CHROM. 5547

THIN-LAYER CHROMATOGRAPHY OF OLIGOSACCHARIDES WITH TUNGSTIC OR MOLYBDIC ACID AS IMPREGNANT

T. MEZZETTI
Istituto di Chimica Farmaceutica e Tossicologia dell 'Università degli Studi di Perugia (Italy)
AND
M. LATO*, S. RUFINI AND G. CIUFFINI
Istituto di Puericultura dell 'Università degli Studi di Perugia (Italy)
(Received July 14th, 1971)

SUMMARY

Thin-layer chromatography of carbohydrates on silica-gel layers impregnated with molybdic or phospho-tungstic acid has been investigated.

These two impregnants are particularly useful for the separation of oligosaccharides because of their capacity to complex polyols and sugars (a capacity also exhibited by boric acid).

The molybdic acid and phospho-tungstic acid complexes, however, showed higher R_F values than the corresponding boric acid complexes.

A total of nine oligosaccharides (six of which were disaccharides), plus six monosaccharides, were separated on a two-development, one-dimensional chromatogram.

The number of separated carbohydrates was increased to twelve oligosaccharides (nine disaccharides), plus seven monosaccharides with a third development at 90° to the first two.

The effect of solvents, impregnants and the thickness of the layer on separation was also thoroughly investigated.

INTRODUCTION

The present work is a development of our general interest in the separation of carbohydrates by thin-layer chromatography (TLC)[1-7]. It specifically concerns the influence exerted on the separation of oligosaccharides by changes in adsorbents, solvent mixtures and impregnants that complex with carbohydrates. Separation of 12 oligosaccharides was achieved by combining two developments in the same direction with a third run at 90° to the first two. The procedure herein described seems more successful than previous ones as far as the number of identifiable oligosaccharides is concerned[8-11], but it is rather more cumbersome than the methods we previously developed, in that three developments are necessary. Moreover, the increase in spacing between the faster- and slower-running sugars is achieved at the expense of individual R_x variations with the different solvents. This flattening of response to the solvents

* Director of the Istituto di Puericultura.

appears to be the chief impediment to thin-layer separation of a larger number of oligosaccharides on silica gel or alumina.

EXPERIMENTAL

Adsorbents

Three types of adsorbents were tested: (a) silica gel, TLC grade with binder (Fluka 5/D); (b) silica gel (as above)–alumina, TLC grade (Fluka) (1:1); (c) silica gel–alumina (3:1); and (d) alumina, TLC grade (Fluka).

Impregnants

The following impregnants were employed: (A) none (water); (B) 0.03 *M* boric acid in water; (C) molybdic acid, saturated solution in water; (D) 0.08 *M* phosphoric acid; (E) 0.04 *M* phosphoric acid; (F) 0.05 *M* phosphoric acid–0.2 *M* sodium tungstate (1:1), pH 7.7; (G) 0.2 *M* phosphoric acid–0.2 *M* sodium tungstate (1:1), pH 6.8; (H) 0.1 *M* phosphoric acid–0.05 *M* sodium tungstate (1:1), pH 3.1; (I) 0.2 *M* phosphoric acid–0.05 *M* sodium tungstate (1:1), pH 2.5; (J) silica gel was mixed with 10 % (w/w) of sodium tungstate, wetted on a filter with a stoichiometric quantity of HCl in water and washed under suction until no further chloride was detectable in the filtrate; the material on the filter was then suspended in water and applied to the plates; (K) As J, with 5 % of sodium tungstate; (L) As J, with 3.75 % of sodium tungstate; (M) As J, with 2.5 % of sodium tungstate; (N) As J, with 5 % of sodium tungstate and 0.02 *M* H_3PO_4 instead of HCl, and (O) As J, with 5 % of sodium tungstate and an excess of H_3PO_4.

Solvents

The following solvents were used: (1) *n*-butanol–1 *M* boric acid (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–1 *M* boric acid (60:3:20:17); (6) *n*-butanol–isopropanol–water (3:5:2); (7) *n*-butanol–ethanol–water (2:1:1); (8) *n*-butanol–ethanol–0.1 *M* phosphoric acid (1:10:5); (9) *n*-butanol–ethanol–0.1 *N* hydrochloric acid (1:10:5); (10) *n*-butanol–methanol–water (5:3:1); (11) *n*-butanol–ethyl acetate–1 *M* boric acid (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-1 *M* boric acid (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); (23) ethyl acetate–pyridine–water (3:3:2); (24) ethyl acetate–pyridine–water (4:3:2); (25) ethyl acetate–pyridine–0.1 *M* boric acid (2:1:2); (26) ethyl acetate–acetic acid– 0.1 *M* boric acid (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–0.03 *M* boric acid (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); (35) acetone–water

(9:1); (37) acetone–water–chloroform–methanol (8:0.5:1:1); (38) methanol–chloroform–acetone–28 Bè ammonia (5:2:3:2); (39) methanol–chloroform–28 Bè ammonia (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); (43) *n*-butanol–methanol–water (5:4:1); (44) *n*-butanol–methanol–water (50:40:15); (45) *n*-butanol–methanol–water (5:5:2); (46) *n*-butanol–methanol–water (70:30:25); (47) *n*-butanol–methanol–water (10:3:3); (48) *n*-butanol–methanol–acetic acid–water (100:25:10:20); (49) ethyl acetate–acetone–isopropanol–water (3:3:4:3); (50) ethyl acetate–acetone–isopropanol–water (30:20:40:35); (51) ethyl acetate–acetone–isopropanol–water (3:2:4:6); (52) ethyl acetate–isopropanol–water (6:4:3); (53) ethyl acetate–isopropanol–water (12:5:6); (54) ethyl acetate–isopropanol–water (50:50:25); (55) ethyl acetate–isopropanol–water (60:50:25); (56) ethyl acetate–methanol–acetic acid–water (80:15:15:10); (57) ethyl acetate–methanol–acetic acid–water (60:30:15:10); (58) ethyl acetate–methanol–acetic acid–water–isopropanol (60:30:15:10:20); (59) ethyl acetate–methanol–acetic acid–water–isopropanol (60:30:15:10:40); (60) ethyl acetate–methanol–acetic acid–water–*n*-butanol; (60:30:15:10:5); (61) *n*-butanol–ethyl acetate–isopropanol–water (30:85:75:30); (62) *n*-butanol–ethyl acetate–isopropanol–water (30:75:85:30); (63) *n*-butanol–ethyl acetate–isopropanol–water (30:80:80:30); (64) *n*-butanol–ethyl acetate–isopropanol–water–ethylene glycol (25:40:100:10:25); (65) *n*-butanol–ethyl acetate–isopropanol–water–ethylene glycol (25:50:90:10:25); (66) *n*-butanol–ethyl acetate–isopropanol–water–ethylene glycol (20:40:100:20:20); (67) *n*-butanol–ethyl acetate–isopropanol–water–ethylene glycol (30:75:65:30:10); (68) *n*-butanol–ethyl acetate–isopropanol–water–ethylene glycol (30:75:85:20:10); (69) *n*-butanol–ethyl acetate–isopropanol–water–ethylene glycol (25:60:85:20:10); (70) *n*-butanol–ethyl acetate–isopropanol–water–ethylene glycol (35:50:85:20:10); (71) *n*-butanol–ethyl acetate–isopropanol–water–ethylene glycol (30:60:80:10:20); (72) *n*-butanol–ethyl acetate–isopropanol–water–ethylene glycol (35:70:70:10:15); (73) *n*-butanol–ethyl acetate–isopropanol–water–0.03 *M* boric acid–ethylene glycol (35:70:70:8:2:15); (74) *n*-butanol–ethyl acetate–isopropanol–water–0.03 *M* boric acid–ethylene glycol (70:35:70:8:2:15); (75) ethyl acetate–acetic acid–methanol–water–0.03 *M* boric acid (60:15:15:9:1); (76) ethyl acetate–methanol–water–0.03 *M* boric acid–ethylene glycol (60:20:9:1:10); (77) *n*-butanol–ethyl acetate–isopropanol–water–0.03 *M* boric acid–ethylene glycol (60:45:70:8:2:15); (78) isopropanol– 0.01 *N* phosphoric acid (4:1); (79) isopropanol–0.05 *N* phosphoric acid (4:1); (80) isopropanol–0.02 *N* phosphoric acid (4:1); (81) isopropanol–0.03 *N* phosphoric acid (4:1); (82) *n*-butanol–methanol– 0.02 *N* phosphoric acid (5:3:1); (83) *n*-butanol–pyridine–0.02 *N* phosphoric acid (8:4:3); (84) *n*-butanol–methanol– 0.02 *N* phosphoric acid (3:4:3); (85) *n*-butanol–acetone–0.02 *N* phosphoric acid (4:5:1); (86) *n*-butanol–methanol–0.02 *N* phosphoric acid (5:1:3); (87) *n*-butanol–acetic acid–0.02 *N* phosphoric acid (4:1:5); (88) *n*-butanol–isopropanol–0.02 *N* phosphoric acid (3:5:2); (89) ethyl acetate–isopropanol–0.02 *N* phosphoric acid (100:60:30); (90) ethyl acetate–isopropanol–0.02 *N* phosphoric acid (65:22:11); (91) *n*-propanol–0.02 *N* phosphoric acid (85:15); (92) *n*-butanol–ethanol–0.02 *N* phosphoric acid (1:10:5); (93) *n*-butanol–ethanol–water (1:10:5); (94) *n*-butanol–ethanol–water (3:10:5); (95) *n*-butanol–methanol–water (4:3:1); (96) *n*-butanol–methanol–water (4:2:1); (97) *n*-butanol–methanol–sodium tungstate (4:2:1); (98) *n*-butanol–ethyl acetate–isopropanol–water–0.03 *M* boric acid (45:70:75:8:

2)); (99) *n*-butanol–ethyl acetate–isopropanol–water–0.03 *M* boric acid (40:70:80:8:2)); (100) ethyl acetate–pyridine–0.6 % boric acid (2:1:2); (101) ethyl acetate–pyridine–1 % boric acid (2:1:2)); (102) ethyl acetate–pyridine–2 % boric acid (2:1:2); (103) *n*-butanol–methanol–0.02 *N* phosphoric acid (7:2:1); (104) ethyl acetate–isopropanol–water (5:5:1)); (105) ethyl acetate–isopropanol–water–*n*-butanol (50:50:25:5)); (106) isopropanol–*n*-propanol–water (42:42:15); and (107) *n*-butanol–ethyl acetate–pyridine–saturated boric acid (80:20:50:100).

Standard carbohydrates tested

The following sugars were examined.

Monosaccharides: Ribose, arabinose, xylose, fucose, rhamnose, glucose, mannose, levulose, sorbose, mannoheptulose, sedoheptulose and α-methyl-D-mannoside.

Disaccharides: sucrose, turanose, maltose, trehalose, lactose, lactulose, melibiose, gentiobiose, cellobiose and isomaltose.

Trisaccharides: Raffinose, maltotriose and melezitose.

Tetrasaccharide: Stachyose.

For one-dimensional chromatography, 10 mg of each sugar were dissolved in 2-ml vials of distilled water; 1 μl of each solution (= 5 μg of carbohydrate) was applied to the chromatoplate with a micropipette. For multiple development and two-dimensional chromatography, a mixture of 10 mg of each of the sugars selected was dissolved in 2 ml of distilled water; 1 μl of the solution contained 5 μg of each sugar in the mixture. The solution was applied with a micropipette to the lower right-hand corner of the chromatoplate about 2 cm from each of the edges.

One-dimensional chromatography

The chromatoplates were prepared by mixing 35 g of adsorbent with 70 ml of the specific impregnant and the suspensions obtained were applied in the usual manner as a 0.4-mm layer to the glass plates. These plates were dried for 24 h at room temperature and then activated for 1 h at 110°.

Before deposition of the sugar, the plates were allowed to cool for a few minutes. Development was effected in a tank lined with filter-paper which was wetted with the developing solvent. Room temperature was kept at about 20°. All the adsorbents, impregnants, solvents and results of the 20-cm one-dimensional development are summarized in Table I.

The adsorbents, impregnants, solvents and results of the simple 30-cm one-dimensional runs are summarized in Table II.

Multiple development and two-dimensional chromatography

Preparation of the chromatoplates and general operating conditions were kept as close as possible to those described in the preceding section on one-dimensional chromatography.

After each development, the plates were dried in an oven at 50–60°. All the adsorbents, impregnants and results of multiple-development and two-dimensional chromatography are summarized in Table II.

Detection of spots

The developed plates, dried at room temperature for a short time, were heated

TABLE I

RESULTS FOR THE 20-cm ONE-DIMENSIONAL DEVELOPMENT

Solvents	*Adsorbents (lower-case letters) and impregnants (capital letters)*	*Shape of spots* *A = sharply defined* *B = slightly diffused* *C = diffused and/or tailed* *Resolution* +++ = *very good* ++ = *good* + = *insufficient* − = *no resolution*
1	aC	B (+)
2	aC; aB	C (++); C (−)
3	aC	B (−)
4	aC; aI; cI	B (−); B (++); B (+)
5	aC	C (++)
6	aC; aI	B (−); A (+)
7	aC; aN	C(+); B (+)
8	aC; aI; cI; aK; aO	B (−); B (−); B (+); B (−); A (−)
9	aC; aI; cI; aN	B (−); B (−); A (++); B (−)
10	aC; aI; bI; cI; aK; aN	B (−); B (++); B (−); B (+); C (−); B (+)
11	aC	B (−)
12	aC	B (++)
13	aC; aB; aH; aF; aG; aN; aI	B (+); A (++); B (+); B (+); B (+); B (−); B (+)
14	aC; aI; aN	B (++); C (++); C (+)
15	aC; aI; aN	A (++); C (+); B (+)
16	aC; aI; cI; aN	A (−); C (−); A (−); B (+)
17	aC; aI; cI; aN	A (−); B (−); B (−); B (−)
18	aC; aI; cI	A (−); C (+); B (++)
19	aC; aI; aN	B (−); B (−); B (−)
20	aC; aN	B (++); B (+)
21	aC; aI; aB	B (+); B (−); B (+)
22	aC	A (+)
23	aC; aI; cI	C (−); B (−); B (++)
24	aC; aB	B (−); B (−)
25	aC; aB; aI	C (−); C (+); C (−)
26	aC; aN	B (−); B (−)
27	aC; aN	A (++); A (−)
28	aC; aI; cI; aN	A (−); B (++); B (+); B (+)
29	aC; aN; aI; aO	A (++); A (+); A (+); B (+)
30	aC; aB	C (++); B (+)
31	aC; aI; aB	A (++); A (++); A (+)
32	aC; aI; aB	A (+); A (++); A (+)
33	aC; aI	C (−); A (+)
34	aC	C (+)
35	aC; aI; cI; bI; aB	B (−); A (+); A (++); A (+); B (++)
36	aC; aI; cI; aB; aO	C (++); C (++); B (−); C (++); C (−)
37	aC; aI; aB	C (+); B (++); C (+)
38	aC	B (+)
39	aC; aI	B (+); C (−)
40	aC	B (−)
41	aC; aI	B (++); B (+)
42	aC; aN; aI; aF; aH	A (++); B (+); A (+); B (+); A (+)
43	aI	B (+)
44	aI	B (++)
45	aI	A (+)
46	aI	A (+)
47	aI	A (++)
48	aI	A (+)
49	aI	C (+)
50	aI	C (−)

(*Continued on p. 334*)

TABLE I (*continued*)

Solvents	*Adsorbents (lower-case letters) and impregnants (capital letters)*	*Shape of spots* *A = sharply defined* *B = slightly diffused* *C = diffused and/or tailed* *Resolution* +++ = *very good* ++ = *good* + = *insufficient* − = *no resolution*
51	aI	B (−)
52	aI	B (−)
53	aI; bI; cI; dI	B (−); B (++); B (++); B (++)
54	aI	A (++)
55	aI	A (++)
56	aC	B (+)
57	aC	C (+)
58	aC	B (++)
59	aC	C (+)
60	aC	B (+)
61	aC	A (++)
62	aC	A (++)
63	aC	A (+)
64	aC	B (+)
65	aC	B (+)
66	aC	B (+)
67	aC	B (+)
68	aC	B (+)
69	aC	B (+)
70	aC	B (+)
71	aC; aA; aB	A (+); C (+); B (+)
72	aC; aB	A (+); B (++)
73	aA	B (+++)
74	aA	B (++)
75	aA	C (++)
76	aA	B (+)
77	aA	A (++)
78	aK; aM	B (+); C (−)
79	aK	C (+)
80	aK; aM; aJ; aL	B (−); B (−); B (−); B (−)
81	aK	C (−)
82	aK	C (++)
83	aJ; aK	C (−); C (−)
84	aK	A (−)
85	aK	A (−)
86	aK	B (−)
87	aK	B (−)
88	aK	B (−)
89	aK; aN; aL	C (+); C (+); C (+)
90	aN	B (−)
91	aN	C (−)
92	aN	A (−)
93	aO	A (−)
94	aN	B (+)
95	aN	B (−)
96	aN; aE; aD	B (++); B (+); B (+)
97	aE; aD	B (+); B (+)
98	aA	B (−)
99	aA	B (−)
100	aA	C (−)
101	aA	C (−)
102	aA	C (−)
103	aK	B (−)

TABLE II

RESULTS FOR THE SINGLE AND DOUBLE MONODIMENSIONAL AND BIDIMENSIONAL DEVELOPMENTS

a = silica gel; I = 0.2 *M* phosphoric acid–0.05 *M* sodium tungstate (1:1), pH 2.5; C = molybdic acid, saturated solution in water; A = water; N = 5% sodium tungstate and 0.02 *M* H_3PO_4.

Adsorbent	*Impregnant*	*Thickness (cm)*	*Solvent, first run*	*Distance, first run (cm)*	*Solvent second run, one-dimensional*	*Solvent second or third run two-dimensional, 20 cm*	*Resolution*	*Maximum number of spots oligosaccharides*
a	I	0.4	4	30			++	7
a	I	0.4	54	30			++	7
a	I	0.4	55	30			++	7
a	I	0.4	106	30			++	7
a	C	0.4	15	30			++	6
a	C	0.4	15	20	15		++	7
a	C	0.4	29	20	29		+++	9
a	C	0.4	42	20	42		++	8
a	C	0.4	28	20	28		++	7
a	I	0.4	54	20	54		++	8
a	I	0.4	54	20	106		++	7
a	I	0.6	54	20	54		++	8
a	I	0.4	48	20		4	+	7
a	I	0.4	48	20		54	+	6
a	I	0.4	106	30		10	++	8
a	A	0.4	73	20		13	−	5
a	A	0.4	73	20		107	−	4
a	N	0.4	82	20		92	−	5
a	I	0.4	54	20	104	31	++	10
a	I	0.4	54	20	104	4	++	10
a	I	0.4	15	20	105	10	++	9
a	I	0.4	105	20	106	13	++	9
a	I	0.4	54	30	54	4	++	10
a	I	0.4	54	20	54	4	+++	12
a	I	0.4	54	30	54	13	+	8
a	I	0.4	54	30	54	10	++	9
a	I	0.6	54	20	54	4	+++	11
a	I	0.6	54	20	54	10	++	10
a	I	1	54	20	54	4	+	9
a	I	1	54	20	54	10	+	8
a	A	0.4	54	20	54	4	+	7

in an oven at 110° until no trace of the solvents could be detected. The heated chromatograms were sprayed with a freshly prepared solution of 20 mg of naphthoresorcinol, 10 ml of ethanol and 0.4 ml of concentrated sulphuric acid. The plates were then reheated for 5–10 min at 110°. The sugars appeared as brightly coloured spots.

Alternatively, plates were sprayed with an 0.5% solution of potassium permanganate in a 1 *N* sodium hydroxide solution and heated at 100° for a few minutes[11].

A third spray that gave good results was prepared as follows: 4 g of diphenylamine, 4 ml of aniline, 20 ml of orthophosphoric acid (80%) and 0.66 g of benzidine were dissolved in 200 ml of acetone. The plates were sprayed with this mixture and then heated in an oven at 100° for 10 min to produce the characteristic colours[9].

Glyceraldehyde and dihydroxyacetone were not clearly visible after two developments, because they probably decompose too easily during the drying process. They were visible, on the other hand, after the first development. If glyceraldehyde

and dihydroxyacetone are among the carbohydrates to be identified, we advise drying the plates in a vacuum.

Gentiobiose and maltotriose are two other sugars that gave weak coloration with the naphthoresorcinol reagent, even after a 20-cm run. These sugars can be detected more easily if potassium permanganate or diphenylamine spray are used.

Trehalose gives good blue-green coloration, but it shows up only after 5 min of heating in an oven at 110–120°.

RESULTS AND DISCUSSION

We started with the same boric acid impregnation with which we had worked previously, since it gave a basis with which to compare the other impregnants to be investigated. It had already been established that this impregnant was excellent with monosaccharides[1,2], but not very selective with oligosaccharides. Changes in the boric acid concentration did not improve the resolution[12]. Lower concentrations yielded much lower resolutions with diffused spots, while higher concentrations accentuated the shortness of migration typical of this impregnant.

We then started to work with other impregnants capable of complexing polyhydroxy compounds, such as molybdic and tungstic acid[8]. The best results were obtained when low-pH plates impregnated with phosphoric acid–sodium tungstate or saturated molybdic acid solution were used. After testing all the solvents, it was evident that the variety of relative R_x variations that we had previously obtained with the boric acid impregnant was significantly reduced or lost, but this work enabled us to obtain good separations of up to 15 mono- and oligosaccharides with developments in the same direction (20 + 30 cm) on a layer impregnated with molybdic acid (Fig. 1), and up to 20 carbohydrates (12 oligosaccharides) with the same development plus an additional third development run at 90° to the first two on a phospho-tungstic acid-impregnated layer (impregnant I).

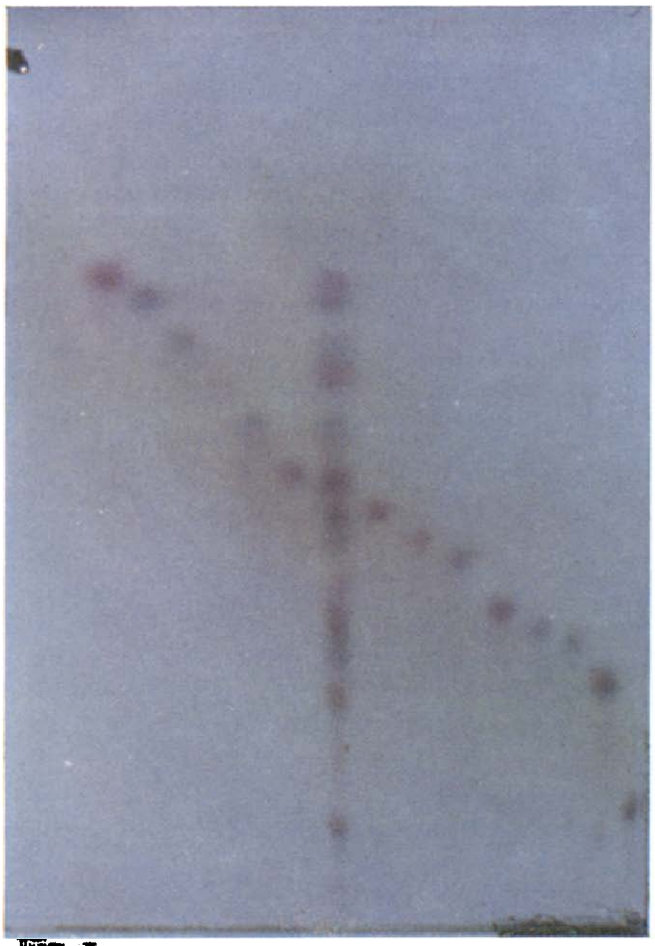
We were also able to make a number of observations on the effect of the impregnant on resolution and diffusion, and to establish the optimum thickness of the layer.

When a phospho-tungstic acid-impregnated plate was developed together with a silica gel and water plate, the difference between the two was evident. The latter showed twice as much diffusion and much less selectivity than the former, and molybdic acid plates showed even sharper spots than those impregnated with tungstic acid.

Increased thickness of the layer presented advantages and disadvantages. The greatest advantage was increased resolution and less diffusion. A 0.6-mm layer shortened the migration of stachyose and the other slow-running sugars (with respect to the optimum 0.4-mm layer), but speeded up the R_x of sucrose and other fast-running spots, while the intermediate-range sugars moved more slowly or more rapidly in proportion to their original tendencies.

This phenomenon was probably caused by some kind of continuous gradient that the multi-solvent system established within the layer during the second run, because of incomplete elimination of the more polar solvents of the first run.

Increased resolution was offset, however, by diminished visibility of the spots. Increases in the amount of sugar spotted at the origin did not yield a proportional increase in colour intensity. Moreover, the time required for the three developments



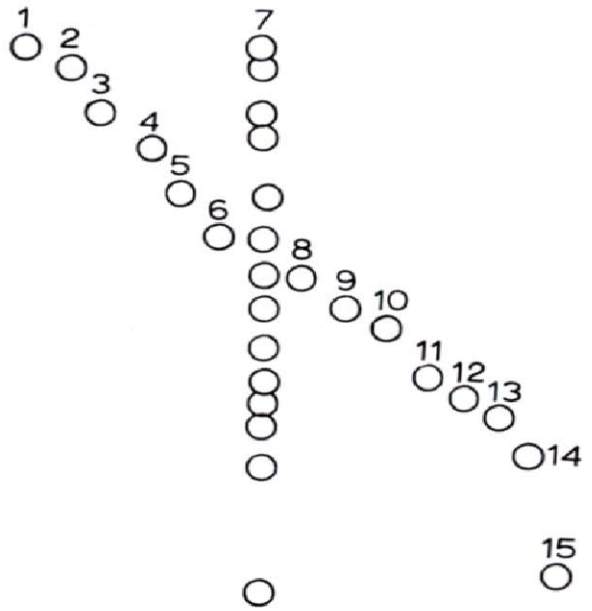
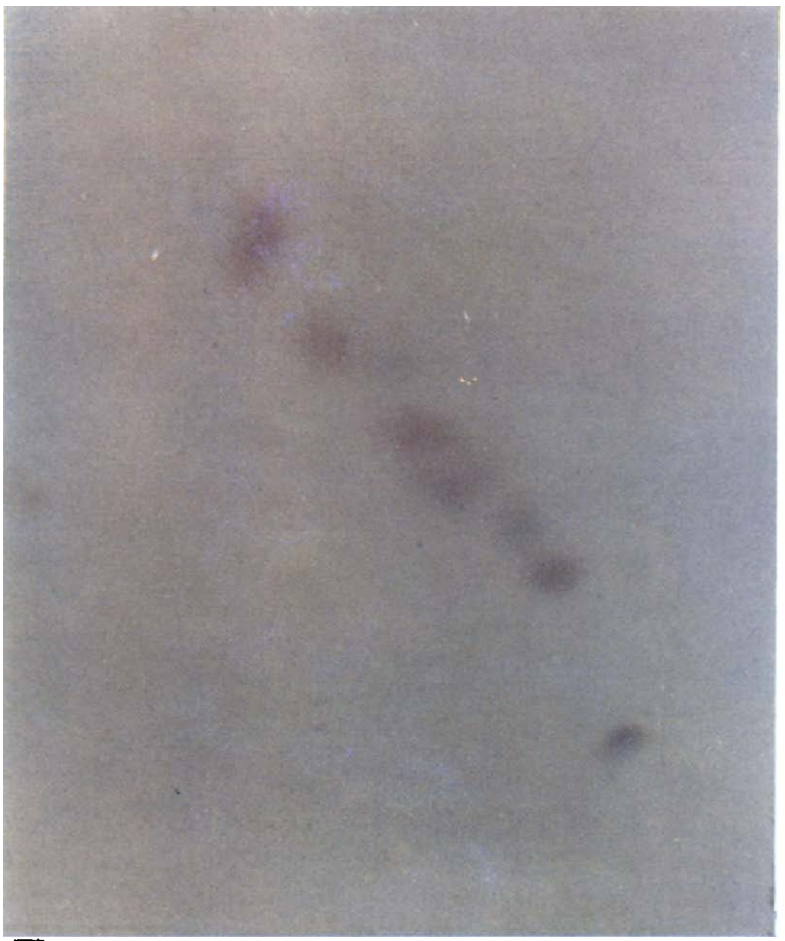


Fig. 1



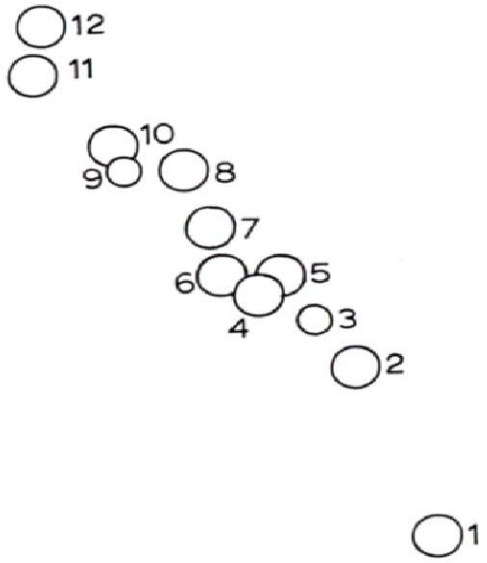


Fig. 2

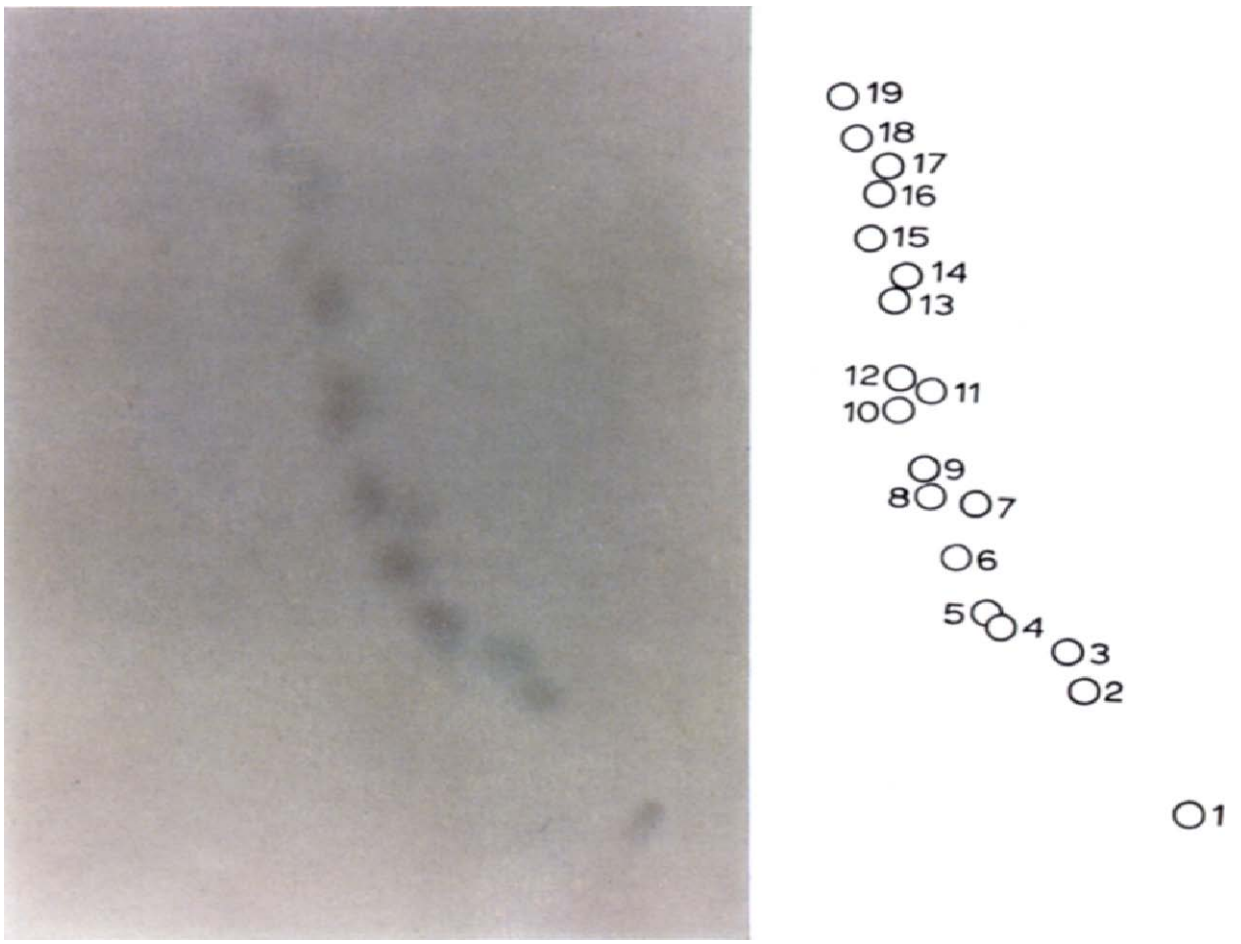


Fig. 3

Fig. 1. One-dimensional chromatography of standard mixture of carbohydrates on a 20 × 35 cm plate. From left to right: 1 = rhamnose; 2 = α-methyl-D-mannoside; 3 = ribose; 4 = fucose; 5 = mannoheptulose; 6 = sucrose; 7 = standard mixture; 8 = turanose; 9 = maltose; 10 = trehalose; 11 = melezitose; 12 = lactose; 13 = melibiose; 14 = raffinose; 15 = stachose. First run (20 cm) = solvent 29; second run (30 cm) = solvent 29. Impregnant C, molybdic acid.

Fig. 2. Two-dimensional chromatography of oligosaccharides. 1 = stachyose; 2 = raffinose; 3 = melibiose; 4 = lactose; 5 = gentiobiose; 6 = lactulose; 7 = melezitose; 8 = trehalose; 9 = cellobiose; 10 = maltose; 11 = turanose; 12 = sucrose. First run (20 cm) = solvent 54; second run (30 cm) = solvent 54; third run (two-dimensional: 20 cm) = solvent 4. Impregnant I, 0.2 *M* phosphoric acid–0.05 *M* sodium tungstate (1:1) pH 2.5.

Fig. 3. Two-dimensional chromatography of carbohydrates on a 20 × 35 cm plate. 1 = stachyose; 2 = raffinose; 3 = melibiose; 4 = lactose; 5 = lactulose; 6 = melezitose; 7 = trehalose; 8 = cellobiose; 9 = maltose; 10 = turanose; 11 = galactose; 12 = sucrose; 13 = mannoheptulose; 14 = arabinose; 15 = fucose; 16 = ribose; 17 = xilose; 18 = α-methyl-D-mannoside; 19 = rhamnose. First run (20 cm) = solvent 54; second run (30 cm) = solvent 54; third run (two-dimensional: 20 cm) = solvent 4. Impregnant I, 0.2 *M* phosphoric acid–0.05 *M* sodium tungstate (1:1) pH 2.5.

with a 0.6-mm layer was 6–7 h for the first, 17 h for the second and 6 h for the third, compared with 3–3.5, 9–10 and 3 h, respectively, for the 0.4-mm layer.

A third, important disadvantage was the slow, incomplete drying of the plate between runs. Higher drying temperatures caused partial oxidation of the sugars, the products of which were strongly adsorbed by the silica gel, giving rise to an artificial multiple image for each sugar when revealed.

Phospho-tungstic acid impregnation

The most striking feature of phospho-tungstic acid impregnation was the very limited diffusion exhibited by the spots. This characteristic allowed us to utilize two developments in the same direction. The first development was allowed to run for 20 cm. The plate was then dried in an oven at 50–60° and again developed for a 30-cm run. When solvent 54 was used for both runs, this technique (see Table II) enabled us to separate the following oligosaccharides: stachyose, raffinose, melibiose, lactose, melezitose, trehalose, maltose and sucrose, to which we then added six other monosaccharides: mannoheptulose, fucose, ribose, α-methyl-D-mannoside, rhamnose and glyceraldehyde. This method is particularly useful for comparing a mixture of oligosaccharides with internal standards. Other solvents that gave good results with phospho-tungstic acid impregnation were 106, 55 and 4 (double development with the same solvent) and the following pairs: 105–106, 15–105 and 54–104 (see Table II).

Phospho-tungstic acid impregnation does not resolve sucrose and turanose with the one-dimensional techniques. Only the three-run, two-dimensional procedure achieved a certain degree of separation, with solvent 54 used for the first two runs, as above, and solvent 4, in the two-dimensional procedure, used for the third run. The 12 oligosaccharides separated by this procedure were stachyose, raffinose, melibiose, lactose, gentiobiose, lactulose, melezitose, trehalose, cellobiose, maltose, turanose and sucrose (Fig. 2), to which the following monosaccharides could be added for a total of 20 sugars: galactose, mannoheptulose, arabinose, fucose, ribose, α-methyl-D-mannoside, rhamnose and glyceraldehyde.

Molybdic acid impregnation

Another impregnant which, in many cases, gave similar results to phospho-tungstic acid, and with which we experimented extensively, was molybdic acid (impregnant C). Several solvents yielded good separations; some of these were slightly superior to their phospho-tungstic acid equivalents (for example, solvent 28). The solvents that gave good separations with molybdic acid impregnation were 2, 5, 15, 20, 28, 29, 31, 32 and 42 (Table I) and the best of these were 15, 29 and 42. Molybdic acid impregnation usually held back sugar migration less than boric acid (impregnant B), (although there were a few striking exceptions such as solvents 21, 22, 27 and 28), but like boric acid, it produced some slight inversions of the migration patterns of a few sugars with some solvents. For example, solvents 31 and 39 showed a higher R_X for maltose than turanose.

The best solvents with molybdic acid impregnation gave more highly resolved multiple-development, one-dimensional chromatograms than the best solvents with phospho-tungstic acid impregnation (see Table I). The best chromatogram obtained with the latter (see above) separated eight oligosaccharides and six monosaccharides. Since the former exhibited less diffusion, the same sugars, plus turanose, were separated

with solvent 29. It should be noted, however, that two-dimensional chromatography with molybdic acid impregnation yielded poorer separation than with phospho-tungstic acid. Solvents containing pyridine that had been used successfully for the third run with phospho-tungstic acid-impregnated plates, had to be excluded because they produced poor spots and no resolution. Other solvents did not modify the general migration pattern enough to justify a two-dimensional run.

CONCLUSION

Although the chromatographic procedures investigated in this work yielded the highest number of oligosaccharides yet separated by TLC, they involve too much manipulation for rapid analysis of many samples, and this restricts their use.

On the other hand, we believe that no better results can be achieved by simple chromatography on silica-gel layers impregnated with sugar-complexing substances.

Our current experimentation, therefore, is directed towards TLC of sugars on new ion-exchange materials.

REFERENCES

1 M. LATO, T. MEZZETTI, B. BRUNELLI AND G. CIUFFINI, *J. Chromatogr.*, 34 (1968) 26.
2 M. LATO, T. MEZZETTI, B. BRUNELLI AND G. CIUFFINI, *J. Chromatogr.*, 36 (1968) 191.
3 M. LATO, T. MEZZETTI, B. BRUNELLI AND G. CIUFFINI, *J. Chromatogr.*, 39 (1969) 407.
4 M. LATO, T. MEZZETTI, B. BRUNELLI, P. LEONE AND G. CIUFFINI, *Quad. Nutr.*, 28, No. 3–4, (1968).
5 M. LATO, T. MEZZETTI, P. LEONE, G. CIUFFINI AND C. BRUNELLI, *Riv. Pediatr. Sicil.*, 24, No. 4 (1969).
6 M. LATO, T. MEZZETTI, S. RUFINI, P. LEONE AND G. CIUFFINI, *Atti XXXIV Congr. Soc. Ital. Ped.*, Vol. III (1970) 310.
7 M. LATO, T. MEZZETTI, S. RUFINI AND G. CIUFFINI, *Conv. Interr. Ped. Puer., Sez. Ligure e Piemontese Soc. Ital. Ped. Imperia-San Remo, 7–8 December, 1970* (communication).
8 E. GUILLAUX AND S. BEAUGIRAUD, *Bull. Soc. Chim. Fr.*, 5 (1965) 259.
9 D. C. JEFFREY, J. ARDITTI AND R. ERNST, *J. Chromatogr.*, 41 (1969) 475.
10 R. F. POWNING AND H. IRZYKIEWIEZ, *J. Chromatogr.*, 29 (1967) 115.
11 SHEIKH SAIF-UR-RAHMAN, C. R. KRISHNAMURTI AND W. D. KITTS, *J. Chromatogr.*, 38 (1968) 400.
12 YU. S. OVODOV, E. V. EVTUSHENKO, V. E. VASKOVSKY, R. G. OVODOVA AND T. F. SOLOV'EVA *J. Chromatogr.*, 26 (1967) 111.
13 H. MAYER AND O. WESTPHAL, *J. Chromatogr.*, 33 (1968) 514.