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Visualization reagents for quantitation of carbohydrates on thin-layer chromatograms by transmission densitometry

The quantitation of carbohydrates by transmission densitometry of thin-layer chromatograms has been reported recently. MIZELLE *et al.*[1] described an aniline–oxalic acid reagent for identifying and quantitating the sugars of flavonoid glycosides, and LAMKIN *et al.*[2] an aniline phthalate reagent for quantitation of the neutral monosaccharides in glycoproteins and glycopeptides. Unfortunately, the use of these aniline reagents was limited to reducing sugars. HUBER *et al.*[3] reported the quantitation of D-glucose and its oligomers that were visualized by spraying with 50% sulfuric acid and heating at 140° for 30 min. LEHRFELD[4] quantitated trimethylsilylated carbohydrates that were visualized by spraying with 5% sulfuric acid in ethanol and heating. Although sulfuric acid reagents are applicable to both reducing and nonreducing carbohydrates, no study to determine the optimum application of sulfuric acid has been reported. Other charring techniques for visualization of chromatograms are known[5–8]. This investigation was undertaken to evaluate four sulfuric acid charring techniques for their suitability in the quantitation of carbohydrates by transmission densitometry.

Experimental

Cleaning of glass plates. Grease on our glass TLC plates caused spots on our chromatoplates and also a flaking of the adsorbent layer from apparently good chromatoplates. A careful scouring of each plate eliminated this problem but was tedious, time-consuming, and often scratched the plates. Boiling the glass plates in a detergent solution followed by rinsing with distilled water effectively eliminated all grease spots.

The cleansing procedure is simple, fast, and efficient when the plate holder and tank shown in Fig. 1 are used. The holder separates the plates and ensures that all surfaces are exposed to the detergent solution. The holder containing the plates is placed in a boiling detergent solution for one-half hour. After the solution cools, the plate holder is removed; the plates are rinsed with distilled water and left to dry. The entire cleaning is carried out with the plates in the holder.

The holder and tank were fabricated from stainless steel. The tank is 9 in. wide × 6 in. deep × 11 in. high. The basic dimensions of the holder are 8 $\frac{3}{4}$ in. wide × 5 $\frac{3}{4}$ in. deep × 7 in. high. A handle projects 7 in. above the top plate of the holder.

The holder consists of two slotted plates and one perforated plate (8 $\frac{3}{4}$ × 5 $\frac{3}{4}$ in.) separated by 6 in. and $\frac{1}{4}$ in., respectively, and held by four $\frac{3}{8}$ × 7 $\frac{3}{8}$-in. rods threaded and bolted at the ends. The handle is a bent $\frac{3}{8}$ × 32 $\frac{1}{2}$-in. rod threaded at the ends and bolted to hold and separate the perforated plate from the lower slotted plate. The perforated plate supports the TLC plates and facilitates drainage.

This holder accommodates ten 8 × 8-in. plates or forty 2 × 8-in. plates. Although the slots in this apparatus are $\frac{1}{4}$ × 8 $\frac{1}{8}$ in., their size or the space between them may be reduced to accommodate more plates and still use the same basic design and dimensions.

Preparation of chromatoplates. Glass plates (20 × 20 cm) were coated with a

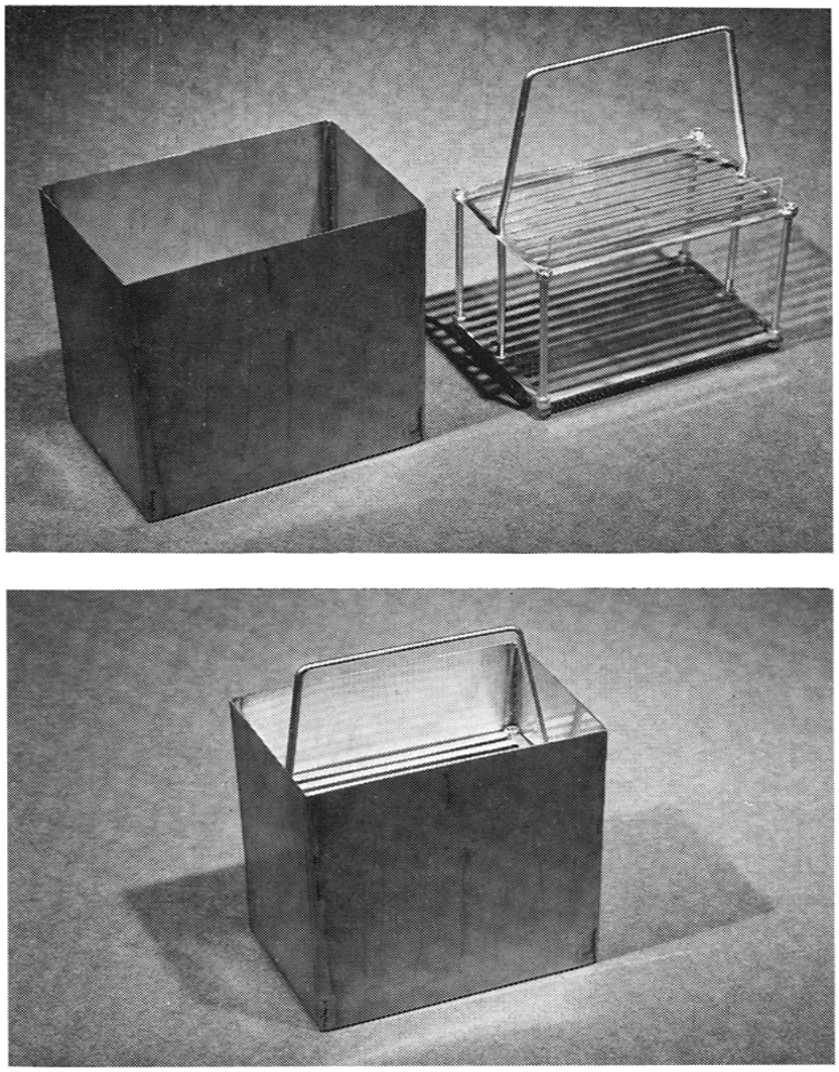

Fig. 1. Plate holder and tank for cleaning glass thin-layer chromatography plates.

0.25 mm layer of Silica Gel G*. The slurry used to coat the plates was prepared from two parts water to one part Silica Gel G[9]. The plates were dried overnight at room temperature and used without activation.

Procedure. Standard solutions of maltose, D-glucose, and methyl α-D-glucopyranoside, containing 1, 3, and 5 μg/μl, respectively, were prepared in (60%) ethanol. Aliquots of 3 μl were applied to the plate in duplicate with a 10-μl Hamilton microsyringe.

Chromatograms were developed by the ascending method in a tightly closed rectangular tank lined with filter-paper wicks[10]. The chromatogram was doubly developed to a distance of 15 cm with butanone–water azeotrope/ethanol (23:2, v/v). The plates were allowed to dry 30 min in a forced-air hood.

* Mention of firm names or trade products does not constitute endorsement over similar firms or products not mentioned by the U.S. Department of Agriculture.

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TABLE I

DENSITOMETRY OF SOME CARBOHYDRATES BY FOUR INDICATOR REAGENTS

Reference compounds (μg)	*3.3% sulfuric acid*				*Ammonium sulfate*				*50% sulfuric acid*				*Sulfuryl chloride*			
	Peak area (cm^2)		*Slope*	*Standard deviation*	*Peak area (cm^2)*		*Slope*	*Standard deviation*	*Peak area (cm^2)*		*Slope*	*Standard deviation*	*Peak area (cm^2)*		*Slope*	*Standard deviation*
	Found[a]	*Calc.*[b]			*Found*[a]	*Calc.*[b]			*Found*[a]	*Calc.*[b]			*Found*[a]	*Calc.*[b]		
Maltose																
3	4.45	4.52	1.04	0.184	5.65	5.80	1.03	0.367	1.90	1.95	0.61	0.122	2.75	2.65	0.70	0.245
9	10.95	10.8			12.30	12.0			5.70	5.6			6.65	6.85		
15	17.0	17.08			18.05	18.2			9.20	9.25			11.15	11.05		
D-Glucose																
3	5.3	5.32	0.87	0.061	5.35	5.52	1.25	0.429	1.80	1.73	0.69	0.163	2.25	2.21	0.75	0.102
9	10.6	10.55			13.35	13.0			5.75	5.88			6.60	6.68		
15	15.75	15.77			20.30	20.47			10.1	10.03			11.20	11.15		
Methyl α-D-gluco-pyranoside																
3	4.50	4.62	0.98	0.306	5.92	6.02	1.07	0.163	2.0	1.83	0.56	0.408	2.25	2.32	0.65	0.184
9	10.75	10.5			12.55	12.42			4.85	5.18			6.40	6.25		
15	16.25	16.37			18.75	18.82			8.70	8.53			10.10	10.17		

[a] Average of two determinations.

[b] Calculated from least squares fit curve.

The plates were sprayed evenly by a crisscross method to near translucence with the following visualization indicators described under (1), (2), and (3); or they were saturated sequentially with vapors of sulfuryl chloride and water (4) below:

(1) 3.3% sulfuric acid in 33% ethanol; heating time, 30 min at 150°.

(2) 20% aqueous ammonium sulfate, 4% sulfuric acid[5]; heating time, 1 h at 210°.

(3) 50% sulfuric acid (aqueous, v/v)[6]; heating time, 30 min at 260°.

(4) Sulfuryl chloride and water vapor chambers[8]. The plates were placed in a tank saturated with sulfuryl chloride vapor for 15 min. Then the plates were rapidly removed (the operator using rubber gloves in forced-air hood) and placed in a second tank saturated with water vapor for 15 min. In the first tank sulfuryl chloride saturates the adsorbent layer and, in the second, is hydrolyzed to sulfuric and hydrochloric acids; the plates are then heated at 150° for 30 min. The density of the spots was determined 1 h after the plates had cooled.

R_F values were as follows: maltose, 0.16; D-glucose, 0.32; and methyl α-D-glucopyranoside, 0.43.

Densitometer. A Photovolt TLC densitometer, Model 530, equipped with a collimating slit aperture of 0.1 × 15 mm was used. The TLC stage was driven 1 in./min. The signal from the photometer was recorded on a 10-in. Beckman linear–log recorder on the log scale with a chart speed of 2 in./min. Peak areas (spot densities) were measured with a Keuffel and Esser compensating polar planimeter.

Results and discussion

For each of the visualization reagents a linear relationship was established between the weight of the standard reference compounds (3–15 mg) and the area of the peaks (Table I). The lines do not coincide, are not parallel, and do not go through the origin. Even though the basic visualization reagent is sulfuric acid, its form and the temperature of charring markedly affect the degree of color development. The color intensity for a given amount of reference compound decreased in the following order: ammonium sulfate, 3.3% sulfuric acid, sulfuryl chloride, 50% sulfuric acid.

Spot intensity decreased with time (Table II). The rate of fading is related to the spray reagent. The per cent decrease after 22 h is greatest with sulfuryl chloride and least with 50% sulfuric acid. The spots from the three sugars visualized by 3.3% sulfuric acid faded at about the same rate. Consequently, an analysis that extends over any period of time would best be carried out with 3.3% sulfuric acid.

The reference compounds are also a significant source of variation. The lines

TABLE II

PER CENT DECREASE IN SPOT INTENSITY AFTER 22 HOURS

Reference compounds	*Visualization indicators*			
	Sulfuryl chloride	*50% sulfuric acid*	*3.3% sulfuric acid*	*20% ammonium sulfate*
D-Glucose	33	7.1	13.2	16.8
Maltose	23	2.5	14.7	11.8
Methyl α-D-glucopyranoside	25	1.5	14.1	13.5

derived from the three reference compounds with any one reagent spray do not coincide (see Table I). The least squares difference between the slopes is 0.0668 (95% level).

The average standard deviation is largest for the ammonium sulfate spray. In this series the largest deviations occurred with the two reducing sugars. Therefore, ammonium sulfate is the least satisfactory reagent for quantitation.

Larger deviations occur when data from several plates are compared. The effect is random and unpredictable. Consequently, it is advisable to run an internal standard. When a series was run on one plate, duplicate spots varied about $\pm$ 3% in recorded peak area. Deviations from a curve constructed by a least squares fit varied about $\pm$ 2.8%. The standard error was 0.26.

Spraying technique markedly affects color development. Either overspraying or underspraying a section of the plate causes color intensity to vary. Because of this variation, the sulfuryl chloride procedure should give the most consistent results. JONES *et al.*[8] suggested holding the sulfuryl chloride saturated plate over a steam bath to hydrolyze the sulfuryl chloride to sulfuric and hydrochloric acids. We found that a chamber saturated with water gave more consistent results without the danger of splattering from a steam bath.

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1 J. W. MIZELLE, W. J. DUNLAP AND S. H. WENDER, *J. Chromatog.*, 28 (1967) 427.
2 W. M. LAMKIN, D. N. WARD AND E. F. WALBORG, JR., *Anal. Biochem.*, 17 (1966) 485.
3 C. N. HUBER, H. SCOBELL AND HAN TAI, *Cereal Chem.*, 43 (1966) 342.
4 J. LEHRFELD, *J. Chromatog.*, 32 (1968) 685.
5 T. ZIMINSKI AND E. BOROWSKI, *J. Chromatog.*, 23 (1966) 480.
6 O. S. PRIVETT AND M. L. BLANK, *J. Lipid. Res.*, 2 (1961) 37.
7 O. S. PRIVETT AND M. L. BLANK, *J. Am. Oil Chemists' Soc.*, 39 (1962) 520.
8 D. JONES, D. E. BOWYER, G. A. GRESHAM AND A. N. HOWARD, *J. Chromatog.*, 24 (1966) 226.
9 K. RANDERATH, *Thin-Layer Chromatography*, Academic Press, New York, 1963, p. 23.
10 H. G. WALKER, B. A. RICCI AND J. C. GOODWIN, *J. Am. Soc. Sugar Beet Technol.*, 13 (1965) 503.

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