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Note

High-performance thin-layer chromatographic separation of sugars: preparation and application of aminopropyl bonded-phase silica plates impregnated with monosodium phosphate

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Thin-layer chromatographic (TLC) analysis of sugars is conducted using cellulose, and more commonly, silica gel as the stationary phase; the extensive literature in this area has been reviewed[1]. Until recently, satisfactory separations could not generally be achieved on silica gel unless it has been impregnated with boric acid[2], disodium hydrogen phosphate[3], or monosodium dihydrogen phosphate[3,4]. Some excellent resolutions of free sugars have been achieved, especially by using silica impregnated with monosodium dihydrogen phosphate and including lactic acid in the mobile phase[4]. More recently, unimpregnated silica gel plates have been applied to sugar separations, by using boric and phenylboronic acids[5] in the mobile phase. This concept has been extended to high-performance thin-layer chromatography (HPTLC) on silica, and an effective quantitative method has been developed[6]. A disadvantage common to all procedures which employ an acidic component in the mobile phase is the lengthy time periods required to irrigate the plates.

Aminopropyl-bonded silica has been widely used in high-performance liquid chromatography (HPLC) for the separation of sugars[7–9]. Preparations of this phase from pre-coated silica TLC plates have been reported[10,11], and some sugars were effectively resolved. High-performance TLC plates with aminopropyl-bonded silica stationary phase have recently become commercially available (E. Merck, Darmstadt, F.R.G.) and were shown to separate closely related compounds in classes other than carbohydrates[12].

The separation of sugars by HPLC on aminopropyl-bonded silica is most often conducted using acetonitrile–water mobile phases; normal-phase HPLC is the mode of separation. Due to the basicity of the column packing, the pH of the mobile phase in these HPLC systems is close to 9.5, favorable for covalent interactions between reducing sugars and the aminopropyl functionality on the silica. It has been established[13] that not only the lifetime of aminopropyl-bonded silica HPLC columns, but also their capacity to quantify certain sugars is compromised by such interactions. Those sugars especially prone to interacting covalently with the aminopropyl groups[9] are those containing appreciable levels of acyclic (aldehydic) form[14] in tautomeric equilibrium with their ring (furanose and pyranose) forms. HPLC conditions were developed[13] to inhibit reactions by adjusting the pH of the mobile phase to 5.9 with phosphate buffer.

In our earlier report[11] on the preparation and application to sugar separation of aminopropyl-bonded silica, we described encountering difficulty with certain sugars. Sugars containing more than 0.05% aldehydic form remained at the origin of the plate as a result of glycosylamine formation after spotting the sample. The problems associated with HPLC of certain sugars (especially pentoses) are magnified in TLC, because of the higher concentration of the solutes and aminopropyl phase. In this report we describe the preparation of aminopropyl-bonded silica HPTLC plates impregnated with monosodium dihydrogen phosphate and the chromatographic mobilities of sugars on these plates.

EXPERIMENTAL

Reagents

Lactulose, maltulose, and cellobiulose were provided by Dr. K. B. Hicks, and laminaribiose by Dr. E. T. Reese. Kojibiose was obtained from Koch-Light* (Colnbrook, U.K.), and a hydrolyzed corn starch (maltodextrin) sample (M-250) from Grain Processing Corporation (Muscatine, IA, U.S.A.). Other sugars were purchased from Sigma (St. Louis, MO, U.S.A.) and Aldrich (Milwaukee, WI, U.S.A.). Pre-coated silica gel (4.5-μm particle size) HPTLC plates (10 × 10 cm), type HP-KF, were purchased from Whatman (Clifton, NJ, U.S.A.), and 3-aminopropyltriethoxysilane (3-APTS) from Aldrich.

Plate derivatization and characterization

Aminopropyl-bonded silica HPTLC plates were prepared by totally immersing silica gel plates for 15 min in a 1.0% solution of 3-APTS in hexane. This procedure was carried out in a TLC developing tank (27.5 × 8.0 × 12.6 cm) and the details of rinsing and drying the plates were described previously[11]. As many as 48 plates, four at a time, were derivatized in a single solution of 3-APTS in hexane. The aminopropyl-bonded silica plates were then impregnated with monosodium dihydrogen phosphate by immersing in a 0.2 *M* aqueous solution of this salt for 15 min. After draining the plates on paper towels in a fume hood for 30 min, they were dried for 2 h in a 70°C vacuum oven. Plates were stored in a plate storage cabinet over calcium chloride desiccant. Samples were scraped from the plates and submitted to Microanalysis Inc. (Wilmington, DE, U.S.A.) for ash, carbon, hydrogen, nitrogen and phosphorus determination. The pH values of aqueous slurries (12%, w/v) of derivatized silica were determined after stirring 15 min.

Development of plates and visualization of sugars

Mobile phases consisting of acetonitrile–water mixtures were used. Increasing the polarity by changing from acetonitrile–water (90:10) to acetonitrile–water (60:40) greatly increases R_F values. The more polar mobile phases are required for the higher oligosaccharides, *i.e.*, those sugars containing numerous hydroxyl groups.

The spray reagent was prepared by mixing 4.0 g diphenylamine hydrochloride, 4.0 ml aniline, 120 ml *tert.*-butyl alcohol, 80 ml ethanol, and after cooling in an ice

* Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

bath, stirring in 30 ml of sulfuric acid over a period of 15 min. This reagent could be stored indefinitely at −6°C, and spots were visualized by heating the plates at 120°C for 5 min after spraying. Distinctions among classes of sugars can be made on the basis of spot color; colors are the same as traditionally observed[15] when using aniline–diphenylamine spray reagents, even though sulfuric acid was substituted for phosphoric acid.

RESULTS AND DISCUSSION

Microanalysis of aminopropyl-bonded silica impregnated with monosodium dihydrogen phosphate gave the following results (%): ash, 84.90; C, 4.35; H, 1.94; N, 1.07; and P, 0.91. From these data, the molar ratios of C:N and N:P are 4.74 and 2.60, respectively. The greater-than-theoretical (3:1) ratio of C:N is possibly due to some ethoxy substituents in 3-APTS being retained after its reaction with silica. From the ash determination, the level of bonded phases is calculated to be about 15%, somewhat higher than we obtained earlier[11] using the same derivatization conditions with silica of larger particle size.

An aqueous slurry (12% w/v) of underivatized silica gave a pH value of 7.11. This value rose to 9.89 after reaction with 3-APTS, a value favoring covalent interactions with reducing sugars and glycosylamine formation. Impregnation with 0.2 *M* monosodium dihydrogen phosphate resulted in neutralization of aminopropyl groups on the bonded-phase plates, and the pH dropped 3.76 units, to 6.13. A pH of 7.67 resulted when the plates were impregnated with 0.1 *M* monosodium dihydrogen phosphate. In our previous report[11] we discussed the reactivity of sugars containing more than 0.05% acylic (aldehydic) form with the aminopropyl functionality on bonded-phase plates. Reactive sugars included the tetroses, pentoses, deoxy sugars, and some hexoses (mannose and galactose) and glucose derivatives. These sugars formed glycosylamines at the plate origin as they were spotted, and were rendered immobile. By lowering the pH of the aminopropyl-bonded silica to 6.13 by monosodium dihydrogen phosphate impregnation, we have now successfully inhibited glycosylamine formation. These results are analogous to those of Porsch[13] who, by lowering the mobile phase pH in HPLC on aminopropyl-bonded silica, inhibited on-column glycosylamine formation by pentoses.

In Table I are listed R_F values for monosaccharides (including aldoses and ketoses), sugar derivatives, and di- and higher saccharides. The mechanism of separation is presumably the partitioning of solute between the aqueous environment of the amine-functionality on the stationary phase and the less polar acetonitrile–water mobile phase. For sugars that display mobility whether or not the plates are impregnated with monosodium dihydrogen phosphate, such as non-reducing sugars, R_F values are 10–20% less after impregnation. The procedure described here was applicable to the conventional TLC plates modified in our previous report[11]; *i.e.*, even sugars with a high proportion of acyclic form in solution were mobile.

Fig. 1 includes chromatograms of various individual sugars and mixtures of sugars on aminopropyl-bonded silica (panel A), aminopropyl-bonded silica impregnated with 0.1 *M* monosodium dihydrogen phosphate (panel B), or 0.2 *M* monosodium dihydrogen phosphate (panels C and D). While in our previous report[11] (no monosodium dihydrogen phosphate impregnation) sugars with more than 0.05%

TABLE I

HPTLC OF SUGARS AND SUGAR DERIVATIVES ON AMINOPROPYL SILICA BONDED-PHASE PLATES IMPREGNATED WITH MONOSODIUM PHOSPHATE*

Sugars and derivatives were all of the D-form, unless designated L

Sample	R_F
*Pentoses**	
L-Arabinose	0.30
L-Lyxose	0.35
2-Deoxyribose	0.50
Ribose	0.39
Ribulose	0.40
Xylose	0.35
*Hexoses**	
2-Amino-2-deoxyglucose	0.04
2-Deoxyglucose	0.42
Fructose	0.27
L-Fucose	0.37
Galactose	0.21
Glucose	0.22
Mannose	0.25
Psicose	0.33
L-Rhamnose	0.44
L-Sorbose	0.29
Tagatose	0.30
Talose	0.31
*Heptose**	
Sedoheptulose	0.32
Sugar derivatives	
2-Acetamido-2-deoxyglucose*	0.38
1,6-Anhydro-glucose**	0.49
4,6-O-Benzylidene-glucopyranose**	0.56
4,6-O-Ethylidene-glucopyranose**	0.46
Glucose diethyl dithioacetal**	0.31
1,2-O-Isopropylidene-α-glucofuranose**	0.49
1,2:5,6-Di-O-isopropylidene-α-glucofuranose**	0.77
Methyl-β-L-arabinopyranoside*	0.55
Methyl-β-glucopyranoside*	0.44
3-O-Methylglucose**	0.55
Methyl α-mannopyranoside*	0.50
*Disaccharides**	
Cellobiose	0.15
Cellobiulose	0.17
Gentiobiose	0.12
Isomaltose	0.12
Kojibiose	0.14
Lactose	0.14
Lactulose	0.16
Laminaribiose	0.18
Maltose	0.14
Maltulose	0.17
Melibiose	0.11

TABLE I (*continued*)

Sample	R_F
Palatinose	0.17
Sucrose	0.18
Trehalose	0.14
Turanose	0.18
*Trisaccharides**	
Maltotriose	0.10
Melezitose	0.12
Raffinose	0.09
*Tetrasaccharide**	
Stachyose	0.04
*Malto-oligosaccharides****	
Glucose	0.47
Maltose	0.41
Maltotriose	0.34
Maltotetraose	0.29
Maltopentaose	0.24
Maltohexaose	0.20
Maltoheptaose	0.16

* Mobile phase: acetonitrile–water (70:30 v/v).
** Mobile phase: acetonitrile–water (90:10 v/v).
*** Mobile phase: acetonitrile–water (60:40 v/v).

acyclic form in solution reacted at the origin and showed no mobility, here these sugars react at the origin and as they proceed up the plate (panel A). In lanes a–e were spotted 2-deoxyglucose, xylose, rhamnose, galactose, and mannose, sugars all having appreciable levels of acyclic form. Other sugars which behave similarly are the tetroses and other pentoses and deoxy sugars. Some tailing is indicated in lanes g (glucose) and h (fructose), even though these sugars possess very low levels of acyclic form. The sugars spotted in lanes i, j, and k are non-reducing and do not covalently interact with the bonded phase, even at the elevated pH.

Impregnation with 0.1 *M* monosodium dihydrogen phosphate resulted in sufficient lowering of pH (to 7.67) so that spot tailing was greatly minimized (panel B). Doubling the concentration of monosodium dihydrogen phosphate in the impregnating solution eliminated the tailing, as shown in panel C. A mobile phase consisting of acetonitrile–water (70:30) was used in the chromatograms represented in panels A, B and C, while 60:40 was used for that in panel D. The latter panel shows that maltodextrins of D.P. (degree of polymerization) 1 through 7 can be resolved. Maltotriose (lane f) appears to contain maltopentaose (D.P. 5) and maltoheptaose (D.P. 7) as trace contaminants.

An additional advantage of impregnating the aminopropyl-bonded silica HPTLC plates with monosodium dihydrogen phosphate is that the sugars are visualized more readily after spraying, as the background is much cleaner. This can be seen in Fig. 1 by comparing panel A with panels B–D. We have found the traditional spray reagents for visualizing sugars to be rather ineffective on the monosodium dihydrogen phosphate impregnated aminopropyl-bonded silica plates. By substitut-

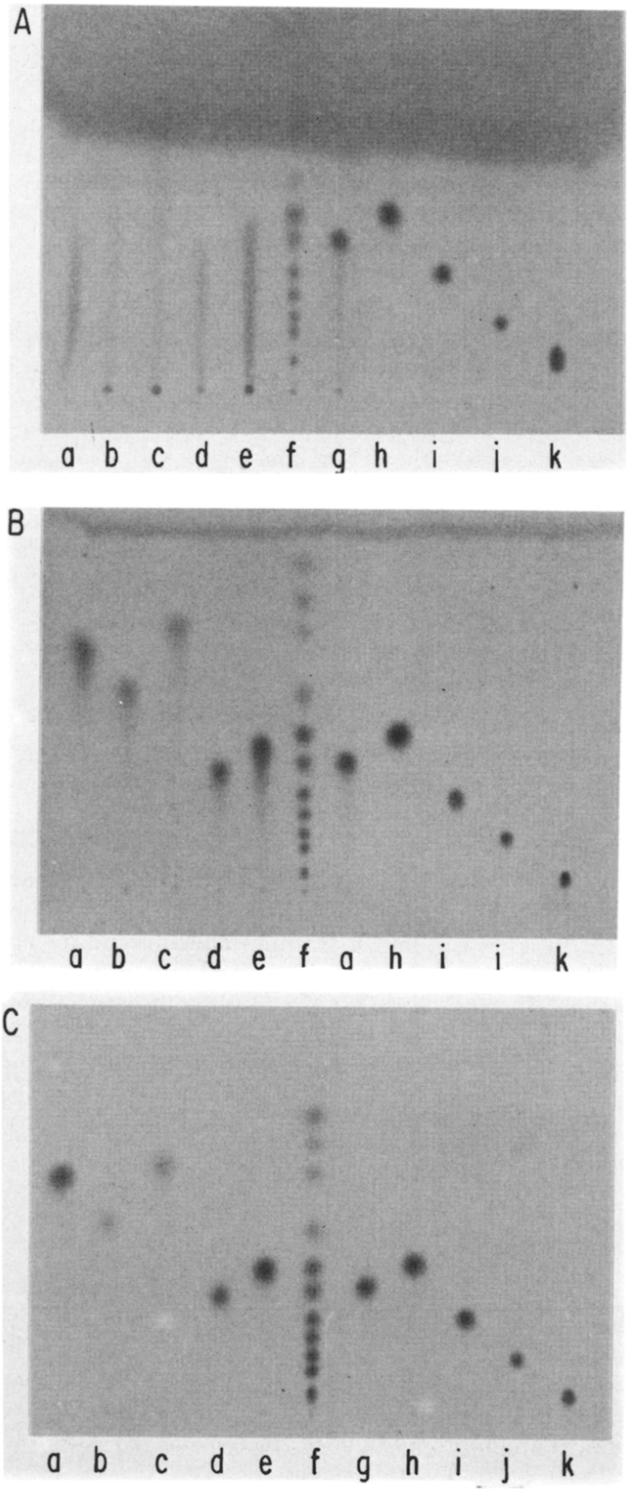

Fig. 1.

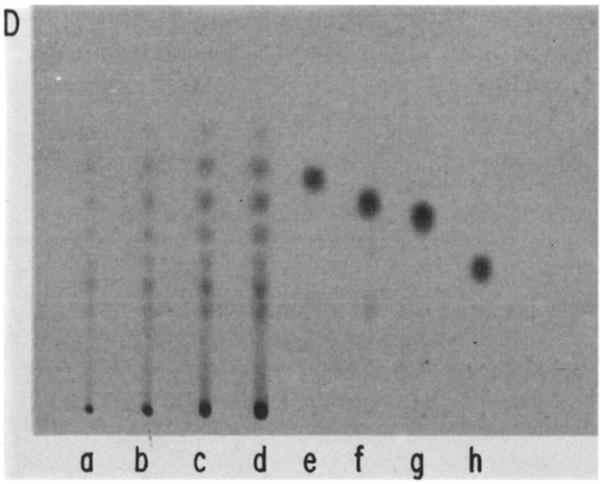

Fig. 1. Chromatograms of sugars and sugar derivatives on aminopropyl bonded-phase HPTLC plates. Mobile phases: panels A, B and C, acetonitrile–water (70:30); panel D, acetonitrile–water (60:40). Stationary phases: panel A, no monosodium phosphate impregnation; panel B, impregnated by 5-min immersion in 0.1 *M* NaH_2PO_4; panels C and D, impregnated by 15-min immersion in 0.2 *M* NaH_2PO_4. Samples (4 μg) spotted: panels A, B and C; a, 2-deoxyglucose; b, xylose; c, L-rhamnose; d, galactose; e, mannose; f, mixture (ascending order of R_F values) of stachyose, raffinose, melezitose, maltose, sucrose, glucose, fructose, psicose, methyl β-glucopyranoside; methyl α-mannopyranoside; and methyl α-arabinopyranoside; g, glucose; h, fructose; i, sucrose; j, melezitose; k, stachyose. Samples spotted on panel D: a, malto-oligosaccharide mixture M-250 (1 μg); b, M-250 (2 μg); c, M-250 (4 μg); d, M-250 (6 μg); and 4 μg each of e, melezitose; f, maltotriose; g, raffinose; and h, stachyose.

ing phosphoric acid with sulfuric acid in the aniline–diphenylamine reagent, all sugars are readily detected, and distinctions among classes of sugars can be made on the basis of spot color.

In conclusion, impregnation of aminopropyl-bonded silica HPTLC plates with monosodium dihydrogen phosphate allows the separation of closely related sugars, without glycosylamine formation by aldehydic sugar forms. By modifying the polarity of acetonitrile–water mobile phases, separations can be achieved in less than 30 min for classes ranging from closely related monosaccharide derivatives to higher oligosaccharides. Since different selectivities for sugars are exhibited when using the TLC procedure described here and in previous[4–6] reports, each has its advantages in resolving certain pairs of sugars. The monosodium dihydrogen phosphate impregnated aminopropyl-bonded silica HPTLC plates described here, however, are more effective for some sugars, require much less time for plate irrigation, and nicely parallel HPLC systems.

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