THIN-LAYER CHROMATOGRAPHY OF CARBOHYDRATES IN THE PRESENCE OF BISULFITE

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INTRODUCTION

In the course of studies on the rapid quantitative determination of lactulose (4-O-β-galactopyranosyl-D-glucose) in milk products which are under way in this laboratory, it was necessary to devise a thin-layer chromatography method capable of separating and identifying tagatose, lactulose and sucrose. During the past few years a considerable amount of work has been reported in the literature on the thin-layer chromatographic analysis of carbohydrates[1–7]. However, it was found in a preliminary experiment that the three carbohydrates described above are not well separated by these methods. As a means of eliminating the disadvantages, the chromatographic development in the presence of bisulfite as reported in previous papers[8,9], was investigated for its applicability to thin-layer chromatography.

The present paper describes the adaptation of the principles of chromatographic development in the presence of bisulfite to the thin-layer chromatographic separation of carbohydrates. Clear separation is achieved by development on a chromatoplate of silica gel G mixed with a small amount of bisulfite. After the development the carbohydrates are detected by four different spray reagents: *o*-aminodiphenyl–orthophosphoric acid, carbazole–sulfuric acid, phenol–sulfuric acid and thymol–sulfuric acid. Keto-sugars are selectively detected by a modification of procedure of ADACHI[10].

EXPERIMENTAL

Preparation of the chromatoplates

A slurry of 40 g of "Kieselgel (silica gel) nach Stahl" (E. Merck, A.G., Darmstadt, Germany) in 80 ml of 0.1 *M* sodium bisulfite solution was applied to the glass plates (20 cm × 20 cm × 0.3 cm) at a thickness of about 0.25 mm, using a Toyo Kagaku Sangyo Co. Model HC-20 Spreader. These plates were allowed to stand for 30 min at room temperature, and then dried in an oven at 110° to 120° for 1 h. The coated chromatoplates were cooled and used for chromatography as described.

Development of the chromatoplates

An aliquot containing 5 to 10 μg of pure carbohydrates and of their mixtures in water was applied to the plates with a micropipette in the conventional manner. The plates were developed by the ascending technique, without prior equilibration,

until the solvent front had reached a distance of 13 cm past the spotting position. The following solvent systems were used:

Solvent A: Ethyl acetate–acetic acid–methanol–water (6.0:1.5:1.5:1.0).
Solvent B: Isopropanol–ethyl acetate–water (7:1:2).
Solvent C: Methyl ethyl ketone–acetic acid–water (3:0.5:1.5).
Solvent D: Propanol–water (8.5:1.5).

When the solvent had reached the limiting line, the plates were removed and air-dried in a horizontal position.

Detection of the spots

After the application of the spray reagent, the carbohydrates were detected on the chromatoplates by heating in an oven for the time required by the reagent used.

(1) *o-Aminodiphenyl–orthophosphoric acid reagent.* The chromatoplates were sprayed with a solution containing 0.3 g of *o*-aminodiphenyl and 5 ml of orthophosphoric acid (sp. gr. 1.88 at 20°) in 95 ml of ethyl alcohol. The plates were then heated in the oven for 15 to 20 min at 110°. The carbohydrates appeared as brown spots.

(2) *Carbazole–sulfuric acid reagent.* The chromatoplates were sprayed with a solution containing 0.5 g of carbazole and 5 ml of concentrated sulfuric acid in 95 ml of ethyl alcohol. After heating the plates for 10 min at 120°, the carbohydrates appeared as violet spots on a blue background. Freshly prepared reagent is preferable.

(3) *Dimedone–orthophosphoric acid reagent*[10]. When the chromatoplates sprayed with this reagent were heated for 15 to 20 min at 110°, ketose-containing carbohydrates appeared as dark greenish grey spots.

(4) *Phenol–sulfuric acid reagent.* The chromatoplates were sprayed with a solution containing 3 g of phenol and 5 ml of concentrated sulfuric acid in 95 ml of ethyl alcohol, and then heated for 10 to 15 min at 110°. The carbohydrates appeared as brown spots which could be intensified by further heating. The reagent could still be used when several days old.

(5) *Thymol–sulfuric acid reagent.* The chromatoplates were sprayed with a solution containing 0.5 g of thymol and 5 ml of concentrated sulfuric acid in 95 ml of ethyl alcohol. After heating for 15 to 20 min at 120°, most carbohydrates appeared as dark pink spots on a white background, changing to faint violet on further heating.

RESULTS AND DISCUSSION

The separations of the carbohydrates are shown in Table I together with the colors developed after the application of the spray reagents. Eighteen solvent systems were tested on the chromatoplates using an eight-component mixture of fructose, glucose, lactose, lactulose, maltose, rhamnose, sucrose and xylose as the sample, but on the basis of separation efficiency, good separations of these carbohydrates were only obtained with the solvent systems given in Table I. Solvent D gave the best separation, though some of the carbohydrates were no longer resolved under these conditions. Fig. 1 shows a typical chromatogram developed with the solvent D. Addition of increasing amounts of acetone to solvent D increases the speed of the development, but also increases the migration distance, and changes the shape of the spots from an exaggerated oval type to a cucumber type with the tail of the cucumber towards the origin. Slight variation in migration was occasionally noted from plate to

TABLE I

R_F VALUES AND SPOT COLORS OF CARBOHYDRATES

Carbohydrate	*R_F in solvent**				*Color with reagent***				
	A	*B*	*C*	*D*	*I*	*II*	*III*	*IV*	*V*
L-Arabinose	0.32	0.63	0.51	0.51	B	V	None	DB	PDC
D-Lyxose	0.46	0.68	0.61	0.59	B	V	None	DB	PDC
D-Ribose	0.50	0.69	0.61	0.57	B	V	None	DB	PDC
D-Xylose	0.34	0.68	0.50	0.59	B	V	None	DB	PDC
L-Fucose	0.49	0.62	0.66	0.55	B	V	None	B	PDC
L-Rhamnose	0.57	0.68	0.53	0.62	B	V	None	YB	B
D-Galactose	0.32	0.53	0.47	0.39	B	V	None	B	P
D-Glucose	0.28	0.61	0.47	0.48	B	V	None	B	P
D-Mannose	0.41	0.60	0.57	0.53	B	V	None	B	P
D-Fructose	0.28	0.57	0.53	0.48	YB	V	DGG	GB	PDC
L-Sorbose	0.43	0.56	0.58	0.47	YB	V	DGG	GB	PDC
D-Tagatose	0.46	0.61	0.58	0.53	YB	V	DGG	GB	PDC
2-Deoxy-D-glucose	0.68	0.79	0.75	0.73	DB	V	DP	DB	DG
Lactose	0.08	0.36	0.21	0.23	B	V	None	B	P
Lactulose	0.10	0.40	0.24	0.27	YB	V	DGG	GB	PDC
Maltose	0.11	0.50	0.22	0.35	B	V	None	B	P
Sucrose	0.20	0.55	0.28	0.40	YB	V	DGG	GB	PDC
Trehalose	0.05	0.38	0.08	0.23	None	V	None	B	P
Melezitose	0.10	0.49	0.16	0.30	B	V	None	GB	P
Raffinose	0.04	0.28	0.10	0.13	B	V	None	GB	P

* A = ethyl acetate–acetic acid–methanol–water (6.0:1.5:1.5:1.0); B = isopropanol–ethyl acetate–water (7:1:2); C = methyl ethyl ketone–acetic acid–water (3.0:0.5:1.5); D = propanol–water (8.5:1.5).

** B = brown; DB = dark brown; DGG = dark greenish grey; DG = dark grey; DP = dark pink; GB = greenish brown; P = pink; PDC = pink with dark center; V = violet; YB = yellowish brown. I = *o*-Aminodiphenyl–orthophosphoric acid reagent; II = carbazole–sulfuric acid reagent; III = dimedone–orthophosphoric acid reagent; IV = phenol–sulfuric acid reagent; V = Thymol–sulfuric acid reagent.

plate with the four different solvent systems, thus making it necessary to chromatograph known compounds simultaneously with unknown. STAHL AND KALTENBACH[1] reported that they had been able to separate ribose and xylose on silica gel G plates in the presence of sodium acetate, using the solvent system ethyl acetate–isopropanol (65:35). This system, however, did not give sufficiently good separation of the two pentoses on the chromatoplates described in this paper.

Since the nature of the reactions involved in the separation of the carbohydrates on the thin-layer chromatoplate cannot be presented with assurance, the theoretical implications of these results have not been fully explored. However, it is of interest to point out that such a separation is consistent with the present theory of sugar-bisulfite addition compounds[11,12]. According to the data of INGLES[11] and BRAVERMAN[13], concentrated mixtures of aldo-sugars and an alkaline metal bisulfite in water should give addition compounds at room temperature. Although addition compounds of keto-sugars and bisulfite have not been isolated, it has been suggested that keto-sugars give unstable addition compounds on a strong anion exchanger in the bisulfite form at a high ethyl alcohol or propanol concentration[8,14]. In the case of the separation of carbohydrates presented here, the conditions for the formation of such addition compounds are fulfilled, and thus it seems reasonable to assume that definite equili-

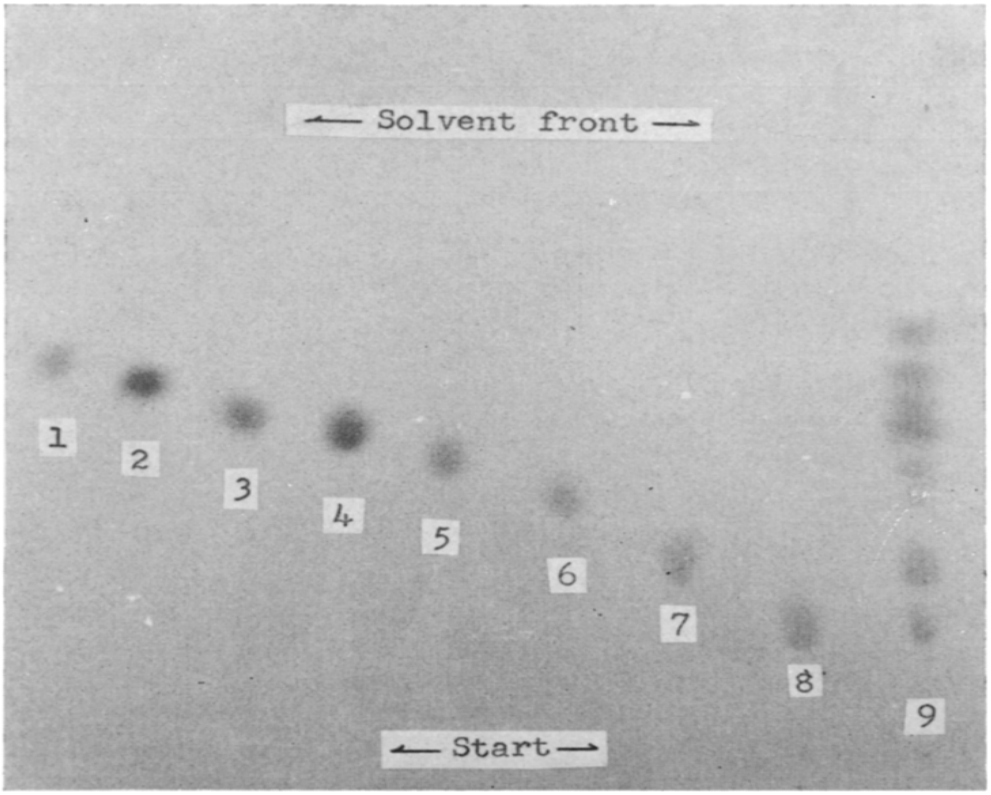

Fig. 1. Thin-layer chromatogram of carbohydrates. The chromatogram was developed in solvent system propanol–water (85:15). The spots were detected with the *o*-aminodiphenyl–orthophosphoric acid reagent. Carbohydrates: (1) rhamnose, (2) xylose, (3) mannose, (4) glucose, (5) galactose, (6) maltose, (7) lactose, (8) raffinose and (9) mixture of (1)–(8).

bria are involved between the adsorbent layer and developing solvent as the sugar-bisulfite compounds move up on the plates. The differences in the development pattern of carbohydrates on plates and on resin columns may be due to factors affecting the migration.

Concomitantly with the experiments on separation various color reagents were being tested for the thin-layer chromatographic detection of carbohydrates. As seen in Table I, the *o*-aminodiphenyl–orthophosphoric acid and thymol–sulfuric acid reagents are of special interest because of the different colors obtained with aldo- and keto-sugars, some of these sugars having R_F values which do not always permit unequivocal identification. The phenol–sulfuric acid reagent gives wider color differences between various carbohydrates. To test the sensitivity of these reagents, aliquots of 0.1, 1, 5 and 20 μg each of fructose, glucose and xylose were spotted on a plate and sprayed with the reagents. Even at a level of 0.1 μg of the carbohydrates the colors were clear and identifiable. The speed of color development of fructose has been found to be generally faster than that of the aldoses. Another distinct advantage of these spray reagents is the lack of background color. The reagents react with the test carbohydrates and the resultant color stands out clearly against a white background. The characteristic colors obtained with keto-sugars by dimedone–orthophosphoric acid reagent are also noteworthy. The reagent reacts only with the test keto-sugars and not with the aldo-sugars, the one exception being 2-deoxy-D-glucose. The minimum sensitivity of the reagent was also 0.1 μg of fructose. On the other hand, with the

carbazole–sulfuric acid reagent a violet color on a blue background was observed for all of the test carbohydrates. The reaction was less sensitive than with the other reagents.

The reactions of carbazole[15], phenol[16] and thymol[17] with carbohydrates in the presence of strong sulfuric acid have been modified for use as methods for the estimation of carbohydrates and these reactions give characteristic colors which have a different absorption maximum for pentose and hexose. It is therefore of interest to note the similarities and differences between pairs of carbohydrates when these reagents were applied to colorimetric determination and detection in thin-layer chromatography. TIMELL *et al.*[18] have demonstrated that *o*-aminodiphenyl in glacial acetic acid gives a characteristic color reaction with aldo-sugars but not with fructose and sucrose in comparable concentration. The results for the color reaction of the dimedone–orthophosphoric acid reagent with carbohydrates parallel the experience of ADACHI using paper chromatography[10].

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SUMMARY

A rapid method for separating carbohydrates by means of thin-layer chromatography on silica gel G mixed with a small amount of sodium bisulfite has been developed. The solvent system propanol–water (85:15) gave the best resolution of the carbohydrates but did not separate some pentoses. Five spray reagents, *viz.* *o*-aminodiphenyl–orthophosphoric acid, carbazole–sulfuric acid, dimedone–orthophosphoric acid, phenol–sulfuric acid and thymol–sulfuric acid, were used for the detection of carbohydrates. Quantities as small as 0.1 μg could be easily detected with these reagents, with one exception, *viz.* carbazole–sulfuric acid reagent. The carbohydrates gave characteristic colors with the reagents, thus making identification easier.

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