CHROM. 4021

Determination of di-, tri-, and tetrasaccharides in mixtures with their component moieties by thin layer chromatography

Thin layer chromatography (TLC) is a useful technique by which many compounds of biological interest, such as carbohydrates, may be separated. However, separation of many oligosaccharides from their hydrolyzation products on TLC has proved to be difficult. Consistently poor separation of fructose, glucose and galactose has been reported[1–14]. Many sugars separate better on kieselguhr than on silica gel. However, on kieselguhr there is unsatisfactory separation of D-glucose and D-galactose[9]. Some sugars separate well on plates made with 0.01 *N* boric acid solution[13–15] or on 0.02 *M* borate buffer[11]. However, consistent R_F values, good separation and compact spots in conjunction with distinct and brilliant colors upon visualization are difficult to obtain. Furthermore, methods for determination of sugars separated on TLC are either insensitive to microquantities (1–50 μg) or else involve time-consuming processes such as synthesis of organic derivatives, wet combustion and electrophoresis[1,4–7,10,12–14,16].

We have obtained good separation of di-, tri-, and tetrasaccharides from their component monosaccharides using Silica Gel G impregnated with 0.02 *M* boric acid by doubly developing in one direction. Excellent separation of D-glucose, D-fructose and D-galactose can be achieved using two-dimensional development with two solvent systems. A modified visualization reagent made possible a simple determination accurate for microquantities (1–50 μg).

Experimental

Qualitative analysis. The sorbent was prepared by mixing 30 g of Silica Gel G (Merck) with 60 ml of 0.02 *M* boric acid[3,13], the mixture being spread with a Desaga applicator on glass plates (20×20 cm) to a thickness of 0.25 mm. Plates were activated in a forced draft chromatography oven for 1 h at 100° and stored in a desiccator until used. Samples were spotted 2 cm from the bottom of the plates with micropipettes (1–5 μl, Drummond Scientific Co.). Mixtures used are presented in Table I.

Two solvent systems were employed: (A) Methyl ethyl ketone–acetic acid–methanol (6:2:2)[13] was used most frequently. (B) Acetone–water (9:1)[3] was used in only one case. The plates were developed in standard enclosed TLC jars (8×15×15 cm) containing 150 ml of solvent to a height of 12–15 cm, requiring 45 min to 1 h.

Double development in all cases enhanced separation and eliminated much of the trailing. It was carried out by air-drying the plates for 1 h after the first development and then developing them a second time in the same direction with the same solvent (Table I; Fig. 1). Two difficulties involving incomplete separation were resolved by double development in both of two dimensions. Melibiose generally separates well from raffinose but the two spots may be cofluent due to trailing. Complete separation of these sugars can be obtained by using solvent A in both dimensions (Fig. 2). Fructose, glucose, and galactose were easily partitioned by development first with solvent B and then in the second dimension with solvent A (Fig. 3).

The sugars were visualized by spraying with a mixture of 4 g diphenylamine, 4 ml aniline, and 20 ml orthophosphoric acid (80%) in 200 ml acetone[17] (DPA). The

TABLE I

SEPARATION OF SUGAR MIXTURES ON SILICA GEL PLATES

Silica Gel G plates were impregnated with 0.02 *M* boric acid. Double development with (A) methyl ethyl ketone–acetic acid–methanol (6:2:2), or (B) acetone–water (9:1), in one or two dimensions.

Mixture		*Dimensions*	*Developing fluid*
No.	*Components*		
1	Lactose, glucose and galactose	1	A
2	Maltose and glucose	1	A
3	Melibiose, glucose and galactose	1	A
4	Raffinose, fructose and melibiose	2	A in both directions
5	Raffinose, galactose and sucrose	1	A
6	Raffinose, fructose, galactose and glucose	2	B followed by A
7	Sucrose, fructose and glucose	1	A
8	Melezitose, turanose and glucose	1	A
9	Stachyose, manninotriose and fructose	1	A
10	Cellobiose and glucose	1	A
11	Gentiobiose and glucose	1	A

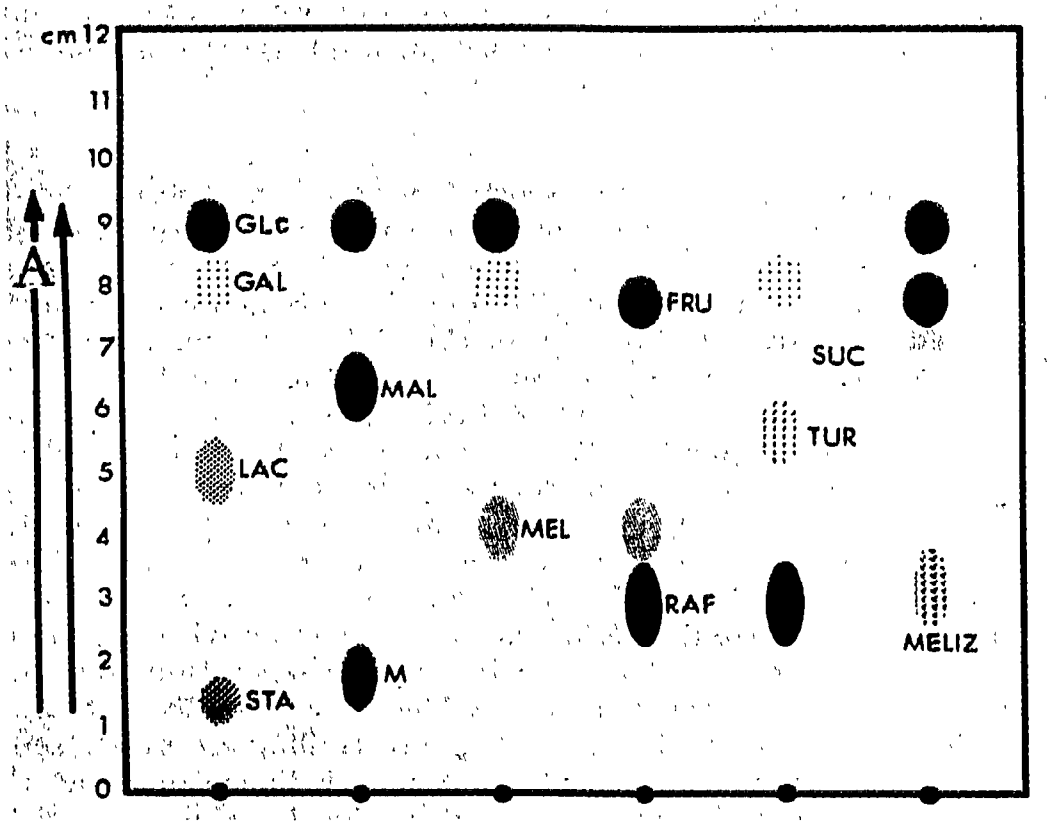

Fig. 1. Separation of fructose (FRU), galactose (GAL), glucose (GLC), lactose (LAC), maltose (MAL), manninotriose (M), melibiose (MEL), melizitose (MELIZ), raffinose (RAF), stachyose (STA), sucrose (SUC) and turanose (TUR) on borate-impregnated Silica Gel G plates. Double development with methyl ethyl ketone–acetic acid–methanol (6:2:2).

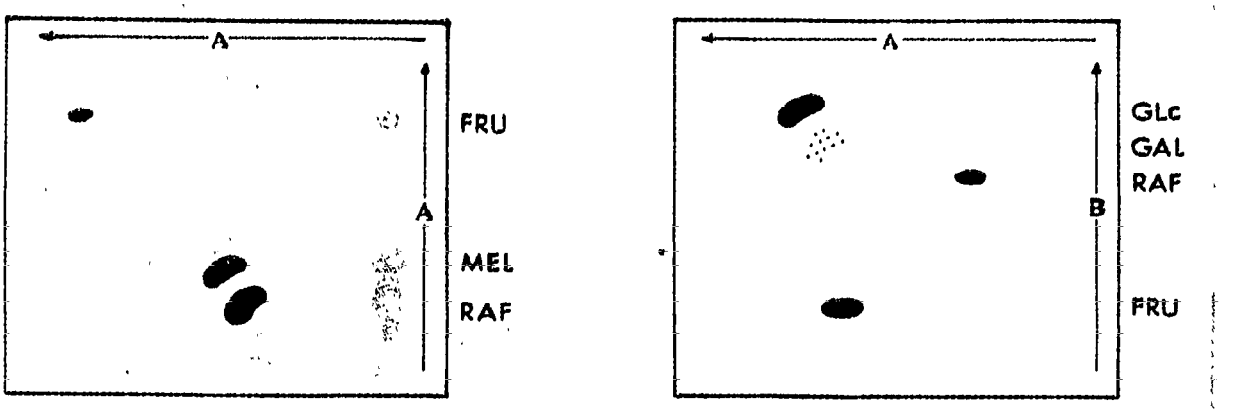

Fig. 2. Separation of fructose, melibiose and raffinose on borate-impregnated plates of Silica Gel G. Double development in both directions with methyl ethyl ketone–acetic acid–methanol (6:2:2).

Fig. 3. Separation of fructose, galactose, glucose and raffinose on borate-impregnated plates of Silica Gel G. Double development in each direction with (A) methyl ethyl ketone–acetic acid–methanol (6:2:2); (B) acetone–water (9:1).

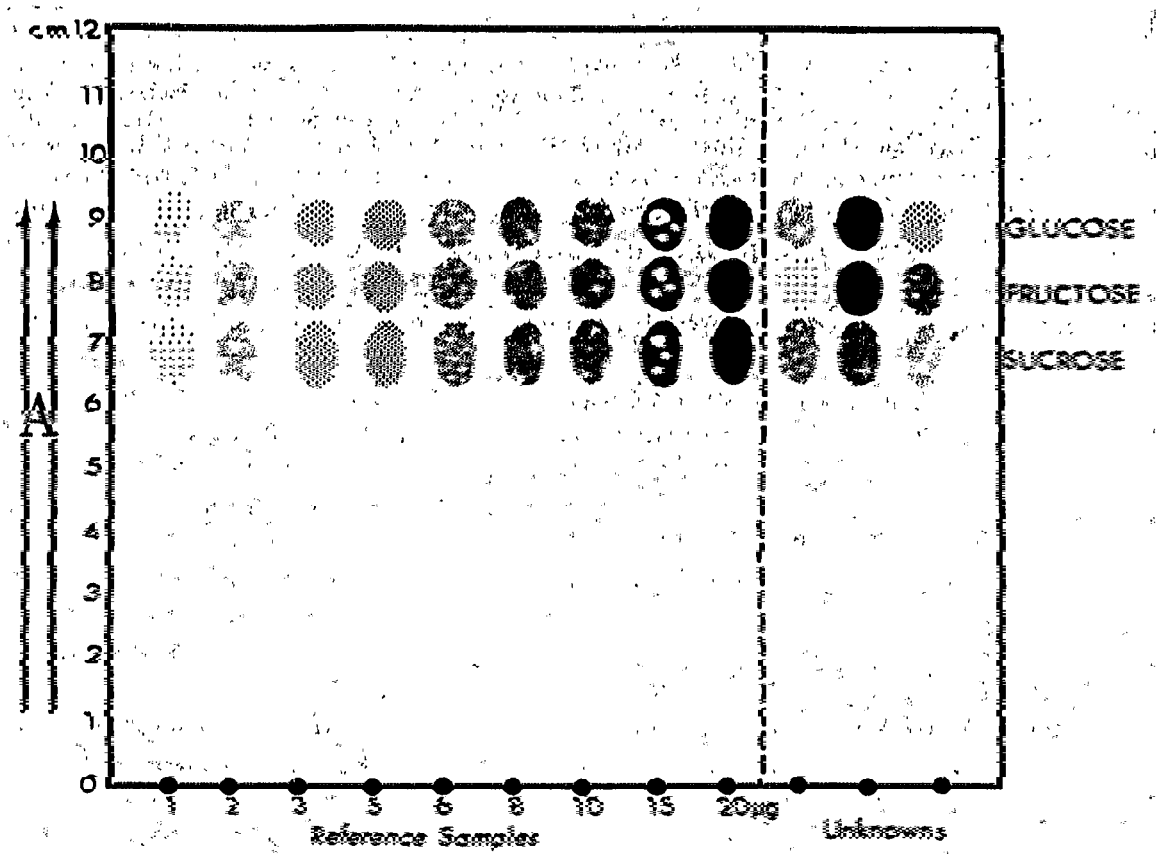

Fig. 4. Spotting pattern of fructose, glucose and sucrose for quantitative analyses on a borate-impregnated plate of Silica Gel G. Double development with methyl ethyl ketone–acetic acid–methanol (6:2:2).

plates were then heated to 100° for 10 min to produce the characteristic colors[17].

Quantitative analysis. On a single plate a sugar solution of known volume but unknown concentration was spotted together with a number of reference samples (Fig. 4). Plates were doubly developed in one dimension with solvent A. The visualization reagent used was DPA[17] modified by the addition of 0.66 g of benzidine dissolved in 110 ml acetic acid[1]. Colors were developed by heating the plates at 100° for 10 min. Immediately after heating, the spots were scraped off the plates and dissolved in 1 ml of 1 % HCl and 0.1 % ascorbic acid in methanol (HAM). The silica gel was separated from the colored supernatant by centrifugation at 1800×*g* for 15 min in covered test tubes. Since a slight background color appeared on the plates after heating, a blank was prepared in the same manner by eluting an unspotted region of the plate. Because this background color intensifies with time, it is important to elute the samples as quickly as possible.

Using the entire supernatant in 1.5 ml cuvettes, the absorption maxima of some sugar–reagent complexes were determined in a Beckman DB-G Spectrophotometer (Table II) and subsequently used in optical density determinations. A standard curve was plotted for each sugar from the eluates of the reference spots (Fig. 5). The quantity of each sugar in the unknown sample was then determined from this curve. Due to lack of uniformity between plates it was necessary to develop both the reference samples and the unknown on the same plate to ensure reliability.

TABLE II

ABSORPTION MAXIMA, COLORS AND R_F VALUES OF SUGARS

Double development with (A) methyl ethyl ketone–acetic acid–methanol (6:2:2) and (B) acetone–water (9:1) on 0.02 *M* borate-impregnated Silica Gel G thin layer plates. Visualization with a mixture of 4 ml aniline 4 g diphenylamine and 20 ml 80% orthophosphoric acid in 200 ml acetone plus 0.66 g benzidine in 110 ml acetic acid[1,17].

Sugar	λ_{max}(mμ)	*Color*	R_F *values*	
			Solvent A	*Solvent B*
Cellobiose		blue	0.68	
D-Fructose	648	orange-brown	0.65	0.39
D-Galactose	643	dark blue	0.67	0.74
B-Gentiobiose		powder blue	0.49	
B-D-Glucose		dark blue	0.74	
α-D-Glucose	643	dark blue	0.74	0.78
Lactose	647	blue-grey	0.43	
Maltose	645	violet-blue	0.55	
Manninotriose		blue	0.15	
α-Melibiose	645	blue-grey	0.37	
Melizitose		grey	0.19	
Raffinose	645	grey	0.30	0.65
Stachyose		grey-green blue	0.11	
Sucrose	642	grey-green	0.59	
Turanose		violet	0.33	

Discussion

The qualitative technique described here can solve the vexing problem of separating glucose, galactose and fructose on TLC. Also, it allows for the rapid separation of several sugar mixtures through the use of only one developing fluid and

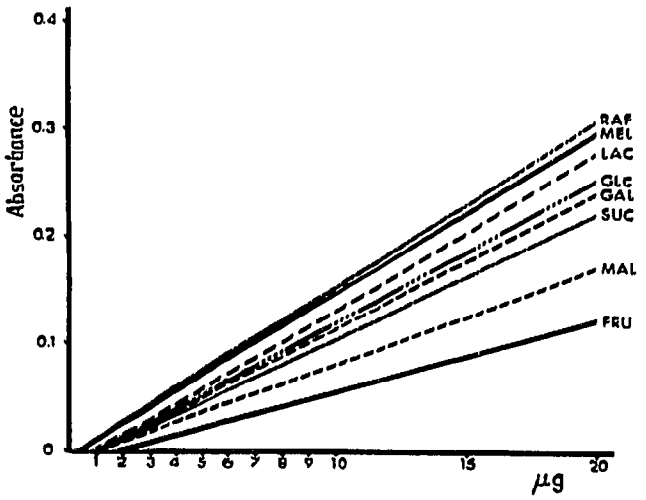

Fig. 5. Sample standard curves for fructose, galactose, glucose, lactose, maltose, melibiose, sucrose and raffinose visualized with aniline–diphenylamine–orthophosphate–benzidine–acetic acid in acetone. Eluting solvent: 0.1 % ascorbic acid and 1 % HCl in methanol.

adsorbent. The spots are well defined and sufficiently separated due to the double development, and have distinct colors permitting fast identification. Addition of acetic acid and benzidine[1] to DPA[17] increases the distinctness, the stability and the intensity or brilliance of the colors. Unfortunately, however, neither DPA, nor the modified reagent can be used for the visualization of trehalose since no color is formed. Quantities and differences as low as 1 μg of sugar within the range of 1–20 μg can accurately be resolved and measured with the modified visualization reagent when 1 ml of HAM is used to elute the spots. If quantities greater than 20 μg are separated for quantitative determinations the spots should be eluted with 3 ml of HAM. The linearity obtained for 20–80 μg with 3 ml of HAM is equal to that of 1–20 μg and 1 ml of eluant. It should be noted that although the standard curves (Fig. 5) are based on the amount of sugar spotted, optical densities are proportional to the amount of color complex present in the eluate.

Two-dimensional (double or single) development requires use of the entire plate for a single sample leaving no space for reference spots. Hence, we have not used these systems (Figs. 2 and 3) for quantitative analysis.

Both the qualitative and quantitative techniques have been used successfully for several recent studies in this laboratory. They have proved useful in assays of sugars in orchid nectars[18,19] while quantitative determinations of sugar content in di-, tri-, and tetrasaccharide solution following hydrolysis by incubated orchid seedlings were greatly simplified. Double development in one or two directions combined with a modified visualization reagent was found suitable for fast qualitative analysis of sugar quantities in the 0.1–0.2 μg range. Quantitative determinations of 1 μg amounts are also possible.

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