THE APPLICATION OF SPECTRAL REFLECTANCE TO THIN-LAYER CHROMATOGRAPHY

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INTRODUCTION

Although the thin-layer chromatographic technique offers many distinct advantages, its analytical utility is restricted by two shortcomings. In the first instance, the difficulty experienced in obtaining reproducible R_F values with thin plates usually makes it necessary to run standards alongside the samples for comparison purposes. Secondly, the quantitative removal and extraction of individual spots from plates is a tedious process which often cannot be accomplished without decomposition occurring. Both of these operations would become superfluous if it were possible to effect the *in situ* identification and determination of chemical species separated on thin plates.

The use of spectral reflectance for these purposes was suggested by various studies[1–4] which demonstrated its utility with respect to paper chromatography. Furthermore, it has been shown that the reflectance spectra of substances concentrated on particulate adsorbents can be used for their identification,[5] and that spectral reflectance can be employed to determine the concentration of dyes scavanged from solution by the batchwise addition of starch[6]. A critical evaluation of the application of reflectance measurements to the direct analysis of solid mixtures has established the fact that analytically useful data can usually be obtained with samples in powdered form[7]. In view of these results, it was decided to investigate the analytical applications of spectral reflectance to thin-layer chromatography using, as a pilot system, water-soluble dyes and aluminum oxide plates prepared according to a method devised by MOTTIER[8]. This system, in that it is stable and absorbs in the visible portion of the spectrum, lent itself most conveniently to the purpose at hand.

EXPERIMENTAL

Stock solutions containing 50 mg of the dyes studied (aniline blue, eosine B, basic fuchsin, malachite green, naphthol yellow S, and rhodamine B) per 100 ml of solvent were applied as spots by means of a 10 μl Hamilton microsyringe. Except for the aqueous eosine B, the solvent used was 95% ethanol. The 10 × 7 × 0.15 cm plates were cut from ordinary window glass and were coated with adsorbent by distributing the adsorbent–water mixture with a glass rod which rested on one thickness of masking tape affixed to the ends of the plates. This technique gave a uniform coating 0.2–0.3 mm thick. The plates were dried at 180° for 2 h and stored in a desiccator. Merck aluminum oxide G and silica gel G were used as adsorbents.

The dyes were chromatographed in *n*-butanol–ethanol–water (80:20:10 by volume) by the ascending technique according to MOTTIER[8], and the plates were then dried at 110° for 15 min. Direct spectral examination of these plates was accomplished by covering them with a clean glass plate of identical dimensions, fixing the ends together with masking tape, and then introducing them into the reflectance attachment of the Beckman Model DK-2 Spectrophotometer employed for this purpose. A sheet of paper, resembling in color the adsorbent material being used as a reference standard, was inserted behind the plate to serve as a reflecting background. The reference standard was prepared by grinding some of the adsorbent from the plate under examination and packing it into the cell described by BARNES *et al.*[9].

A Beckman Model DU Spectrophotometer fitted with a standard attachment for the measurement of diffuse reflectance was employed to examine spots scraped off chromatographic plates. The cells used to hold both sample and reference material consisted of white paper, of a size that permitted its introduction into the sample holder of the reflectance attachment, to which a microscope cover glass had been affixed by two pieces of tape. Fifty milligrams of material were carefully compressed between the cover glass and the paper until a thin layer having an approximate thickness of 0.3 mm and an approximate diameter of 1.8 cm was obtained. This last was necessary, since the impinging beam of light had an approximate diameter of 1.4 cm. As before, the reference standard consisted of adsorbent from the plate under examination.

RESULTS AND DISCUSSION

Direct examination of chromatographic plates

As indicated in Fig. 1, which contrasts the transmittance spectrum of an aqueous solution of eosine B with the reflectance spectra of $2.5 \cdot 10^{-3}$ mg of the dye adsorbed on

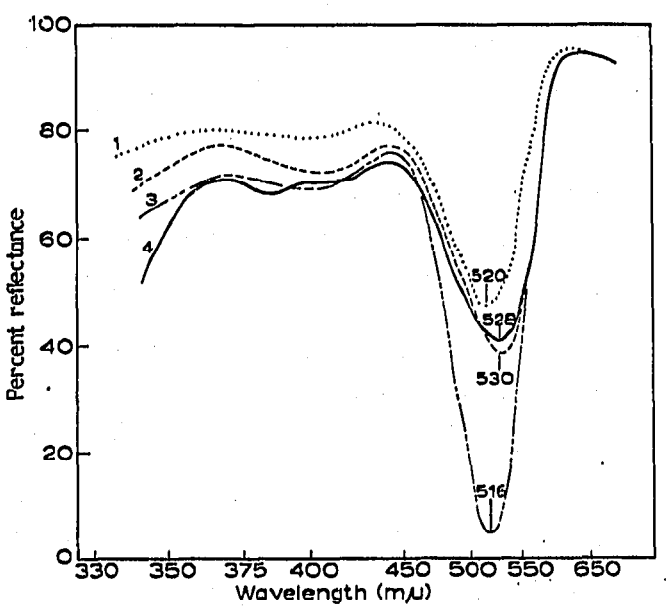

Fig. 1. Reflectance spectra of eosine B adsorbed on filter paper, alumina, and silica gel compared with the transmittance spectrum of an aqueous solution of the dye. (1) Silica gel (Merck thin-layer chromatography grade). (2) Filter paper (Whatman No. 42). (3) Alumina (Merck thin-layer chromatography grade). (4) Transmittance spectrum.

filter paper, alumina and silica gel, the spectra obtained for the different dyes were influenced by the nature of the adsorbent employed. The positions of the absorption maxima obtained for the dyes under various experimental conditions are summarized in Table I. In all cases the absorption maxima obtained for transmittance shifted to

TABLE I

ABSORPTION MAXIMA OF TRANSMITTANCE AND REFLECTANCE SPECTRA OF DYES

Dye	Transmittance*		Reflectance*		
	H_2O	EtOH	Filter paper	Alumina	Silica gel
Aniline blue	—	600	615	594	592
Eosine B	516	524	530	528	520
Basic fuchsin	—	549	553	550	540
Malachite green	—	620	628	615	615
Naphthol yellow S	—	435/389	443/394	436/391	431/390
Rhodamine B	—	546	550	550	547

* Readings are given as mμ.

higher wave-lengths when the reflectance spectra of the dyes adsorbed on Whatman No. 42 filter paper were determined. These results agree substantially with those of YAMAGUCHI *et al.*[2]. That no such general trend is observed in the case of the alumina or the silica gel is probably attributable to the larger number of experimental variables introduced by their employment. For example, ZEITLIN AND NIIMOTO[10] have shown that spectral displacements depend, in part, upon the extent of subdivision of the adsorbent. For these reasons, the standardized procedure described above was employed for the preparation and development of the plates. There was no discernible change in the reflectance of dyes separated on plates which had been stored in a desiccator over silica gel for periods up to three days following their development. It might also be noted that a complete inhibition of the fluorescence exhibited by eosine B and rhodamine B in transmittance measurements occurs when the dyes are adsorbed on filter paper, alumina or silica gel.

With proper precautions it is possible by direct examination of chromatographic plates to obtain spectra suitable for identification purposes as shown in Fig. 2a and 2b, which depict the reflectance spectra for the various dyes adsorbed on alumina. Spots having diameters as small as 5 mm could be centered by using the red portion of the visible spectrum and measured with ease. In the resolution of complex dye mixtures, a spot separation of one centimeter sufficed to permit spectral identification of the component dyes.

The quantitative potential of the technique was demonstrated by examining plates spotted with dilution series of the dyes. Fig. 3, which shows the reflectance spectra of various concentrations of eosine B adsorbed on alumina, typifies the data obtained during this study. The solutions were added in 5 μl increments to give spots whose diameters approximated 1 cm. As indicated above, the thickness and particle size of the adsorbent and the drying temperature of the plates were kept fairly constant. It was found that excessive tailing decreased the precision of measurements, and an effort was made to keep it to a minimum. Precision was likewise affected by the im-

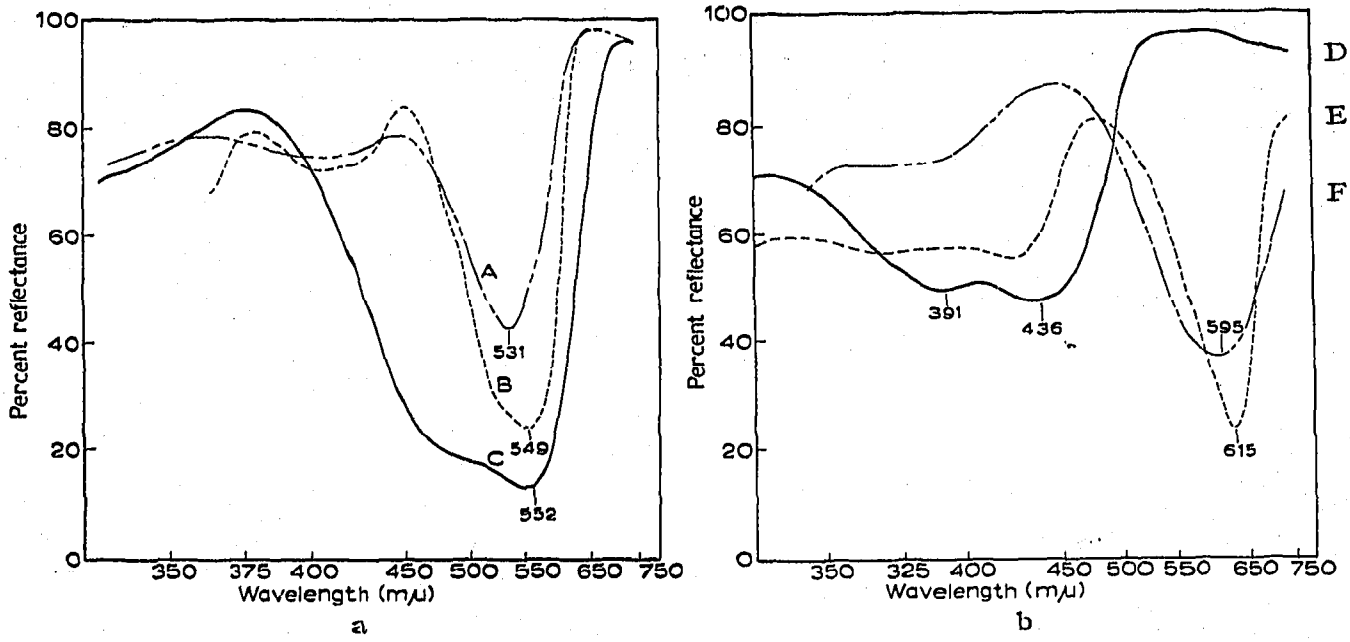

Fig. 2. (a) Reflectance spectra of dyes adsorbed on alumina. (A) Eosine B. (B) Rhodamine B. (C) Fuchsin. (b) Reflectance spectra of dyes adsorbed on alumina. (D) Naphthol yellow S. (E) Malachite green. (F) Aniline blue.

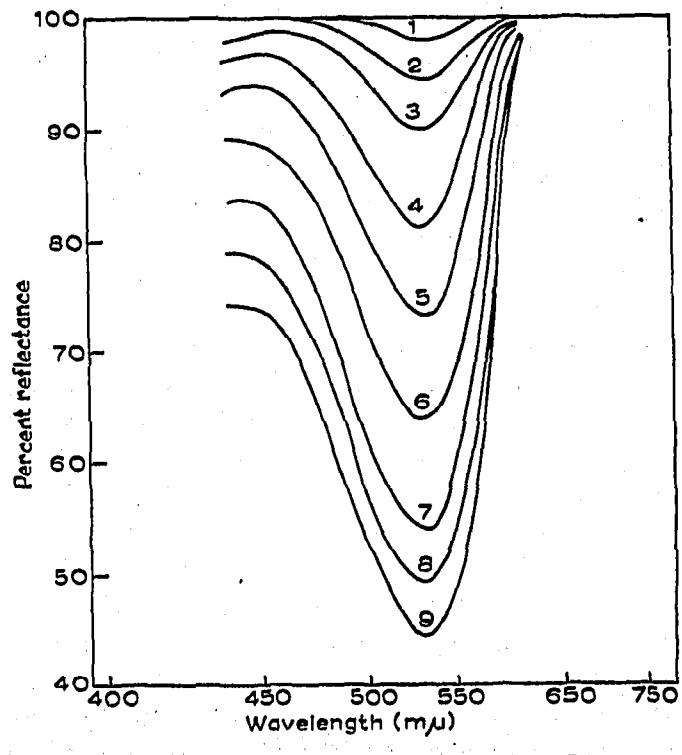

Fig. 3. Reflectance spectra of various concentrations of eosine B adsorbed on alumina. Concentrations in mg/100 ml: (1) 0.78; (2) 1.56; (3) 3.12; (4) 6.25; (5) 12.5; (6) 25.0; (7) 50.0; (8) 75.0; (9) 100.0.

proper positioning of the spot in the impinging light beam, and care was taken to center it in the manner described previously. No significant changes in reflectance were observed over a period of a week when the plates were stored in a desiccator in the dark. When these precautions were taken, the largest difference in reflectance found between any two members of thirteen sets of triplicate samples of eosine B was 4.0 units on the 100-unit reflectance scale. These samples represented a concentration range of $5 \cdot 10^{-3}$ to $4 \cdot 10^{-5}$ mg of added dye. The data for this study are presented in Table II, which indicates the reproducibility that can be expected for reflectance

TABLE II

REPRODUCIBILITY OF REFLECTANCE READINGS AT 530 mμ OBTAINED FOR DIFFERENT SPOTS OF THE SAME CONCENTRATION OF EOSINE B ADSORBED ON ALUMINA

Concentrations of dilution series (mg dye/100 ml soln.)	*Trial*		
	1 (%R)	*2* (%R)	*3* (%R)
0.78	98.0	98.5	98.4
1.56	94.5	93.8	93.5
3.12	89.5	91.0	91.0
4.30	87.0	88.0	87.3
6.25	83.0	82.0	81.5
8.50	79.0	77.5	78.5
12.50	76.5	74.0	73.5
18.00	71.0	71.5	69.0
25.00	66.5	62.5	63.5
37.50	60.0	59.3	59.5
50.00	55.0	55.5	54.0
75.00	49.0	50.0	49.0
100.00	44.5	47.0	45.0

readings obtained for different spots of the same concentration. A consideration of Fig. 4, in which these data are plotted in the form 2—log % *R versus* concentration, reveals that Beer's law holds only as a limiting law for reflectance and that a linear relationship obtains only for concentrations less than 5 mg of dye/100 ml of solution. As may also be seen in Fig. 4, plotting the same data in the form 2—log % *R versus* the square root of the concentration extends this linear relationship ten-fold to an upper limit of 50 mg/100 ml. The same relation between reflectance and concentration was found to exist for rhodamine B. These results are in agreement with those obtained by YAMAGUCHI *et al.*[3] for different food dyes adsorbed on filter paper.

Examination of spots removed from chromatographic plates

The precision attained in the determination of the adsorbed dyes by the direct examination of the chromatographic plates was improved by scraping the spots off the plates and measuring the reflectance of this material with the cell described above. Such a device proved to be necessary, as the amount of material removed from the plates was insufficient to fill the cells available for this purpose. The addition of more alumina was undesirable, since it introduced a considerable dilution factor that decreased the sensitivity of the method. Using this improved cell and taking precautions to insure a homogeneous sample of relatively uniform particle size, it was possible to

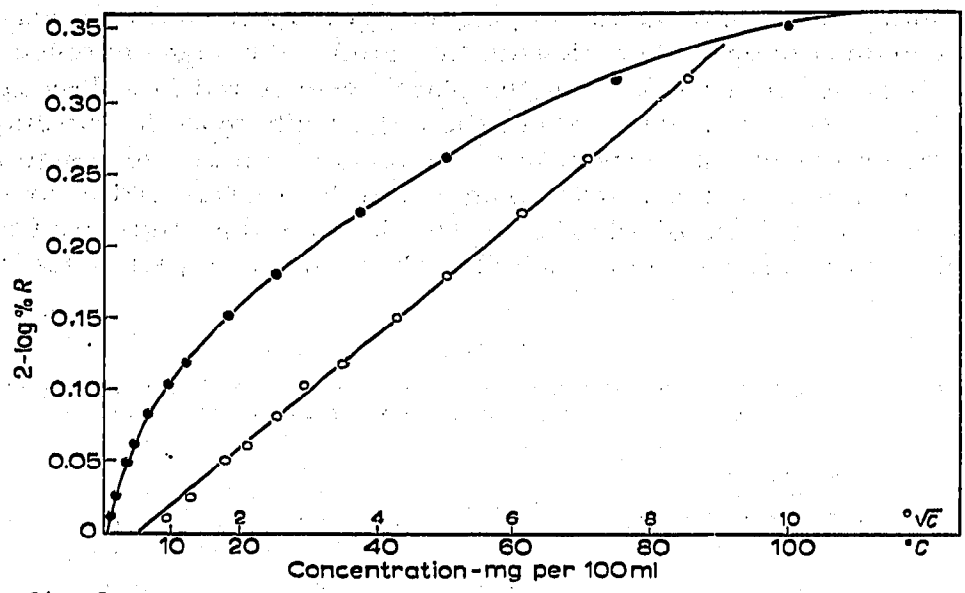

Fig. 4. 2—log % reflectance at 530 mμ of eosine B adsorbed on alumina as a function of concentration. ●——● C; ○——○ $C^{\frac{1}{2}}$.

get reflectance readings for replicate samples which differed by no more than 0.6 reflectance units. This is indicated in Table III, which lists readings obtained at various wave lengths for three different samples of eosine B of identical concentration.

The procedure employed was identical with that followed in the determination of the dyes by direct examination up to the point the sample material was removed from the plate. The 50 mg comprising the sample were weighed to ± 0.2 mg and then ground in a small agate mortar for two periods of 15 sec each to insure homogeneity and uniform

TABLE III

REPRODUCIBILITY OBTAINED FOR DIFFERENT SAMPLES OF IDENTICAL CONCENTRATIONS OF EOSINE B ADSORBED ON ALUMINA

Wave length (mμ)	*Sample I (%R)*	*Sample II (%R)*	*Sample III (%R)*
540	85.7	86.1	86.3
530	85.2	85.4	85.7
520	85.9	86.3	86.4

particle size. The grinding procedure was standardized, as it was found that a measurable difference in reflectance was produced by varying the operation. LERMOND AND ROGERS[7] reported similar results for the screening of sample materials. The greatest difficulty encountered was the attainment of reproducible packing of the sample in the cell. To assure the degree of precision achieved in the test of reproducibility which is summarized in Table IV, it is essential that the samples have approximately the same diameter and thickness and possess a uniformly smooth surface. The largest observed difference for any pair of readings obtained for the same sample repacked in the same holder was 0.7 reflectance units. When one considers that these differences are of the same order as the ones obtained for the replicate samples listed in Table III, it becomes

TABLE IV

TEST OF REPRODUCIBILITY OF PACKING REFLECTANCE CELL. EOSINE B ADSORBED ON ALUMINA

Wave length (mμ)	*1st packing (%R)*	*2nd packing (%R)*	*3rd packing (%R)*
620	98.4	98.4	99.0
600	97.2	96.8	97.2
580	93.3	93.7	93.8
560	89.0	89.1	89.5
540	86.9	87.2	86.5
530	86.7	86.8	86.3
520	87.2	87.2	86.9
500	89.6	89.6	89.6
480	91.8	91.9	91.9
460	93.4	93.7	93.8

apparent that the precision of the technique is limited by the reproducibility of packing the sample.

The relationship between reflectance and the concentration of eosine B adsorbed on alumina was investigated again, this time by means of the spot removal technique. A plot of the data obtained in the form 2—log % *R versus* concentration gave the same type of smooth curve as was obtained by direct examination and depicted in Fig. 4. The only notable difference was the upward extension of the linear relationship to a dye concentration of 20 mg/100 ml of solution. It is possible to extend this upward even further to 40 mg/100 ml if, as is done in Fig. 5, the concentration is plotted

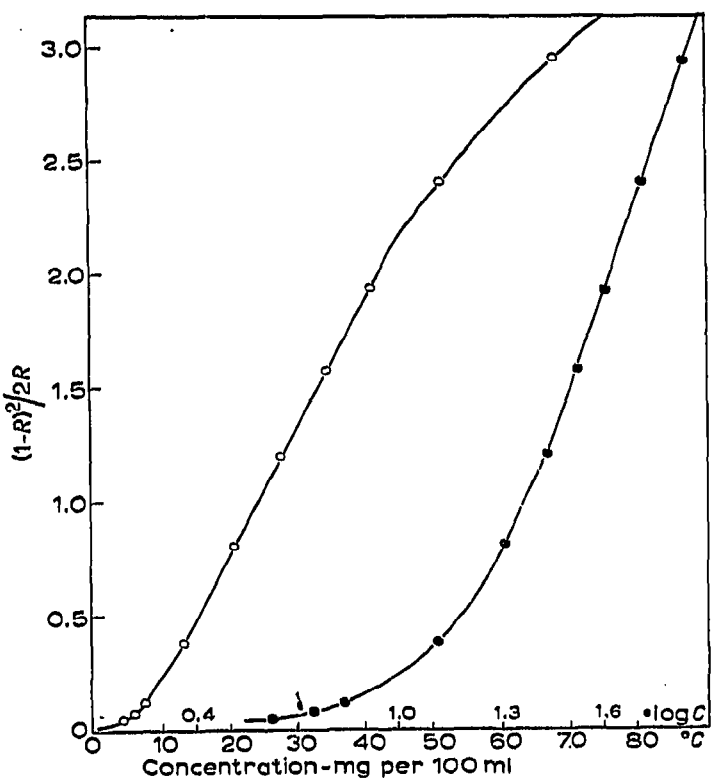

Fig. 5. Kubelka-Munk values at 530 mμ for eosine B adsorbed on alumina as a function of concentration. O——O C'; ●——● log C'.

against $(I-R)^2/2R$, the form of the Kubelka-Munk expression most often used[11]. Beyond this point, the curve is so smooth that it can serve a quantitative function. By plotting the logarithm of the concentration *versus* $(I-R)^2/2R$, as shown in Fig. 5, it is possible, if this is desired, to expand the linear relationship to the highest concentration studied, 350 mg/100 ml.

Finally, some measurements were made on a dilution series using the cell without the glass cover, which increased readings as much as 6.0 reflectance units at 530 mμ. For samples having a smooth surface, the precision was unaffected by the removal of the glass. Utilization of the cell without the glass cover might be desirable when dealing with samples of low reflectance or with spectral regions requiring a quartz plate.

The above results show that the components of complex mixtures of dyes separated on thin-layer plates can be identified rapidly, without recourse to R_F values, by direct examination of the plates by spectral reflectance. The same operation is capable of providing quantitative data having a precision of approximately ± 5%. A degree of precision identical to that afforded by transmittance is attained if the reflectance measurements are carried out on spots removed from the chromatographic plates and packed in an appropriate cell. By applying the Kubelka-Munk function, linear reflectance-concentration relationships can be obtained for concentration ranges of interest to the analytical chemist. Although the present study was restricted to the visible portion of the spectrum, other regions can be used as well[12] if the cell is provided with a quartz plate or employed without a cover. In general, one can say that the application of spectral reflectance to thin-layer chromatography would enhance its utility greatly by simplifying and expediting the analysis of complex mixtures.

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SUMMARY

The components of dye mixtures resolved on thin-layer plates were identified by direct examination of the plates by spectral reflectance The amounts of adsorbed dye were determined at the same time with a precision of approximately ± 5%. Reflectance measurements carried out on spots removed from the plates and packed in an appropriate cell afforded a degree of precision identical to that attained with transmittance.

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