

Mass Spectrometric Method To Determine the Chain Length of Oligosaccharides Attached to Phenolic Polymers by Nonglycosidic Linkages^{1,2}

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In many plants, a portion of the polysaccharides appears to have a very low degree of cross-linking with aromatic polymers such as lignin or flavolans. The proportion of cross-linked units may be enriched for study by enzymatically hydrolyzing the nonbonded carbohydrates. A convenient method is described for the simultaneous analysis of sugar content and apparent chain length of the oligosaccharidic fragments remaining after enzymatic hydrolysis. The analysis assumes attachment to the phenolic polymers by nonglycosidic linkages. The hemiacetal ends of the oligosaccharidic fragments are reduced with sodium borohydride while the fragments are still attached to the phenolic polymer. After acid hydrolysis, monomeric sugars are reduced with sodium borodeuteride. The mixed isotopic products are analyzed as their alditol acetate derivatives by capillary GC/MS with selected ion monitoring. The isotopic ratioing procedure is detailed and the precision determined. The method is accurate to 1 monomeric unit for oligosaccharide chain lengths of less than 10.

During a study of chemical bonding between lignin and carbohydrates in woody plants, the relatively few bonding sites were enriched by enzymatic hydrolysis of the major portion of the carbohydrate units not involved in the interpolymer bonding (Minor, 1982). The resultant material is primarily lignin with oligosaccharidic units attached. To completely characterize the bonding, it was necessary to develop a procedure to analyze the chain length of the attached oligosaccharides. The procedure involves an initial reduction of the attached oligosaccharides with sodium borohydride followed by hydrolysis and reduction of the released sugars with sodium borodeuteride. The alditols are then analyzed as their acetates by capillary gas chromatography/mass spectrometry (GC/MS) using chemical ionization and selected ion monitoring. The apparent chain length is obtained from the ratio of deuteriated to nondeuteriated alditol acetates.

This paper describes a study of the isotopic ratioing, its limitations, and the precision to be expected. The procedure is useful for oligosaccharides containing up to 10

monomeric sugar units. An alternative procedure, which can be used if a mass spectrometer is not available, involves conversion of the released sugars to their aldonitrile derivatives and analysis of both the alditol acetates and aldonitriles by gas chromatography (Baird et al., 1973).

EXPERIMENTAL SECTION

Preparation of Standards and Samples. With chloroform as solvent, two stock solutions were prepared from measured amounts of the five wood sugar alditol acetates, one in the protio and the other in the monodeuterio form. Standard mixtures of deuteriated and undeuteriated alditol acetates were prepared by mixing aliquots from the stock solutions. The extent of deuteriation from a given lot of NaBD₄ was checked as follows by mass spectrometry. A deuteriated derivative was prepared by reducing 1.2 mmol of sugar with 3 mmol of NaBD₄ in 2 mL of 2 M NH₄OH at 60 °C for 1 h. The reaction was stopped, and sodium ions were removed with cation-exchange resin (H⁺). Borate was removed as the methyl ester. The residue was acetylated by the procedure of Harris et al. (1984). The yield from a satisfactory lot of NaBD₄ contained less than 2.0% undeuteriated compound. (It is noted that one unsatisfactory lot yielded about 30% undeuteriated compound and was replaced by the vendor.)

Kraft pulp enzyme lignins were obtained from loblolly pine kraft pulps by enzymatic hydrolysis of the pulp polysaccharides (Minor, 1986) and then were suspended in

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Table I. Integrated Ion Counts for Masses 375 and 376 from Solutions Containing Mannitol Hexaacetate, Showing the Variation of Percent ^{13}C (Solution 1) and the Ratio of P/D (Solutions 2 and 3) with Electron Multiplier Detector Voltage

EM. V	solution 1 ^a			solution 2 ^b			solution 3 ^b		
	integrated counts for		% ^{13}C	obsd integrated counts for		ratio (P/D)	obsd integrated counts for		ratio (P/D)
	mass 375	mass 376		mass 375	mass 376		mass 375	mass 376	
1000	416 520	30 898	7.4	4 947	23 306	0.216	64 367	65 498	1.060
1100	960 184	101 212	10.3	102 490	241 022	0.445	444 750	439 045	1.131
1200	2 380 610	323 520	13.6	204 707	451 687	0.483	1 187 600	1 151 380	1.200
1300	4 748 250	711 529	15.0	304 256	633 479	0.517	1 353 070	1 306 140	1.227
1400	11 237 200	1 725 610	15.4	1 008 780	1 867 560	0.589	2 115 040	2 018 490	1.249
1500	26 364 800	3 287 060	12.5	1 998 920	3 570 970	0.602			

^aContains only the protiated species. ^bContains a mixture, of deuteriated and protiated species. The ratio (P/D) was calculated after correcting the integral counts for mass 376 for ^{13}C . The correction formula is $(\text{mass } 376)_{\text{cor}} = (\text{mass } 376)_{\text{obsd}} - (\text{mass } 375)_{\text{obsd}}(\% \text{ } ^{13}\text{C}/100)$.

0.1 M NaOH and treated with NaBH_4 for 3 h at room temperature. The reaction mixture was acidified with acetic acid and the precipitate recovered by centrifuging, washing, and freeze-drying. The reduced samples were hydrolyzed by the standard method for wood sugars (Saeman et al., 1954). The insoluble lignin was removed by filtration, and the sugar solution was neutralized with BaCO_3 or anion-exchange resin (HCO_3^-). The sugars were then reduced with NaBD_4 and acetylated without the removal of borate by the combined procedure (Harris et al., 1984), which is performed in one centrifuge tube.

Gas Chromatography/Mass Spectrometry. A Finnigan Model 4510 GC/MS was used to separate the sugar derivatives and analyze their respective mass fragments for amount of deuteriation. The chromatograph was fitted with an SP-2340, 30-m fused silica capillary column (Supelco, Bellefonte, PA). The following temperatures were maintained: injection port, 240 $^\circ\text{C}$; column oven, 240 $^\circ\text{C}$; ion source, 220 $^\circ\text{C}$. The carrier gas was helium with a flow rate of 1.0 mL/min; the split ratio was 80:1. The five wood sugar alditol acetates eluted with base-line separation in less than 9 min: arabinitol, 4.21 min; xylitol, 5.23 min; mannitol, 7.29 min; galactitol, 8.10 min; glucitol, 8.94 min. Methane was used as a chemical ionization reagent at a source thermal gauge pressure of 0.30 Torr. The electron energy was 70 eV, and the electron multiplier detector voltage was optimized at 1200–1300 V. Two mass range windows were scanned at m/z 302–307 and 374–379. These windows permitted detection only of the major fragments from the acetylated pentitols and hexitols, respectively. Total scan time was 0.22 s, divided equally between the two mass ranges.

RESULTS AND DISCUSSION

Mass Spectrometer Parameters. Methane chemical ionization (CI) of alditol acetates yields the dominant fragment $[(M + 1 - \text{HOAc})^+ = M']$ and the less abundant isotopic fragments at $M' + 1$ and $M' + 2$. These fragments account for 95% or more of the total ion count in the mass range 100–500. The precision and sensitivity of the intensity data can be improved by restricting data acquisition to the narrow range of these mass fragments. Borodeuteride reduction of the sugars introduces one nonexchangeable deuterium atom, and the masses of interest are extended one nominal mass higher to $M' + 3$ and range from 303 to 306 for acetylated pentitols and 375 to 378 for acetylated hexitols.

The carbon isotopic ratios are consistent for the pentitol acetates (12–14%) and hexitol acetates (15–17%) but are somewhat lower than the theoretical value calculated from the natural abundance of ^{13}C . (The theoretical values are 14.3% for the major fragment from pentitol acetates and 17.6% for the major fragment from hexitol acetates.) Experimental values were used to correct for the presence

of ^{13}C when ratios of protio to deuterio fragments were computed for the alditol acetates.

We noticed that the ratio of ion counts for two masses of interest changed with detector voltage. For example, the percent ^{13}C measured from a solution containing only protiated mannitol hexaacetate is low (7–10%) at lower EM voltages (1000–1100 V) and levels off to about 15% at 1300–1400 V (Table I, solution 1). At 1500 V, the measured percent of ^{13}C drops because the detector saturates on the ion count for mass 375. Solutions 2 and 3 (Table I) contain mixtures of the protiated and deuteriated compounds, and the P/D ratios show the same trend as the percent ^{13}C measured for solution 1. The ion count for mass 376 in the mixtures was corrected with the corresponding measured percent % from solution 1. Solution 3 contains more similar amounts of the protiated and deuteriated compounds than does solution 2, and the relative change in the P/D ratio is not as great. It is apparent that an adequate ion count level must be achieved to obtain an accurate ratio and that, at lower counts, the less abundant fragment has a disproportionately lower count than the more abundant fragment. Zeroing of electronic noise and the mass resolution were both checked by collecting profile data. Base-line separation was noted between the M' and $M' + 1$ fragments, and background counts were 1–2 as desired. Practically, a ratio of 0.05 or less (or $\overline{DP}_n$ greater than 20) cannot be determined with great accuracy because of the low ion count from the less abundant fragment. Also, because the absolute value of the detector voltage setting is determined by the condition of the multiplier, voltage vs. ratio measurements should be checked if increased voltages are required to maintain count rates or if another multiplier is installed.

Response Factors. Although in the earlier work (Minor, 1982, 1986), quantitative sugar analyses were obtained by injecting the alditol acetate solutions into an instrument equipped with a flame ionization detector, the mass spectrometer can be used as a quantitative detector. When a measured amount of undeuteriated standard is analyzed under CI conditions and the ion counts for the major fragments at masses M' and $M' + 1$ are integrated, it is found that the response factor, as total counts/mole, varies inversely with the molecular weight, as shown below for a four-, five-, and six-carbon alditol acetate series. [Data are the integrated ion counts per mole injected for masses $(M + 1 - \text{HOAc})$ plus $(M + 2 - \text{HOAc})$, average of six injections.] The observed response decreases by a factor of about 2 for each increase of one carbon atom in the acetylated series: tetritol, pentitol, hexitol. This could be partly explained if stable $(M + 1 - \text{HOAc})^+$ fragments result only from protiation of an acetyl group at either end of the molecular chain with subsequent loss of acetic acid.

Table II.

A. Summary of Protio/Deuterio Ion Fragment Ratios for Alditol Acetate Standards (Observed Values Are the Mean of Three Measurements; Standard Deviations in Parentheses)

std	arabinitol pentaacetate, mass ratio 303/304		xylitol pentaacetate, mass ratio 303/304		mannitol hexaacetate, mass ratio 375/376		galactitol hexaacetate, mass ratio 375/376		glucitol hexaacetate, mass ratio 375/376	
	obsd	calcd ^a	obsd	calcd ^a	obsd	calcd ^a	obsd	calcd ^a	obsd	calcd ^a
1	0.554 (0.013)	0.529	0.526 (0.009)	0.490	0.559 (0.002)	0.506	0.551 (0.003)	0.498	0.481 (0.002)	0.481
2	0.278 (0.014)	0.265	0.255 (0.021)	0.245	0.264 (0.020)	0.253	0.254 (0.021)	0.249	0.223 (0.019)	0.241
3	0.0861 (0.0054)	0.1014	0.0941 (0.0092)	0.1106	0.0690 (0.0112)	0.1015	0.0623 (0.0121)	0.0972	0.0672 (0.0116)	0.1044
4	0.0493 (0.0077)	0.0507	0.0540 (0.0106)	0.0553	0.0411 (0.0145)	0.0508	0.0371 (0.0145)	0.0486	0.0433 (0.0148)	0.0522
5	0.0297 (0.0041)	0.0254	0.0317 (0.0049)	0.0277	0.0261 (0.0056)	0.0254	0.0245 (0.0057)	0.0243	0.0299 (0.0060)	0.0261
6	0.0201 (0.0025)	0.0127	0.0212 (0.0046)	0.0139	0.0173 (0.0060)	0.0127	0.0163 (0.0065)	0.0122	0.0220 (0.0066)	0.0131

B. Results of Linear Regression Analysis of Data

	arabinitol pentaacetate		xylitol pentaacetate		mannitol hexaacetate		galactitol hexaacetate		glucitol hexaacetate	
	unweighted	weighted	unweighted	weighted	unweighted	weighted	unweighted	weighted	unweighted	weighted
intercept	-0.0025	0.0036	-0.0048	0.0013	-0.0141	0.0051	-0.0080	0.0004	-0.0158	0.0059
slope	1.049	1.007	1.072	1.059	1.118	1.114	0.996	0.998	1.119	1.117
std dev of slope	0.023	0.048	0.030	0.030	0.044	0.017	0.047	0.018	0.048	0.020
std dev of intercept	0.003	0.0045	0.0118	0.0050	0.0104	0.0077	0.0106	0.079	0.0111	0.008
correln coeff	0.99900	0.99543	0.99847	0.99837	0.99688	0.99952	0.99560	0.99933	0.99639	0.99936

^a Based on weighed values.Table III Observed and Calculated (from Calibration, Table II) Values of Protio/Deuterio Ion Fragment Ratios for Alditol Acetates and the Apparent Number Average Degree of Polymerization ($\overline{DP}_n$) of the Corresponding Oligomeric Fragments Derived from Loblolly Pine Samples (Standard Deviations in Parentheses)^a

sample	arabinitol pentaacetate		app $\overline{DP}_n$	xylitol pentaacetate		app $\overline{DP}_n$	mannitol hexaacetate		app $\overline{DP}_n$	glucitol hexaacetate		app $\overline{DP}_n$	galactitol hexaacetate		$\overline{DP}_n$
	obsd	calcd		obsd	calcd		obsd	calcd		obsd	calcd		obsd	calcd	
180A	0.463 (0.010)	0.444	3.3	0.380 (0.011)	0.359	3.8	0.280 (0.014)	0.263	4.8	0.123 (0.016)	0.131	7.6	0.187 (0.015)	0.181	6.5
180B	0.245 (0.008)	0.236	5.2	0.333 (0.010)	0.315	4.2	0.242 (0.014)	0.229	5.4	0.163 (0.016)	0.172	6.8	0.072 (0.015)	0.078	13.8
180C	0.552 (0.012)	0.529	2.9	0.405 (0.011)	0.382	3.6	0.366 (0.016)	0.340	3.9	0.195 (0.016)	0.204	5.9	0.142 (0.015)	0.141	8.1

^a Measurements using a 60-m DX4 fused silica capillary column. See the text.

The relative number of acetyl groups/molecule that would lead to positive fragments after protiation and loss of acetic acid would decrease with increasing carbon chain length.

compound	CI (CH ₃)	EI
erythritol tetraacetate	9.8×10^7	2.6×10^4
xylitol pentaacetate	4.6×10^7	3.0×10^4
glucitol hexaacetate	2.4×10^7	3.3×10^4

For electron impact (EI) ionization, there is a slight increase in the total fragmentation response factor with increasing molecular weight for the same series of compounds. (Data are the integrated counts for fragments in mass range 41–400/mole injected; average of four injections.) This behavior is consistent with the concept of EI cross sections increasing with increasing molecular size for the same class of compounds (Fitch and Sauter, 1983). From these results, it is apparent that chemical ionization causes a different pattern of response factors and the usual EI relation of ionization cross section and molecular size is not applicable.

Isotopic Separation. No isotopic separation was observed with the SP2340 column. However, peak height retention times were consistently 5–9 s less for the deu-

teriated alditol acetates than for the corresponding undeuteriated compounds on a 60-m DX4 fused silica capillary column used in preliminary work. In this case, retention times varied from 15 min for arabinitol pentaacetate to 33 min for galactitol hexaacetate at an oven temperature of 230 °C. Ratios were carefully determined on each column, and no significant difference was found. Data from the SP2340 are preferred because of the shorter retention time and better resolution of galactitol and glucitol hexaacetates. Isotopic separation has been observed for trimethylsilyl derivatives of glucose and glucose-*d*, using a packed SE-30, 2-m column (Sweeley et al., 1966). The deuteriated glucose derivative elutes before the undeuteriated derivative.

Measurement of Standards. Standard solution mixtures of the alditol acetates were injected into the GC/MS, ion intensities were measured, and isotopic ratios were computed. Calibration curves were prepared for the five major wood sugar alditol acetates. The data for all five sugar acetates are shown in Table IIA. Each standard was measured three times. From the standard deviations we calculated a weighted calibration curve, but this (Table IIB) shows no significant difference from the unweighted

Table IV. Number of Monomer Sugar Units in a Carbohydrate Oligomer Calculated from Theoretical Values of P/D

P/D	no. sugar monomers	P/D	no. sugar monomers
0.0	1	0.167	7
1.0	2	0.143	8
0.5	3	0.125	9
0.33	4	0.111	10
0.25	5	0.071	15
0.20	6	0.053	20

curve, in either slope or intercept.

Measurement of Unknowns. A satisfactory analysis of end groups by the present method should be possible for most unknown samples. As mentioned, each lot of NaBD₄ must be tested by reduction of a known sample to determine the extent of deuterium incorporation. Also, there must be no interference from the sample in the mass region of interest. Interference is minimized by capillary chromatography and by restricted data acquisition at a relatively high ratio of ion mass to charge. If there is a suspicion of impurity or background interference, the sample can be tested by performing both reductions on a portion of the sample with sodium borodeuteride and both reductions on a second portion with sodium borohydride. The preferred sequence is first to reduce the oligomer reducing end with borohydride and, after hydrolysis, to reduce with borodeuteride. This method produces less protio than deuterio material and lessens the correction for protiated fragments containing the ¹³C isotope (the M' + 1 peak).

We tested the analytical method on several samples of loblolly pine kraft pulp that had been treated with polysaccharidases. The resultant "enzyme" lignin contained oligomeric fragments of wood polysaccharides. The results for these samples are presented in Table III. Calculated amounts and their standard deviations were determined from the unweighted data of Table IIB. An apparent number average degree of polymerization ($\overline{DP}_n$) can be calculated from these data by $\overline{DP}_n = DP + 1$. Further interpretation depends on how much is known about the

original polysaccharide structures. To convert sugar analysis results to percent of the original samples, appropriate factors must be included for hydrolysis losses and anhydride structures as well as the molecular weight factors resulting from derivatization. Usually end group and sugar analyses must be combined with other structure determination techniques, such as methylation analyses, for complete interpretation.

CONCLUSION

The residual oligosaccharidic fragments present in "enzyme lignins" after enzymatic hydrolysis of wood polysaccharides have a very low $\overline{DP}_n$ and are ideally suited to the present type of analysis. For $\overline{DP}_n = 8-9$ or less, the analysis is accurate to one monomer unit (Table IV). Above that, the ratio difference due to a change of one monomer unit is equal to or smaller than the standard deviation for the calculated ratio (Table 111).

Registry No. NaBH₄, 16940-66-2; NaBD₄, 15681-89-7; arabinose, 147-81-9; xylose, 58-86-6; mannose, 3458-28-4; glucose, 50-99-7; galactose, 59-23-4.

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