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CONSTRUCTION AND EVALUATION OF A SCANNING THIN-LAYER SINGLE-DISC MULTI-SLOT PHOSPHORIMETER

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SUMMARY

A device for the direct measurement of phosphorescence from separated components on thin-layer chromatograms is described. An expression relating the dimensions of the single-disc phosphoroscope used to the measured phosphorescence intensity is given. In order to demonstrate the potential application of the device, sulphamerazine, sulphamethazine and sulphadiazine have been separated by thin-layer chromatography and estimated qualitatively and quantitatively.

INTRODUCTION

Since the introduction of phosphorimetry as an analytical technique by Kiers *et al.*[1], numerous workers have shown phosphorimetric measurements to be extremely sensitive and selective. The present paper describes a further improvement in selectivity by the combination of thin-layer chromatography (TLC) with phosphorimetry. Applications of the combined technique have included the determination of *p*-nitrophenol in urine[2], biphenyl in oranges[3] and alkaloids in tobacco[4]. In each of these instances, however, the sample had to be eluted from the thin-layer plate into ethanol prior to measurement. Phosphorescence spectra at 77°K have been observed from materials adsorbed on a variety of supports including silica, alumina, paper, asbestos and glass fibres after immersion in liquid nitrogen in the conventional Dewar flask[5–8]. Despite this, phosphorimetry has yet to achieve general acceptance among analysts partly because of difficulties with the currently commercially available equipment.

The present paper describes a simple device capable of scanning TLC strips directly. Sample strips are cooled by conduction, an advantage over techniques using a Dewar flask in that the number of layers of silica through which radiation must

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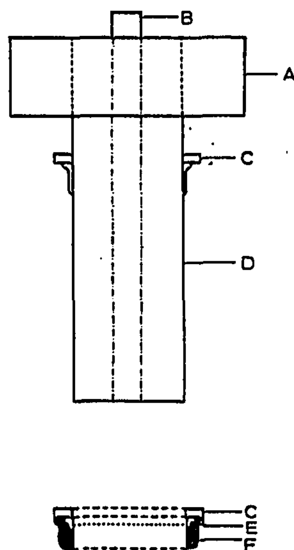
pass is reduced and the absence of liquid nitrogen in the light path reduces scatter. Qualitative and semi-quantitative measurements can easily be made with this device on individual components of complex mixtures such as those encountered in the study of drugs in biological fluids.

EXPERIMENTAL

Thin-layer phosphorimeter

The device was designed for use in a Baird Atomic SF 100E spectrofluorimeter, but with minor modification it could be adapted to most other commercial fluorimeters. It consists of two discrete parts, the drum sample holder (Fig. 1) and the single-disc three-slot phosphoroscope[9–12] (Fig. 2). The sample holder is a hollow copper cylinder closed at its lower end, with a canopy attached at the top as light trap and a hollow rod down the centre of the drum the lower end of which is located by a pivot in the phosphoroscope assembly. The upper end of the hollow rod is connected to a small variable-speed electric motor used to rotate the drum. On the outside of the cylinder close to the top is a brass collar, with an 'O' ring seal and a ring of insulating material. The 'O' ring forms an air-tight seal with the phosphoroscope and allows the sample drum to rotate freely at liquid nitrogen temperature whilst the insulating ring reduces cooling of the rest of the phosphoroscope.

Fig. 2 shows the combined single-disc phosphoroscope and air-tight compart-



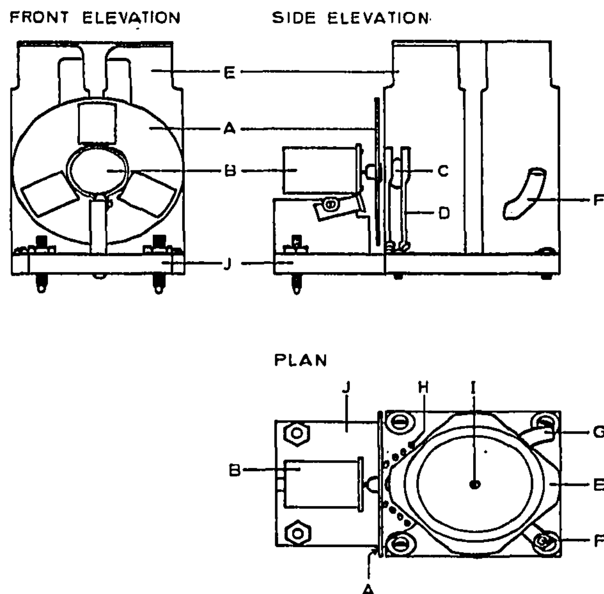


Fig. 1. Copper drum sample device. A = Light trap; B = hollow central rod; C = brass collar; D = outer copper cylinder; E = "O" ring seal; F = insulating ring.

Fig. 2. Single-disc phosphoroscope assembly. A = single disc; B = variable speed motor; C = silica windows; D = slit mountings; E = sample drum compartment; F = nitrogen inlet; G = nitrogen vent; H = upward nitrogen inlet flow; L = sample drum locating pivot; J = base plate.

ment into which the sample holder is placed. The single-disc phosphoroscope is a thin copper disc, painted matt black, with three equally spaced slots, the dimensions of which were determined from an equation given later in the text. The phosphoroscope is driven by a small variable-speed motor mounted in front of the sample drum compartment. To allow the entrance of excitation radiation and exit of emitted radiation two silica windows are placed at right angles in the front of the sample drum compartment. The two vertical slits mounted in front of these windows provide the facility to change resolution. A series of small holes in the base plate allow a constant stream of dry nitrogen to be blown across the silica window to prevent condensation of moisture. A stream of pure dry nitrogen is introduced into the sample drum compartment from an inlet pipe at the rear of the assembly, and vented by an outlet pipe mounted similarly to prevent oxygen quenching of the excited molecules in the triplet state and to prevent condensation of water within the sample drum compartment.

The phosphoroscope assembly is placed in the instrument compartment using locating screws. To facilitate location of the sample drum and allow topping up with liquid nitrogen, a hole is cut in the top of the spectrofluorimeter sample compartment door large enough to allow the phosphorimeter sample drum to pass through into the sample compartment. A small cylinder is fixed round the hole so that when the sample drum is in position the canopy forms an overlapping light trap, ensuring that the instrument sample compartment is light-tight.

To scan a thin-layer strip, it is attached to the lower end of the copper drum (by means of elastic bands) which is then located in the air-tight compartment through the hole in the top of the spectrofluorimeter sample compartment door. Liquid nitrogen is poured into the sample drum and the thin layer cooled by conduction. The sample strip is then irradiated and scanned by slow rotation of the drum.

The single-disc phosphoroscope allows the isolation and observation of phosphorescence because of the prolonged life time of the emission. Excitation radiation passes through the rotating disc and fixed excitation slit to irradiate the sample. As the excitation radiation is cut off by the disc, the fluorescence decays rapidly and only the long-lived phosphorescence remains. The phosphorescence is then allowed to reach the emission monochromator and photodetector as the disc covering the emission slit opens. A further important and useful feature of the phosphoroscope is its minimization of interference from scattered light.

When a luminescent component on the strip is at the focal point of the optical system, a signal is registered in the photodetector circuit. The drum can be stopped at any position and the spectral characteristics of each component measured; the position of the drum relative to its starting position is used to calculate the R_F value of the component. The amplitude of the signal is a quantitative measure of the component. Phosphorescent lifetimes are measured from the rate of decay of the luminescent signal after cut-off of the exciting radiation.

Thin-layer chromatography

2 μl of an ethanolic solution containing 2 μg of sulphadiazine, sulphamerazine and sulphamethazine were chromatographed on an aluminium-backed silica gel thin-layer plate according to the method of Brunner[13]. The plate was developed for 1 h and the separated components examined using the thin-layer phosphorimeter.

Quantitation was studied by applying replicate spots of samples on the same

and also on different thin-layer strips. Phosphorescence from silica gel and aluminium oxide thin layers (with and without fluorescent indicator), filter paper and cellulose acetate were studied by direct application of samples and also after chromatographic development.

Influence of phosphoroscope design on phosphorescence intensity

O'Haver and Winefordner[14] describe the relationship between phosphoroscope design and measured phosphorescence intensity for the conventional rotating cylinder and Becquerel, twin-disc phosphoroscopes. The characteristics of a single-disc multi-slot phosphoroscope such as that described here have not apparently been previously discussed.

The ability of the phosphoroscope to resolve short- and long-lived emissions is directly related to four time periods which are a function of phosphoroscope design and speed of rotation, namely: (i) the cycle time, t_c, the time for one complete cycle of excitation and observation; (ii) the exposure time, t_e, the time during which the sample is excited or observed; (iii) the transit time, t_t, the time taken for the rotating aperture to open fully; and (iv) the delay time, t_d, the time between the end of one excitation period and the beginning of the next observation period. The magnitude of these time periods, relative to the lifetime, τ, of the luminescent species, influences the measured luminescence flux.

The ratio of P_p, the average d.c. photocurrent observed using a phosphoroscope, to P_c, that which would be observed (*i.e.* continuous) without a phosphoroscope is given by α (if prompt fluorescence and scattered light were not to interfere)[14].

$$\alpha = \frac{P_p}{P_c} = \frac{\tau}{t_c} \cdot \frac{e^{-t_D/\tau}(1 - e^{-t_E/\tau})^2}{1 - e^{-t_c/\tau}}$$

where $t_E = t_e + t_t$ and $t_D = t_d + t_t$.

It is convenient to calculate times relative to t_c because such values are independent of the speed of rotation, Θ (rpm), of the phosphoroscope shutter. Relative times are denoted with a prime superscript. Intensities relative to the absolute intensity, I_{p0}, under steady conditions are also denoted by prime superscripts. Thus in relative variables the above equation becomes

$$\alpha' = \frac{P_p'}{P_c'} = \frac{\tau' e^{-t'_D/\tau'}(1 - e^{-t'_E/\tau'})^2}{1 - e^{-1/\tau'}}$$

where $t_D' = t_D/t_c$, $t_E' = t_E/t_c$ and $\tau' = \tau/t_c$.

Characteristics of the single-disc multi-slot phosphoroscope

The dimensions of both the slots in the rotating disc and the fixed slits in the cell holder are critical. The slot in the rotating disc is centered on the fixed slit in the excitation or emission system of the spectrofluorimeter (Fig. 3). If the centre of the slot in the rotating disc and the fixed slits in the cell holder subtend angles x^0 and y^0, respectively, then the dimensions of the disc can be related by simple geometry to the time functions in the equation for the calculation of α' and are given below.

Transit time, t_t (for the case $y^0 > x^0$). The transit time is the time taken for

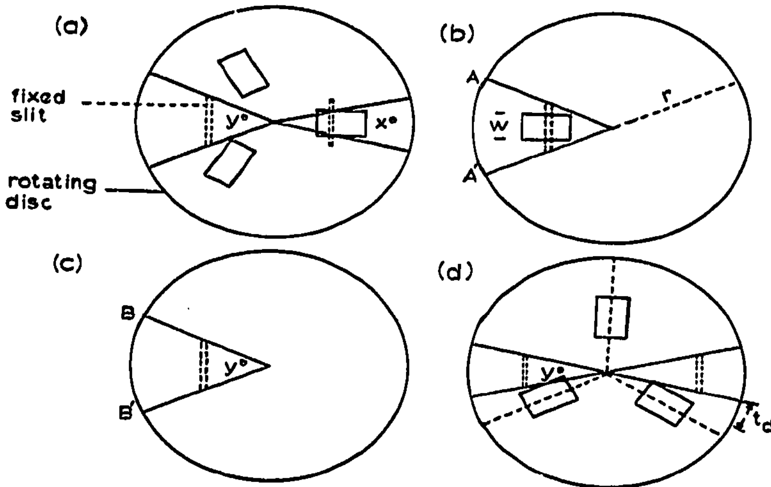


Fig. 3. Schematic representation of time functions of single-disc phosphoroscope. (a) General arrangement of single-disc phosphoroscope; (b) determination of transit time, t_t; (c) determination of exposure time, t_e; (d) determination of dark time, t_d.

the fixed slit to become completely illuminated and is the time for the fixed point A to move to A′.

$$\text{Hence } t_t = \frac{x}{6\Theta}$$

Exposure time, t_e. The exposure time is the time taken for the leading edge of the rotating disc's slot to pass both top and bottom edges of the fixed slit, in which time a point, B, on the edge of the disc will have travelled a distance B–B′ minus the equivalent distance at the circumference of the width of the slit in the rotating disc

$$\text{Hence } t_e = \frac{(y - x)}{6\Theta}$$

Dark time, t_d. The total dark time is that for the trailing edge of one slit in the rotating disc to leave the lower edge of the stationary slit and be replaced by the leading edge of the next slit in the rotating disc.

$$\text{Hence } t_d = \frac{60 - (x + y)}{6\Theta}$$

Therefore, for phosphorescence alone to be observed $x^0 + y^0 \leqslant 60°$, since the maximum slit widths for the rotating shutter and fixed slits which will give $t_d = 0$ are given when $x^0 + y^0 = 60°$.

Cycle time, t_c. The cycle time for a rotating disc with three apertures is

$$t_c = \frac{\text{time for 1 complete revolution}}{3} = \frac{20}{\Theta}$$

For the case $y^0 > x^0$ the times relative to t_c are

$$t_t' = \frac{x}{120},\ t_d' = \frac{60 - (x + y)}{120},\ t_D' = \frac{60 - y}{120},\ t_e' = \frac{y - x}{120},\ \text{and}\ t_E' = \frac{y}{120}$$

and for the case $x^0 > y^0$

$$t_t' = \frac{y}{120},\ t_d' = \frac{60 - (x + y)}{120},\ t_D' = \frac{60 - x}{120},\ t_e' = \frac{x - y}{120},\ \text{and}\ t_E' = \frac{x}{120}$$

In the single disc used in the present experiments $x^0 > y^0$. The relative response of the phosphoroscope as a function of phosphorescent lifetime of samples was calculated using the equation for α'. Plots of α' against τ' for discs of different disc slot dimensions are shown in Fig. 4. In the α'–τ' curve α', as indicated earlier, is proportional to the reading obtained by an integrating d.c. photometer. For values of $\tau' < 10$ and a speed of rotation of 7,000 rpm, $\tau = \tau' \times t_c = 200/7000 \approx 0.03$ sec and the luminescent intensity observed will be minimal. For values $\tau' > 100$ (*i.e.* $\tau > 0.3$ sec) the measured intensity is independent of speed of rotation of the phosphoroscope. Hence, the response will only show variations with speed of rotation for samples with lifetimes in the range 0.3–0.03 sec. However, if the response is a function of shutter speed then the lifetime of the sample may be calculated from intensity readings taken at two different shutter speeds. For each shutter speed a value for τ' is obtained

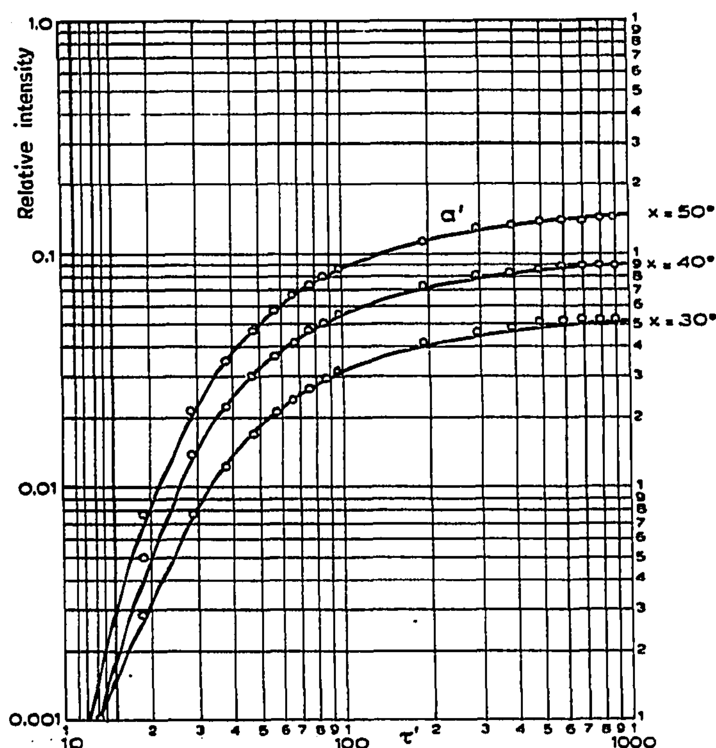


Fig. 4. Relative phosphoroscope response as a function of sample lifetime.

by dividing the unknown value of the sample by t_c as determined for that shutter speed. Each of these values can then be substituted into the equation for a' together with the other relative times and the two resulting simultaneous equations then solved for τ.

RESULTS

A phosphorogram obtained by scanning the sulphonamide thin-layer chromatogram is shown in Fig. 5. The emission characteristics of these compounds were similar to those observed earlier in ethanediol[15]. The reproducibility of the system is indicated by the similarity of the duplicate scans. The use of the phosphoroscope reduces incident light scatter, compared to total luminescence scans, with a resultant reduction in the background signal. There was a reproducible return to a constant baseline between samples, enabling quantitative integration to be performed. Luminescence characteristics and R_F values for each component of the mixture are given in Table I.

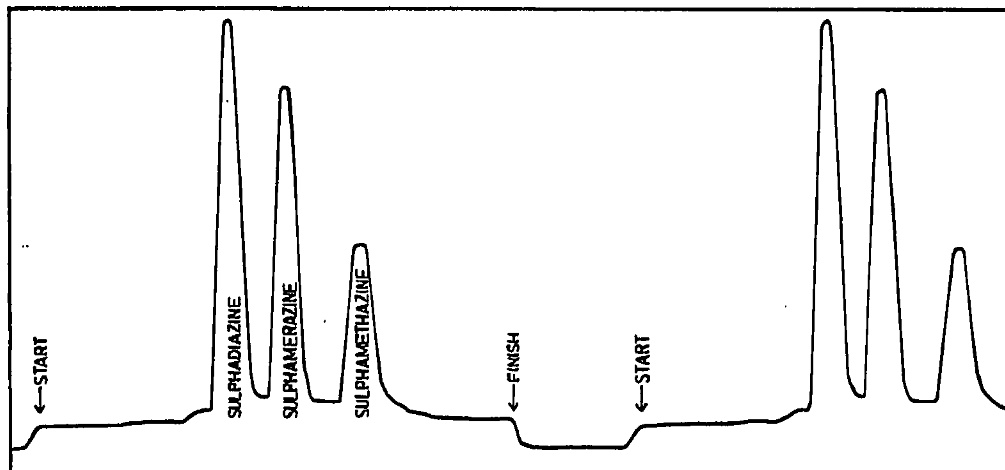


Fig. 5. Phosphorogram of three sulphonamides.

Fluorescence and phosphorescence have also been observed from samples applied to chromatography paper, cellulose acetate, aluminium oxide and silica gel layers. It was also found possible to locate non-luminescent compounds after removal of the phosphorimeter disc by using thin-layer plates containing a fluorescent indi-

TABLE I

THIN-LAYER PHOSPHORIMETRY OF SULPHONAMIDES

Compound	R_F	λ_a *(nm)*	λ_p *(nm)*	τ *(sec)*
Sulphadiazine	0.41	290	420	0.7
Sulphamerazine	0.52	290	420	0.8
Sulphamethazine	0.68	290	420	0.8

cator. The background phosphorescence of the different support materials investigated was found to vary, being minimal for cellulose acetate. Aluminium oxide and silica gel layers were found to give relatively high phosphorescent backgrounds. However, these could be considerably reduced by washing in an appropriate solvent prior to chromatography. Paper was found to have the highest phosphorescent background which is probably due to the presence of organic binders and whiteners in the fibres.

The intrinsic reproducibility of the system was shown by repeated scanning of a single chromatographic zone for up to 1 h, 30 scans. The coefficient of variation of the intensity was found to be less than 2% and frosting in the sample compartment was absent. However, the best reproducibility that could be achieved from replicate samples on a single chromatographic plate was 8%. This was due to the problems of sample application and development inherent in the chromatographic technique. The application of the samples in the form of rectangular zones rather than spots was found to yield more consistent quantitative results. However, the best standard deviation obtained was 7%.

To examine direct quantitation, a series of standard sulphonamide solutions were run on a plate together with unknowns. Results from spot applications were only accurate to 10% and the application of samples as zones rather than spots was not entirely satisfactory since this entailed scanning several spots on the same plate at right angles to the desired direction of scanning. Neither was the application of samples directly on to thin layers in order to produce calibration curves entirely satisfactory because of the widely acknowledged intrinsic inaccuracies in TLC plate sample application, including different degrees of sample spread on the thin layers.

CONCLUSIONS

The combined technique of TLC and phosphorimetry is considered to have great potential for use in pollution control, pharmaceutical and biological analysis and other fields where small quantities are required to be estimated in complex mixtures. Low-temperature fluorescence analysis can also be carried out.

Electrophoretic separations can also be examined by this technique, enabling separations of complex mixtures such as plasma samples containing a drug and its metabolites to be studied. The versatility of the method can also be enhanced by performing multiple scans at different wavelengths of excitation and emission and by careful selection of the disc phosphoroscope scanning speed. Compounds with similar R_F characteristics can be resolved. Furthermore, chemical or physical (*e.g.* pH or solvent) modification reactions on particular zones of the chromatogram can be used to significantly extend the sensitivity and selectivity of many of these analyses. Results from studies of warfarin and its metabolites will be reported shortly.

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