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DEVELOPMENT AND PRACTICAL UTILIZATION OF A LINEAR SPECTRODENSITOMETER

LASZLO R. TREIBER

Department of Macromolecular Science, Case Western Reserve University, Cleveland, Ohio 44106 (U.S.A.)

SUMMARY

The development of a linear detector system for the *in situ* quantitation of thin-layer chromatograms indicates the general applicability of spectrodensitometry, comparable to that of gas–liquid chromatography and liquid column chromatography. There are general requirements to meet, however, regarding chemical methodology and instrumentation, before this technique may be successfully used. A commercially available instrument modified in our laboratories has shown high sensitivity, reliability, versatility, and short analysis times in biological applications and in the monitoring of organic reactions during synthetic work. Statistical calculations indicate that the construction of an automatic linear spectrodensitometer is now feasible. Some predictions appear to be realistic on the basis of the statistical values.

INTRODUCTION

The advances in microanalysis made possible by the advent of gas–liquid (GLC) and liquid column chromatography (LC) are well known. In principle, the development of thin-layer chromatography (TLC) could also have proceeded to the quantitative level. In practice, the development of qualitative and quantitative TLC has not occurred simultaneously since, unlike the GLC and LC methods, the separated fractions lie in an inhomogeneous, intensely light scattering medium. Thus, despite the fact that TLC (or more generally, thin-medium chromatography) is known to be one of the simplest yet most powerful approaches for the separation and purification of small quantities of material, its quantitative application has been hindered by several difficulties based on two particular problems.

Available commercial instruments are designed on the premise that, firstly, either the Beer Law or a special case[1,2] of the theory of Kubelka and Munk[3], are appropriate quantitative representations of concentration and, secondly, that available preparations of plates for photodensitometric scanning are adequate for quantitative purposes. Prior to a densitometric scan, chromatograms may have been visualized by spray reagents, these often provide only semiquantitative conversion and may mechanically affect the adsorbent layer. Although the use of spray reagents

may be an adequate technique for the visualization of the fractions, they do not allow reliable conclusions regarding instrument characteristics.

First after employing stable, light absorbing substances as models we could divert our attention to parameters involving the absorbent layer and instrumentation.

Investigations on the effect of the adsorbent layer have shown that the densitometric pattern of a track containing no light-absorbing substance is constant and reproducible in repeated scans[4]. Moreover, the relationship between the patterns of the transmittance and reflectance scanning of the same track has been recognized. The practical application of this recognition is the simultaneous transmittance and reflectance measurement[5] providing the best elimination of the baseline noise known for the wavelength range of above approximately 310 nm.

Although the increase in sensitivity by one order of magnitude is appreciable, the real importance of this concept lies in its extension. It is obvious that the Beer Law (eqn. 1) does not apply, since the medium is intensely light scattering. Reliable data indicate linear calibrations apply only for the lowest concentrations[6,7].

$$K_1 \cdot C = K_r \cdot \ln \frac{I_0}{I_x} \tag{1}$$

Likewise, the special case of the Kubelka–Munk theory[1,2] (eqn. 2) has limitations for a number of reasons, such as partial transparency of the layer and the non-diffuse illumination of the samples. As a result, significant non-linearity has been found, especially in the low concentration ranges[7,8].

$$k \cdot \varepsilon \cdot C \equiv K_2 \cdot C = K_r \frac{\left(1 - \frac{I_x}{I_0}\right)^2}{2\left(\frac{I_x}{I_0}\right)} \tag{2}$$

if the relative reflectance $R_\infty = I_x/I_0$

where K_1, K_2, k, ε = constants, depending on the substance chromatographed

K_r, K_t = constants, depending on the properties of the adsorbent layer

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 where $C = 0$.

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

From the extension of the simultaneous transmittance and reflectance techniques arose the question as to whether both respective classical theories would complement each other (eqns. 3–5).

$$(K_1 + K_2) \cdot C = K_r \frac{\left(1 - \frac{I_x}{I_0}\right)^2}{2\left(\frac{I_x}{I_0}\right)} + K_t \cdot \ln \frac{I_0}{I_x} \tag{3}$$

After a transformation, if

$$K_R = \frac{K_t}{K_1 + K_2}$$

and

$$K_T = \frac{K_t}{K_1 + K_2}$$

then

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

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

The first manual application[5] of this concept was very encouraging and resulted in linear calibrations and good reproducibility. An analog device based on eqn. 3 made the method easy to use in statistical experiments requiring a large number of data. Relevant results have recently been published[7] comparing the characteristics of the concepts mentioned above. Eqn. 3 has proved to be superior to both classical theories. Linear calibrations were obtained using a commerical instrument modified in accordance with eqn. 3. This system has been utilised routinely since 1972 without any serious trouble. The linearity of the calibration can consistently be reproduced throughout the whole practical range of reflectance (R_∞ values of above 90%)[9].

CURRENT APPLICATIONS

Generally speaking densitometry is the complementary approach to GLC and LC; it is especially useful for the analysis of non-volatile, temperature-sensitive compounds. There are, of course, many overlaps; densitometry, however, is in many cases the only practical method of choice. Some methodological aspects are demonstrated by the following representative examples which have been studied in detail in our laboratories.

Clinical chemistry

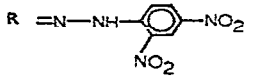
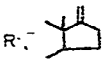
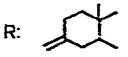
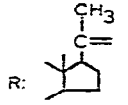
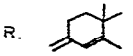
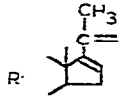
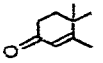
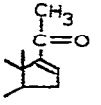
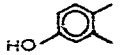
For the *in situ* quantitative evaluation of electropherograms densitometry is virtually the only technique available.

Steroid hormones have been successfully analysed by means of densitometry (Table I). Oxosteroids can readily be labelled by photometric means to form highly absorbing derivatives. The reaction with 2,4-dinitrophenylhydrazine (2,4-DNPH) is simple and quantitative. If the chromatographic systems are properly chosen, the reaction mixture can be applied directly onto the TLC plate. The acetone component in the mixture reacts with excess reagent to give a very unpolar, thus chromatographically easily removable, byproduct[10]. This method is rapid and reliable, as shown in aldosterone estimation.

Steroids containing conjugated double bonds are directly detectable on the plate without the formation of any light-absorbing derivative[11,12]. This feature was utilized for structure elucidation of a steroid derivative isolated from the urine of a

TABLE I

STEROID DERIVATIVES ANALYSED BY *IN SITU* SCANNING OF THIN-LAYER CHROMATOGRAMS

Compound	*Structure of the absorbing site*	λ_{max} *(nm)*	*Reference*
Ketosteroid 2,4-DNPH derivatives			
17-Ketosteroids		365	5
3-Ketosteroids		365	unpublished
20-Ketosteroids		368	unpublished
Δ^4-3-Ketosteroids		385	5, 10
Δ^{16}-20-Ketosteroids		383	12
Conjugated double bonds Δ^4-3-Ketosteroids		252.5	13
Δ^{16}-20-Ketosteroids		240	12
Estrogens		280	11

newborn girl with congenital adrenogenital syndrome[12]. The steroid metabolite was identified as 16-dehydropregnenolone.

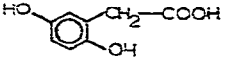
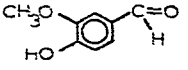
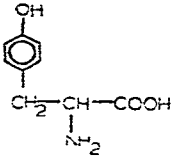
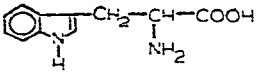
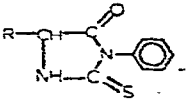
Preliminary investigations on phenolic acids[13], amino acids[14], etc. (Table II) suggest further potential areas of application of densitometry in clinical chemistry.

Pharmaceutical products

Methodologically this section could be discussed together with clinical chemistry, since the main field of application involves work with materials of biological

TABLE II

METABOLITES AND DERIVATIVES OF AMINO ACIDS ANALYSED BY *IN SITU* SCANNING OF THIN-LAYER CHROMATOGRAMS

Compound	*Structure*	λ_{max} *(nm)*	*Reference*
Homogentisic acid		290	13
Vanillin		310	unpublished
Tyrosine		275	unpublished
Tryptophane		220, 280	unpublished
PTH amino acids		269	14

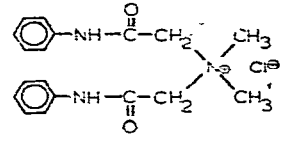
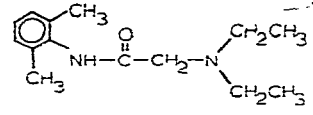
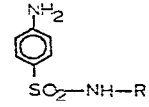
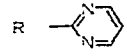
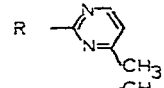
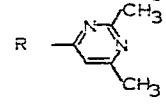
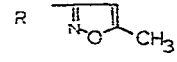
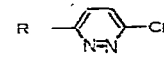
origin. Unlike GLC and LC, the TLC adsorbent is disposable; as a result, irreversibly adsorbed contaminants do not change the performance of the method. In the development and clinical trial of a new antiarrhythmic drug[15] (Table III), we tried first to employ an LC method for determining blood and urine concentration in clinical samples. As a change of the column was necessary after every fifth run, we had no choice but to develop a densitometric method. An unpublished modification of this method is occasionally used for confirmation of xylocaine concentrations in blood which is usually analysed by GLC. Both modifications were employed for determination of the therapeutic concentration of the samples.

In the case of some sulfonamides[15], the urinary excretion of the parent compounds and their main metabolite, the N^4-acetyl derivatives, were monitored. Urine samples (25 μl) were applied directly onto the plate and after development quantitative evaluation was achieved by means of densitometry. A simple scan provided the quantitative data for both the parent drug and its N^4-acetyl derivative. This simplicity resulted in substantial savings in terms of technician time and chemicals.

The instrument has also been used for *ad hoc* analyses without first developing any particular procedure. In fact, this appears to be a potential field of application for densitometers. In toxicology and forensic chemistry the chemist often faces unusual problems. In cases of undefined intoxications or drug abuses, screening of body fluids or extracts is sometimes the only way to proceed. Nevertheless, qualitative

TABLE III

SOME PHARMACEUTICAL PRODUCTS ANALYSED BY *IN SITU* SCANNING OF THIN-LAYER CHROMATOGRAMS

Compound	*Structure*	λ_{max} *(nm)*	*Reference*
"QX-572"		250	15
Xylocaine		270	unpublished
Sulfonamides			
Sulfadiazine		274	7, 16
Sulfamerazine		274	16
Sulfaisodimidine		284	16
Sulfamethoxazole		273	16
Sulfachloropyridazine		278	16

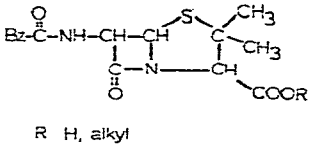
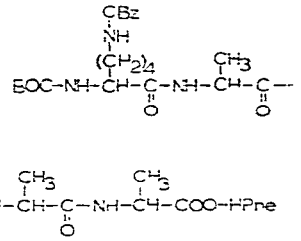
TLC methods have already established themselves in these areas. It seems likely that the use of TLC scanners would increase the specificity and sensitivity of the qualitative methods after appropriate modification.

Monitoring organic reactions

Densitometry has proved to be very useful when monitoring complex reaction mixtures in order to establish reaction kinetics or to achieve optimal yields and purity. We have monitored the synthesis of some peptide-type compounds such as penicillin derivatives and peptide intermediates[17] (Table IV). In these cases, it was useful to have an instrument working in the wavelength region of the peptide bond (about 205 nm).

TABLE IV

UTILIZATION OF THE ABSORPTION OF THE PEPTIDE BOND

Compound	*Structure*	λ_{max}	*Reference*
Benzylpenicillin	 R H, alkyl	205	unpublished
tert.-BOC-Lys(CBz)Ala-Ala-Ala-O-hydroxyphenyl ester		207	17

FUTURE DEVELOPMENT

The results presented above were obtained as a result of a modification to a commercial instrument (Zeiss Chromatogram Spectrophotometer) (Fig. 1). Surprisingly, there is no commercial instrument available that can be employed for quantitative work in a convenient manner without considerable modification, and the addition of various parts. As a matter of fact, customers need to be more familiar with the aspects of instrumentation than manufacturers if they wish to employ quantitative TLC. If we compare the needs of the analytical chemist and the instruments normally available, there is still a long way to go before a reasonable machine will be available. Based on our statistical investigations[7] the following, commerically not available, requirements are justified.

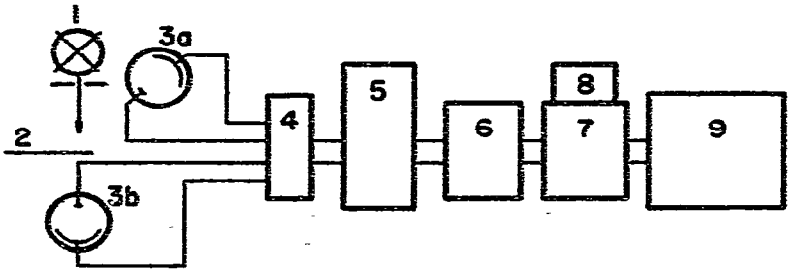


Fig. 1. Schematic representation of a linear chromatogram spectrophotometer. 1 = Light source; 2 = TLC plate; 3 = photo tube; 4 = simultaneous attachment; 5 = amplifier; 6 = analog computer device; 7 = electronic integrator; 8 = printer; 9 = chart recorder.

(1) The instrument should be able to locate chromatographic spots or zones even on a two-dimensionally developed plate in an automatic manner (see Fig. 2).

(2) It should scan over the zones in a truly two-dimensional fashion[7,13], the light spot area being negligibly small compared to the chromatographic zone. An x–y plotter could be used for the visual presentation (see Fig. 2).

(3) The relationship between concentration and instrument response should be rendered linear[7,13].

(4) The instrument should accommodate several plates and scan them successively in an automatic manner.

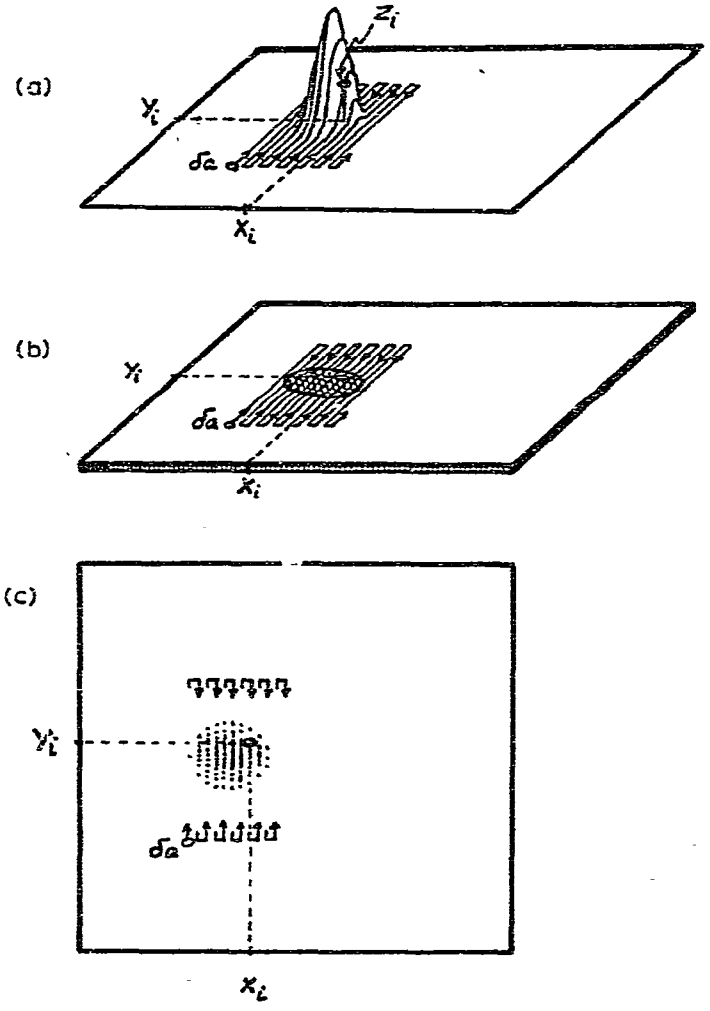


Fig. 2. Two-dimensional integration. (a) Three-dimensional presentation of the quantitative scan. x_i and y_i are the coordinates describing the position of the light spot (δa); Z_i is absorbance. (b) TLC plate. (c) Two-dimensional presentation of the quantitative scan by using an x–y plotter.

(5) The digital output of the instrument should give the location of the zones (x and y coordinates), the respective integral, and the wavelength of the scanning.

(6) The instrument should be capable of recording absorbtion spectra automatically (see Fig. 3).

Considering the ability of the minicomputers, the features listed above are feasible. A commercial instrument satisfying all these demands would certainly stimulate further research into the methodology of quantitative TLC and photometry in inhomogeneous media.

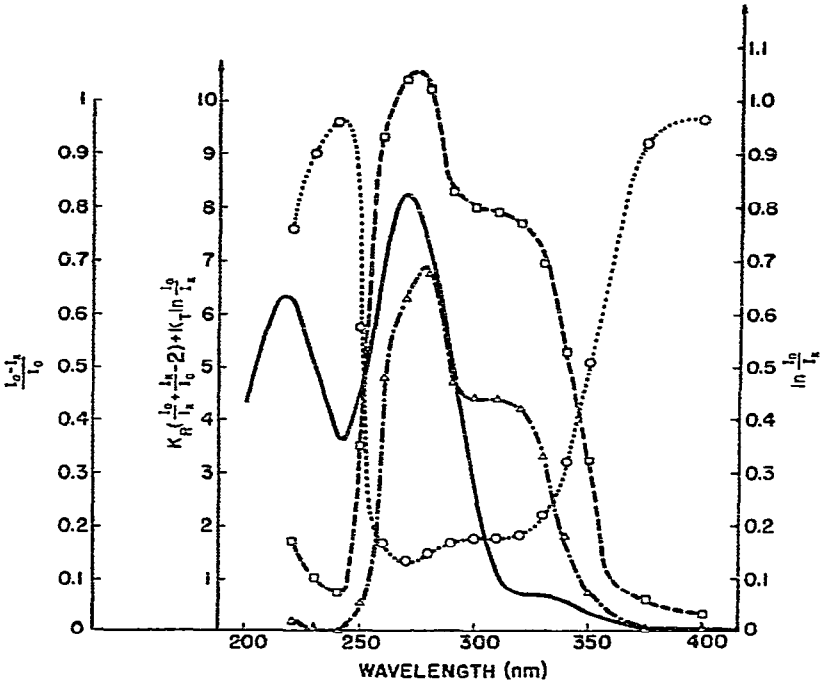


Fig. 3. Absorption spectra of sulfapyridine in solution (———, 33 nmoles/ml ethanol) and on the TLC plate (10 nmoles) in reflectance according to the formula $(I_x - I_o)/I_o$ (○···○), eqn. 1 (□–·–□), and eqn. 5 (△–·–△).

GLC and LC would also probably be affected by the development of better techniques of photometry in inhomogeneous media. As the bulk of the material chromatographed is in the stationary phase, photometric sensors directed onto the chromatographic medium would greatly increase the sensitivity of detectors compared to those used today.

Further data with respect to the above will be presented elsewhere. Ongoing projects include the testing of the conditions necessary to linearise the photometer response as obtained from a variety of chromatographic media.

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REFERENCES

1 P. Kubelka, *J. Opt. Soc. Amer.*, 38 (1948) 448.
2 G. Kortüm and J. Vogel, *Z. Phys. Chem. (Frankfurt am Main)*, 18 (1958) 110.
3 P. Kubelka and F. Munk, *Z. Tech. Phys.*, 12 (1931) 593.
4 H. Jork, *Z. Anal. Chem.*, 236 (1968) 310.
5 L. R. Treiber, R. Nordberg, S. Lindstedt and P. Stöllnberger, *J. Chromatogr.*, 63 (1971) 211.
6 J. C. Touchstone, S. S. Levin and T. Murawec, *Anal. Chem.*, 43 (1971) 858.
7 L. R. Treiber, *J. Chromatogr.*, 100 (1974) 123.
8 H. Keuker, *Chromatographia*, 4 (1971) 40.
9 M. Sandberg and K. A. Hansson, *Internal Report on Zeiss Chromatogram Spectrophotometer*, Pharmacia Fine Chemicals, Uppsala, 1973.
10 L. R. Treiber, *Clin. Chim. Acta*, 38 (1972) 171.
11 G. J. Krol, G. R. Boyden, R. H. Moody, J. C. Comeau and B. T. Kho, *J. Chromatogr.*, 61 (1971) 187.
12 N. E. Brandstrup and L. R. Treiber, *J. Steroid Biochem.*, 2 (1971) 133.
13 L. R. Treiber, B. Örtengren, R. Lindsten and T. Örtegren, *J. Chromatogr.*, 73 (1972) 151.
14 G. K. Zwolinski and L. R. Treiber, *J. Chromatogr.*, 107 (1975) 311.
15 L. Rydén, A. Berlin and L. R. Treiber, *Eur. J. Clin. Pharmacol.*, 8 (1975) 277.
16 B. Örtengren and L. R. Treiber, *Res. Commun. Chem. Pathol. Pharmacol.*, 9 (1974) 339.
17 D. B. Wender, L. R. Treiber, H. B. Bensusan and A. G. Walton, *Biopolymers*, 13 (1974) 1929.