Ledl, F.; Fritsch, G. *Lebensm. Unters. Forsch.* **1984**, *178*, 41.
Ledl, F.; Fritsch, G.; Severin, Th. *Z. Lebensm. Unters. Forsch.* **1982**, *175*, 208.
Mills, F. D. *J. Agric. Food Chem.* **1978**, *26*. 894.
Mills, F. D.; Weisleder, D.; Hodge, J. E. *Tetrahedron Lett.* **1970**, *15*, 1243.
Paulsen, H.; Pflughaupt, K. W. In *Carbohydrates IB*; Pigman, W., Horton, D., Eds.; Academic: New York, London, Toronto, Sydney, San Francisco, 1980; p 881.
Peer, H. G.; van den Ouweland, G. A. M.; De Groot, C. N. *Recl.: J. R. Neth. Chem. Soc.* **1968**, *87*, 1011.
Pisarnitskii, A. F.; Egorov, I. A.; Egofarova, R. Kh.; Erygin, G. D. *Prikl. Biokhim. Mikrobiol.* 1979, 15, 902.
Severin, Th.; Seilmeier, W. *Z. Lebensm. Unters. Forsch.* **1967**, *134*, 230.
Severin, Th.; Seilmeier, W. *Z. Lebensm. Unters. Forsch.* **1968**, *137*, 4.
Severin, Th.; Krönig, U. *Chem. Mikrobiol. Technol. Lebensm.* **1973**, *2*, 49.
Simon, H.; Heubach, G. *Chem. Ber.* **1965**, *98*, 3703.
Tatum, J. H.; Shaw, E. P.; Berry, R. E. *J. Agric. Food Chem.* **1967**, *15*, 773.
Tonsbeek, C. H. T.; Plancken, A. J.; van de Weerdhof, T. J. *J. Agric. Food Chem.* **1968**, *16*, 1016.
Van den Ouweland, G. A. M.; Peer, H. G. *Recl.: J. R. Neth. Chem. Soc.* **1970**, *89*, 750.
Weygand, F.; Simon, H.; Bitterlich, W.; Hodge, J. E.; Fisher, B. E. *Tetrahedron* **1958**, *6*, 123.

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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 aldononitrile derivatives and analysis of both the alditol acetates and aldononitriles 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 $NaBD_4$ was checked as follows by mass spectrometry. A deuteriated derivative was prepared by reducing 1.2 mmol of sugar with 3 mmol of $NaBD_4$ in 2 mL of 2 M NH_4OH 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 $NaBD_4$ 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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[2] Forest Products Laboratory maintained at Madison, WI, in cooperation with the University of Wisconsin.

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

	solution 1[a]			solution 2[b]			solution 3[b]		
	integrated counts for			obsd integrated counts for			obsd integrated counts for		
EM, V	mass 375	mass 376	% ^{13}C	mass 375	mass 376	ratio (P/D)	mass 375	mass 376	ratio (P/D)
1000	416520	30898	7.4	4947	23306	0.216	64367	65498	1.060
1100	980184	101212	10.3	102490	241022	0.445	444750	439045	1.131
1200	2380610	323520	13.6	204707	451687	0.483	1187600	1151380	1.200
1300	4748250	711529	15.0	304256	633479	0.517	1353070	1306140	1.227
1400	11237200	1725610	15.4	1008780	1867560	0.589	2115040	2018490	1.249
1500	26364800	3287060	12.5	1998920	3570970	0.602			

[a] Contains only the protiated species. [b] Contains 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 $(\text{mass } 376)_{cor} = (\text{mass } 376)_{obsd} - (\text{mass } 375)_{obsd}(\%\ ^{13}C/100)$.

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 °C, column oven, 240 °C; ion source, 220 °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 $[(M + 1 - HOAc)^+ = M']$ 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 iostopic 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 ^{13}C 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 − HOAc) plus (M + 2 − 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 $(M + 1 - HOAc)^+$ 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)

	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	
std	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]

	arabinitol pentaacetate			xylitol pentaacetate			mannitol hexaacetate			glucitol hexaacetate			galactitol hexaacetate		
sample	obsd	calcd	app $\overline{DP}_n$	obsd	calcd	app $\overline{DP}_n$	obsd	calcd	app $\overline{DP}_n$	obsd	calcd	app $\overline{DP}_n$	obsd	calcd	$\overline{DP}_n$
180A	0.463	0.444 (0.010)	3.3	0.380	0.359 (0.011)	3.8	0.280	0.263 (0.014)	4.8	0.123	0.131 (0.016)	7.6	0.187	0.181 (0.015)	6.5
180B	0.245	0.236 (0.008)	5.2	0.333	0.315 (0.010)	4.2	0.242	0.229 (0.014)	5.4	0.163	0.172 (0.016)	6.8	0.072	0.078 (0.015)	13.8
180C	0.552	0.529 (0.012)	2.9	0.405	0.382 (0.011)	3.6	0.366	0.340 (0.016)	3.9	0.195	0.204 (0.016)	5.9	0.142	0.141 (0.015)	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_4)	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 deuteriated 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_7, 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 $NaBD_4$ 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 ^{13}C 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 $\overline{DP}_n = D/P + 1$. 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 III).

Registry No. $NaBH_4$, 16940-66-2; $NaBD_4$, 15681-89-7; arabinose, 147-81-9; xylose, 58-86-6; mannose, 3458-28-4; glucose, 50-99-7; galactose, 59-23-4.

LITERATURE CITED

Baird, J. K.; Holyroyde, M. J.; Ellwood, D. C. *Carbohydr. Res.* **1973**, *27*, 464–467.
Fitch, W. L.; Sauter, A. D. *Anal. Chem.* **1983**, *55*, 832–835.
Harris, P. J.; Henry, R. J.; Blakeney, A. B.; Stone, B. B. *Carbohydr. Res.* **1984**, *127*, 59–73.
Minor, J. L. *J. Wood Chem. Technol.* **1982**, *2*, 1–16.
Minor, J. L. *J. Wood Chem. Technol.* **1986** *6*, 185–201.
Saeman, J. F.; Moore, W. E.; Mitchell, R. L.; Millett, M. A. *Tappi* **1954**, *37*(8), 336–343. Technical Association of the Pulp and Paper Industry, TAPPI Standard T250, Atlanta, GA 30348.
Sweeley, C. C.; Elliott, W. H.; Fries, J.; Ryhage, R. *Anal. Chem.* **1966**, *38*(11), 1549–1553.

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Caloric Utilization of Sorbitol and Isomalt in the Rat

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Sorbitol and isomalt are modified saccharides used to substitute for the physical properties of sucrose in various prepared foods. The merits of the various methods for determining caloric availability were reviewed. Balance and growth curve methods are inaccurate and inappropriate for determination of the caloric availability of these substances when present in diets at low concentrations, whereas the radiolabel disposition method is a direct and precise measure of utilization. Accordingly, we administered uniformly ^{14}C-labeled material to rats and collected excreta and expired air. The appearance of about half of the label in CO_2 indicated that, by comparison with labeled glucose, about 80% of the orally administered sorbitol and isomalt was calorically available to the rat. The high caloric availabilities of these materials were confirmed by the appearance in feces of only 14 and 12% of the administered label from sorbitol and isomalt, respectively.

In addition to providing essential nutrients, the food we consume supplies energy for physiological maintenance and growth. To avoid the undesired weight gain resulting from energy intake beyond these needs, high-intensity sweeteners are gaining popularity as a replacement for sucrose. Modified saccharides (bulking agents) have also been developed to provide the texture and bulk necessary in many prepared foods but not provided by high-intensity sweeteners alone. These modified saccharides can replace the bulk of sucrose, and sometimes that of other ingredients, approximately on a 1:1 (w/w) basis. Because they

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