

CHROM. 5785

A CRITICAL STUDY OF SOME PARAMETERS IN QUANTITATIVE *IN SITU* INVESTIGATIONS OF THIN-LAYER CHROMATOGRAPHY BY LIGHT ABSORPTION METHODS

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(Received September 9th, 1971)

SUMMARY

Reflectance spectroscopy has been found to be superior to transmission measurements for the *in situ* evaluation of thin-layer chromatograms, contrary to several other published results. Explanations for these experimental facts are given. In the visible region of the spectrum, simulation of infinite layer thickness by inserting empty chromatoshets as a background, shows advantages in the reflectance mode. A double beam instrument is recommended when chromogenic sprays have to be used for visualization of the spots. With regard to time, cost and simplicity of a method, scanning of chromatographic zones with a slit is still considered the optimum approach to deal with inhomogeneity problems. The potential of adjustable lightbeams for single measurement techniques has been critically evaluated and found promising in the absence of tailing and if used with a modified Kubelka-Munk function. In conclusion *in situ* reflectance spectroscopy is considered a truly quantitative method, with errors between 2–5% to be expected. Chromatographic rather than instrumental parameters are likely to be responsible for errors of this magnitude.

INTRODUCTION

Densitometric techniques involving the measurement of both transmitted light¹ and diffusely reflected light^{1,2} have become quite common for the *in situ* quantitative investigation of thin-layer chromatograms. The utilization of light transmission phenomena for the evaluation of paper chromatograms has also been practised extensively³. Considerable controversy exists on the question of whether transmittance or reflectance techniques are to be preferred⁴ and it is the purpose of this study to critically compare the two modes by a suitable choice of experiments. Other parameters of interest include a comparison of double beam versus single beam scanning; an investigation of the effects of sample homogeneity and thickness on analytical results, and a statistical error analysis of such a method is given.

The system chosen for this work consisted of cobalt (II) ions separated on silica gel layers and sprayed with 4-(2-thiazolylazo)resorcinol. The resulting complex

is purple on a yellow-orange background. The advantage of this system is its high stability and the fact that it absorbs in the visible region of the spectrum which is the only region in which the two modes can really be compared objectively. Due to background fluctuations arising from the spraying step, the system is also well suited for a comparison of double and single beam modes.

EXPERIMENTAL

Reagents and equipment

Solvents, acids and cobalt nitrate stock solution were reagent grade. A spray solution of 0.1% 4-(2-thiazolylazo)-resorcinol (TAR) (Aldrich Chemical Co. Inc., Milwaukee, Wisc.) in 95% ethanol was used. The spray solution was filtered prior to use. Doubly distilled water was used throughout.

The quantitative reflectance and transmittance studies were carried out with a Zeiss Chromatogram Spectrophotometer with a Servogor Recorder RE 511 and with a Farrand VIS-UV Chromatogram Spectrophotometer. Detailed instrumental conditions for the direct scanning of chromatograms are given in the relevant sections.

Chromatography

The separation of cobalt was carried out on silica gel, Eastman precoated sheets No. 6064, with a solvent resistant polyvinyl alcohol binder (Eastman Organic Chemicals, Eastman-Kodak Co., Rochester, N.Y.). Prior to actual use, the sheets were purified by ascending development in a methanol-acetone-6 N HCl (5:5:1) solvent mixture. The chromatographic separation was carried out as described previously⁵. An aerosol atomizer was employed for the spraying procedure. The elution of the chromatograms with the acetone-6 N HCl-acetic acid-water (8:1:1:1) solvent system, the drying of the developed chromatograms in a cold airstream and the complexation of the cobalt spots with TAR, have already been reported in a previous paper⁵.

RESULTS AND DISCUSSION

A comparison of reflectance versus transmission

For these experiments, the same chromatogram sheet containing eight spots of 0.2 μg of cobalt each, were scanned as the TAR complexes at 580 nm after spraying. The Zeiss Chromatogram Spectrophotometer was used in the transmission and reflectance modes. Interference from the background due to the spraying with the chromogenic reagent was minimal under these conditions. The same slits and scanning speeds were used in both modes. The reproducibility study yielded a 4% rel. S.D. for the transmission measurements and approximately 2% rel. S.D. for the reflectance measurements.

For an investigation of dilution series, multiple spotting with micropipettes was used and initial spots were kept approximately 5 mm in diameter prior to development. The calibration curves for both the reflectance and transmission modes are shown in Fig. 1 and 2. Plotting peak area $\exp 2$ vs. concentration yields linear calibration curves for both modes up to 0.8 $\mu\text{g}/\text{spot}$.

The relationship

$$A^2 = kc \quad (1)$$

is actually a simplified but adequate version of the Kubelka-Munk function

$$F(R) = \frac{(1 - R)^2}{2R} = \frac{k}{s} \quad (2)$$

where k would be the absorption coefficient and s the scattering coefficient which is constant under existing experimental conditions. Function (1) applied to transmission measurements yields a linear plot over approximately the same concentration range

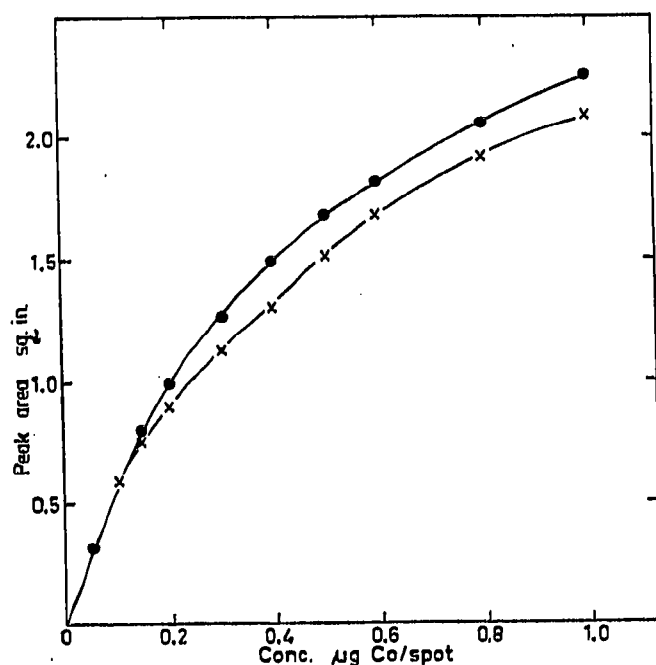


Fig. 1. Calibration curves peak area vs. concentration of cobalt obtained by *in situ* reflectance spectroscopy (●—●) and transmission spectroscopy (×—×). (Measured at 580 nm.)

but the plots do not extend through the origin. The use of Beer's law does not improve the situation for the transmission data. Contrary to statements made previously^{4,6,7}, the sensitivity has been found to be better for the reflectance than for the transmission mode (see Figs. 1, 2) in spite of the fact that the transparency of the chromatogram sheet is quite good at 580 nm. The superior reproducibility of reflectance measurements over transmittance measurements is another factor in favor of the former technique. This can be attributed to an enhanced influence of layer irregularities and scattering effect in the transmission mode. In this set of experiments the Zeiss instrument was chosen, since the same collection device is used for diffusely reflected, transmitted and scattered radiation. In the transmission mode the optical tube device is mounted in a vertical position directly underneath the sample spot, and in the reflectance mode directly on top of the spot in a slightly slanted position. The arrangement is therefore such that all parameters can be kept the same for both modes of operation or slightly in disfavour of reflectance, since the tube is slanted to permit elimination of specular reflectance. For instruments which

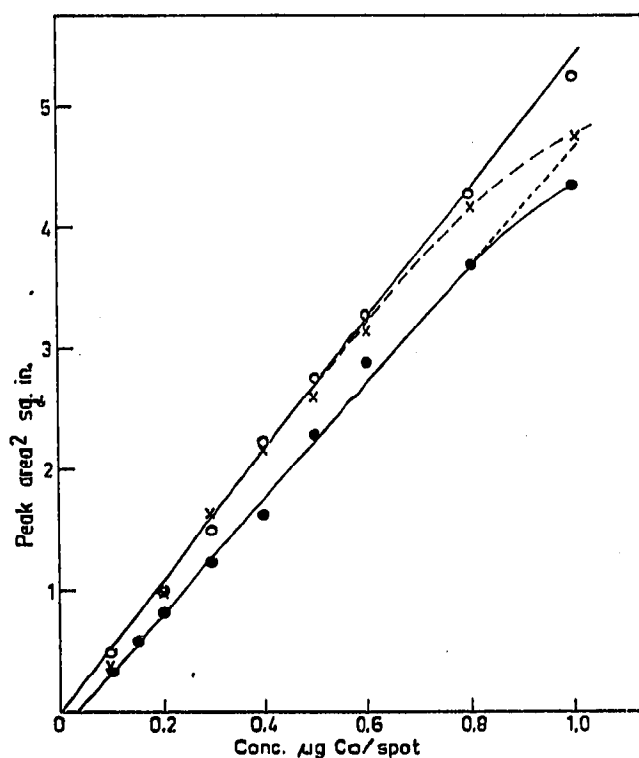


Fig. 2. Calibration curves peak area exp 2 vs. concentration, obtained by reflectance spectroscopy without backing sheets (x—x) for infinite layer thickness (2 backing sheets) (O—O) and by transmission spectroscopy (●—●).

are claimed to be more sensitive in the transmission mode⁷, it has to be assumed, that their design for reflectance measurement (usually added as a later feature) is inferior to the design for transmission work. From investigations of actual transparencies of thin-layer chromatograms⁸ one finds, for example, that at 500 nm about 2.2% light is transmitted through a silica gel layer 300 μ thick. For a 160 μ thickness, which is closer to the experimental conditions at hand this is between 5 and 10%. These data seem to support our opinion that under appropriate instrumental design conditions (efficient collection device) reflectance spectroscopy should be more sensitive, as evidenced by this study. The situation becomes even more drastic in the UV region, where absorption of UV light by chromatographic adsorbents is appreciable⁸, so that UV measurements become practically unfeasible in the transmission mode or require very costly and cumbersome instrument designs (high power energy source, very sensitive detectors) as evidenced by a recent study⁹. With reflectance spectroscopy none of these difficulties are experienced. As an alternative, fluorescence quenching is often recommended for UV active compounds. The disadvantages of this approach are however, well known. Fluorescence quenching could at best be of equal sensitivity as UV reflectance¹⁰ but is usually less, since it is very difficult to achieve ideal experimental conditions, such as absorption of the compound of interest at or near the excitation maxima for the phosphor. Other disadvantages are nonspecificity and strong background fluctuations due to irregular distribution of fluorescence indicator.

GOLDMAN AND GOODALL⁴, on the basis of a theoretical study have decided in favour of the transition mode but have never verified this fact experimentally. While the theoretical approach, utilized by these authors, which is based on the Kubelka-

Munk theory of radiative transfer, is basically correct, it remains doubtful whether Fig. 3 and 4 of their publication can be compared to each other meaningfully. The concentration ranges (KX) used for these two modes of operation respectively differ by a factor of almost 40. Statements (i) and (ii) in their paper, which are based on these data, are therefore objectionable.

The criterion of infinite layer thickness

Since it appears from the previous discussion that the reflectance mode is superior to transmission, further critical studies have been carried out by diffuse reflectance spectroscopy. One condition necessary for a system to adhere to the Kubelka-Munk theory is that the layer of the scattering material be of infinite thickness.

This condition is usually not fulfilled when a chromatogram is scanned directly, as is evident from the previous discussion. In earlier work it has therefore repeatedly been recommended² to use a white backing paper as a reflecting back-

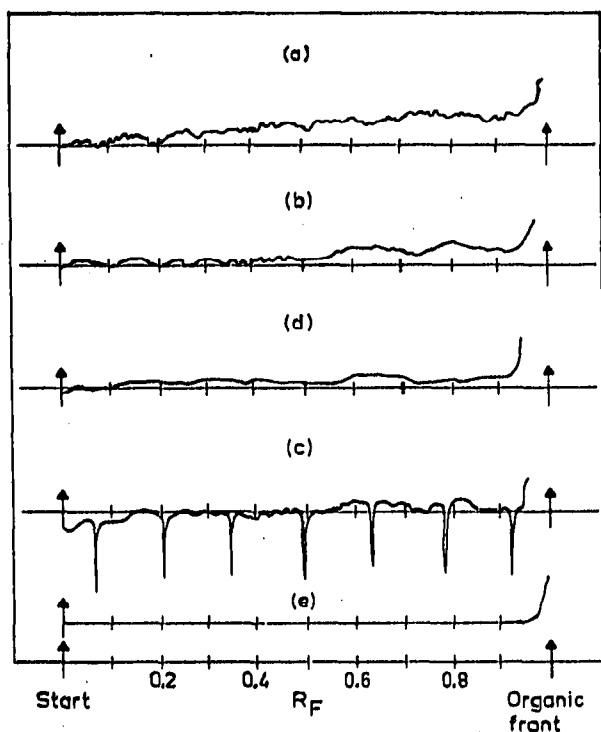


Fig. 3. Scans of reagent background (580 nm) at different conditions with the Zeiss instrument. (a) by transmission measurement; (b-d) by reflectance measurement; (b) on black chromatogram stage; (c) with infinite layer thickness (2 empty sheets); (d) on black stage with white lines; (e) double beam recording with the Farrand instrument.

ground. In the present study purified silica gel sheets were used to simulate an infinite layer thickness. Two sheets placed underneath the chromatogram were sufficient to eliminate the background interference from the black chromatogram stage. A system thus measured under these conditions, adheres more closely to the Kubelka-Munk theory as can be seen from Fig. 2 (○—○) which shows an extension of the linear concentration range and a calibration plot which goes nicely through the point of origin. Another advantage in using the white background is the increased stability of the

TABLE I

REPRODUCIBILITY AS A FUNCTION OF CONCENTRATION OF COBALT DEVELOPED ON SILICA GEL LAYERS

Conc. $\mu\text{g Co/spot}$	Number of determinations (n)	Average reading and S.D. ^a (sq. in.)	Variation coefficient (% S.D.) ^a	Relative error $\frac{dc}{c} \times 100$ (%)
0.05	9	0.33 \pm 0.026	7.81	16
0.2	9	1.01 \pm 0.020	2.02 (4.54) ^b	6.6
0.4	9	1.50 \pm 0.021	1.39	1.66
0.6	7	1.77 \pm 0.025	1.41	3.88
0.8	7	2.06 \pm 0.062	2.98	6.6
1.0	9	2.25 \pm 0.108	4.83 (7.40) ^b	12

$$^a \text{ S.D.} = \pm \sqrt{\frac{\sum (X_i - \bar{X})^2}{n - 1}}; \quad \% \text{ S.D.} = \pm \frac{\text{S.D.} \times 100}{\bar{X}}$$

^b No sheets for infinite layer thickness.

measurements as can be seen from curves d and b of Fig. 3 and consequently a better reproducibility of measurements was obtained (see Table I). For a spot of 0.2 μg concentration for example, 2.02% S.D. was observed for infinite layer thickness condition as compared to 4.54% S.D. if measured just with the black background of the chromatogram stage. The infinite layer condition also enhances the sensitivity of the method. The transmitted light portion, which would otherwise be absorbed by the black background, is reflected back by the white backing sheets hence increasing the reflectance yield.

Black chromatogram stages which have white coordinate lines, such as in the case of the Zeiss instrument, are unsuitable for reflectance work in the visible region without the use of white backing sheets, since the white lines show up as small reflectance peaks (see Fig. 3, curve c).

For work in the UV region, all these considerations are of little or no importance, since the amount of radiation transmitted through the layer is very small due to absorption by the adsorbent.

Homogeneity of the sample

Another condition necessary for adherence of a system to the Kubelka-Munk theory is that the distribution of adsorbing material be uniform over the area illuminated. The best method to achieve this is the spot-removal technique² which in removing the spot from the plate and then mixing it homogeneously prior to packing, ensures both infinite layer thickness and homogeneity of the sample. Accordingly this method gives the best reproducibility (between 1 and 2% rel. S.D.) and adherence to the Kubelka-Munk law; but it is also the most time consuming. For *in situ* measurement of spots this condition is difficult to achieve and one way of getting around this problem is to illuminate only a very small area of the spot at one particular time. It could then be assumed that the area under test would be fairly homogeneous. Scanning of the spot and integration of the individual values would then lead to the desired result. In view of this, the closest approach to an ideal system would be the "Flying spot method" proposed by GOLDMAN AND GOODALL⁹ which

utilizes a light spot of very small area oscillating over the entire chromatographic spot. The disadvantage of such a system is the long scanning time and the cumbersome data processing⁹. Most commercial instruments on the market use a compromise solution by scanning the spot with an optical slit slightly wider than the chromatographic zone. The data obtained in this manner are sufficiently reliable for most analytical applications (2–5% rel. S.D. under good chromatographic conditions.)^{1,2,8}

In this study the effect of homogeneity of the sample has been further investigated by measuring the reflectance of a spot in one single measurement with an adjustable light beam slightly larger in cross-section than the largest spot in a dilution series. The Zeiss Chromatogram Spectrophotometer was ideal for this type of investigation since it is equipped with an iris blende which permits adjustment of the circular light beam to the diameter of the spots. BRAUN AND KORTÜM^{11–13} have investigated this problem previously in conjunction with paper chromatography and have shown that the measured reflectance depends on the irregularity of the spot shape and the empty space around the spot which is included in the measurement. Simulation of layer thickness was achieved in their experiment by using several clean sheets of chromatogram paper as a background for the spot.

The Kubelka-Munk equation (eqn. 3)

$$F(R_{\infty}) = \frac{(1 - R_{\infty})^2}{2R_{\infty}} = \frac{ec}{s} \quad (3)$$

has been modified by the same workers for such a single beam approach to the following form:

$$F'(R_{\infty}) = \frac{b^2}{2n(1 - b)} - \frac{F(R_{\infty})}{n} \quad (4)$$

which is proportional to the absorbing material on the spot.

From the mean observed diffuse reflectance \bar{R}_{∞} of the complete area under the light beam (spot and surrounding matrix) and the diffuse reflectance of the paper itself R'_{∞} one can compute the term b as follows:

$$b = 1 - n\bar{R}_{\infty} + (n - 1)R'_{\infty} \quad (5)$$

n is the ratio of the area under observation to the actual spot area. This relationship was tested with the Co-TAR complex system on silica gel sheets and infinite layer thickness was simulated by using two clean sheets as a background. The Zeiss instrument was used with a light beam adjusted to 11 mm diameter. The monochromator slit was 0.3 mm. The results are presented in Fig. 4.

The plot % R vs. concentration shows the usual bent shape. Plotting the Kubelka-Munk function $F(R_{\infty})$ (eqn. 3) vs. concentration results in considerable deviation from linearity particularly at low concentrations ($\bar{R}_{\infty} = 70\%$). This phenomenon which is attributed to the non-homogeneity factor, has been confirmed previously¹³. The self-absorption of the background is quite small at the working wavelength of 580 nm (about 0.39 R_{∞} , hence $F(R'_{\infty}/n) = 0.002$). For the modified function (eqn. 4) the reflectance measurements were therefore made vs. a background adjusted

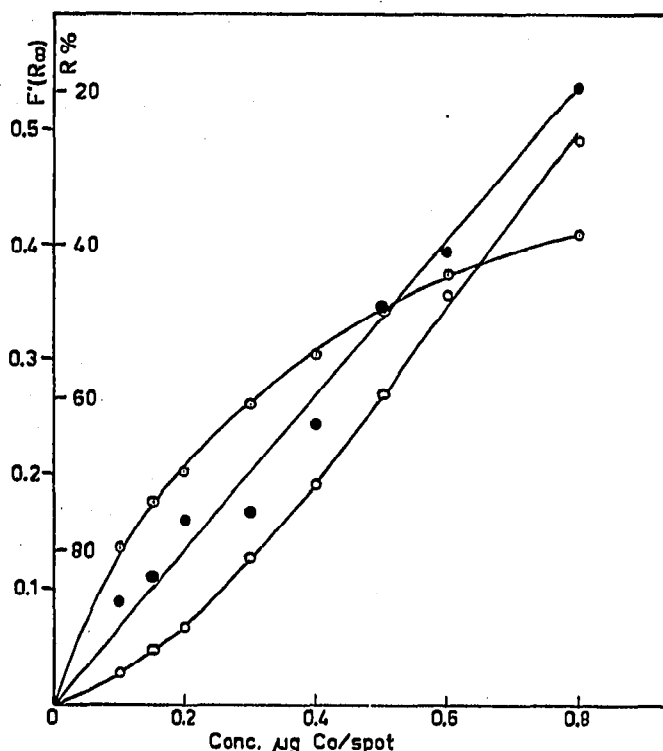


Fig. 4. Calibration curves obtained by reflectance spectroscopy with adjustable beam. Kubelka-Munk values (○—○); modified Kubelka-Munk values (●—●) and % reflectance (○—○) as a function of concentration of cobalt. (Measured at 580 nm; adjustable beam $d = 11$ mm.)

to 100% R . Thus in neglecting this self absorption of the background, the second term of eqn. 4 can be dropped and accordingly the simpler relationship

$$F'(R_{\infty}) = \frac{b^2}{2n(1-b)} = \text{prop. } c \quad (6)$$

was used for the evaluation of the data. Adherence to the Kubelka-Munk theory via this modified function is quite good up to 0.8 $\mu\text{g Co/spot}$ (see Fig. 4). The rather large spread of values at lower concentrations is due to the difficulty encountered in measuring the areas of the often elliptically shaped spots either by planimetry or geometrical formulas.

Double beam versus single beam operation

The system investigated in this study constitutes one of the best examples for a demonstration of the distinct advantage of double beam operation since fluctuations in the spray background can be easily compensated for. This has actually been tested (see Fig. 3, curve e) and a perfectly linear baseline has been obtained for the same chromatoshet measured with a Farrand Vis-UV spectrophotometer in the double beam mode. Vastly superior reproducibilities were also found by recording the same spots on the Farrand Instrument first in the single beam mode (3.5% S.D.) and then in the double beam mode (1.5% S.D.)¹⁴.

The advantage is not so obvious for plates which do not require spraying, and particularly if measurements are made in the UV region, except for a slight compen-

sation for irregularities in the coating, or if UV active impurities in the solvent front would interfere.

Error analysis

The reproducibility for low concentrations of cobalt obtained by reflectance measurements with an adjustable beam of light is presented in Table II. From these results it can be seen that with proper data handling, the above method can be quantitative. A comparison of transmittance *vs.* reflectance spectroscopy (infinite

TABLE II

REPRODUCIBILITY AS A FUNCTION OF CONCENTRATION OF COBALT MEASURED BY REFLECTANCE SPECTROSCOPY WITH ADJUSTABLE BEAM

Slit 0.3/10 adj. beam $d = 6$ mm.

Conc. $\mu\text{g Co/spot}$	Average reading and S.D. ^a (% R)	Variation coefficient (% S.D.) ^a
0.05	79.19 ± 1.66	2.09
0.2	49.97 ± 1.85	3.70

^a For calculation of S.D. and % S.D., see Table I; $n = 9$.

layer thickness) shows about a 2% better standard deviation for the latter. If no simulation for infinite layer thickness is used, the reproducibility drops significantly (Table I).

It was decided that diffuse reflectance spectroscopy with infinite layer thickness simulation and scanning with an optical slit, offered more advantages than any other *in situ* operation mode. A complete error analysis was therefore carried out under these conditions with the cobalt-TAR system, using the Zeiss instrument for measurements.

For systems adhering to the Kubelka-Munk theory, optimum concentration with regard to accuracy can be deduced by computing the relative error dc/c (ref. 15). In terms of the Kubelka-Munk equation (eqn. 3) the error in c would be

$$dc = \frac{K'(R_{\infty} - 1) dR_{\infty}}{2R_{\infty}^2} \quad (7)$$

and the relative error in c is

$$\frac{dc}{c} = \frac{(R_{\infty} + 1) dR_{\infty}}{(R_{\infty} - 1) R_{\infty}} \quad (8)$$

Assuming a reading error amounting to 1% R , *i.e.* $dR_{\infty} = 0.01$ then

$$\frac{dc}{c} \times 100 = \frac{(R_{\infty} + 1)}{(R_{\infty} - 1) R_{\infty}} = \% \text{ error in } c \quad (9)$$

Percent error $dc/c \times 100$ as computed with the use of eqn. 9 and plotted as a function of % R will result in an error analysis curve similar to the one observed in Fig. 5 (O—O). The values for $dc/c \times 100$ can also be computed from the slope of a calibra-

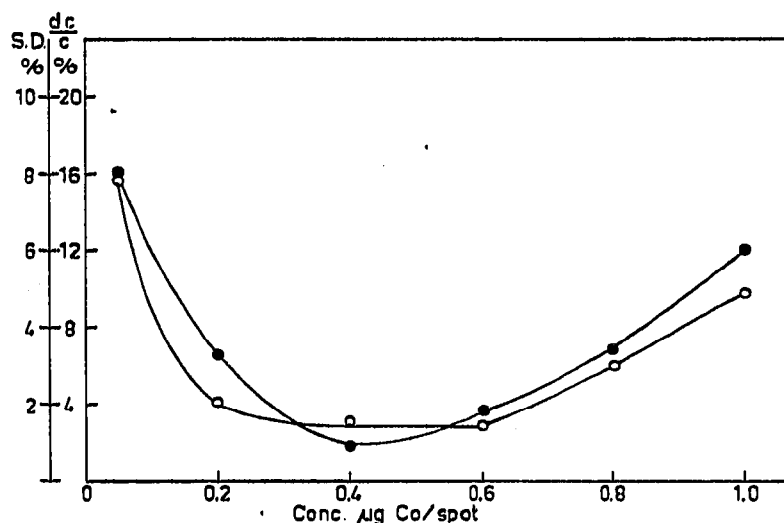


Fig. 5. Error analysis curves. Variation of % S.D. (○—○) and % dc/c (●—●) as a function of cobalt concentration.

tion curve. In this instance, they have been computed from curve 1 in Fig. 2 and plotted as a function of concentration of Co/spot, since reflectance was measured *in situ* as peak area and could not be reproduced as meaningful reflectance values. Obviously this optimum concentration of $0.4 \mu\text{g/spot}$ would vary somewhat with experimental fluctuations. A plot of % S.D. as a function of concentration (corresponding to Table I,) is also given in Fig. 5. It can be seen that in the area $0.2\text{--}0.8 \mu\text{g/spot}$ the % S.D. values remain fairly low (2% S.D.); this comes close to the instrumental and planimetric error for repetitive scanning of one spot, e.g. for a spot of $0.2 \mu\text{g}$ concentration S.D. = 1.3%.

Conclusions

It appears from this study that diffuse reflectance spectroscopy is to be preferred to *in situ* transmission techniques for the evaluation of chromatograms even in the visible region of the spectrum. Apparent advantages of the transmission mode are probably due to poorer instrument design for the reflectance mode. In the UV region transmission work is quite unfeasible due to the high self-absorption of the chromatographic adsorbents.

From these points of view it would then seem appropriate to construct an instrument which works only in the reflectance mode and satisfactorily⁴ covers the UV and visible range, whereas a transmission instrument would not offer the same versatility. Of the various modes of measurement by reflectance *i.e.* slit, flying spot, adjustable beam and spot removal technique, the first seems to offer the best compromise as to reproducibility and efficiency, although the flying spot and spot removal techniques would be more appropriate in the sense of adherence to theoretical principles. Greatest accuracy can still be achieved by the spot removal technique or by double beam scanning, particularly for systems such as those investigated. The single measurement approach with adjustable beam has some merits when the spots are relatively small and compact. Since it could be used with simple instruments such as the Spectronic 20 (large beam modification) or Beckman DU, it would be a relatively simple and inexpensive approach with potential as a field method.

The problem of infinite layer thickness can be solved elegantly by simulation techniques, using two clean TLC sheets as background. Better adherence to the Kubelka-Munk theory and better sensitivity and accuracy can thus be achieved.

Finally this work clearly shows that *in situ* reflectance techniques in conjunction with thin-layer chromatographic work can be a truly quantitative method. The quality of the methods would depend primarily on the quality of the chromatographic techniques rather than instrumental factors:

ACKNOWLEDGEMENTS

This work has been supported by a grant of the National Research Council of Canada. The author is grateful for the experimental assistance of V. MIKETUKOVA and helpful discussions with S. CRAVITT.

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