0273-2289/88/1700-0137/$02.60

Comparison of Colorimetric and HPLC Techniques for Quantitating the Carbohydrate Components of Steam-Treated Wood

Scientific Note

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Index Entries: Carbohydrate quantitation; steam-treated wood; high performance liquid chromatography; colorimetric assays.

INTRODUCTION

Any economic and technical evaluation of a bioconversion process is dependent upon the reproducibility of analytical techniques that provide a reliable and quantitative characterization of substrates and products. The composition and eventual distribution of the carbohydrate content is of prime importance since the accurate calculation of conversion yields and overall efficiency are most influenced by these values.

Many traditional wood chemistry methods for the analysis of pentosan and hexosan in pulps are based on colorimetric assays such as the TAPPI standard method for pentosans in pulp. The anthrone reagent has been used for the quantitative determination of cellulose for many years (*1–3*). These assays are well established for typical pulping processes, but analysis of substrates for biomass conversion processes is not as straightforward since these materials have a less uniform composition as a result

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of either pretreatment conditions (steaming time, catalyst addition) or interfering components such as decayed wood and bark. Paper chromatography has been applied to the quantitative determination of carbohydrate in wood pulps (*4*). In addition, gas chromatography has been used for carbohydrate quantification in wood samples after derivatization to volatile compounds (*5–7*). Both the paper- and gas-chromatographic methods require lengthy and tedious procedures.

More recently, HPLC has been applied to the analysis of wood sugars. With the commercial availability of packed ion-exchange columns, applications in the pulp and paper industry have been established (*8,9*). Analytical columns with different counter-ions have been developed and used for the quantitative analysis of sugars in wood samples as well as for the identification of decomposition compounds involved in biomass conversion processes (*10–12*).

This paper shows a comparison between results obtained for the carbohydrate analysis of steam-pretreated aspenwood using colorimetric assays and HPLC. The limitations of the techniques are discussed as well as possible explanations for discrepancies obtained when the various methods are used.

METHODS

Klason and Acid-soluble Lignin

Klason lignin was determined according to the TAPPI standard method (T 222 os-74).

TAPPI useful method 250 describes a method for the determination of any lignin solubilized during the Klason lignin determination.

Pentosan Determination

Pentosan analyses were performed by the TAPPI standard method T 223 os-71.

Hexosan Determination

Hexosans were analyzed using the filtrate from the secondary hydrolysis of the Klason lignin determination. The hydrolysate of a 1 g sample of wood or pretreated wood was collected and diluted to a volume of 1 L in a volumetric flask. A 1-mL portion of this solution was pipeted into a test tube containing 3 mL of water and 1 mL of 72% sulfuric acid. Standard glucose solutions, containing 0.06 to 0.6 mg/mL, were treated similarly. A blank was prepared for each run consisting of 4 mL of water and 1 mL of 72% sulfuric acid.

The anthrone solution containing 0.5000 g of anthrone in 250 mL of slightly diluted concentrated sulfuric acid (34.8*N*) was prepared daily and used once it had sat for 4 h following preparation. Ten mL aliquots of this

anthrone solution were added to 5 mL of the diluted Klason lignin filtrates at 1 min intervals. The test tubes were then loosely covered and immersed in a vigorously boiling water bath for exactly 4 min in the procedure finally adopted. The tubes were then immersed in cold water at 20°C. The absorbance of the solutions was read against the blank at 625 nm using a Beckman DB-GT spectrophotometer.

The values for hexosan were, in some cases, corrected for interference attributable to the pentosan content. For most of the water-washed, steam-pretreated samples, no correction was necessary. The 4-min reaction time minimizes the influence of the pentosan while maximizing hexosan absorption. In the case of samples with greater amounts of pentosan, a correction was made based on a contribution curve developed from standard mixtures of glucose and xylose.

High Performance Liquid Chromatography

Two different ion-exchange columns were used for the analysis of hydrolyzed carbohydrates in the Klason lignin filtrates. The Aminex HPX 87P column (300 × 7.8 mm id; Bio-Rad Laboratories Ltd.) with lead as a counterion was operated with deashing microguard cartridges (Bio-Rad) using deionized water (Milli-Q system, Waters Scientific) which was degassed before use. The mobile phase for the HPX 87 H column (300 × 7.8 mm id; Bio-Rad) which contains a cation-exchange resin in the hydrogen form was 0.01*N* sulfuric acid. The flow rate was 0.6 mL/min. The column temperature was 85°C for the HPX 87P column and 70°C for the HPX 87 H column.

The HPLC system consisted of a Varian 5000 LC pump, a rheodyne injector (both 20 and 100 μL loops were used), a column oven and a Varian RI-3 refractive index detector. A Varian Vista 401 data control station was used for data acquisition and calculations.

Total glucan in the wood samples is estimated by combining the peak areas of cellobiose and glucose obtained from the chromatograms of the acid hydrolysates. Cellobiose and glucose are then converted into glucan equivalents, divided by the oven dry wt of material and multiplied by a hydrolysis loss factor. The conversion factor from glucose to glucan is 0.90. The conversion factor from cellobiose to glucan is 0.95. Following a similar calculation, xylose may be converted to xylan by multiplying by a factor of 0.88 and the corresponding hydrolysis loss factor.

In the case of the HPX 87 P column, where neutralization with saturated barium hydroxide to pH 5.5 was required, erythritol was used as an internal standard. When using the HPX 87 H column, however, the acidic hydrolysate was injected directly onto the column and no internal standard was used.

Steam Pretreatment

Steam pretreatment of aspenwood (*P. tremuloides*) was performed using the Forintek gun as described by Brownell et al. (*13*). The alkali ex-

traction of steam-treated aspenwood has been described by Levitin (*14*). H_2O_2-treated substrates were prepared by exposing the SHA-WI to a 1% (w/v) H_2O_2 solution according to the procedure given by Gould (*15*).

Abbreviations used with respect to steam pretreatments are:

SHA-WI	Steam-heated aspenwood, water-washed
SHA-WIA	Steam-heated aspenwood, water-washed, alkali-extracted
SHA-WI/H_2O_2	Steam-heated aspenwood, water-washed, treated with hydrogen peroxide.

RESULTS

Analysis of wood sugars by chromatographic and colorimetric methods requires an acid hydrolysis step to convert polysaccharides into monomeric sugars. During this procedure, some of the monomers decompose to products such as 5-hydroxymethyl furfural and furfural. The anthrone method includes these compounds in the total amount of carbohydrate observed since the technique is based on a color reaction with the decomposition products. Fig. 1 shows that for solutions of pure glucose and xylose, the absorbance observed is the same before and after acid hydrolysis, similar to that of the Klason lignin analysis. With HPLC analysis, however, decomposition of a small amount of sugars during acid hydrolysis must be taken into account, since only the sugars are analyzed and not the decomposition products.

Fig. 1 also indicates the linearity of absorbance with respect to concentration for solutions of glucose and xylose, which are the main sugar components in hydrolysates of hardwoods such as aspenwood. Other sugars such as galactose, arabinose, and mannose may also be present, but these do not amount to more than a few percent of the material. Although the anthrone reaction is carefully timed to optimize the survival of the hexosan with respect to pentosan, there is a contribution by the pentosan (mainly xylose) to the total absorbance. Consequently, a correction for the pentosan content must sometimes be made. This correction is significant for the analysis of untreated wood, where the ratio of xylose to glucose is quite large. However, in most of the steam-pretreated wood samples which have been water-and/or alkali-washed, the majority of the hemicellulose has been removed and the correction for xylose is negligible.

We have found that the contribution of xylose to the total absorbance of glucose–xylose mixtures varies with the amount of glucose present in solution. The xylose contribution to total absorbance is the observed absorbance obtained from a glucose–xylose mixture minus the absorbance that would have been observed if the glucose had been alone. Data in Fig. 2 show that the xylose contribution to the absorbance increases in a linear manner as the concentration of glucose in solution is

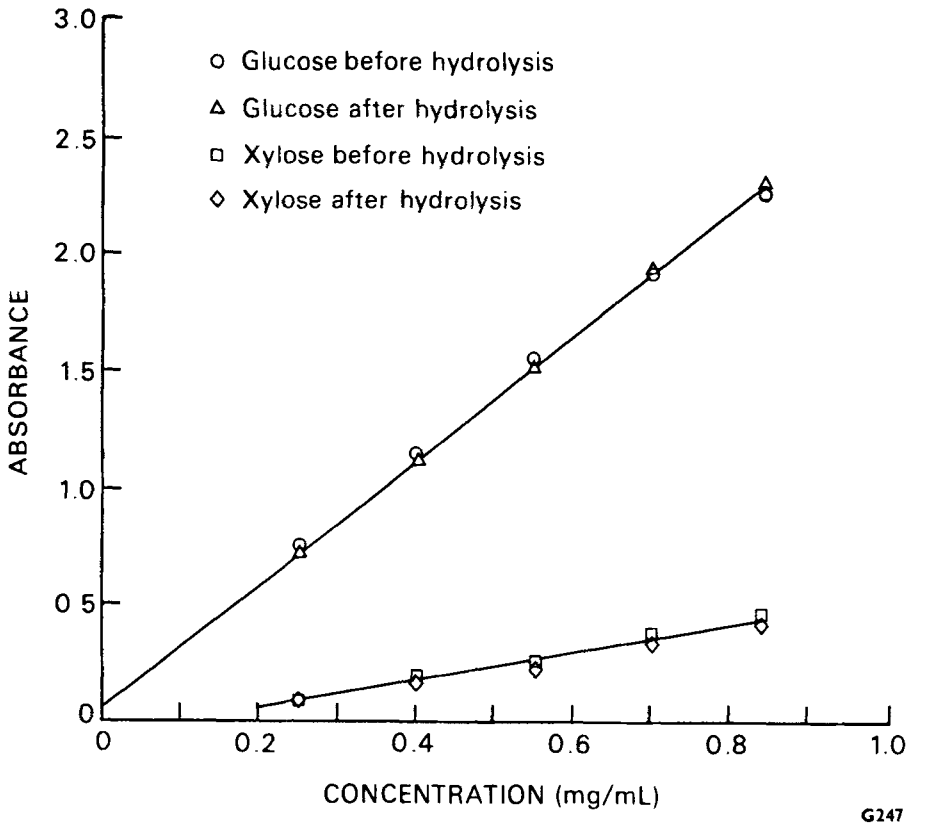

Fig. 1. Effect of acid hydrolysis of glucose and xylose solutions on absorbance produced from reaction with anthrone.

increased. It would seem beneficial therefore to use diluted Klason lignin filtrates in order to minimize errors for calculated hexosan values contributed by xylose.

Analysis of wood carbohydrates by HPLC eliminates the problems associated with interfering sugars. Wood sugars can be analyzed in a single analysis, whereas the colorimetric methods may require different analyses for different sugars (e.g., pentosan analysis is required in addition to the anthrone method for total carbohydrate analysis of wood substrates unless the pentosan content is known to be low).

Both of the HPLC columns which were used in this work are capable of separating and quantitating wood sugars. The HPX 87 P column allows separation of all the common wood sugars (glucose, xylose, galactose, arabinose, and mannose). The HPX 87 H column, on the other hand, does not resolve galactose and mannose from xylose. This does not present a problem in the case of most steam pretreated aspenwood samples since the levels of mannose and galactose are negligible.

Analysis of acidic Klason lignin hydrolysates on the lead column requires neutralization and concentration steps prior to chromatographic analysis. On the other hand, acidic hydrolysates may be directly injected onto the HPX 87 H column, which speeds up sample preparation time

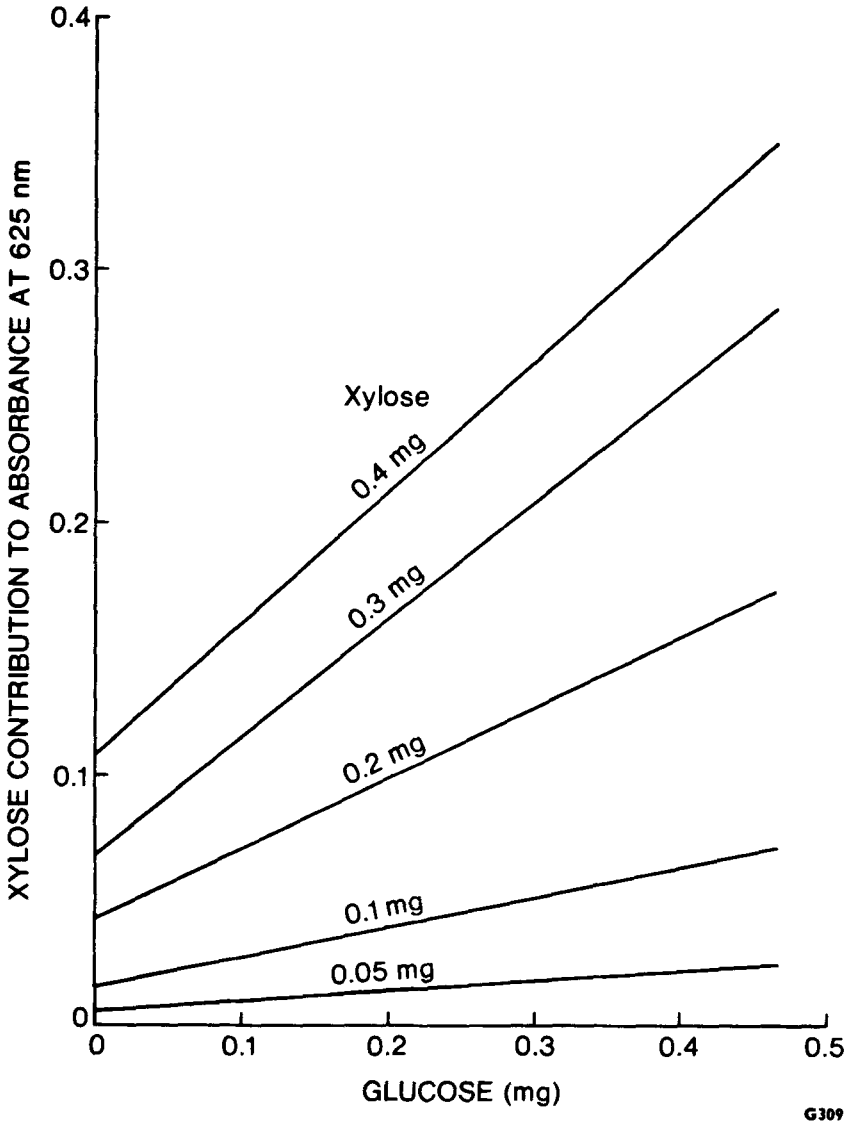

Fig. 2. Effect of concentrations of glucose and xylose on the xylose contribution to absorbance in anthrone assay.

considerably. Therefore, as long as there is no interference with galactose or mannose (as is the case with most hardwoods), then the HPX 87 H column is preferred over the HPX 87 P column for analysis of wood sugars.

Table 1 shows the chemical analysis of both extracted and unextracted aspenwood by HPLC and colorimetric methods. The agreement for glucan and xylan is very good when the 87 H and 87 P columns are compared. Likewise, the pentosan values determined colorimetrically are in good agreement with the xylan values determined by HPLC. However, the hexosan values from the anthrone method are slightly higher than the glucan values obtained by HPLC analysis. This may be explained for

Table 1
Chemical Composition of Aspenwood (percent oven-dried material)

	Klason lignin	Acid soluble lignin	Total apparent lignin	HPLC		Anthrone hexosan	TAPPI pentosan
				Glucan	Xylan		
Aspenwood (*P. tremuloides*)	20.3	3.0	23.3	43.7	20.5	47 .4	19.0
Extractive-free aspenwood	18.7	3.2	22.0	47.3 (45.2)[a]	21.2 (20.5)	49.8	19.9

[a]Parentheses indicate HPX 87P column, all other HPLC analysis HPX 87H.

these whole, unsteamed woods by the fact that mannose, galactose, and glucuronic acid all contribute to the absorbance produced by reaction with the anthrone reagent.

The chromatograms in Fig. 3 show the separation of a standard solution and a hydrolysate of steam pretreated aspenwood using the 87 H column. This column is capable of resolving cellobiose, glucose, and xylose as well as acetic acid, furfural, and hydroxymethyl furfural. With the 87 P column, analysis of five common wood sugars is possible (Fig. 4). Glucose and xylose are the major sugars present in steam treated aspenwood. Other material is present in small quantities, but does not appear to interfere with glucan and xylan analysis.

As was mentioned before, values for glucan and xylan on the basis of HPLC analysis must take into account the decomposition of sugars during acid hydrolysis of the material. Hydrolysis loss factors may be estimated by performing acid hydrolysis on solutions of pure sugars and analyzing the resulting solutions by HPLC. By this method, a hydrolysis loss factor of 1.055 was determined for glucose. In other words, about 95% of the glucose survives acid hydrolysis. This agrees well with the value given by Moore and Johnson (*16*). A hydrolysis loss factor of 1.155 for xylose was used in the present work.

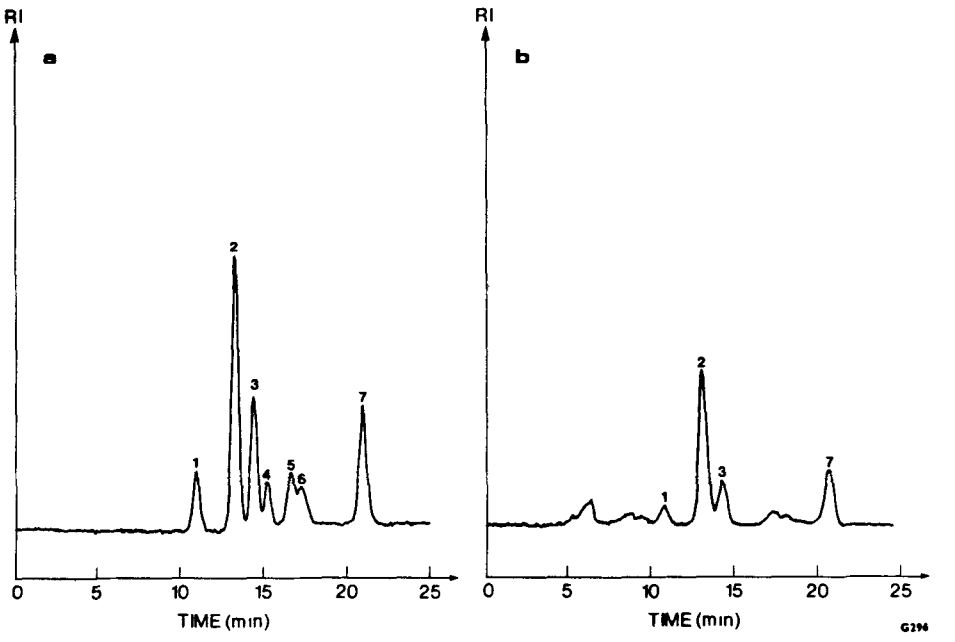

Fig. 3. HPLC chromatograms. Column: HPX 87 P + deashing microguard cartridges (Bio-Rad). Mobile phase: deionized water. Flow rate: 0.6 mL/min. Column temperature: 85°C. Detection: refractive index (RI). Peaks: 1 = cellobiose, 2 = glucose, 3 = xylose, 4 = galactose, 5 = arabinose, 6 = mannose, 7 = erythritol. 3(a), standard sugar solution; 3(b), steam pretreated aspenwood hydrolysate (SHA 25 min/190°C).

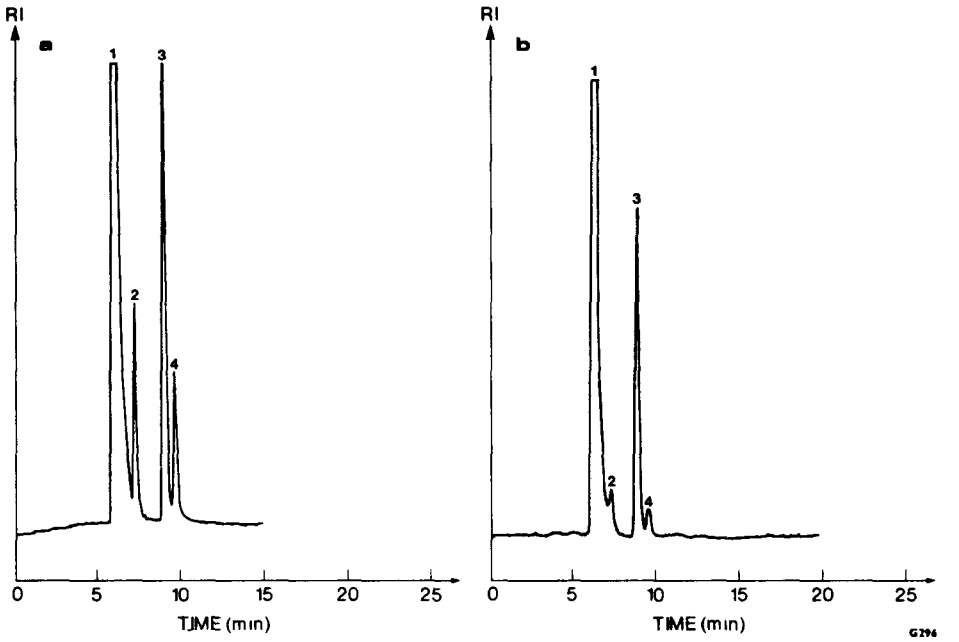

Fig. 4. HPLC chromatograms. Column: HPX 87 H + cation microguard cartridge (Bio-Rad). Mobile phase: 0.01*N* sulfuric acid. Flow rate: 0.6 mL/min. Column temperature: 70°C. Detection: refractive index (RI). Peaks: 1 = sulfuric acid, 2 = cellobiose, 3 = glucose, 4 = xylose. 4(a), standard sugar solution; 4(b), steam pretreated aspenwood hydrolysate (SHA-WI 40 s/240°C).

Table 2 and Fig. 5 show the chemical composition of a series of steam-heated aspenwood samples (*P. tremuloides*). The Klason lignin, acid soluble lignin, and carbohydrate contents of the samples are reported. Carbohydrate was analyzed by HPLC, using the 87 H column, and the anthrone method for hexosan content. None of the hexosan values was corrected for xylose contribution since there was not suffificent pentosan present to interfere with quantitation. The three sets of steam-treated aspenwood (steam temperature 240°C) include water-washed material (SHA-WI), water- and alkali-washed material (SHA-WIA), and water-washed material that had been given an additional treatment with peroxide (SHA-WI/H_2O_2). Increased steaming time resulted in lower xylan values being determined by HPLC, indicating the enhanced removal of the hemicellulose component at prolonged steaming.

In general,the anthrone values for hexosan and the HPLC values for glucan show identical trends with steaming time in all three cases. On average, however, the hexosan values from anthrone are slightly higher than the glucan values from HPLC. In particular, the substrates that were only water-washed show a relatively bigger difference in comparison to alkali-washed and peroxide-treated samples at longer steaming times. The differences observed with the SHA-WI samples remained rel-

Table 2
Chemical Composition of Steam-Heated Aspenwood (percent oven-dried material)

Sample	Steaming time at 240°C	Klason lignin	Acid-soluble lignin	Total apparent lignin	Glucan[a]	Xylan[a]	Hexosan[b]	Hexosan minus glucan
SHA-WI	20	23.0	2.3	25.3	57.4	12.1	60.8	3.4
	40	25.6	2.0	27.6	60.1	8.8	62.3	2.2
	80	28.9	1.8	30.7	62.5	3.0	63.4	0.9
	120	30.7	1.9	32.6	58.6	1.4	62.9	4.3
	180	33.6	2.4	36.0	56.1	<1	60.4	4.3
SHA-WIA	20	15.5	1.7	17.2	66.6	9.3	72.9	6.3
	40	12.6	1.3	13.9	78.0	6.8	79.9	1.9
	80	8.4	0.6	9.0	84.4	1.7	86.6	2.2
	120	3.5	0.4	3.9	90.1	1.0	92.5	2.4
	180	2.1	0.3	2.4	91.9	<1	91.8	−0.1
SHA-WI/H_2O_2	20	13.8	1.9	15.7	70.3	9.0	73.6	3.3
	40	10.0	1.5	11.5	77.6	7.1	80.3	2.7
	80	3.4	0.8	4.2	89.9	1.7	90.7	0.8
	120	3.0	0.5	3.5	92.0	1.1	92.6	0.6
	180	2.1	0.3	2.4	96.0	<1	94.9	−1.1

[a]Determined by HPLC, HPX 87 H column.
[b]Determined by anthrone method.

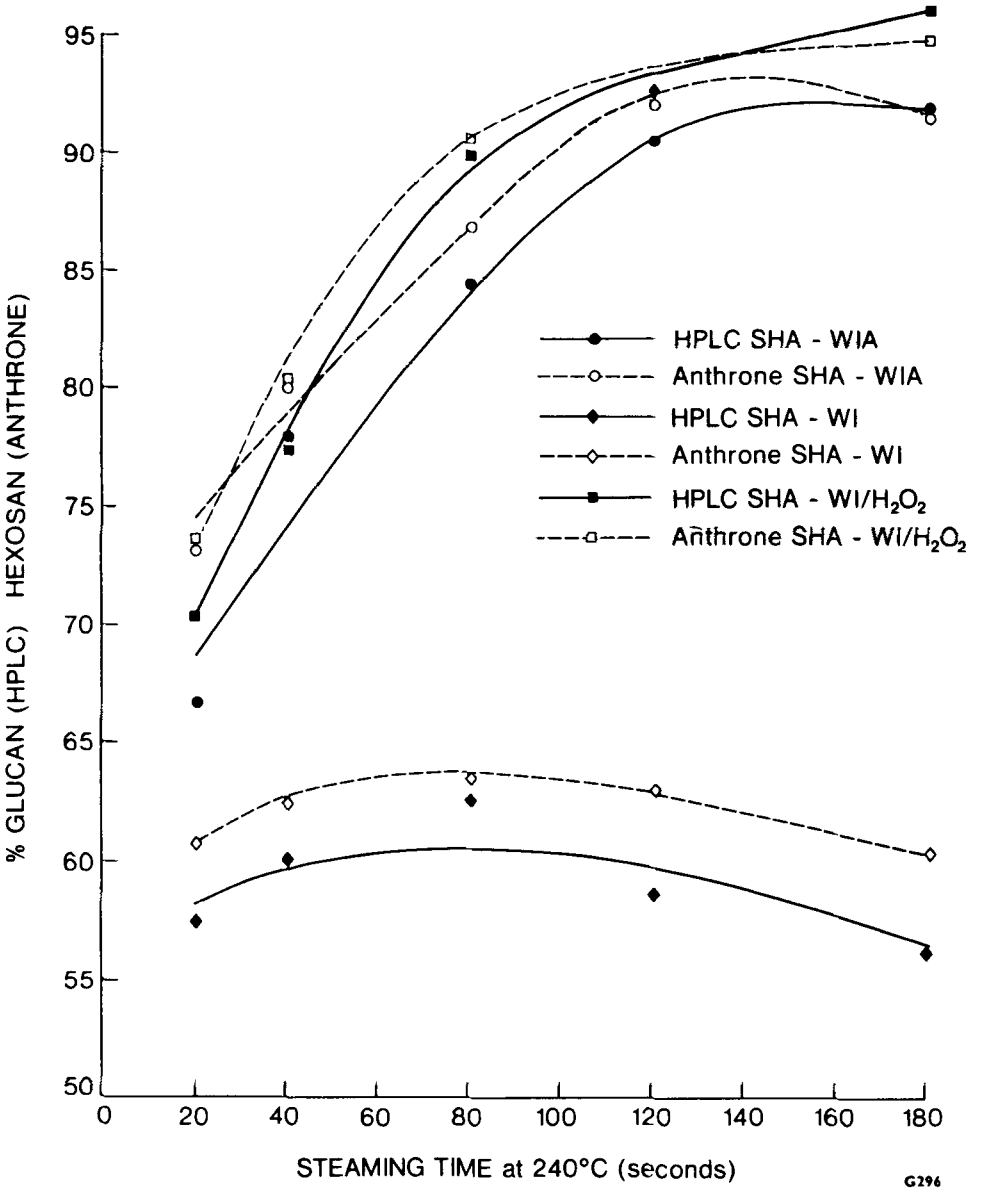

Fig. 5. Comparison of glucan analysis of steam-pretreated water-washed aspenwood by HPLC and anthrone methods.

atively constant with steaming time. On the other hand, the values for hexosan by anthrone and glucan by HPLC moved closer together at long steaming times in the case of the alkali-washed and peroxide-treated samples. This may be a consequence of the removal and/or destruction of material that interferes with the anthrone assay.

DISCUSSION

Both the anthrone and HPLC methods are useful for quantitating the amount of cellulose within steam-pretreated wood substrates. The

anthrone methode is well suited to laboratories without access to chromatographic methods. It has been shown that a correction must be made to account for pentosan contribution to the total absorbance in samples which contain a significant amount of xylose with respect to glucose. The TAPPI standard method for pentosan content may be used together with a xylose contribution curve to make the necessary correction. As long as the substrate is a hardwood which gives primarily glucose and xylose, the anthrone is a suitable technique. In the case of softwoods, which give large amounts of mannose, a correction for mannose would be necessary to obtain a meaningful value for glucan from the anthrone method. However, the anthrone method would also be useful with softwoods if the steam treatment and water washing removed most of the glucomannan and the method was applied to the water-insoluble fraction, as is commonly the case.

Both HPLC columns used were suitable for analysis of wood sugars obtained after acid hydrolysis of wood. In the case of the 87 P column, a relatively tedious and time-consuming neutralization step is required prior to analysis. The 87 H column, on the other hand, allows direct injection of acidic wood hydrolysates. Thus, the 87 H column is preferable when the principle sugars are glucose and xylose. With softwoods, however, large amounts of mannose would interfere with the xylose peak and make quatitation of the xylan impossible. However, very often the hemicellulose would be removed after water washing and the 87 H column would be suitable for analysis of the water-insoluble substrate.

The facts that both the anthrone and HPLC methods show similar trends with steaming times and in most cases agree within 2% of the weight of material suggest that both techniques are suitable for many bioconversion applications. However, there are limitations to both techniques, especially when substrates such as softwoods and agricultural residues are used. It is essential that every effort is made to quantitate and characterize the carbohydrate components of biomass substrates, since attempting to calculate the amounts by difference or by using one analytical method can result in nonrepresentative values.

ACKNOWLEDGMENTS

This work was supported financially by the Canadian Forestry Service. Many valuable discussions with Dr. Roger Sutcliffe are gratefully acknowledged. Dr. Wolfgang Schwald is grateful to the "Fonds zur Foederung der wissenschaftlichen Forschung," Vienna, Austria for granting an "Erwin-Schroedinger scholarship" (Proj. No. J0128C).

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