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BIDIMENSIONAL THIN-LAYER CHROMATOGRAPHY OF CARBOHYDRATES ON SILICA GEL IMPREGNATED WITH BORIC ACID

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SUMMARY

The separation of a large number of sugars by means of bidimensional thin-layer chromatography is described.

Factors which influence separation of carbohydrates on silica gel impregnated with boric acid, and the limitations of the monodimensional technique are investigated and discussed.

Many attempts to separate sugar mixtures by thin-layer chromatography have been made since STAHL's first experiments[1,2]. Results obtained with all known adsorbents and with many different types of impregnant and solvent mixtures have proved unsatisfactory[3–21]. Identification of many of the most important sugars was highly dubious.

OVODOV *et al.*[17] have recently stressed that "poor separation of some of the more common sugars and the low capacity of the chromatoplates" are responsible for the lack of general acceptance of thin-layer chromatography in research work on carbohydrates.

The purpose of our work was to explore the limits and possibilities of thin-layer chromatography on silica gel impregnated with boric acid for carbohydrates.

EXPERIMENTAL

Preparation of the chromatoplates

30 g of silica gel, TLC grade, without binder (Fluka DO), was mixed with an 0.03 *M* H_3BO_3 solution (2.1 g of H_3BO_3 in 1 l of distilled water). The suspension obtained was applied in the usual manner to the glass plates (0.3 mm layer). These plates were dried for 24 h at room temperature. They were then activated for one hour at 110°.

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Samples

The following pure sugar samples* were used:

Monosaccharides	*Oligosaccharides*	*α-Methyl derivatives*
Ribose	Sucrose	α-Methyl-D-glucoside
Arabinose	Turanose	α-Methyl-D-mannoside
Xylose	Maltose	
Lyxose	Lactose	
Fucose	Trehalose	
Rhamnose	Raffinose	
Glucose		
Galactose		
Mannose		
Levulose		
Sorbose		
Mannoheptulose		

Monodimensional chromatography

10 mg of each sugar were dissolved in 2 ml distilled water; 1 μl of each solution (= 5 μg of sugar) was applied to the chromatoplate with a micropipette in the usual manner. The chromatoplate was then placed in a tank containing the developing solvent system, the walls being lined with filter paper impregnated with the developing solvent. The plates were so slightly tilted that they were practically upright. The room temperature was kept at 22°.

The following solvent systems were employed:

(1) *n*-Butanol–boric acid, 1 *M* (9:1)
(2) *n*-Butanol–acetone–water (4:5:1)
(3) *n*-Butanol–acetic acid–water (4:1:5)
(4) *n*-Butanol–pyridine–water (8:4:3)
(5) *n*-Butanol–pyridine–acetone–boric acid, 1 *M* (60:3:20:17)
(6) *n*-Butanol–isopropanol–water (3:5:2)
(7) *n*-Butanol–ethanol–water (2:1:1)
(8) *n*-Butanol–ethanol–phosphoric acid, 0.1 *M* (1:10:5)
(9) *n*-Butanol–ethanol–hydrochloric acid, 0.1 *N* (1:10:5)
(10) *n*-Butanol–methanol–water (5:3:1)
(11) *n*-Butanol–ethyl acetate–boric acid, 1 *M* (70:15:15)
(12) *n*-Butanol–ethyl acetate–pyridine–water (2:3:2:3)
(13) *n*-Butanol–ethyl acetate–isopropanol–acetic acid–water (35:100:60:35:30)
(14) *n*-Butanol–ethyl acetate–isopropanol–acetic acid–water (3:10:6:6:3)
(15) *n*-Propanol–water (85:15)
(16) *n*-Propanol–acetic acid–water (4:1:5)
(17) *n*-Propanol–acetic acid–boric acid, 1 *M* (4:1:5)
(18) *n*-Propanol–pyridine–water (5:3:2)
(19) *n*-Propanol–ethyl acetate–water (1:4:2)
(20) Ethyl acetate–acetic acid–water (6:3:2)
(21) Ethyl acetate–pyridine–water (2:1:2)
(22) Ethyl acetate–pyridine–water (3:1:2)

* Nutritional Biochemicals Co., Cleveland, Ohio, U.S.A.

(23) Ethyl acetate–pyridine–water (3:3:2)
(24) Ethyl acetate–pyridine–water (4:3:2)
(25) Ethyl acetate–pyridine–boric acid, 1 *M* (2:1:2)
(26) Ethyl acetate–acetic acid–boric acid, 0.1 *N* (3:1:3)
(27) Ethyl acetate–isopropanol–water (65:22:11)
(28) Ethyl acetate–isopropanol–acetic acid–water (100:60:35:30)
(29) Ethyl acetate–isopropanol–water (100:60:30)
(30) *n*-Butanol–methanol–boric acid, 0.03 *M* (5:3:1)
(31) *n*-Butanol–acetic acid–water (5:4:1)
(32) Ethyl acetate–acetic acid–methanol–water (60:15:15:10)
(33) Isopropanol–ethyl acetate–water (7:1:2)
(34) Chloroform–methanol (6:4)
(35) Isopropanol–water (4:1)
(36) Acetone–water (9:1)
(37) Acetone–water–chloroform–methanol (8:0.5:1:1)
(38) Methanol–chloroform–acetone–ammonia (28 Bè) (5:2:3:2)
(39) Methanol–chloroform–ammonia (28 Bè) (6:4:0.7)
(40) *n*-Butanol–ethyl ether–water (4:5:1)
(41) *n*-Butanol–ethyl acetate–isopropanol–water (200:100:70:35)
(42) *n*-Butanol–ethyl acetate–isopropanol–water (35:100:60:30).

The R_F values relative to each solvent are reported in Table I.

Detection of the spots. After development, the chromatoplate was dried at room temperature for a short time. It was then heated in a 110° oven until the odour of the solvent could no longer be detected (~ 1 h).

TABLE I

R_F VALUES (× 100) OF CARBOHYDRATES WITH EACH SOLVENT SYSTEM LISTED IN THE TEXT

Carbohydrate	*R_F values (× 100)*															
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*
Ribose	23	17	42	39	32	33	39	57	53	44	20	42	48	53	36	68
Arabinose	21	33	39	44	37	39	40	58	48	46	22	43	43	40	45	68
Xylose	28	36	34	41	37	42	41	58	59	48	25	44	50	57	44	72
Lyxose	29	36	29	47	34	43	41	57	64	50	26	47	45	48	48	68
Fucose	25	49	25	51	36	42	39	59	67	54	24	48	44	46	47	65
Rhamnose	33	60	37	52	43	45	43	60	67	64	34	51	54	58	51	69
Glucose	20	36	46	52	40	38	41	62	60	55	18	46	37	39	45	68
Galactose	18	23	44	47	36	35	40	61	61	51	16	46	33	35	41	68
Mannose	26	33	32	51	46	42	45	59	57	47	25	45	54	38	49	70
Levulose	19	11	37	34	26	31	34	57	51	37	13	41	38	41	32	69
Sorbose	12	10	47	29	17	25	30	54	41	25	10	29	44	40	29	70
Mannoheptulose	17	13	30	38	24	33	35	55	57	41	12	38	37	39	38	71
Sucrose	15	36	45	56	40	37	40	65	66	55	13	48	28	29	44	69
Turanose	15	32	37	44	25	35	31	60	65	47	9	40	25	22	40	63
Maltose	11	33	41	49	31	37	35	63	56	48	8	41	26	25	43	68
Lactose	7	18	44	47	27	27	31	62	63	42	5	44	17	18	33	67
Trehalose	5	36	20	46	27	34	30	68	65	47	7	38	21	22	38	68
Raffinose	3	15	26	42	20	26	25	61	62	32	2	41	13	16	32	74
α-Methyl-D-glucoside	22	61	29	51	36	43	38	63	64	58	25	48	44	49	49	66
α-Methyl-D-mannoside	28	64	36	51	42	46	43	65	66	62	31	52	50	54	51	68

The heated chromatoplate was then sprayed with a freshly prepared solution of 20 mg of naphthoresorcinol, 10 ml of ethanol and 0.2 ml of conc. H_2SO_4. After a few minutes at room temperature, well-defined and vivid spots appear against a white background. The white background darkens if the chromatoplates are heated after spraying (as is generally advised in the literature)[3,16,21].

The colours that appear are given in Table II. It should be noted, however, that the colours change slightly depending on the concentration of the sugar.

Bidimensional chromatography

A mixture of 10 mg of each of the 20 sugars was dissolved in 2 ml distilled water. 1 μl of the solution (containing 5 μg of each sugar) was applied with a micropipette to the lower right-hand corner of the chromatoplate about 2.0 cm from each of the edges. The chromatoplate was then placed in the tank as described in the section on monodimensional separations.

The following solvent systems were employed:

a { Run I: Solvent No. 35
  { Run II: Solvent No. 13

b { Run I: Solvent No. 42
  { Run II: Solvent No. 13.

The results obtained with the above pairs of solvents are illustrated in Figs. 1 and 2.

After the first development, the chromatoplate is dried for 1 h in an oven at 40–50°.

18	*19*	*20*	*21*	*22*	*23*	*24*	*25*	*26*	*27*	*28*	*29*	*30*	*31*	*32*	*33*	*34*	*35*	*36*	*37*	*38*	*39*	*40*	*41*	*42*
39	44	43	45	71	48	47	31	13	52	60	22	29	68	54	33	9	35	30	16	19	8	5	12	25
51	51	41	57	73	68	71	51	16	44	65	30	52	63	43	53	28	55	52	36	33	18	3	24	33
57	52	48	49	44	65	62	44	21	57	74	38	40	51	64	48	22	48	46	31	35	19	4	26	39
50	53	47	53	62	63	65	51	24	55	74	41	49	53	59	52	35	50	56	36	37	18	4	26	37
62	58	46	59	67	74	75	54	25	53	74	41	55	51	59	58	49	58	66	37	35	33	3	29	42
64	65	57	62	69	78	79	57	35	63	75	52	62	54	69	63	50	62	70	50	37	34	12	41	57
59	50	35	55	68	74	74	51	8	40	66	26	53	65	44	53	29	61	54	35	19	15	2	25	33
51	48	31	56	70	63	63	43	9	40	65	24	46	60	40	48	17	52	40	22	11	11	2	17	26
55	48	40	53	60	68	66	66	14	45	69	31	53	65	42	56	23	57	52	31	20	16	3	24	36
35	47	33	45	71	40	44	19	6	46	64	16	24	68	43	26	7	33	22	9	12	5	2	8	16
33	56	42	57	64	36	33	11	4	47	66	11	18	65	45	21	6	24	16	8	6	5	1	3	8
33	41	35	26	62	46	44	44	7	43	75	25	27	46	48	33	8	29	24	10	2	5	1	9	16
61	49	26	62	71	84	82	52	6	34	66	23	55	54	34	62	20	70	59	32	9	17	1	19	27
58	59	25	47	68	71	71	49	4	34	68	25	45	31	35	51	20	51	38	19	3	9	0	13	24
58	49	41	56	68	84	82	48	3	28	69	19	52	46	25	62	23	63	53	27	9	14	0	15	21
56	50	15	51	73	67	67	34	3	24	64	12	39	42	21	46	11	52	34	13	2	10	0	6	12
68	63	18	46	70	80	77	42	3	30	75	21	43	26	65	64	25	58	41	10		19	11	13	20
62	55	11	39	69	66	62	20	1	23	48	12	26	29	16	52	6	47	25	9		7	0	3	8
75	60	52	66	69	84	82	57	29	55	69	45	62	52	62	66	60	67	74	61		55	4	37	53
72	63	56	66	69	85	82	59	37	62	71	53	63	54	67	66	62	62	75	61		51	10	44	59

TABLE II

COLOURS DEVELOPED BY SPRAYING THE CHROMATOGRAM WITH A NAPHTHORESORCINOL REAGENT

Sugar	*Colour*	*Sugar*	*Colour*
Ribose	Blue-green	Sorbose	Red
Arabinose	Blue-green	Mannoheptulose	Violet
Xylose	Blue-green	Sucrose	Crimson
Lyxose	Blue-green	Turanose	Purple-red
Fucose	Purple-red	Maltose	Gray-violet
Rhamnose	Cherry-red	Lactose	Blue-violet
Glucose	Gray-violet	Trehalose	Blue
Galactose	Gray	Raffinose	Orange-red
Mannose	Violet	α-Methyl-D-mannoside	Blue
Levulose	Dark red	α-Methyl-D-glucoside	Violet

Detection of the spots. Detection of the spots was effected in the same manner as for the monodimensional chromatography.

RESULTS AND DISCUSSION

All 42 solvent systems used with the monodimensional technique were defective in some way. It was consistently observed that the resolution of the carbohydrates was such that only a limited number of sugars could be separated. Even in the best chromatograms, where spots were well-defined and sharp, the relative differences among R_F values were never important enough to permit the identification of more than 6 to 7 sugars at a time.

It was only by resorting to 35 × 20 cm chromatoplates, developed with solvent systems 13 or 14, that we were able to obtain 9 to 10 well-separated sugars. This method, however, was time-consuming, since development took about 12 h.

As far as the shape of the spots was concerned, it was observed that systems containing pyridine produced diffused and tailed spots. Solvents containing methanol or acetone often gave similar results. These same phenomena were accentuated when the solvent mixture combined either methanol and chloroform or methanol and acetone. Some of these solvents did, however, result in good separations, but, as stated above, the shape of the spots was poor.

Developing time with the various solvents was between 2.5 and 5 h; the solvents that permitted a shorter developing time (whose principal components were always methanol or acetone, either combined with water or chloroform, or combined with each other) consistently yielded deformed and diffuse spots.

All the solvents that contained boric acid gave well-shaped spots, but low R_F values and poor separation.

The best results were obtained with solvent systems containing isopropanol, which favours the migration of carbohydrates with a wider spectrum of R_F values. Addition of acetic acid improved separation and sharpened the spots.

The influence of other solvents, such as *n*-butanol, ethyl acetate, ethanol, etc., is more difficult to assess.

The choice of an 0.03 *M* boric acid solution for the impregnation of the silica gel was made on the basis of the fact that lower molarity solutions did not have a good effect on the shape of the spots and higher molarity solutions only reduced the R_F values.

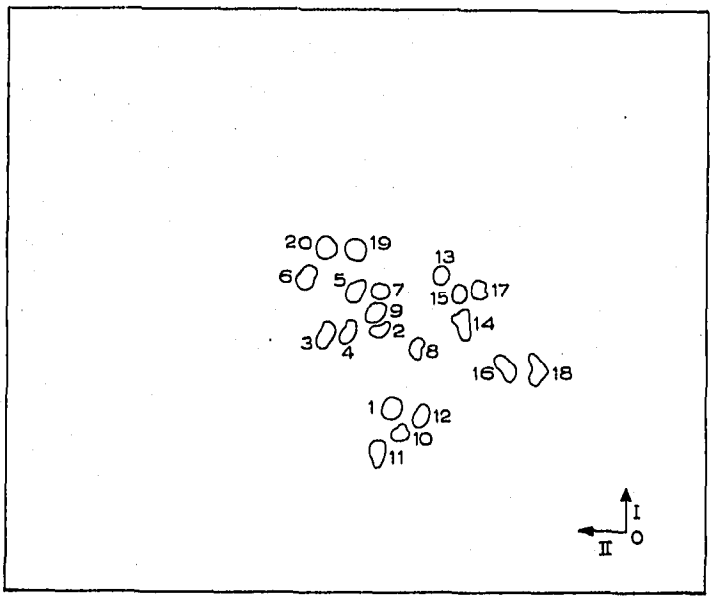

Fig. 1. Bidimensional chromatography of carbohydrates. 1 = Ribose; 2 = arabinose; 3 = xylose; 4 = lyxose; 5 = fucose; 6 = rhamnose; 7 = glucose; 8 = galactose; 9 = mannose; 10 = levulose; 11 = sorbose; 12 = mannoheptulose; 13 = sucrose; 14 = turanose; 15 = maltose; 16 = lactose; 17 = trehalose; 18 = raffinose; 19 = α-methyl-D-glucoside; 20 = α-methyl-D-mannoside. 1st run = isopropanol–water (4:1); 2nd run = *n*-butanol–ethyl acetate–isopropanol–acetic acid–water (35:100:60:35:30). The remarkable separation of monosaccharides and oligosaccharides should be noted.

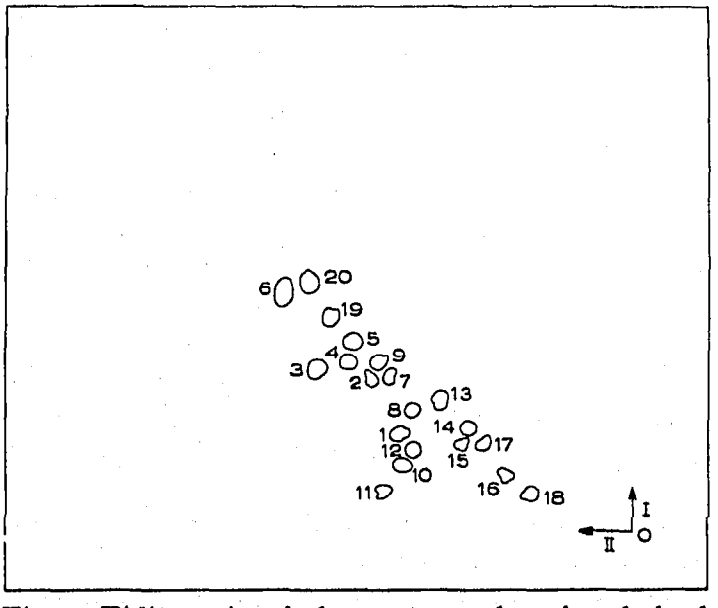

Fig. 2. Bidimensional chromatography of carbohydrates: Same sugars as in Fig. 1. 1st run = *n*-butanol–ethyl acetate–isopropanol–water (35:100:60:30); 2nd run = *n*-butanol–ethyl acetate–isopropanol–acetic acid–water (35:100:60:35:30).

Double development in one direction was also tried[7], but the results did not justify the doubling of the developing time. Modifications of the positions of the spots were obtained, but, very often, the improvement in separation of a specific pair of sugars was counterbalanced by the reduced separation of others.

This method can be successfully applied only to the separation of a few specific mixtures of carbohydrates.

A 30 cm double development run gives better results, but the time needed for development is about 24 h (which cancels the time advantage of the TLC method itself), and the sugars with higher R_F values are excessively diffused.

Continuous flow chromatography[13] was even less satisfactory for the separation of a complex mixture of carbohydrates.

Excellent results were obtained with the bidimensional technique, which, we think, greatly increases the use that can be made of TLC in carbohydrate research. All possible combinations of the best solvents described in Table I were tried.

The best were as shown in Table III.

TABLE III

BEST SOLVENT SYSTEMS FOR BIDIMENSIONAL TLC OF CARBOHYDRATES

Solvent mixture		*Pattern of the spots*
First run	*Second run*	
(a) No. 35	No. 13	see Fig. 1
(b) No. 42	No. 13	see Fig. 2
(c) No. 6	No. 13	similar to (a)
(d) No. 29	No. 13	similar to (b)
(e) No. 41	No. 13	similar to (b)
(f) No. 30	No. 13	similar to (a)

Of all these combinations, the best by far were the first two (also described in the Experimental section).

We observed that if a solvent system containing acetic acid is used in the first development, separation of the spots is impaired, since, under experimental conditions, it is very difficult to completely eliminate the acetic acid which is strongly bound to the silica gel. On the other hand, as seen above, each of the best combinations uses solvent No. 13, which contains acetic acid, for the second run. If solvent No. 13 is substituted by any other solvent system in the second run, the spots obtained are not sharp or well-shaped.

The apparent contradiction implicit in the above statements can probably be explained by the fact that the molecules of acetic acid bound to the silica gel during the first run, occupied a sufficient number of sites to disturb the normal partition on the adsorbent of the solvents of the second run.

Certain sugars, or oligosaccharides, present a special problem in monodimensional thin-layer chromatography: they have very similar mobilities with the majority of the solvents used. When a specific solvent, such as, for example, No. 25, succeeds in separating arabinose from mannose and levulose (one of the most difficult triads to resolve), it cannot separate glucose, sucrose and lyxose from arabinose, nor can it separate raffinose from levulose.

Even our best solvent for a monodimensional run, No. 13, separates the majority of the carbohydrates studied into 4 spots: (1) arabinose, mannose, sorbose, fucose, lyxose, α-methyl-D-glucoside; (2) glucose, levulose, mannoheptulose; (3) sucrose, maltose, turanose; (4) xylose, α-methyl-D-mannoside, ribose.

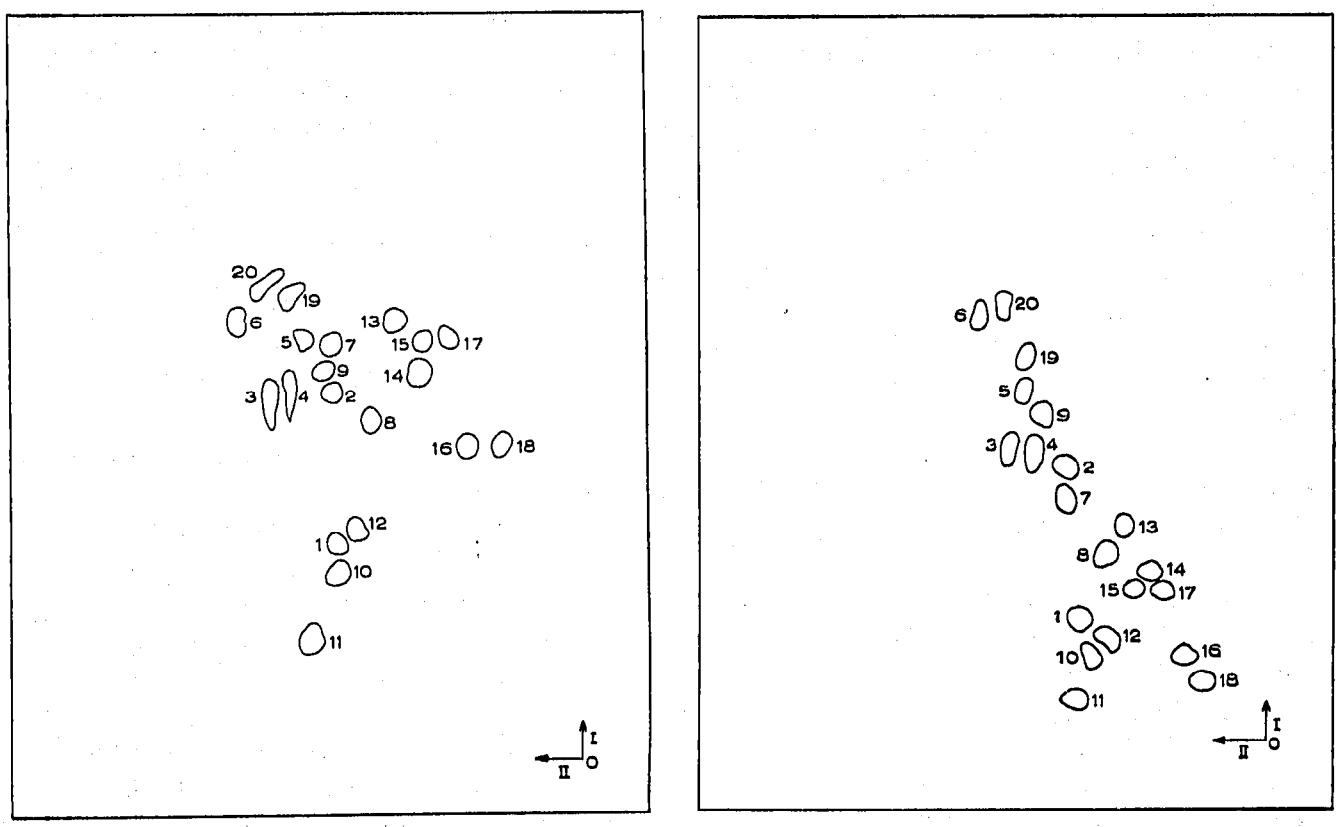

Fig. 3. Bidimensional chromatography of carbohydrates on a 20 × 30 cm chromatoplate: Sugars and solvents same as in Fig. 1.

Fig. 4. Bidimensional chromatography of carbohydrates on a 20 × 30 cm chromatoplate: Sugars and solvents same as in Fig. 2.

For this reason, monodimensional chromatography is too limited to be used as a tool in qualitative analysis involving complex sugar mixtures. Our experience has shown that even the comparison of several chromatoplates developed with different solvents is insufficient for the separation and identification of an unknown complex mixture.

This is obviously not a problem in bidimensional chromatography, which is clearly a more advanced and complete technique. But although the number of sharply separated carbohydrates is much higher than with the monodimensional method, the limitation in this case arises from the fact that slightly larger amounts of each sugar are needed to compensate for the greater diffusion of the spots, and, when a complex sugar mixture is used, the total amount of the substance placed at the origin exceeds the capacity of the silica gel. Different brands of silica gel have slightly different capacities, but the average maximum load on a layer of 250 microns should not exceed 35–50 μg.

Experimentally, we were able to load a 350 micron layer with up to 120 μg of substance, and were still able to obtain good separation of the sugars involved. In fact, we advise the use of a layer thicker than the standard. Commercially prepared chromatoplates are not suitable because the layer is too thin and therefore low in capacity.

A layer thicker than 300–350 microns is not advisable, however, if some of the sugars contained in the mixture are present in a lesser amount than 2–3 μg, because of the difficulties involved in revealing small quantities in thick layers.

It should be noted that quantities of some particular sugars should not exceed specific amounts (4–5 μg for a 350 micron layer) since, even in bidimensional chromatography, they tend to run close together. The sugars with this tendency in the chromatogram illustrated in Fig. 1 are: mannoheptulose–levulose; maltose–trehalose; glucose–arabinose–mannose; rhamnose–α-methyl-D-mannoside.

In short, the quantities of some of the single sugars that can be applied to a chromatoplate, apart from the total capacity of the gel, is a limiting factor that must be added to those mentioned above, when sugars with very similar mobilities are involved.

This limitation can be partially or wholly eliminated by resorting to bidimensional chromatography on a rectangular 30 × 20 cm plate (Figs. 3 and 4).

In this case, the carbohydrates separate much better than with the standard 20 × 20 cm plate. It is necessary, however, for the first run to be the longer of the two. The positions of the sugars, in this case, are similar to the corresponding positions on the standard plate. We strongly advise this modification of the technique whenever the quantities of those sugars that tend to run closely together vary widely in the sample mixture.

For the detection of spots, it is preferable to use the naphthoresorcinol reagent, because the great variety of colours it produces particularly helps in the identification of the carbohydrates (see Table II). This is especially pertinent when two sugars are very close together.

For specific applications, such as the differentiation of aldoses and ketoses, or of the aldohexoses, aldopentoses and ketohexoses, several other reagents have been reported[11,18].

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