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DIRECT QUANTIFICATION OF MICRO-THIN-LAYER CHROMATOGRAMS

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SUMMARY

A variety of compounds have been quantified after thin-layer electrophoresis and/or thin-layer chromatography on microplates by means of the triple product of the largest and smallest diameters and the maximum absorbance of each spot. The diameters were measured with a graduated magnifier and spot absorbance was determined by means of a fiber optic light pipe fitted to a standard spectrophotometer. Precision and accuracy of $\pm$ 5 % or less were obtained using amino acids, glucose, and cholesterol in the range of 10^{-11}–10^{-9} moles. A total of six different reagents were used on silica gel and cellulose, with glass plates and Mylar film supports after both mono-dimensional and two-dimensional separations. Except for the lipids which were applied with a modified syringe, all solutions were spotted by means of calibrated Nichrome wire loops delivering 36–126 nl. The procedure was found to be simple and economical and reliable in routine use.

INTRODUCTION

Substances on paper and thin-layer chromatograms have been quantified directly by a variety of methods based on measurements of the spot size or its color density[1,2], and various empirical mathematical manipulations. These techniques are subject to numerous limitations even when applied to conventional paper chromatography (PC) and thin-layer chromatography (TLC)[3]. None of the methods in current use have been suitable for the quantification of microplates where the spots are less than 10 mm^2 in area and contain nanogram amounts of non-fluorescent substances.

The procedure presented here was first proposed by BLOCK in 1948 for the quantification of two-dimensional amino acid patterns on PC[4]. The method is based on the principle that the product of the area of a spot and its maximum color density approximates a total integration of the spot color, and is directly proportional to the quantity of material. Unlike measurements of area or color density alone, this technique is relatively unaffected by differences in the applied sample volume[5]. Despite its effectiveness[5–7] BLOCK discontinued use of the method after a very short time and recommended that it should not be used[5,7,8]. This was because, in his view, the double determination of area and color density required an investment of time and effort not justified by the increased precision compared to that obtained from maximum spot density alone.

In large part, this was due to the problems attending area measurement by planimetry. Consequently the method was hardly ever used, is not even mentioned in most standard references[1–3,9–12] and virtually had to be rediscovered. Fortunately, by being unaware of the history of the technique at the start of our research, we were also unaware of the strong recommendation that it should not be used.

The present successful use of the method is based on a different technique for gauging spot area so that rapid, precise measurements can be made even of elliptical spots smaller than 2 × 2 mm in size. Maximum color density is also measured quickly and precisely, without the need for expensive specialized equipment. The speed of the quantification procedure is appropriate to that of the separation time on 5 × 5–10 × 10-cm microplates, and the precision and accuracy are comparable to standard colorimetry.

The origin spot on a paper or thin-layer chromatogram can be thought of as a cylindrical volume in which the third dimension is equal to the thickness of the medium. The quantity, Q, of a substance in the cylinder is equal to the concentration of the material per unit volume times the total volume. In classical colorimetry, the concentration can be related to the absorbance, provided the analytical reaction observes the Lambert–Beer law. On a solid medium the Kubelka–Munk equation[1] more accurately describes the proportionality between the spot color and the quantity of material present. Empirically, however, the Lambert–Beer law has been regularly used in the design of commercial densitometers and has been shown to give linearity over a sufficiently useful range[1]. As a substance migrates, the concentration and absorbance decrease and become non-uniform while the spot size increases and becomes more diffuse. Somewhat simplistically, the situation is analogous to a cuvette of constant light path but progressively increasing cross-sectional area. For maximum precision both the absorbance and the area would need to be measured and their product related to the quantity of material. The area of an elliptical spot is directly proportional to the product of the largest and smallest diameters.

Therefore, the quantity, Q, of a substance in a chromatographic spot is related to the product of its maximum absorbance (A) and its two diameters (D and d):

$$Q = K \cdot A \cdot D \cdot d$$

For any given substance, under the same chromatographic conditions, K should be the same and equal to the slope of the line Q *vs.* $A \cdot D \cdot d$.

This quantitative relationship should be limited only by the capacity of the medium and the detection reagent, and should be valid for one- or two-dimensional chromatograms. In practice, an intercept correction must be added to the right-hand side of the equation.

METHODS AND MATERIALS

Quantification

Spot absorbance was measured with a simple accessory, developed to fit the photomultiplier unit of the Hitachi-Coleman 139 spectrophotometer. This attachment could be adapted to fit other instruments equally well. Essentially, the device consisted of a small fluorescent light source and a fiber optic light guide which conducted light transmitted through the chromatogram to the photomultiplier. A surplus monocular

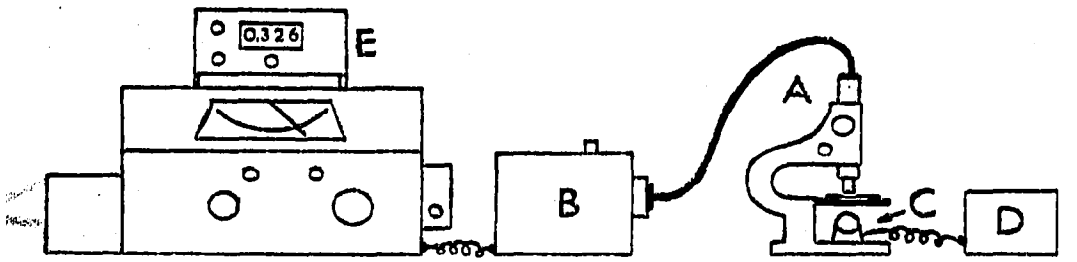

Fig. 1. Photometric system: (A) microscope stand with fiber optic light guide; (B) photomultiplier; (C) light source; (D) voltage stabilizer; (E) digital readout unit.

microscope was used as a support frame (Fig. 1). The light source was constant and the spectrophotometer sensitivity controls were used to adjust the response magnitude.

The light source was a 4-W fluorescent lamp (General Electric F4T5-CW) resting on the microscope base in the usual substage position and fastened in place. The input voltage was stabilized with a constant voltage transformer. No filters were used. The mirror, mirror-mount, and condenser assembly of the microscope were all removed as were the eyepiece and objective lenses. A fiber optic light guide (American Optical Co. LGM-3X24) 61 cm (24 in.) long with 3.2-mm (1/8 in.) polished end windows passed through the body tube of the microscope, and was held in place by two rubber stoppers of appropriate size. At the bottom of the tube, the stopper and light guide were covered with a circle of black vinyl electrical tape with a 1-mm circular hole in the center which served as a fixed aperture. A larger aperture could be used if required by the size of the series of chromatographic spots to be measured. At its other end, the light guide was fitted snugly into a one-hole rubber stopper which fit tightly into the light port of the photomultiplier unit. A ground glass plate, on the microscope stage, with its smooth side up and cross "hairs" scratched on its lower surface, was securely mounted so that the reference lines intersected directly under the aperture. This was easily set by measuring the maximum absorbance of a black ink spot superimposed over the cross point.

In practice a TLC plate was placed, coated side down, on the glass surface and the microscope tube was lowered until the aperture touched the chromatogram. In conventional fashion, zero transmittance was set with the shutter of the photomultiplier closed and 100% transmittance was set with the shutter open on a blank area. A spot to be read was then centered on the cross lines, the aperture again placed in contact with the surface and the maximum absorbance read. The digital readout unit of the spectrophotometer was a particularly convenient accessory.

Accurate measurements of the diameters of small chromatographic spots were obtained by means of a measuring magnifier (Bausch & Lomb 81-34-38 with metric scale) which gave an effective linear magnification of 4.5 times.

A fluorescent light box was used for illuminating the chromatograms without heating them. Average time required for reading the absorbance and two diameters of a series of six spots was about 15–30 min depending on their intensity and diffusiveness.

Chromatograms were quantified by calculating the slope and intercept of the standard curve for each substance individually, and using these to calculate the Q's corresponding to the observed ADd values of the unknowns. The standard error

of estimate (S_E), correlation coefficient (r), and coefficient of variation (V_E)* were determined for each standard curve. The mean, standard deviation (S_D) and coefficient of variation (V_D)**, were determined for the unknowns[13].

Chromatography

Two-dimensional amino acid separations were carried out on Eastman 6064 cellulose "Chromagrams" cut to 7.5 × 7.5 cm. Samples and standards were applied with a 126-nl Nichrome wire loop[14,15] calibrated by the spot area method to ± 1 nl. Separations were two-dimensional[16] with high-voltage electrophoresis (TLE) at pH 1.9, 2000–2200 V, in the first direction, and TLC in *n*-propanol–methyl ethyl ketone–formic acid–water(10:6:1:3) in the second. The approximate separation times were about 3 min for TLE, and about 15 min for TLC. The chromatograms were dried at room temperature and stored (when necessary) over concentrated sulfuric acid in a closed chamber. They were quickly dipped in ninhydrin solution[16] (2.0 % (w/v) ninhydrin–0.5 % 2,4,6-trimethyl pyridine–0.5 % glacial acetic acid in acetone) and placed in the acid cabinet for color development at room temperature. After 2 h the plates were dipped into an acetone solution of acidified cupric nitrate[17]. The spot colors are stable for at least 5 days provided the chromatograms are kept in the dark when not being used. Samples (in triplicate) and a five- or six-point standard curve were always processed together for maximum uniformity.

TABLE I

SEPARATORY PROCEDURES USED FOR QUANTIFICATION

The ranges given are those that were used, and not necessarily the limits of any of these methods. Systems: (a) ethyl acetate–pyridine–water (12:5:4)[18]; (b) petroleum ether–diethyl ether–glacial acetic acid (90:10:1)[19]; (c) acetic acid–formic acid–water (12:6:982)[20]; (d) *n*-propanol–methyl ethyl ketone–formic acid–water (10:6:1:3)[21].
Media: (CS) Cellulose, Schleicher & Schuell No. F1440 (without binder); (SA) Silica Gel G, Analtech "Uniplate" activated at 165° for 30 min; (CE) Cellulose, Eastman Kodak Co. "Chromagram" No. 6064.
Reagents: (A) (1) saturated ethanolic silver nitrate diluted 1/5 with ethanol; (2) 0.5% ethanolic KOH; (3) 9% sodium thiosulfate in 50% aqueous ethanol. Sprayed in sequence. (B) 5% H_3PO_4–5% H_2SO_4 in 95% ethanol. Sprayed and heated at 100° for 10 min. (C) Diazotized sulfanilic acid[22]. Sprayed. (D) Cupric nitrate[23]; (2) ninhydrin[16]. Sprayed, then dipped, in sequence. (E) (1) Ninhydrin[16]; (2) cupric nitrate[17]. Dipped in sequence. (F) 0.04% Bromocresol green[24]. Sprayed.

Substance	*Range (nmoles)*	*System*	*Medium*	*Size (cm)*	*Reagent*
Sugars	1.26 – 3.78	TLC (a)	CS	7.5 × 7.5	A
Cholesterol	1.70 –12.9	TLC (b)	SA	10 × 10	B
Histamine	0.060– 0.178	TLE (c)	CE	7.5 × 10	C
δ-Aminolevulinic acid	0.123– 0.978	TLE (c)	CE	7.5 × 10	D
Amino acids	0.63 – 2.53	TLE (c)/TLC (d)	CE	7.5 × 7.5	E
	0.090– 0.363	TLE (c)/TLC (d)	CE	5.0 × 5.0	E
3-Methylhistidine	2.53 –10.1	TLE (c)	CE	5.0 × 7.5	F

To test the general applicability of the quantitative procedure with other reagents, mono-dimensional separations were used as shown in Table I. Chloroform–methanol (2:1) solutions of cholesterol were applied with a modified 10-μl Hamilton

* $V_E = S_E/\text{mean} \times 100$.
** $V_D = S_D/\text{mean} \times 100$.

701 microsyringe and Sage 237-1 syringe drive unit[25]. All other solutions were applied with the Nichrome loops. Standard and unknown solutions were always prepared in the same solvent and spotted with the same loop or syringe. Precision was assessed by re-reading amino acid chromatograms as well as by the re-analysis of various unknown samples. Accuracy of the method was evaluated by comparison with standard analytical methods for free amino acids and glucose.

TABLE II

AMINO ACID ANALYSIS BY MICRO-TLE/TLC AND BY AN AMINO ACID ANALYZER (AAA)[a]

Amino acid	*Value (mM)*		*Error (%)*[b]
	TLE/TLC	*AAA*	
Aspartic acid	2.99	2.85	+ 4.91
Threonine and glutamate	11.2	10.0	+ 12.0
Valine	2.30	2.28	+ 0.87
Phenylalanine	1.29	1.30	− 0.76
Lysine	2.90	3.08	− 5.84
Leucine and isoleucine	4.88	5.23	− 6.52
Serine, glycine and alanine	6.15	5.95	+ 3.36
		Total	+ 8.02
		Mean	± 4.89

[a] Analysis performed by Dr. PAUL MANOWITZ.
[b] Based on the AAA value as the true concentration.

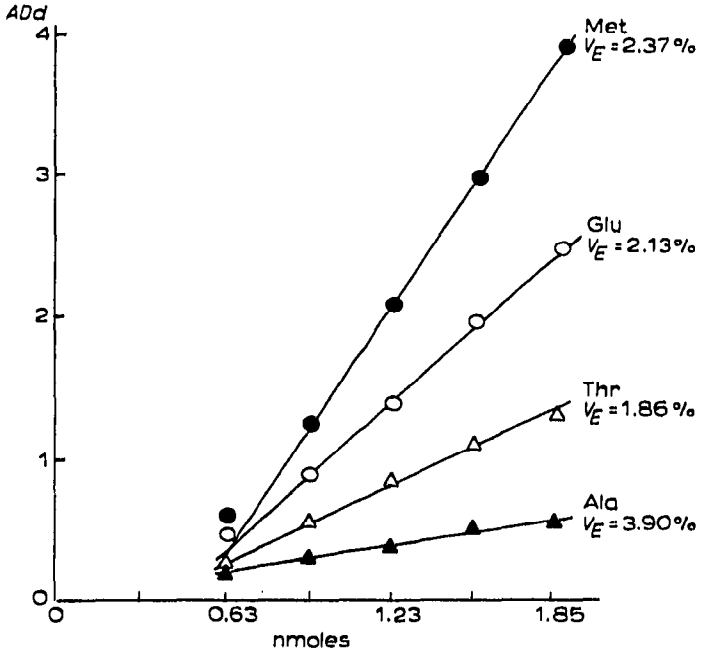

Fig. 2. Amino acid standards, 2.5 m*M*, spotted with a 123-nl loop; two-dimensional TLE/TLC as described in Table I.

RESULTS

Analysis of a solution of a commercial acid hydrolysate of casein (Atlas Chemical Industries, Stuart's amino acids) by the method as described above and by means of a amino acid analyzer (Technicon Corp.) gave the results shown in Table II. The same reference standard (Calbiochem AA-5) was used in both procedures (Fig. 2).

The glucose concentration of a pooled standardized normal human plasma was determined by micro-TLC to be 6.44 mM (typical values from one of three separate duplicate determinations: $r = 0.993$, $V_E = \pm 4.25\,\%$, mean concentration = 6.57 mM). The concentration as measured by an automated ferricyanide procedure* was 6.22 mM (3.5 % error, based on the ferricyanide value as the true concentration).

Free cholesterol in the standardized plasma was analyzed by micro-TLC and found to be 41.8 mg/100 ml ($V_D = \pm 7.5\,\%$ on two separate triplicate determinations). As can be seen from Fig. 3, variation in the amount of acid sprayed and the duration of heating resulted in very different standard curves in the two separate analyses. Nevertheless, the precision of the results was excellent.

Linearity was equally good with a variety of other compounds using reagents yielding diverse spot and background colors (Table III).

The procedure was also used to measure black spots on a transparency film Polaroid Type 46-L). The photograph of a chromatogram of cholesterol standards and

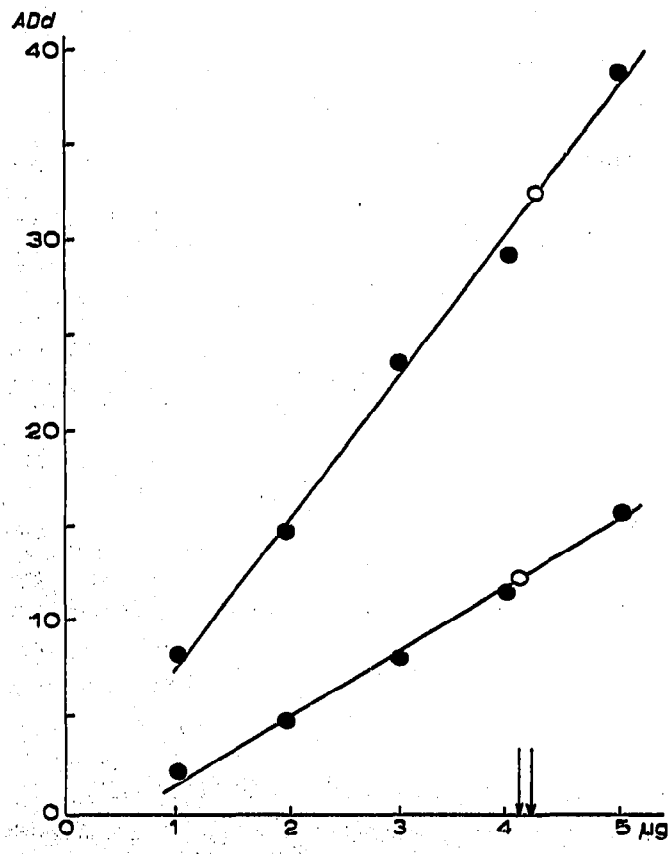

Fig. 3. Cholesterol analysis showing the precision in measuring a serum free-cholesterol (in triplicate) on two different days. ●, Standards; ○, unknown.

* Provided by Dr. Louis Rosenfeld.

TABLE III

THIN-LAYER QUANTIFICATION USING VARIOUS REAGENTS

Thin-layer procedures and reagents are described in Table I.

Compound	*Spot color*	*Background color*	*r*	S_E(*nmoles*)	V_E(%)
Inositol	brown-black	light tan	0.997	0.175	2.8
Ribose	brown-black	light tan	0.997	0.148	2.7
Histamine	rose	white	0.999	0.002	1.3
δ-Aminolevulinic acid	yellow	blue-green	0.999	0.014	2.8
3-Methylhistidine	blue	green	0.998	0.199	3.2
Cholesterol	black	white	1.000	0.302	1.2
Valine	pink	white	0.993	0.036	4.2

quadruplicate extracts from 28 mg of mouse brain tissue, was placed between glass plates for protection and the spots measured in the usual way. The brain extract was found to be 5.14 μmoles/mg of fresh weight tissue. Another chromatogram scanned directly produced a value of 5.06 μmoles/mg of fresh weight tissue.

Measuring the maximum absorbancy and dimensions of a 4-mm diameter chromatographic spot by means of a 1-mm light probe and a reticle graduated to 0.1 mm, proved to be a procedure of very high precision (Table IV). The natural tendency to read the values that are expected is greatly minimized by the fact that either the absorbance (A) or spot size ($D \cdot d$) alone can give a poor correlation with the quantity of material (Fig. 4).

TABLE IV

ADd VALUES OF ALANINE AND SERINE

0.924 nmoles each per 5 × 5-cm TLE/TLC pattern. The three chromatograms were each read three times during a 7-h period.

Amino acid	*Chromatogram (No.)*	*Mean ADd value*	S_D(*ADd*)	V_D(%)
Alanine	1	2.105	0.068	3.2
	2	2.431	0.074	3.0
	3	1.819	0.118	6.5
Serine	1	2.018	0.020	1.0
	2	1.725	0.067	3.9
	3	1.555	0.040	2.6

The sensitivity of the method was explored only to a limited extent since it is primarily a function of the quality of the chromatographic resolution and the sensitivity of each particular reagent. When amino acid standards (Hamilton Co., Amino acid calibration standard Type P-AN) were separated on 5 × 5-cm layers, phosphoserine which appears as a sharply defined spot close to the origin could be quantitatively measured in the range of 0.090–0.463 nmoles with a standard error (S_E) of ± 0.0073 ($r = 0.997$, $V_E = \pm 3.6\%$). A 0.046-nmoles spot was visible but its dimensions could not be measured. On the other hand, a histamine standard which was separated by high-voltage electrophoresis alone could be quantitatively measured at a level of 0.059 nmoles (Fig. 5).

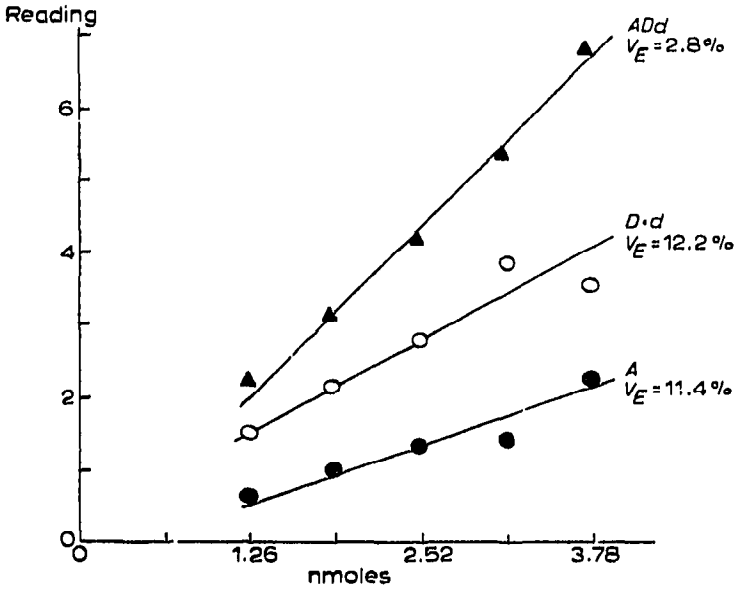

Fig. 4. Quantity of inositol as a function of spot absorbance alone (A), spot area alone ($D \cdot d$) and the product of the absorbance and area (ADd).

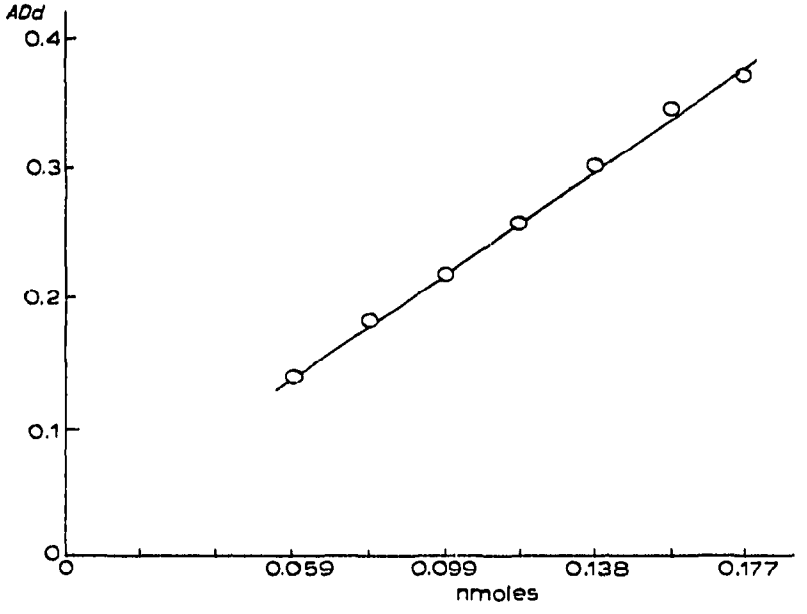

Fig. 5. Histamine standard, 0.16 mM, spotted with a 123-nl loop; conditions given in Table I.

DISCUSSION

TLC on microplates is a rapid, sensitive technique of great versatility. Simple and economical, as a quantitative method it can be a powerful tool. Substances in the range of 10^{-11}–10^{-9} moles can be separated in one or two dimensions by electrophoresis and/or chromatography. They can be detected with an appropriate reagent and the amount of material measured with precision and accuracy.

Chromatographic spots of 2–3 mm in diameter cannot be measured with commonly available commercial instruments and in general contain too little material for elution analysis. Flying spot scanning is possible[26] but the apparatus is costly. In contrast, a fiber optic light pipe (with randomly oriented fibers) is a very inexpensive device. By means of a simple mechanical coupling it can be used to convert a standard laboratory spectrophotometer into a microdensitometer suitable for measuring the absorbance of small chromatographic spots. The Hitachi-Coleman 139 spectrophotometer can be easily converted from one function to another in less than 10 sec. Other instruments can probably be adapted just as easily.

As shown in Fig. 4, the molar content of a series of spots correlates better with the product of the maximum absorbance and area of the spots than with either the absorbance or area alone. While a double measurement is required, the routine precision of the method easily justifies this.

High resolution with micro-TLC requires an extremely small origin spot. This can be achieved either by the incremental application of small minute volumes, best done with a Nichrome wire loop[15], or by means of a syringe drive unit operating a modified microsyringe at a very slow speed[25]. This latter method is practical only with very volatile solvents, since otherwise sample application is inordinately slow in relationship to the rest of the procedure.

The procedure appears to have general applicability. It has been successfully used with cellulose and silica gel, on glass plates and on Mylar plastic. It is as appropriate for a series of two-dimensional chromatograms as for a single mono-dimensional plate and is equally suitable for lipids, amino acids, sugars, or (acidic and) basic compounds. Neither the color of the chromatographic spots nor the background appears to be limiting. Since the method has been used for measuring black spots on a phototransparency, there is no reason to believe that it could not be used for scanning autoradiograms or photo-negatives of fluorescent patterns. The technique appears to be usable as the basis of a general scheme of microanalyses.

Since this technique has been used for measuring spots on paper chromatograms[6] and for quantifying TLC plates as small as 5 × 5 cm, it should be usable for virtually any size chromatogram. The general high sensitivity and small sample volume required makes the TLC method particularly suitable for analyses where the quantity of material is severely limited. This procedure is currently being used for topographic chemical studies of mouse brain.

Although the area–absorbance product can correct for discrepancies in spot size, it cannot correct for the disparity that occurs when the standard and sample are prepared in different solvents and applied in grossly unequal amounts. It is necessary, therefore, for the standard and unknown solutions to differ not more than ten-fold in concentration if one does not wish to apply more than ten loop increments per chromatogram.

As in any other quantitative microanalysis, strict attention must be paid to the accuracy and precision of the various technical manipulations. Color development in TLC is primarily a surface phenomenon. A more diffuse spot can have a greater ADd value (*i.e.* total color) than the same quantity of material in a compact spot. Serious non-uniformity in TLE or TLC can, therefore, affect color development and result in non-linearity of the data. Various sources of error in common with other methods of chromatographic quantification are discussed elsewhere[27]. Errors due to

refraction or scattered reflection of the light, are thought to be minimal, however, because of the good uniformity of the commercial plates, the perpendicular alignment of the light path, and the small size of the aperture which is in direct contact with the plate.

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