thereafter to effect a separation of the components in question.

CHOICE OF SOLVENT FOR DEVELOPMENT

Thin-layer chromatography is a very simple technique which normally imposes the limitation that separation be effected by the rise of the developer to the top of the plate. In practice R_F values of the order of 0.5 are typical. Extension of the development is less straightforward than in the techniques of paper or column chromatography, where with no extra elaboration, the throughput of flowing phase can be much greater. Consequently, effective separations are possible on the paper or the column when the R_F values are of lower order, and the R_F differences less than those required for thin-layer operation.

When the polarity of a single solvent is too high to effect a required separation on the plate, it is customary to try solvent mixtures where the most polar component may act as a displacement developer (*cf.* ref. 6). The use of component solvents of differing vapour pressure (non-isobaric systems, see below) and of differing affinity for the static phase, may cause the appearance of a second front at which, fortuitously, a desired separation takes place. Isobaric solvent mixtures have been employed as developers by Roeder *et al.*[7]. If this approach can be more widely applied, it will be a major advance towards a reproduceable technique for interlaboratory comparisons. However, the second front phenomenon may be the result of another interaction, *i.e.*, differential adsorption of the components in the developing mixture.

FACTORS AFFECTING THE MEASUREMENT OF TRANSMISSION IN THE UV AND NOTES ON THE AUTHOR'S APPARATUS

With the chromatogram completed and residual solvent removed, the significant factors which affect objective measurement of the zone may now be considered. If the compound in question has a light-absorbtion in the visible part of the spectrum and assuming access to a densitometer with wavelength selection, the attainment of a favourable signal-to-noise ratio —preferably a maximum of 1 absorbance unit above background— should present little difficulty either in the reflection or the transmission mode on silica gel G plates. When the compound shows no light absorbtion either in the visible or the UV spectrum, colour-forming reagents are frequently applied. The use of iodine vapour in this context is always worth considering as it readily penetrates into the solute zones and has little affinity for silica gel G. In a stirred glass vessel one can continue the treatment until the test and standards appear to have attained a convenient range of optical density. It is then necessary to clamp on a cover-glass and seal the edges with wax or tape. The iodine adducts exhibit a high light absorbtion maximum around 350 nm, which opens the possibility of a very sensitive estimation. However, there is a tendency for the iodine to evaporate from the zones, at a rate dependent on temperature and on the length of the path from each zone to the periphery of the plate. Other workers have published a comparable technique (*e.g.*, Owen[8]), but they do not comment on the rate of loss of iodine, which presumably is related to the class of compound. This author's experience is that the iodine merely dissolves in the zone.

Many compounds of both academic and commercial importance show absorbtion spectra in the UV and it is in this region that the densitometry of thin layers becomes difficult because of the steep rise in the apparent light absorbtion of the media in common use[2]. Magnesium carbonate, however, should be more suitable than silica gel G for reflection work at these lower wavelengths. Goldman and Goodall[5] have shown why, according to the Kubelka–Munk equation, the transmission mode can give rise to a higher response–dose curve than the reflection mode, and for this reason they decided to measure transmission in the UV. A synthetic fused silica base-plate of high optical transmission is necessary and for economic reasons the size was fixed at 75 mm × 75 mm. On these plates a method of miniature thin-layer chromatographic procedure has been developed.

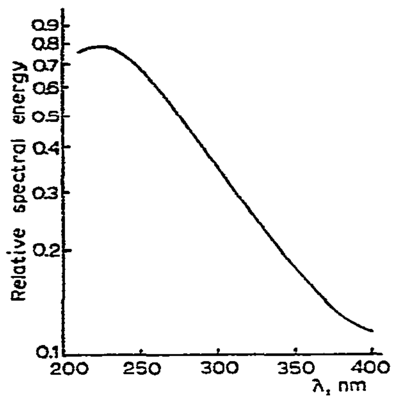
The optical system employed by the author was suggested by the late Mr. Fichter, then of Hilger and Watts, who demonstrated privately in a preliminary way that the transmission of UV light through the silica gel G plates at such low levels of $\%T$ as 0.01 could probably be measured to a precision of $\pm$ 0.0001 with the use of a powerful deuterium source and at least one beam condenser. The completed apparatus was first described in the Journal of Chromatography in conjunction with a data-processing program (Goldman and Goodall[1]), then with alternative circuitry for analogue recording (Goodall[3]). However, there are some aspects of the optical and electronic design which may be of general interest and hitherto have not been discussed.

Light flux from the deuterium source

The emission from the deuterium lamp although not of very high intensity rises to a peak at about 250 nm and this tends to compensate for the falling transmission of the silica gel G and the declining response of the photomultiplier cathode (*cf.* Figs. 1 and 2). The increasing effect of stray light in a monochromator when low orders of transmission are to be measured is well known. In our apparatus a 10-mm cell containing a concentrated nickel–cobalt salt solution showing a transmission band in the range 230–350 nm was inserted before the first beam-condenser to filter out the stray light components in the visible. Below 230 nm some alternative compound filter would be preferable.

More intense sources such as the xenon discharge or mercury vapour lamps also emit in the visible and presumably a high degree of filtration of the stray light is required when such sources are contemplated for use in the scanning of thin-layer chromatograms in the UV.

In practice, it was found that when the 125-W water-cooled deuterium lamp



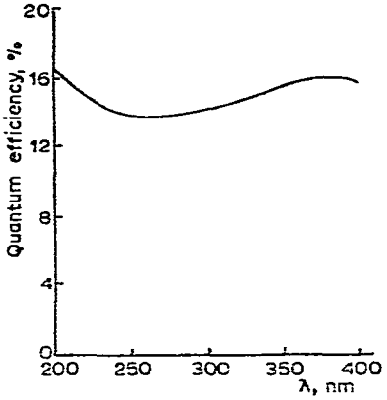


Fig. 1. Approximate spectral energy of the deuterium lamp in relative terms. (Data supplied by Quarzlampen Gesellschaft M.P.H., Hanau, G.F.R.).

Fig. 2. Spectral response of photomultiplier E.M.I. 6256S. (Reproduced from manufacturer's data, Electrical and Musical Industries Ltd., Hayes, Middlesex, Great Britain).

with a 1-mm aperture in the envelope was focused by the first beam condenser on to the layer, there resulted a lower response than that from a comparable arrangement with a 25-W air-cooled lamp. Thus the source was changed to a regular stock lamp and power-pack as used for solution spectrophotometry. This unexpected result was observed by attaching a cathode-ray oscillograph directly to the photomultiplier output and positioning it in the line of sight when adjusting the first beam-condenser. It was clear that the image of the aperture from the water-cooled lamp was not condensed down to as small an area (nominally 0.25-mm diameter), nor was the focusing as precise as with the alternative lamp.

Photomultiplier

In 1969 an end-window photomultiplier with a cathode coating suitable for UV work and an aperture of 10 mm was selected. After helpful discussion with the Technical Service Department of the makers (E.M.I., Hayes, Middlesex, Great Britain) it was anticipated that the output from the 13-stage Venetian-blind Type 6256S would be above the level at which shot-noise, etc., becomes troublesome. It was noted that signal amplification by increasing the photomultiplier voltage, rather than by a following amplifier, introduces less noise.

Optimising signal-to-noise ratio

With experience, especially below 250 nm, it became clear that the main cause of signal variation is that due to the particulate nature of the layer. At the lower wavelengths, as in solution spectrophotometry, the signal-to-noise ratio is better maintained by opening the slit of the monochromator rather than by increasing the photomultiplier voltage, or by boosting the subsequent amplification. Reduction of layer thickness from 0.25 to 0.14 mm is the alternative and preferred way of maintaining the light-flux for a favourable signal-to-noise ratio.

Amplification system

A closely stabilised power supply to the lamp and high voltage to the photomultiplier are essential in order to avoid the exponential effects of voltage variation. The beam is chopped at 1213 Hz. and the signal is gated at this frequency in the phase-sensitive detection system (Goldman and Goodall[1]). The low noise amplifier has an input filter for the rejection of low frequencies and the band width of the signal to be amplified is 1213 ± 50 Hz, which is a familiar way of excluding interference, for example from mains-frequency ripple. As a further refinement the band-width can be reduced —at some sacrifice of speed— by a time-averaging option. The above electronic units are commercially available items and have been assembled as shown in Fig. 2 of ref. 1.

One of the hazards of measuring transmission in the above system is the risk of damage to the photomultiplier cathode by accidental exposure to an excessive light flux. Hence a safety amplifier and cut-out was designed and constructed for the author by the Electronic Services Department of I.C.I. Pharmaceuticals Division. The cut-out is adjusted to trip when the digital display rises above 9.98 V.

Finally, a recent modification is the optional circuit for analogue recording, where the novel features are a pulse generator to provide an alternative source of stepping-signals and a logarithmic amplifier. A further improvement would be the addition of an integrating circuit. Results obtained by analogue recording in this way have been reported and discussed in a recent paper (Goodall[3]).

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