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

LUCA ZOIA, ALISTAIR W. T. KING, and DIMITRIS S. ARGYROPoulos

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 abies) and Eucalyptus grandis wood, and corn stover (Zea mays L.) to examine the molecular weight distributions by gel permeation chromatography (GPC) of the solubilized materials, recovered during the different steps of the...
EMAL procedure. Due to the lack of any significant chromophore in the polysaccharide portions (cellulose and hemicellulose) of lignocellulosic materials, we employed a novel benzylation methodology in solvating IL media. During this procedure, essentially all lignocellulose hydroxyl groups (from both polysaccharides and lignin) were functionalized as benzyol esters using benzyol chloride and pyridine as the acid acceptor in [amin][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 (Norwegian 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 eucalyptus 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 to alkaline extraction with [amin][Cl] as the solvent medium. This facilitated visualization of all components by GPC with UV detection (9, 10). The values reported are the averages of three analyses ± 1.0% (P = 0.05, n = 3).

1-allyl-3-methylimidazolium chloride ([amin][Cl]) Synthesis. [amin][Cl] was prepared according to a general procedure, provided by Zhao et al. (20), with a slight modification. Allyl chloride and 1-methylimidazole were distilled prior to use. [amin][Cl] was further purified to remove trace color by dissolving the crude [amin][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 [amin][Cl] as a pale yellow oil (99% purity by ¹H NMR): δH (300 MHz; CDCl₃; Me₂Si) 10.39 (1 H, s, NCHN), 7.65 (1H, s, C=CH₂), 7.40 (1H, dd, J = 16.9, 10.3, 6.5 Hz, C=CH₂), 5.33–5.26 (2 H, m, C=CH−C), 4.86 (2 H, d, J = 6.4 Hz, NCH₂), 3.97 (3 H, s, NCH₂). Water content was initially followed by ¹H NMR during drying and finally confirmed to be 0.4% by K₂ titration; pH was tested by dissolving 1 g in 20 mL of deionized water to ensure neutrality.

Typical Benzoylation from [amin][Cl]. [amin][Cl] (950 mg) was added to lignocellulosic material (90 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 disappeared. 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 cellulose materials and 14 mmol g⁻¹ of –OR 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 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 × 10⁶ g mol⁻¹. Millenium 32 GPC software (Waters) was used for data processing to obtain as numerical output *Mₚ* (peak molecular weight), *Mₚₚ* (number-average molecular weight), and *Mₚₚₚ* (weight-average molecular weight). The ratio *Mₚₚₚ*/*Mₚₚ* (polydispersity) has been calculated as well.

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

**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 sodium chloride (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 suspension was filtered and washed in previous milled and sized glass filter (grade C). The filtrate was oven-dried overnight to give the HOLOs. The yield of holocellulose was calculated by using the formula

\[
\text{yield} (%) = \frac{\text{EMAL (g) × EMAL lignin content (%)}}{\text{MW (g) × MW lignin content (%)}} \times 100
\]
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 cellulose 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.

As the planetary milling treatments result in an increase of both the cellulose 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 (CDCl3) and dimethyl sulfoxide (DMSO-d6) 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 1H NMR, and the integration ratios for aryl (8.66–6.66 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.

Table 1. Solubility of IL Media Benzoylated Corn Stover, Milled for Different Times, in Chloroform (CDCl3) and Dimethyl Sulfoxide (DMSO-d6), and Integration Results from 1H NMR

<table>
<thead>
<tr>
<th>MW corn stover</th>
<th>CDCl₃</th>
<th>DMSO-d₆</th>
<th>H-aryl/H-alkyl</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 h</td>
<td>−</td>
<td>+</td>
<td>na</td>
</tr>
<tr>
<td>10 h</td>
<td>−</td>
<td>+</td>
<td>1.47</td>
</tr>
<tr>
<td>20 h</td>
<td>++</td>
<td>+</td>
<td>1.89</td>
</tr>
<tr>
<td>30 h</td>
<td>+++</td>
<td>+</td>
<td>1.91</td>
</tr>
</tbody>
</table>

*The solubility has been evaluated by visual inspection: (−) not soluble, (+) slightly soluble, (++) almost soluble, (+++) completely soluble.*
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 10^7 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_w values slightly increase with the milling time, whereas the M_n 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.

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

<table>
<thead>
<tr>
<th>Sample</th>
<th>First Peak</th>
<th>Second Peak</th>
<th>M_n</th>
<th>M_w</th>
<th>M_w/M_n</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 h</td>
<td>50200</td>
<td>2670</td>
<td>3330</td>
<td>191000</td>
<td>57.36</td>
</tr>
<tr>
<td>10 h</td>
<td>46000</td>
<td>2930</td>
<td>3300</td>
<td>96500</td>
<td>29.24</td>
</tr>
<tr>
<td>20 h</td>
<td>34200</td>
<td>4350</td>
<td>3630</td>
<td>42100</td>
<td>11.60</td>
</tr>
<tr>
<td>30 h</td>
<td>31500</td>
<td>3950</td>
<td>3950</td>
<td>24000</td>
<td>6.08</td>
</tr>
</tbody>
</table>

The M_w values reported for the second peak in the 20 and 30 h samples were calculated by deconvolution from Figure 2, 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 benzyl 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
precipitation of high molecular weight material, without redis-
solution, 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 com-
position) 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 representa-
tive 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
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
the lignin in the starting MW samples was still present in the CEL samples. This was achieved by verifying the equation

\[
\text{MW (100 g)} \times \frac{\text{MW lignin content (%)}}{100} = \frac{\text{CEL (g)} \times \text{CEL lignin content (%)}}{100}
\]

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 chloride 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.

**Table 3. Results from Application of the Isolation Procedures on the Different Starting Materials**

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

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.

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 × 10^4 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 × 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 × 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 × 10⁴ and 6.0–7.0 × 10⁴ 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 × 10^4 g mol⁻¹ and a shoulder at 4.0–3.0 × 10^3 g mol⁻¹. The HOLO (81% of the wood) shows a unimodal distribution
with molecular weight centered at $4.0-3.0 \times 10^3$ g mol$^{-1}$; this peak matches the main peak of the MW. The CEL and EMAL samples show a distribution around $5.0 \times 10^3$ 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.

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.

<table>
<thead>
<tr>
<th>Table 4. Numerical Output from the GPC Analyses of Norway Spruce, Eucalyptus grandis, and Corn Stover Preparates</th>
</tr>
</thead>
</table>
| sample       | $M_\text{p}$ | $M_\text{n}$ | $M_\text{w}$ | $M_\text{w}$/
| Norway spruce|            |            |            | $M_\text{n}$     |
| 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      |            |            | |
| MW           | 34200       | 3630       | 42100       | 11.60            |
| HOLO         | 31000       | 15400      | 49600       | 3.22             |
| CEL          | 4900        | 1450       | 7080        | 4.87             |
| EMAL         | 4900        | 1450       | 7080        | 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 $M_\text{p}$ values. The $M_\text{p}$ values for the HOLO fraction are slightly lower than for...
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 disprove 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**

<table>
<thead>
<tr>
<th></th>
<th>peak 1 (HOLO)</th>
<th>peak 2 (EMAL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Norway spruce</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MW</td>
<td>0.49</td>
<td>0.51</td>
</tr>
<tr>
<td>simulation</td>
<td>0.41</td>
<td>0.59</td>
</tr>
<tr>
<td>Eucalyptus grandis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MW</td>
<td>0.64</td>
<td>0.36</td>
</tr>
<tr>
<td>simulation</td>
<td>0.52</td>
<td>0.48</td>
</tr>
<tr>
<td>corn stover</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MW</td>
<td>0.44</td>
<td>0.56</td>
</tr>
<tr>
<td>simulation</td>
<td>0.43</td>
<td>0.57</td>
</tr>
</tbody>
</table>
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 due to the incorporation of the lignin to the higher molecular weight polysaccharide fraction.

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 microfibril cellulose 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 microfibrillation process. However, the lower molecular weight fraction of the original pulp seems to be removed to a greater extent during the microfibrillation 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 pre-treated 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 chloride 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.

LITERATURE CITED


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