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JOURNAL OF WOOD CHEMISTRY AND TECHNOLOGY, 13(3), 429-437 (1993)

SAMPLE PREPARATION FOR ANALYSIS OF WOOD SUGARS BY ANION CHROMATOGRAPHY

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ABSTRACT

We investigated the degradation of sugars during acid hydrolysis and the effects of other aspects of sample preparation on measurement of wood sugars by anion exchange HPLC using pulsed amperometric detection. Loss of sugars in standards ranged from 6.4% for arabinose to 15.7% for mannose. Measurements of wood samples to which standard was added before hydrolysis were very close to the sum of wood samples and standards hydrolyzed and analyzed separately except in the case of galactose, which was in significantly lower amounts (4.7% lower) in the combined samples. There was greater variance among injections of individual replicates than among replicates. Other, minor procedural details were investigated.

INTRODUCTION

Methodologies for analysis of wood sugars have undergone rapid advances in recent years. To replace paper chromatography and photometric analysis, gas chromatography following derivatization was developed.[1] Cation-exchange HPLC coupled with refractive index detection was found to be simpler and faster.[2,3,4] Acid hydrolysis in

sealed vessels was devised to retain volatile constituents, which can be analyzed along with the major sugars by HPLC.[5] Recently, anion-exchange HPLC with pulsed amperometric detection (PAD) was found to have the advantages of greater sensitivity and easier sample preparation.[6]

Quantitative data on the reproducibility, detection limit, and the need for sample neutralization are available,[6] but the effects of hydrolysis and other sample parameters have not been thoroughly explored. This work was conducted to clarify these aspects of the procedure.

EXPERIMENTAL

Wood samples of southern yellow pine (*Pinus taeda*) were ground in a Wiley Mill to pass a 40-mesh screen. After determination of moisture content, sufficient wood meal was weighed into tared hydrolysis tubes to yield 200 ±10 mg dry weight. The tubes were dried overnight at 40 C and 20 mm Hg and reweighed to determine the exact dry weight of the sample.

The hydrolysis followed procedures for determination of Klason lignin.[7,8] Two ml 72 ±0.1% H_2SO_4 (w/w) were added and the tubes were kept at 30 ±0.2 C in a circulating water bath with frequent stirring. One hour after addition of acid, 56 ml water was added, giving approximately 4% H_2SO_4 (w/w). Tubes were then covered with foil and autoclaved at 120 C for 1 hr.

The hydrolysate was poured and rinsed through glass fiber filters (Whatman 934-AH) and 1 ml fucose internal standard (20 mg/ml) was added. The hydrolysate was brought to 100 ml and one volume was diluted with 7 volumes water. An aliquot was passed through a Sep-Pak C_{18} cartridge followed by a 0.2 µm nylon particle filter. Samples were stored frozen before HPLC analysis.

The chromatographic equipment (Dionex) consisted of an eluant degas module, a GPM-II pump, a microinjection valve with a 25-µl sample loop, a Carbopac PA1 column and guard column, a post-column reagent delivery module, a PAD-II pulsed amperometric detector, and a 4400 integrator. The three pulsed voltages and their durations were E_1=0.05 V (480 ms), E_2=0.6 V (120 ms), and E_3=-0.6 V (60 ms); response time was 1 sec, sampling time was 200 ms, and output range was 1000 nA. Eluant was 2 mM NaOH; flow rate was 1.0 ml/min. To enhance detector response and stabilize the baseline, 0.7 ml/min 300 mM NaOH was introduced after the column. An excess of sample was loaded through the sample loop. The integrator was operated in internal standard mode. A run sequence consisted of 8 min of column regeneration (see below), 5 min of equilibration with eluant, followed by sample injection. The time from beginning of the run to elution of the last peak (mannose) was about 55 min.

The main experiment involved four treatments: a) unhydrolyzed standard; b) hydrolyzed standard; c) wood meal; and d) wood meal spiked with standard before hydrolysis. The standard stock solution contained (in mg/ml) arabinose (2), galactose (3), glucose (84), xylose (24) and mannose (10). The hydrolyzed standard was prepared by drying 1 ml stock solution in hydrolysis tubes in the vacuum oven and subjecting to the hydrolysis and sample preparation as described above. The unhydrolyzed standard was prepared with 1 ml of stock solution and 1 ml of fucose internal standard diluted directly to 100 ml and then following the same dilution, Sep-Pak and particle filtering as the hydrolyzed samples. The wood meal was hydrolyzed as described above. For the last treatment, 1 ml stock solution was added to hydrolysis tubes containing wood meal before drying. They were handled just as the wood meal alone except that, before injection, these samples were further diluted with an equal volume of water.

Each treatment was replicated with five analyses, and each replicate was injected three times. One of the five replicates of the unhydrolyzed standard was used for all calibration injections.

Values for samples with wood were adjusted to a sample weight of 200.0 mg to correct for the minor variations in actual sample weights. For wood samples to which standard was added, the correction was applied only to the portion of the value attributable to the wood, based on the corrected values for wood alone.

Analyses of variance were conducted with SAS (Statistical Analysis System, SAS Institute, Cary, North Carolina). The NESTED procedure, designed for hierarchical data sets, was used where there were equal numbers of replicates in the treatments being compared. In this case, replicates were nested within treatments and the variation among injections represents the "error." This procedure provides a calculation of the percent of variance attributable to each level of the hierarchy. Because one replicate of the hydrolyzed standard was lost, for that analysis it was necessary to use the GLM procedure (General Linear Models), which is a more general analysis but which can accomodate an unbalanced design. GLM uses the method of least squares. In this case, a nested model (concentration = treatment (replicate)treatment) was specified.

RESULTS

Initially, hydrolyzed samples and standards (but not unhydrolyzed standards) caused progressive decrease of retention times. Lengthy periods of column regeneration with 250 mM NaOH were necessary to maintain consistent retention times in successive injections. Conducting the hydrolysis with purified acid (Fisher Optima grade) did not resolve the problem, nor did neutralization of samples to pH 4 with NaOH. The problem was resolved by a combination of greater sample dilution and higher sensitivity settings on the detector (as described under Experimental) along with higher concentration of NaOH (300 mM) as column regenerant. We suspect that the sulfate binds rather strongly to the column, so that lesser loading and stronger regenerant may be necessary to maintain performance.

Under these conditions, separation of sugars (Fig. 1) is almost identical to that shown by Pettersen and Schwandt.[6] We have noted, however, that with wood species containing rhamnose, it co-elutes with arabinose under these conditions but is separated with higher concentrations of eluant. Unfortunately, mannose and xylose run together under the higher concentrations.

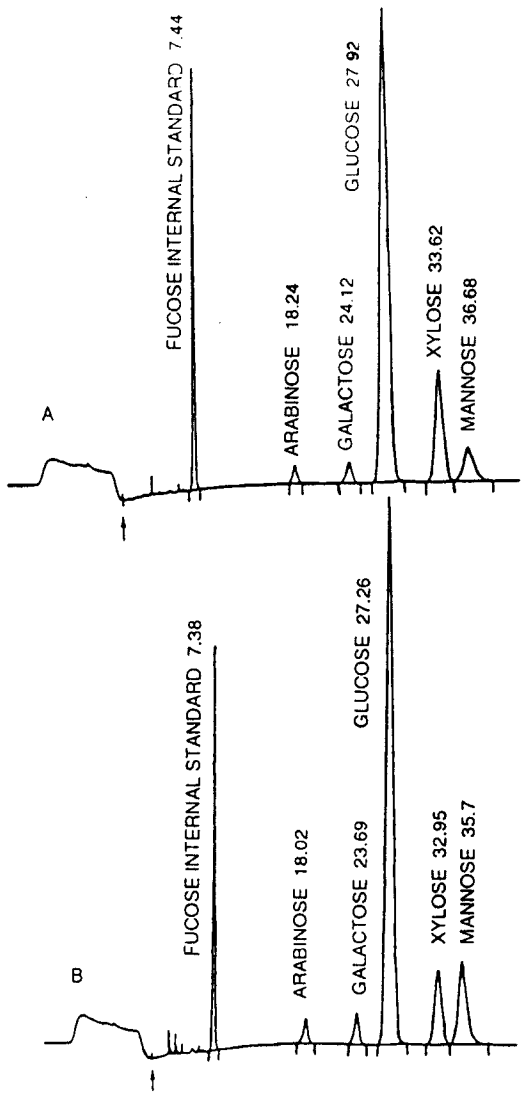

Fig. 1. Chromatograms of hydrolyzed standard (A) and pine sample (B). Column regeneration is followed by sample injection at time 0 (arrow). Retention times (minutes) are indicated with each component.

TABLE 1
Effect of Hydrolysis on Measured Amounts of Sugars in Standard

	Sugar amount (mg/sample)[a]				
	Glu	Man	Xyl	Gal	Ara
Standard: Unhydrolyzed (SU)	83.19	9.88	23.50	2.95	1.93
Hydrolyzed (SH)	77.11	8.33	19.88	2.70	1.81
% Loss (SU-SH)*100/SU	7.3	15.7	15.4	8.5	6.4
Probability$_{SU\ vs.\ SH}$[b]	<0.001	<0.001	<0.001	<0.001	0.056
Coefficient of Variation (%)	0.6	4.8	1.2	4.7	8.4

[a]Values are the means of 5 replicates, each injected 3 times, except that only 4 replicates were used for hydrolyzed standard (SH).
[b]Significance levels of the differences between SU and SH, calculated using the GLM procedure and a nested model.

As expected, there was a loss of sugars in the standard during the hydrolysis procedure (Table 1). The magnitude of the loss varied among sugars, but was not related to concentration. The loss was highly significant for all sugars except arabinose, which was present in the lowest concentration. The coefficients of variation (= relative standard deviation, standard deviation as percentage of the mean) were inversely related to concentration, being highest for arabinose.

The measurements for pine samples to which standard had been added before analysis were very close to the sum of values for hydrolyzed standards plus pine alone (Table 2). The difference was less than 2% except for the minor components arabinose and galactose, and was statistically significant only for galactose. The experiment was repeated with similar results.

With such a nested and balanced experimental design, it was possible to calculate the percent of variance attributable to the treatment (sum of pine (P) plus hydrolyzed standard (SH) run separately versus the two combined in a single sample (PS)), the replicates, and the injections. Much more of the variance was among injections than among replicates (Table 2). Only for galactose was there a substantial variance due to the treatment.

TABLE 2
Sugar Measurements in Pine Samples With and Without Added Standard

		Sugar amount (mg/sample)[a]				
		Glu	Man	Xyl	Gal	Ara
Pine:	Alone (P)	90.93	22.39	12.76	4.15	2.61
	With Standard (PS)	169.04	30.16	32.35	6.53	4.24
% Diff.:						
(P+SH-PS)*100/(P+SH)		-0.6	1.8	0.9	4.7	4.0
Probability$_{P+SH\ vs.\ PS}$[b]		0.41	0.46	0.24	0.003	0.21
% Variance from:	Treatment	0	0	4	26	4
	Replicate	0	1	0	0	0
	Injection	100	99	96	74	96

[a]Values are the means of 5 replicates, each injected 3 times.
[b]Significance levels of the differences between P+SH and PS, calculated using the NESTED procedure.

Based on the data for pine alone, the polysaccharide contents of the pine wood were calculated. Values were corrected for the hydrolysis loss and then multiplied by the conversion factor for monosaccharides to polysaccharides (0.88 for pentoses, 0.9 for hexoses).[1] The results were 44.1% glucan, 12.0% mannan, 6.6% xylan, 2.0% galactan, and 1.2% arabinan. Coefficients of variation were calculated using the replicate means as variates. These were 0.7% for glucose, 1.6% for mannose, 1.2% for xylose, 1.7 % for galactose, and 8.3% for arabinose.

DISCUSSION

Losses during hydrolysis vary among investigations using similar hydrolysis procedures.[3,9] One source of variation may be the temperature of secondary hydrolysis, which is only coarsely controlled in an autoclave. The loss may be less when 3% H_2SO_4 (w/w) is used in the secondary hydrolysis.[10] In case of possible variation in hydrolysis losses among

experiments, we routinely hydrolyze the standard solution with each batch of samples and use it as calibration standard without correction.

The close correspondence between measurements of pine spiked with standard and the sum of measurements of sample plus standard alone indicates that, in general, the effect of hydrolysis on sugars is probably the same in wood. Thus, other wood components apparently do not protect sugars or enhance their degradation during hydrolysis. However, almost 5% less galactose was recovered in the pine with standard than in the sum of pine and standard analyzed separately, and the difference was highly significant. This suggests that galactose degradation during hydrolysis may be somehow enhanced by other wood components, and that galactose concentration may be slightly underestimated in such analyses.

The coefficients of variation in our analyses of pine are similar to those determined by others with the method.[6] The much higher variance among injections than among replications suggests that chromatographic technique and consistency in integration are very important for precise results. The components in lower concentration, particularly arabinose, have peaks so small relative to glucose that separate runs with different output ranges may be necessary in studies where precision is important for those minor components.

ACKNOWLEDGEMENTS

R. C. Pettersen and V. H. Schwandt, Forest Products Laboratory, Madison, were very generous in helping us to establish this procedure in our laboratory. D. H. Griffin provided helpful advice in the design and analysis of the experiments. The research was supported in part by a grant from USDA-CSRS.

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