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FLYING-SPOT SCANNING Ddensitometry IN THE ULTRAVIOLET REGION FOLLOWED BY DATA PROCESSING

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SUMMARY

The optical properties of some common thin-layer chromatographic adsorbent layers are compared. Silica Gel G is much more transparent above 280 nm than are similar thicknesses of other adsorbents. Below 280 nm, the attenuations of these adsorbents rise more or less steeply.

Improvements to the author's apparatus for densitometry by transmission in the UV region are described, followed by off-line data processing on an I.B.M. 360 computer. Examples shown are: preliminary tests of the precision of a steroid determination at 238 nm where the background "absorbance" is about 2.8; separation and determination of minor impurity zones at 340 nm on glass support plates; and at 298 nm the active agent remaining after the decomposition of a 0.2% aqueous solution.

Fig. 1 shows the absorption:scattering (K/S) ratios of some commercially available thin-layer chromatographic (TLC) adsorbents. These have been determined approximately in the spectral range 340–230 nm by means of a Unicam SP800 diffuse reflectance attachment.

More directly useful here are measurements of attenuation in the transmission mode using the scanning apparatus[2] and assuming a 30% collection efficiency. Calibration is by reference to a liquid dispersion of Indian ink of known absorbance. Fig. 2b is a plot of the attenuation of Silica Gel G against wavelength and layer thickness. The "absorbance" axis in this measurement is dependent upon absorption (K), scattering power (S) and layer thickness (X). Silica Gel G shows lower "absorbance" than the other sorbents (*e.g.*, $MgCO_3$ in Fig. 2a) in the 280–320 nm range presumably because it is much less scattering. Below 280 nm there is a marked rise, probably due to selective absorption.

Although high background absorption is commonly regarded as a barrier to direct *in situ* densitometry either by transmission or reflection scanning in the UV region, recent work in our laboratories after minor modifications to the system already described[2] has shown considerable promise. The use of a 25-W air-cooled deuterium lamp (Quarzlampen Ges. D102, 1 mm aperture) supplied from a Unicam power-pack (Model SP500) allows more precise focusing and shows an improved signal-to-noise ratio when tested by cathode-ray tube display of the photomultiplier output. Hence, it is now possible to scan sub-microgram amounts of, for example,

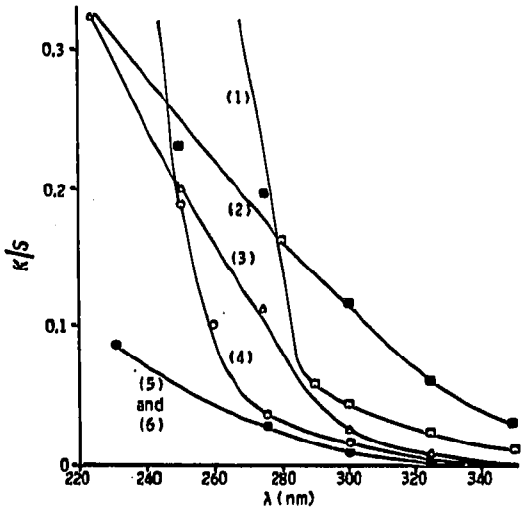


Fig. 1. Absorption: scattering ratios of various adsorbents in the spectral range 225–350 nm. The percentage reflectance is determined, from which $(1 - R_\infty^2)/2R_\infty = K/S$ is calculated[1]. Finely ground Suprasil powder is used as a standard in preference to magnesium oxide. The data presented are only approximate. (1) Silica Gel G (Merck); (2) aluminium oxide (Woelm, basic); (3) magnesium silicate (Woelm); (4) Silica Gel HR (Merck); (5) and (6) magnesium carbonate and calcium sulphate.

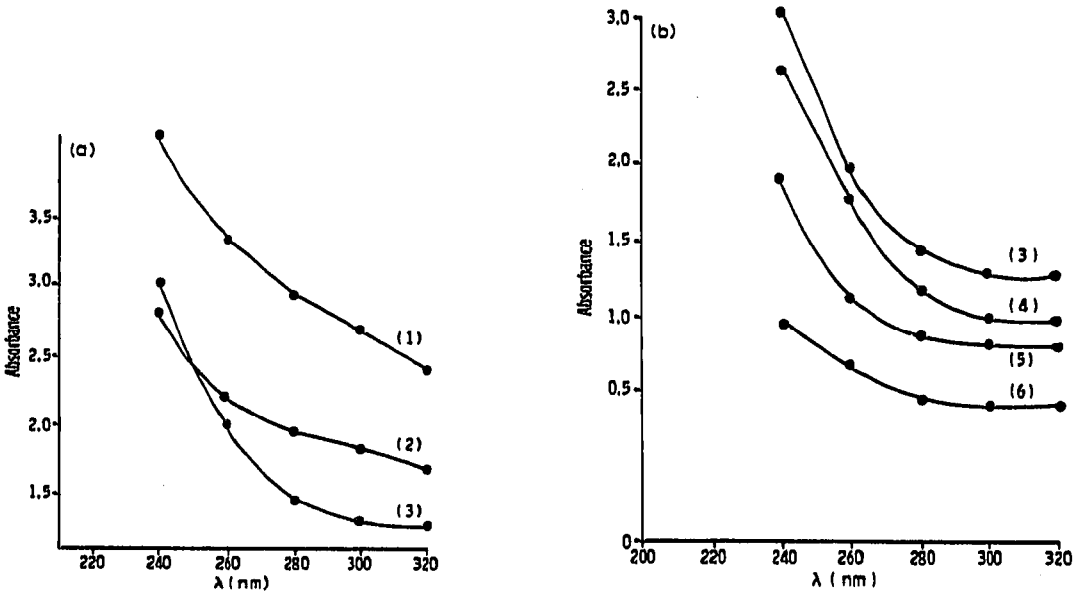


Fig. 2. (a) Absorption spectra of magnesium carbonate (levis) and Silica Gel G layers on a UV-transparent base plate. The data were obtained with the spot scanner at slit widths of 0.4–0.75 mm and 1000 V H.T. Calibration was by dispersions of Indian ink of known absorbance. (1) $MgCO_3$ levis + 14% gypsum, layer thickness 0.23 mm; (2) $MgCO_3$ levis, layer thickness 0.17 mm; (3) Silica Gel G, layer thickness 0.41 mm. (b) Silica Gel G layer thickness *versus* absorbance in the UV region. The data were obtained with the spot scanner as in Fig. 2a. (3) Merck 0.41 mm; (4) Merck 0.24 mm; (5) Merck 0.14 mm; (6) Woelm 0.13 mm.

steroids having peak absorbance levels from 0 to 1 on top of a background level of 3–4. To attain 1 % precision, the absorbance should be readable to 6–7, and this is the aim.

The record of the signal to noise ratio is a generally useful criterion of densitometer performance. A well designed system keeps the electronic noise small. The main noise and distortion in scanning records from transmission arise from variation in substrate thickness across the plate, hence the importance of adequate correction for background absorption and scattering according to the Kubelka–Munk equation.

Our background corrections are computed by interpolation from the recorded boundary value around the spot. POLLAK AND BOULTON[3], on the other hand, used a differential scanner to observe the same part of the zone at absorbing and non-absorbing wavelengths (the advantages and disadvantages of both systems are open to discussion).

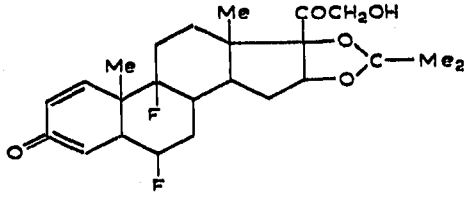
In practice, a layer of silica gel on a small quartz plate (77 × 77 × 1.1 mm) is loaded with four samples and two standards using a suitable template. After chromatography, the solvent is removed under vacuum and the zones of interest are scanned at the appropriate wavelength selected by the monochromator. The stray light also tends to be absorbed by the layer but a liquid Co–Ni solution filter after the deuterium light source and an additional 240-nm interference filter after the monochromator are available for maximising signal-to-noise ratio at 240 nm.

The scanner records on to paper tape at 110 characters/sec (7 characters per 3-digit reading) and the six zones of interest are usually recorded in 4 min. If the light level is very low, the signal-averaging time is increased by factors of 5–10, so that scanning may occupy up to 40 min. Data tapes from as many as six plates

TABLE I

PRELIMINARY TESTS OF PRECISION ON A HIGHLY ABSORBING SUBSTRATE BACKGROUND (SILICA GEL G AT 238 nm)

A 1 µl volume of the steroid solution in methanol is loaded on the plate according to a template. The Drummond microcapillary pipette is filled with 1 µl of methanol and this wash discharged on the spot. The results below show the dose: response of five standards chromatographed on one plate in the year 1970. Recent improvements in the apparatus, technique, and data processing would be expected to increase the precision. The background absorbance is about 2.8 at this wavelength.

Compound	*Load (ng)*	*Found (µg × absorptivity)*	*Load (%)*	*Found (%)*
Fluocinolone acetonide	1010	43.9	100	99.0
	1010	44.9	100	101.4
	758	33.0	75	74.5
	505	22.8	50	51.5
	253	10.8	25	24.3
	1010	38.8	100	88.0

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MICROGRAMS X ABSORPTIVITY= 38.347
MEAN SCATTERING POWER= 9.63
WITH MAX OF 9.9% AND MIN OF 9.36
LENGTH OF SCAN USED= 10.5 MM
WITH 2.0 MM SPARE AT START
AND 50.5 MM SPARE AT END
WIDTH OF SCAN= 14.5 MM
 10  10  10  10  10  10   9  10   9   9   9   9   9   9   9   9   9   9   9   9
 10  10  10  10  10   9   9   9   9   9   9   9   9   9   9   9   9   9   9   9
 10  10   0   0   0   0   0   0   0   2   2   0   0   0   0   0   0   0   9   9
 10  10   0   0   0   0   0   0   2   0   2   1   0   2   0   0   0   0   9   9
 10  10   0   0   0   3   3   3   5   5   2   2   0   3   2   0   0   0   9   9
 10  10   0   0   0   2   3   6   5   9   3   5   4   5   2   2   0   0   2   9
 10  10   2   2   2   2   3   9  11  17  13  11  11   5   3   2   3   3   9   9
 10  10   5   0   2   3   9  16  20  25  23  23  19  14   8   3   0   5   3   9
 10  10   3   0   5   6  12  20  23  28  28  27  26  27  13   9   1   2   2   9  10
 10  10   0   2   5   8  13  23  36  39  33  36  31  30  17   6   0   2   2  10  10
 10  10   0   2   8   9  15  31  39  41  39  44  43  33  17   9   2   2   2  10  10
 10  10   0   3   8   9  22  34  42  38  39  42  44  34  18   9   1   2   0  10  10
 10  10   0   0   5  10  22  34  41  39  36  42  39  33  19  11   0   0   0  10  10
 10  10   0   0   2   8  22  31  42  38  36  39  41  33  21   9   4   2   0  10  10
 10  10   2   2   5   8  20  31  38  36  36  36  42  27  19   9   5   2   0  10  10
 10  10   0   0   5   5  17  27  38  27  31  30  36  21  17   8   3   0   0  10  10
 10  10   0   0   2   5  14  22  30  23  27  24  27  19  11   3   3   2   0  10  10
 10  10   0   0   0   6  14  16  25  22  17  17  20  13   5   3   3   0   0  10  10
 10  10   0   0   0   2   9  11  19  16  14  15  16  11   5   2   5   2   0  10  10
 10  10   0   0   0   2   5   9  16  11  13  13  14   9   2   0   0   0   0  10  10
 10  10   0   0   0   0   3   5  10  10   9  11  13   6   2   0   0   0   0  10  10
 10  10   0   0   0   0   2   2   5   6   5   4   9   5   0   0   2   0   0  10  10
 10  10   0   2   0   0   0   0   2   2   3   0   4   5   2   0   1   0   0  10  10
 10  10   0   0   0   0   0   0   2   0   4   3   4   2   2   0   0   0   0  10  10
 10  10   1   0   0   0   0   0   1   0   2   2   2   0   0   0   1   0   0  10  10
 10  10   3   0   0   0   0   0   2   2   0   0   0   0   0   0   0   0   0  10  10
 10  10   0   0   0   0   0   0   0   0   0   0   0   0   0   0   0   0   0  10  10
 10  10  10  10  10  10  10  10  10  10  10  10  10  10  10  10  10  10  10  10  10
 10  10  10  10  10  10  10  10  10  10  10  10  10  10  10  10  10  10  10  10  10
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Fig. 3. Computer print-out of processed data from scanning a chromatogram of a 1 μl spot ≡ 1010 ng of Fluocinolone acetonide B.P. Scanning wavelength was 238 nm. The surrounding numbers 9, 10 refer to the scattering power, SX, of the background silica gel. Further details are in Table I.

TABLE II

REPEAT DETERMINATIONS OF MINOR IMPURITIES ON TWO TLC PLATES (AT 340 nm)

These are single tests, five on each plate. A 1 μl volume is loaded from a concentrated solution of the sample in formic acid. The mono-N-oxide (II) runs ahead of major constituents (I). The scanning track must be wide enough to allow for some variations in R_F between samples and standards. As all the zones are fluorescent, a preliminary brief inspection under a UV lamp at 366 nm facilitates marking boundaries of the scan. Glass plates are suitable for transmission work down to 320 nm.

Compound	*Load* (*ng*)	*Found* (*μg* × *absorptivity*)		% *Mono-N-oxide*	
		Plate (*i*)	*Plate* (*ii*)	(*i*)	(*ii*)
Quinoxaline mono-N-oxide (II), an impurity in the di-N-oxide (I)	46 000	12.9	11.4	0.35	0.32
	52 000	22.4	26.2	0.54	0.64
	9 000	36.3	31.3	5.0	4.4
		Standards of mono-N-oxide			
	500	40.3	39.3		
I II	250	—	18.9		

TABLE III

ACCELERATED STABILITY TEST (ACTIVE AGENT REMAINING IN A 0.2% W/V AQUEOUS SOLUTION) (AT 298 nm)

Two test and two reference spots are compared on the same plate. The mean $\mu g \times$ absorptivity for the standard is 39.2, hence absorptivity = 39.2/0.72 = 54.5. Therefore μg in test solution 1 = 28.3/54.5 = 0.52. A 1 μl methanol rinse through the 1 μl Drummond micropipette is added to the spot.

Arrangement on plate	*Found* ($\mu g \times$ *absorptivity*)	*Active agent (ng)*	
		Initial	*Remaining*
Standard solution (1 μl = 720 ng)	39.9	—	—
Test solution 1 (1 μl)	28.3	2,000	520
Test solution 2 (1 μl)	30.3	2,000	560
Standard solution (1 μl = 720 ng)	38.5	—	—

can be processed as a single job on an I.B.M. 360 computer. As the programme is fairly elaborate and as there are about 3500 data points per plate, a small computer does not have an adequate core-store to hold the data. The I.B.M. 360 has first to convert the tape code data to "card images", which is a slow step that takes several minutes owing to the low speed of the paper tape reader. Actual data processing is very rapid on this "number crunching machine"; 20000 data points are processed through the programme in a few minutes.

A computer print-out from a six-spot scanning run is shown in Fig. 3, *i.e.*, a contour "grid" or map of one spot, the surrounding background, and the total of $\mu g \times$ absorptivity.

Applications to the determination of precision and of minor impurity levels at appropriate wavelengths in the range 340 nm downwards are described in Tables I–III. The dose/response of the standard is linear.

In conclusion, what of future developments? Quantitative assays based on TLC may evolve in two ways, as already predicted[4], *i.e.*:

(*a*) The observed optical data will be recorded on tape, and then processed by computer according to an appropriate programme, just how appropriate being dependent on the skill and expertise of the systems analyst. Time-sharing computers are not suited to this task as the data-input rate and capacity of the available computers are too low for the purpose. Access to larger computers of adequate capacity should become commonplace within the next 5 years.

(*b*) The optical data points will pass into an assembly of electronic converter circuits, designed to apply functional corrections to the input signal, so that the final output is linearly related to concentration. After this stage, the output will be summed and the running total presented on a chart or digital recording.

A useful field of application is in the UV region, where many natural and synthetic compounds show high absorptivity.

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