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STANDARDIZATION OF THE DIRECT SPECTROPHOTOMETRIC QUANTITATIVE ANALYSIS OF CHROMATOGRAMS

IV. A COMPARATIVE STUDY ON THE MOST COMMON MATHEMATICAL TREATMENTS OF THIN-LAYER DENSITOMETRIC PROBLEMS

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SUMMARY

A study comparing the linearity of the Lambert–Beer law, the Kubelka–Munk function and the equation of Treiber *et al.* was carried out on the same thin-layer chromatographic plate. A series of standard amounts of sulfadiazine covering the range of 0 to 10 μg was employed. For the three treatments mentioned above, the following parameters were found in the two-dimensional scanning manner: Lambert–Beer: $a = 1153$, $b = 577$, $r = 0.99585$, $s_a = \pm 3.2\%$; Kubelka–Munk: $a = 1073$, $b = -596$, $r = 0.99778$, $s_a = \pm 2.4\%$; Treiber *et al.*: $a = 2088$, $b = -37.1$, $r = 0.99991$, $s_a = \pm 0.46\%$ (a: slope, b: y-intercept, r: correlation coefficient, s_a: relative standard deviation of the slope in the equation $y = ax + b$ calculated by the least squares method). In the first two cases, the parameters were highly dependent on the different regions of the concentration range considered. However, they were constant when the equation of Treiber *et al.* was used.

INTRODUCTION

For a very long time spectrophotometric methods have been the most essential and indispensable tool of the analytical chemist. It is not at all surprising that spectrophotometers have also found application in liquid column chromatography as one of the most important detector systems. The ease with which the requirements of the classical Lambert–Beer law can be fulfilled in this particular technique made possible a rather rapid expansion of quantitative liquid column chromatography. Yet, the same quantitative approach to thin-layer chromatography (TLC) was encumbered with serious difficulties. The mathematical treatments of different research groups show to date deep disagreement on several points. They have only one main feature in common: they claim a strict and reproducible linearity for a variably wide concentration range.

The most frequently used methods for the mathematical treatment are closely related to the classical theories of spectrophotometry, more specifically the Lambert–

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Beer law and the function of Kubelka and Munk[1] for intensely light-scattering and infinitely thick media[2,3]. Although many papers published claim linear calibration curves of the thin-layer chromatograms for both the above mentioned functions, we were able to show that the *non-linearity* is significant and reproducible in all of these cases on carrying out the whole procedure in our laboratories with appropriate control of each step.

It was our opinion that the best way of forming a correct picture in this controversial situation might be the direct comparison of the most common techniques.

The present paper thus reports a study on the direct spectrophotometric quantitation of thin-layer chromatograms based upon the Lambert–Beer law, the theory of Kubelka and Munk[1] for intensely light-scattering and infinitely thick media[2,3] and a combination of these classical equations according to Treiber and coworkers[4,5].

INSTRUMENT AND MATERIALS

The basic components and the principle of operation of the apparatus employed for the quantitation *in situ* of thin-layer chromatograms were described in a previous article[5]. Since then, all the accessories mentioned in this work have become commercially available. The statistical calculations were carried out by means of a minicomputer of Type Wang 600 (Wang Labs., Tewkesbury, Mass., U.S.A.). The solvents for the chromatography and the chemicals were of analytical grade. Their purity was checked by IR, NMR and chromatographic methods.

Sulfadiazine (N′-2-pyrimidinylsulfanilamide) was purchased from Pharmacia (Uppsala, Sweden). Its N^4-acetyl derivative was prepared in our laboratories by acetylation of the parent drug with acetic anhydride in acetic acid. The concentrations of the stock solutions were 0.100 mg/ml and 1.00 mg/ml.

The pre-coated TLC plates of silica gel 60 were manufactured by E. Merck (Darmstadt, G.F.R.). Plates of standard size (20 × 20 cm) and thickness (0.25 mm) on glass backing were employed. Before being used, all plates were purified by ascending chromatography in absolute ethanol, dried and reactivated at 110–120° for 1 h. In order to prevent mixing of the samples with each other due to migration across the tracks, the chromatographic fields were divided by scoring of thin lines.

EXPERIMENTAL

Appropriate volumes of the stock solutions were applied to the TLC plate by a Hamilton microsyringe to give a series of increasing amounts of sulfadiazine and N^4-acetylsulfadiazine covering a range of 0 to 10.0 μg. The developing chromatographic system was ethyl acetate–dioxane–acetic acid (8:2:0.1) resulting in a clear separation of both compounds.

After development of the chromatogram the plate was dried. The wavelength at which the absorbance was a maximum was determined on the plate by densitometric scanning and found to be 274 nm. Further chromatographic properties of these and other related derivatives are given in another paper[6]. Each experiment was carried out on the same plate to assure a reliable comparison.

The calibration curves were then estimated in accordance with the Lambert–

Beer law (eqn. 1), the theory of Kubelka and Munk[1] for intensely scattering and infinitely thick media[2,3] (eqn. 2) and the function of Treiber *et al.*[4,5] (eqns. 3–6):

$$K_x \cdot C = K_t \cdot \ln \frac{I_0}{I_x} \tag{1}$$

$$K_x \cdot C = K_r \cdot \frac{\left(1 - \frac{I_x}{I_0}\right)^2}{2 \cdot \frac{I_x}{I_0}} \equiv \frac{(1 - R_x)^2}{2 \cdot R_x} = k \cdot \varepsilon \cdot C \text{ (refs. 2 and 3)} \tag{2}$$

$$K_x \cdot C = K_r \cdot \frac{\left(1 - \frac{I_x}{I_0}\right)^2}{2 \cdot \frac{I_x}{I_0}} + K_T \cdot \ln \frac{I_0}{I_x} \tag{3}$$

after a simple transformation:

$$K_x \cdot C = K_r \cdot \frac{1 - 2\frac{I_x}{I_0} + \left(\frac{I_x}{I_0}\right)^2}{2 \cdot \frac{I_x}{I_0}} + K_T \cdot \ln \frac{I_0}{I_x} \tag{4}$$

$$K_x \cdot C = K_r \cdot \left(\frac{1}{2} \cdot \frac{I_0}{I_x} - 1 + \frac{1}{2} \cdot \frac{I_x}{I_0}\right) + K_T \cdot \ln \frac{I_0}{I_x} \tag{5}$$

$$\left(\frac{1}{2} \cdot K_r = K_R\right)$$

$$K_x \cdot C = K_R \cdot \left(\frac{I_0}{I_x} + \frac{I_x}{I_0} - 2\right) + K_T \cdot \ln \frac{I_0}{I_x} \tag{6}$$

where

K_x = constant depending on the substance chromatographed;
$K_R(K_r)$, $K_T(K_t)$ = constants depending on the properties of the adsorbent layer;
k = constant depending on the adsorbent layer;
ε = molar absorbance of the substance chromatographed;
C = concentration of the substance chromatographed in weight per surface unit;
I_x = intensity of the light leaving the sample;
I_0 = constant, maximal light intensity on the adsorbent layer free from any substance chromatographed.

Thus $0 \leqslant I_x \leqslant I_0$ is the possible range.

The constants K_x, K_R and K_T should be empirically estimated. The light intensities appear on the recorder attached to the indicator unit of the photometer as voltage. The maximal recorder response ($U_{max.}$) and its change ($U_{max.} - U_x$) due to light absorbance correspond to I_0 and I_x, respectively.

TABLE I

CONVERSION OF THE PHOTOMETER RESPONSES $\left(\frac{U_{max.}-U_x}{U_{max.}}\right)$ TO VALUES ACCORDING TO THE FOLLOWING FUNCTIONS: KUBELKA–MUNK FOR INTENSELY LIGHT-SCATTERING MEDIA[2,3] (K–M), LAMBERT–BEER (L–B) AND THE COMBINATION OF BOTH ACCORDING TO TREIBER *et al.*[4,5] (K–M + 0.45 × (L–B))

$\frac{I_x}{I_o}$	$\frac{U_{max.}-U_x}{U_{max.}}$	*L–B*	*K–M*	*0.45 × (L–B)*	*K–M + 0.45 × (L–B)*
1.00	0	0	0	0	0
0.95	0.05	0.05129	0.00132	0.02308	0.02440
0.90	0.10	0.10536	0.00556	0.04741	0.05297
0.85	0.15	0.16252	0.01324	0.07313	0.08637
0.80	0.20	0.22314	0.02500	0.10042	0.12542
0.75	0.25	0.28768	0.04167	0.12946	0.17112
0.70	0.30	0.35667	0.06429	0.16050	0.22479
0.65	0.35	0.43078	0.09423	0.19385	0.28808
0.60	0.40	0.51083	0.13333	0.22987	0.36321
0.55	0.45	0.59784	0.18409	0.26903	0.45312
0.50	0.50	0.69315	0.25000	0.31192	0.56192
0.45	0.55	0.79851	0.33611	0.35933	0.69544
0.40	0.60	0.91629	0.45000	0.41233	0.86233
0.35	0.65	1.04982	0.60357	0.47242	1.07599
0.30	0.70	1.20397	0.81667	0.54179	1.35845
0.25	0.75	1.38629	1.12500	0.62383	1.74883
0.20	0.80	1.60944	1.60000	0.72425	2.32425
0.15	0.85	1.89712	2.40833	0.85370	3.26204
0.10	0.90	2.30258	4.05000	1.03616	5.08616
0.05	0.95	2.99573	9.02500	1.34808	10.37308
0	1.00	∞	∞	∞	∞

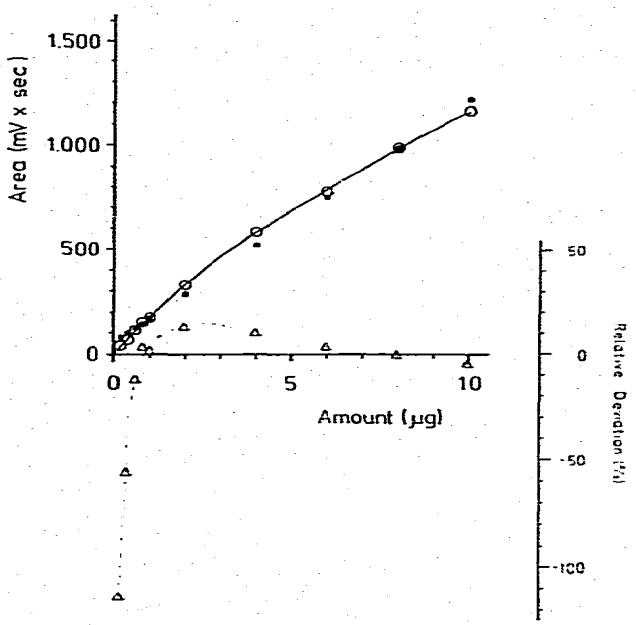

Fig. 1. Sulfadiazine: calibration curve (○—○) and the best fitting straight line (●—●) according to the Lambert–Beer law (eqn. 1) by two-dimensional automatic integration. Relative deviation (△---△) of the experimental values from the best fitting line.

A series of the I_x/I_0 values was transformed corresponding to the functions 1, 2 and 6 and listed in Table I. Each of these functions could be conveniently selected by proper adjustment of the function converter[4,5]. The three calibration curves of sulfadiazine were also estimated in the two-dimensional (Figs. 1, 3, 5; Table II) as well as in the one-dimensional (Figs. 2, 4, 6; Table II) manner[5]. The best linearity was obtained with a K_T/K_R ratio of 0.45 (eqn. 6).

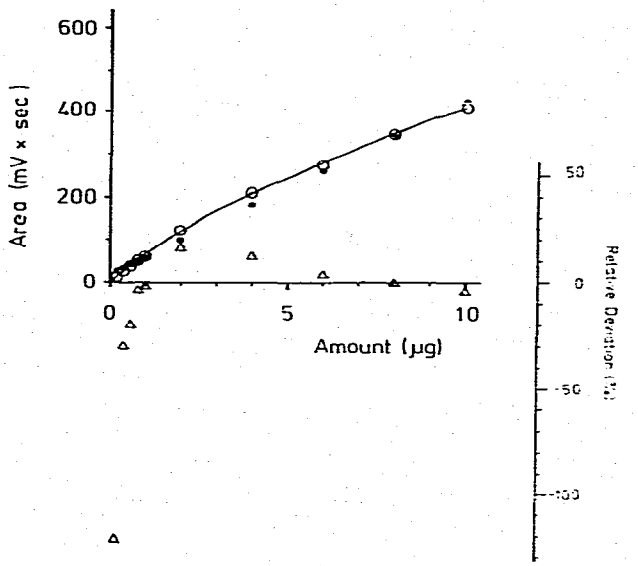

Fig. 2. The same as Fig. 1 by one-dimensional automatic integration.

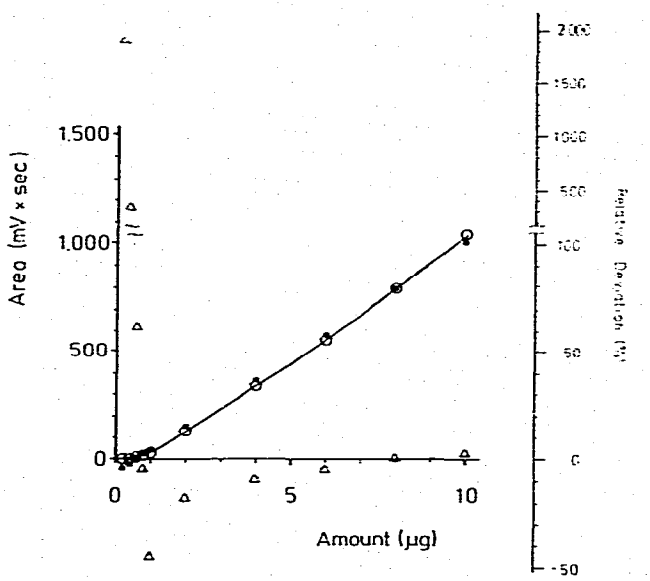

Fig. 3. Sulfadiazine: calibration curve (○—○) and the best fitting straight line (●—●) according to a special case[2,3] of the theory of Kubelka and Munk[1] (eqn. 2) by two-dimensional automatic integration. Relative deviation (△---△) of the experimental values from the best fitting line.

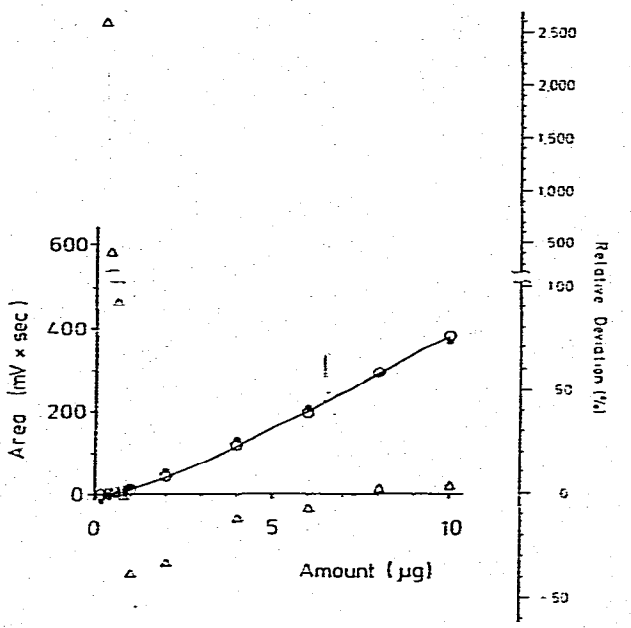

Fig. 4. The same as Fig. 3 by one-dimensional automatic integration.

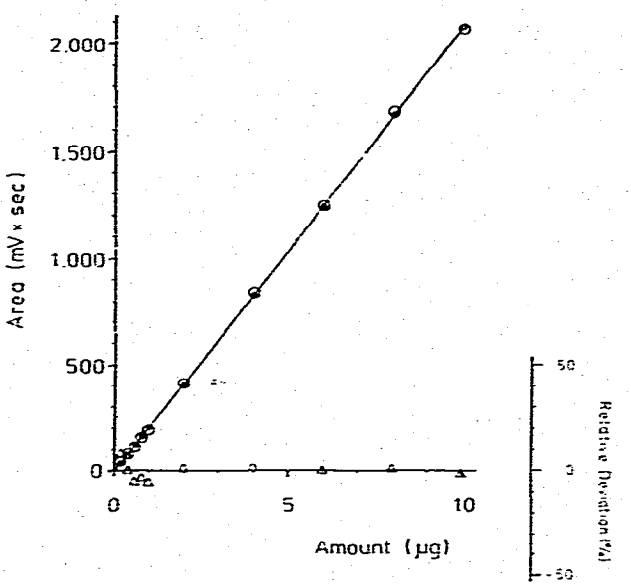

Fig. 5. Sulfadiazine: calibration curve (○—○) and the best fitting straight line (●—●) according to Treiber *et al.*[4,5] (eqn. 6) by two-dimensional automatic integration. Relative deviation (△---△) of the experimental values from the best fitting line.

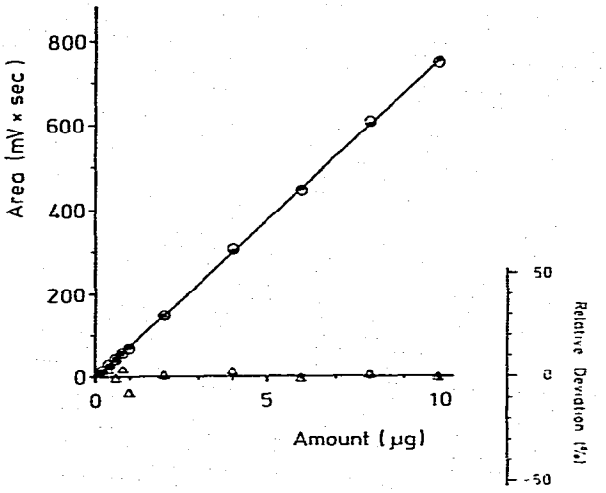

Fig. 6. The same as Fig. 5 by one-dimensional automatic integration.

TABLE II

STATISTICAL CALCULATIONS OF THE CALIBRATION LINES

Function	*Conversion mode* *Scan mode (dimensional)*	*Range (µg)*	*a (slope)*	*b (y-intercept)*	*r (correlation coefficient)*	s_a *(relative standard deviation)*
Sulfadiazine						
L–B	automatic	0–10	1153	577	0.99585	37.3 (± 3.2‰)
	(two)	0–2	1636	103	0.99679	65.7 (± 4.0‰)
		2–10	1030	1493	0.99825	35.3 (± 3.4‰)
	automatic	0–10	404	222	0.99482	14.6 (± 3.6‰)
	(one)	0–2	599	31.9	0.99916	12.3 (± 2.1‰)
		2–10	352	605	0.99829	11.9 (± 3.4‰)
K–M	automatic	0–10	1079	−596	0.99778	25.5 (± 2.4‰)
	(two)	0–2	733	−268	0.97005	91.7 (± 12.5‰)
		2–10	1154	−1142	0.99941	22.9 (± 2.0‰)
	automatic	0–10	388	−229	0.99647	11.6 (± 3.0‰)
	(one)	0–2	230	−78	0.97970	23.5 (± 10.2‰)
		2–10	425	−500	0.99890	11.5 (± 2.7‰)
K–M +	automatic	0–10	2088	−37.1	0.99991	9.6 (± 0.46‰)
0.45 × L–B	(two)	0–2	2088	−58.0	0.99876	52.1 (± 2.5 ‰)
		2–10	2069	114	0.99988	18.3 (± 0.88‰)
	automatic	0–10	752	−17.3	0.99984	4.7 (± 0.63‰)
	(one)	0–2	752	−26.6	0.99892	17.8 (± 2.4 ‰)
		2–10	744	44.9	0.99969	10.7 (± 1.4 ‰)
N⁴-Acetylsulfadiazine						
K–M +	automatic	0–10	558	27.2	0.99902	8.7 (± 1.6 ‰)
0.45 × L–B	(one)	0–2	553	49.3	0.99655	23.0 (± 4.2 ‰)
		2–10	572	−85	0.99803	20.8 (± 3.6 ‰)
	manual	0–10	0.717	0.104	0.99885	0.012 (± 1.7 ‰)
	(one)	0–2	0.789	0.018	0.99904	0.017 (± 2.2 ‰)
		2–10	0.687	0.329	0.99803	0.025 (± 3.6 ‰)

The calibration curves of N^4-acetylsulfadiazine were assessed in the one-dimensional mode only by using both the automatic (Fig. 7; Table II) and manual (Fig. 8; Table II) techniques. A detailed presentation of the manual technique without any function converter is provided below.

The substance amount applied to the plate gave the integral of the left-hand side of the eqn. 6 because the distribution of the substance concentration (C) is a function of the chromatographic track. For the integral (A) of the right-hand side the densitometric peak appearing on the chart paper (Fig. 9) was used. Multiplication of the peak height (H) with the peak width at half the height ($W_{h/2}$) gave A:

$$A = H \cdot W_{h/2} \tag{7}$$

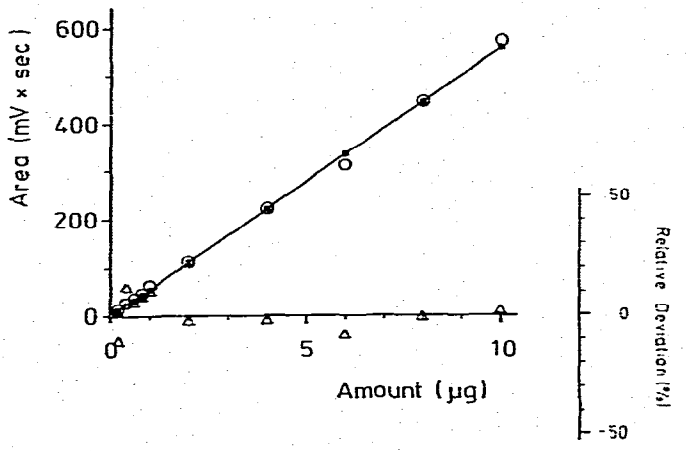

Fig. 7. N^4-Acetylsulfadiazine: calibration curve (△—△) and the best fitting straight line (●—●) according to Treiber *et al.*[4,5] (eqn. 6) by one-dimensional automatic integration. Relative deviation (△---△) of the experimental values from the best fitting line.

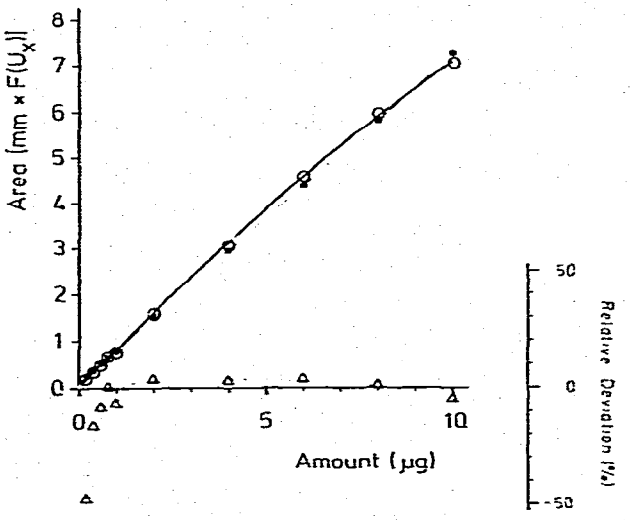

Fig. 8. The same as Fig. 7 by one-dimensional manual integration.

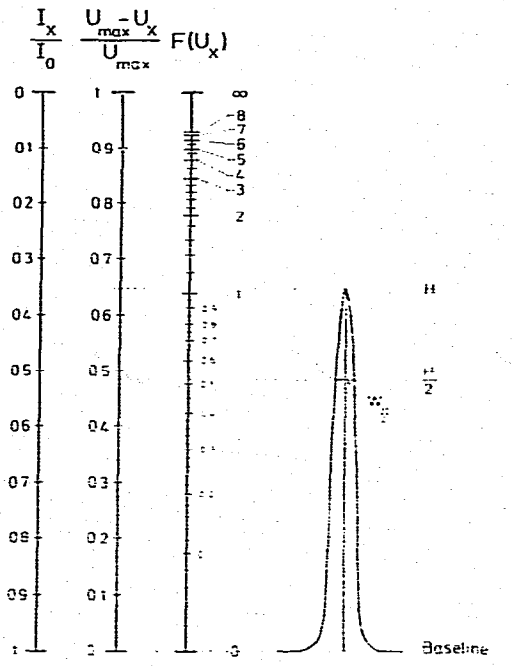

Fig. 9. Scales for the manual transformation of $R_x = \frac{I_x}{I_0} \equiv \frac{U_{max.} - U_x}{U_{max.}}$ values according to Treiber *et al.*[4,5]. The $F(U_x)$ values were thus calculated from eqn. 6 for a K_T/K_R ratio of 0.45. Illustration of a manual transformation and integration of the densitometric chart of 10.0 μg of N^4-acetylsulfadiazine.

The values H and $W_{H/2}$ were measured by means of a special scale (Fig. 9) established by transformation of a proper series of the recorder responses ($U_{max.} - U_x$). These data are collected in Table I.

The steps of the manual conversion and calculation of integrals were carried out as follows (*cf.* Fig. 9):

(1) Estimation of the $(U_{max.} - U_x)/U_{max.}$ ratio at the top of the peak.

(2) Determination of the corresponding $F(U_x) \equiv H$ value.

(3) Calculation of $\frac{1}{2} H$.

(4) Determination of the $(U_{max.} - U_x)/U_{max.}$ value corresponding to $\frac{1}{2} H$.

(5) Measurement of the peak width at the $(U_{max.} - U_x)/U_{max.}$ value obtained under 4.

(6) Calculation of the integral (A) according to the eqn. 7.

This procedure was subsequently repeated for each peak to obtain the calibration curve. It can be realized by using a scale individually made for the chart paper of the recorder attached to any type of densitometer.

The straight lines best fitting the calibration points were calculated by statistical methods. For the estimation of the constants in the general formula $y = ax + b$, the least squares method was used. These data determined the exact position of the lines. Furthermore, the correlation coefficients (r) and relative standard deviations of the slopes (s_a) were calculated. Table II shows the numerical results. The percentage differences between the calculated and experimentally found

integrals were individually estimated for each point of each calibration curve (Figs. 1–8).

RESULTS AND DISCUSSION

The criteria absolutely required in choosing the model substances used for the examination of the calibration curves were considered carefully. Stability during the whole procedure and a characteristic light absorbance are those properties that make our conclusions reliable from the point of view of chemical methodology.

The scanning technique was also kept under satisfactory control because the whole investigation was begun using two-dimensional* integration[5]. The thickness of the adsorbent layer is negligible compared to the other dimensions of a developed chromatographic spot, which in turn can be considered, at least in this connection, as a two-dimensional formation. Therefore, any one-dimensional method, before being routinely used, must be compared with the results of the two-dimensional integration.

All the experimental conditions described above have been examined in earlier studies[4,5].

However, the statistical treatment of the calibration curves might be subject to critical consideration. Although the calculation of the best fitting line by the least squares method is justified if the pairs of observed values are randomly distributed along a straight line, the same method must not be generally employed if the relationship between both groups of data is obviously non-linear. On the other hand, we had to examine the experimental values by the same approach in order to compare the practical usefulness of the functions involved. That was the reason why we decided to calculate in each case the best fitting line. It was felt that in spite of the incorrect choice of the statistical method, the comparison might be realistic, especially after considering other data discussed below. In fact, the incorrect choice is one piece of evidence that the linearity of functions 1 and 2 is highly limited in thin-layer densitometry. The estimation of the best fitting lines (Table II) resulted in the second piece of evidence indicating an excellent linearity provided by function 6, while functions 1 and 2 gave no straight calibration lines for the same concentration range. On calculating the a and b values for different parts of the standard series, a drastic change occurred in eqns. 1 and 2, even if some of the regions happened to be nearly linear.

The same technique caused no significant change in a and b when eqn. 6 was used. That means that the deviations have not exceeded the usual degree of experimental uncertainty. Moreover, b was negligibly small compared to a in the cases where eqn. 6 was utilized. In other words, the calibration lines passed through the origin. The y-intercepts appeared to have a random deviation from the origin probably caused, at least partly, by statistical fluctuation of the baseline. They did usually not run above 0.5% of a, which corresponds to the least detectable amount of the substances examined.

For eqns. 1 and 2, however, the y-intercepts never amounted to less than 50% of a on considering the same concentration range.

Using function 6 no tendency to non-linearity was observed. The manual transformation and integration might be the only exception depending on the non-

* Generally known as "flying-spot" technique or zig-zag mode.

Gaussian distribution of the substance chromatographed. But even in this case all of the parameters were better than those of either of the eqns. 1 and 2.

As Figs. 1–8 and Tables I–II show, even special considerations were introduced into the discussion in order to illuminate practical problems better from several directions. The percentage differences in the calculated and experimentally found intergrals are given in the figures showing the calibration curves. The systematic errors exhibit the third piece of evidence to prove the non-linearity of functions 1 and 2 (Figs. 1–4). The distribution of the differences by use of function 6 is rather random and the error decreases quickly on increasing concentration (Figs. 5–8). All these aspects gathered from the experimental data are collected numerically in Table II. It might be of great interest to demonstrate whether our results correspond to or disagree with earlier data available.

First, let us survey the situation in the case of the Lambert–Beer law. For small amounts we obtained calibration curves of reasonable linearity (*cf.* Table II, L–B, 0–2 μg). Parameters, such as b/a, r and s_a were quite satisfactory too. Similar results have been reported from other laboratories[7,8] also. These papers, however, have not discussed the form of the calibration curves for higher amounts. Touchstone and coworkers[9] have more recently shown, that the linearity by this technique is acceptable for low concentrations only, in contrast to earlier results from the same laboratory[10,11]. Goldman and Goodall[12] introduced a correction factor to extend the linearity to the higher concentration ranges. The standard curves without any correction have shown the same form[13] as ours (Figs. 1 and 2). The correction resulted in an improvement of the linearity up to 5 μg of β-carotene[13].

In contrast to the Lambert–Beer law, we found a better linearity by use of the theory of Kubelka and Munk[2,3] when the higher concentration regions were investigated (Figs. 3 and 4, Table II). In this context it has to be noted that a special case[2,3] of the Kubelka–Munk function is commonly used for the purposes of the quantitative TLC. To our knowledge, there are no data available estimated by the general theory of Kubelka and Munk[1]. The assumptions are intensely light-scattering medium and an infinitely thick adsorbent layer. Our calibration curves (Figs. 3 and 4) support the results of Keuker[14], strongly bent calibration curves in the low concentration region improving with increasing amounts. However, in contrast to the results by Hezel[15], the straight section of the calibration curves do not even nearly pass through the origin. Several authors[16–18] have recognized this failure of this technique in an early stage and tried to eliminate the errors. These efforts have mainly consisted of modifying the experimental conditions to the requirements of the special case[2,3] of the Kubelka–Munk function[1]. The adsorbent layer containing the samples has been scratched from the plate and the powder obtained has been studied by reflectance spectrophotometry.

From the practical point of view, we preferred to keep the TLC plates intact and to look for an appropriate mathematical function[4,5]. The observations gained by the simultaneous measurements[19] in transmittance and reflectance have then initiated to employ both classical theories of the spectrophotometry at the same time. These efforts resulted in useful results[4,5] demonstrating the general validity of eqn. 6 for objects, such as standardized TLC plates. Published and unpublished investigations on various types of compounds (steroids[4,5], phenolic acids[4,5], dyes[19], peptides[20], pharmaceuticals[6], etc.) covering a wavelength range of 200 to 600 nm have provided

an excellent and reproducible linearity with negligible y-intercepts and standard deviations, regardless of the scanning technique used. Kubelka[2] has already shown, that the scattered light leaving the scattering medium behaves independently of the travelling direction (assuming of course an intensely light-scattering medium).

The idea of using both classical theories at the same time has a strong connection to the general problems of spectrophotometry. As Kortüm and Vogel[3] demonstrated, the theory of Kubelka and Munk[1] is generally valid for any kind of spectrophotometric problem. However, the demanding mathematical treatment can be considered as the most effective draw-back of the practical application of the theory in its original form. Kubelka himself has stated[2] the necessity of elaborating mathematically simpler formulae for practical purposes. The possibility, whether any direct mathematical connection exists between the theory of Kubelka and Munk[1] and eqn. 6 (refs. 4 and 5), has not yet been investigated.

The sulfonamide derivatives used in this study belong to a group of therapeutically important compounds. The importance of the present work for practical pharmacology, metabolic investigations and clinical chemistry will be presented elsewhere[6] in connection with a number of sulfonamide derivatives.

CONCLUSIONS

(1) Reports claiming linearity for the Lambert–Beer law and the special case of the Kubelka–Munk function[3] mentioned above should be taken with reservation in their separate use for the *in situ* quantitation of thin-layer chromatograms.

(2) The Lambert–Beer law provides a nearly linear relationship and reasonably small y-intercepts for substance amounts *vs.* integral for the lowest concentration range.

(3) The function of Kubelka and Munk[1] for intensely light-scattering and infinitely thick media[2,3] shows a severe non-linearity for the lowest regions of the substance amounts. A nearly linear relationship can be obtained for amounts *vs.* integrals in the higher concentration ranges. However, the deviation of the y-intercept from the origin remains significant.

(4) A perfect linearity was achieved by use of the eqn. 6 (refs. 4 and 5) for a wide concentration range. The calibration lines pass through the origin.

(5) The execution of the comparative examination and quantitative measurements can be also carried out manually. This method is time consuming and tedious, but does not require any additional costs if a densitometer of any type is present. Small laboratories of limited financial resources might take practical advantage of this possibility.

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