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DENSITOMETRY OF MINIATURE THIN-LAYER CHROMATOGRAMS

APPROXIMATE RESULTS BASED ON FLYING SPOT SCANNING WITH A NEWLY DEVELOPED APPARATUS

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SUMMARY

An alternative circuit for the output from the author's flying spot scanner has been developed so that transmission in the two dimensions may be presented continuously as a negative logarithm on a flat-bed recorder. Thus, a rapid visual survey of all the detectable information present in as many as six chromatograms on one 75 × 75 mm plate can now be made. The proportion of each resolved zone can be estimated approximately from manual calculations based on the sum of peak heights. In a binary mixture scanned at 330 nm the coefficient of variation found was about 4%. The chromatography, recording and manual calculation of, for example, twelve impurities in terms of two standards occupy about 2 h. When scanning at wavelengths below 280 nm, a reduction in layer thickness to 0.14 mm is made to reduce the increase in light absorption of silica gel G. In the examples shown, $-\log T$ recordings in the UV are more sensitive than visual or visualisation methods for testing the resolution of mixtures. The effect of tailing of standards in the estimation of impurities in a manufactured intermediate product is also discussed.

INTRODUCTION

In a previous paper[1], the authors described the development of an apparatus for scanning thin-layer chromatograms which absorb light in the visible or in the UV where the substrate generally has a significant background absorbance. The intention of the design and computation of the apparatus was to improve accuracy by allowing for such factors as local changes in layer thickness and point-to-point changes in the absorbance of the chromatogram. This called for a degree of sophistication not then available in commercial equipment.

With the apparatus, scanning in two dimensions is effected by moving the plate (size 75 × 75 × 1.1 mm) in 0.5-mm steps with stepping motors about a fixed point of light. The light transmitted through the chromatogram is focused on to a monochromator which is adjusted to the wavelength of maximum absorbance. After am-

plification, the signal is digitised and recorded on paper tape. Each plate can accommodate six chromatograms and the resulting data tape carries many thousands of data points directly proportional to transmission. A complex computer program is applied to each point to evaluate parameters in the Kubelka–Munk equation, *i.e.* scattering power of background (SX) and absorbance of background (LX) and of sample (KX) by iterative approximation, where L/S is defined independently for each batch of substrate. The concentration of absorbing substance at each 0.25 mm^2 segment is $C = KX/2.303a$ (where a is the absorptivity and X is the layer thickness). Finally, the micrograms of compound in the whole chromatographed zone follow by integration (for details see p. 387 of ref. 1).

Off-line communication with a computer such as an IBM 360 is the least expensive way of data-processing the tapes, but delays due to postal links sometimes amounted to 48 h before return of the results. Thus, it became apparent that an instantaneous graphical (*i.e.*, analogue) recording would be a quicker and simpler way of reviewing the UV absorbance of these chromatograms prior to deciding whether the more accurate computer processing was required. Such a modification is described below as an option. The work of Treiber *et al.*[2] is relevant (see Discussion and conclusions).

A technique for the preparation and use of chromatograms supported on 75 × 75 × 1.1 mm plates of glass, or synthetic fused silica for use in the measurement of transmission below 320 nm, has already been described[3]. Advantages are claimed in speed and economy of material over the usual 200 × 200 mm size. These have been used for scanning in the UV followed by data-processing[3,4]. The procedure now proposed should enhance the convenience of the technique in cases where approximate results are acceptable.

As a conclusion to the author's work in this field, some ancillary experiments using analogue recordings are included. These examine the effect of varying applied volume on dose *vs.* response curves and the problem of quantitating a tailing zone.

MODIFICATIONS TO THE APPARATUS

The following units, connected as shown in Fig. 1, are additional to those defined in ref. 1, Fig. 2.

Logarithmic amplifier (Fig. 2)

The input range of the logarithmic amplifier is 0.1–10.0 V, with a negative-going output of 300–0 mV and a variable offset. An additional co-axial socket on the front panel of the phase-sensitive detector is a convenient connection.

Flat-bed recorder

The flat-bed recorder used is Bryans Model 2700, or equivalent, with a mV range and speed selections.

Pulse generator (Fig. 3)

In order to avoid running the tape punch continuously as the source of stepping pulses it is necessary to construct a pulse generator as shown to provide an alternative

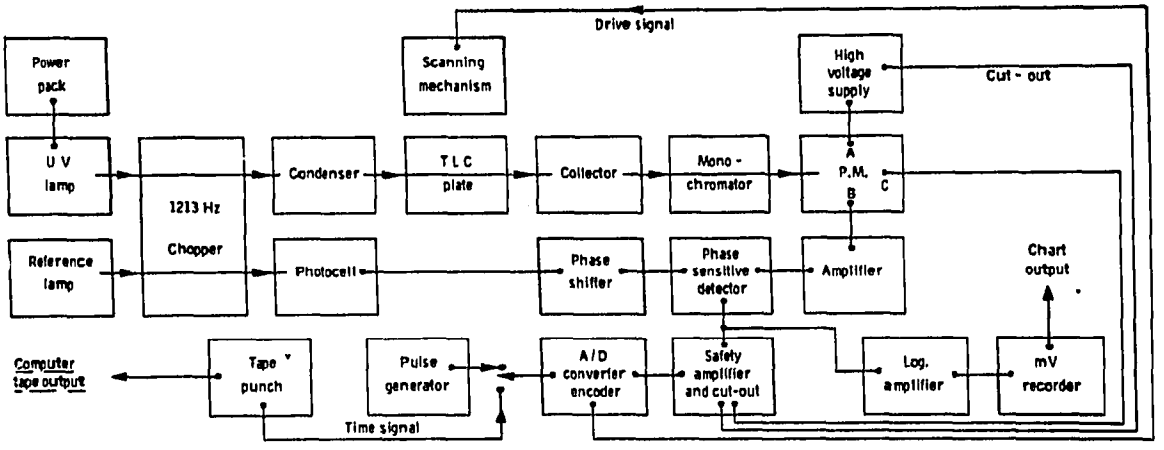
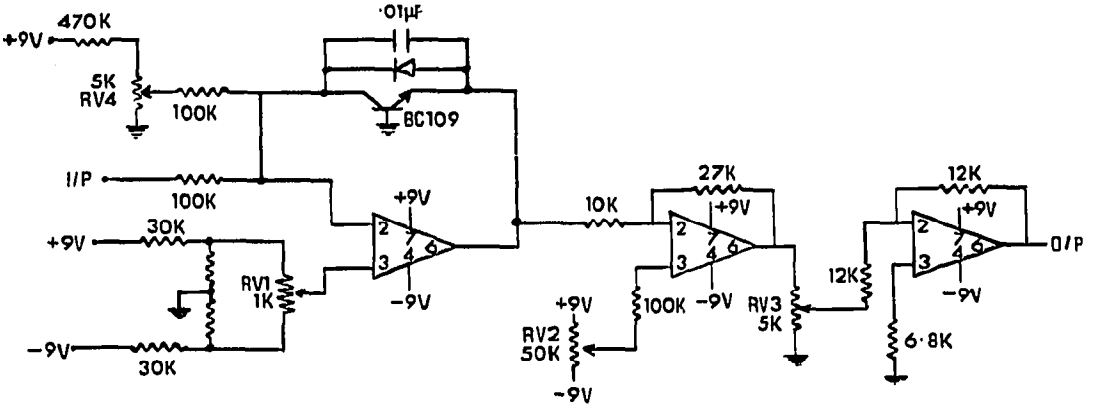


Fig. 1. Diagram of the revised instrument which now offers either encoded digital tape or analogue output on a chart.

source of signals. Pulses at 200-msec intervals (see later) allow the pen to reach equilibrium between each pulse.

Amplifier and safety circuit (Fig. 4)

A refinement on the original assembly is the provision of a safety circuit to trip out the high-tension voltage supply to the photomultiplier when the digital dis-



100mV to 10V - 300mV out
RV1 Fine control for Log O/P
RV2 Set offset
RV3 Set gain
RV4 Log Amp offset

Fig. 2. Circuit of the logarithmic amplifier.

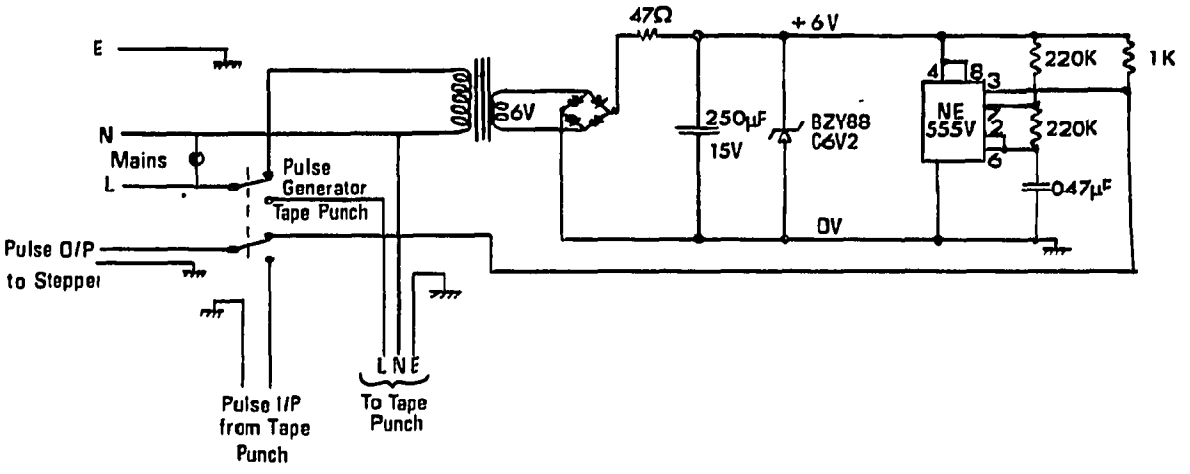


Fig. 3. Circuit of the pulse generator which was used to simulate tape punch synchronising pulses.

play, *i.e.* the output, rises above 9.98 V. If the photocathode is accidently exposed to a clear part of the plate, the cut-out prevents any possibility of a catastrophic rise in the photocathode current leading to "burn-up".

Stray-light

A 1-cm nickel-cobalt sulphate solution filter transmitting between 230–350 nm is placed between the source and first beam condenser to reduce the stray light in the visible when operating in the UV. The 240-nm or other interference filter is not required. The solution filter must be removed before work in the visible, and below 230 nm a liquid filter —with a higher transmission below 230 nm— is desirable.

Deuterium lamp

The 150-W water-cooled deuterium lamp and power-pack is now replaced by a 25-W air-cooled lamp of 1-mm aperture with a power-pack as supplied commercially for use with a Unicam SP 500 spectrophotometer.

MODEL COMPOUNDS

Tetrazobenzene-β-naphthol (Sudan III)

Sudan III is a red solvent-soluble dye readily visible at sub-microgram levels on silica gel G, but it tends to form a slight deposit at the higher loadings. Results are not reported in this paper as the following examples cover the same ground. Sudan III is useful for comparing the dose *vs.* response relationship in the visible with that in the UV. The absorption spectrum in methanol solution shows $\lambda_{max.}$ at 510, 350, 228 and a plateau at 200 nm with $E^{1\%}_{1cm}$ of approximately 900, 400, 840 and 400, respectively. It may be loaded from and developed with toluene; the R_F value on silica gel G is ≈0.33.

Diethyl- and dimethyldinitrophenylhydrazones

Diethyl- and dimethyldinitrophenylhydrazones form yellow- and orange-coloured spots (respectively) on silica gel G which show light absorption maxima in the range 360–370 nm. Mixtures of the two in a wide range of proportions can be observed to separate when developed with toluene; the R_F values are approximately 0.52 and 0.32, respectively. Six hours after loading the absorbance on the silica had declined by 4%.

Diethyl ketone dinitrophenylhydrazone (DNPH) standard sets A and B in differing volumes

Diethyl ketone DNPH set A was prepared as follows: from a stock solution of 1.86 mg in 10 ml toluene, 1.00-ml aliquots were diluted with 0, 0.2, 0.5, 1.00 and 2.00 ml of toluene in 4-ml vials; a 0.50-ml aliquot was also diluted with 2.50 ml of toluene. Set B was prepared similarly from a stock solution containing 4.66 mg in 10 ml toluene.

Two-component mixture

Diethyl ketone DNPH standard solutions S_1 and S_2 for chromatography were prepared by adding 8.0 and 12.0 ml of toluene, respectively, to 1.00-ml aliquots from a stock solution (E) of diethyl ketone DNPH containing 6.76 mg in 10 ml toluene.

Of dimethyl ketone DNPH 5.68 mg were dissolved in 10 ml toluene (solution M). A mixture (T) of 0.50 ml of solution E + 5.0 ml of solution M was prepared for chromatography.

Fluocinolone acetonide ("Synalar")

Synalar is a colourless steroid defined in the British Pharmacopoeia. The peak absorption lies at 238 nm in the UV ($E^{1\%}_{1cm} \approx 360$) in methanol. Chromatograms on silica gel G are well reproduced after loading from chloroform and developing with butyl acetate, $R_F \approx 0.5$.

Standard solutions

3.89 mg of the acetonide were dissolved in 4.0 ml of chloroform. 1.00-ml aliquots and one 0.50-ml aliquot were diluted with 0, 0.20, 0.50, etc. ml of chloroform to form six standard solutions as directed above for diethyl ketone DNPH.

PROCEDURE

Chromatography

A silica gel G plate is prepared as described by Goodall[3]. The dimensions of the layer are 75 × 75 × 0.25 mm; the layer should be free from pin-holes or obvious changes in thickness when inspected by transmitted light. For work below 320 nm a synthetic fused silica baseplate[3] is required and below 260 nm the layer thickness should be reduced to about 0.14 mm by "packing" the corners of the baseplate with PVC tape 0.17 mm thick before spreading the layer. Details of loading and developing solvent used are described later for each example under Results. The technique of applying the loads at fixed spacings of 10 mm according to a template and the general procedure for the author's form of miniature thin-layer chromatography are detailed

by Goodall[3]. A margin of approximately 1 mm is cleared around the perimeter of the plate and at each corner near the solvent front a transparent triangle of about 5 mm per side is cleared so as to allow passage of the light beam. Just below the solvent front after development and vertically above the points of application according to the template, a spot of ink is placed to mark the top of each chromatographic vertical axis around which scanning will be centred.

Setting up the apparatus

The function of the component parts of the apparatus and the use of the apparatus for digital recording have been described by Goldman and Goodall[1]. The output from the safety amplifier (Fig. 1) in the range 0–9.99 V is displayed continuously in the digital form of 0–999 on the A/D converter panel. In the optional circuit (Fig. 1) the driving signals to the scanning mechanism come from the pulse generator, when selected as indicated by the arrow on the A/D converter. The pulse is audible and is adjusted to 50 per 10 sec, *i.e.* 200 msec per pulse, which is sufficiently long to accommodate a time-averaged signal of 100 msec from the phase-sensitive detector and to allow for the slower response of the chart recorder as compared with the tape punch. The digital display is directly proportional to transmission through the chromatogram. This signal is converted to a negative logarithmic form in the logarithmic amplifier and then recorded on the chart output from a flat-bed recorder. A chart speed of 3 cm/min on the 300-mV range of the recorder is generally suitable. With the spectrophotometer slit closed, a wide range of signal offset is made possible by turning the recorder "shift-knob" to mid-position, then adjusting RV3 and RV4 of the logarithmic amplifier (Fig. 2) until the recording pen is at the middle of the scale. The shift-knob is then turned until the pen lies at 80% of the full-scale deflection (200 mm). This is a convenient infinite absorbance position which avoids overrunning the pen-clutch mechanism during rapid excursions.

A typical plate is inserted in the carriage with the silica gel G layer facing the photomultiplier. The latter is supplied with 1000 V. The appropriate wavelength is selected and the slit control opened slowly until the average silica gel G background obtained by manual movements of the carriage in the two dimensions corresponds to about 400 digits on the A/D display. The pen should now lie in the region of 5–10% of full-scale deflection. The pen response is calibrated by plotting logarithm of digital display against pen displacement, and the result should be linear down the range from 400–20 digits, obtained by closing the slit to record progressive steps as the chart travels at 3 cm/min. The slope referred to later as the "scale-factor" is about 100 mm per tenfold reduction in digital units (*i.e.*, a reduction of one absorbance unit).

The calibration remains constant, but the apparatus is standardised before and after each set of chromatograms to check whether battery or other voltage variation has occurred. A dispersion of Indian ink of known absorbance in the range 1.5–2.0 prepared as described by Goodall[3] is the standard, inserted in the optical path, after the second beam-condenser, when the beam passes through the cleared margin of the plate. During this manipulation the slit must remain undisturbed and to prevent excessive light flux an orange-coloured plastic sheet is inserted before the first beam-condenser. Normally, there is no drift between standardisations. For changes of a few per cent a linear correction is assumed as permissible. A larger drift may indicate the need for new batteries in the logarithmic amplifier. A poor response at wavelengths

above 350 nm will occur if the nickel–cobalt band-pass filter has not been removed (see *Stray light*).

Scanning the plate

The plate may carry as many as six chromatograms, with the solvent front visible as a faint line up to 60 mm from the points of application of the spots. Figs. 5 and 8 show photographs of sections of plates bearing chromatograms; the full scale can be deduced from the fact that the chromatograms are spaced at 10 mm between centres. For economic reasons the digitising procedures previously published were confined to selection and scansion of one zone only in each chromatogram, by traversing across the plate. The new option readily permits the routine scanning of the whole of each chromatogram from a mark near the solvent front down to the origin. The excursions of the carriage are adjusted around the long axis of each chromatogram indicated by the mark and to traverse by ± 6 mm. After each sample has been scanned the carriage is moved by hand and the plate repositioned in the clamping jaws until the traverse is symmetrical about the next mark near the solvent front. A sequence of switching initiates the automatic scanning process which continues until an alarm bell activated by the "down-stepping" rack on the carriage signals the end of scan. The safety amplifier and cut-out (see Modifications to the apparatus and Fig. 4) protect the photomultiplier against damage due to excessive light flux through pin-holes or major reduction in the thickness of the adsorbent layer. Pin-holes may be "spotted-out" so that scanning can be continued after returning the plate and switching the safety amplifier and high-tension supply to the "on" position.

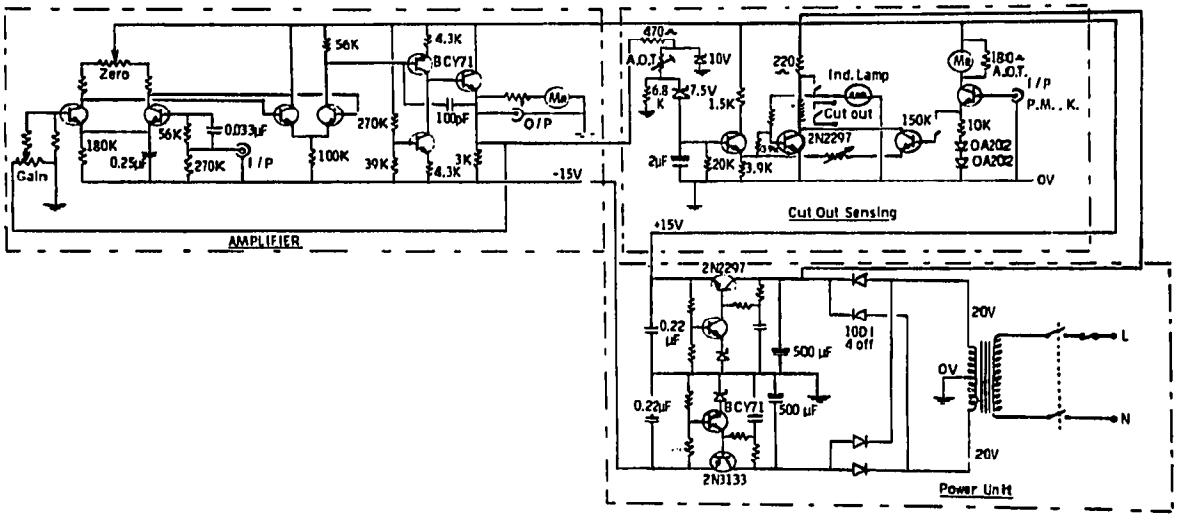


Fig. 4. Amplifier and safety circuits. All transistors are BC 108 unless otherwise defined.

Calculation

The recording from all the chromatograms on the plate is spread out on the bench and a reference baseline is drawn in green ink under all the peaks, at a level

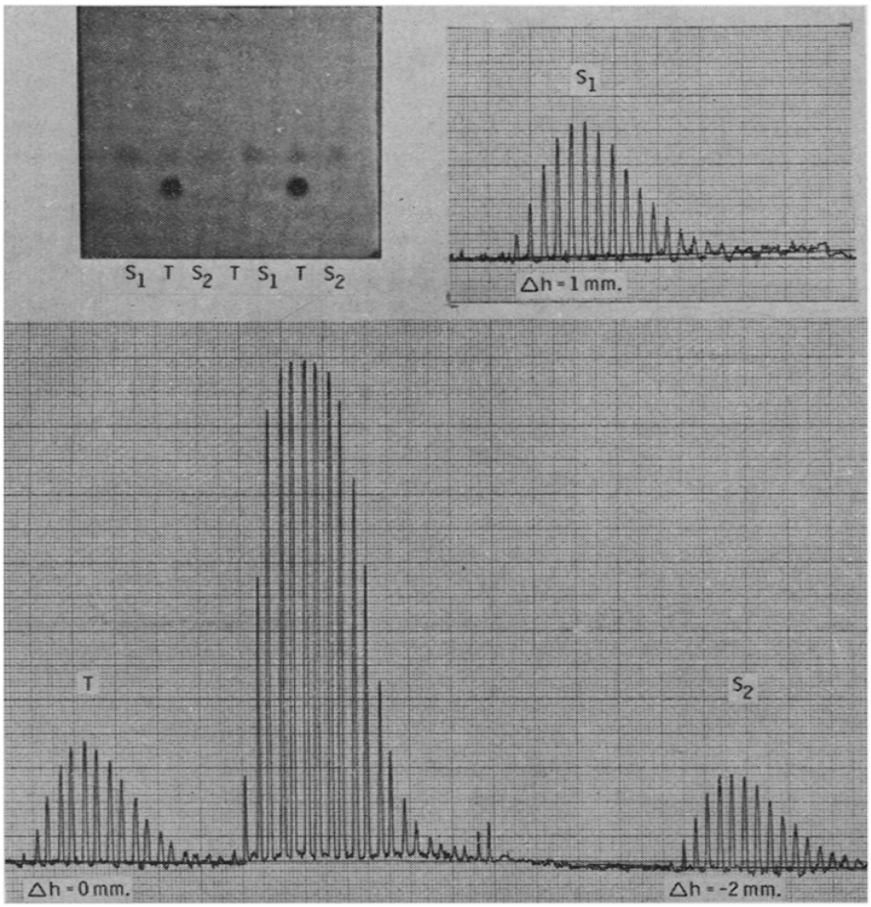


Fig. 5. Diethyl and dimethyl ketone DNPHs in admixture. Analogue recorded scans (λ = 330 nm are shown to the same scale as a subsequent life-size copy photograph in the visible. The estimation of the diethyl component in T in terms of S_1 and S_2 is detailed in Table I.

corresponding to the base of the lowest peak recorded (compare Fig. 5 and Fig. 8, t_3). Lines in red ink are drawn to define the base of each group of peaks (*e.g.* the sloping line under Fig. 5, S_1). Where there is a small cyclical change of level at the base of each peak the approximate mean point is used. In the case of partially separated zones as in Fig. 8, t_2 (r_3, r_2, r_1), each is divided at the position of absorption minimum. In the zones of interest the height of each peak is measured to the nearest 0.5 mm with an engraved rule. Let Σh signify the total of heights (mm) in each zone. Σh is converted to absorbance units when multiplied by the "scale-factor" determined under *Setting up the apparatus* and the product ΣA is noted as in Table I. The mean deviation in mm of the zone baseline from the reference baseline is measured and denoted as Δh_0. $\Delta h_0 \times$ scale factor is recorded as ΔA_0 in the next column. Exponent ($-2\Delta A_0$) is found from a set of exponential tables and the product $\Sigma A \cdot \exp(-2\Delta A_0)$ is entered under the column entitled ΣA corrected. The exponential term is a correction for change in scattering power due to change in layer thickness previously pub-

lished (ref. 5, p. 34, and ref. 6). It is usual to run two or more standards from which a graph is drawn of μg loaded against the response ΣA corrected. From this graph and the ΣA corrected from each test zone the μg equivalent of the test is found and then expressed as a proportion of the weight loaded. If the effect of curvature of the graph is likely to be significant, the response may be linearised by introducing the squared term $\Sigma(A + 0.4\,A^2)\exp(-2\Delta A_0)$, as illustrated in Fig. 7.

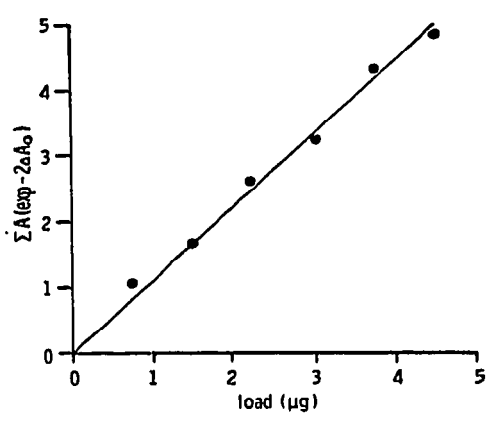
RESULTS

Dose vs. response relationship

Fluocinolone acetonide. Preliminary experiments showed that a reduction in layer thickness from approx. 0.25 to 0.14 mm was advantageous in overcoming fluctuations due to the high absorbance of silica gel G at lower wavelengths. The thinner layer was prepared on a synthetic fused silica plate 1.1 mm thick with "Lassotape" backing as described above. No activation at 100° was necessary.

4.63-μl aliquots from the range of standards solutions (see above) diluted in chloroform were loaded on the plate to form loads of from 0.75–4.5 μg. These were then developed over a distance of 30 mm by allowing butyl acetate to ascend to the top of the plate using the techniques described by Goodall[3]. The butyl acetate was removed *in vacuo* and the plate was scanned transversely at λ = 238 nm and recorded as described above. After measurement and calculations the response *vs.* dose relationship was as shown in Fig. 6.

Diethyl ketone DNPH —effect of load volume. Approximately equal quantities of diethyl ketone DNPH in toluene solution (standard sets A and B, see Model compounds) were loaded from nominal 2- and 5-μl pipettes in pairs on the same plate so as to form two sets of standards, interspersed in the range 0.17–0.46 μg and starting with different spot diameters (approx. 3 and 4 mm, respectively). After developing with toluene the spots had travelled 29 mm and the diameters were now both in the



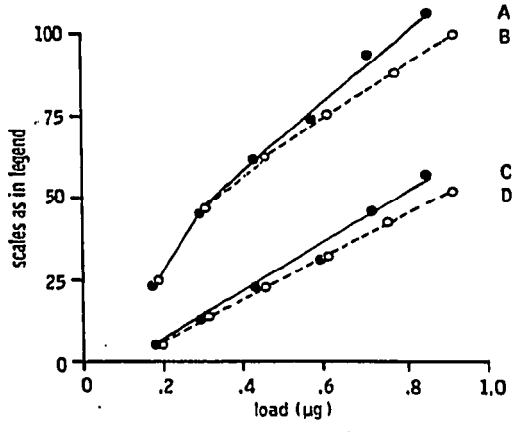


Fig. 6. Response *vs.* dose relationship of fluocinolone acetonide after developing 5-μl spots on a silica gel G layer 0.14 mm thick. Scanning wavelength, λ = 238 nm.

Fig. 7. Effect of load volume. Diethyl ketone DNPH standards are loaded from 4.63 μl (●) and 1.97 μl (○), then developed through 29 mm with toluene and scanned at λ = 360 nm. Vertical axes: A and B, $\Sigma A \cdot \exp(-2\Delta A_0) \times 10$; C and D, $\Sigma(A + 0.4\,A^2)\cdot\exp(-2\Delta A_0)$.

range 4–4.5 mm. The plate was pumped *in vacuo* till dry, then scanned at the peak maximum $\lambda = 360$ nm as described above, except that the scanning was done in one sweep transversely from left to right across the spots using a track 14 mm wide.

The experiment was extended upwards from 0.57–92 μg on another plate of similar overall layer thickness. Overall layer thickness is readily measured by micrometer as the difference between baseplate and a "clipped-on" cover plate when the layer is present and when absent. After scanning each plate, digital readings of the background ($T \times$ const.) were taken at six approximately equally spaced points on either side of each scanning track. The percentage transmissions were determined by reference to a grey liquid standard placed behind a cleaned area of each plate (see *Setting up the apparatus*). There was no significant difference between the averaged background absorbances of the two plates so that no alignment adjustment between the plates was necessary.

The results in Figs. 7A and B show a divergence at the higher loadings due to difference in load volume. The divergence tends to be overemphasised by the use of a $\times 10$ factor on the vertical axis $\Sigma A \cdot \exp(-2\Delta A_0)$ used for convenience in graphical comparison with Figs. 7C and D, which incorporate the term $0.4A^2$ for curvature correction[5]. Also in this experiment there is a high slope on the response *vs.* dose curve so that peak heights corresponding to the higher standards exceed one absorbance unit. This is the preferred working limit attained here from the 0.46-μg loading. For further comment, see Discussion and conclusions.

Analysis of a mixture of dinitrophenylhydrazones. 4.63 μl of standard solution S_1, a solution of mixture T and standard solution S_2 (prepared as above) were loaded on a silica gel G plate, then replicated to form two sets in the order S_1 T S_2 S_1 T S_2. The plate was developed with toluene. When the solvent front had travelled about 60 mm the diethyl ketone zone had run ahead with a clear space of about 4 mm between the zones. The plate was dried *in vacuo* then scanned at $\lambda = 330$ nm. The electronic set-

TABLE I

REPEAT ESTIMATIONS OF DIETHYL KETONE DNPH IN ADMIXTURE

Spot No.	*Title*	*Load (μg)*	ΣA*	ΔA_0**	$e^{-2\Delta A_0}$	ΣA *corrected***	*Found (%)*
1	Standard S_1	0.38	3.20	0.01	0.98	3.13	
2	Test T_1	2.89	2.62	0.0	1.0	2.62	10.6
3	Standard S_2	0.26	2.20	−0.02	1.04	2.29	
4	Standard S_1	0.38	3.35	−0.02	1.04	3.48	
5	Test T_1	2.89	2.50	−0.04	1.08	2.70	9.8
6	Standard S_2	0.26	2.14	−0.08	1.17	2.50	
Rescanned 1–6			3.30	0.04	0.92	3.04	
			2.60	0.02	0.96	2.49	10.5
			2.15	0.0	1.00	2.15	
			3.15	0.0	1.00	3.15	
			2.55	−0.02	1.04	2.65	10.8
			2.05	−0.045	1.09	2.24	
						Theory	10.7

* ΣA = Sum of peak heights in mm $\times$ 0.01, where one absorbance unit is 100 mm on the chart.
** ΔA_0 = Mean deviation from baseline of ΣA.
*** ΣA corrected = $\Sigma A \cdot \exp(-2\Delta A_0)$.

tings were: 1000 V, 100-msec delay, recorder scale at 3 × 100 mV.

A photograph of the resulting pale yellow chromatograms taken in the visible with a polaroid camera and enlarged to life size is included in Fig. 5 for comparison with selections of chart recordings at $\lambda = 330$ nm from a similar experiment. The line drawn 12 mm above the foot of the chart is the reference baseline. The base of each zone is defined by a second line (see Fig. 5, S_1) which may drift slightly above or below the reference line. The mm drift at the centre of each zone is recorded and converted to ΔA_0 ($\times 0.01$). The calculations and results are summarised in Table I.

Technical application —impurities in an intermediate product. The intermediate product has a high affinity for silica gel G and so a solvent system containing either a volatile acid or ammonia is obligatory. Samples of different "strength" from works manufacture were used in preliminary screening experiments to test various systems for resolution of impurities and ease of operation. The results were made visible by an iodine absorbtion technique. The solvent system diethyl ether–acetic acid–water (6:2:1) used as described by Goodall[3], but without cover glass, appeared to be the one of choice. Fig. 8 shows a life-size copy photograph from a "Polaroid" print which is on the same scale as the corresponding recording selections made from these samples by scanning at $\lambda = 257$ nm before iodination, etc. A load of 20 μg in 1 μl is required to chart the separation of a leading impurity (f_1) and the partial separation of three impurities (r_3, r_2 and r_1 in t_2) following the main zone.

The method of calibration by reference to two standards S_1 and S_2 (2 and 1% of the total load) has proved to be less satisfactory than at first thought. The response *vs.* dose line by the above calculations no longer passes through the origin, presumably due to the disproportionate amount of tailing in S_2. In view of this and the range of impurity levels (f_1, r_3, r_2, r_1) it is necessary to avoid extrapolation by making further comparisons in which one type of impurity is selected and compared with an appropriate spread of standards. For example, the level of the dominant impurity t_2, r_2, was estimated from a further comparison as follows. Two smaller loadings, 5.25 and 10.50 μg of t_2 in 1 μl were chromatographed with three standards of 0.24, 0.47 and 0.71 μg in 1 μl. ΣA corrected was 2.68 and 4.39 for t_2, r_2 against 3.46, 6.26 and 8.00, respectively, for the standards. From a graph of the standards and the weights taken the relative percentages of r_2 found were 3.2 and 3.05%. For further comment, see Discussion and conclusions.

DISCUSSION AND CONCLUSIONS

The experimental approach used with the apparatus described conforms to the well-known principles of optimising the signal-to-noise ratio. The signal is a function of the light flux, the intrinsic absorptivity of the sample, the smallness of the spot diameter and the optical properties (SX, LX) of the silica layer. Electronic noise is not difficult to overcome, but any variation in layer thickness, particularly in the lower UV region, can have a significant effect on the light flux. The present correction for change in layer thickness uses the mean departure of any group of peaks from the common baseline. This approximation is avoided in the point-by-point correction employed in the computer program[1]. Even from sub-microgram loadings, absorbances in excess of one unit above background are frequently attainable, but at such levels a progressive departure from linearity of response was observed.

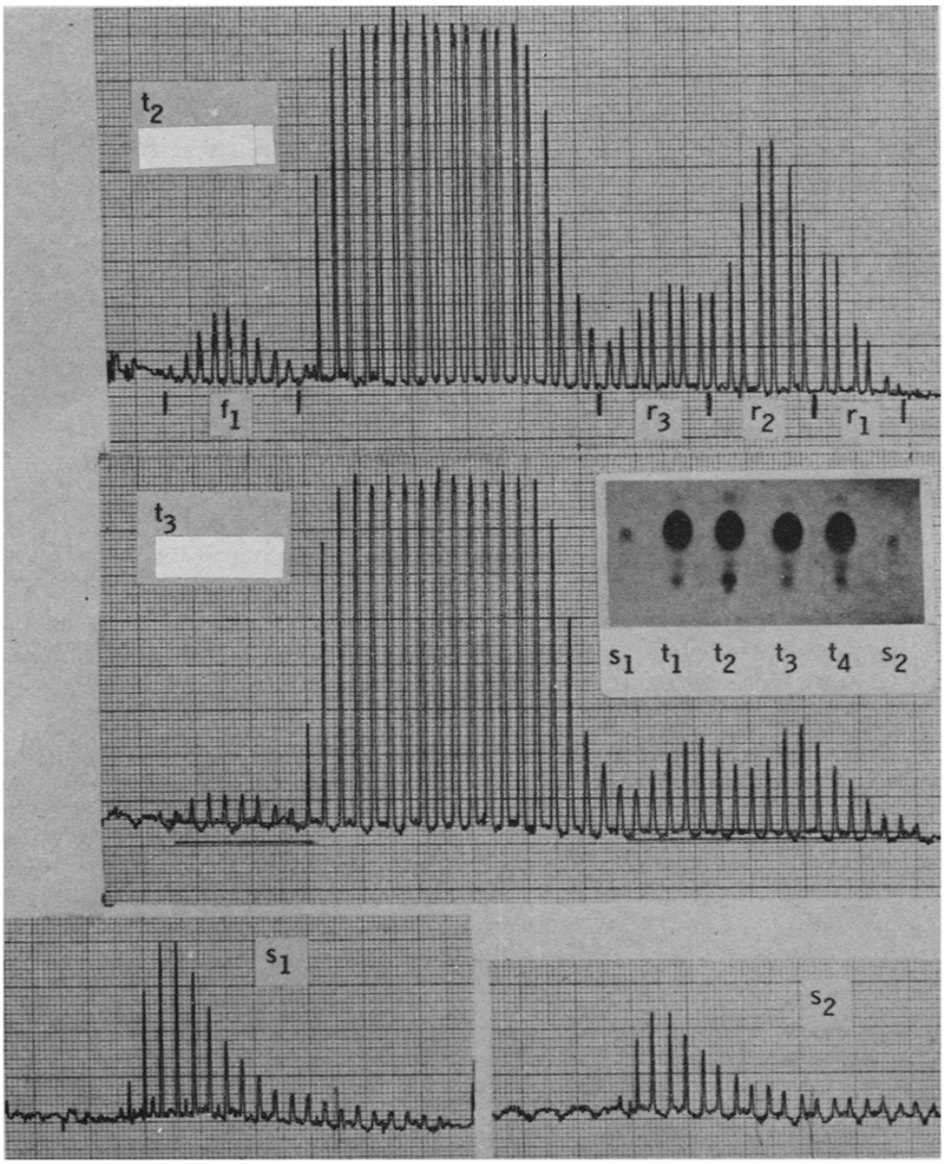


Fig. 8. Impurities in an intermediate product. Abstracts from recorded scans at $\lambda = 257$ nm of tests $t_1 - t_4$ and reference amounts S_1 and S_2 of the purified principal component are shown to the same scale as a life-size copy photograph of the plate after an iodine reaction.

A scanning record resembling that illustrated in Fig. 5 has been published by Treiber *et al.*[2]. However, there are considerable differences between the two approaches both in respect of procedure and derivation of results. Treiber *et al.* do not use, as far as we know, an automatic drive for the flying-spot in the second dimension, but have the advantage of electronic integration of the signal output, which can be recorded both in the reflection and the transmission mode. They consider that the

ideal conditions assumed in the Kubelka–Munk equation do not apply in thin-layer chromatography and instead have developed an empirical equation incorporating terms for both reflection and transmission. In their second communication an electronic "function transformator" is described for use with their expression and a suitable choice of constants to obtain a linear response.

The apparatus shown in Fig. 1 continuously records constant·($-\log T$) from a scanning raster travelling to and fro across each zone. The recorded height of each peak is directly proportional to the maximum absorbance, where the limit is not in excess of one absorbance unit. The recorded diameters of the absorbance "slices" will vary according to the shape of the zone. Generally, zones are circular or elliptical so that the diameters increase progressively from zero to a maximum, then decrease. In the absence of an electronic integrating circuit, a manual summation of the areas under the triangular records of each scan is the obvious procedure. The "slices" are all 0.5 mm apart so that this third dimension will cancel out in the comparison. For accurate measurement of each base width a scale enlargement factor of 10–20 is necessary and can be achieved by running out the paper at maximum speed. However, the very large output of paper corresponding to each set of chromatograms and the time required to handle and measure each individual base is quite uneconomic. On the scale adopted (*e.g.*, the x axis of Fig. 5) any differences in base diameter between the test and standard slices are too small for simple measurement.

To simplify the procedure, measurement of base width was omitted and only the heights of the triangles were noted. After correcting for any averaged change in layer thickness, the assessment is based on the ratio of total absorbance (ΣA corrected) in the test *vs.* the standards. The relatively low absorbances at the start and finish of each zone scanned will tend to reduce the proportional error from the smaller "slice" diameter. In criticism this procedure embraces those comparative aspects and limitations inherent in conventional quantitative thin-layer chromatography, and obviated by the initially developed flying spot scanning technique with digital output. A further development is clear, *i.e.* the design of a suitable integrating circuit.

Where the chromatograms are symmetrical as in most of the present examples, a linear response *vs.* dose relationship has been found, provided that the individual peak maxima do not exceed one absorbance unit above the background. The coefficient of variation of the levels found in the mixture (Table I) is 4%.

Where the chromatogram is asymmetric and shows a tail which tends to occupy an increasingly higher proportion of the whole as the load decreases (as in S_2 of Fig. 8) the response *vs.* dose relationship is not a linear function and standardisation is less straightforward. The occurrence of tailing in any type of chromatography usually prompts the chromatographer to look for another, non-tailing system. In the present case tailing could be inhibited by adding 10% (w/v) of sodium acetate trihydrate to the water used for making the slurry. However, the resolution of the impurity zones in the tests was distinctly inferior. Possibly a better course would be to isolate and characterise sufficient of the impurity r_2 for use as a standard which would not travel far enough to show a tail.

The basis of the above calculations is the empirical expression[5] $0.434\,KX = 2\exp(-2\Delta A_0)(A + 0.4A^2)$, where the exponential term allows for changes in thickness of the layer which produce changes in scattering power (SX) and the squared term allows for curvature of response. The latter was presumed to be unnecessary in these

calculations (Table I), but the allowance should be applied to more widely separated comparisons.

In addition to the scattering parameter S the background L of the silica gel becomes increasingly significant below $\lambda = 280$ nm. L and S are not separable variables, but an experimental measurement of infinite reflectance R_∞ gave the following ratio: $(1 - R_\infty)^2/2R_\infty = L/S$. This ratio has been evaluated at different wavelengths and should remain unchanged for a particular batch of silica gel G. In the computer program of ref. 1 the independence of SX with respect to LX is removed by interpolating T_0 from regions where $KX = 0$ to all other regions. Having evaluated L/S and interpolated T_0, L can be eliminated from the Kubelka–Munk equation (ref. 1, eqn. 3). Finally from the interpolated T_0 and observed T values, SX and then KX are computed by iterative approximation.

Thus, the simple empirical expression in terms of A and A_0 above does not apply to cases where L is significant, *e.g.* for normal layer thickness at wavelengths below 280 nm. The use of a thinner layer for work in this region is an attempt to minimise the magnitude of L and S. The experimental points as in Fig. 6 show less scatter when a 0.14-mm layer rather than a 0.25-mm one was scanned at 238 nm; also the response is linear. Further tests to establish the lowest wavelength suitable for the use of the empirical expression are required.

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