UNDERSTANDING THE LIMITATIONS OF REMOVAL OF HEMICELLULOSES DURING AUTOHYDROLYSIS OF A MIXTURE OF SOUTHERN HARDWOODS

Mehmet Sefik Tunc, Martin Lawoko,* and Adriaan van Heiningen

Extraction of hemicelluloses from wood prior to pulping is being pursued to generate more value-added products, while still producing high quality pulp from the pre-extracted wood. For a better understanding of the factors limiting selective extraction, enzymatic hydrolysis in combination with size exclusion chromatography (SEC) was applied to milled wood and hydrothermally treated wood. Complete dissolution was achieved in a lithium chloride/dimethylacetamide solvent system after mild ball-milling of a Southern Hardwood Mixture (SHM), of SHM extracted using auto-hydrolysis, and of enzyme-treated SHM. SEC tests showed that severe degradation of wood polymers occurred after a milling time of 3 hours. The SEC data also confirmed the presence of lignin-carbohydrate complexes. Based on the results, it is suggested that linkages between lignin and polysaccharides may play an important role in limiting extraction of hemicelluloses.

Keywords: Southern hardwoods; Autohydrolysis; Lignin carbohydrate complex (LCC); Prehydrolysis factor (P-factor); Endoglucanase; Endoxylanase; Size exclusion chromatography (SEC)

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INTRODUCTION

Stimulated by global environmental concerns and the growing cost of fossil fuels, there has been a dramatic increase in research and development of alternative products obtained from renewable resources. The forest product industry, which is based on a renewable feedstock, is well placed to produce some of these new products. For example, the production of value-added products from hemicelluloses extracted from wood prior to the pulping has recently been described (van Heiningen 2006). Significant amounts of hemicelluloses (40-80% on hemicellulose basis, depending on the wood type and extraction conditions) can be extracted with hot water. The extraction is autocatalytic, resulting from the production of acetic acid from the deacetylation of xylan (Brasch and Free 1965). The resulting acidity has also been reported to cause degradation of the wood polymers and their subsequent dissolution (Garrote et al. 1999; Casebier et al. 1969, 1973). In a recent study, however, it was reported that the dissolved xylans from hardwood were still significantly acetylated (Tunc and van Heiningen 2008a), and that deacetylation occurred after the dissolution of xylan. Thus, the sequence of reactions leading to the dissolution of hemicellulose still requires further study. Another issue requiring further clarification is why the removal of hemicelluloses is incomplete, even

after severe autohydrolysis conditions. Yang and Wyman (2008) discussed the importance of the degree of polymerization (D.P) for the solubilization of xylo-oligomers, and reported a D.P. in the range of 30 for corn stover during autohydrolysis in the temperature range of 200-240 °C. The same authors also emphasized the importance of role of lignin-xylan complexes in the hydrolysis of lignocellulosic biomass.

Several studies have proposed the presence of lignin-carbohydrate complexes in native wood, as summarized by Koshijima and Watanabe (2003) and Lawoko (2005). Recently, a new quantitative method to determine the amount of lignin covalently bound to carbohydrates (so called LCC) was developed for softwood (Lawoko et al. 2006), as well as chemical pulps made from them (Lawoko et al. 2003). However when applied to hardwood species, the LCCs were isolated in semi-quantitative yields (Henriksson et al. 2007). The occurrence of covalent bonds between lignin and carbohydrates may therefore be another factor responsible for the incomplete removal of hemicelluloses during pre-extraction with hot water (autohydrolysis).

In the present work, enzymatic hydrolysis in combination with size exclusion chromatography is applied to further understand the changes occurring in wood as a result of autohydrolysis.

EXPERIMENTAL

Materials

Endoglucanase (Novozyme 476) was purchased from Novozyme, Denmark. The xylanase used was a product of Fluka that was purified from *Trichoderma viride*.

A mixture of Southern hardwoods was used for the autohydrolysis experiments. The Southern hardwood mixture (SHM) contained sweet and black gum (35%), oak (35%), maple (15%), poplar and sycamore (12%), and southern magnolia (3%). The SHM chips were air-dried, ground in a Wiley mill, and the fraction passing 2 mm holes was stored in double plastic bags for later experimental use.

The wood extractives were removed from the ground wood particles by dichloromethane extraction to eliminate interference on the analysis of the carbohydrates and lignin in the extracted wood. The chemical composition of extractives-free SHM is summarized in Table 1.

Methods

Autohydrolysis of wood

Hemicelluloses were extracted with water at 150 ºC for 15 to 500 min and for 100 min at 130 ºC to 170 ºC in a modified Dionex ASE-100 extractor. The heat-up time varied with desired temperature and was 6 minutes for 130 ºC, 7 minutes for 150 ºC, 8 minutes for 160 ºC, and 9 minutes for 170 ºC. The time at temperature was corrected for heat-up time using the Dionex program of the modified ASE-100 (Tunc and van Heiningen 2008a). The liquor to wood ratio (L/W) was approximately 3.7:1. The amount of water required to displace the extract in the ASE-100 after autohydrolysis was found to be 1.5 cell volumes, as determined from the outlet concentration profile.

Table 1. Chemical Composition of Extractives-Free Southern Hardwood Mixture (% on extractive free original wood)

Chemical Component	Original Wood	Extracted wood, 150C, 100 minutes	Extracted wood, 170C, 100 minutes
Arabinan	0.52	0.14	0.04
Galactan	1.00	0.61	0.15
Glucan	43.66	42.39	41.81
Xylan	15.48	13.58	5.02
Mannan	2.18	1.94	1.22
Lignin	28.60	26.15	24.46
Ash	0.38	0	0
Acetyl groups	3.30	1.87	1.07
Uronic Acid Groups	4.47	1.79	0.78
Total	99.59	88.47	74.55

Analysis of wood particles

The moisture content of the milled wood particles was determined by drying a representative sample at 100 ±5 ºC in an oven overnight. The ash content was determined according to TAPPI standard method T211 om-85. Acid insoluble lignin concentration i.e., Klason lignin, was determined according to a method by Effland (1977), while the acid soluble lignin content was determined by TAPPI method 250. The uronic anhydride content was determined using the chromophoric group analysis method developed by Scott (1979). The mono sugar content was determined by High Performance Anion Exchange Chromatography with Pulse Amperometric Detection (HPAEC-PAD) of the hydrolysate, which was produced by two-steps acid hydrolysis with 72 and 4% sulfuric acid (Davis 1998). Acetic acid in the hydrolysate was determined by HPLC using a refractive index detector and BIO-RAD Aminex HPX-87H column. The mobile phase used was 5 mM H_2SO_4 with a flow rate of 0.6 mL/min and the oven temperature was 60 ºC. Pectins were not determined. However, omitting pectins results in minor mass balance errors, since the content of pectins in hardwoods is less than 1% (Fengel and Wegener 1984).

Enzymatic hydrolysis

The cellulase treatment was performed as described by Lawoko et al. (2006). 9.5 ml of 100mM sodium acetate at pH 5 was added to 150 mg of ball-milled wood or to ball-milled hydrothermally treated wood. After 10 minutes of stirring at ambient temperature, 0.5 ml of Novozyme 476 was added. The mixture was incubated at 60°C with gentle stirring for 12h.

In the case of the xylanase treatment 100 mg of the milled samples was used. To these samples, 10ml of 100mM sodium acetate at pH 5 was added, stirred for 10 minutes, and then 3 mg of xylanase was added. Incubation was performed under identical conditions as the cellulase treatment.

After enzymatic hydrolysis, the samples were centrifuged and the hydrolysate separated from the solid residue. The latter was washed, and the first wash was added to

the original hydrolysate and stored for further sugar analysis after secondary acid hydrolysis. The solid residue was washed 3 times with centrifugation. The wash water was discarded. The washed solids were then dried in a vacuum oven at 40°C for 3 hours prior to SEC analysis.

Ball-milling of wood

About 200 mg of extractive-free wood was ball-milled in a laboratory vibratory mill (stainless steel milling jar dimensions: 18mm wide, 48mm long) for 3 hours at 100 vibrations per second.

Pure lignin analysis

To determine the presence of any carbohydrate-free lignin generated by ball-milling, the ball-milled wood samples were extracted with dioxane:water (96:4), a common lignin solvent for milled wood lignin (MWL) (Bjorkmann 1957), followed by UV-Vis analysis at 280nm.

Dissolution of ball-milled wood in Lithium Chloride/Dimethylacetatmide (LiCl/DMAc) solvent system

The first part of the procedure was as described by Sjoholm et al. (2000) for pulp dissolution. However, in the present study, ball-milled wood and hydrothermally treated wood were used. To 30mg of ball-milled SHM and hydrothermally treated SHM, 3ml of 8% LiCl/DMAc were added. The solution was then gently stirred at 4°C for 24 hours. The resulting activated wood was then diluted with DMAc to a total volume of 30ml. At this stage, no significant dissolution was observed. Then, in this modification, the samples were left to stand at 25°C for 4 days with occasional shaking. Complete dissolution was observed for all SHM samples.

Size Exclusion Chromatography (SEC)

The centrifuged and clear solutions were analyzed using a Viscotek GPC max system equipped with a VE 3580 refractive index (RI) detector, VE3210-UV-Vis detector, and an IV-DP viscometer (intrinsic viscosity-differential pressure) detector. The RI detector was operated at 25°C. Three PLgel columns (300 x 7.5mm) equipped with a guard column (50 x 7.5mm) from Polymer Laboratories, were used coupled in series. The separations were performed at a column temperature of 80°C with 0.8% LiCl/DMAc as eluent operating at a flow rate of 0.7 ml/min. For calibration, Pullullan standards from Polymer Laboratories 342 Da, 1320 Da, 5900 Da, 11800 Da, and 22800 Da were used.

RESULTS AND DISCUSSION

Autohydrolysis of Wood

Hemicelluloses were extracted from the mixture of southern hardwoods (SHM) using hot water at extraction times varying from 15 min to 500 min at 150 °C and at different temperatures of 130 °C to 170 °C for 100 minutes. The solid wood yield after autohydrolysis is plotted against P-factor in Fig. 1. The P-factor describes the effect of

time and temperature during autohydrolysis and is given by the following expression (Sixta 2006),

$$P - factor = \int_0^t \frac{k(T)}{k_{100^o C}} dt = \int_o^t e^{40.48 - \frac{15106}{T}} dt \quad (1)$$

where t is reaction time in hours, T is temperature in Kelvin, and k is the rate constant. The activation energy used in equation (1) was 125.6 kJ/mol (or 30 kcal/mol).

Figure 1 shows that the effect of time and temperature on the solid yield of SHM during autohydrolysis (extraction with hot water) can be expressed by a single relationship as a function of P-factor, as shown earlier by Tunc and van Heiningen, (2008b). The solid yield decreased rapidly with increasing P-factor up to 600 hours and slowed down at more severe conditions.

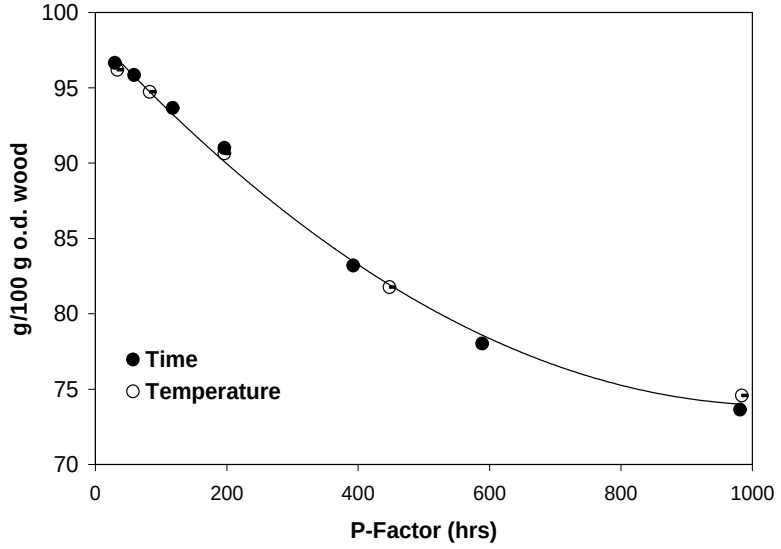

Fig. 1. Solid yield of southern hardwood mixture after autohydrolysis as a function of P-factor

The major wood components (cellulose, hemicellulose, and lignin) remaining in the wood after autohydrolysis are depicted in Fig. 2. A small amount of the total glucan was dissolved, likely resulting from the degradation of amorphous regions in cellulose, while a small amount of delignification took place. The majority of wood dissolution was due to hemicellulose removal. The exact composition of the original and extracted woods was presented in Table 1. It is clear that some hemicelluloses were still present in the extracted wood.

Effect of Ball-Milling on Residual Lignin Content

The total lignin content, i.e. the sum of the Klason and acid soluble lignin of original and pre-extracted wood, was determined. The pure lignin content of the ball-milled wood was determined by extraction with 96% dioxane. Both total and pure lignins are plotted against P-factor in Fig. 3. It is shown that between 25% and 50% of the lignin in the original ball-milled wood was recovered in pure form (i.e. 96% dioxane soluble)

after ball-milling. This is consistent with reports citing significant degradation of beta aryl ether linkages during the ball-milling procedure (Ikeda et al. 2002; Onnerud and Gellerstedt 2003; Guerra et al. 2006; Lechinsky et al. 2008). The formation of pure lignin has also been attributed to the degradation of LCCs during the ball-milling process (Lawoko et al. 2006). It is interesting that the amount of pure lignin (Fig. 3) slowly decreased with increasing severity, reaching a plateau at a P-factor of about 200 hrs.

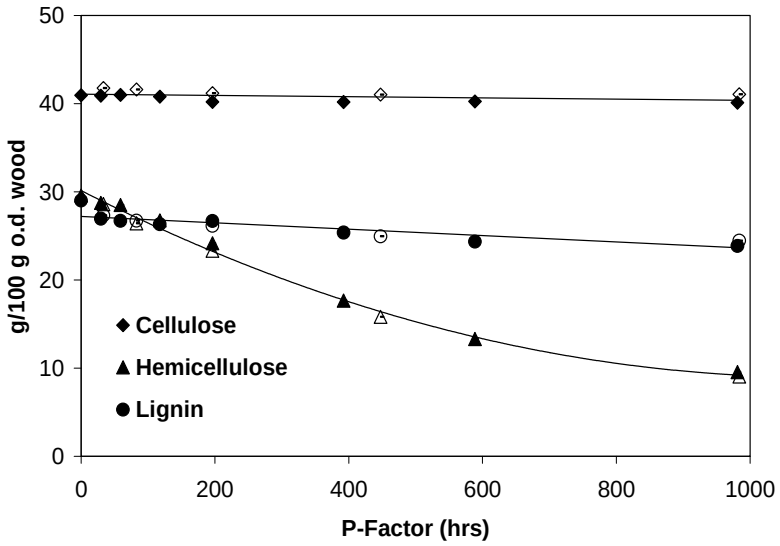

Fig. 2. Wood components remaining in southern hardwood mixture after autohydrolysis as a function of P-factor

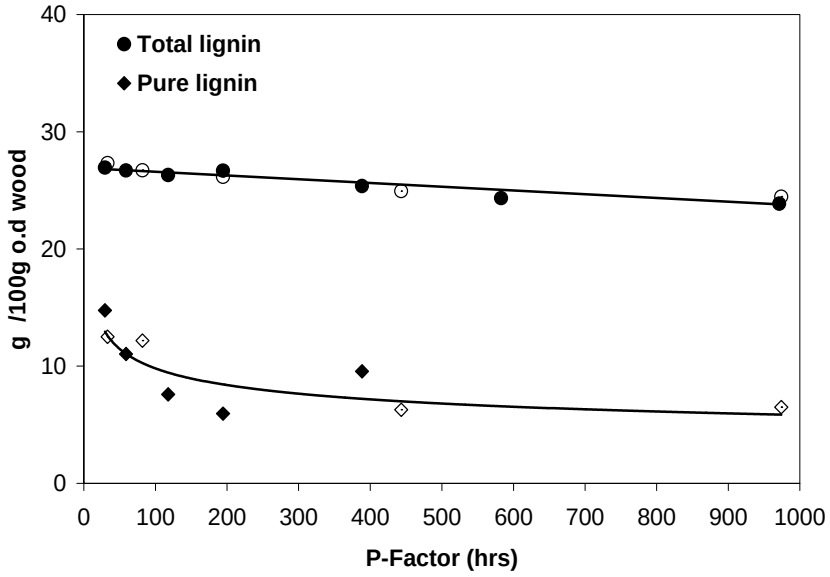

Fig. 3. Total lignin content of extracted wood and pure lignin in ball-milled wood as a function of P-factor

Size Exclusion Chromatography

Complete dissolution of ball-milled original Southern Hardwood Mixture (SHM), hydrothermally treated SHM, and enzyme treated SHM was achieved in the LiCl/DMAc system. When we tried to dissolve ball-milled Norwegian Spruce, no significant dissolution was obtained. This may indicate that softwood lignin was not reduced to soluble size by the milling condition. Alternatively, the insolubility may be attributed to the high content of glucomannan in softwoods as has been reported in the literature (Sjoholm et al. 1997).

Size Exclusion Chromatography with triple detection, applying a RI, IV-DP, and UV (set at 295nm) detectors, was used to study the behavior of carbohydrates and lignin during auto hydrolysis of SHM.

It must be emphasized that we observed that at the elution volume region investigated in this study, the IV-DP detector only responded to fractions containing carbohydrates. This finding is based on the observation that a commercial kraft lignin (indulin AT) did not give an IV-DP signal (Fig 4a), while birch xylan did at the same concentrations and SEC conditions as those applied to the ball-milled hydrothermal wood (Fig 4b). This is not surprising, since the intrinsic viscosity of branched polymers is generally lower than that of linear ones of the same molar mass. Thus, the IV-DP can be used selectively to monitor elution patterns of carbohydrates and their derivatives, e.g. lignin carbohydrate complexes in ball-milled hardwoods. The UV set at 295 nm monitors the lignin, and the RI is a universal concentration detector.

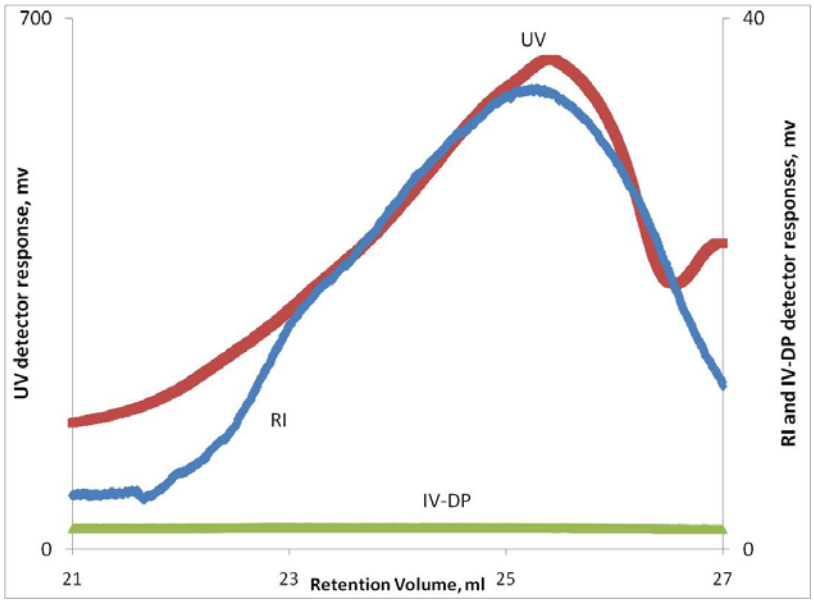

Fig 4a. SEC of Indulin AT

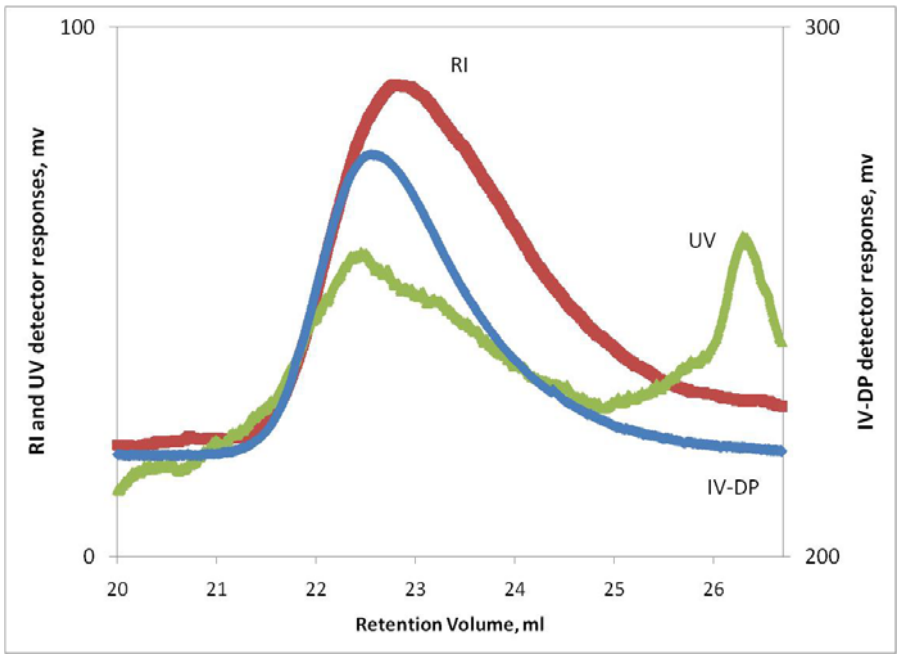

Fig 4b. SEC of commercial birchwood Xylan

In Fig. 5a, the SEC for the original ball-milled wood is presented.

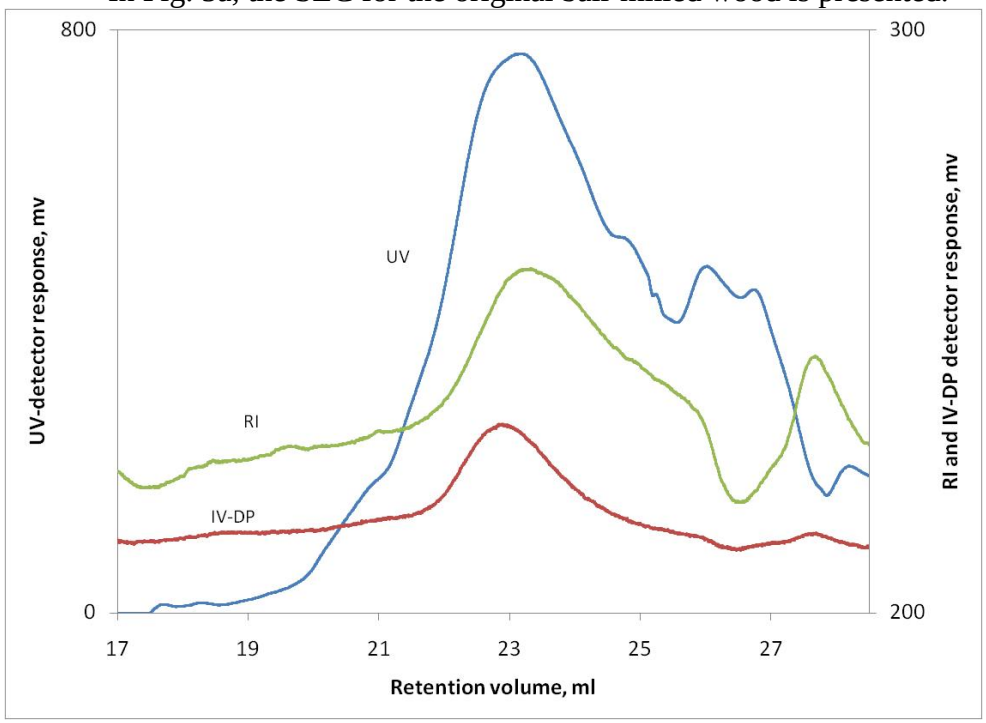

Fig. 5a. SEC of the original ball-milled SHM

The distribution shows that a majority of the polymers (reflected in the RI distribution) were of uniform hydrodynamic size and co-eluted at about 23ml. The co-

elution of lignin (reflected by UV-distribution) with the carbohydrates (IV-DP signal) may suggest inter-linkage with one another in LCC. The UV signal is multimodal, while that of the RI and IV-DP signal are bimodal. Bimodality in connection with the SEC of hardwood kraft pulp solutions in LiCl/DMAc has been reported (Westermark and Gustafsson 1994; Berggren et al. 2001), where the cellulose eluted in the higher molar mass region (lower retention volume) and hemicelluloses in the lower molar mass region (higher retention volume). Since we have observed that the IV-DP will only respond to polysaccharides in the investigated region, the bimodality of the IV-DP (Fig. 5a) indicates that the carbohydrates were co-eluting with lignin (represented by the UV signal) over the entire distribution.

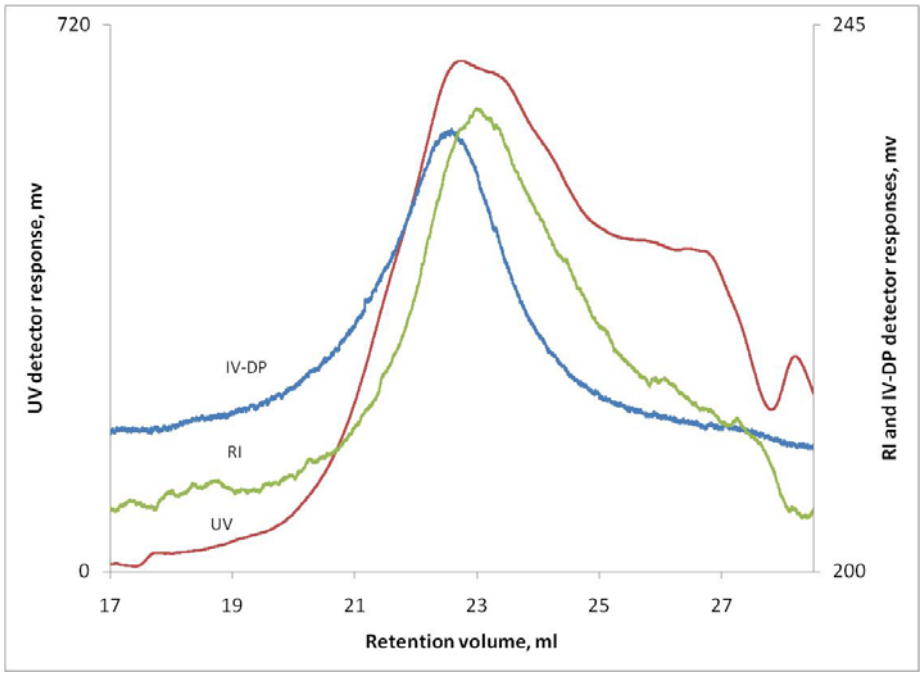

Fig. 5b. SEC of hydrothermally treated wood at 150 °C for 100 minutes

Comparing the SEC of the original ball-milled wood (Fig. 5a) with that of the hydrothermally treated wood at 150 °C (Fig. 5b), it was observed that the multimodality of the UV signal decreased, the RI showed a trend towards unimodality, and the IV-DP showed a unimodal distribution. The peaks at lower molar mass distributions (higher retention volume) in the original milled wood diminished, consistent with dissolution of hemicelluloses occuring during hydrothermal treatment of wood (Garrote et al. 1999; Tunc and van Heiningen 2008a). The absence of an IV-DP signal in the region between 25 and 28 ml, where a UV signal was present, indicates that carbohydrate-free lignin was eluted at this volume; however, the concentration was quite low, as manifested in the RI signal.

It is also interesting to note that the main UV peak in the original wood (eluting at about 23ml) began to split into two, indicating either the occurrence of lignin degradation at the prehydrolysis condition or the degradation of polysaccharide in LCC.

One of the emerging UV-peaks coincided with the IV-DP peak and may indicate enrichment of lignin carbohydrate complexes.

In Fig. 5c, the SEC of the hydrothermally treated wood at 170 ^{o}C is shown. The aforementioned UV peak splitting observed in Fig. 5b, is more pronounced, indicating that the reactions leading to this behavior were favoured by the increased pre-treatment temperature.

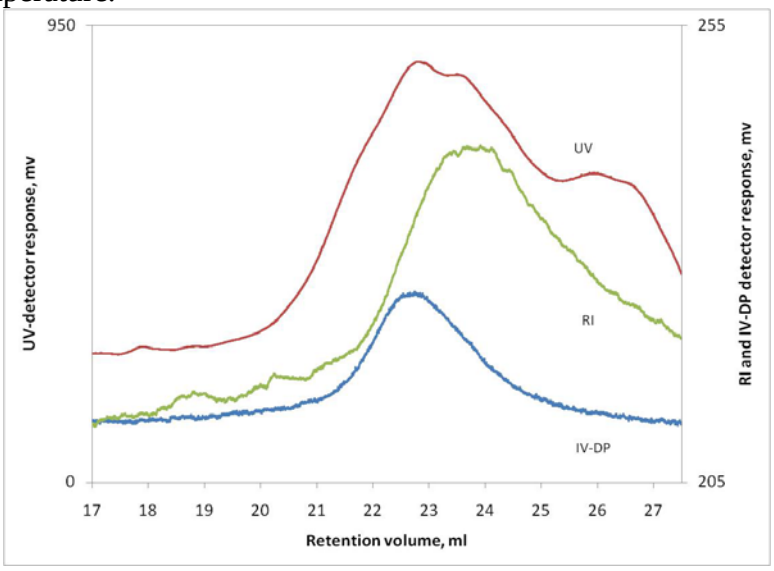

Fig. 5c. SEC of hydrothermally treated wood at 170 ^{o}C for 100 minutes

Using the Mark-Houwink constants for cellulose in Licl/DMAc (Bikova and Treimanis (2002), the molar mass distrubitions (MMD) of the original ball-milled wood was determined and plotted against the IV-DP detector response (Fig 5d).

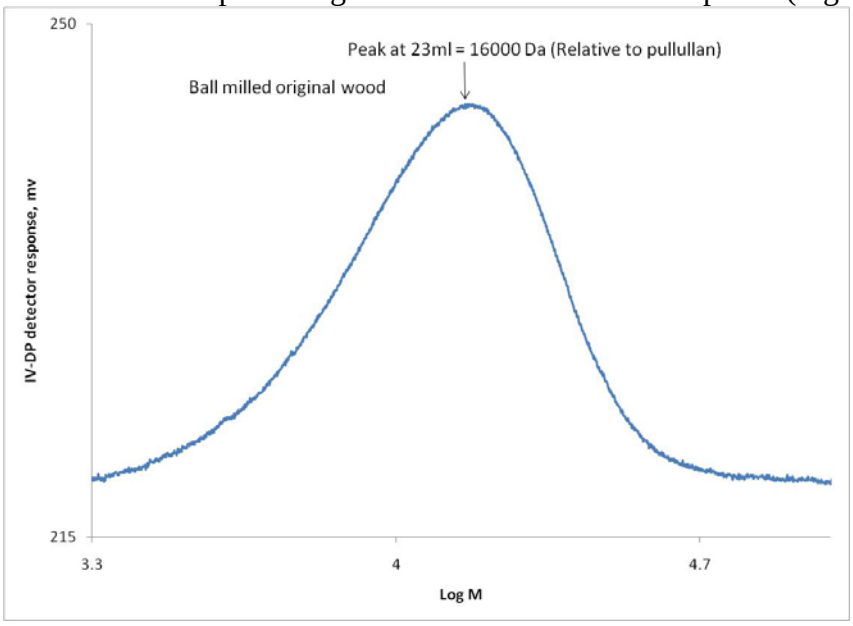

Fig 5d. Molar mass distribution of the original ball-milled wood

As observed in the figure, the ball-milled material eluting at 23ml had a peak value of about 16 000 Da. This indicates that severe degradation of the cellulose occurred during the 3 hour ball-milling, since wood since wood celluloses has a degree of polymerization of about 10,000. In connection with our discussion on the lack of IV-DP signal for pure lignin samples (Fig 4a), it is reasonable to assume that lignin molecules in the size range in proximity to 16,000 dalton do not give an IV-DP signal.

To test the hypothesis that co-elution of lignin with carbohydrates indicated in Figs. 5a-5c was due to the existence of chemical bonds between the two polymer types and not simply similarity in hydrodynamic volumes of the chemical components, the ball-milled and hydrothermally treated- woods were subjected to two separate enzymatic treatments with endoglucanase and endoxylanase. The yield loss in the solid phase on enzymatic hydrolysis and sugar yields on secondary acid hydrolysis of the enzymatic hydrolysate are shown in Table 2. It is interesting that cellulase treatment of SHM and hydro-thermally treated wood at 170 ^{o}C also led to significant solubilisation of the xylan, small amounts of the other sugars, and significant dissolution of lignin, apart from the expected degraded cellulose, and yet the enzyme (purified grade) lacks both hemicellulase and ligninase activities (Lawoko et al. 2006). In a similar manner, xylanase treatment led to significant disolution of cellulose and lignin, yet only minimal cellulase activity (<1%) is reported for this enzyme (Technical data from Fluka). Simultaneous solubilization of xylan, cellulose, and lignin upon treatment with xylanase has been reported by Furano et al. (2006). This observation may be a result of cross-linking of cellulose to xylan through lignin. The cross-linking of two different carbohydrates by lignin has been reported for softwoods (Lawoko et al. 2006).

The solid residue after the enzymatic hydrolyses was dissolved in the LiCl/DMAc system in the same manner as the ball-milled wood. The SEC was studied with special emphasis on UV detection to monitor the effects of the enzyme hydrolyses on fractions containing lignin. Since the enzymes lacked ligninase activity, the enzyme hydrolysis should have had no effect on the size of lignin, rather an effect on the size of lignin-carbohydrate complexes.

In Fig. 5d, the results of the SEC study for enzyme treatment of the original wood is shown. Notable differences were observed when compared with the reference orignal milled wood UV chromatogram. It can be seen that the enzymatic hydrolysis led to removal of the UV absorbance peak at high retention volume in the treated samples. Since the enzymatic treatment dissolves sugars, this is indicative of solubilisation of lignin-carbohydrate complexes. At the lower retention volume, it was observed that the broad UV peak associated with the untreated milled wood became narrower on enzymatic treatment, especially with the xylanase treatment. This supports the assertion that at least part of the polymers eluted in this region were also lignin-carbohydrate complexes. This interpretation is supported by the compositional analysis of the enzyme hydrolysate in Table 2, which shows that the removal of sugars by enzymatic hydrolysis was accompagnied by a large removal of lignin as well.

Table 2. Composition of the Enzyme Hydrolysate

	SHM original	T170°C original	SHM-cellulase hydrolysate	SHM-xylanase hydrolysate	T170°C - cellulase hydrolysate	T170°C - xylanase hydrolysate
Arabinan %*	0.52	0.05	0.3	0.35	0.02	0.06
Galactan %*	1.00	0.20	0.40[c]	0.44	0.22	0.13
Glucan %*	43.66	56.07	21.05 (53.6)[d]	31.7 (80.7)[d]	11.63	9.4
Xylan %*	15.48	6.73	10.28 (75.5)[d]	13.2 (96.9)[d]	2.46	3.1
Mannan %*	2.18	1.64	1.64	2.1	0.75	0.7
Total carbohydrate dissolved %*			33.67	51.7	15.1	13.3
Yield Loss (on solids basis, %[a])			55.7	72.4	42	38
Estimated lignin in hydrolysate (%)[b]			22.1 (76.2)[d]	20.7 (71.4)[d]	26.9	24.7

* Expressed as a % of solid material before enzymatic treatment. [a]Determined by weighing the dry solid before and after the hydrolysis. [b]Determined by subtraction of the total hydrolysed carbohydrates from the yield loss on solid basis. [c]Corrected for the amount of galactose present in the enzyme. [d]The percentage of the component in the solid material that was solubilized by enzyme. For lignin, the estimated amount in Table 2 was divided by the sum of Klason and acid soluble lignin in Table 1.

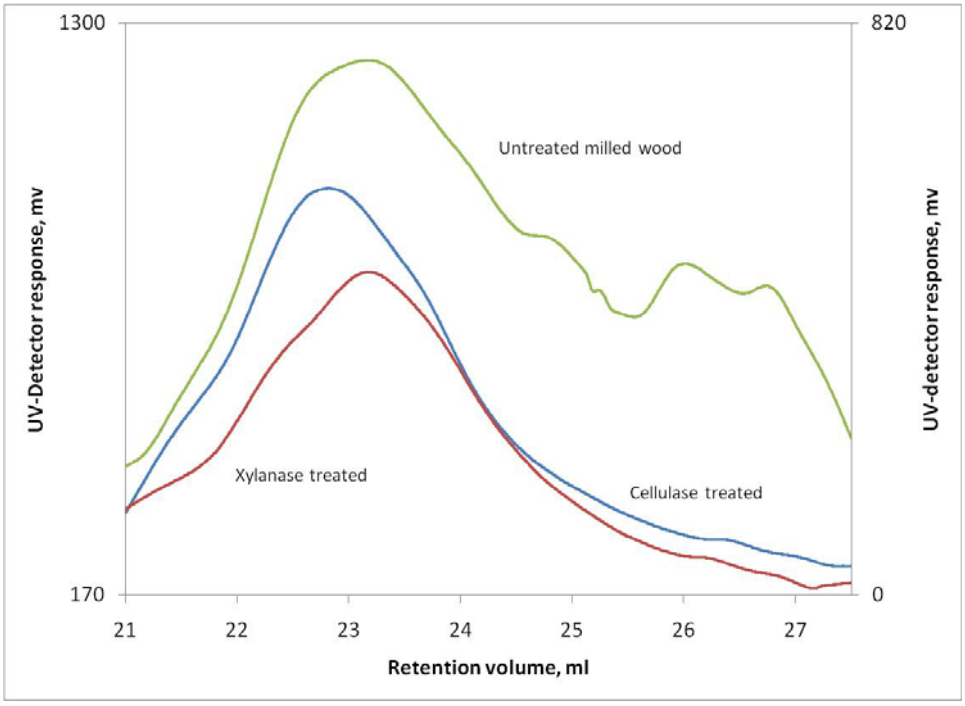

Fig. 5e. SEC of enzymically treated original wood

The ball-milled hydrothermally treated wood at 170 °C was also subjected to the enzymatic hydrolyses. From the SEC data (Fig. 5f), similar trends were observed in the high retention volume (lower molar mass fraction) section as in the enzyme-treated ball-milled SHM (Fig. 5d). Upon xylanase treatment, however, the double peak present in the untreated sample between 22.5 and 23.5 ml, shows some interesting features: it is narrowed to a single peak, placed in between the two peaks in the untreated sample. This indicates that a partial degradation of LCC is achieved, shifting the peak of lower retention volume observed in the untreated sample to the right. The abovementioned phenomena was also observed upon cellulase treatment, albeit to a lesser extent.

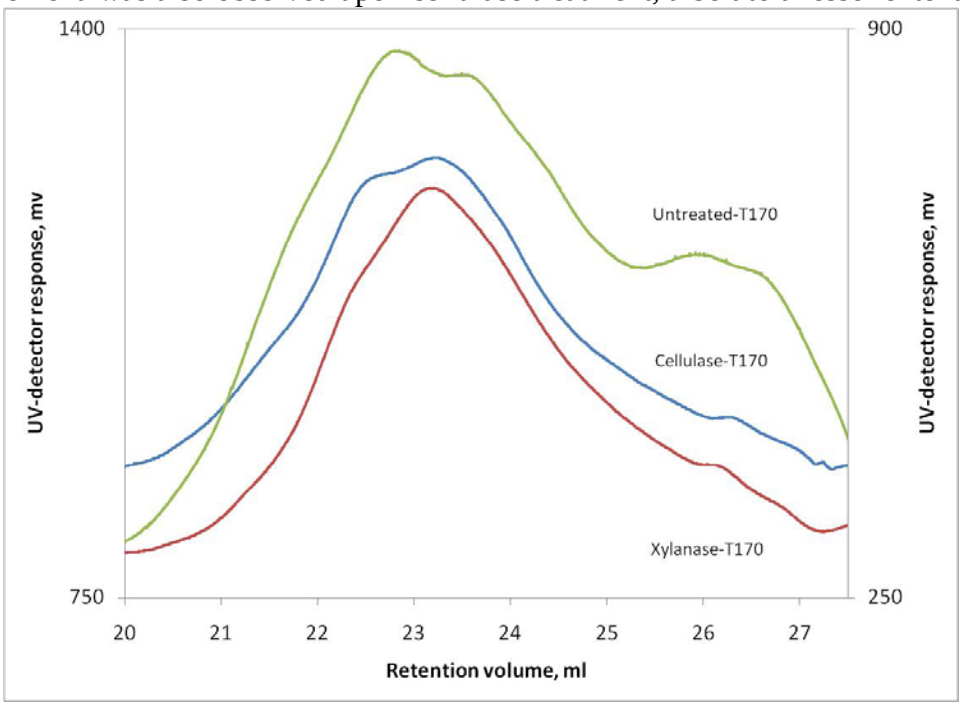

Fig. 5f. SEC of enzymically treated wood after hydrothermal treatment at 170 °C for 100 minutes.

CONCLUSIONS

The results described above indicate the presence of lignin-carbohydrate bonds in ball-milled Southern US hardwood mix and its pre-extracted solid residue. It is our tentative belief that the lignin-carbohydrate complexes in undegraded (un-milled) wood constitute insoluble macromolecular structures, which are enriched during auto-hydrolysis. This may explain the recalcitrance of hemicelluloses to complete dissolution during autohydrolysis of wood. This result is consistent with reports by Yang and Wyman (2008), who suggested that lignin-xylan complexes play an important role in the hydrolysis of lignocellulosic biomass. The present study, however, indicates that lignin-cellulose complexes may also be present in hardwoods, consistent with recent reports (Henriksson et al. 2007). The pre-hydrolysis of these bonds may play an important role when quantitative pre-extraction of hemicelluloses is desired.

ACKNOWLEDGMENTS

This research was made possible by financial support of the US Department of Energy and International Paper Corporation under contract FC36-04GO14306 and National Science Foundation under Grant No. 0554545.

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Article submitted: 26th October 2009; Peer review completed: Dec. 21, 2009; Revised version received and accepted: Jan. 3, 2010; Published: Jan 4, 2010.