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DENSITOMETRY OF THIN-LAYER CHROMATOGRAMS USING POLAROID PROJECTION FILM

APPLICATION TO THE ANALYSIS OF MONOSACCHARIDES

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SUMMARY

A method for quantitative densitometry of thin-layer chromatograms using Polaroid Type 46-L projection film and a conventional thin-membrane densitometer is described. Standard curves prepared from fucose and glucose visualized with aniline–diphenylamine–phosphoric acid showed a linear relationship between the peak areas and the amount of sugar present on the chromatographic plate. The applicability of the method to biological materials is demonstrated by its successful use in determining plasma fucose concentrations. The method is applicable to the quantitative densitometry of chromatographic and electrophoretic systems where the properties of the substances to be analyzed and the physical characteristics of the separation system make direct densitometric analysis technically difficult.

INTRODUCTION

Materials separated on thin-layer chromatograms can be quantified either following elution from the chromatogram adsorbent or by an analytic method performed directly on the chromatographic plate, such as densitometry[1]. Densitometry has the advantage of simplicity but does suffer from limitations due to the physical characteristics of the chromatographic system. Densitometry of thin-layer chromatograms has been described for the analysis of amino acids[2], carbohydrates[3], lipids[4], steroids[5], alkaloids[6], drugs[7] and dyes[8], and the methods have been reviewed recently[9]. A number of instruments designed for thin-layer chromatogram densitometry are commercially available[10].

This communication describes a method of thin-layer chromatographic densitometry using photographs made on Polaroid Type 46-L Land projection film and a conventional thin-membrane strip densitometer. The method provides both a means of quantifying materials on thin-layer chromatograms and a permanent record of the appearance of the chromatogram. Photography on Polaroid projection film does not require a dark room, permits an immediate evaluation of the quality of the photograph

and provides a positive image of the chromatogram for scanning without the necessity of image reversal. The photograph can be taken immediately after color development and the densitometry performed when convenient since chromogen fading and background darkening of the original chromatogram with time are eliminated. The method requires a minimum amount of manipulation of the chromatogram, reducing the likelihood of disturbing the adsorbent surface. The photographs are resistant to mechanical damage and are stable for at least two years without special storage. The sensitivity of this method is as good as direct densitometry and the linearity of the response extends through a greater range of sample quantity than has been reported with direct densitometry of carbohydrates[3,11–13].

MATERIALS AND METHODS

L-Fucose, D-glucose, D-galactose, D-mannose, D-glucosamine, sialic acid and aniline were purchased from Sigma Chemical Co. (St. Louis, Mo.). L-[$^{14}C_6$]Fucose was purchased from Calbiochem (Los Angeles, Calif.). Analytical grade methyl ethyl ketone, methanol, diphenylamine, 1-butanol, pyridine, hydrochloric acid, phosphoric acid and glacial acetic acid were purchased from Fisher Scientific Company (Boston, Mass.). Silica Gel G and Aluminum Oxide G were obtained from Brinkmann Instruments, Inc. (Westbury, N.Y.).

Two thin-layer chromatographic systems were used in this study[14]. Silica Gel G suspended in 0.1 *N* boric acid or Alusil (aluminum oxide–Silica Gel G, 1:1 w/w) suspended in distilled water was spread in 250-μ layers on 20 × 20 cm glass plates using an adjustable thin-layer chromatogram spreader. The plates were dried at room temperature and stored in a desiccator until used. Synthetic mixtures of sugars, reference standards of fucose and glucose, and neutralized acid hydrolysates of plasma proteins were applied along a line 2 cm from the lower edge of the plate in a volume of 1 μl using capillary microliter pipets. The plasma samples, the addition of L-[$^{14}C_6$]fucose to the plasmas for isotope dilution determination and the method for preparing the glycoprotein hydrolysates have been described[15]. In certain experiments, radioactively-labeled fucose was added to the fucose standards at a ratio of 100 d.p.m. of L-[$^{14}C_6$]fucose per microgram of fucose. The chromatographic plate was then placed in a developing tank containing a small amount of the solvent system, and allowed to equilibrate for 16 h. The Alusil plates were developed with 1-butanol–pyridine–0.1 *N* HCl (5:3:2) and the Silica Gel G plates were developed with methyl ethyl ketone–methanol–glacial acetic acid (6:2:2). Following equilibration, additional solvent was added until the fluid level rose sufficiently so that it was in contact with the adsorbent layer on the glass plate. The chromatogram was developed until the solvent had risen 15 cm from the lower edge of the plate. The chromatogram was removed from the tank and dried at room temperature. The chromatograms were sprayed evenly with the aniline–diphenylamine–phosphoric acid reagent[16] and heated in an oven at 105° for 15 min. This reagent gave good visualization with all the sugars used in this study; however, the sensitivity was somewhat greater for the hexoses than for fucose[12], while glucosamine was approximately 50% as reactive as the neutral sugars.

Photographs of the plates were made with a standard MP-3XL Industrial View Land Camera (Polaroid Corporation, Cambridge, Mass.) fitted with 150-W, 2800-K bulbs and the 127-mm lens. Photographs were made on Polaroid 4 × 5 Land Type 52

film (black and white prints) and on Polaroid Type 46-L Land projection film (black and white continuous contrast transparencies) for densitometry. The plates were placed on the surface of an unlighted conventional X-ray view box since this type of surface resulted in even base lines in the densitometric scans. A lens opening of F-22 to F-32, at $\frac{1}{2}$-sec exposure time, yielded optimal contrast and even base lines. The densitometer available for this study required that each plate be photographed in three sections in order to provide a large enough densitometric peak image for accurate measurement. Exposure of the chromatogram to the flood lights for 1 to 2 min did not change its appearance; however, after approximately 10 min exposure the chromatogram darkened. Identifying information was written on the Type 46-L Land projection film immediately after development, prior to drying of the emulsion. The Polaroid films were processed according to the manufacturer's instructions except that the Type 46-L Land projection film was fixed with Kodak fixer (Eastman Kodak Co., Rochester, N.Y.) for 30 sec because the Polaroid Dippit resulted in fine lines appearing on the film surface which adversely affected the quality of the densitometric scans. After rinsing for 1 h in running tap water, the projection film photographs were dried and cut into approximately 3-cm-wide strips and scanned in an Analytrol Recording Densitometer–Integrator (Beckman Instruments, Inc., Fullerton, Calif.) fitted with a 500-mμ peak wave length light filter, modified with a Scan-a-Tron attachment (Gelman Instrument Co., Ann Arbor, Mich.) and a B-2 logarithmic balancing cam. The slit size was 0.5 mm wide by 11 mm long; this length extended slightly beyond the visible boundaries of the chromogen images. All results included in this report are from scans that were carried out in the direction of development starting at the solvent front. Scanning could also be performed at right angles to the direction of development.

Curve areas were measured by planimetry. Following densitometry, those samples containing L-[$^{14}C_6$]fucose were scraped from the plate into counting vials, 0.5 ml of water was added and the radioactivity was measured with the liquid scintillation mixture of PROCKOP AND EBERT[17] and a Packard Tricarb liquid scintillation spectrometer. Control experiments indicated that the aniline–diphenylamine–phosphoric acid spray and the Silica Gel G particles did not interfere with the radioactivity determinations. The counting efficiency for chromatographed and unchromatographed L-[$^{14}C_6$]fucose was 50%. Recovery of L-[$^{14}C_6$]fucose from the chromatogram was 95% and the radioactivity was located entirely in the fucose spot.

Statistical analysis of differences among serum fucose levels was performed using standard methods with $P < 0.05$ as the level of significance. Linear regression analysis was employed to test for significance of the relationship between the area under the densitometry peaks and the amount of the substances on the chromatographic plate.

RESULTS

The Alusil plates yielded good results in resolving mixtures of the sugars present in plasma glycoproteins including fucose, mannose, galactose, glucosamine and sialic acid. Fig. 1 shows the separation of the sugars chromatographed in this system as recorded by scanning on Polaroid Type 46-L Land projection film. This sample contained a mixture of fucose, mannose, galactose, glucose, glucosamine and sialic

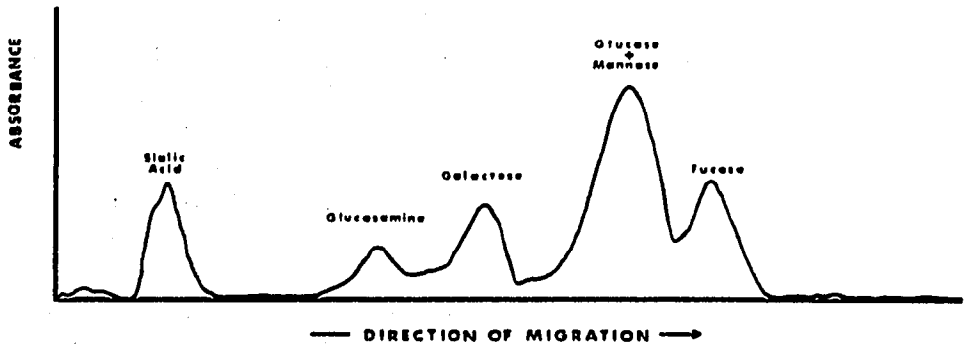

Fig. 1. Polaroid Type 46-L Land projection film densitometric analysis of a standard mixture of fucose, glucose, mannose, galactose, 5 μg/μl each, and glucosamine and sialic acid, 10 μg/μl each. Sample volume, 1.0 μl; adsorbent, Alusil; solvent, 1-butanol–pyridine–0.1 N HCl (5:3:2).

acid. The above sugars, except for glucose and mannose which had very similar R_F values, were well separated. A four-fold increase of the amount of the mixture placed on the chromatographic plate was accompanied by a similar increase in the areas of each densitometric peak, with the exception of sialic acid, which has an R_F of 0 (Fig. 2). Substances having low or high R_F values do not show a linear relationship between the amount on the chromatogram and the peak area when they are studied by direct densitometry[6,8].

Quantitative densitometric data were obtained for fucose and glucose chromatographed on Silica Gel G plates developed with methyl ethyl ketone–methanol–glacial acetic acid (6:2:2). Test solutions of each of the individual sugars were made at concentrations of 1–50 μg/μl. Known amounts of the sugars were placed on the

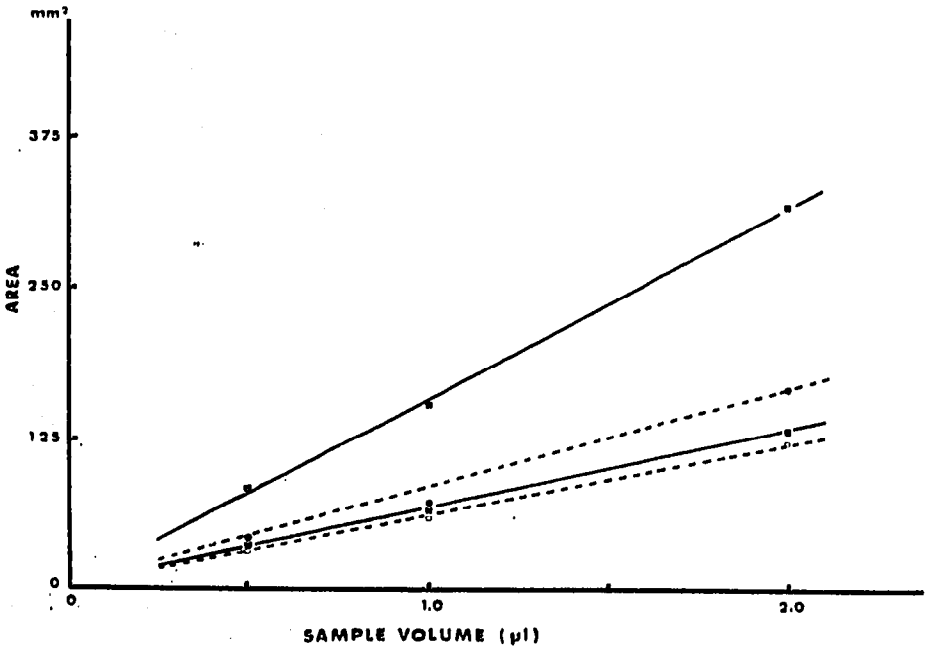

Fig. 2. Relationship of the Polaroid projection film densitometric peak areas to amount of sugar present on the chromatogram. The sugar mixture, adsorbent and solvent system were the same as in Fig. 1. Glucose–mannose peak (□—□), fucose peak (○— — —○), glucosamine peak (■—■), galactose peak (●— — —●).

chromatographic plates in a total volume of 1 μl, by using solutions of different sugar concentrations. Following chromatographic separation of the sugars, spraying with the aniline–diphenylamine–phosphoric acid reagent, Polaroid projection film photography and scanning, the areas under the curves were measured by planimetry, each value being the result of five planimeter readings, performed on duplicate scans. The data were analyzed to determine the relationship of the curve area to the amount of each sugar on the chromatographic plate. Fig. 3 shows the results of a study of

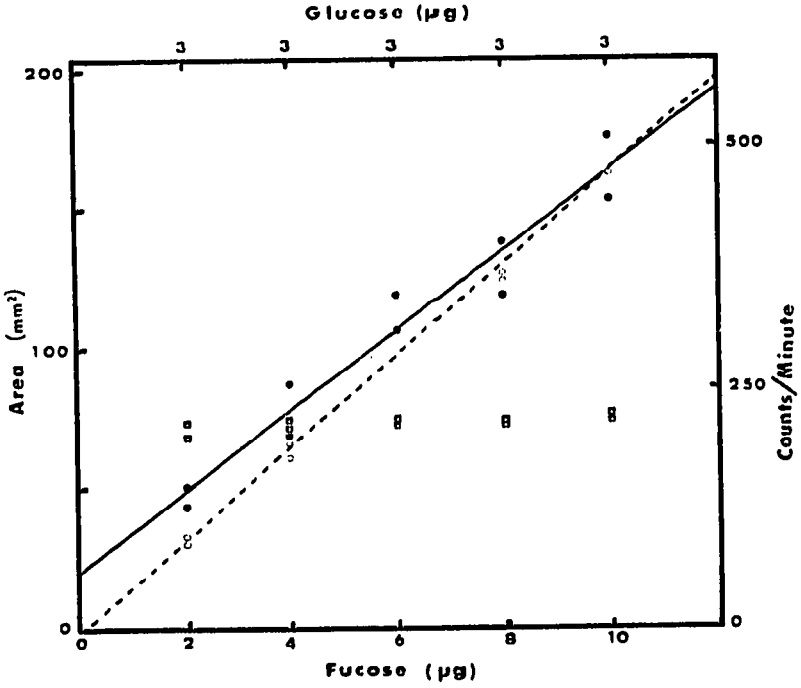

Fig. 3. Relationship of the Polaroid projection film densitometric peak area to the amount of fucose and glucose present on the chromatogram. Glucose (□), 3 μg and fucose (●), 2–10 μg per sample; L-[$^{14}C_6$]Fucose (○), 100 d.p.m. per μg fucose. Each point represents an individual determination. Densitometry and planimetry were performed in duplicate on each sample. The mean area of ten determinations of glucose was 74 mm^2 (S.D. = $\pm$2.0, S.E. = 0.8). The regression lines were fitted to the fucose peak area determinations (————) and L-[$^{14}C_6$]fucose radioactivity counts (— — —) by the method of least squares. The linear relationships were highly significant ($P < 0.001$).

standard fucose and glucose solutions with added L-[$^{14}C_6$]fucose. The amount of glucose placed on the chromatogram in each sample application was 3 μg and the amount of fucose on the chromatogram was varied as indicated. There is a linear relationship between the amount of fucose present and the areas under the densitometric peaks, and the regression line fitted to these points passes close to the origin although the origin did not fall within the 95% confidence limits of the slope. The reproducibility of the method on an individual chromatogram is demonstrated by the uniform peak areas obtained for the 3 μg of glucose present in each sample. As little as 1 μg of fucose or glucose could be measured by this method. When more than 25 to 30 μg of either sugar were placed on the chromatograms, the relationship between the amount of the sample and the area under the densitometric peak was no longer linear. In all our experiments with fucose and glucose standards performed as above,

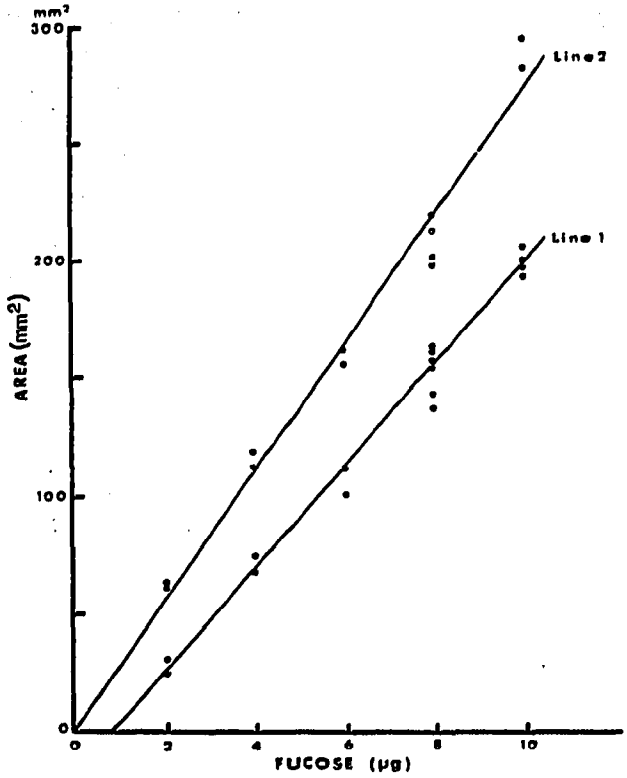

Fig. 4. Variation of the relationship between the Polaroid projection film densitometric peak areas to the amount of fucose present on the chromatogram following single and double spraying of the chromatogram with the aniline–diphenylamine–phosphoric acid spray reagent. Fucose (●), 2–10 µg per sample. Line 1, first spraying; line 2, second spraying. The regression lines were fitted to the experimental data by the method of least squares. The linear relationships were highly significant ($P < 0.001$).

a similar linear relationship was obtained and the regression lines passed close to the origin. The slope of the regression line was, however, variable.

Fig. 4 shows the results when a single chromatographic plate which contained standard amounts of fucose was sprayed, heated, photographed and scanned, then these steps repeated. In both instances, the relationship between the amount of fucose present and the area under the densitometric peaks was linear. The origin fell within the 95% confidence limits of the slope of line 2 but it was just outside of the 95% confidence limits of the slope of line 1. The slopes of the two regression lines were different, indicating that a primary source of variation between plates was in the staining reaction.

To test the usefulness of this method on biological material, human plasma protein-bound fucose levels were determined by combining radioisotope dilution and Polaroid 46-L projection film densitometry of plasma fucose isolated by thin-layer chromatography. In the experiments reported in this paper, the fucose-containing solutions were chromatographed on plates that also contained known amounts of fucose standards. The amount of fucose present on the chromatographic plate in the serum samples was determined by Polaroid 46-L Land projection film densitometry. The radioactivity present in the plasma fucose spots was counted and the fucose specific activity was calculated. As shown in Fig. 3, the ratio of radioactivity recovered

to the chromatographic peak area was constant when varying amounts of L-[$^{14}C_6$]-fucose and unlabeled fucose were chromatographed in a constant ratio. The plasma fucose concentration was determined by isotope dilution based on the amount of L-[$^{14}C_6$]fucose added to the plasma sample, the volume of plasma and the fucose specific activity. Table I shows that the levels of plasma fucose determined by the method of DISCHE AND SHETTLES[18] and by this method are not significantly different ($P > 0.20$).

TABLE I

PLASMA PROTEIN-BOUND FUCOSE CONCENTRATION MEASURED BY THE METHOD OF DISCHE AND SHETTLES AND BY THE POLAROID PROJECTION FILM DENSITOMETRY/ISOTOPE DILUTION METHOD
Significance of the difference in the fucose concentration determined by the two methods, $P > 0.20$.

Sample No.	*Fucose concentration (mg/100 ml)*	
	Dische and Shettles	*Densitometry/ isotope dilution*
1	5.8	8.4
2	5.8	7.2
3	8.0	7.1
4	5.6	7.7
5	9.2	8.0
6	10.5	9.7
7	6.2	5.7
8	9.8	7.0
9	13.8	9.9
10	18.0	8.2
11	9.0	11.7
12	8.6	11.9
13	9.6	7.9
14	8.6	8.2
Mean	9.2	8.5
S.D.	±3.4	±1.8
S.E.	±0.9	±0.5

DISCUSSION

Direct and conventional photographic[19] densitometric analysis of thin-layer chromatograms has been successful on a number of substances despite the problems inherent in using these methods with a non-transparent supporting material[20]. Direct transmittance and reflectance densitometry have both given satisfactory results in comparative studies[6,8]. The factors which affect the results of direct densitometry of thin-layer chromatograms include the following: the nature, thickness and moisture content of the absorbent layer, the solvent system, the rate of solvent flow, the R_F of the compounds, sample application size and shape, the direction of scanning, the measurement of the densitometric curve areas and the detection processes used for colorless compounds[5,8–10]. These factors were not systematically investigated, but the potential effects of these variables were taken into account in conducting the experi-

ments and no evidence was seen for these variables' causing unique problems in Polaroid projection film densitometry. This method gave satisfactory results in two thin-layer chromatographic systems with differing stationary and mobile phases and, while each chromatographic system would require a separate evaluation, it is probable that the method would be applicable to other systems.

Linear relationships have been found between the area of the densitometric peak and the amount of substance on the chromatographic plate in a number of systems[3,4,7,11–13,21]. Other investigators have obtained linearity by relating the square root of the area of the peak and the log of the amount of material on the chromatogram plate[5], the absorbance and the log of the amount of material on the chromatogram plate[22] or area of the peak and the square root of the amount of material on the chromatogram plate[6]. Our finding of a linear relationship between the peak areas and the amount of material on the plate, unless a certain maximum amount of substance is exceeded, is in agreement with direct densitometric studies of thin-layer chromatograms of carbohydrates[3,11–13,23]. We cannot predict the results of Polaroid projection film densitometry on chromatograms of materials where direct densitometry has not yielded linear relationships between peak area and amount of sample. It is of interest to note, however, that SHELLARD AND ALAM[6], working with a number of alkaloids, found that a linear relationship between peak area and amount of material on the plate was seen only when the chromatogram was rendered transparent and densitometry performed by transmittance. Photographing a chromatogram with Polaroid projection film may result in a similar transformation and therefore may yield linear relationships different from direct densitometry on the same chromatogram.

While the relationship between the amount of material present and the peak area was linear, there was a variation in the slopes of the regression lines fitted to the data from individual chromatograms. Differences in the degree of color development from plate to plate appeared to account for most of this variation; therefore each chromatogram must contain standards, in addition to the unknown samples. Investigators using direct densitometry have also reported that colorimetric reactions are the least reproducible step in the method[8,9]. In instances where the chromatographed compounds are colored, the results from replicate chromatograms would be more reproducible since variations due to photography and scanning are small. Differences in the exposure time, lens setting and illumination will affect the area of the curves obtained by densitometry from individual chromatograms, but if conditions are selected so that the photographs are representative of the actual appearance of the plate, and then the photographic conditions kept constant, duplicate photographs made with different film lots show good quantitative agreement. Differences in developing or fixing do not affect the results of this method. Scans performed on photographs before and after fixing gave identical results.

We did not do an extensive investigation of the effect of different colors on this method of densitometry, but the Polaroid projection film Type 46-L is panchromatic and would therefore be expected to record the visible spectrum satisfactorily. The colors of the glucose and fucose spots were blue when sprayed with the aniline–diphenylamine–phosphoric acid reagent. The color of glucosamine was yellow-brown and, while the spray reagent sensitivity was less than for neutral sugars, a linear relationship similar to that of neutral sugars was obtained with Polaroid projection film densitometry. Additional evidence that this method is applicable to materials

of other colors has been obtained by its use for quantitative lipoprotein electrophoresis stained red with Oil Red O[24].

The sensitivity of Polaroid projection film densitometry is similar to that of direct densitometric methods. Other workers have found the lower limits of sensitivity of the densitometry of carbohydrate thin-layer chromatography to be approximately the same as in our experiments[3,11,13] although direct densitometry of carbohydrates on thin-layer chromatograms with as little as 0.25 μg has been reported[12]. Spots barely visible on the chromatograms were easier to see in the photographs made with either Polaroid Land Type 52 black and white film or on the Polaroid Type 46-L Land projection film. The sensitivity of the method is most likely limited by the chromatographic system, the substance studied and the colorimetric reaction rather than by the photographic process.

The background against which the chromatogram is photographed is an important factor in the densitometric results. LUGG[20] presented the theoretical reason for the importance of background in the densitometry of non-transparent, non-homogeneous supporting media. He suggested that in reflectance densitometry, a material behind the object which reflected the portion of the incident light that was transmitted and scattered would improve contrast and the results of densitometry. Our findings and those reported by DALLAS[8] are consistent with this suggestion. Polaroid projection film densitometry was not tested with the use of transmitted light since uniform background lighting is required in order to avoid uneven backgrounds.

The use of a densitometer designed for analysis of small scale separations, such as the Microzone Densitometer (Beckman Instruments, Inc., Fullerton, Calif.), would save time and expense since an entire 20 × 20 cm chromatographic plate could be photographed on a single frame.

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