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THIN-LAYER CHROMATOGRAPHY OF CARBOHYDRATES

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CONTENTS

1. INTRODUCTION

Not long ago Scherz *et al.*[1], in a clear and concise review, discussed the thin-layer chromatography (TLC) of sugars and related compounds which had been developed before 1967. Our review is intended as a continuation of the work of Scherz *et al.*, and deals with the techniques and the results which have been obtained since 1967. However, in some cases, we have re-examined, more critically and in greater detail, those results and techniques which we feel were dealt with too summarily and, therefore, incompletely in the previous review.

2 GENERAL REMARKS ON THE CHROMATOGRAPHIC MODEL

In the chromatography of sugars on layers of cellulose, it is probable that the separation mechanism is based essentially on the liquid–liquid partition principle, according to a model in which the distribution of each sugar between the mobile phase and the "water–cellulose complex"[2,3] or the "liquid gel"[4,5] is expressed quantitatively by the partition coefficient. The relative ease with which the carbohydrates can enter the structure of the complex between water and the solid support reflects the differences in molecular volume and in steric configuration between the various

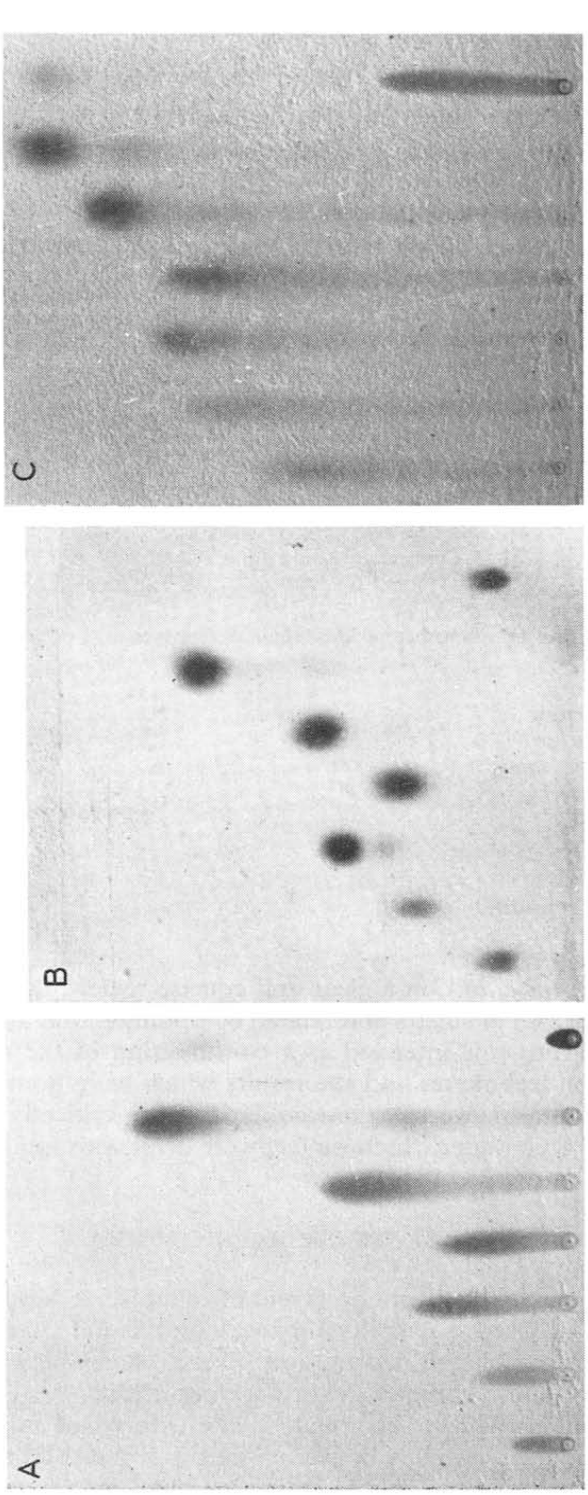


Fig. 1. Chromatography of galactose, glucose, mannose, fructose, ribose, rhamnose and sucrose (from left to right) on silica gel (Merck) impregnated with 0.15 *M* NaH_2PO_4. Solvent systems: A = *n*-butanol–acetone (0.9:1.1); B = *n*-butanol–acetone–water (4:5:1); C = *n*-butanol–methanol–acetone (2:2:1). Solvents B and C are of equivalent polarity. From unpublished results of Lato *et al.*[17].

compounds, and has a fundamental rôle in the separation, together with the differences in solubility of the sugars in a given elution system.

On layers of silica, the partition processes seem to be replaced, to some extent, by adsorption phenomena, so that the chromatography of carbohydrates on this material is clearly influenced by the concentration and type of inorganic salts present in the silica matrix. In this case, the separation is determined not only by the partition coefficient but, also, by the selectivity of the solid matrix.

3. SOLVENTS

The elution systems in the chromatographic separation of sugars are usually organic solvents of binary or ternary composition. Water is an indispensable component, since water-free solvents, or solvents having low water contents, give somewhat diffused spots which compromise the separation (Fig. 1). The content of water varies from 10 to 20% in the solvents for use on cellulose or silica gel, the optimum being 15%. Kieselguhr systems require less than 10% of water.

The binary, water–active solvent, mixtures are efficient eluents, especially for use on layers of silica gel. An active solvent is one which is sufficiently polar to be water-soluble and, at the same time, capable of interacting, through weak solvation bonds, with solutes. Systems such as 2-propanol–water (17:3) and acetone–water (9:1) are among those most frequently used in the analysis of monosaccharides. The ternary systems are made up of water–active solvent–diluting solvent mixtures. The diluting solvent is usually a, or a mixture of two, water-insoluble, or almost insoluble, organic solvent. It influences the flow velocity of the eluting band, whilst the resolving power of the system depends largely on the water–active solvent ratio, which varies from 1:6 to 1:2. For good reproducibility of the results, the ternary system must remain monophasic for the entire chromatographic process. For this purpose, a small quantity of a polar solvent or an organic acid may be added.

The addition of an organic acid to the elution system, other than as a solving agent, has a particular significance in two-dimensional chromatography of carbohydrates, which will be explained later. The best elution systems found in the literature, together with comments, are given in Table 1.

4. SORBENTS

The materials most often used as solid supports in the TLC of carbohydrates are silica, cellulose and Kieselguhr. Some workers have experimented with the use of other adsorbents such as gypsum[6], polyamide[7], aluminium oxide[8] and polycarbonate[9]. The choice of support must be made according to certain criteria: (a) the chromatoplate capacity; (b) the possibility of using at least one of the reagents for the detection of the sugars, and of obtaining a high degree of sensitivity on a clear background; (c) the possibility of resolving a sufficiently high number of carbohydrates.

A. Silica gel

Silica does not give a satisfactory separation of sugars unless it is impregnated with inorganic salts such as bisulphite[10], boric acid[11–13], tetraborate, mono- and dibasic phosphate[14–16] and sodium acetate[13,16], which are capable of interacting with the

TABLE 1

SOLVENT SYSTEMS FOR THE TLC OF CARBOHYDRATES AND THEIR DERIVATIVES

Solvent system	*Comments*	*Reference*
To be used on cellulose		
1 Ethyl acetate–pyridine–water (2:1:2)	Galactose, glucose, mannose, fucose and rhamnose are well separated in 3 h	35
2 Formic acid–ethyl methyl ketone–*tert.*-butanol–water (3:6:8:3)	D-Arabinose shows different R_F value from L-arabinose	36
3 Ethyl acetate–pyridine–acetic acid–water (5:5:1:3)	Differentiation of β-methylglucoside from its α-enantiomer, and of α-methylgalactopyranoside from its β-methylgalactofuranoside	37
4 *n*-Butanol–acetic acid–water (3:1:1)		
5 Ethyl acetate–pyridine–water (20:7:5)	Quantitative determination of mono- and oligo-saccharides	39
6 *n*-Butanol–pyridine–0.1 *N* HCl (5:3:2)	Separation of glucosamine and galactosamine together with some hexoses and pentoses	40
7 *n*-Butanol–ethanol–water (3:2:2)	Analysis of oligosaccharides (DP 2–8)	
8 *n*-Butanol–ethanol–water (1:1:1)	Analysis of oligosaccharides analysis (DP 8–14)	42
9 *n*-Butanol–acetic acid–water (4:1:2)	Analysis of ionic charged oligosaccharides	
To be used on silica gel		
10 Acetone–*n*-butanol–water (5:4:1)	Chromatoplates impregnated with inorganic phosphate salts (Soerensen buffer, pH 8)	14
11 Dioxane–*n*-butanol–water (5:4:1)		
12 Ethyl acetate–acetic acid–methanol–water (6:1.5:1.5:1)	Bisulphite impregnation; fair separation of some mono- and di-saccharides	10
13 *n*-Propanol–water (8.5:1.5)		
14 2-Propanol–ethyl acetate–water (7:1:2)		
15 *n*-Butanol–ethanol–water (2:1:1)	Various inorganic salts are used as impregnants; fair separation of some uronic acids	17
16 *n*-Butanol–pyridine–water (8:4:3)		
17 *n*-Butanol–ethanol–0.1 *M* H_3PO_4 (1:10:5)		
18 *n*-Butanol–ethanol–0.1 *M* HCl (1:10:5)		
19 Ethyl methyl ketone–acetic acid–methanol (3:1:1)		78
20 Acetone–water (9:1)	Designed for the resolution of the most common mono- and di-saccharides; impregnation with boric acid and monobasic phosphate	16, 20, 78
21 2-Propanol–water (4:1)		
22 Acetone–water–chloroform–methanol (8:0.5:1:1)		16, 20, 27
23 Ethyl acetate–2-propanol–acetic acid–water (10:6:3.5:3)		27
24 Ethyl acetate–2-propanol–water (2:1:1)	Suitable for the analysis of oligosaccharides on silica gel impregnated with tetraborate, tungstate or molybdate	19, 20, 30, 31

25 *n*-Butanol–2-propanol–water (3:5:2)	0.2 *M* NaH_2PO_4 as impregnant; glucose, galactose, and mannose are separated from each other and from some disaccharides	16
26 *n*-Butanol–ethyl acetate–2-propanol–water (3 5:10:6:3)	Boric acid impregnation; common aldo- and keto-hexoses are well separated (see Table 5)	20
27 Acetone–*n*-butanol–water (7:1.5:1.5)	Separation of common hexoses, pentoses and deoxy-sugars	27
28 Acetone–*n*-butanol–water (8:1:1)		
29 Acetone–*n*-butanol–acetic acid–water (8:0.5:0.5:1)		
30 *n*-Butanol–acetone–formic acid–water (60:17:8:15)		
31 2-Propanol–acetone–1 *M* lactic acid (2:2:1)	Designed for the separation of oligosaccharides (DP 2–6)	53, 56
32 2-Propanol–acetone–0.1 *M* lactic acid (2:2:1)		
33 *n*-Propanol–water (7:1)	Hexosamine separation on unimpregnated silica gel	49
34 *n*-Propanol–water (7:1.5)		
35 *n*-Propanol–ethyl acetate–water (5:1:4)		
36 Methyl acetate–2-propanol–water (18:1:1)		
37 2-Propanol–ethyl acetate–water (1:1:2)	For the analyses of cello-oligosaccharides	55
38 2-Propanol–ethyl acetate–water (6:1:3)		
39 *n*-Propanol–water (7:3)		54
40 *n*-Propanol–water–ammonia (7:3:0.1)		54
41 *tert.*-Butanol–water (2:1)		54
42 *tert.*-Butanol–water–ammonia (70:35:1)	Prepared for the analysis of chitin-oligosaccharides	54
43 *n*-Butanol–ethanol–water (5:7:4)		54
44 *n*-Butanol–ethanol–water–ammonia (50:70:40:1)		54
45 Isopentyl alcohol–ethanol–water (5:6:3)		54
46 Isopentyl alcohol–ethanol–water–ammonia (50:60:30:1)		
To be used on Kieselguhr		
47 Ethyl acetate–2-propanol–water (4:1:0.5)	Sodium acetate impregnation; quantitative determination of some pentoses of biological interest	34
48 Ethyl acetate–methanol–*n*-butanol–water (16:3:3:2)	Suitable for the analysis of sugars of clinical interest	57
49 Ethyl acetate–methanol–propanol–*n*-butanol–water (8:1:1:1:1)		
50 Ethyl acetate–methanol–*n*-butanol–water (16:3:3:1)		
51 *n*-Butanol–ethyl acetate–2-propanol–water (20:10:7:3)	Fair separations of hexoses, pentoses and deoxy-sugars	27
52 *n*-Butanol–pyridine–water (45:33:22)	Separation of malto-oligosaccharides	58
53 *n*-Butanol–pyridine–water (20:19:11)		

carbohydrates by reasonably well-known mechanisms. In this way, according to the type of impregnating salt and the solvent system used, a wide range of R_F values is obtained on the chromatoplate, and the resolution of the more common sugars becomes possible.

Ovodov *et al.*[17] systematically analyzed the effects of the type of impregnating salt and its concentration on the chromatographic behaviour of certain carbohydrates by using a number of solvents. Their studies showed that a satisfactory separation can be obtained on silica gel impregnated with phosphates. For monosaccharides and uronic acids, the best phosphate concentrations are between 0.2 and 0.3 *M*, and for oligosaccharides between 0.05 and 0.1 *M*. Another important influence of phosphate salts on silica gel is that they cause it to be sufficiently insensitive to the negative interferences of inorganic salt impurities[14,17] and non-saccharide organic compounds such as urea, amino acids, carboxylic acids, etc.[18].

Boric acid and tetraborate, known for their ability to form unstable ionic complexes with carbohydrates, were not among the many impregnants examined by Ovodov *et al.* Earlier, neither Pastuska[11] nor Prey *et al.*[12] obtained satisfactory results with these impregnants, perhaps because of the inadequacy of the acid elution systems used. In fact, as we have pointed out[18,19], the acidity of the solvent system greatly reduces the impregnant effect in the borate chromatoplates. Lato *et al.*[20] carried out extensive studies on the chromatographic possibilities of silica gel impregnated with boric acid. Of the 42 solvent systems examined by these workers, none gave results as good as those obtained with other systems. The resolutions were such that a limited number of carbohydrates could be analyzed by a normal one-dimensional technique. Although, by using larger chromatoplates (20 × 35 cm), it was possible to increase the number of resolvable carbohydrates, the long development time of 12 h proved to be a limiting factor.

Boric acid and tetraborate, when used as impregnants, are the only compounds whose mechanism of action on sugars is well understood. Their capacity to form anionic complexes with polyhydroxy compounds in general, and carbohydrates in particular, has been widely exploited in ion-exchange chromatography[21–26]. A similar mechanism certainly operates in the TLC of sugars in the presence of borate ions. In fact, an examination of Table 2 shows that the chromatographic behaviour of sugars on a layer of non-impregnated silica appears to be compatible with the degree of solubility, and the molecular weight, of the substances examined. When borate ions are present on the silica layer, this behaviour is substantially altered. This suggests that the selectivity of the sorbent (on which the borate ions, insoluble in the mobile phase, develop a primary action in the resolution of the sugars by a complexing–decomplexing mechanism) is a major factor in the chromatographic process[18].

When the borate impregnant is replaced by dihydroxyphenylborane which is soluble in the mobile phase, the selectivity of the solvent assumes a primary rôle in the resolution of the sugars (Table 3). In a two-dimensional technique it is, therefore, important to couple a layer of non-impregnated sorbent (or one impregnated with acetate, phosphates or other salts), in which the separation model is that of partition chromatography, to a borate-impregnated sorbent layer. Following this concept, Mezzetti *et al.*[19] applied the coupled-layer technique of Berger and co-workers[29,30] to the two-dimensional separation of sugars and obtained resolutions of mixtures of monosaccharides, their derivatives and oligosaccharides (Fig. 2).

TABLE 2

R_F VALUES OF SUGARS AND DERIVATIVES, SEPARATED ON (A) UNIMPREGNATED AND (B) 0.05 *M* $Na_2B_4O_7$-IMPREGNATED SILICA GEL (POLYGRAM)

Solvent system: ethyl acetate–2-propanol–water (6:3:1) (ref. 27).

Sugar	R_F	
	A	*B*
α-Methylxyloside	0 68	0.59
2-Deoxyribose	0.65	0.52
Xylose	0.63	0.19
Arabinose	0.54	0.21
Glucose	0.48	0.22
Galactose	0.40	0.15
Levulose	0.51	0.08
Sucrose	0.34	0.20
Melibiose	0.18	0.07
Lactose	0.18	0.08
Melezitose	0.22	0.09
Raffinose	0.13	0.04

TABLE 3

R_F VALUES[28] OF CARBOHYDRATES AND RELATED COMPOUNDS ON WHATMAN NO. 1 PAPER

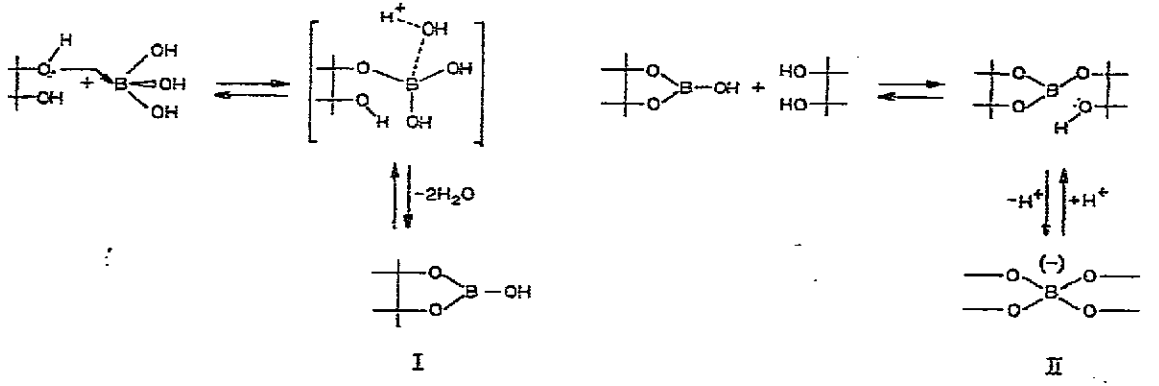
Solvent systems: A = ethyl acetate–acetic acid–water (9:2:2); B = a 0.55% solution of dihydroxyphenylborane in A.

Compound	R_F	
	A	*B*
Erythrose	0.31	0.84
Erythritol	0.23	0 31
Arabinose	0 12	0.11
Arabitol	0.14	0.50
1-Deoxyarabitol	0.45	0.71
5-Deoxyarabitol	0.46	0.85
Ribose	0.25	0.50
Ribitol	0.14	0.48
Xylose	0.15	0.15
Xylitol	0.14	0.45
Galactose	0.06	0.08
Galactitol	0.07	0.47
Glucose	0.08	0.08
Glucitol	0.08	0.45
Levulose	0.11	0.12
Sorbose	0.10	0.16

The selectivity of the sorbent due to the presence of borate ions may be lost when acid, generally organic, is added to the elution system[19]. This suggests that the sugar–borate complexes are less stable in an acidic environment, and that the complex-formation equilibrium has little influence on the chromatographic separation. However, Bourne *et al.*[28] propose that in acid solution the reaction stops at the stage

Fig. 2. Coupled-layer chromatogram of a carbohydrate mixture. Impregnation, P–R (see Table 8): solvents, ethyl acetate–2-propanol–water (2:2:1) and 32. From ref. 19.

of the neutral boric ester (I), whilst in a basic medium the complex-forming reaction proceeds until anionic complexes are formed. The conversion of the neutral ester (I) into the anionic borate (II) is dependent on the presence of a Brønsted base (proton acceptor). In order to explain the chromatographic behaviour shown in Fig. 3, we must assume that the neutral esters (I) have chromatographic characteristics similar to those of free sugars. Thus the chromatography of sugars on a thin layer of borate-impregnated silica gel must exhibit a considerable solvent dependence, since, according to the pH of the elution system, two substantially different mechanisms of separation can be obtained on the same layer, favouring a high resolution capacity.



I II

Taking into account these points, we carried out extensive investigations of the possibilities of two-dimensional chromatographic analysis of sugars on thin layers of silica gel impregnated with borate and obtained excellent separations of carbohydrate

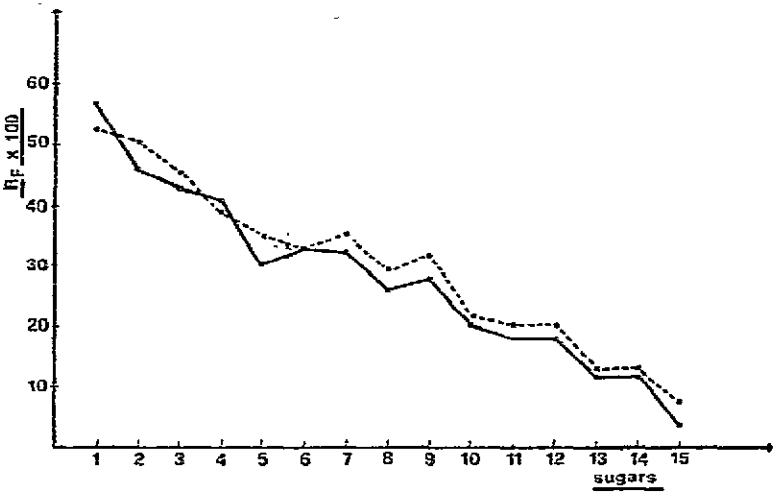


Fig. 3. $R_F \times 100$ values of carbohydrates separated on non-impregnated (— — —) and sodium tetraborate–sodium tungstate-impregnated (———) silica gel plates. Solvent system: ethyl acetate–acetic acid–methanol–water (12:3:3:2). For description of the sugars, see Fig. 9. From ref. 18.

mixtures of clinical interest by simple and relatively brief procedures[18,27]. Mezzetti *et al.*[31] also studied molybdate and tungstate as impregnant salts in the separation of sugars. Bisulphite, which is known for its characteristic addition reactions with aldoses and chetoses, has been used for the analysis of certain monosaccharides and oligosaccharides with good results[10].

B. Cellulose

Cellulose has the same chromatographic characteristics as paper, with the advantage that elution times are shorter and the sensitivity to detection reagents is enhanced. Unlike silica gel, this support gives good resolution of carbohydrates, even without the use of impregnants, and, in addition, is superior for the analysis of oligosaccharides having high degrees of polymerization (DP).

C. Kieselguhr

Compared with silica and cellulose, the diatomaceous earth Kieselguhr has a lower water take-up and a lower surface activity. The elution systems used on this support must therefore contain less water. However, the chromatographic patterns obtained for sugars on Kieselguhr plates that have been impregnated with a specific inorganic salt are not very different from the patterns obtained on silica layers impregnated with the same salt. Furthermore, perhaps owing to its lower activity, Kieselguhr has a limited capacity, 5 μg being the maximum quantity of the sample that can be chromatographed[32]. Another of the limitations of Kieselguhr is the difficulty in revealing the sugar spots with the more common reagents. Despite the statements made by Stahl and Kaltenbach[33], anisaldehyde–sulphuric acid reagent does not show satisfactory sensitivity to the sugars on Kieselguhr.

Recently Bell and Talukder[34] reported a new reagent which is specific for the carbohydrates on Kieselguhr. We have observed that the naphthoresorcinol–sulphuric

acid reagent is sufficiently sensitive, even on Kieselguhr, when the acid and the aromatic alcohol are sprayed separately on to the plate (first a 4% solution of H_2SO_4 in alcohol, then a 0.2% solution of naphthoresorcinol in alcohol). We have also observed that 2-deoxy-sugars give dark grey spots on a Kieselguhr plate when the plate is heated to 120° for 15–20 min. However, other sugars, even if held at this temperature, do not develop any colour. These sugars are sufficiently sensitized to be readily detectable with ethanol–sulphuric acid (19:1) (ref. 27). Kieselguhr may contain organic bases or other types of impurities that may interact with certain carbohydrates at high temperatures to give chromogens.

5. ONE-DIMENSIONAL SEPARATION OF MONOSACCHARIDES, THEIR DERIVATIVES AND OLIGOSACCHARIDES

In one-dimensional TLC, the area available for the separation of sugars is approximately that between the sample application points and 70% of the height of the chromatoplate. The distribution of these substances on this area is such that only some of them can be effectively resolved.

A. Separation on cellulose

For adequate separation of carbohydrates, cellulose systems almost always require multiple elutions. In this way, the more common hexoses and pentoses can be readily separated together with certain di- and trisaccharides (Table 4). Thus the solvent system 1 of Schweiger[35] on MN 300 cellulose is useful for the analysis of the group of monosaccharides formed by galactose, glucose, mannose, fucose and rhamnose. This system needs two successive developments each of 90 min duration. Vomhof and Tucker[36] investigated solvents suitable for cellulose, but of the nine elution systems they reported only one (2) was of interest. It is worth noting that, with this solvent, D-arabinose may be distinguished from its L-isomer, the R_G values being 1.45 and 1.51. Wolfrom *et al.*[37] extended the analysis of the more common hexoses and pentoses to the methylglycosides and their amino derivatives on the microcrystalline cellulose Avirine using solvents 3 and 4. These workers also reported the separation of some α-methylglycosides from their β-enantiomers. Petre *et al.*[38] used solvent 2 on Chromedia CC-41 (W. & R. Balston, Maidstone, Great Britain) for the analysis of carbohydrates and uronic acids in plant extracts. According to these workers, 12 saccharides and three uronic acids can be separated with their system (Table 4). More recently, Raadsveld and Klomp[39] suggested solvent 5 for the complete resolution, in three successive developments, of glucose, galactose, mannose, arabinose, xylose and rhamnose, together with sucrose, maltose and lactose.

In the study of the structures of glycoproteins, glycopeptides and polysaccharides it is often important to use a chromatographic system which is capable of resolving the amino-sugars and the acetylamino-sugars, together with the most common monoses, in the hydrolyzates of the substances being studied. Although, in this respect, few results have been obtained with TLC, one can, nevertheless, mention the system butanol–pyridine–0.1 *N* hydrochloric acid (5:3:2) by which glucosamine and galactosamine, together with galactose, mannose, fucose and rhamnose, can be resolved[40]. Although the method of Günther and Schweiger[41] has given good results in the resolution of glucosamine, galactosamine and their N-acetyl derivatives, it

TABLE 4

R_G AND R_F VALUES OF SUGARS AND THEIR DERIVATIVES ON CELLULOSE CHROMATOPLATES

For solvent systems, see Table 1. $R_G = R_F$ value relative to glucose.

Carbohydrate	*MN 300*			*Chromedia CC-41*	*Microcrystalline (Avirine) (R_F values)*	
	1	*2*	*3*	*2*	*4*	*5*
L-Arabinose		1 51				
D-Arabinose	1.11	1.45	1.31	1.30	0.46	0.31
Ribose	1.42	1.91		1.49	0.59	0.39
Xylose	1.25	1.60	1.55	1.34	0.52	0.33
Lyxose		1.70				
Galactose	0.90	0.91	0.83	0.93	0.36	0.31
Glucose	1.00	1.00	1.00	1.00	0.39	0.25
Mannose	1.09	1.23	1.15	1.17	0.44	0.30
Levulose		1.30	1.15	1.21		0.29
Sorbose		1.23				
6-Deoxygalactose	1.31			1.44		
6-Deoxymannose	1.52		2.10	1.58	0.60	0.46
α-Methyl-D-galactopyranoside					0.54	
β-Methyl-D-galactofuranoside					0.72	
β-Methyl-D-galactopyranoside					0.56	
α-Methyl-D-glucoside					0.57	
β-Methyl-D-glucoside					0.61	
β-Methyl-D-arabinoside					0.65	
α-Methyl-D-xyloside					0.70	
α-Methyl-D-lyxoside					0.75	
Sucrose		0.65	0.66	0.64		
Maltose		0.38	0.53	0.42	0.29	0.15
Cellobiose		0.32	0.30		0.25	0.13
Lactose		0.26				

loses its practical importance because these workers did not compare the separations with those of the monosaccharides which are often present together with the aminic derivatives in the analytical samples.

The one-dimensional analysis of oligosaccharides having DP 4–12 can be more effectively carried out on cellulose chromatoplates. In their study of the enzymatic hydrolysis of the complex polysaccharides of Agar, Duckworth and Yaphe[42] described the one-dimensional separation of the neoagarobioses (DP 2–12) on thin layers (Table 6). These workers used microcrystalline 0.25-mm cellulose layers (Camag, Mutenz, Switzerland) and solvent systems 7 and 8. After being separated from the neutral compounds on a column of DEAE-Sephadex A-25 (Cl^-), the ionic oligosaccharides of the Agar hydrolyzates were analyzed on cellulose using solvent system 9 in a single development.

The chromatographic system suggested by Damonte *et al.*[43] requires three successive elutions, and does not offer very good separation possibilities.

B. Separation on silica gel

The complete resolution of glucose, galactose, mannose, levulose, sorbose and

TABLE 5

$R_F \cdot 100$ VALUES OF SUGARS AND THEIR DERIVATIVES ON SILICA GEL AND KIESELGUHR LAYERS

SG(?) = Silica gel (trade mark not specified) impregnated with a 2 *M* NaH_2PO_4 solution; SG = silica gel (Merck) impregnated with 0.1 *M* sodium bisulphite; SG* = silica gel KSK impregnated with 0.3 *M* NaH_2PO_4; SG** = silica gel (Fluka DO) impregnated with 0.03 *M* boric acid; PSG = precoated silica gel (Merck, art. 5715) unimpregnated when solvent 31 is used (with solvent 32 the layer is impregnated with 0.5 *M* NaH_2PO_4, allowing the plates to stand overnight in a bath of aqueous alcohol); SG-H = non-impregnated silica gel (Merck); KGA = Kieselguhr (Merck) + 0.02 *M* sodium acetate; KGP = Kieselguhr (E. Merck) impregnated with 0.15 *M* NaH_2PO_4. The R_F values in parentheses, corresponding to the KGP/48 system, were obtained by a double development with the same solvent. For solvent systems, see Table 1.

Carbohydrate	*SG(?)*		*SG*			*SG**		*SG***				*SG-H*				*PSG*		*KGA*	*KGP*	
	10	*11*	*12*	*13*	*14*	*17*	*18*	*20*	*21*	*25*	*26*	*27*	*28*	*29*	*30*	*31*	*32*	*47*	*48*	*50*
Arabinose	28	47	32	51	63			52	55	39	33					67	47	32	25(42)	61
Ribose	37	58	50	57	69			30	35	33	25					72	60	66	39(62)	83
Xylose	40	58	34	59	68			46	48	42	39					76	63	45	38(60)	77
Lyxose			46	59	68			56	50	43	37						65			
Xylulose																	76			
Galactose	16	30	32	39	53			40	52	35	26	54	55	72	30	58	30	11	10(18)	28
Glucose	22	41	28	48	61			54	61	38	33	62	61	75	42	65	41	14	14(25)	42
Mannose	28	49	41	53	60			52	57	42	36					69	50			
Levulose	26	46	28	43	57			22	33	31	16					64	46	25	20(34)	53
Sorbose			43	47	56			16	24	25	8					69	52			
Tagatose			46	53	61												60			
Mannoheptulose								24	29	33	16									
2-Deoxyribose																86	83			
2-Deoxyglucose			68	73	79															
6-Deoxyglucose																	80			
Fucose			49	55	62			66	58	42	42					75	65	47	55(77)	91
Rhamnose	55	72	57	62	68			70	62	45	57					85	83	78	80(97)	98
α-Methylglucoside								74	67	43	53									
α-Methylmannoside								75	62	46	59									

Galacturonic acid						8	16										
Glucuronic acid						27	26										
Mannuronic acid						23	33										
Glucurone						50	55										
Mannurone						36	46										
Galactosamine												13	19	22	7		
Glucosamine												21	26	31	10		
N-Acetylglucosamine												54	59	74	33		
N-Acetylgalactosamine												45	51	66	26		
N-Acetyl-Neuraminic acid												3	5	2	1		
Sucrose	14	36	20	40	55	42	53	59	70	37	27					66	47
Turanose								38	51	35	24						
Maltose	12	32	11	35	50	35	47	53	63	37	21					57	34
Isomaltose																	17
Trehalose			5	23	38	31	45	41	58	34	20					57	31
Gentiobiose																	20
Cellobiose						39	49									56	
Lactose	6	22	8	23	36			34	52	27	12					47	23
Lactulose			10	27	40												
Melibiose																36	14
Panose																35	14
Melezitose			4	30	49											56	36
Raffinose	4	12	4	13	28	29	40	25	47	26	8					42	17
Maltotriose																46	26
Isomaltotriose																25	6

tagatose, which are the more common constituents of clinical analysis samples such as urine and plasma, and of extracts from other biological materials, is of great practical interest. The three aldohexoses can be resolved with relative simplicity (Table 5). Complications arise when the aldoses have to be examined together with the hexoketoses, since the hexoketoses generally tend to give spots which overlap those of glucose and mannose. The separation of these two groups of isomers can be achieved on boric acid-impregnated silica gel plates, although a clear-cut separation among the aldoses and ketoses is not always achieved (Table 5). With the elution systems 20, 25 and 26, the quartet galactose, mannose, fructose and sorbose can be separated on 0.03 *M* H_3BO_3-impregnated silica gel layers (Fluka, Buchs, Switzerland). Glucose can be resolved together with the other carbohydrates when solvent 21 is used[27]. Using the acetone–water (9:1) solvent system and its variants (Table 8), we were able to separate galactose, glucose, fructose, sorbose and tagatose on silica gel layers (Merck, Darmstadt, G.F.R.) (Fig. 4). Although there was overlapping of mannose and fructose, we also obtained good resolution with solvent system 23 (ref. 27).

As far the pentoses are concerned, it has been observed that arabinose and ribose can usually be resolved easily also in the presence of the aldohexoses (Table 5). Arabinose, ribose and xylose are clearly resolved, together with glucose and galactose, by the chromatographic system of Ragazzi and Veronese[14], *i.e.*, silica, 2 *M* NaH_2PO_4 and solvent 10 or 11. Adachi[10] reported good separations of these three compounds, as well as of other monosaccharides and oligosaccharides, by use of silica gel impregnated with 0.1 *M* sodium bisulphite and solvent 12. Lombard[44] described a method which necessitates multiple successive elutions for the complete separation of the four aldopentoses, together with fructose, rhamnose and glucose, in 1 *N* H_2SO_4 solution. This worker used silica gel G (Merck) impregnated with a Soerensen phosphate buffer (pH 8), and methyl ethyl ketone–acetic acid–saturated solution of boric acid (9:1:1) as the solvent system. Complete separation of arabinose, ribose, xylulose and ribulose can be obtained on silica gel impregnated with 0.35 *M* NaH_2PO_4 by use of solvents 27, 28 and 29. By use of the same systems, the deoxysugars fucose, rhamnose, 2-deoxyglucose and 2-deoxyribose can also be resolved on phosphate-impregnated silica gel (Fig. 4).

The analysis of the more common uronic acids can be carried out with solvent systems 17 and 18 on silica gel KSK impregnated with monobasic sodium phosphate (0.3 *M*) (ref. 17).

Until now, there have been few results on the chromatographic analysis of amino-sugars on thin layers of silica gel, owing to the practical necessity of a clear-cut resolution of the more common amino derivatives, together with the hexoses and non-amino derivatives, including glucose, mannose, galactose, fucose, rhamnose, glucuronic acid and mannuronic acid, which are the most frequently occurring constituents in the hydrolyzates of glycoproteins and mucoid substances. The elution systems that, generally, give good separations of the last substances, do not cause the amino-sugars to migrate satisfactorily on silica gel impregnated with phosphates or borates. Some workers have used silica gel supported on glass fibre and impregnated with monobasic phosphate[45] or copper sulphate[46] for the separation of this class of compounds. However, their ammonium-containing solvent systems were unsuitable for the simultaneous study of the neutral monosaccharides and the aminic derivatives, because the silica gel catalyzes the amination reaction of the monosaccharides in the

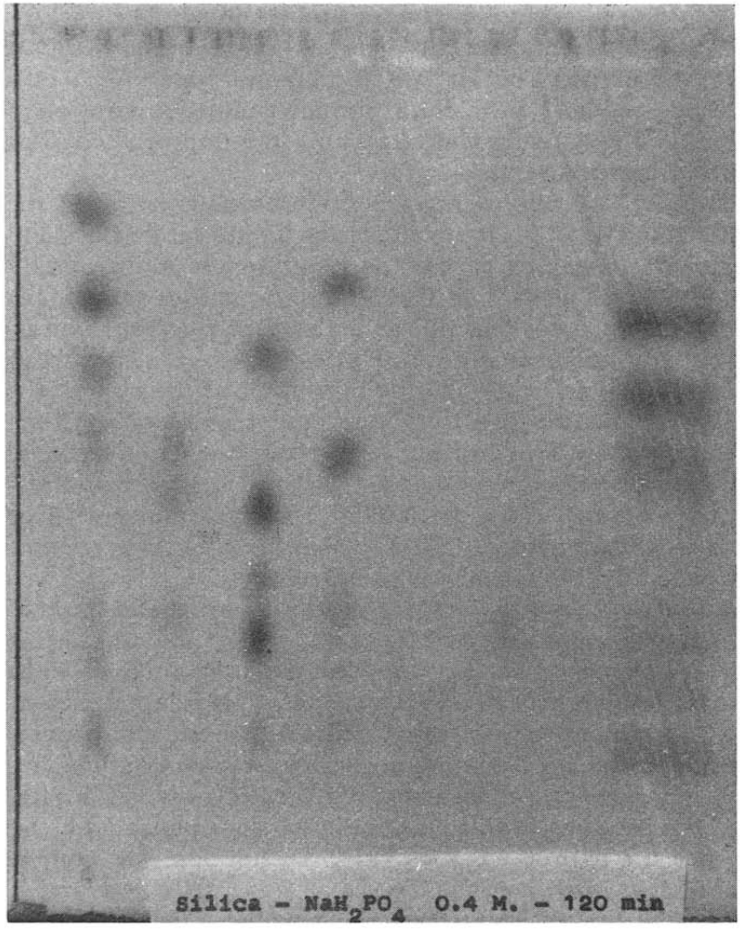


Fig. 4. Chromatogram of monosaccharide mixtures on NaH_2PO_4-impregnated silica gel 60 (Merck). Solvent system, 28; development time, 2 h; spray reagent, NPR. Samples (from left to right and from the lower part of the chromatogram): galactose, glucose, mannose, fucose, 2-deoxygalactose, 2-deoxyglucose, rhamnose, digitoxose; arabinose, ribose, fucose; galactose, glucose, fructose, sorbose, tagatose, 2-deoxygalactose; galactose, mannose, ribulose, xylulose; galactose; mannose. From unpublished results of Lato *et al.*[27].

presence of the ammonium ions. Thus the neutral monosaccharides migrate on the chromatoplate in part unchanged and in part as their amino derivatives, thereby giving double spots[46–48].

Non-impregnated silica gel layers, therefore, seem to be more suitable for the separation of amino-sugars, even if the number of monoses that can be examined by this method is somewhat limited. The study of Gal[49] on the separation and identification of the amino-sugars galactosamine and glucosamine and their N-acetyl derivatives, together with glucose, galactose and the N-acetyl neuraminic acid, on a non-impregnated silica gel layer is noteworthy in this context.

As far as the chromatographic examination of polyalcohols is concerned, the one-dimensional method of Weinstein and Segal[50] gave good resolution of sorbitol,

galactitol and mannitol, in addition to galactose, glucose and mannose. The analysis was performed on a silica gel layer impregnated with a borate buffer (0.02 *M* $Na_2B_4O_7$ + 0.02 *M* H_3BO_3); the elution solvent was 2-propanol–water (4:1) in a triple development. The method used by Němec *et al.*[51], which enabled separation of a large number of aldonic acids and polyhydroxy alcohols on a non-impregnated silica gel, seems to be more rapid and more efficient.

The use of impregnants in TLC represents a considerable limitation in the analysis of oligosaccharides having DP < 3. However, for the lighter saccharides, impregnated sorbents give high selectivity and permit the resolution of numerous di- and tri-saccharides. Thus, the system silica–0.1 *M* $NaHSO_3$–solvent 13 is suitable for the analysis of saccharides, including lactose, lactulose, maltose, saccharose and raffinose. Solvent 14 gives an equally good separation of these oligosaccharides when used on bisulphite-impregnated silica gel, although the resolution of the monoses is somewhat poorer[10]. Jeffrey *et al.*[52] described a technique that, besides offering the possibility of analysis of certain monosaccharides, gave a rapid one-dimensional resolution of cellobiose, saccharose, maltose, gentiobiose, lactose, melibiose and raffinose. The chromatographic system was silica gel–0.02 *M* boric acid–solvent 19 or 20. The non-aqueous solvent 19 caused spot diffusion, which these workers tried to avoid by a double development of the plate in the same solvent.

Mezzetti *et al.*[31] conducted a systematic study of solvents, sorbents and impregnants suitable for the separation of common di- and tri-saccharides. The best systems permitted the resolution of 9–12 oligosaccharides. However, the long time (8–20 h) needed for the entire chromatographic process seriously limited its adaptability to rapid routine analysis. The oligosaccharides isomaltose, maltose, panose, maltotriose, isomaltotriose and saccharose, as well as some monosaccharides, can be examined by the Hansen method[53] (Fig. 5). The separation is carried out on ready-made silica gel plates (Merck, art. No. 5715) impregnated with monobasic sodium phosphate; the plates are immersed for 15–20 h in a bath of a 0.5 *M* NaH_2PO_4 solution. The elution system is 2-propanol–acetone–0.1 *M* lactic acid (2:2:1).

The separation and identification of heavy fragments produced by the hydrolysis of polysaccharides cannot be carried out with the systems so far described, owing to the low migration rate of these saccharides on silica layers impregnated with inorganic salts. Non-impregnated silica gel layers and solvents with a relatively high water content are the most suitable systems for the chromatographic examinations of these compounds[54–56]. Powning and Irzykiewicz[54] used silica gel G plates (Merck) and solvents containing 25–30% water (Table 6) in their chromatographic studies on the hydrolyzates of chitin. These workers found a linear relation between the R_M values of the saccharides examined and their DP values, thus showing that the separation process for these compounds is based mainly on partition. On the other hand, with the system used[56] for the identification of the oligosaccharides produced by starch hydrolysis, there does not seem to be a similar relation; the isomers maltotriose, isomaltotriose and 6-α-glucosylmaltotriose, as well as maltotetraose and isomaltotetraose, gave different R_F values (Table 6).

C. Separation on Kieselguhr

The limited number of reports on the chromatography of sugars on thin layers of diatomaceous earth can, perhaps, be attributed to the poor capacity of this sorbent.

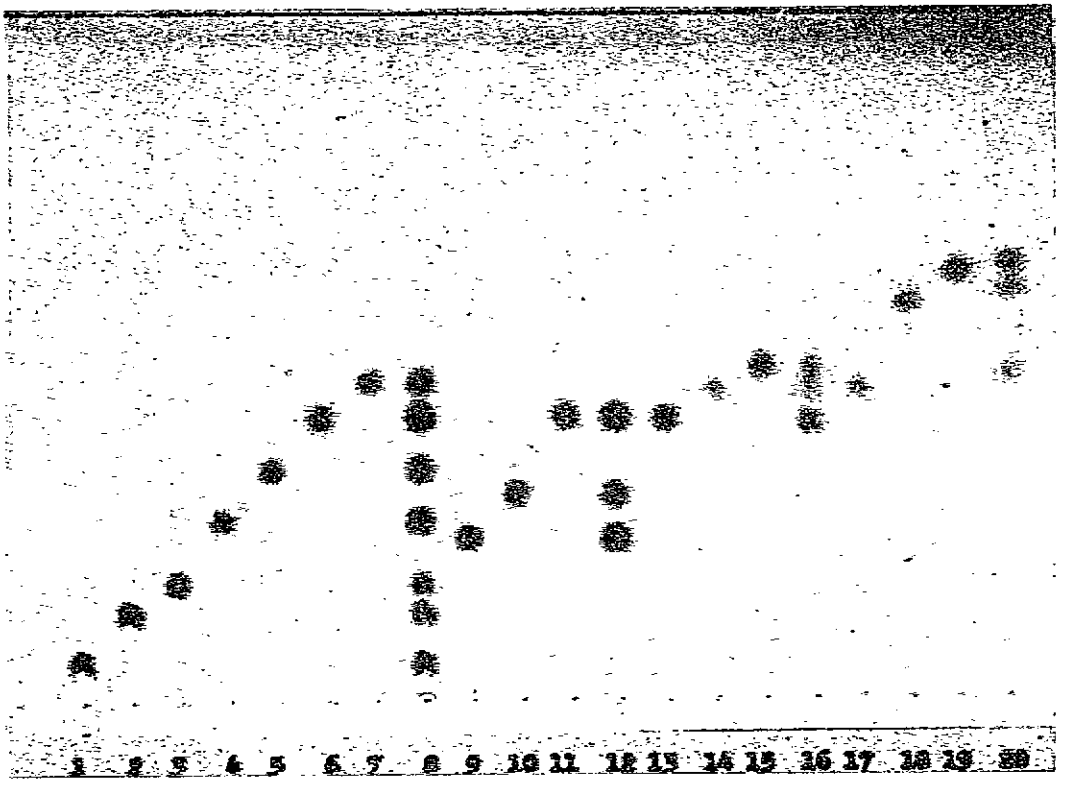


Fig. 5. Chromatogram of mono-, di- and trisaccharides on 20 × 20 cm pre-coated TLC silica gel 60 plates (Merck, Art. No. 5715) impregnated with NaH_2PO_4. Solvent system, acetone–isopropanol–0.1 *M* lactic acid (40:40:20); spraying reagent, aniline–diphenylamine–acetone–80% H_3PO_4 (4 ml:4 g: 200 ml:30 ml). Samples, 1 μl of (1) isomaltotriose, (2) panose, (3) isomaltose, (4) maltotriose, (5) maltose (6) glucose, (7) sucrose, (8) 1–7, (9) lactose, (10) galactose, (11) glucose, (12) 9–11, (13) glucose, (14) fructose, (15) mannose, (16) 13–15, (17) arabinose, (18) ribose, (19) xylose, (20) 17–19. From ref. 53.

However, Kieselguhr offers a separation of monoses which is comparable to that obtained on silica gel (Table 7).

Kieselguhr G (Merck) impregnated with 0.15 *M* monobasic sodium phosphate permits the resolution of the monoses galactose, glucose, fructose, arabinose, xylose, fucose and rhamnose with solvents 34, 35 and 37. Double development increases the degree of separation without altering the quality of the spots. Xylose and ribose can be resolved by use of solvent system 37 in a double development on a 20 × 35-cm plate[57]. Furthermore, the elution system 38 applied on Kieselguhr chromatoplates (Merck) has given excellent separations of hexoses, pentoses and deoxy-sugars in our laboratory. These results could not, however, be reproduced with impure samples, for example urine.

Shannon and Creech[58] showed that Kieselguhr is very suitable for the chromatographic separation of oligosaccharides having high DP values. The fact that these workers stated that they were unable to reproduce the results previously obtained by Weil and Hanke[59] and Huber *et al.*[60] on the same support suggests that although Kieselguhr gives interesting results it is not suitable for standard methods.

TABLE 6

R_G AND $R_F \cdot 100$ VALUES OF SOME OLIGOSACCHARIDES (DP 2–16) SEPARATED ON CELLULOSE, SILICA GEL AND KIESELGUHR

NAG- = N-Acetylglucosamine. For supports see Table 5. For solvent systems, see Table I. $R_{Gal} = R_F$ value relative to galactose.

Saccharide	*Microcrystalline cellulose*			*PSG*	*SG-H*										*KG*	
	7	*8*	*9*	*31*	*37*	*38*	*39*	*40*	*41*	*42*	*43*	*44*	*45*	*46*	*52*	*53*
Cellobiose				56	33	36										
Neoagarobiose	1.11		1.10													
NAG-biose							63	45	58	39	57	41	42	27		
Maltobiose				57											92	89
Cellotriose					23	30										
NAG-triose							42	24	48	29	48	32	30	16		
Maltotriose				46											86	84
Cellotetraose					15	23										
Neoagarotetraose	0.87		0.80													
NAG-tetraose							33	17	39	21	40	23	22	10		
Maltotetraose				34											80	81
Isomaltotetraose				17												
Cellopentaose					8	18										
NAG-pentaose							25	12	31	15	32	17	14	6		
Maltopentaose				27											74	77
Cellohexaose					5	13										
Neoagarohexaose	0.65		0.51				19	8	23	11	25	11	9	3		
NAG-hexaose																
Maltohexaose				23											69	73
Maltoheptaose															64	68
Maltooctaose															58	62
Neoagarooctaose	0.41	0.57	0.32													
Maltononaose															50	55
Maltodecaose															44	49
Neoagarodecaose		0.42	0.18													
Malto – 11 DP															35	42
Malto – 12 DP															29	36
Neoagaro – 12 DP		0.30														
Malto + 13 DP															22	29
Malto – 14 DP		0.18													18	24
Neoagaro – 14 DP																
Malto – 15 DP															15	20
Malto – 16 DP															12	16

TABLE 7

$R_F \cdot 100$ VALUES OF SUGARS AND THEIR DERIVATIVES ON SILICA GEL AND KIESELGUHR LAYERS[27]

SG.B = Silica gel (Merck) impregnated with 0.1 *M* boric acid; SG.P = silica gel (Merck) impregnated with 0.2 *M* NaH_2PO_4; SG.P* = silica gel (Merck) + 0.3 *M* NaH_2PO_4; SG.P** = silica gel (Merck) + 0.35 *M* NaH_2PO_4; KG.P = Kieselguhr (Merck) impregnated with 0.3 *M* NaH_2PO_4. For solvent systems, see Table 1.

Carbohydrate	*SG.B*	*SG.P*			*SG.P**		*SG.P***	*KG.P*	
	26	*23*	*26*	*28*	*28*	*29*	*27*	*48*	*51*
Galactose	20	15	8	22	12	11	12	10	6
Glucose	24	21	12	33	20	21	21	18	12
Mannose	26	27	18	43	28	27	30	26	23
Levulose	12	25	17	41	26	26	27	28	23
Sorbose	6	30	18	47	32	33	32	31	29
Tagatose	12	25	34	55	43	43	40	38	41
Mannoheptulose		21	13	36	27	25	24		
Sedoheptulose		22	13	40	30	31	25		
Arabinose	25	27	17	42	30	30	28	29	26
Ribose	18	38	32	59	47	46	43	46	44
Xylose	26	39	32	61	49		45	44	46
Lyxose	28	39	35		52	50	48	46	50
Ribulose	3	38	38	62	54	58	48		53
Xylulose	2	45	48	70	65	66	60		61
2-Deoxyribose	46	55	58	75	73	72	66	88	87
2-Deoxygalactose	36	40	41	65	60	58	55	63	68
2-Deoxyglucose	45	50	52	72	69	67	62	78	80
Rhamnose	40	55	58	75	73	72	66	87	87
Fucose	28	34	30	58	51	50	47	60	63
Digitoxose	58	73	75	83	81	82	77		

6. TWO-DIMENSIONAL SEPARATION

Two-dimensional chromatography usually implies amphoteric behaviour of the substances under examination, in the sense that such substances should exhibit distinct characteristics in the two successive developments, and produce a wide distribution of spots over the chromatographic plate. Such a requirement is, obviously, related to a difference between the solute–solvent and/or solute–support interactions in the two successive chromatographic processes, and this can be easily obtained for substances which are naturally amphoteric, such as the amino acids and the indole derivatives that migrate on the chromatoplate both as positively charged molecules and negative ions, according to the pH of the elution system. In this case the separation depends on the p*K* values.

For non-amphoteric substances, such as carbohydrates, a similar differentiation in the two developments is feasible on the borate-impregnated silica gel layers previously discussed. Juxtaposition of two layers of differently impregnated silica gel produces a good separation of monosaccharides and their derivatives and oligosaccharides, because the migration of the sugars takes place in each of the two layers according to different chromatographic mechanisms. The results obtained with this technique are summarized in Table 8. Despite the fact that these separations are inter-

TABLE 8

CHROMATOGRAPHIC DATA FOR SUGARS SEPARATED ON COUPLED LAYERS OF SILICA GEL G (FLUKA DO), IN A TWO-DIMENSIONAL TECHNIQUE

Impregnants: P = 0.132 *M* sodium tetraborate–0.204 *M* boric acid–0.06 *M* sodium tungstate (1:1:1); Q = 0.036 *M* boric acid; R = 0.24 *M* sodium acetate; S = 0.24 *M* monobasic sodium phosphate; T = saturated solution of molybdic acid. Solvents: A = *n*-butanol–ethyl acetate–2-propanol–acetic acid–water (7:20:12:7:6); B = ethyl acetate–2-propanol–water (10:6:3); C = 2-propanol–*n*-propanol–water (14:14:5); D = methyl acetate–2-propanol–water (2:2:1). The remaining solvents are defined in Table 1.

Impregnant		*Solvent system*		*No. of sugars separated*	*Comments*
Narrow layer	*Wide layer*	*First run*	*Second run*		
P	Q	D	A	26	Generally, chromatography on P–Q coupled layers results in glucose–mannose and maltose–trehalose–cellobiose overlapping; nevertheless fair separation of many hexoses, pentoses and disaccharides is achieved. Solvent 12 and A are almost equivalent with respect to their resolution capacity, but the former elutes more rapidly (1.5 h) than the latter (3–4 h)
		D	12	34	
		24	A	28	
		24	12	31	
		24	12	31	
		C	A	28	
		C	12	28	
P	R	24	A	32	Maltose–trehalose overlap, and so do arabinose–glucose and galactose–sedoheptulose
		24	12	31	
		C	A	24	
		C	12	26	
		D	A	32	
		D	12	25	
R	P	22	D	28	The solvents of the first run have the advantage of moving at very fast rates (60–90 min); some oligosaccharides give double spots
		20	D	34	
T	Q	13	12	22	Sharply defined spots
		B	A	20	
T	R	B	12	20	Slightly diffuse spots
		B	22	22	
		13	20	22	
T	S	13	20	19	Slightly diffuse spots
		B	20	21	
		B	22	22	
R	T	22	B	21	Solvent systems 22–13 and 20–13 give sharply defined spots
		22	13	22	
		20	B	23	
		20	13	21	

esting (Fig. 2), the length of time required for the double development (7–8 h) is a limiting factor.

A new method reported by Lato and his co-workers[18,27] overcomes this limitation while retaining the same type of separation (Fig. 6). It is based on the different properties of the borate-impregnated silica gel layer according to the pH of the eluent systems (Fig. 7). The results obtained by Günther and Schweiger[41] in the two-dimensional separation of certain amino-sugars are, in our opinion, based on the same principle, since between the first and the second chromatographic runs the cellulose chromatoplate is sprayed with a borate buffer at pH 8.

7. VISUALIZATION OF THE SUGAR SPOTS

There is a large number of reagents for the detection of carbohydrates on thin layers and these have been well documented in various papers and reviews[10,14,31,43,44,61,62]. Here we report only those reagents which, because of their sensitivity, specificity and capacity for polychromatic staining, are useful not only for revealing the sugars but also for their identification.

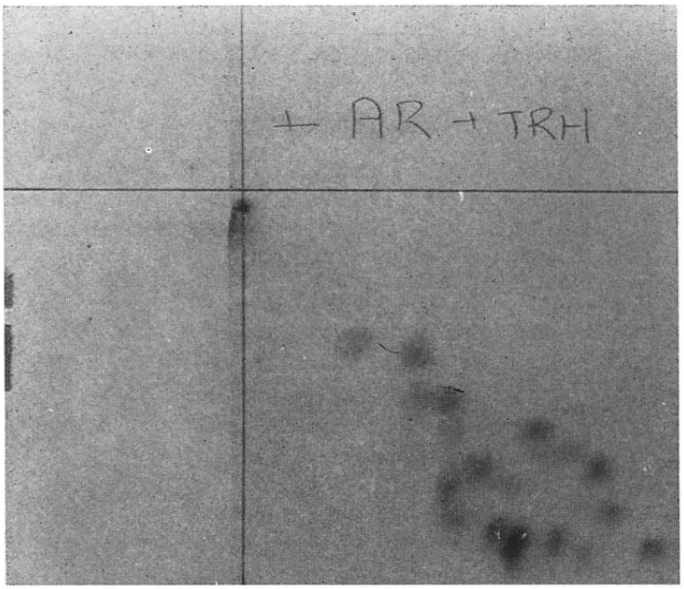


Fig. 6. Two-dimensional chromatogram of mono- and oligosaccharides on tetraborate–phosphate (0.01 *M* H_3PO_4 + 0.028 *M* $Na_2B_4O_7$)-impregnated mono-layer of silica gel 60 (Merck). Solvent systems, 24–30. From unpublished results of Lato *et al.*[37].

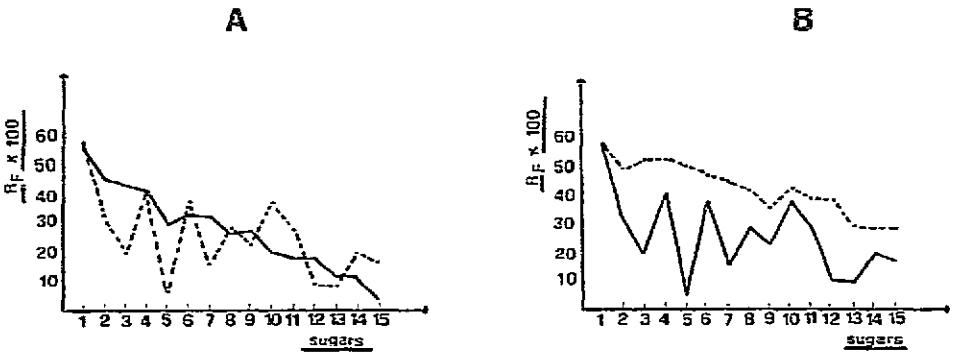


Fig. 7. A, $R_F \times 100$ values of sugars separated on a sodium tetraborate–sodium tungstate-impregnated silica gel layer by the neutral solvent 24 (— — —) and by the acidic solvent 12 (———). B, $R_F \times 100$ values of the same sugars separated on a sodium tetraborate–sodium tungstate-impregnated silica gel layer (———) and on a non-impregnated silica gel layer (— — —) by solvent 24. For description of the sugars, see Fig. 9. From ref. 18.

TABLE 9

DETECTION OF SUGARS AND THEIR DERIVATIVES ON CHROMATOGRAPHIC THIN LAYERS

For detection reagents, see Table 10; col. = colours developed; sens. = sensitivity limits in μg. Bl = blue; V = violet; PuR = purple red; Lg = light; Gr = green; Pk = pink; Or = orange; Y = yellow; Br = brown; Ol = olive; I = intense; Bk = brick; Gy = grey; F = faint. (———), not reported in the original reference. The reaction colours in ref. 27 were obtained on silica gel (Merck); solvent systems which did not contain acetic acid or pyridine were used as elution systems.

Sugar	*NPR*[27]		*DPA*[43]	*DPA*[27]	*APT*[43]	*APT–NPR*[27]		*TBA*[27]	
	col.	*sens.*	*col.*	*col.*	*col.*	*col.*	*sens.*	*col.*	*sens.*
Galactose	Bl	1.5	Gy	BlGy	Y	YBr	0.5	LgBl	2.0
Glucose	V	4.0	GyBl	BlGy	Y	YBr	0.5		
Mannose	Bl	1.5	BlGy	BlGy	Y	YBr	0.5		
Fructose	PuR	0.5	YOr	LgR		PuR	0.1	Y	0.5
Sorbose	PuR	0.1	—	Y	—	PuR	0.1	Y	0.5
Tagatose	R	0.5	—	LgR	—	PuR	0.1	Y	0.5
Mannoheptulose	PuR	0.5	—	PuR	—			Y	0.1
Sedoheptulose	PuR	4.0	—	PuR	—			Y	2.0
Arabinose	LgBl	1.5	Ol	FGyBl	Pk-BkR	BrY	0.5		
Ribose	LgBl	1.5	Ol	FGyBl	Pk-BkR	BrY	0.5		
Xylose	Br	1.5	Ol	IGyBl	Pk-BkR	BrY	0.5		
Lyxose	LgBl	1.5	Ol	IGyBl	Pk-BkR	BrY	0.5		
Ribulose	Gr	0.5	—	Y	—	Or	0.1	Y	4.0
Xylulose	Or	0.2	—	Y	—	Or	3.0	Y	4.0
2-Deoxyribose	PeaGr	0.5	Pk	Br-IV*	Y	BlGr	1.0		
2-Deoxygalactose	LgBl	0.5	—	V-IBr*	—	Gr	2.0		
2-Deoxyglucose	LgBl	0.5	—	V-IBr*	—	Gr	2.0		
6-Deoxymannose	Pk	0.5	YGr	Ol-IGy*	Y	BrY	1.0		
6-Deoxygalactose	Pk	0.5	—	Ol-IBl*	—	BrY	1.0		
Galacturonic acid			—		—				
Glucurone			—		—				
α-Methylglucoside	Bl	0.5	—		—	R	2.0		
α-Methylmannoside	Pk	0.5	—		—	Bl	1.5		
α-Methylarabinoside	Bl	0.5	—		—	V	0.5		
α-Methylxyloside	Bl	0.5	—		—	Bl	0.5		
Sucrose	PuR	0.5	BrY	Or-V*		R	0.5	Y	
Maltose	Bl	4.0	Bl	Bl		BrY	1.0		
Lactose	Bl	4.0	GyBl	Bl	Y	BrY	1.0		
Melibiose	Bl	4.0	GyBl	Bl	Y	BrY	1.0		
Turanose	PuR	0.2	YBr	R		R	0.5		
Raffinose	PuR	0.5	YGr	V		R	0.5		

* Colours immediately after detection and some hours after detection.

The furfural-yielding reagents, such as naphthoresorcinol–sulphuric acid[20,63], α-naphthol–phosphoric acid[62], orcinol–hydrochloric acid[62], aniline–diphenylamine–phosphoric acid[62], *p*-anisidine–phthalate[35,44], aniline–phthalate and aniline–phosphate[64], under specific conditions, permit the recognition, through colour differences, of spots belonging to various classes of carbohydrates. Furthermore, under certain conditions, anisidine–phthalate and aniline–phthalate reagents are specific for aldoses, since ketoses and oligosaccharides containing ketoses do not show marked reactivity at low concentrations (10 μg). The limit of sensitivity of these reagents to aldoses is 0.5 μg.

APT[27]		*TBA–NPR*[27]		*URE*[27]		*URE–NPR*[27]		*AMP*[27]		*ANP*[27]	
col.	*sens.*	*col.*	*sens.*	*col.*	*sens.*	*col.*	*sens.*	*col.*	*sens.*	*col.*	*sens*
Y	0.5	LgBl	2.0			Bl	0.5				
Y	0.5	LgBl	2.0			Bl	0.5				
Y	0.5	LgBl	2.0			Bl	0.5				
Y	4.0	BrY	0.5	Bl	0.5	PuR	0.5	Y	1.0		
Y	4.0	BrY	0.5	Bl	0.5	PuR	0.5	Y	1.0		
Y	4.0	BrY	0.5	Bl	0.5	PuR	0.5	Y	1.0		
				BrY	1.0			Gr	1.5	V	1.0
								Gr	1.5	V	1.0
BkR	0.5	LgBl	1.0							Pu	0.5
BkR	0.5	LgBl	1.0							Pu	0.5
BkR	0.5	LgBl	1.0							Pu	0.5
BkR	0.5	LgBl	1.0							Pu	0.5
Y	3.0	V	0.1	BrY	0.5			Y	0.5	Bl	0.5
Y	3.0	V	0.5	BrY	0.5			Y	2.0	Bl	2.0
Y	3.0	BrY	2.0					Bl	3.0	Or	2.0
Y	3.0	BrGr	2.0					Bl	0.5	Or	2.0
Y	3.0	BlGr	2.0					Bl	0.5	Or	2.0
Y	1.0	V	2.0							Or	0.5
Y	1.0	V	2.0							Or	0.5
				Bl	0.5			BlV	1.0		
				Bl	0.5			BlV	1.0		
				Bl	0.5						
Y	2.0										
Y	2.0										
Y	2.0										
				Bl	1.0						
				Bl	1.0						

Dimedone[65], thiobarbituric acid[66] and urea[67] form another category of detection agents which are of considerable interest owing to their selectivity for ketoses, which are easily detected even at levels of 0.5 μg, whilst the lower limits of sensitivity for the aldoses is considerably higher.

The data necessary for the identification of the spots after their visualization on the chromatoplate are shown in Table 9. It should be borne in mind that if the temperature and duration of exposure of the plate to the heat source are not exactly reproduced, both the colour, essential for the identification of the sugars, and the selectivity of the reagents may be absent. For example, when the chromatoplate is

sprayed with the naphthoresorcinol–sulphuric acid reagent and heated for more than 15 min the carbohydrates, invariably, give red spots. Colour differentiation is influenced by the amount of acid, as well as by the type of acid. The concentration of phosphoric acid in the thiobarbituric acid reagent[66] is critical for the selectivity of ketoses and deoxy-sugars. High levels of phosphoric acid also cause the aldoses to give coloured spots. The naphthoresorcinol reagent becomes inefficient for the differentiation of ketoses and aldoses when the acid content is greater than 4–5%. When the same reagent is acidified with trichloroacetic acid instead of sulphuric acid it becomes specific for the visualization of uronic acids and ketoses[32].

Another factor which may influence the specific coloration is the eluent composition. Trace amounts of some components of the elution systems, such as acetic acid, pyridine and butanol, have a tendency to persist on the layer, even after the plate has undergone heating. These trace solvents may cause the limit of sensitivity of the sugars to be raised, or may alter the colours produced after the reaction. For this reason, the anisidine–phthalate reagent, which according to Schweiger[35] should give a green coloration with hexoses, caused the aldohexoses to give similar yellow spots to the deoxy-sugars in the experiments of Damonte *et al.*[43]. We tested this reagent on a silica G layer (Merck) impregnated with monobasic phosphate, after elution with solvent system 17, and obtained the same colorations as reported by Damonte *et al.* However, the sensitivity to ketoses and ketose-oligosaccharides was equal to or somewhat higher (2.5 μg) than that of the aldoses.

Other reagents, according to our experience, are less sensitive and do not detect the special category of carbohydrates for which they were proposed. According to Stahl and Kaltenbach[32], the anisaldehyde reagent should reveal sugars at a concentration of 0.5 μg on silica or Kieselguhr layers; however, under the conditions of Bell and Talukder[34], the same chromatographic system did not give good results. We tested this reagent and obtained poor results[27]. According to Brockman *et al.*[68], the *o*-aminophenol reagent should visualize the amino-sugars. Under our conditions, this reagent was useful for the differentiation of the deoxy-sugars (Table 9), but its sensitivity to hexoses, oligosaccharides and amino-sugars was too low. Amino-sugars are, on the other hand, not only sensitive to ninhydrin and the Morgan–Elson reagent, but also to anisidine–phthalate.

8. IDENTIFICATION OF THE SPOTS

The R_F values given by the different chromatographic methods assume importance in the identification of the spots only when they are evaluated together with other criteria such as the selectivity of the detection reagent, the differences in colorations and the use of internal standards. The R_F values are not constant parameters, but vary from one experiment to another with the variations in temperature and humidity of each laboratory, according to the mark or the batch number of the silica gel used and with many other factors such as irregularities in the thin layer, tiny differences in the thickness of the layer, etc. Thus R_F values have only an indicative value, and each group of workers must adapt the chromatographic methods described to his own conditions in order to obtain good results.

In the identification of sugars by means of chemical reactions on the thin layers, one of the problems that can be most easily solved is the differentiation of the

TABLE 10

DETECTION REAGENTS FOR SUGARS AND THEIR DERIVATIVES ON THIN LAYERS OF SILICA AND CELLULOSE

Reagents		*Treatment*	*Reference*
NPR	(a) 0.2% Naphthoresorcinol in ethanol (b) Conc. H_2SO_4 Mix (a) and (b) before use (1:0.04)	100°, 5 min For revealing sugars on Kieselguhr (E. Merck) first spray with (a) and heat the plate to 120° for 10–15 min, then spray with (b) and heat to 100° for 5 min	20, 63 27
DPA	(a) 2% Diphenylamine in acetone (b) 2% Aniline in acetone (c) 85% H_3PO_4 Mix (a), (b) and (c) before use (5:5::1)	100°, 10 min	43, 62
APT	0.1 *M* solution of *p*-anisidine and phthalic acid in 96% ethanol	100°, 10 min	35
APT–NPR		First spray with APT, heat to 100° for 10 min, record the developed colours and then spray with NPR and heat to 100° for 5 min	27
TBA	(a) 0.5% Thiobarbituric acid in ethanol (b) 85% H_3PO_4 Mix (a) and (b) (1:0.02)	100°, 10 min	66
TBA–NPR		First spray with TBA, revealing the ketoses, then spray with NPR	27
URE	5 g of urea in 20 ml of 2 *M* H_2SO_4 Mix with 100 ml of ethanol. (The original urea reagent contained HCl instead of H_2SO_4, refs. 32 and 67)	100°, 20–30 min	27
URE–NPR		First spray with URE, heat and then spray with NPR	27
AMP	1% *o*-Aminophenol in methanol + 10 ml of 85% H_3PO_4 + 5 ml of water. This reagent was originally devised for hexosamines[68]. Under our conditions, it was useful for detecting pentoses and deoxy-sugars	100°, 10 min	27
ANP	10 vol. 0.5% α-Naphthol in 50% ethanol + 1 vol. H_3PO_4	90°, 10–15 min	62

ketose series from the aldose series. This is made possible by the availability of reagents which are specific for only one of these two series of carbohydrates, and of reagents which reveal the sugars of both series by means of two distinctly different colours (Tables 9 and 10). Aminoguanidine sulphate has been proposed[69] as a new reagent for the selective differentiation of fucose. We have tested[27] urea–sulphuric acid (Table 10), a variant of the reagent specific for ketoses and uronic acids[32], and have found it to be equally useful for the differentiation of 2-deoxy-glucose and 2-deoxy-galactose from fucose, rhamnose and 2-deoxy-ribose, as well as from pentoses and hexoses. We have also found[27] that the *o*-aminophenol reagent[68] is suitable for the selective detection of 2-deoxy-glucose and 2-deoxy-galactose; rhamnose and fucose do not react. The series of 2-deoxy-sugars is also easily detected by use of α-naphthol–sulphuric acid which gives colours which are clearly distinct from those of

the other sugars; naphthoresorcinol–sulphuric acid is useful for the detection of ketoses from both their aldo-isomers and other sugars.

An interesting procedure of successive coloration is the association of reagents specific for the aldoses with those for ketoses, and the multiple-coloration technique of Damonte *et al.*[43] offers very good possibilities for the identification of carbohydrates.

The identification of the spots in two-dimensional chromatoplates is facilitated by the use of internal standards made up of substances which are, if possible, not saccharides. Such standards, as they occupy known positions on the chromatoplate, can be used as fixed points of reference. Straight lines traced through such points will divide the chromatogram into restricted areas and the groups of sugars can be easily characterized. In a recent paper[18] we suggested a method for identifying urinary sugars on two-dimensional chromatograms by use of urea and sucrose, occurring naturally in the analysis samples, as internal standards (Fig. 8). Urea can be used as internal standard because: (a) although it is not a saccharide, it reacts with the naphthoresorcinol–sulphuric acid reagent and yields a very distinct colour; (b) it does not cause negative interference with the chromatographic migration of the sugars; (c) it does not cause overlapping with the sugars examined and so mask their

Fig. 8. Two-dimensional chromatogram of untreated urine from a subject affected by an intestinal absorption disorder. Sorbent: sodium tetraborate–sodium tungstate (0.024 *M* $Na_2B_4O_7$ + 0.0125 *M* Na_2WO_4)-impregnated mono-layer of silica gel G 60 (Merck)–syloid 63 (W. R. Grace) (2:1, w/w). Solvent systems 24–12. From unpublished results of Lato *et al.*[27].

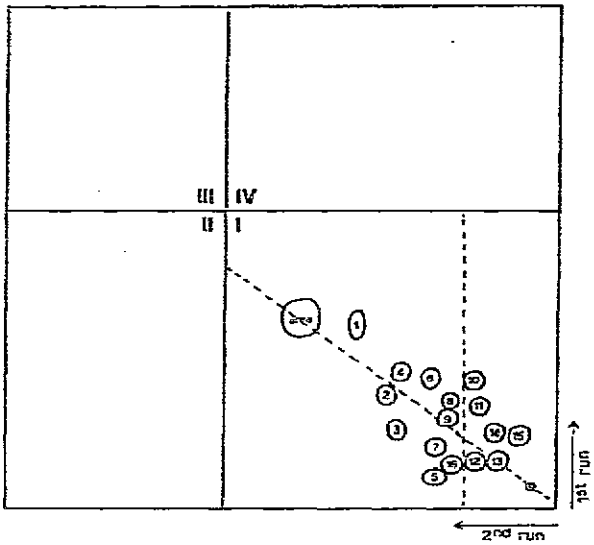


Fig. 9. Two-dimensional chromatogram of urea and sugars of clinical interest on sodium tetraborate–sodiumtungstate-impregnated silica gel mono-layer. Solvent systems 24–12. Spots: 1 = 2-deoxyribose; 2 = xylose; 3 = ribose; 4 = fucose; 5 = xylulose; 6 = glucose; 7 = fructose; 8 = galactose; 9 = sedoheptulose; 10 = sucrose; 11 = maltose; 12 = palatinose; 13 = lactulose; 14 = lactose; 15 = raffinose; 16 = allulose. From ref. 18

visualization. Sucrose has, equally, a chromatographic migration that makes it possible to trace two ideal straight lines at a tangent to the sugar spot, thereby outlining a zone (Fig. 9) in which only oligosaccharides are found.

9. QUANTITATIVE DETERMINATION

Two different procedures can be used with reasonable accuracy to determine well separated sugars on thin layers: (a) analyses *in situ*, in which the quantitative evaluation is carried out directly on the chromatoplate after the spots have been visualized with the appropriate reagent; (b) spectrophotometric analyses after elution of the spots from the solid chromatographic medium.

Of the quantitation methods *in situ*, those based on visual evaluation of the spots for comparison with external standards are unsatisfactory because they result in subjective and approximate responses. Densitometric methods are sufficiently exact and sensitive, although they present some technical difficulties, owing to the irregularities of the spots and the poor reproducibility of the spray detection technique which invariably leads to small differences in the intensities of the spots not associated with the amount of the sugar being tested. For these reasons, the application of these methods is limited to the determination of the spots in one-dimensional chromatograms[70–73].

The spectrophotometric determination of the eluates is more reliable, since the operations required for this technique, even if relatively long and laborious, involve less parameters and error. Furthermore, the quantitative determination of mannose and fructose, the chromatographic separation of which is difficult (Tables 4 and 5),

can be easily performed, even when these compounds are both present on the chromatoplate[39]. The total amount of both of these sugars can be determined with the help of a general reagent (*e.g.*, tetrazole blue) and then fructose alone can be quantitated by the resorcin method[74]. The applications of this method to quantitative analyses are more numerous and they can be used for a large number of carbohydrates. Scott[62] has made a comprehensive illustrated summary of these techniques.

For removing samples from the chromatoplates, the area containing the substances under examination is localized by use of pure standards which are co-chromatographed on to guide strips on the same layers. The detection of these standards is then carried out by heating only the guide strip with a suitable electric resistance slide after the reaction solution has been deposited on the strip by use of a 0.1-ml syringe[75]. Another method of revealing the external standards, without contaminating the substances to be examined, is that of masking the portion of the chromatoplate containing the sample with a glass plate and warming it with a hair-dryer until the spots become visible[76]. After having sprayed only the guide strips, McKelvy and Scocca[77] heated the plate to 100° for a few seconds; the short time of exposure to the heat presumably prevented thermodegradation of the samples. However, the use of a silver salt, *e.g.*, Ag_2CO_3, avoids heating of the plate and, therefore, possible degradation of the carbohydrates.

The use of pure external standards for locating the substances under examination does not always guarantee an acceptable degree of precision, especially when one is dealing with impure biological samples. Inorganic salts such as chlorides may cause interference with the chromatographic process by altering the R_F values of the sample sugars with respect to the pure standards. It should be noted that the use of these procedures is limited to one-dimensional chromatography. On the other hand, Jeffrey *et al.*[52] and Bell and Talukdar[34] visualized the sugars under examination directly on the chromatoplate, and eluted the coloured products for spectrophotometric determination; this has the advantage of greater precision in locating the spots. The reaction product must, however, be stable in order to obtain satisfactory results.

In both methods, the possibility of a quantitative elution of the sample from the chromatographic gel is of major importance, in the sense that a *ca.* 100% recovery aids the sensibility of the methods. Scott's studies[75] on the elution of carbohydrates from silica gel showed that 95% of the sugars glucose, mannose, galactose, arabinose, xylose and sucrose are recovered. McKelvy and Scocca[77] obtained a 100% recovery of fucose, xylose and mannose from a layer of cellulose treated with tetrahydroborate. The degree of sugar recovery is not specified in the methods based on elution of the coloured products.

10. SUMMARY

Thin-layer chromatography, with its inherent simplicity of operation and sufficiently high degree of sensitivity and resolution, can, as a preliminary technique in the analysis of sugars and related compounds, replace other more sophisticated procedures such as gas–liquid and ion-exchange chromatography. Furthermore, as far as the identification of the sugar compounds is concerned, TLC is superior because it permits the use of rapid differential reactions on the layer and the utilization of other criteria such as R_F values and colour variations.

This technique has been widely used in studies of the carbohydrates and their derivatives in the biological and clinical fields. A wide range of monosaccharides, oligosaccharides and derivatives of monosaccharides can be separated on cellulose, silica gel and Kieselguhr layers. Many solvent systems, adaptible to each of these sorbents, have been reported. Two-dimensional separation of sugars and related compounds can be easily and rapidly performed. The samples are applied directly to the chromatographic layer without any pre-treatment. Quantitative determination of the sugars can be thus carried out with sufficient accuracy on crude analytical samples.

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