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Molecular Weight Distributions and Linkages in Lignocellulosic Materials Derivatized from Ionic Liquid Media

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A novel and reproducible method is described for accurately determining the molecular weight distribution by size exclusion chromatography (SEC) of whole lignocellulosic materials. This approach offers the opportunity to compare the molecular weight distributions of intact milled woods and its component fractions, lignins and holocelluloses, all from the same source, thus highlighting the potential of the technique and the contributions of the individual components to the chromatogram. The method is based on the dissolution of the ball-milled samples in the ionic liquid 1-allyl-3-methylimidazolium chloride ([amim]Cl). Under these homogeneous ionic liquid media, a derivatization reaction was performed with benzoyl chloride in the presence of pyridine. The thoroughly benzoylated wood with its associated carbohydrate and lignin components was found to be completely soluble in the THF SEC eluent with marked UV detector sensitivity. This methodology, when applied to the individually isolated holocellulose and lignin (enzymatic mild acidolysis lignin; EMAL) materials from Norway spruce (*Eucalyptus grandis*) wood and corn stover, offered a better understanding as to the possible ways the lignin and the carbohydrates may interact within these three different species. Finally, the applicability of the methodology is shown for a series of pure cellulosic samples under intense mechanical defibration conditions, offering a visualization of the molecular weight distribution changes induced during the production of nanofibrillated cellulose.

KEYWORDS: Ionic liquid; [amim]Cl; lignin; holocellulose; wood; cellulase; benzoylation; gel permeation chromatography; lignin-carbohydrate complex, cellulose, molecular weight distribution

INTRODUCTION

Wood is the most abundant renewable lignocellulosic resource on Earth. It mainly consists of cellulose, hemicellulose, and lignin. Despite extensive investigations, the complex and irregular structure of wood is not completely understood. One of the most difficult problems in elucidating wood structure has been the isolation of the different fractions of wood, as close to their native state as possible, for subsequent chemical analyses. Recently Wu and Argyropoulos (*1*) have proposed a novel procedure for isolating lignin known as enzymatic mild acidolysis lignin (EMAL). The procedure is composed of an initial enzymatic hydrolysis of milled wood (MW), followed by a mild acid treatment stage. During the initial milling procedure, the degree of milling was found to affect the yield of EMAL and its molecular weight range. The cellulolytic action hydrolyzes most of the cellulose carbohydrates, whereas the mild acidolysis is designed to cleave the remaining lignin-carbohydrate bonds, liberating lignin in high yield and purity (*2*,*3*). Moreover, the high molecular weight range and complex structure make it sparingly soluble in most organic solvents. For attempted homogeneous derivatization, and to preserve the native structure of wood for analysis, it is important to find a nonderivatizing solvent, which will provide efficient dissolution and stability to various reagents. Ionic Liquids (ILs) have emerged as such solvents based upon the initial demonstration of efficient cellulose dissolution into 1-butyl-3-methylimidazolium chloride ([bmim]Cl) by Swatloski et al. (*4*). Other dialkylimidazolium chlorides such as 1-allyl-3-methylimidazolium chloride ([amim]Cl) have been shown to be effective media for the solvation of the more complex composite structures of intact wood and subsequent functionalization of the solvated hydroxyl groups as carbamates and acyl esters (*5*−*7*). We have also previously demonstrated that homogeneous functionalization of wood from IL media is possible (*5*, *8*); however dissolution as well as derivatization can be greatly facilitated by application of a preliminary physical treatment such as milling (*8*). This affords the opportunity to reduce artifacts due to the use of milder dissolution conditions required for total dissolution and homogeneous modification.

In the present study we used the opportunity to derivatize Norway spruce wood (*Picea abis*), *Eucalyptus grandis* wood, and corn stover (*Zea mays* L.) and to examine the molecular weight distributions by gel permeation chromatography (GPC) of the solubilized materials, recovered during the different steps of the

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EMAL procedure. Due to the lack of any significant chromophore in the polysaccharide portions (cellulose and hemicellulose) of lignocellulosic materials, we employed a novel benzoylation methodology in solvating IL media. During this procedure, essentially all lignocellulose hydroxyl groups (from both polysaccharides and lignin) were functionalized as benzoyl esters using benzoyl chloride and pyridine as the acid acceptor in [amim]Cl as the solvent medium. This facilitated visualization of all components by GPC with UV detection (*9*, *10*). The possibility of analyzing whole wood samples has previously been found to be useful in deciphering the complex structure of intact wood (*11*−*13*). Using comparative GPC analyses of isolated lignocellulose preparations and intact samples, one may obtain valuable information on the molecular weight distributions (*14*) and, in particular, arrive at information pertaining to the elusive lignin-carbohydrate linkages. There is a consensus among scientists that lignin is linked to different polysaccharides in the cell wall; however, the exact nature of such bonds is still a matter of significant discussion (*15*, *16*).

EXPERIMENTAL PROCEDURES

Lignocellulose Pulverization Procedure, Milled Wood (MW). The wood chips or stovers from different sources (Norway spruce wood chips, *E. grandis* wood chips, and corn stover) were ground to pass a 20-mesh screen in a Wiley mill and Soxhlet extracted with acetone for 48 h. Corn stover was Soxhlet extracted with a 1:2 ethanol/benzene mixture. The resulting Wiley-milled wood powders were air-dried and stored in a desiccator under vacuum. The eucalpytus wood powder was submitted to alkaline extraction with (0.075 mol/L) NaOH for 1 h (liquid-to-wood ratio of 50:1) to remove tannins before drying. The planetary ball milling was performed using a 250 mL zirconium-grinding bowl (zirconium dioxide 95%) in the presence of eight zirconium balls (10 mm in diameter each) using a Microwolf planetary mill (Torrey Hills Technologies, USA). The extractives-free wood powders were loaded into the grinding bowl to the amount of 5 g. The milling process was conducted at room temperature with a rotation speed of 400 rpm. The procedure involved the repetition of 30 min of milling and 15 min of cooling cycles. Total milling times of 5, 10, 20, and 30 h were applied.

Isolation of Lignins (CELs and EMALs). The MW from Norway spruce, eucalyptus, and corn stover was treated with cellulase (Iogen, Canada; filter paper activity, 130 FPU mL^{-1}) in a previously optimized ratio of 40 FPU g^{-1} of wood (*17*). The enzymatic hydrolyses were carried out at 40 °C for 48 h using 50 mM citrate buffer (pH 4.5) at 5% consistency (weight percentage solids content) in an orbital water bath shaker. The insoluble material that remained after the enzymatic hydrolysis was collected by centrifugation (2000*g*), washed twice with acidified deionized water (pH 2), and freeze-dried. The percent weight loss was calculated by dividing the weight of the recovered cellulolytic enzyme lignin (CEL) by the weight of the starting materials. The yield (%) for the CEL materials was calculated simply dividing the amount (g) recovered by the amount of the starting materials (g) and multiplying by 100. The recovered CEL was further submitted to a mild acid hydrolysis using an azeotrope (bp, 86 °C) of aqueous dioxane (dioxane/water 85:15, v/v, containing 0.01 M HCl) under an argon atmosphere. The resulting suspension was centrifuged (2000*g*), and the supernatant was carefully withdrawn, neutralized with sodium bicarbonate, and finally added dropwise to 1 L of acidified deionized water (pH 2). The precipitated lignin was allowed to equilibrate with the aqueous phase overnight, and it was then recovered by centrifugation, washed three times with deionized water, and freeze-dried to give the EMAL samples. The yield of lignin was calculated by using the formula

$$\text{yield (\%)} = \frac{\text{EMAL (g)} \times \text{EMAL lignin content (\%)}}{\text{MW (g)} \times \text{MW lignin content (\%)}} \times 100$$

Extraction of Holocelluloses (HOLOs). Holocellulose was prepared according to the procedure presented by Chang et al. (*18*). Three grams of MW was placed in a 250 mL Erlenmeyer flask, and 120 mL of water was added. The sample was left to soak for 5 min. Six grams of sodium chlorite (80%) and 24 mL of glacial acetic acid were added. The flask was placed into an oil bath at 90 °C, for 1 h, with stirring. The solution was filtered and washed in a preweighed sintered glass filter (grade C). The filtrand was oven-dried overnight to give the HOLOs. The yield of holocellulose was calculated by using the formula

$$\text{yield (\%)} = \frac{\text{HOLO (g)}}{\text{MW (g)} \times (100 - \text{MW lignin content) (\%)}} \times 100$$

Lignin Content. The purities of the samples were calculated by summing the acid-insoluble (Klason lignin) and acid-soluble lignin contents, measured according to the method reported by Yeh et al. (*19*). The values reported are the averages of three analyses ± 1.0% ($P = 0.05$, $n = 3$).

1-Allyl-3-methylimidazolium chloride ([amim]Cl) Synthesis. [amim]Cl was prepared according to a general procedure, provided by Zhang et al. (*20*), with a slight modification; both allyl chloride and 1-methylimidazole were distillated prior to use. [amim]Cl was further purified to remove trace color by dissolving the crude [amim]Cl mixture in water and refluxing with activated charcoal (18 h). The solution was filtered through a Celite plug, water was removed by rotary evaporation, and the concentrate was dried for 2 days under high vacuum to yield [amim]Cl as a pale yellow oil (99% purity by ^{1}H NMR): δ_H (300 MHz; $CDCl_3$; Me_4Si) 10.39 (1 H, s, NC*H*N), 7.65 (1H, s, C═C*H*), 7.40 (1H, s, C═C*H*), 5.86 (1 H, ddt, J = 16.9, 10.3, 6.5 Hz, C═CH_2), 5.33−5.26 (2 H, m, C═C*H*—C), 4.86 (2 H, d, J = 6.4 Hz, NCH_2), 3.97 (3 H, s, NCH_3). Water content was initially followed by ^{1}H NMR during drying and finally confirmed to be 0.4% by K_f titration; pH was tested by dissolving 1 g in 20 mL of deionized water to ensure neutrality.

Typical Benzoylation from [amim]Cl. [amim]Cl (950 mg) was added to the lignocellulosic material (50 mg) in an 8 mL sample bottle. The lignocellulosic material was dispersed with a fine needle and the mixture vortexed until all solid particles had dispersed. The sample was heated at 80 °C with stirring until the solution was clear (~2 h). Pyridine (230 μL, 2.6 mmol) was added, and the solution was vortexed until homogeneous and allowed to cool to about room temperature. Benzoyl chloride (280 μL, 2.4 mmol) was added in one portion and vortexed until a homogeneous white paste formed. The sample was left at room temperature for 3 h. Deionized water (5 mL) and ethanol (15 mL) were added. The mixture was vigorously shaken and vortexed for 5 min. The solid was filtered off through a sintered funnel (grade 3), washed with further ethanol, and triturated with methanol at room temperature for 18 h. The solid was then filtered off and dried under vacuum to give a white powder. Weight percent gain (WPG) was calculated for all samples. Theoretical WPGs were calculated using the values of 18.5 mmol g^{-1} of −OH groups for cellulosic materials and 6.0 mmol g^{-1} of −OH groups for lignins.

Tetrahydrofuran (THF) Solubility. An accurately weighed sample of benzoylated material was suspended in THF for 15 min. The suspension was filtered through a 0.45 μm PTFE HPLC syringe filter (Alltech, USA), and then the solution was dried under argon flow and the remaining material weighed. The percentage of the solubilized material, with respect to the starting mass, was calculated.

Preparation of Microfibrillated Cellulose (MFC). A sample of loblolly pine fully bleached pulp was suspended in water at a 2.0% (w/v) consistency, and it was passed through a microfluidizer processor (model M-110 EH-30). Size reduction of the product took place using a cellule of 400 μm. Three pumping cycles were used for the examined fibrillation process.

Gel Permeation Chromatography (GPC). GPC analysis of samples was performed using a Waters size exclusion chromatographic system with UV detection (280 nm) and differential refractive index (RI) detection. The analyses were carried out at 30 °C using THF as the eluent, at a flow rate of 0.7 mL min^{-1}. A 200 μL volume of the sample dissolved in THF (1 mg mL^{-1}) was injected into HR5E and HR1 columns (Waters) connected in series. The GPC system was calibrated with polystyrene standards in the molecular weight range of 890−1.86 × 10^6 g mol^{-1}. Millennium 32 GPC software (Waters) was used for data processing to obtain as numerical output M_p (peak molecular weight), M_n (number-average molecular weight), and M_w (weight-average molecular weight). The ratio M_w/M_n (polydisperisity) has been calculated as well.

^{1}H NMR Spectroscopy. ^{1}H NMR spectra were collected using a Bruker-300 spectrometer (operating at 300 MHz). The total number of

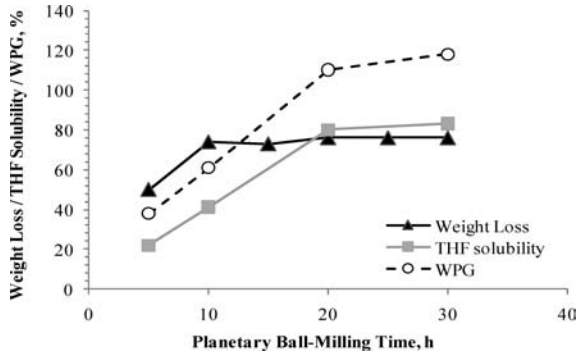

Figure 1. Weight loss during the enzymatic hydrolysis step (dot line), weight percent gain after benzoylation (black line), and THF solubility after benzoylation (gray line), as a function of planetary ball-milling time (h) for corn stover material.

scans for all experiments was 64 with an acquisition time of 1.60 s. $CDCl_3$ and DMSO-d_6 were used as locking solvent with a sample concentration of 25 mg mL^{-1}.

RESULTS AND DISCUSSION

In our efforts to develop the aforementioned methodology, we initially examined a number of variables such as the effect of ball-milling time on the solubility and benzoylation yields in IL, the effect of ball-milling time on the molecular weight distribution, and the effect of temperature during the benzoylation reaction. The optimized procedure was then applied to different lignocellulosic materials from the EMAL isolation procedure. The comparison of chromatograms of whole woods and their fractions (holocellulose and lignin) has permitted us to highlight the presence of lignin-carbohydrate complexes within the starting materials.

Effect of Milling on Solubility and Benzoylation. It is known that pulverization is a fundamental step in the EMAL lignin isolation procedure. Both particle size and degree of crystallinity substantially affect enzymatic digestibility. It was initially determined that the efficiency of enzymatic digestion (expressed by the weight loss, %) for planetary ball-milled corn stover increased as a function of milling time (**Figure 1**). No weight loss was observed when simple Wiley milled wood was directly treated with cellulase. After milling, the accessibility of milled wood to cellulase increased, as shown by the weight loss percent observed during the enzymatic treatment. **Figure 1** shows that the weight percent loss increases from 0 to 10 h of milling time, reaching a plateau at around 75%. The theoretical value of weight loss on the basis of lignin content data for corn stover should be about 81%. The milling allows us to reach almost the maximum theoretical weight loss for the cellulosic fraction after 10 h of treatment. This is in comparison to ca. 20 h of milling time for both the hardwood and softwood species, indicating superior processability for the annual plant material in comparison to the woody material.

The weight percent gain (WPG) and the THF solubility of the same samples, after dissolution in [amim]Cl and benzoylation, are also shown in **Figure 1**. The WPG represents the increase in weight after benzoylation, and it is a measure of the degree of substitution. In a manner similar to that observed for the weight losses, the increase in milling time led to increases in WPG and in THF solubilities for all species. As determined before, the degree of solvation in IL and hence the functionalization of the wood are highly dependent on the particle size of the wood sample. This insolubility is due to the complex and compact structure of the intact wood material, where strong associations between the lignin, cellulose, and hemicelluloses essentially prevent complete swelling and inhibit the diffusion of the ionic liquid into its interior, resulting in only the partial dissolution of wood.

Table 1. Solubility of IL Media Benzoylated Corn Stover, Milled for Different Times, in Chloroform ($CDCl_3$) and Dimethyl Sulfoxide (DMSO-d_6), and Integration Results from ^{1}H NMR[a]

MW corn stover	$CDCl_3$	DMSO-d_6	H-aryl/H-alkyl
5 h	−	+	na
10 h	−	++	1.47
20 h	++	+++	1.89
30 h	+++	+++	1.91

[a] The solubility has been evaluated by visual inspection: (−) not soluble, (+) slightly soluble, (++) almost soluble, (+++) completely soluble.

As the planetary milling treatments result in an increase of both the cellulase accessibility and the degree of functionalization, the solubility of the benzoylated materials was also found to be dependent on the milling time. Solubility in both chloroform ($CDCl_3$) and dimethyl sulfoxide (DMSO-d_6) was qualitatively examined for benzoylated corn stover by visual inspection (**Table 1**). The solubility of this material after 5 h of milling was poor. An increase in the milling time (10, 20, and 30 h) led to increased solubility in both solvents, until almost complete solubility was reached after 20 h of milling and benzoylation.

After partial or complete dissolution in DMSO, the benzoylated milled corn stover samples were analyzed by ^{1}H NMR, and the integration ratios for aryl (8.6−6.6 ppm) to alkyl (6.0−3.5 ppm) resonances were calculated, as a measure of the degree of benzoylation. The data, reported in **Table 1**, show an increase in the aryl/alkyl ratio most probably due to the increase in the degree of benzoylation. This is accompanied by an increase in the solubility of the samples. The ratio is reported to be 2.00 for completely benzoylated samples (*8*). The data are also in agreement with the increase in WPGs, illustrated in **Figure 1**. The increase in milling time permits the ionic liquid to progressively solvate the wood and allow for increased benzoylation. Short milling times are related to a low degree of substitution (low WPG and low aryl/alkyl ratio), whereas increased milling times permit almost complete benzoylation (high WPG and high aryl/alkyl ratio).

Effect of Milling on Molecular Weight. To study the effect of planetary ball-milling on the molecular weight distribution, the samples were then submitted for GPC analyses. The GPC results are illustrated in **Figure 2**, with a comparison between UV detection (280 nm) and RI detection. The relative absorbance of the chromatograms (*y*-axis) is normalized by the representativeness of the samples, expressed by the THF solubility.

It is remarkable that the molecular weight distributions show bimodality during UV detection, which is not very apparent from the RI-detected chromatograms. The two peaks present in the UV chromatograms could be related to the carbohydrate and lignin fractions, respectively. The 5 and 10 h samples showed low solubility in THF, and as such they were not fully representative of the whole fraction. In fact, the THF solubilities for these samples were only ca. 25 and 40%, respectively. The THF-soluble portion seems to be composed mainly of low molecular weight lignin and high molecular weight carbohydrate fractions. It should be considered that lignin has a higher UV mass extinction coefficient at 280 nm. For simple comparison we measured the extinction coefficients for benzoylated holocellulose and benzoylated corn stover EMAL. The values were found to be 43.2 and 129.0 L cm^{-1} g^{-1}, respectively. With longer milling times, the carbohydrate fraction became more soluble, indicative of cell wall fragmentation leading to a relative increase in the absorbance of the carbohydrate fraction. The analyses for the 20 and 30 h

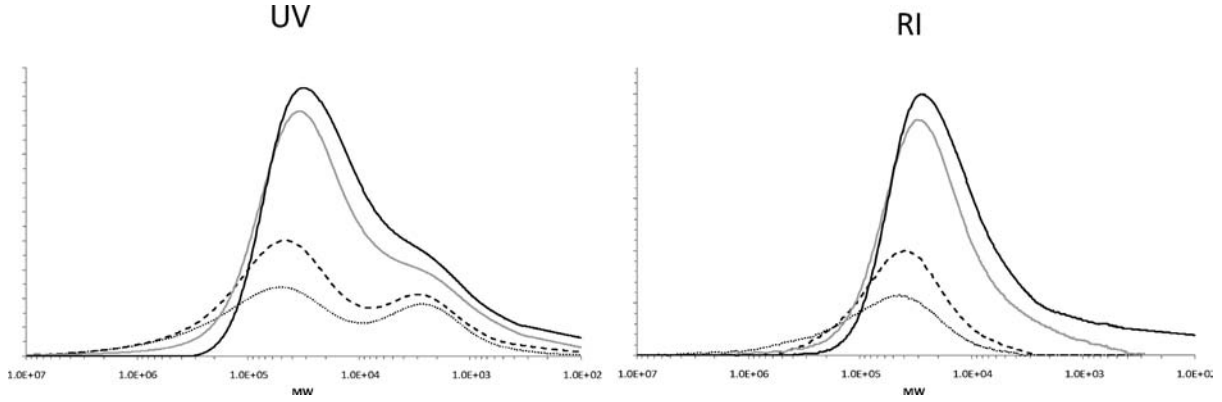

Figure 2. GPC chromatograms for benzoylated corn stover MW samples, milled to different degrees: 5 h (dotted line); 10 h (long dotted line); 20 h (gray line); 30 h (black line). Normalization of the chromatograms has been done by multiplying the absorbance by the THF solubility of each preparation (%, **Figure 1**).

Table 2. Numerical Output from the GPC Analyses for Benzoylated Corn Stovers Milled for Different Planetary Milling Times: 5, 10, 20, and 30 h[a]

	M_p				
sample	first peak	second peak	M_n	M_w	M_w/M_n
5 h	50200	2670	3330	191000	57.36
10 h	46000	2930	3300	96500	29.24
20 h	34200	4350	3630	42100	11.60
30 h	31500	3950	3950	24000	6.08

[a] The M_p values reported for the second peak in the 20 and 30 h samples were calculated by deconvolution from **Figure 2**, UV detection.

samples showed a slight decrease in the molecular weight distribution, and it became possible to note a limited loss of high molecular weight material between 10^6 and 10^5 g mol^{-1}. The main peak of the molecular weight distribution also shifted to a lower molecular weight. This result can be explained on the basis of degradation of the sample by the milling process.

These conclusions are confirmed by analyses of the data using RI detection. As mentioned previously, the second peak related to the lignin fraction (as observable with UV detection) was not detectable at all in the 5 and 10 h samples. The lignin solubilized by this treatment seems not to be connected to carbohydrates. With longer milling times (20 and 30 h) faint shoulders on the main RI chromatogram peaks appear at the low molecular weight range.

The numerical results from GPC analyses of the benzoylated corn stover MW samples are presented in **Table 2**. All of the samples showed a bimodal molecular weight distribution; this is most clear for the 5 and 10 h samples. For the 20 and 30 h samples the second peak, related to the lignin fractions, became a shoulder to the main carbohydrate fraction (**Figure 2**). From the data we calculated the molecular weight peak maxima (M_p), the number-average molecular weight (M_n), the weight-average molecular weight (M_w), and the polydispersities (M_w/M_n). The M_p values reported for the second peak in the 20 and 30 h samples were calculated by deconvolution.

The M_n values slightly increase with the milling time, whereas the M_w values are seen to rapidly decrease, leading to a decrease in polydispersity. The M_p for the carbohydrate fraction decreases with the milling time. The M_p for the second peak, related to the lignin fraction, increases from 5 to 20 h and slightly decreases for the 30 h sample. Taking into account the incomplete solubility in THF for the 5 and 10 h samples, it is likely that partial degradation of the 20 and 30 h samples is observed, and this is a fact responsible for the increased solubility of these samples.

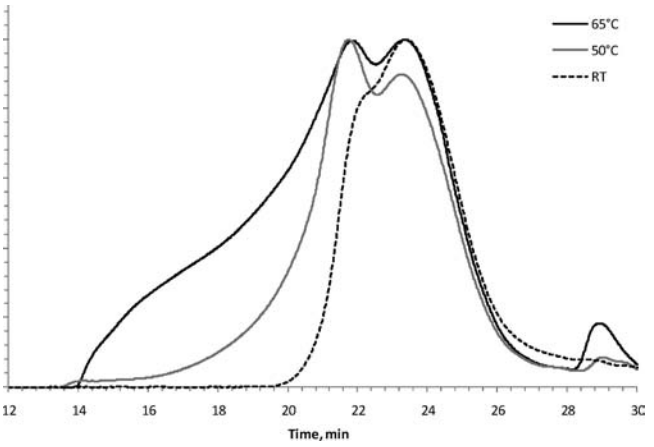

Figure 3. GPC chromatograms for benzoylated Norway spruce EMAL samples, with the benzoylation reaction carried out at different temperatures: room temperature (RT, black dotted line); 50 °C (gray line); 65 °C (black line). UV detection.

Effect of Temperature on Benzoylation. The effect of elevated temperature on the molecular weight distribution of benzoylated EMAL from Norway spruce is presented in **Figure 3**. Interestingly, when the benzoylation reaction was carried out at elevated temperatures, an increase in the molecular weight became apparent. This behavior can possibly arise from a branching reaction. A simple mechanism for this reaction is proposed (**Figure 4**) and is based upon the presence of both carboxylic acid and phenolic or alcoholic functionalities within the lignocellulosic material. During the milling of wood, it has been observed that carboxylic acids are formed, probably as a result of hemicellulose ester cleavages or β-O-4 ether breakage of the lignin (*2*). Upon benzoylation of this material, we anticipate the formation of not only aliphatic and aromatic benzoyl esters but also mixed anhydrides, from the reaction of benzoyl chloride with carboxylic acids. The observed branching could be a result of the reaction of free alcohols or phenols with these mixed anhydrides, resulting in a large increase in the molecular weight and eventually precipitation of high molecular weight material. This is an indication of the liability of the esters under these reaction conditions, and further transesterification is likely catalyzed by the Lewis acidic and nucleophilic pyridinium hydrochloride. This species is produced as a byproduct from the esterification reaction and as a neat molten salt has been known to cleave alkyl aryl ethers (*21*). The eventual

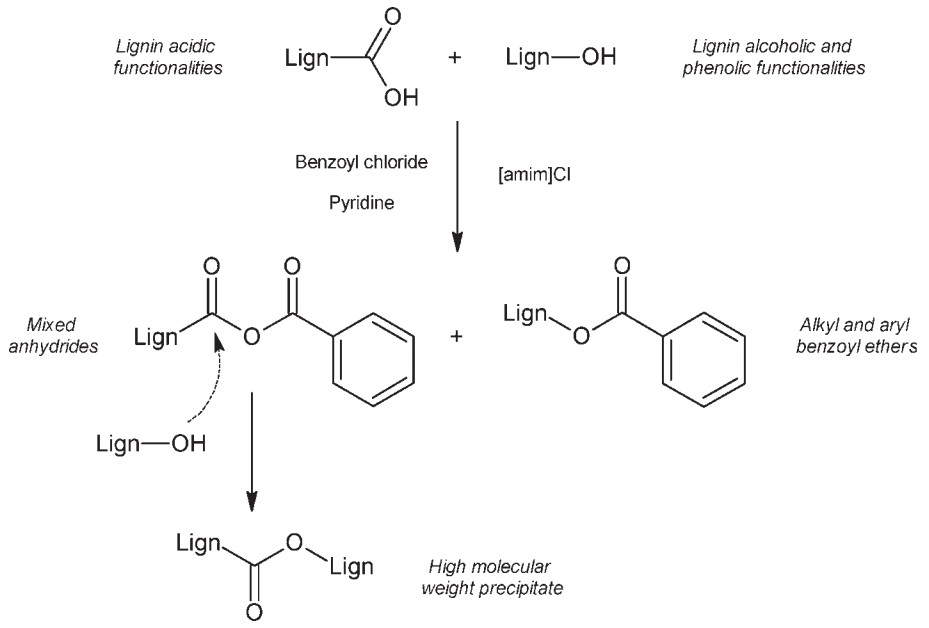

Figure 4. Proposed mechanism to the branching reaction occurring during benzoylation of lignocellulosic materials in [amim]Cl at elevated temperatures.

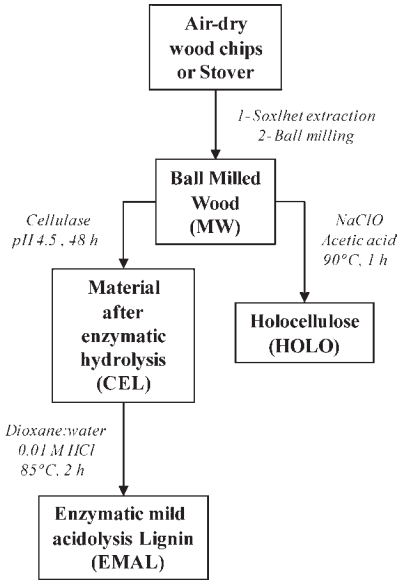

Figure 5. Isolation schemes for the different representative lignocellulose preparates.

precipitation of high molecular weight material, without redissolution, is a possible driving force for this reaction.

It is worth mentioning that the room temperature (RT) chromatogram of **Figure 3** represents Norway spruce EMAL, benzoylated at room temperature, which is in good agreement with a chromatogram for acetylated EMAL (not reported). Of course, there is no guarantee that branching is not occurring during acetylation, but it seems that this observation offers support that by lowering the reaction temperature, one may avoid this effect. Branching during acetylation of lignins has not previously been reported, to the best of our knowledge.

Sample Preparation Procedure and Lignin Balance. The offered GPC methodology was then applied on different lignocellulosic preparations as they arise from the EMAL lignin isolation protocol. The association between lignin and carbohydrates is expected to greatly influence the yield and most likely the structure (molecular weight range, dimer/trimer linkage composition) of lignin that can be extracted from wood. The isolation process requires a physical pretreatment involving the pulverization of wood. In general, the longer the milling times are, the higher the lignin yield. To improve yields while minimizing the degradation of the more unique wood functionalities and linkages, the extent and severity of mechanical action during the milling procedure must be minimal and the chemical treatment should not be harsh. Moreover, a mild acid hydrolysis can liberate lignin from lignin-carbohydrate complexes (known to prevent lignin isolation in high yields). Such a step may facilitate the isolation of previously less-accessible lignin from milled wood. Consequently, the recently developed EMAL procedure offers new opportunities for wood structural elucidation, due to the combination of low-severity milling and mild acidolysis conditions.

Thus, the EMAL procedure was applied to different representative species and samples from each stage collected for analysis. The representative species examined were Norway spruce (NS) as a softwood, *E. grandis* (EG) as a hardwood, and corn stover (CS) as a grass-based feedstock. The corresponding holocellulose (HOLO) samples were also prepared, representing the delignified materials. The isolation schemes for the different fractions are illustrated in **Figure 5**.

In short, EG chips were treated with sodium hydroxide to remove tannins. The extracted EG chips, stover, and NS wood chips were air-dried and submitted to Wiley milling so as to pass a 20-mesh screen. The powders were Soxhlet extracted overnight with acetone, to remove extractives. For CS, the extractives were removed with an ethanol/benzene mixture rather than acetone. The extractive-free powders were then treated by planetary ball milling for 20 h, to obtain the MW preparations. These materials should be considered as the starting reference materials. The lignin contents for all materials were measured, and the data are shown in **Table 3**.

The MW materials were then treated with cellulase to remove the cellulose fractions. The accessibility of the enzyme to the cellulosic fraction is facilitated by the 20 h milling time, resulting in weight losses (NS, 67.3%; EG, 73.5%; and CS, 75.8%) quite close to the theoretical values (NS, 73.9%; EG, 81.0%; and CS, 81.4%). Moreover, it was possible to show that practically all of

Table 3. Results from Application of the Isolation Procedures on the Different Starting Materials[a]

sample		weight of MW (g)	yield (%)	lignin content (%)
Norway spruce	MW	100.0		26.1
	CEL	32.7	32.7	79.4
	EMAL	8.8	29.8	88.5
	HOLO	55.0	74.4	1.0
Eucalyptus grandis	MW	100.0		19.0
	CEL	26.5	26.5	74.8
	EMAL	9.8	46.2	89.6
	HOLO	70.0	86.4	0.9
corn stover	MW	100.0		18.6
	CEL	24.2	26.5	76.9
	EMAL	11.7	50.0	79.4
	HOLO	60.0	73.7	1.1

[a] The weight of MW is the experimental recovery (g) starting from 100 g of MW. The yields are calculated following the formulas given under Experimental Procedures.

the lignin in the starting MW samples was still present in the CEL samples. This was achieved by verifying the equation

$$\frac{\text{MW (100 g)} \times \text{MW lignin content (\%)}}{100} \cong \frac{\text{CEL (g)} \times \text{CEL lignin content (\%)}}{100}$$

The results are NS, 26.1 ≈ 26.0; EG, 19.0 ≈ 19.8; and CS, 18.6 ≈ 18.6. This was strong evidence supporting the fact that the cellulase treatment seems not to affect the total amount of extractable lignin, maintaining a lack of extensive covalent linkages of cellulose to lignin throughout the wood structure.

After the enzyme treatments, the CEL materials were treated using a mild acidolysis step (0.01 M HCl in dioxane/water azeotrope). This treatment is suggested to liberate the lignin from lignin-carbohydrate bonds while minimizing the chemical degradation of the samples. The treatment allows one to obtain the EMALs in moderate yields (NS, 29.8; EG, 46.2; and CS, 50.0) and good purities (NS, 88.5%; EG, 89.6%; and CS, 79.4%). From the same MW materials, bleaching with sodium chlorite allowed the isolation of the HOLO fractions, eradicating almost all of the lignin in these fractions. The yields of HOLO, based on recovery of the predicted lignin-free material, are presented in **Table 3**.

GPC Analysis of Different Preparations and Species. The materials recovered during the EMAL and HOLO isolation procedures were solvated with [amim]Cl and benzoylated using benzoyl chloride in the presence of pyridine. Benzoylation allows for the detection of all components by GPC, with UV detection. The standard milling time was 20 h, and the benzoylation reaction was carried out at room temperature, as optimized in the previous experiments. After dissolution in THF, the samples were examined with GPC. All of the samples showed good solubility in THF. Only the NS CEL sample showed incomplete solubility.

The chromatograms for all preparations (MW, CEL, EMAL, and HOLO) and species (NS, EG, and CS) are presented in **Figure 6**. The scaling on the *y*-axis was normalized by the following factors: MW, 100%; HOLO, 100 − MW lignin content %; CEL, recovery %; EMAL, MW lignin content %. The figure shows the molecular weight distributions as detected by both UV and RI detection.

In general, the lignin (CEL and EMAL) and HOLO preparations present reasonably symmetrical peaks, whereas the MW fractions are asymmetric to a greater or lesser degree, dependent on species. As the average molecular weights show reasonable separation between the lignins and the HOLO, the degree to which the shoulder is present on the MW sample, in relation to the main lignin peaks, is obviously a measure of the connectivity between the lignin and polysaccharide fractions. This can be assessed by approximate convolution of the individual components to emulate the MW molecular weight distribution. Comparison of the convoluted spectra with the actual distribution should offer a qualitative measure of the degree of connectivity of the lignin and polysaccharide fractions. No qualitative information can be obtained from these data, although relative connectivities may be assessed for different species.

The difference in the molecular weight distributions of CELs and EMALs is limited, and the chromatograms are almost identical. The exception for this is the comparison of EG CEL and EMAL samples. This is rationalized on the basis of the larger increase in lignin content, on going through the mild acidolysis treatment, for EG than for the other species. This is an indication of more extensive linkages between lignin and polysaccharides in EG than for the other species.

The chromatograms for NS MW, HOLO, CEL, and EMAL are illustrated in **Figure 6a**. The UV chromatogram of milled wood at first glance shows an asymmetric unimodal distribution, although a minor shoulder is present at $4.0-3.0 \times 10^3$ g mol^{-1}. The HOLO fraction shows a symmetric unimodal distribution corresponding to the main MW peak. The CEL sample presents a poor solubility in THF after benzoylation and could not be considered as completely representative. Although both the CEL and EMAL samples have similar molecular weight distributions that seem to correspond to the shoulder present in the MW chromatogram. This shoulder is not as evident as in the CS MW sample and so is an indication that linkages between lignin and polysaccharides in this species are stronger than in the grass species.

The chromatograms for EG MW, HOLO, CEL, and EMAL are illustrated in **Figure 6b**. The MW shows a unimodal distribution in both the UV and RI chromatograms, with the main peaks around $4.0-5.0 \times 10^4$ g mol^{-1}. The shape of the peak seems to be symmetrical without any shoulder, as was observed for the CS and NS MW samples. The molecular weight distribution of the HOLO fraction also shows a unimodal distribution centered at $4.0-5.0 \times 10^4$ g mol^{-1}. This distribution is very similar in both the UV and RI chromatograms to the MW material. The CEL and EMAL samples were detected as a distribution centered at 1.0×10^4 and $6.0-7.0 \times 10^3$ g mol^{-1} respectively. In the case of EG the MW sample could not be described as a simple sum of the HOLO and EMAL fractions, indicating more extensive links between lignin and polysaccharides in this species over the other two species.

As mentioned previously, the differences observed between the CEL and EMAL samples seem for EG to be connected with a presence of lignin-carbohydrate bonds in the starting wood. The cellulase treatment is able to hydrolyze a large fraction of the carbohydrate present in the wood. The remaining carbohydrate seems to be chemically connected to the lignin and is a further indication of the greater extent of linkages between lignin and polysaccharide in this species, over the softwood and grass species. Only with a mild acidolysis treatment did these bonds seem to be cleaved, allowing the observation of a lower molecular weight fraction by GPC UV analysis.

As shown previously, the UV chromatogram of CS MW (**Figure 6c**) shows a bimodal distribution with a main peak at $4.0-3.0 \times 10^4$ g mol^{-1} and a shoulder at $4.0-3.0 \times 10^3$ g mol^{-1}. The HOLO (81% of the wood) shows a unimodal distribution

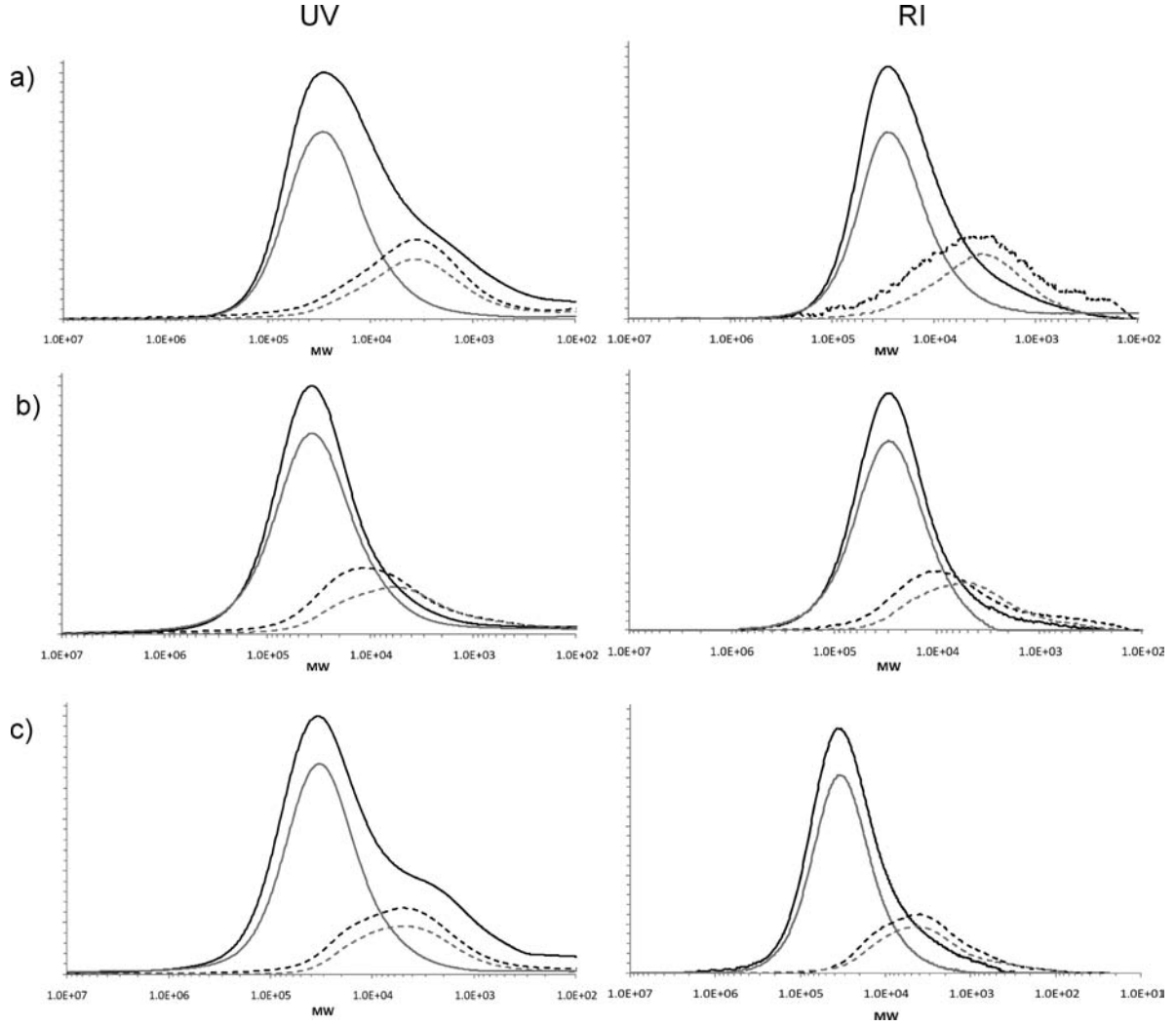

Figure 6. GPC chromatograms for benzoylated MW (black line), HOLO (gray line), CEL (black dotted line), and EMAL samples (gray dotted line): (**a**) Norway spruce; (**b**) *Eucalyptus grandis*; (**c**) corn stover. Detection: UV, left side; RI, right side. Normalization of the chromatograms has been done by multiplying the absorbance by the percentage content of each preparation in the original wood.

with molecular weight centered at $4.0-3.0 \times 10^4$ g mol^{-1}; this peak matches the main peak of the MW. The CEL and EMAL samples show a distribution around 5.0×10^3 g mol^{-1}. The shoulder present in the UV chromatogram of the CS MW was suggested to be the lignin fraction (previous discussion surrounding **Figure 2**). Confirmation for this hypothesis could be observed from the UV chromatograms for the CEL and EMAL. Comparison of the RI chromatograms for the CS MW and HOLO now highlight the presence of a shoulder in the MW sample, which apparently is due to the presence of lignin in MW. The degree of attachment of the lignin to the remaining wood components by comparing the peak and shoulder profiles is not totally apparent at this stage, although bimodality is most apparent for the CS MW sample from the UV chromatograms, in comparison to other species. This again suggests a weak interaction between the bulk of the lignin and polysaccharide components in this material and may allow for easier processability of this material, in comparison to hard- and softwoods. In a comparison between UV and RI detection, UV seems to be the clear winner as the lignin peaks are more emphasized, whereas in the RI detection, bimodality is easily overlooked. The combination of the two detection techniques, however, does offer a way to distinguish the lignin-rich material from polysaccharide in bimodal molecular weight distributions.

Table 4. Numerical Output from the GPC Analyses of Norway Spruce, *Eucalyptus grandis*, and Corn Stover Preparates

sample		M_p	M_n	M_w	M_w/M_n
Norway spruce	MW	36300	2850	36800	12.91
	HOLO	34400	12800	75500	5.90
	CEL	3600	1950	13050	6.69
	EMAL	3700	2000	6100	3.05
	HOLO + EMAL	34200	1900	27600	14.53
Eucalyptus grandis	MW	37000	12500	49400	3.95
	HOLO	35800	16300	64300	3.94
	CEL	11500	2500	18900	7.56
	EMAL	5700	1830	10500	5.74
	HOLO + EMAL	35200	6800	39500	5.81
corn stover	STOVER	34200	3630	42100	11.60
	HOLO	31000	15400	49600	3.22
	CEL	4900	1370	10100	7.37
	EMAL	4900	1450	7060	4.87
	HOLO + EMAL	30500	2870	35600	12.40

The numerical results from GPC analyses of all three species of benzoylated samples are presented in **Table 4**. We can see that of all the samples, the MW samples present the higher M_p values. The M_p values for the HOLO fraction are slightly lower than for

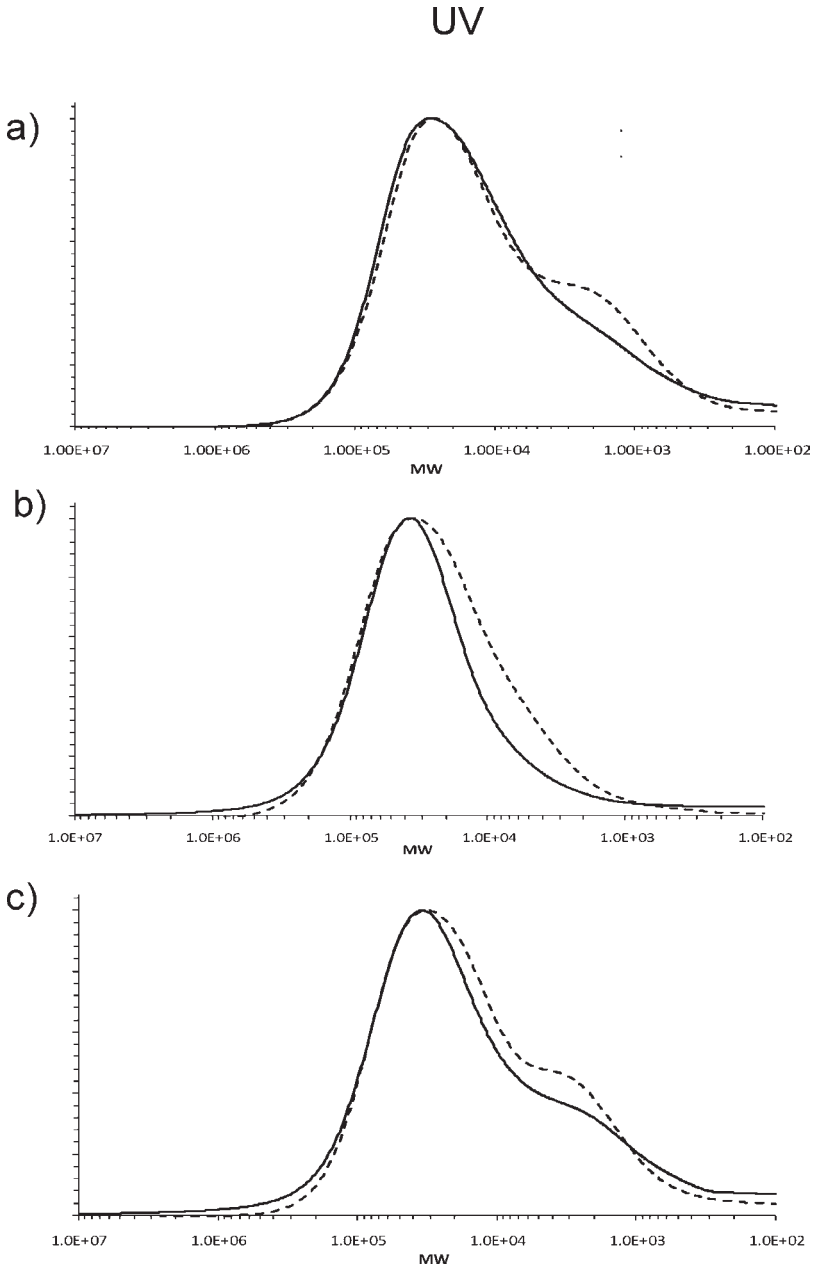

Figure 7. GPC chromatograms for the MW (black line) and simulated wood samples mixing holocellulose and EMAL (black dotted line): (**a**) Norway spruce; (**b**) *Eucalyptus grandis*; (**c**) corn stover. UV detection.

the respective milled woods, but have lower polydispersities (M_w/M_n) than the corresponding MW samples, as expected due to the absence of lignin. The only exception to this is the EG sample, which has nearly identical polydispersities for the MW and HOLO, further indicating the extensive covalent connectivity of lignin and polysaccharide in this species. This differing connectivity is again echoed by comparison of the numerical values for the CELs and EMALs. For NS and CS, the CELs and EMALs have similar values for M_p and M_n. Again, EG is the exception, showing higher molecular weight values for the CELs over the EMALs, indicating the presence of a larger portion of polysaccharides in the CEL, which are cleaved during the mild acidolysis treatment.

GPC, HOLO, and EMAL in the Detection of lignin-carbohydrate Complexes. Convincing data have already been presented concerning the qualitative presence or absence of LCCs in the milled wood samples, using a combination of GPC with UV and RI detection. It is even possible to give an approximate relative assessment of LCCs for the different wood types. To dispel doubt about these conclusions, a simple illustration can be used to demonstrate the presence of this connectivity. The chemical composition (disregarding the connectivity) of the MW samples can be best emulated by mixing the corresponding HOLO and EMAL materials in the correct proportions. Using this

Table 5. Areas of Peak 1 (HOLO) and Peak 2 (EMAL) from Deconvolution Calculation of MW and Simulated Samples with Origin 8.0 Software

		peak 1 (HOLO)	peak 2 (EMAL)
Norway spruce	MW	0.49	0.51
	simulation	0.41	0.59
Eucalyptus grandis	MW	0.64	0.36
	simulation	0.52	0.48
corn stover	MW	0.44	0.56
	simulation	0.43	0.57

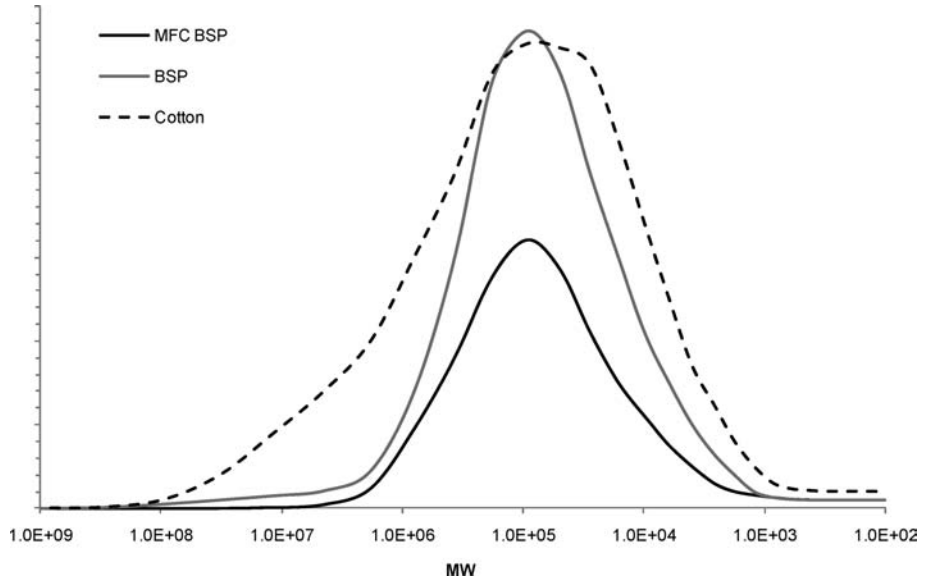

Figure 8. GPC chromatograms for a series of pure cellulose samples dissolved in IL and benzoylated: microfibrilated fully bleached softwood pulp (MFC BSP, black line); bleached softwood pulp (BSP, gray line); cotton (dotted line). UV detection.

methodology, simulated traces for the MW samples were prepared by mixing the HOLO and EMAL data, on the basis of their weight ratios (as determined from the lignin content analyses). These samples were benzoylated using procedures identical to those for the MW samples and submitted for GPC analyses. The chromatograms were compared to the MW samples and are presented in **Figure 7**. From these comparisons it is apparent how similar the simulated versus approximate MW molecular weight distributions are for NS (**Figure 7a**) and CS (**Figure 7c**). Polydispersity for these samples visually seems to be quite similar. Both MW traces exhibit bimodality; the CS MW trace shows a significant shoulder corresponding to lignin, whereas the NS sample shows only a slight shoulder. Both simulated spectra exhibit modest shoulders, strongly suggesting more extensive LCCs for NS than for CS.

An important difference is visible from the EG comparison. Both traces are unimodal. The simulated wood shows an evident low molecular weight tailing, related to the lignin fraction, whereas the MW sample shows lower polydispersity and much smaller tailing in the low molecular weight region (**Figure 7b**). This, as mentioned previously, is a convincing illustration of extensive LCCs due to the incorporation of the lignin to the higher molecular weight polysaccharide fraction.

To confirm the results, deconvolution calculations were performed on the chromatograms by Origin 8.0 software. The deconvolution process is able to decompose a complex peak in an ensemble of simple gaussian peaks. The chromatograms from MW and simulated wood samples (UV detection, **Figure 7**) were deconvoluted in two peaks related to the holocellulose (peak 1) and the lignin (peak 2), respectively. In all of the cases, the fitting reported a R^2 of > 0.99. Then the areas of peaks 1 (HOLO) and 2 (EMAL) were calculated and reported in **Table 5**.

It is possible to note that the relative area of peak 2 related to the lignin fraction (EMAL) is higher for the simulating wood with respect to the MW. This is rationalized by the presence of the lignin-carbohydrate complex. In the MW samples, part of the lignin is chemically linked with carbohydrate and the molecular weight distribution shows a unimodal shape (or shoulder). In the simulated woods, the holocellulose and lignin fraction are chemically not linked, so the molecular weight distribution shows a bimodal shape.

More specifically, in *E. grandis* the difference of the area values of peak 2 is the highest (23 for MW and 31 for simulated EG wood), indicating an important presence of linkage between lignin and cellulose. For Norway spruce the difference between the MW and simulated sample area of peak 2 is still evident (30−37). For corn stover the difference is not significant, indicating limited presence of lignin-carbohydrate bonds.

GPC of Pure Cellulose Samples. In an effort to further validate our conclusions and create a more general and uniformly applicable method, we finally examined a series of pure cellulose samples with the same procedure (**Figure 8**). It is significant that cotton displays a markedly wider molecular weight distribution than a fully bleached softwood pulp from loblolly pine. In addition, subjecting the pulp to an intense mechanical defibration that is causing the delamination of cellulosic fibres in a high-pressure homogenizer results in the formation of microfibrilated cellulosic material (MFC). When this gel-like material was subjected to our methodology, it became apparent that only a minor fraction of the higher molecular weight component of the original pulp is removed during the microfibrilation process. However, the lower molecular weight fraction of the original pulp seems to be removed to a greater extent during the microfibrilation process.

Conclusion. GPC analyses of carefully characterized and benzoylated lignin preparations (MW, HOLO, CEL, and EMAL) can be used to identify LCCs in intact woods. The benzoylation procedure (carried out at room temperature to avoid branching reactions) from [amim]Cl (an efficient solvating media) of pretreated lignocellulose materials (20 h ball-milling time) has proven to be invaluable in this respect. In regard to LCCs in the three representative species examined, the grass species (corn stover) seems to have relatively few linkages between lignin and polysaccharides. This offers increased processability as a first-generation biorefinery feedstock. The softwood Norway spruce qualitatively has a stronger interaction between lignin and polysaccharides, whereas the hardwood *E. grandis* demonstrated the most extensive interactions between lignin and polysaccharide. Due to the pulverization applied to this sample, this interaction is most likely covalent in nature, and apparently these linkages can be cleaved by mild acidolysis treatments.

ABBREVIATIONS USED

MW, milled wood; EMAL, enzymatic mild acidolysis lignin; HOLO, holocellulose fraction from sodium chlorite oxidation of milled wood; CEL, milled wood after enzymatic digestion; CS, corn stover; NS, Norway spruce; EG, *Eucalyptus grandis*; LCC, lignin-carbohydrate complex; MFC, microfibrillated cellulose; GPC, gel permeation chromatography; IL, ionic liquid; BSP, Bleached Softwood Pulp; ([amim]Cl), 1-allyl-3-methylimidazolium chloride; THF, tetrahydrofuran; UV, ultraviolet detection; RI, refract index detection.

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