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Note

Separation of partially methylated alditol acetates on SP-2330 and HP-1 vitreous silica capillary columns

ELAINE M. SHEA and NICHOLAS C. CARPITA*
Department of Botany and Plant Pathology, Purdue University, West Lafayette, IN 47907 (U.S.A.)
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Because of recent appreciation of the wide distribution of glycosyl linkage positions in polysaccharides, glycoproteins, and glycolipids, considerable effort has been devoted to developing improved capillary columns for convenient and efficient separation of most sugar derivatives. Separations of partially methylated alditol acetate derivatives relied on high polarity phases such as ECNSS-M[1] or OV-225[2]. With the advent of capillary columns, these less stable phases were replaced with those of a wide range of polarity, and the increase in resolution afforded by capillary columns made separation of most derivatives possible[3–7]. Generally, the high-polarity phases gave more satisfactory separation of the partially methylated alditol acetates. Bonded phases, such as BP-75 (a bonded-phase OV-275), have been employed to overcome instability of the high polarity phases in vitreous silica columns[3], a problem that ultimately results in column bleed and irreproducible retention times. Despite these advantages, there are problems in resolving some common derivatives. Derivatives of hexoses are separated well on BP-75, but derivatives of the deoxysugars, rhamnose and fucose, are not[3]. SP-2100 is a low-polarity column that separates derivatives of deoxysugars and pentoses fairly well but is less useful for derivatives of hexoses. Derivatives of mannose and glucose are poorly resolved[5]. Several investigators, including ourselves, have reported that the high-polarity phase, SP-2330, is particularly useful in general analysis[8–10], but no systematic evaluation of its capabilities has been reported. A lesser known low-polarity methyl silicone column, HP-1, has recently been made available, and its usefulness is compared to SP-2330. We found SP-2330 to be an excellent general purpose column that resolves partially methylated alditol acetates of virtually all deoxysugars, pentoses, and hexoses.

EXPERIMENTAL

Materials

The *p*-nitrophenyl ethers of the following sugars were obtained from Sigma (St. Louis, MO, U.S.A.): α-L-arabinopyranoside, α-L-arabinofuranoside, β-L-fucopyranoside, β-D-galactopyranoside, β-D-glucopyranoside, β-D-mannopyranoside, α-L-rhamnopyranoside, β-D-xylopyranoside. *n*-Butyllithium (1.6 *M* solution in hexanes) was obtained from Aldrich (Milwaukee, WI, U.S.A.).

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Preparation of partially methylated alditol acetate standards

n-Butyllithium is a useful alternative to potassium or sodium hydride for formation of the methylsulfinylmethanide ion[11], and several recent reports have encouraged its use as a safe and convenient method for permethylation of oligo- and polysaccharides[12,13]. The monosaccharides were deliberately undermethylated by a modification of the *n*-butyllithium method of Parente *et al.*[14]. Each *p*-nitrophenyl glycoside (5 mg) was placed in a 15 ml Corex tube and dried over phosphorus pentoxide overnight. The tubes were sealed with rubber serum sleeve stoppers and 1 ml of dimethyl sulfoxide was added with a syringe. The tubes were evacuated and sonicated for 4 h. Ultra-high-purity argon was introduced via a syringe needle, with a second needle inserted for escape flow. *n*-Butyllithium (250 μl) was added to each tube, and the solution was stirred under continuous argon flow. When the solution cleared after evaporation of the hexane, an amount of methyl iodide equal to one third the molar amount of replaceable H^+ was added to each tube. The argon flow was removed and the samples were left stirring overnight under argon.

The partially methylated *p*-nitrophenyl glycosides were recovered by the method of Ciucanu and Kerek[15]. A 3-ml volume of chloroform–methanol (2:1) was added to each tube and the samples were mixed well. Water (2 ml) was added to each tube and the samples were mixed. The tubes were centrifuged for 5 min at 1000 *g* and the aqueous layer was removed. The organic phase was washed four more times with water, transferred to 1-dram vials and evaporated under a stream of nitrogen at 30°C.

The samples were hydrolyzed in 2 *M* trifluoroacetic acid (TFA) for 90 min at 120°C. The mixture was cooled to 30°C, and the TFA was removed by evaporation under a stream of nitrogen. The methyl glycosides were reduced with sodium [$^2H_4$]tetrahydroborate, and acetylated according to the method of Blakeney *et al.*[16].

Gas chromatography–mass spectrometry (GC–MS)

Separations were carried out with a Finnigan/MAT 9610 gas chromatograph coupled to a Finnigan/MAT 4021 quadrupole mass spectrometer interfaced to a Finnigan/MAT 2100C INCOS data system. Samples in dichloromethane were introduced via a Varian split/splitless capillary injector operated in the split mode (split ratio *ca.* 50:1). Derivatives were separated on a 30 m × 0.25 mm vitreous silica, 0.20 μm film thickness wall-coated open tubular capillary column of SP-2330 (Supelco), temperature programmed from 160 to 210°C at 2°C/min, then from 210 to 240°C at 5°C/min. Derivatives were also separated on a 25 m × 0.32 mm column, 1.05 μm film thickness (cross-linked methylsilicone gum) HP-1 (Hewlett-Packard), temperature programmed from 150 to 210°C/min, then from 210 to 240°C at 5°C/min. Helium was used as the carrier gas at 180 kPa. The injector temperature was 225°C, the oven temperature 240°C and the ion source was at 300°C. Mass spectra were recorded at 70 eV. The scan time was 0.95 s (0.05 s reset) over the *m/z* range of 41–350. Identification of derivatives by electron impact (EI) MS was as described[17].

RESULTS AND DISCUSSION

Methylation analysis of complex carbohydrate polymers depends on the clear separation and identification of the sugar derivatives. In the past, researchers have

used homo-polymers or hetero-polymers of known structure to generate standards for analysis of mixtures of partially methylated alditol acetates by GC–MS[18], while others have deliberately undermethylated the corresponding monosaccharides or sugar alcohols to generate a more complete mixture containing sugars representing most of the possible derivatives[19]. Deliberate undermethylation of *p*-nitrophenyl glycosides provided us with such a mixture.

Retention times for partially methylated alditol acetates relative to 2,3,4,6-tetra-O-methylglucitol are listed in Table I for SP-2330 and in Table II for HP-1. Most of the derivatives of deoxysugars and pentoses were resolved, but 2,3-O-methyl arabinose co-eluted with 2,4-O-methyl xylose and 2-O-methyl arabinose co-eluted with 3,4,6-O-methyl galactose (Table I). Members of each pair were distinguished easily by EI-MS[17]. Symmetrical derivatives, such as 2,3- and 3,4-O-methyl xylose or 3- and 4-O-methyl hexoses, are distinguished with EI-MS only by introduction of deuterium at C-1 upon reduction of the permethylated monosaccharides with sodium [$^{2}H_{4}$]tetrahydroborate. Except for the symmetrical derivatives just mentioned, virtually all of the hexoses were resolved from each other. By comparison, the low-

TABLE I

RELATIVE RETENTION TIMES OF PARTIALLY METHYLATED ALDITOL ACETATES ON A SP-2330 CAPILLARY COLUMN

Retention times are relative to 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol. *myo*-Inositol hexaacetate = 2.688.

Position of O-methyl[a]	*Parent monosaccharide*						
	Rhamnose	*Fucose*	*Arabinose*	*Xylose*	*Mannose*	*Galactose*	*Glucose*
2,3,4,6-	–	–	–	–	0.984[b]	1.120	1.000
2,3,4-	0.583	0.732	0.748	0.779	1.545	1.781	–
2,3,6-	–	–	–	–	1.491	1.587	1.637
2,4,6-	–	–	–	–	1.387[c]	1.484	1.406[d]
3,4,6-	–	–	–	–	1.409[d]	1.566	1.437
2,3-	0.980[b]	1.103	1.170[e]	1.274[h]	1.991	2.171	2.146
2,4-	–	1.052	1.184	1.169[e]	2.050	2.195	–
2,6-	–	–	–	–	1.760	1.848[f]	1.904
3,4-	0.945	1.149	1.199	1.274[h]	2.091	2.246	–
3,6-	–	–	–	–	1.934[g]	1.983	1.968
4,6-	–	–	–	–	1.746	1.848[f]	1.934[g]
2-	1.241	1.304	1.567	1.729[i]	2.229	2.332	2.370
3-	1.392[c]	1.469	1.623	1.715	2.376[j]	2.480[k]	2.439[l]
4-	1.320	1.450	1.604	1.729[i]	2.376[j]	2.480[k]	2.439[l]
6-	–	–	–	–	1.960	2.125	–
2,3,5-	–	–	0.628	–	–	–	–
2,5-	–	–	1.041	–	–	–	–
3,5-	–	–	0.944	–	–	–	–
5-	–	–	1.750	–	–	–	–
None	1.420	1.503	1.839	2.143	2.396	2.500	2.600

[a] 2,3,4,6-O-methyl mannitol = 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl mannitol.
[b–g] Unresolved derivatives distinguished by EI-MS.
[h–l] Symmetrical derivatives distinguished by EI-MS when sodium [$^{2}H_{4}$]tetrahydroborate is used for reduction.

TABLE II

RELATIVE RETENTION TIMES OF PARTIALLY METHYLATED ALDITOL ACETATES ON A HP-1 CAPILLARY COLUMN

Retention times are relative to 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol. *myo*-Inositol hexaacetate = 2.688.

Position of O-methyl[a]	*Parent monosaccharide*						
	Rhamnose	*Fucose*	*Arabinose*	*Xylose*	*Mannose*	*Galactose*	*Glucose*
2,3,4,6-	–	–	–	–	0.991	1.049	1.000
2,3,4-	0.678	0.732	0.668[b]	0.665[b]	1.326	1.417	–
2,4,6-	–	–	–	–	1.242[c]	1.243[c]	1.284[f]
2,3,6-	–	–	–	–	1.264[g]	1.290[h]	–
3,4,6-	–	–	–	–	1.230	1.290	1.238[i]
2,3-	0.890[j]	0.910	0.870	0.889[j]	1.565	1.615	1.607
2,4-	–	0.948	0.893[j]	0.863	1.637[k]	1.689	–
2,6-	–	–	–	–	1.423	1.441	1.465
3,4-	0.890[j]	0.975	0.893[d,j]	0.889[d,j]	1.610	–	–
3,6-	–	–	–	–	1.497	–	1.509[l]
4,6-	–	–	–	–	1.447	1.491	1.505[l]
2-	1.063[m]	1.074	1.083	1.100[n,p]	1.751	1.798[e]	1.803[e]
3-	1.132	1.140	1.118	1.109	–	–	–
4-	1.102[n]	1.150	1.091	1.100[n,p]	–	–	–
6-	–	–	–	–	1.624	1.635[k]	1.656
2,3,5-	–	–	0.578	–	–	–	–
2,5-	–	–	0.780[o]	–	–	–	–
3,5-	–	–	0.780[o]	–	–	–	–
5-	–	–	1.061[m]	–	–	–	–
None	1.238[i]	1.278[f]	1.265[g]	1.323	–	–	2.014

[a] 2,3,4,6-O-methyl mannitol = 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl mannitol.
[b–e] Identical derivatives unresolved by chromatography and not resolved by EI-MS.
[f–o] Unresolved derivatives distinguished by EI-MS.
[p] Symmetrical derivatives distinguished by EI-MS when sodium [$^2H_4$]tetrahydroborate is used for reduction.

polarity HP-1 column was unsuitable because all pentoses and hexoses either had considerable overlap or were unresolved. The BP-75 column was comparable to the SP-2330 column, but was unable to resolve 2,3- and 2,4-O-methyl rhamnose or 2,4- and 3,4-O-methyl mannose[3]; these pairs were well resolved on the SP-2330. Highly-polar Silar 9CP[4] and Silar 10C[20] phases possibly are capable of separation of many partially methylated alditol acetate derivatives, but an insufficient range of derivatives was tested; separation of derivatives of hexose by Silar 9CP was similar to that of SP-2330, but those of pentoses were not evaluated[4]. It should be noted that both Silar 9CP[4] and SP-2330 (data not shown) are such highly polar phases that 2-deoxy-2-(N-methyl)acetamidohexitol acetate derivatives typical of glycoproteins either decompose or do not elute at temperatures higher than recommended for the column. In this regard, Dexsil 410 or OV-101 are recommended for separation of derivatives of glycoprotein oligosaccharides or N-acetylated amino sugars[4].

The SP-2330 is an unbonded phase, but all separations were carried out below the recommended temperature limit with low bleed. By our estimation, this highly

polar phase is quite stable, and when improved techniques to generate clean derivative preparations are used, well over one hundred runs have been made before loss in resolution was noted. Retention times of derivatives shorten noticeably from initial values obtained in the first few runs, but then are consistent throughout the life of the column. Because of the great number of derivatives and despite near baseline resolution of most of them, a multiple column approach was recommended to verify analysis based solely on retention time, particularly for those without routine access to MS[7]. MS is still necessary for discrimination of symmetrical derivatives, many of which are encountered naturally. Coupled with the excellent resolution provided by SP-2330, unequivocal identification of virtually all partially methylated alditol acetate derivatives of neutral sugars normally found in polysaccharides and glycoproteins would be permitted.

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