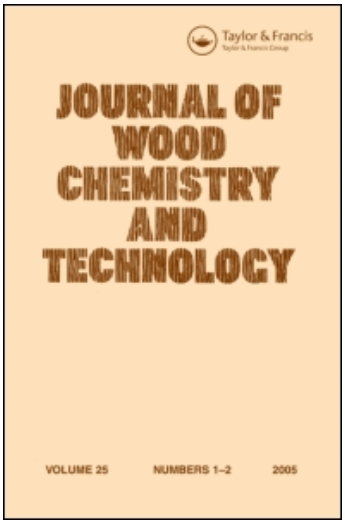

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THE COMPLETE ANALYSIS OF WOOD POLYSACCHARIDES USING HPLC

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ABSTRACT

The paper describes an analytical method whereby the chemical composition of the polysaccharide fraction of woody materials, including the uronic acids and carbohydrate acid degradation products, can be completely determined. Sealed vessels, termed bombs, are used during the high temperature acid hydrolysis of the materials to retain volatile constituents that would otherwise be lost. High-Performance Liquid Chromatography (HPLC) is used for all of the analyses. The sample preparation procedures are simple and easily adopted into a routine. To demonstrate the reproducibility of the method, triplicate samples of Quaking aspen (*Populus tremuloides*) and White fir (*Abies concolor*) were analyzed. No significant peaks in the chromatograms were left unidentified.

INTRODUCTION

The increased use of lignocellulosic materials as feed-stocks for chemical conversion processes has placed a heavy emphasis on the need for a rapid, reliable summative analysis method for these materials. The objective of this study was the development of a suitable analytical method for woody materials that would not only produce a complete accounting of the chemical components, but would also provide the analysis in the most time efficient manner possible. The resulting Bomb/HPLC analytical method proposes

improvements in the hydrolytic and analytical procedures that are currently practiced in the field of wood chemistry. This paper addresses the analysis of the polysaccharide fraction.

In order to analyze the components of a wood sample, the material must first be digested chemically. In wood analysis, the most commonly used method for this purpose involves hydrolysis of comminuted wood with mineral acid. The Klason procedure, named after the Swedish chemist Peter Klason, utilizes a two stage sulfuric acid hydrolysis in which the wood material is first treated with concentrated acid, usually 72%, at or around room temperature. This is followed by dilution to 4% acid and further hydrolysis at reflux or a higher temperature. There have been many variations of the Klason procedure proposed; among them TAPPI Standards T 222-om-83, "Acid-insoluble Lignin in Wood and Pulp", and T 249-cm-85, "Carbohydrate Composition of Wood and Pulp by Gas-Liquid Chromatography".[1] Of the various Klason-type procedures that abound, TAPPI Standard T 249 has the distinct advantage of rapid processing time. The procedure requires treating comminuted wood with 72% sulfuric acid at 30 °C for 1 h, followed by dilution to 4% acid and further reaction in an autoclave at 103 kPa (120 °C) for an additional hour. Unfortunately, the method specifies that the sample be contained in a loosely covered beaker during the high temperature hydrolysis. Since the beaker is not a sealed environment, the loss of volatile degradation products, such as 2-furaldehyde (2-F) and acetic acid, is allowed to occur. Thus, a complete analysis is not possible using this method specifically. However, by utilizing a sealed container, or bomb, during the high temperature hydrolysis step, the volatile compounds are contained and therefore can be accounted for in the subsequent analyses. Furthermore, because all of the degradation products are retained in the procedure, the minimization of their production is no longer a primary concern and slight variations in autoclave temperature and/or heating time become insignificant. The use of a bomb during the wood hydrolysis, which was otherwise performed according to TAPPI Standard T 249, was the hydrolytic technique developed for use in the Bomb/HPLC analytical method.

In a wood hydrolytic solution, the polysaccharide moieties that are present include the neutral carbohydrates, the organic acids, such as uronic acids and acetic acid, and any acidic degradation products. All of these moieties must be analyzed in some fashion. The traditional means of carbohydrate analysis has been by Gas Chromatography (GC).[1-7] However, the advent of High-Performance Liquid Chromatography (HPLC), and its continued refinement and development, has resulted in a tool that is almost ideal for the analysis of carbohydrate mixtures.[8-24] Not only are the elution times shorter when compared to identical analyses by GC, but when using HPLC there is no need to derivatise the hydrolyzed sugars to their related volatile alditol acetates (in the case of TAPPI Standard T 249) or other derivatives.[5,7] Thus, the necessary sample preparations are also greatly simplified. HPLC using pulsed amperometric detection (PAD) is an especially promising method for carbohydrate analysis[11,12,16-18], though PAD was not the detection device used in this study.

The satisfactory analysis of the organic acids from wood, most notably the uronic acids, is one of the important advantages that can be attributed to HPLC in the area of wood analysis. Previously, the analysis of uronic acids involved complicated procedures, with colorimetric, CO_2 adsorption, and mass spectroscopy techniques being employed.[2,3,12,25-27] Furthermore, the two most widely used of these procedures, the colorimetric (carbazole) and decarboxylation methods, could not be used to differentiate between uronic acids and were insensitive to the presence of monosaccharides that might be bonded to a uronic acid moiety. Since at least half of the 4-O-methylglucuronic acid present in wood persists through hydrolysis glycosidically linked with xylose[29], a large error can be incurred when using either of these two methods on wood hydrolysates. HPLC, however, does not have this analytical limitation.

Finally, the analysis of 2-F and 5-(hydroxymethyl)-2-furaldehyde (HMF), two common acid degradation products of monosaccharides, has been overlooked in most summative analysis procedures in which acid hydrolysis has been utilized.[1]

Fortunately, HPLC provides an ideal tool to quantify these compounds[9,29,30], as well as other wood acidic degradation products such as acetic acid and levulinic acid. To demonstrate the reproduciblity and applicability of the Bomb/HPLC method, three samples each of Quaking aspen *(Populus tremuloides)* and White fir sapwood *(Abies concolor)* were analyzed and the chemical composition of the polysaccharide fraction of each sample was determined.

EXPERIMENTAL

Extraction

Approximately 80 g of air-dried comminuted wood was extracted in a Soxhlet extractor using 600 ml of a 2:1 benzene:95% ethanol solution followed by extraction with 600 ml of 95% ethanol. The extracted sample was then transferred to a 2000-ml beaker and 1400 ml of reverse osmosis (RO) water was added. The wood slurry was then heated to boiling, with mechanical stirring, for a period of 4 h. The beaker was left uncovered during the hot water extraction to allow residual solvent to be distilled away; additional water was added as needed to maintain the original volume. At the conclusion of the 4 h time period, the extractive-free comminuted wood was recovered by vacuum filtration through a coarse sintered glass filter funnel. The wood sample was washed with 1 L of fresh RO water and allowed to air dry for two weeks before analysis.

Hydrolytic method

The hydrolysis of the extracted comminuted wood specimens was performed, in triplicate, according to TAPPI Standard T 249 cm-85. However, the samples were contained in bombs during the hydrolysis rather than beakers. Corning 220-ml centrifuge bottles with threaded necks were used to fabricate the hydrolysis bombs. The tops of the bottles were ground on a polishing stone to insure a flat, sealable surface. Plastic caps for the bottles were obtained and fitted with teflon liners. Rubber liners were used under the teflon to provide flexibility and insure a tight seal. Since the

centrifuge bottles had narrow bases, each bomb that was to be used for any run was placed in a 250-ml beaker. The beaker was readily numbered and provided additional stability for the bomb.

The procedure used for all of the extractive-free specimens was identical. Approximately 0.35 g of comminuted wood of known moisture content was weighed to 0.0001 g. This sample was then quantitatively transferred to a hydrolysis bomb. The transfer of the wood specimen to the bomb was done with care to ensure that no stray wood particles adhered to the sides of the bomb vessel. Three milliliters of a 72% sulfuric acid solution, at room temperature, was added onto the comminuted wood, rinsing the lower sides of the vessel with the acid. A glass stirring rod was used to homogenize the wood/acid mixture and the bomb containing the mixture was placed in a 30 °C water bath. Over the course of 1 h, the mixture was intermittently stirred. After 1 h in the water bath, the beaker containing the bomb was removed and the acid slurry was diluted to 4% acid by the addition of 84 ml of RO water. The water was added in two parts: approximately one-half was added and mixed with the wood/acid slurry; the remainder was added while cleansing the stirring rod with the aid of a rubber policeman. Once the water addition was completed, the vessel was sealed tightly with its fitted cap and the beaker holding the bomb was placed in an autoclave. The bomb with its contents was then heated by live steam at 103 kPa (120 °C) in the autoclave for a period of 1 h. The heating was discontinued and the autoclave was allowed to cool to 70 °C. At this point, the bomb was removed from the autoclave and allowed to continue cooling to room temperature. Once the bomb had cooled, the insoluble residue was removed from the hydrolysate solution by filtration through tared, medium porosity, sintered glass filter crucibles. The retained solids were washed with 100 ml of fresh RO water. The filtrate was then quantitatively transferred to a 250-ml volumetric flask and the volume was brought up to the mark with additional RO water. This solution became the stock solution for all subsequent analyses.

2-F and HMF Analysis

The method used for the determination of the 2-F and HMF concentrations in the wood hydrolysates utilized HPLC. A direct injection of the hydrolysate was made from a 10-µl sample loop injection valve onto a Brownlee Labs 3-cm PPH-GU guard cartridge without internal standard (IS) or additional sample preparation. Although this cartridge was designed to be used as a guard column for the organic acids column (see "Acids Analysis" section), it gave satisfactory separations of 2-F and HMF. The eluant, 0.01 M sulfuric acid, was used at a flow-rate of 0.8 ml/min; the total analysis time was approximately 2.5 min. Detection was made using an UV detector set at 280 nm. The system was calibrated using an external standard mixture of freshly distilled 2-F and fresh analytical grade HMF. Representative chromatograms of 2-F/HMF analyses for hardwoods and softwoods are presented in Figure 1.

Analysis of Carbohydrates

The carbohydrates of the wood samples were analyzed using a Bio-Rad "Polypore" HPX-87P column, 7.8 x 300 mm, which was kept isothermally at 85 °C. A Brownlee Labs 3-cm PPP-GU guard cartridge was used before the main column. Degassed and 0.2-µm-filtered "Nanopure" water was used as the eluant at a flow rate of 1.2 ml/min. A refractive index (RI) detector set at range 0.25 RIU was used for peak detection. The sugars samples from the hydrolysates were applied to the column using a 20-µl sample loop injection valve.

The procedure for the preparation of the sugars samples included pipetting 20 ml of hydrolysate and 2 ml of a 2 mg/ml erythritol IS solution into a 50 ml beaker. This solution was adjusted to pH 5.3 with a sat. $Ba(OH)_2$ solution, then centrifuged and the clear supernatent decanted into a 100 ml round-bottom flask. The supernatent was concentrated, using a rotary evaporator, to approximately 2 ml. This concentrate was then applied to a mixed-bed ion-exchange column using a Pasteur pipet. The ion-exchange column was prepared using a Bio-Rad disposable "Poly-prep" column

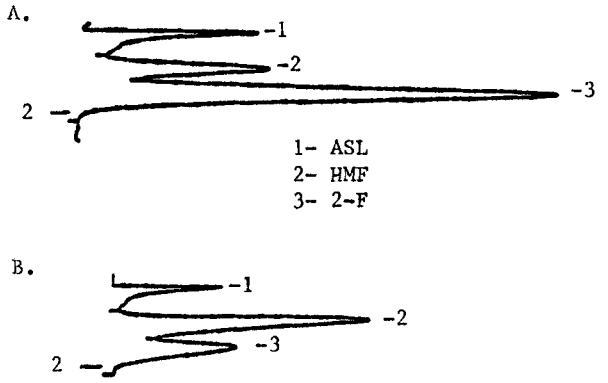

Figure 1. HPLC analyses of aspen (A) and white fir (B) to quantify 2-F and HMF contents. The detection was by UV at 280 nm. As expected, the aspen sample contained more 2-F than HMF, whereas in the white fir sample the reverse was true. Also, more ASL (acid soluble lignin) was detected in the aspen sample.

and charged with a lower layer of 0.2 ml of Bio-Rad AG 50W-X8, 100-200 mesh H^+ resin covered by a layer of 0.4 ml of Dowex 1X-8, 200-400 mesh $CO3^{2-}$ resin. As the sample was applied the eluate was collected in a small vial. After the sample had been eluted, a 0.5-ml water wash was applied to the resin bed and collected with the eluate in the vial. The sample was then analyzed by HPLC. Examples of sugars chromatograms from the aspen and white fir are presented in Figures 2 and 3, respectively.

Acids Analysis

Two sample preparation procedures, acids #1 and acids #2, were used in the analysis of the acidic components in hydrolysates. All of the samples prepared by these procedures were analyzed using the same chromatographic conditions. The column used was a 7.8 x 300 mm Bio-Rad "Polypore" HPX-87H with a Brownlee Labs 3-cm PPH-GU guard cartridge. Samples were injected onto the column through a 20-µl sample loop injection valve. The eluant was degassed 0.01 M sul-

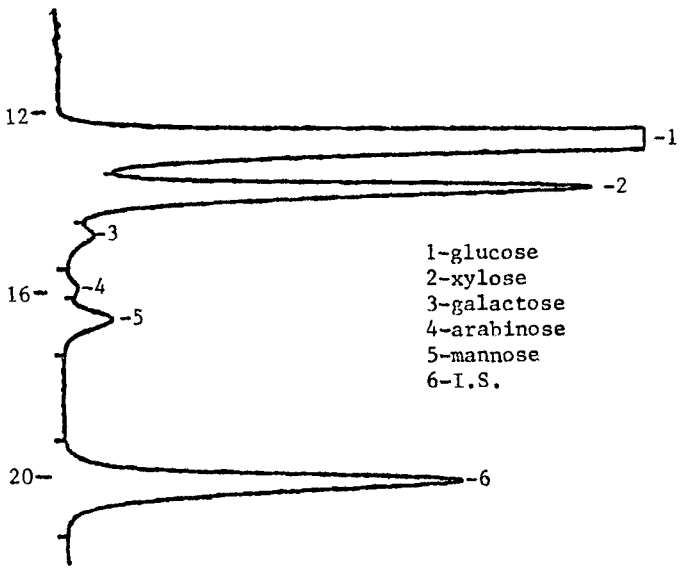

Figure 2. HPLC analysis of aspen to quantify monosaccharides.

furic acid, prepared from Nanopure water and used at a flow rate of 1.2 ml/min. Peak detection was accomplished using an RI detector.

The acids #1 sample preparation procedure was used for the uronic acids determination in the wood samples. In this procedure, a 20-ml aliquot of a hydrolysate solution and 1 ml of a 0.5% succinic acid IS solution were pipetted into a 50-ml beaker, neutralized to pH 2.5 with a sat. $Ba(OH)_2$ solution, and centrifuged. The pH was kept low to prevent the precipitation of the uronic acids that would occur in the presence of excess Ba^{2+} ion. The supernatent was decanted and concentrated on a rotary evaporator to approximately 2 ml.

The retention time of glucose on the acids column is within one to two minutes of the retention times for the uronic acids. As a result, the accurate determination of the uronic acids in a hydrolysate sample can be compromised by the presence of a relatively large amount of glucose. Since the wood hydrolysates

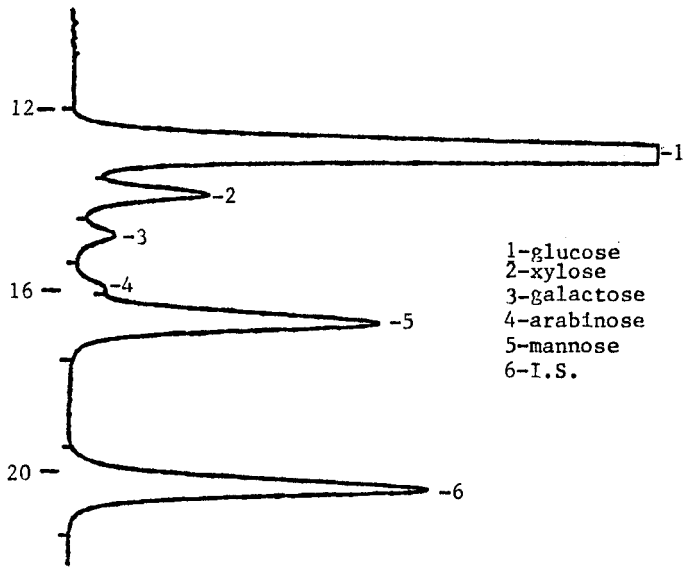

Figure 3. HPLC analysis of white fir to quantify monosaccharides. This chromatogram should be compared to the identical analysis for aspen (Figure 2) in which the relative amount of xylose to mannose is reversed.

contained large amounts of glucose, each concentrated acids #1 sample from the wood hydrolysates was further treated by applying the sample to a fresh ion-exchange column to adsorb acids as described in the section "Carbohydrate Analysis". The column was then washed five times with 1-ml aliquots of RO water. The initial eluate and the washes containing the sugars were discarded. Thereby, the glucose and the other sugars were eliminated from the sample, thus avoiding any interference with the uronic acids in the subsequent HPLC analysis. The adsorbed acids were then eluted from the ion-exchange column by treating the resin bed with five successive 1-ml aliquots of 5% sulfuric acid, followed by one 1-ml wash of RO water. The eluates and washings, collected in a 50-ml beaker, were neutralized to pH 2.5 with sat. $Ba(OH)_2$ solution, centrifuged, and the supernatent concentrated to 2 ml by rotary

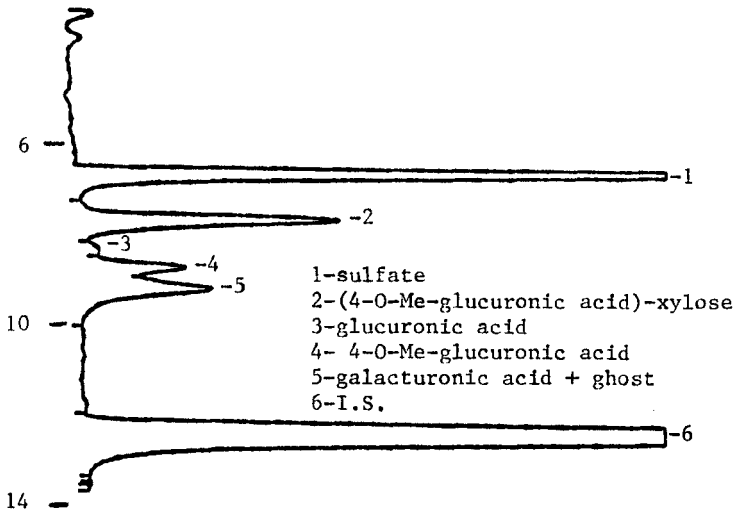

Figure 4. HPLC analysis of an acids #1 sample from aspen. This analysis quantifies the uronic acids.

evaporation. The sample was then analyzed by HPLC. An acids #1 chromatogram from a wood sample is illustrated in Figure 4. It should be noted that, in the acids #1 HPLC analysis, a "ghost" peak, that is generated during the sample preparation or the subsequent chromatography, co-elutes with galacturonic acid. The galacturonic acid contributions were estimated by subtracting a normalized blank peak from the 9.1 minute peak area. This blank was obtained by preparing two acids #1 samples from a solution containing only sulfuric acid of the same acid concentration as a hydrolysate. The peak areas that were generated from these blanks were then averaged. A chromatogram of an acids #1 blank analysis is given in Figure 5.

The acids #2 procedure was designed to prevent the loss of volatile acids, such as acetic acid, during the rotary evaporation step. In this procedure, a 20-ml aliquot of hydrolysate and a 1-ml aliquot of 0.5% butyric acid IS solution were pipetted into a 50-ml beaker. The sample was then neutralized to pH 9-10 with a sat. $Ba(OH)_2$ solution. After centrifuging to remove the precipitate, the

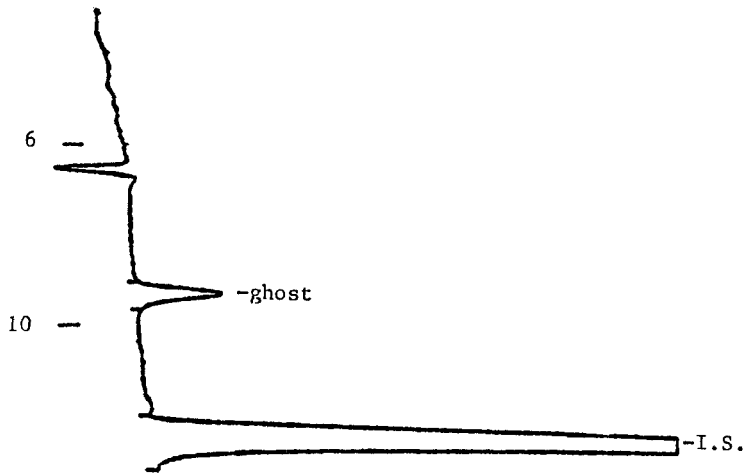

Figure 5. HPLC analysis of an acids #1 blank sample. Two of these samples were prepared and the peak areas were averaged. The average peak area was subtracted from the appropriate peak in all of the acids #1 samples to better estimate the galacturonic acid fraction.

supernatent was concentrated to approximately 2 ml using a rotary evaporator. The sample was acidified by adding three drops of 5% sulfuric acid solution before analysis by HPLC. A sample acids #2 chromatogram is presented in Figure 6. The acids #2 chromatogram compared to the acids #1 chromatogram (Figure 4) illustrates the effect that sugars in high concentration can have on the chromatography of the uronic acids.

RESULTS AND DISCUSSION

The results of the polysaccharide analyses for each of the three samples from the aspen wood and white fir wood are given in Tables 1 and 2, respectively. An explanation of the procedure used and rational for the calculation results given in Tables 1 and 2 is necessary. In the hydrolysis reaction, the glycosidic linkages of the wood polymers are cleaved with the addition of water. The

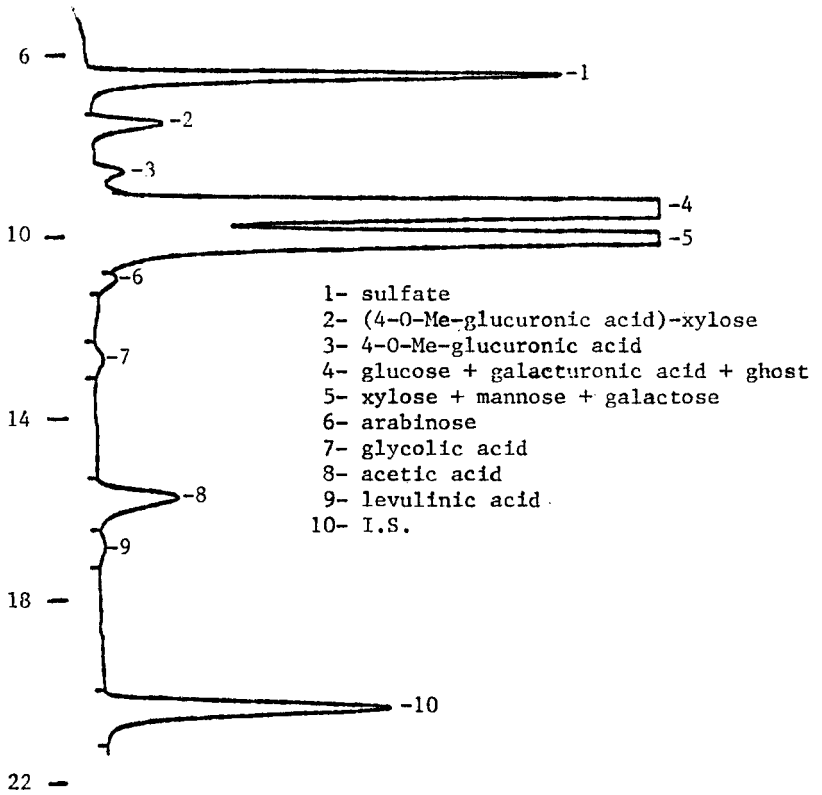

Figure 6. HPLC analysis of an acids #2 sample from aspen. This analysis quantifies the volatile acids, principally acetic acid.

cleavages taking place produce monomeric compounds such as glucose and xylose. It is these monomers that are then determined quantitatively by HPLC. In order to ascertain its true contribution to the original wood, the weight of each constituent determined quantitatively in the hydrolysate must be calculated stoichiometrically as the weight of that moiety as it occurs in the wood. All monosaccharides in the hydrolysates of extractive-free wood are present in the polysaccharides of the cell wall bonded through glycosidic linkages. Thus, each monosaccharide can be reported in

TABLE 1
Polysaccharide Summative Analyses for Quaking Aspen

	1	2	3	average
glucan	47.92	47.92	48.05	
HMF	0.45	0.43	0.46	
levulinic acid	0.41	0.24	0.41	
total glucan	48.79	48.59	48.93	48.77 ± 0.17
xylan	14.44	14.35	14.54	
2-F	0.99	0.89	0.99	
[4-O-Me-GUA]-X[1]	0.89	0.97	0.94	
total xylan	16.33	16.20	16.47	16.33 ± 0.13
arabinan	0.41	0.42	0.44	0.42 ± 0.02
galactan	1.09	1.22	1.11	1.15 ± 0.09
mannan	1.60	1.68	1.57	1.62 ± 0.06
4-O-Me-GUA	0.99	0.81	1.01	
4-O-Me-GUA-[X][2]	1.28	1.39	1.35	
total 4-O-Me-GUA	2.28	2.20	2.36	2.28 ± 0.08
galacturonic acid	0.60	0.60	0.60	0.60
glucuronic acid	0.13	0.12	0.17	0.14 ± 0.03
acetyl	3.60	3.54	3.62	3.57 ± 0.05
total	74.83	74.57	75.27	74.70 ± 0.18

[1]4-O-Me-glucuronic acid-xylose, xylan contribution
[2]4-O-Me-glucuronic acid-xylose, uronic anhydride contribution

the summative analysis as its pure theoretical homopolymer. Specifically, these are glucan, mannan, and galactan for the three hexoses and xylan and arabinan for the two pentoses. The sugars are represented as the glycosidically linked monomer, i.e. upon polymerization (ignoring the reducing end groups of the resulting polysaccharide) one mole of water is lost per mole of monosaccharide. On cleavage of the side chains of the hemicelluloses by the addition of water, i.e. in hydrolysis, it may be considered as addition of a hydroxyl to the glycosidic residue and of a hydrogen to the aglycone group. Thus, the group contributing the hydroxyl is considered to be present as an anhydride. The same relationship exists between the sugar contributing the alcoholic group and the acyl group of the acid, e.g. acetyl groups in the hemicelluloses. The stoichiometric factors used to calculate the weight of the moieties occurring in the wood based upon the weight of the

TABLE 2
Polysaccharide Summative Analyses for White Fir

	1	2	3	average
glucan	44.98	44.87	44.98	
HMF	0.54	0.47	0.59	
levulinic acid	0.46	0.47	0.57	
total glucan	45.98	45.81	46.16	45.98 ± 0.17
xylan	4.36	4.18	4.16	
2-F	0.26	0.26	0.35	
[4-O-Me-GUA]-X[1]	0.50	0.48	0.48	
total xylan	5.12	4.92	4.98	5.01 ± 0.10
arabinan	0.81	0.84	0.82	0.83 ± 0.02
galactan	1.47	1.44	1.48	1.46 ± 0.02
mannan	12.12	11.71	11.90	11.91 ± 0.20
4-O-Me-GUA	0.46	0.56	0.48	
4-O-Me-GUA-[X][2]	0.73	0.69	0.69	
total 4-O-Me-GUA	1.18	1.26	1.16	1.20 ± 0.06
galacturonic acid	0.50	0.50	0.50	0.50
glucuronic acid	0.10	0.07	0.06	0.08 ± 0.02
acetyl	1.25	1.27	1.26	1.26 ± 0.01
total	68.53	67.82	68.32	68.22 ± 0.36

[1]4-O-Me-glucuronic acid-xylose, xylan contribution
[2]4-O-Me-glucuronic acid-xylose, uronic anhydride contribution

TABLE 3
Stoichiometric Factors Used in Tables 1 and 2

compound	reported as:	factor used:
glucose	glucan	162/180
HMF	glucan	162/126
levulinic acid	glucan	162/116
xylose	xylan	132/150
2-F	xylan	132/96
4-O-Me-GUA-X	xylan	132/340
4-O-Me-GUA-X	uronic anhydride	190/340
4-O-Me-GUA	uronic anhydride	190/208
glucuronic acid	uronic anhydride	176/194
galacturonic acid	uronic anhydride	176/194
arabinose	arabinan	132/150
galactose	galactan	162/180
mannose	mannan	162/180
acetic acid	acetyl	43/60

constituents in the hydrolysates are given in Table 3. The general factor is given by the ratio: $(MW_{compound}-MW_{water})/(MW_{compound})$. Thus, in the case of the hexoses, the ratio is (180 - 18)/(180)=0.9. As an example, if the percentage of glucose was determined by HPLC to be 50%, the contribution of glucan to the summative analysis would be 0.9*50%=45%. The degradation products formed from hexose sugars, HMF and levulinic acid, are reported as glucan since it represents the majority of hexose in the wood. Similarly, the contribution due to 2-F is reported as xylan. The presence of glycolic acid in the acids #2 chromatogram (Figure 6) illustrates the effect that even a short exposure of sugars to alkaline conditions can have on composition. The acids #2 procedure involves concentrating, with the application of vacuum and heat, hydrolytic samples that have been neutralized to a pH in the range of 9 to 10. These reaction conditions are those that promote the ß-hydroxy carbonyl elimination reaction characteristic of carbohydrates. Numerous organic acids can be formed as products in this reaction[31]; glycolic acid is one. Thus, it is clear that this acid was produced as an artifact during the acids #2 procedure, was not produced during the original acidic hydrolytic procedure, and should not be included in the material balances.

CONCLUSIONS

As can be seen by the low standard deviations (Tables 1 and 2), the Bomb/HPLC analytical method offers reproducible quantitation of wood polysaccharide moieties. The use of the bomb during the autoclave hydrolysis retains the volatile constituents, thereby allowing for their accounting. The sample preparation procedures are straight forward and easily adapted to a routine. The ability to individually differentiate the uronic acids, including those still bonded to a xylose moiety, is an especially important advance in wood analysis.

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California, Berkeley. The current address for WEK is VPI & SU, Dept. of Wood Science, 210 Cheatham Hall, Blacksburg, VA 24061-0323.

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