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DUAL-WAVELENGTH POINT ZIG-ZAG SCANNING OF ZONES ON THIN-LAYER CHROMATOGRAMS AS A TOOL FOR QUANTITATIVE ASSAY

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SUMMARY

Dual-wavelength difference photometry has been applied to zones on chromatograms. A zone was scanned, in two dimensions alternately, by two very narrow wavelength-selected pulses of light; the difference in the logarithm of the reflectance measurements between the two wavelengths was converted electronically into an absorption coefficient proportional to concentration according to the Kubelka–Munk equations, and this converted value was integrated two-dimensionally across the whole zone area. For zones of various sizes, shapes and concentrations, the integrated value was proportional to the applied solute concentration. Additional compensation was provided for coloured or UV-absorbing backgrounds that could not be compensated for by the dual-wavelength difference-photometric procedure.

INTRODUCTION

Thin-layer chromatography is a modern analytical technique, which permits the rapid and clear separation of a mixture into its components. For quantitative assay, however, each component separated as a zone is eluted, usually to give a transparent extract, which is assayed by spectrophotometry. This is because spectrophotometry of transparent materials is a well established technique and because the proportionality between absorbance and concentration (Beer's law) is obeyed for transparent extracts. Direct measurement of transmission, reflection or fluorescence of zones *in situ* on chromatograms is more useful if the direct assay gives more sensitive and more accurate results for the concentration of substance in the zones; in addition, the tedious elution procedure can be omitted. The advantages of these types of *in situ* measurement were stressed by Jork[1], Frei *et al.*[2] and Touchstone *et al.*[3],

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and achievements in the early stages of development were reviewed by Bush[4] and by Boulton[5,6]. Despite these promising advantages, the technique of direct photometry has not become fully established because of several difficulties, which are briefly summarized below.

(1) Beer's law is not obeyed for coloured zones on translucent thin-layer materials. The Kubelka–Munk equation[7–9], which is much more intricate with respect to the relationship between concentration and attenuance [$-\log T$ (transmittance) or $-\log R$ (reflectance) as a measure of light attenuation by both absorption and scattering][10], is expected to be obeyed for such translucent materials. A useful approximation of the general Kubelka–Munk equation to simpler analytical functions was reported by Goldman and Goodall[11,12] and by Treiber[13], with discussions on the errors included in the approximation. The Kubelka–Munk equation for the reflectance of infinitely thick layers has been applied by Hezel[14] for direct photometry. On the other hand, more general equations (including fluorescence, as well as scattering and absorption) were derived by Goldman[15].

(2) In order to examine the applicability of these general or approximate equations to zones on chromatograms, the photometric readings must be accurate and precise. However, the reading of attenuance at a single wavelength when a chromatogram is scanned along a line (denoted as the x axis) fluctuates considerably because the background of the white adsorbent on the plate is not completely homogeneous. This interferes with precise measurements and precludes further analysis of data.

(3) A light beam passed through a slit is commonly used as incident radiation. Such a slit produces a beam that illuminates a long narrow area in a round or elliptical zone in which the sample is distributed non-uniformly. The light transmitted through, or reflected from, the long narrow area is therefore composed of light fluxes of different intensities from different local densities along the y axis; the photo-current from a detector placed behind or above the zone thus gives a reading of average intensity. To eliminate the effect of local variations in density, a much smaller uniform area should be measured, or else the sample should be applied as a band before development in order to obtain uniform density along the y axis. Further, variations in size and shape of the zone introduce additional errors in readings by one-dimensional slit-beam scanning.

The second difficulty arising from background fluctuation due to scattering was overcome by using difference photometry with alternate dual-wavelength light pulses. This technique, which was developed by Chance[16,17] for reducing the fluctuation of the reading with time during a biochemical reaction in a translucent sample, was applied by Salganicoff *et al.*[18] and by Shibata[19] to reduce fluctuation in reading while scanning a translucent material along a line. The fluctuation due to scattering changes at a light-absorbing wavelength (λ_2) was compensated for by subtracting the fluctuation at a different wavelength (λ_1) at which the separated chromogen exhibited no absorption but experienced the same scattering fluctuations. In this way a very weak spot was recorded as a distinct peak on a straight horizontal base-line in an expanded full scale of the order of 0.1 as absorbance difference. The great improvement in sensitivity and accuracy attainable by dual-wavelength photometry of chromatograms has been discussed theoretically by Boulton and Pollak[20–23]. The use of an opal-glass plate behind the sample further improved the result when scattering

by the sample was dependent on wavelength[10,19], although such a diffuser was not necessary for thin-layer chromatograms, where the scattering was independent of wavelength. The precise measurements thus achieved made it possible to solve the remaining problems.

In the third problem, due to local density variation, a zone was scanned in two dimensions[21,24]. In the present experiment, the stage on which the chromatogram was mounted was moved in a zig-zag manner in order to scan the zone two-dimensionally with dual-wavelength light pulses having a very minute cross-section. The light reflected from the small area was measured to obtain a signal of $D = \log(1/R_2) - \log(1/R_1)$, in which R_1 and R_2 are the reflectances at λ_1 and λ_2, respectively. These wavelengths were so selected that λ_2 included the absorption maxima of the substance chromatographed, but λ_1 did not include absorption by the separated chromogen.

In the present study, the applicability of the general Kubelka–Munk equations was examined without approximation. To achieve this, an electronic device referred to in this paper as a "linearizer" was used. The Kubelka–Munk equations for R_1 and R_2 can be expressed as follows:

$$R_1 = \frac{S}{S + 1} \tag{1}$$

$$R_2 = \frac{\sinh bS}{a \sinh bS + b \cosh bS} \tag{2}$$

$$D = \log(R_1/R_2) \tag{3}$$

where

$$K = kd \text{ and } S = sd$$

$$a = \frac{S + K}{S} \text{ and } b = (a^2 - 1)^{\frac{1}{2}}$$

d being the thickness of the thin layer, s the scattering coefficient per unit thickness, and k the absorption coefficient per unit thickness, which is proportional to the substance concentration.

The two curves in Fig. 1 are examples showing the relationship between D and K at $S = 3$ and 7, respectively. It was assumed in the calculation that the scattering coefficient was identical at the two wavelengths, and that the light-scattering layer did not absorb light. The linearizer is an electronic device that converts the signal of D for the minute uniform area into a signal proportional to K for a pre-set value of S according to the above forms of the Kubelka–Munk equations; similar conversion by means of a computer was made by Goldman and Goodall[12], and the theoretical basis for its electronic simulation was reported by Pollak and Boulton[25,26]. Recording of K during the zig-zag scanning of a zone in the present experiments gave many spikes, as shown by curve A in Fig. 2b. The value of K for the minute square area was integrated to obtain the area (denoted as Ky) of each spike, and the values of Ky for many spikes were summed to obtain the zone volume (denoted as Kxy; see curve B in Fig. 2b) with the K value taken as the height. The parameter S was so

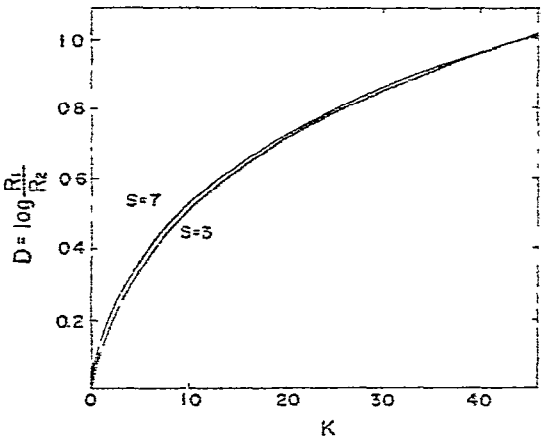

Fig. 1. Theoretical curves relating D and K calculated according to the Kubelka–Munk equations (see eqns. 1, 2 and 3 in the text) at values of $S = 3$ and 7.

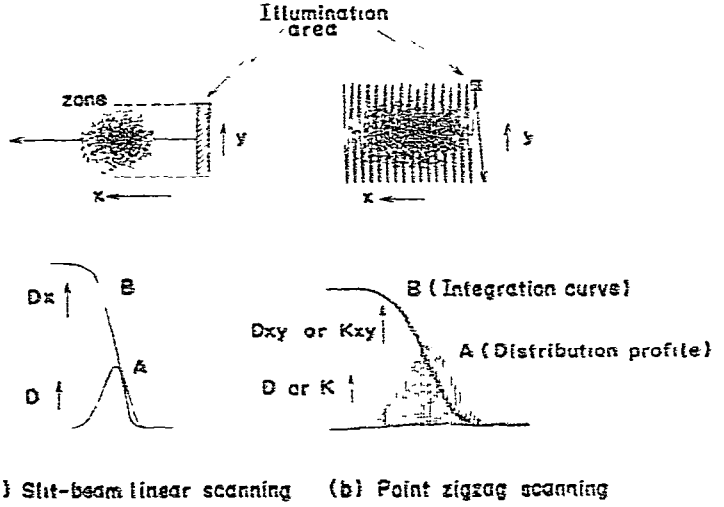

Fig 2. Distribution profile and integration curve obtained by point zig-zag scanning (b) as compared with those obtained by slit-beam linear scanning (a).

varied as to obtain proportionality between Kxy and substance content. When the layer thickness (d) is constant, the value of K should be proportional to the substance concentration in the minute area, so that the value of Kxy integrated over the whole zone area should be proportional to the substance content in the zone. Two other methods of measurements were used for comparison; in one, the value of D for the minute area before the conversion was integrated to estimate the zone volume (Dxy) with the D value taken as the height, and in the second, the average reading of D for the long area in the zone of the slit-beam linear scanning system (Fig. 2a) integrated along the x axis to obtain a reading denoted as Dx for the whole zone[27].

The adsorbents for thin-layer chromatography sometimes contain impurities that run with the solvent front during chromatography and are distributed non-

uniformly on the plate. Treatment with a spray reagent after chromatography often causes a similar non-uniform coloration of the background. Non-uniform background colour of UV absorption, which cannot be cancelled by dual-wavelength difference photometry because of its wavelength-dependent absorption, distorts the base-line. An example of such distortion by contamination near the solvent front is shown in Figs. 3a and 3b (in Fig. 3a, the pitch along the x axis in the point zig-zag scanning relative to the actual zone dimensions is much exaggerated for illustrative purposes). The fluctuation of the reading of D at the starting points of integration (dots in Figs. 3a and 3b) is due to such contamination. An electronic unit called a "background compensator" was developed to nullify the value of D at the starting points so as to obtain spikes on a straight base-line, as shown in Fig. 3c.

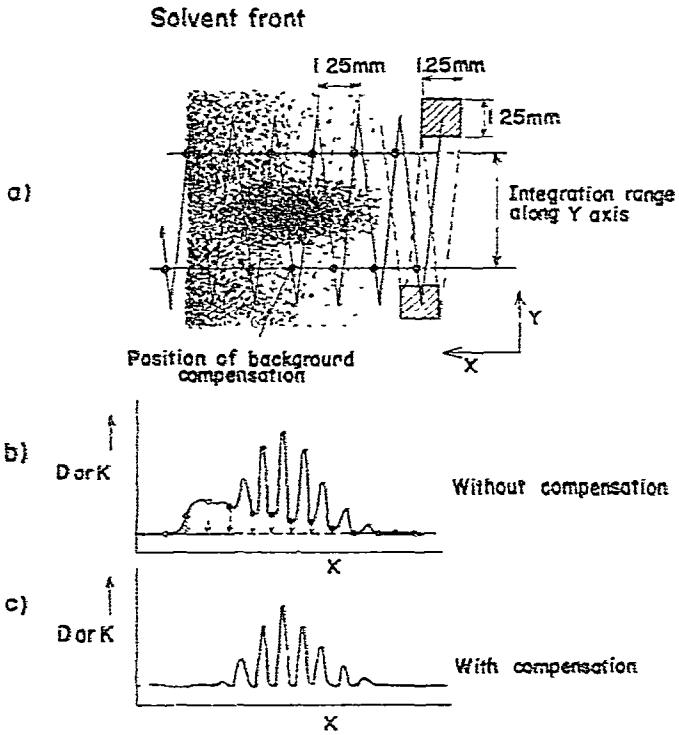

Fig. 3. Zig-zag scanning and compensation for contaminated background: (a) trace of the zig-zag scanning light beam across a zone near a contaminated solvent front; (b) distribution profile obtained without compensation; (c) distribution profile obtained with compensation. The hatched 1·25-mm-square blocks refer to the size and shape of the light beam cross-section, and the continuous and broken lines refer to the traces of the block centre and edges, respectively. The pitch of scanning was set to be identical with the dimension (1.25 mm) of the square block, so that the entire area including the sample zone was scanned exactly twice. The pitch along the x axis is exaggerated for illustrative purposes.

EXPERIMENTAL

Instrument and method

The dual-wavelength zig-zag scanner developed from the one described earlier[19] is illustrated schematically in Fig. 4. The optical systems are the same as before,

except that two small grating monochromators were specially designed to illuminate a very small area vertically and alternately with two wavelength-separated light pulses. Part of the reflected light fell on a photomultiplier (Hamamatsu TV R446), and the alternate photo-currents for the two wavelengths were led to a logarithmic amplifier, background compensator, linearizer, integrator and, finally, recorder. For the two-dimensional scanning, the light beam was fixed, and the stage carrying the thin-layer plate was moved in a zig-zag manner. The area illuminated by the incident beam measured 1.25 × 1.25 mm (the pitch along the x axis on each scanning along the y axis was 1.25 mm), so that the beam scanned the entire area including the sample zone exactly twice (see Fig. 3a).

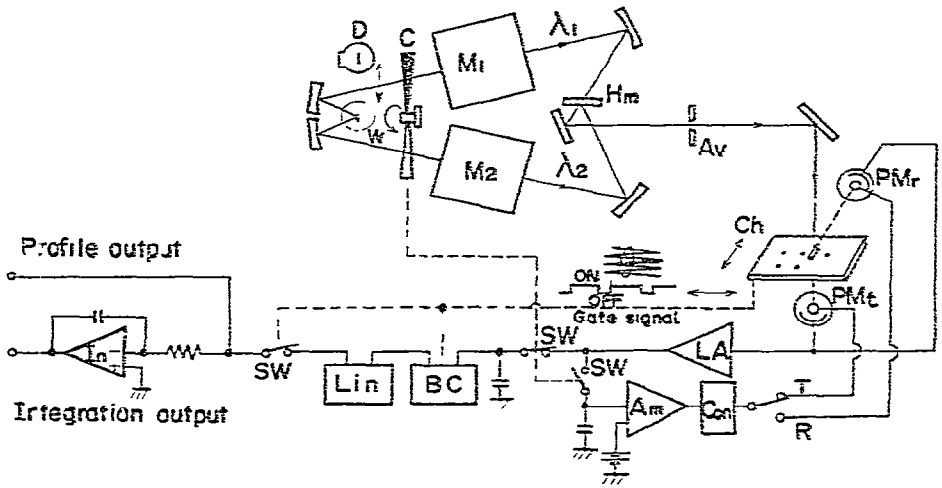

Fig. 4. Schematic diagram of a dual-wavelength zig-zag scanner. W = tungsten lamp; D = deuterium lamp; C = chopper; M_1, M_2 = monochromators; Hm = half mirror; Av = variable aperture, Ch = chromatogram; PMr, PMt = photomultipliers (PMr for reflectometry as applied in the present study); Con = d.c.–d.c. converter; Am = amplifier; LA = logarithmic amplifier; SW = gate switch; BC = background compensator; Lin = linearizer; In = analogue integrator.

The signal D was converted into a signal of K for a fixed value of S pre-set on the linearizer (the appropriate value of S was dependent on the adsorbent material, as will be demonstrated later). Five points were chosen at intervals on the theoretical curve (Fig. 1) for the appropriate value of S, and the five pairs of D and K values for these points were pre-set on the linearizer. The linearizer was so constructed that the output signal was proportional to the values of K corresponding to the signal of D on the curve passing through the five points and the origin. When the background was contaminated, the signal of D was passed through the background compensator before being led to the linearizer. The reading of D at each starting point of integration, which had been memorized in the compensator, was subtracted from the D values during scanning along the y axis, so that a profile on a straight zero-line (Fig. 3c) was obtained.

The K value was recorded with one of the dual pens on the recorder to obtain a distribution profile composed of many spikes, such as shown by curve A in Fig. 2b.

The signal of K from the linearizer was led to an integrator to obtain a signal of Kxy, which was recorded with another pen to obtain an integration curve such as shown by curve B in Fig. 2b. The plateau height of the integration curve was examined for its proportionality to the substance content in the zone. For comparison, the D values on the zig-zag scanning and the D values on the slit-beam linear scanning were integrated without the conversion to estimate the plateau heights, Dxy and Dx, respectively.

Materials for chromatography

The samples examined by the various techniques were caffeine, phenacetin and methyl yellow (Wako Junyaku, Osaka, Japan), erythrosine and Rose bengal (Tokyo Chemical Industry, Tokyo, Japan), various steroids (Applied Science Labs., State College, Pa., U.S.A.) and nicotinamide (Wako Junyaku). These compounds, and the solvents used for development, were of reagent grade. Plates pre-coated with silica gel, alumina or cellulose powder (layer thickness 0.25 mm: Merck, Darmstadt, G.F.R., or Wako Junyaku), or home-made plates coated with silica gel (thickness = 0.25 mm; Wako Junyaku) by means of a spreader (Mitsumi Kagaku Sangyo) were used for thin-layer chromatography. Paper chromatography was carried out on Toyo No. 51 paper.

RESULTS AND DISCUSSION

Effects of zone shape and size

The two-dimensional scanning was effective in obtaining reproducible readings for a zone of irregular shape. The data in Fig. 5 show an example obtained for a deformed elliptical zone of methyl yellow that had been developed on a layer of silica gel. The zone was scanned by dual-wavelength reflectometry in two ways (both one- and two-dimensionally), through its centre along three lines at different angles (A, B and C in Fig. 5). The linear scanning was made with a slit-beam with its length in cross-section adjusted to the width of the zone for each angle of scanning, and the two-dimensional scanning was made with a spot-beam with a minute square cross-section by moving the stage carrying the thin layer in a zig-zag manner. The values of D in these measurements were integrated over the whole area without being converted into K values. The distribution profiles and integration curves obtained by these measurement techniques are shown in the upper and lower parts, respectively, of Fig. 5. As expected, the point zig-zag scanning gave much better reproducibility. The integrated levels of Dxy obtained by the zig-zag scanning at the three angles agreed with each other, whereas the integrated levels of Dx obtained by slit-beam linear scanning at the three angles differed considerably.

This consistency in Dxy values measured at different angles for the same zone does not necessarily assure consistency of Dxy values for different zones containing the same amount of sample, but distributed differently. The D value for the minute area is not proportional to the substance content, so that a change in distribution may result in a change in the integrated Dxy value. Values of Dxy without the conversion (shown in Fig. 6 for three zones containing the same amount of methyl yellow) exhibit great variation; the Dxy level for the largest zone (C) is higher by 25% than the Dxy level for the smallest zone (B). The conversion into a K value

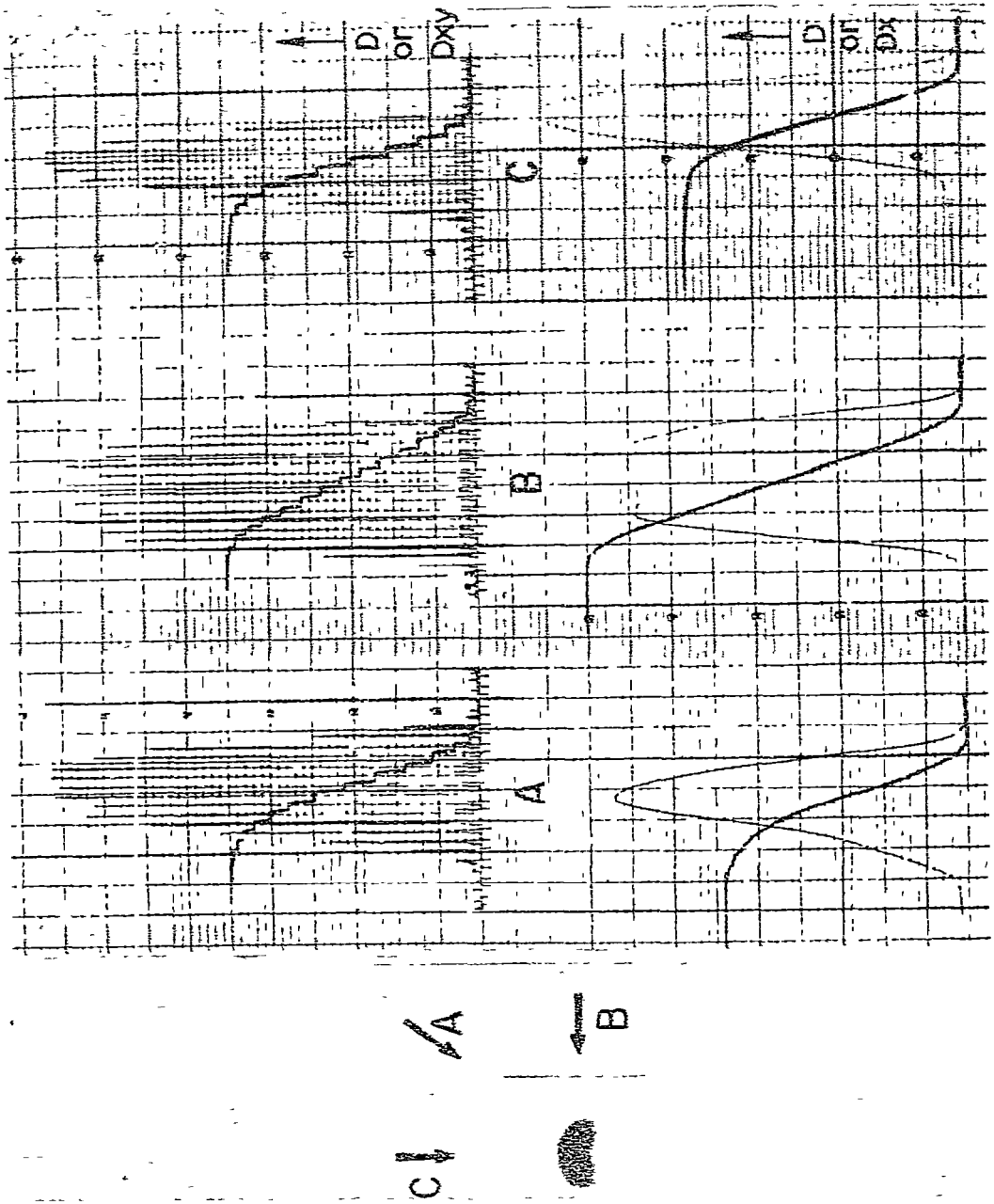

Fig. 5. Distribution profile in D and its integrated Dxy curve (upper figures) of an irregularly shaped zone of methyl yellow measured by the point zig-zag scanning method as compared with the profile and its integrated Dx curve (lower figures) for the same zone measured by slit-beam linear scanning. Scanning was by dual-wavelength reflectometry at 700 nm (λ_1) and 420 nm (λ_2) along three axes (A, B and C as shown on the left of the figure). The methyl yellow was separated on a silica gel plate (Merck F254), with methanol as developing solvent.

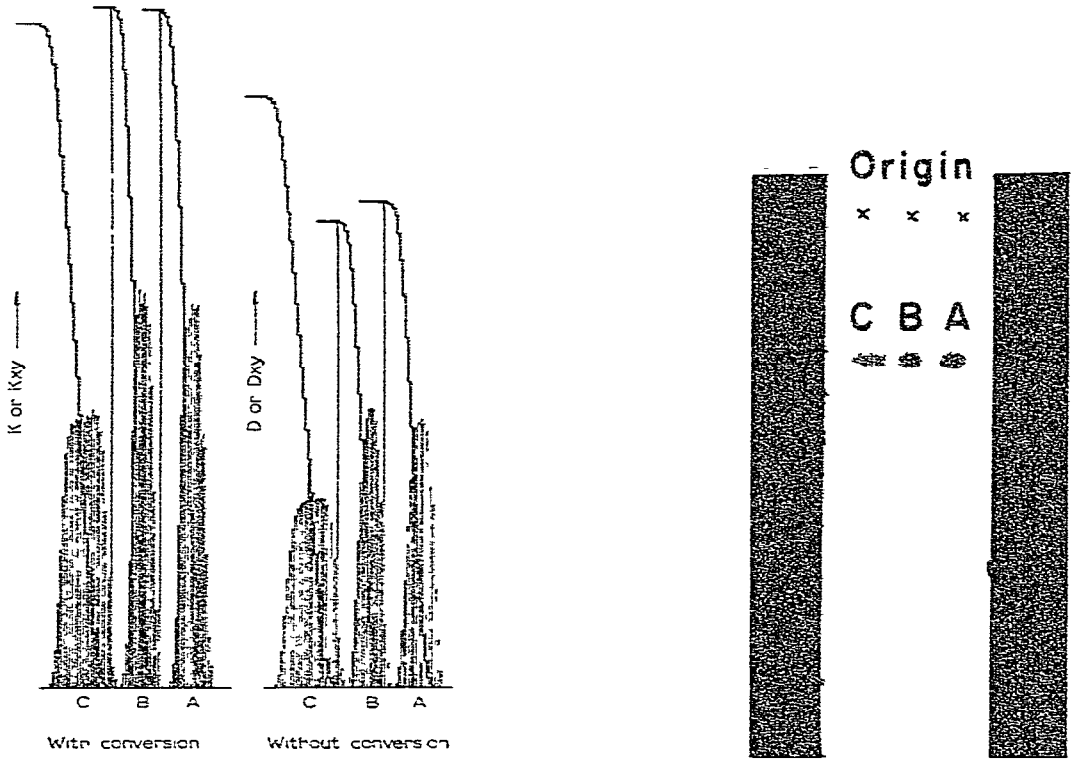

Fig. 6. Integrated *Kxy* values converted at $S = 3$, and *Dxy* values integrated without conversion, for three zones containing the same amount of methyl yellow but in different distributions. The methyl yellow was separated on a silica gel plate (Merck F254), with methanol as developing solvent. Dual wavelength reflectometry was carried out at 700 nm (λ_1) and 420 nm (λ_2)

proportional to the content before integration was necessary to obtain constancy of reading for such zones; the variation between *Kxy* levels for the same three spots (shown on the left of Fig. 6) is at most 2%. The constancy of reading for zones containing the same amount of sample thus achieved, together with the very low fluctuation in reading by dual-wavelength difference photometry, provided the basis for further precise analysis of data.

Dependency of Dx. Dxy and Kxy values on sample content

Fig. 7 shows the improvement in proportionality to the sample content that was achieved by zig-zag scanning of the spots in combination with conversion into *K* values. The data obtained by this combination technique for zones of caffeine (line A) and phenacetin (line C) developed on a silica gel plate (Merck F254) were compared with the data (curves B and D) for the same zones measured by slit-beam linear scanning without conversion. The conversion was made at $S = 3$, and the wavelengths for dual-wavelength reflectometry were 350 nm (λ_1) and 270 nm (λ_2); caffeine and phenacetin on silica gel show an absorption maximum ($\lambda_{max.}$) at 270 and 255 nm, respectively, and no absorption at 350 nm. The *Kxy* values obtained by integration of the *K* values ($S = 3$) measured by zig-zag scanning were proportional

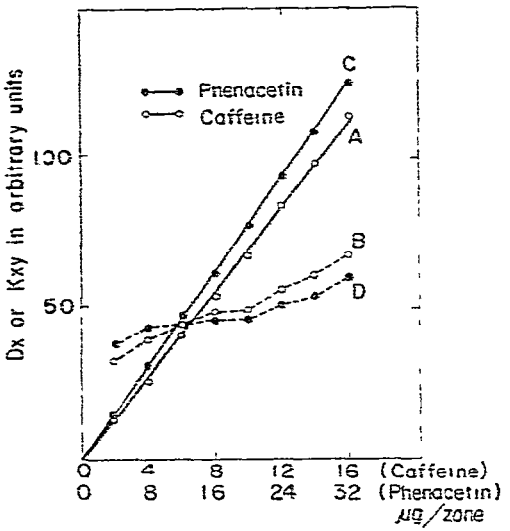

Fig 7. Relationship between *Dx* value measured by slit-beam linear scanning, *Kxy* values ($S = 3$) measured by point zig-zag scanning and zone concentration. Open circles on line A and curve B are values of *Kxy* and *Dx*, respectively, for zones of caffeine; solid circles on line C and curve D are values of *Kxy* and *Dx*, respectively, for zones of phenacetin. These substances were separated in chloroform–acetone (6:1; v/v) on a Merck F254 silica gel plate and measured by dual-wavelength reflectometry at 350 nm (λ_1) and 270 nm (λ_2).

to the sample content, but the *Dx* values obtained by integration without conversion of the *D* values measured by linear scanning showed a non-linear relationship. Similar measurements were made for testosterone ($\lambda_{max.} = 250$ nm), progesterone ($\lambda_{max.} = 260$ nm), corticosterone ($\lambda_{max.} = 255$ nm), cortisone ($\lambda_{max.} = 250$ nm) and hydrocortisone ($\lambda_{max.} = 250$ nm) on a silica gel plate at $\lambda_1 = 350$ nm and $\lambda_2 = 250$ nm, and for food dyes such as Rose bengal ($\lambda_{max.} = 550$ nm) and erythrosine ($\lambda_{max.} = 530$ nm) on the same plate at $\lambda_1 = 700$ nm and $\lambda_2 = 545$ nm. The *Kxy* values at $S = 3$ for these samples also showed close proportionality to the sample contents, but the dependencies of the *Dx* values showed curves.

The data in Fig. 8 show the effect of the conversion of *D* into *K* for the two-dimensional measurements. Zones containing different amounts of caffeine separated on a Merck silica gel plate were measured by point zig-zag scanning with and without the conversion, followed by integration at wavelengths of 350 nm (λ_1) and 270 nm (λ_2). The *Kxy* values obtained with the conversion at $S = 3$ (solid circles on line A) indicate proportionality to caffeine content, while the *Dxy* values (curve B) obtained without conversion are not proportional to the content.

The value of *S* appropriate for obtaining proportionality was dependent on the chromatographic adsorbent. Zones of the same sample of caffeine, but on a different plate (Wako FM pre-coated), were measured by zig-zag scanning reflectometry at the same wavelengths (350 and 270 nm). Proportionality in this instance occurred with *Kxy* values converted at $S = 7$, but not with values converted at $S = 3$, as can be seen from line C and curve D, respectively, in Fig. 8. Similar experiments with caffeine on a home-made silica gel (Wako B-5UA) plate at $\lambda_1 = 350$ nm and

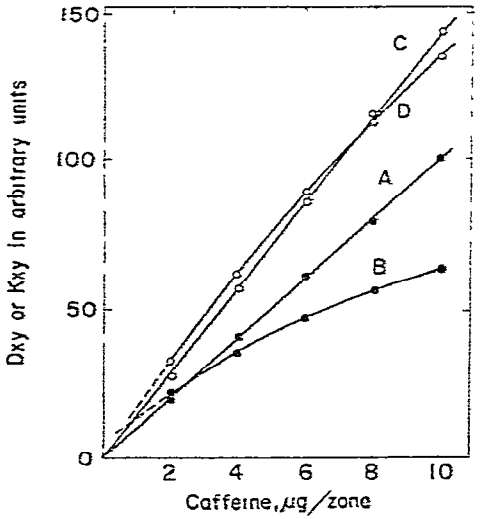

Fig. 8. Relationship between *Kxy* and *Dxy* values, with and without conversion, measured by point zig-zag scanning and caffeine concentration; *Kxy* values converted at $S = 3$ (line A) and *Dxy* values without conversion (curve B) for caffeine zones developed with chloroform–acetone (6:1) on a Merck F254 silica gel plate, and *Kxy* values converted at $S = 7$ (line C) and $S = 3$ (curve D) for caffeine zones developed with the same solvent mixture, but using a Wako FM plate. Measurements were made by dual-wavelength reflectometry at 350 nm (λ_1) and 270 nm (λ_2)

$\lambda_2 = 270$ or 285 nm, and with methyl yellow zones on a Merck cellulose plate at $\lambda_1 = 700$ nm and $\lambda_2 = 420$ nm, showed proportionality for *Kxy* values converted at $S = 3$. Fig. 9 shows data for methyl yellow zones ($\lambda_{max.} = 420$ nm) on Toyo No. 51 paper. In this instance, conversion at $S = 3$ gave *Kxy* values proportional to

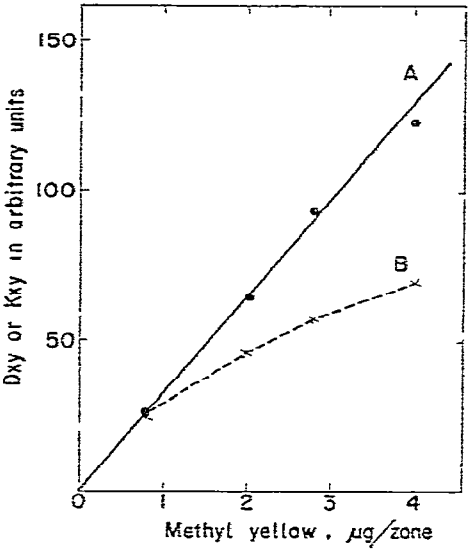

Fig. 9. *Kxy* values converted at $S = 3$ (line A) and *Dxy* values (curve B) measured by point zig-zag scanning for methyl yellow zones developed with chloroform on Toyo No. 51 paper, dual-wavelength reflectometry at 700 nm (λ_1) and 420 nm (λ_2)

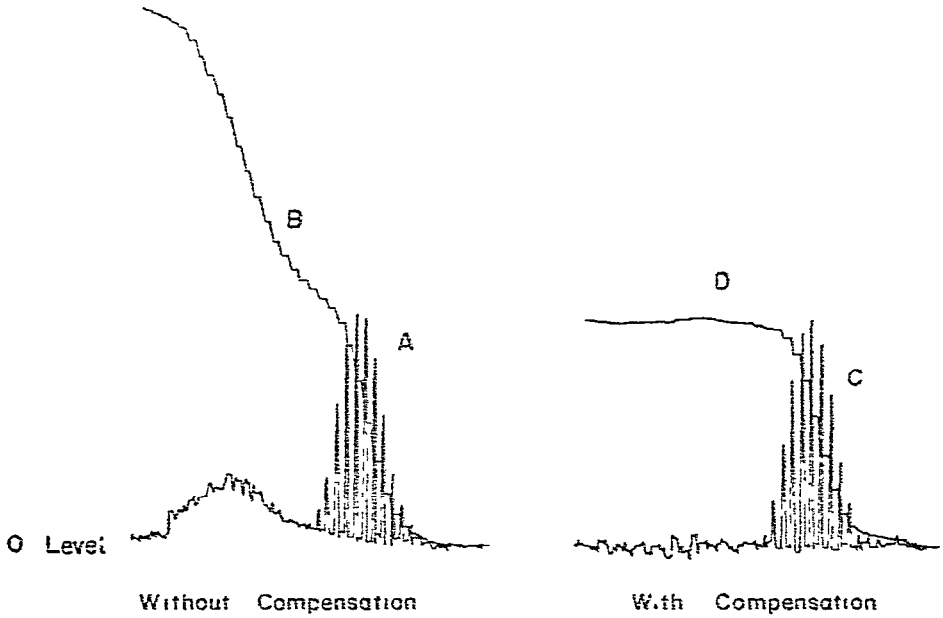

Fig. 10. Effect of background compensation in zig-zag scanning of a zone of nicotinamide on a contaminated background near the solvent front on a Merck silica gel plate; dual-wavelength reflectometry at 350 nm (λ_1) and 265 nm (λ_2). The nicotinamide was separated with methanol. Curves A and B are the K and Kxy signals, respectively, recorded without background compensation, curves C and D are the same signals recorded with compensation. The conversion into K was made at $S = 3$.

methyl yellow content (see line A). The Dxy values obtained by integration of D values without conversion are shown by curve B, which is bent at higher concentrations.

The two integration curves in Fig. 10 show the effect of background compensation observed for a zone of nicotinamide ($\lambda_{max.} = 265$ nm) developed on Merck silica gel. The integration curve (D) obtained with compensation shows stepwise increases in Kxy value to a plateau, whereas curve B (obtained without compensation) shows a further increase in Kxy value over the range of the plateau. This increase in Kxy value near the solvent front results from positive K values due to contamination around the zone, as can be seen from the distribution profile obtained without compensation.

All the data presented in this paper indicate the applicability of the general Kubelka–Munk equations for the measurement of absorbing zones separated on thin-layer chromatograms. This was demonstrated from the precise data obtained by the combined techniques of dual-wavelength difference reflectometry, two-dimensional zig-zag scanning and integration, conversion of D to K, and compensation for coloured background.

A similar investigation with transmission photometry as opposed to reflection photometry was also carried out, and will be reported elsewhere; the results may also be of value.

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