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RAPID ANALYSIS OF SIMPLE CARBOHYDRATES BY MEANS OF VAPOUR-PROGRAMMED THIN-LAYER CHROMATOGRAPHY AND DENSITOMETRY AND USING A NEW SPOTTING DEVICE

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SUMMARY

A rapid method is described for the separation of common naturally occurring mono- and disaccharides by means of vapour-programmed thin-layer chromatography; the development time is 3 h, and quantitation is obtained by *in situ* reflectance spectrometry. A new spotting apparatus with syringes in the horizontal position has been developed, which allows perfectly reproducible sample delivery in the microlitre range. The coefficient of variation for the total procedure is about 4–8 %. The total analysis time is 5 hours.

INTRODUCTION

The separation and quantitation of carbohydrate mixtures still remains cumbersome, owing to the highly polar character and the structural similarity of the sugars. Gas, liquid, paper and thin-layer chromatography have all been applied to carbohydrate analysis, and each method has disadvantages. Gas–liquid chromatography (GLC) requires derivatization of the carbohydrates into more volatile components, such as trimethylsilyl ethers or alditol acetates. However, this may result in multi-peak formation from a single carbohydrate, as each anomer can form its own derivative, or identical derivatives may be obtained from two different carbohydrates. Further, GLC is limited to one sample per run. High-pressure liquid chromatography offers advantages in terms of sample preparation and speed, but is also limited to one sample per run[1].

Of the multi-sample methods, paper chromatography is slow and does not allow the use of aggressive spray reagents. Thin-layer chromatography (TLC) is useful, but has a major drawback in that normal TLC appears to lack sufficient resolution for complex mixtures and can also be a long operation.

Lato and co-workers have recently carried out an extensive study on carbo-

hydrate separations by TLC[2–6]. For one-dimensional work, they recommend buffer-impregnated sorbents and long (40 cm) plates, resulting in development times of 6–8 h. Two-dimensional TLC on 20 × 20-cm plates with buffer-impregnated sorbents and/or with two sorbents side by side (coupled-layer TLC[6]) substantially increased the possibilities for resolution, but the total analysis time was not shortened. Excellent separations can be obtained, but the long development times and the fact that two-dimensional TLC allows only one sample per run prevent rapid analysis. We have tried to incorporate Lato's principles into a fast one-dimensional procedure with adequate resolving power for use in the routine analysis of simple carbohydrate mixtures. This objective has been achieved by means of vapour-programmed TLC (VP-TLC), coupled with *in situ* quantitation by reflectance spectrometry.

EXPERIMENTAL

Solvents and standards

All solvents were certified ACS grade (Fisher) and solvent compositions are given in percentages by volume. Standard carbohydrates were commercial samples and were shown by TLC to be at least 99% pure, *i.e.*, TLC of 20 μg of each sugar did not reveal any spots of decomposition products or other impurities (detection limit for carbohydrates 0.1–0.2 μg). Single carbohydrates or carbohydrate mixtures were dissolved in deionized water to give solutions containing 2 μg/μl of each component.

TLC procedures

Silica gel G* (E. Merck, Darmstadt, G.F.R.) was used as the sorbent; 32 g of sorbent were suspended in 64 ml of 0.2 *M* sodium dihydrogen phosphate solution; this yielded sufficient slurry for 5 plates (20 × 20 cm) with a wet-layer thickness of 0.3 mm.

After spreading, the plates were air dried for 15 min, heated for 30 min at 100° in a circulating oven, then cooled and stored in a desiccator. Samples ranging in volume from 1 to 10 μl were spotted with 10-μl syringes [Hamilton 701 N* (Hamilton, Reno, Nev., U.S.A.), point style No. 3], 2.5 cm from the bottom of the plate and at least 1 cm apart by means of a mechanical multi-sample spot applicator. A band of sorbent 5 mm wide was stripped from the side and bottom edges of the plate.

The 20-cm VP-chamber (Desaga, Heidelberg, G.F.R.) was used for development[7]. The ambient temperature was 24–25°, and the VP-chamber was thermostatically controlled at 24°. The room relative humidity ranged from 40–70% and did not adversely affect the reproducibility of separation. The saturation time was 10 min, and the development time was 3 h, with acetone–methanol–water (80:15:5) as the solvent.

The vapour programme in the troughs was as follows (A = acetone, M = methanol, W = water):

trough 3 = A–M–W (70:20:10)
trough 6 = A–M–W (60:20:20)
trough 8 = A–M–W (40:30:30)

* Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture, and does not imply its approval to the exclusion of other products that may also be suitable.

trough 10 = A–M–W (20:40:40)
troughs 12, 14, 16, and 18 = A–M–W (20:25:55)
troughs 1, 2, 4, 5, 7, 9, 11, 13, 15, 17, 19, 20 and 21 = A.
The spacers were 1 mm.

Visualization and quantitation

After development, the plates were dried for 30 min at 100° in a circulating oven. For qualitative purposes, plates were sprayed while hot with a freshly prepared solution of 20 mg of naphthoresorcinol in 10 ml of ethanol and 0.2 ml of concentrated sulphuric acid (NR reagent[3]), then re-heated for 5 min at 110° to intensify the spot colours.

For quantitative purposes, the plates were either sprayed with a mixture of 4 g of diphenylamine, 20 ml of orthophosphoric acid (80%), 4 ml of aniline, 200 ml of acetone, 0.66 g of benzidine and 100 ml of acetic acid (DPA reagent[8]), followed by heating for 10 min at 100°, or with a mixture of 1 g of thiobarbituric acid, 4 ml of orthophosphoric acid (80%) and 100 ml of ethanol (80%) (TBA reagent[9]), followed by heating for 10 min at 100°. The latter two reagents were freshly prepared each day. Plates were scanned with a Zeiss chromatogram-spectrophotometer M4Q II, coupled with a Servogor recorder with disc integrator (Goerz Electro). Reflection was measured at 640 nm (for DPA) or at 440 nm (for TBA). A standard calibration curve was plotted for each carbohydrate, and unknown samples were quantitated from these curves.

Spot applicator

The apparatus is shown in Figs. 1 and 2. The aluminum base contains a plate holder (1) for 20 × 20-cm plates; the holder is hinged at one end and can be pivoted about the hinge by raising or lowering the opposite end of the plate by means of a jacking device underneath. The position of the plate in the holder can be varied, as the bottom end rests on an adjustable bar (2), which can slide up and down the side edges of the holder. Behind the plate holder is the syringe tray, which can hold up to 9 syringes. The syringes (5) are firmly held by two pairs of aluminum clamping racks mounted on the tray. Each half of the rack has semicircular grooves, the groove diameter depending on the type and size of syringe used. The lower arms of the clamping racks are attached to the syringe tray, whereas the upper arms have two vertical holes that fit on two pins on the lower arms. The two arms are kept together by means of

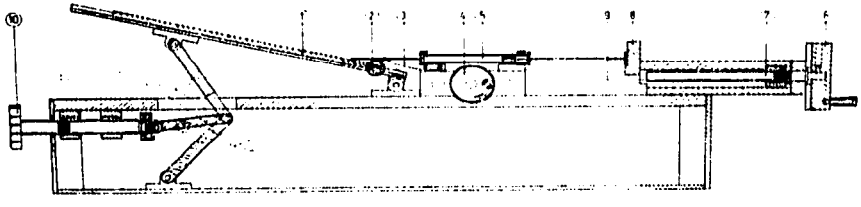

Fig. 1. The spot applicator. 1 = Plate holder; 2 = bar for plate positioning; 3 = supporting bar for needles; 4 = syringe-tray-driving wheel; 5 = syringe; 6 = ram-driving wheel; 7 = finely threaded spindle; 8 = ram; 9 = dove-tail construction to guide ram movement; 10 = knob operating jacking device. Dotted lines indicate plate holder and jacking device in retracted position.

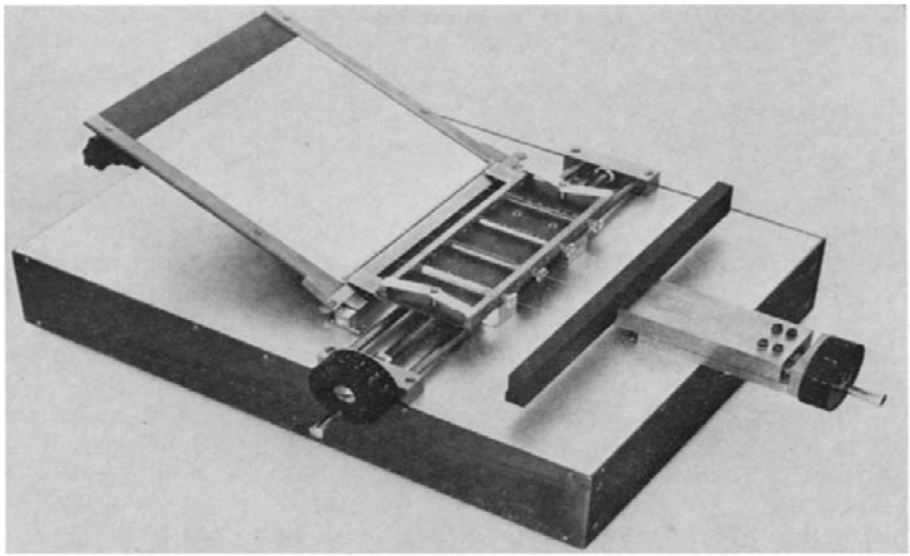

Fig. 2. Spot applicator prepared for dispensing 4 samples.

two spring-loaded wings. The syringe tray is mounted on a finely threaded shaft and two guiding rails.

By turning the wheel (4), the tray can be accurately positioned laterally for spotting. The syringe needles protrude from the small openings in the front clamping rack and rest in small grooves on a supporting aluminum bar (3). Behind the syringe tray is a brass ram (8) to drive the plungers. The ram is mounted on a finely threaded shaft (7) and guided by a long dove-tail construction (9) to obtain perfectly reproducible forward and backward movement. The ram is driven by turning the wheel (6).

Spotting is done as follows: the plate holder is lowered (dotted position) and a plate (hand-made or prefabricated) is put in the holder and positioned by means of the sliding bar (2) at the bottom. The syringes (5) are filled properly and clamped on the syringe tray with the ram in the retracted position. The tray is then positioned with respect to the plate, and the top end of the plate holder is raised by turning the knob (10) until the needle tips just touch the sorbent.

The ram is put forward so as just to make contact with the plungers and is then driven carefully to deliver the samples. After spotting, the plate holder is lowered and the plate is removed.

RESULTS AND DISCUSSION

Our investigations were limited to the common naturally occurring carbohydrates rhamnose, xylose, arabinose, mannose, fructose, glucose, galactose, sucrose, maltose and lactose. We investigated the acetone–water–chloroform–methanol (80:5:10:10) system (*cf.* Lato *et al.*[4]) using sodium dihydrogen phosphate-impregnated plates in the VP-chamber. Results were best with acetone–methanol–water (80:15:5) as solvent, combined with the acetone–methanol–water vapour programme described in Experimental.

Fig. 3. VP-TLC of simple carbohydrates. 1 = Lactose; 2 = sucrose; 3 = galactose; 4 = glucose; 5 = mannose; 6 = arabinose; 7 = xylose; 8 = mixture of 1–7 plus fructose.

The increased proportions of methanol and water in the higher troughs enabled us to separate and spread the spots across the entire plate. The total amount of water, *i.e.*, that in the solvent plus that in the troughs, was critical. In the systems used in this work, at lower water concentrations glucose and mannose tended to coincide, and at higher water concentrations, sucrose and galactose would not separate. The sodium dihydrogen phosphate impregnation produced compact, round spots, thereby reducing chances for overlap and allowing adequate analysis. The resolution and spot shape are illustrated in Fig. 3, and R_F values are listed in Table I. The VP-separation is as effective as that obtained with system 37 of Lato *et al.*[4], but the major advantage of the VP-chamber is the reduction of the development time from 8 to 3 h. A disadvantage of this VP-TLC system is its inability to separate fructose from mannose. None of the vapour programmes we tried effected this separation. The separation of these two components by means of system 37, as reported by Lato *et al.*[4], has been questioned[10]. Impregnation of the sorbent with boric acid instead of sodium dihydrogen phosphate, or the use of a solvent containing boric acid, can be used to resolve fructose

TABLE I

R_F VALUES (× 100) OF SIMPLE CARBOHYDRATES AFTER VAPOUR-PROGRAMMED TLC

Carbohydrate	$R_F \times 100$	*Carbohydrate*	$R_F \times 100$
Rhamnose	84	Glucose	36
Xylose	71	Galactose	27
Arabinose	53	Sucrose	18
Mannose	44	Maltose	11
Fructose	44	Lactose	5

and mannose. However, we have found that boric acid systems cause tailing of the fructose spots and we would not recommend their use for general carbohydrate analysis.

Detection with NR reagent is convenient for qualitative analysis. Ketoses yield crimson to brown–red colours, whereas aldoses give blue to grey spots[3]. The colours of fructose (red) and mannose (bluish-grey to brownish-grey) help to differentiate between these two compounds; the colour intensities and the spot sizes can also be used for semi-quantitative analysis, provided that reference compounds are available on the same plate. The detection limit is 0.1–0.2 μg.

The NR reagent is not suitable for quantitative purposes, as the colours are unstable. The DPA reagent[8] is less sensitive (0.2–0.4 μg) but produces colours that are stable for at least 1 h. All carbohydrates, except fructose, showed up as bluish spots with absorption maxima between 640 and 650 nm; for fructose, the absorption maximum was at 530 nm. The working range for quantitation by reflectance spectrometry at 640 nm was about 1–40 μg (see Fig. 4, curve B). For fructose at 530 nm, the sensitivity is about one-third of that of the other carbohydrates at 640 nm (see Fig. 4, curve C). A more sensitive reagent for fructose is TBA[9], which can also be used for other ketoses, as it does not react with aldoses. The sensitivity is 0.1–0.2 μg, the absorption maximum of the yellow spots is at 440 nm, and the working range is about 1–30 μg. As can be seen from curves A and B in Fig. 4, a linear relationship is obtained when plotting peak area *versus* the square root of amount of carbohydrate[11]. In general, transverse scanning was carried out. The coefficients of variation for recovery of the different carbohydrates were within the range 4–8%, which is about the same or slightly better than those from normal TLC development[12–14]. Transverse scanning is facilitated by the compact spots and the near-Gaussian distribution of the carbo-

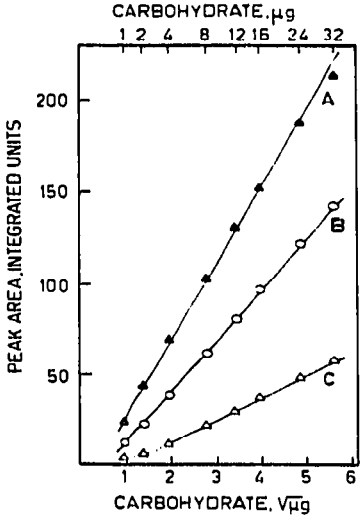

Fig. 4. Calibration curves for reflectance spectrometry of carbohydrates. Curve A, fructose, with TBA reagent and measurement at 440 nm; curve B, mannose, with DPA reagent and measurement at 640 nm; curve C, fructose, with DPA reagent and measurement at 530 nm.

TABLE II

QUANTITATIVE RESULTS OF CARBOHYDRATE ANALYSIS BY *IN SITU* REFLECTANCE SPECTROMETRY WITH TRANSVERSE SCANNING

In each experiment, 10 μg of each carbohydrate was used. Fructose determinations were done with an aqueous solution of this carbohydrate (2 μg/μl), of which 5 μl was spotted on 10 different plates. Colour development was with TBA reagent, with measurement at 440 nm. All other experiments were done with a mixture of rhamnose, xylose, arabinose, mannose, glucose, galactose, sucrose, maltose and lactose in water (2 μg/μl of each carbohydrate), of which one 5-μl sample was spotted on 10 different plates. Colour development was with DPA reagent, with measurement at 640 nm.

Carbohydrate	*Carbohydrate recovery (%) in experiment No.*										*Average recovery (%)*	*Coeff. of variation (%)*
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*		
Rhamnose	98	95	102	92	96	99	100	106	96	104	98.8	4.3
Xylose	103	102	99	104	95	98	92	100	104	98	99.5	3.9
Arabinose	96	96	102	103	98	94	106	102	103	109	100.5	4.8
Mannose	106	93	100	102	95	101	91	99	98	104	98.9	4.8
Fructose	98	101	103	97	90	108	94	103	102	97	99.3	5.2
Glucose	104	106	93	105	99	92	107	103	97	95	100.1	5.6
Galactose	100	98	101	95	97	107	107	95	89	98	98.7	5.5
Sucrose	97	93	94	102	108	106	88	111	96	98	99.3	7.3
Maltose	92	101	104	95	107	92	96	103	110	96	99.6	6.3
Lactose	102	94	93	109	87	109	107	105	95	104	100.5	7.7

hydrate over the spot area. In general, components with low R_F values had the larger coefficients of variation, which may be due to small irregularities in carbohydrate distribution over the total spot area. The quantitative data are summarized in Table II.

Spray application of colour-development reagents can introduce variation into this analytical procedure. We found that a light spray produced weak colour development, presumably because the reagent did not fully penetrate the sorbent layer. Another important factor in reproducibility is the spotting of the samples. Because of their polar character, carbohydrates have to be dissolved in equally polar solvents, such as water, but aqueous solutions are difficult to work with in spotting procedures. We therefore developed the multi-sample spot applicator, the basic features of which are that the syringes are positioned horizontally and that spotting is done with the needles making a wide angle with the plate. This offers a number of advantages: (1) there is no "creep-back" of sample along the needle stem because the needles touch the layer; (2) no damage is done to the layer, even after successive applications of samples, because the needles do not penetrate the sorbent but rest on its surface; (3) the spot size can be fully controlled and is independent of sample solvent or sample size; (4) the delivery of sample is reproducible, with a coefficient of variation of about 2% for 10-μl syringes; (5) the needles do not clog with sorbent particles, nor did we observe withdrawal of sample from the needle as was reported by Fairbairn[15]; (6) the apparatus allows application of different sample volumes in one run; and (7) the construction allows the syringe tray to be adapted to syringes of other size, make or model. Use of the apparatus does not require any particular skill, and it has already been used successfully in other areas of TLC analysis[16,17].

The total time for a complete analysis is about 5 h. For routine analysis, 2 plates, each containing 10–12 samples, can be handled per day per analyst.

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