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Determination of molecular weight distributions in native and pretreated wood

Timo Leskinen, Stephen S. Kelley, Dimitris S. Argyropoulos*

Department of Forest Biomaterials, North Carolina State University, NCSU, Campus Box 7212, Raleigh, NC 27695-8005, USA

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ABSTRACT

The analysis of native wood components by size-exclusion chromatography (SEC) is challenging. Isolation, derivatization and solubilization of wood polymers is required prior to the analysis. The present approach allowed the determination of molecular weight distributions of the carbohydrates and of lignin in native and processed woods, without preparative component isolation steps. For the first time a component selective SEC analysis of sawdust preparations was made possible by the combination of two selective derivatization methods, namely; ionic liquid assisted benzoylation of the carbohydrate fraction and acetobromination of the lignin in acetic acid media. These were optimized for wood samples. The developed method was thus used to examine changes in softwood samples after degradative mechanical and/or chemical treatments, such as ball milling, steam explosion, green liquor pulping, and chemical oxidation with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ). The methodology can also be applied to examine changes in molecular weight and lignin-carbohydrate linkages that occur during wood-based biorefinery operations, such as pretreatments, and enzymatic saccharification.

1. Introduction

Wood and other lignocellulosic feedstocks are an abundant source of polymeric raw materials, amenable to modification into value-added chemical and biofuel applications (Ragauskas et al., 2006; Naik, Goud, Rout, & Dalai, 2010; Belgacem & Gandini, 2008). Their effective utilization requires a clear understanding of their polymeric structure and the implication of alteration caused by various pretreatment or isolation processes. It is also important to understand how these changes affect the subsequent utilization of the biomass and its individual components.

The molecular weight (MW) determination of lignocellulosic materials tends to be laborious and time consuming. Isolated preparations of wood components are typically needed for good solubility of samples and a selective analysis (Sjöholm, 2003; Hortling, Turunen, & Kokkonen, 2003). Overall, the literature demonstrates that the compositional and structural heterogeneity of the intact cell wall imposes significant limitations for analysis of the molecular weights directly from wood. The selective and controlled alteration of the cell wall components *in situ*, and the application of two complementing solvent systems, may provide an effective route to the analysis of wood in its native form. Our aim here is to demonstrate that it is possible to use distinct dissolution/reaction chemistries on lignin and carbohydrates to produce soluble derivatives that represent the native components.

Ionic liquids (ILs) provide a new analytical tool in the field of cellulose molecular weight analysis (Hallac & Ragauskas, 2011). Recently IL's have been successfully applied for the size-exclusion chromatography (SEC) as the mobile phase for chromatographic analyses (Kuroda, Fukaya, & Ohno, 2013). ILs also have the unique capacity to be used as solvents for the analysis of cellulosic substrates when derivatization is needed to obtain readily soluble samples (El Seoud, Koschella, Fidale, Dorn, & Heinze, 2007).

Zoia, King, and Argyropoulos (2011) have introduced a novel derivatization method based on benzoylation of ball milled wood in 1-allyl-3-methylimidazolium chloride ([amim]Cl). Salanti, Zoia, Tolppa, and Orlandi (2012) have modified the procedure and added acetylation as a secondary step aimed toward the selective analysis of lignin. Ball milling was used in these studies to enhance the overall solubility of wood in IL, but there is evidence that the MW of wood components may be altered during such preparative treatment (Fujimoto, Matsumoto, Chang, & Meshitsuka, 2005; Howsmon & Marchessault, 1959). For this reason the ball milling may limit the usefulness of utilizing IL as tool for component

* Corresponding author. Tel.: +1 919 5157708; fax: +1 919 5156302.
E-mail address: dsargyro@ncsu.edu (D.S. Argyropoulos).

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selective MW analyses. Recent studies have concluded that the dissolution of non-milled wood sawdust in certain ILs proceeds via an extraction type mechanism where the cellulose is the primarily dissolved component (Leskinen, King, Kilpeläinen, & Argyropoulos, 2013; Casas, Alonso, Oliet, Santos, & Rodriguez, 2013). On this basis an effective method to directly dissolve cellulose for MW determination can be developed. The poor solubility of the lignin from non-milled wood and the uncertainties related to the use of ball milled wood for lignin MW analyses highlights the need for other solvent systems.

Instead of the derivatization in IL, an alternative approach for lignin acetylation is the acetobromination procedure that has been used to dissolve and analyse wood (Iiyama & Wallis, 1990). Recently this approach has been successfully applied for the analysis of isolated lignin preparations (Asikkala, Tamminen, & Argyropoulos, 2012). Both the acidic reaction media and the strongly acidic HBr by-product from the acetyl bromide, can degrade the carbohydrates in wood. At the same time, the native structure of lignin is retained and it becomes soluble with the introduction of acetyl and bromide functionalities.

The objective of this study was to create a facile methodology for the determination of molecular weight distribution (MWD) of native and pre-treated woods. To do this we have examined and optimized the analytical procedure and investigated the effects of preparative milling and possible side reactions known to occur during the various derivatization steps. The work, detailed below, has led to the development of an optimized protocol for such analyses.

2. Experimental

2.1. Materials

The solvents were purchased from Sigma-Aldrich and Fischer scientific and used without purification, except allyl chloride and 1-methylimidazole that were distilled prior to use. The fibrous cellulose powder was received from Whatman International Ltd. (grade CF1). enzymatic mild acidolysis lignin (EMAL) was prepared from ball-milled Norwegian Pine sawdust following the original procedure by Wu and Argyropoulos (2003). Enzymatic hydrolysis (48 h, 40 FPU of Viscozyme® enzyme cocktail from Novozymes, USA) was followed by mild hydrolysis in dioxane-water (85:15 v/v) containing 0.01 M HCl. The product was collected by precipitation from pH 2 HCl solution, and washed with deionized water.

Thermomechanical pulp (TMP) was obtained from a pulp mill located in Sweden. Material was extracted with acetone for 18 h, Wiley milled using 0.85 mm screen, and then a fraction below 0.40 mm size fraction was sieved off. Planetary ball milling of the resulting material was performed using a 250 ml zirconium bowl, loaded with eight zirconium balls (10 mm in diameter) and 2 g of TMP using a Microwolf planetary mill (Torrey Hills Technologies, USA) at 400 rpm. The milling sequences were set to 20 min milling and 20 min break times, totaling 1, 10 and 20 h. Samples of Norwegian Pine and Birch sawdust were received from BioOil AS (Norway), extracted with dichloromethane for 24 h, and sieved to particle size fractions of 0.85–0.25 mm and <0.25 mm. Bleached softwood pulp was received from a pulp mill located in the USA. Green liquor pulp from Pine (lignin content approx. 20 wt.%) was produced in-house according to the procedure described elsewhere (Wu, Chang, Jameel, & Philips, 2010). Hydrolysis was done in acetate buffer at 50 °C during 72 h in presence of 40 FPU of cellulolytic enzymes.

Synthesis of 1-allyl-3-methyl-imidazolium chloride ([amim]Cl) was performed as described elsewhere (Leskinen, King, Kilpeläinen, & Argyropoulos, 2011), with the exceptions that synthesis was carried out with 1.05 molar equiv. of 1-methylimidazole in relation to allyl chloride, and the charcoal purification was done using acetonitrile as solvent during 24 h stirring at room temperature. Purity of the final product was confirmed by ^{1}H NMR.

2.2. Benzoylation

For the dissolution of wood prior to reaction, 1 g of ionic liquid was first weighed into a 8 ml screw cap vial with a magnetic stirring bar. Then 200 μl of 1-methylimidazole/pyridine 3:1 co-solvent mixture was added and the solvent was homogenized with vortex mixing. Vacuum-oven dried (24 h, RT) wood sawdust (particle size <0.25 mm) was added in 10 mg portion and mixed well using a vortex mixer. The sample was then placed in an oil bath at 60 °C (or 80 °C as specified in the text), and allowed to dissolve using stirring speed of 200 rpm. After an incubation time of 66 h (or specified times reported in the text), the viscous solution still containing some solids was taken out of the bath and cooled for a few minutes, and then 112 μl of benzoyl chloride was added. The reagent was mixed with the wood solution on the vortex mixer (~5 s), and then the sample was left to react for 4 h at room temperature using slow magnetic stirring. The reaction was stopped by the addition of 4 ml of 75% ethanol, and again mixed on the vortex mixer for 1 min. The precipitated mixture then was transferred into a centrifuge tube using the 75% ethanol solution and finally the solvent volume was adjusted to 20 ml. The washing solvent was removed using centrifuge, and the resulting solids were washed twice with 20 ml of ethanol by shaking and centrifuging the solvent. The solid product was left to dry under low vacuum overnight, and further dried in a room temperature vacuum oven. The experiments where benzoylation procedure was carried out without co-solvent, were done as described by in the original publication (Zoia et al., 2011). The dextran standards were benzoylated similarly as the wood samples, but the dissolution step was done at 40 °C temperature. The samples were fully dissolved after 66 h, and then benzoylated.

2.3. Acetobromination

The procedure from Asikkala et al. (2012) was optimized for this work. A dried 10 mg wood sample (particle size 0.85–0.25 mm) was weighed into a 8 ml screw cap vial and then dispersed to 2 ml of glacial acetic acid. The reaction was started by adding 218 μl of acetyl bromide. The sample was protected from light and left to stir at room temperature, maintaining 300 rpm magnetic mixing. Reaction time of 42 h was used for all samples, if not specified otherwise. After the specified reaction time, the dissolved sample was transferred to a 50 ml round bottom flask, and the solvent was evaporated using a high vacuum rotary evaporator. When the solvent had evaporated completely, the solid sample was dissolved in 30 ml of dimethylformamide (DMF) for the SEC analysis.

2.4. UV-spectroscopy

UV-absorption was measured from the same sample solutions of benzoylated and acetobrominated samples that were used for SEC analysis after 1/5 dilution with DMF. Exact concentrations of 0.5 mg/ml were used for benzoylated CF1 cellulose and lignin. Absorption spectra were recorded with a Beckman DU 640 spectrophotometer using quartz cuvettes.

2.5. Size-exclusion chromatography (SEC)

The samples were dissolved directly after derivatization and solvent removal in 20 ml (benzoylated) or 30 ml (acetobrominated) of DMF. Concentrations were empirically optimized based on the average soluble fractions of the samples and corresponding strength of the UV signal. Samples were allowed to dissolve for 1 h for benzoylated samples and 15 min for acetobrominated

samples prior to filtration through a 0.45 μm PTFE filter. Injection volume of 50 μl was used with the manual injection. The SEC system consisted of a HP G1312A pump connected to Waters HT6E and HT2 styragel columns in series with a Waters 484 UV-absorbance detector. Detection wavelengths were 275 and 285 nm for benzoylated and acetobrominated samples, respectively. The mobile phase used was DMF with 0.05 M of lithium bromide (LiBr) as an additive. Calibration was done using either narrow polystyrene standards ranging from 1860 kDa to 0.82 kDa, or benzoylated dextran and model saccharides from 1360 to 0.18 kDa (see Supporting information Fig. S1). Empower software was used for controlling the system operation and preparation of the calibration curve.

Due to the multimodal nature of the chromatograms we use retention times instead of the average molecular weights. Further details about calibration and approximate peak MW values of cellulose, hemicellulose, and lignin in a typical wood chromatogram are provided in the supporting information.

3. Results and discussion

3.1. Targeting the benzoylation and acetobromination methods toward carbohydrates and lignin

When whole wood is being analysed, it is useful for the analysis to distinguish the individual components. In this work this attribute is termed as "component selectivity". The proposed methodology is based on selective solubilization, derivatization, and selective UV detection of the two major wood components measured with SEC.

The two solvent systems used have different mechanisms of dissolving components, and this can be used to gain component selectivity. For the benzoylation reaction we use an ionic liquid reaction system that can extract and preserve both the cellulose component of wood, while the lignin rich fraction with majority of the hemicelluloses remains undissolved. The preferential solubility of the cellulose, over the less soluble hemicelluloses and lignin (Leskinen et al., 2013), was found to be related to the type of the wood preparation and the dissolution temperature. In the case of native sawdust, the non-benzoylated cellulose and hemicelluloses were found to have limited solubility in IL, yet the individual components could be resolved from the chromatograms. Pretreatment (such as steam explosion) of the wood samples enhanced the solubility of the cellulose and hemicellulose in IL relative to lignin. Therefore, a greater proportion of the carbohydrates from wood in relation to lignin could be benzoylated, resulting better resolved chromatograms.

The acetic acid/acetyl bromide 9:1 solvent system used for acetobromination is known to dissolve wood under mild conditions. Unlike what is seen with non-derivatizing ILs, the dissolution of wood is a result of partial hydrolysis and acetylation, and subsequent solubilization of the carbohydrate, while the relatively resistant lignin moieties slowly become solubilized by the acetylation of the hydroxyl groups, and the resulting elimination of association and hydrogen bonding. Sequential acetobromination and benzoylation analyses (Supporting information Section SI2) revealed that the carbohydrates are not fully converted to low molecular weight products under the proposed ambient temperature conditions, unlike reported by Iiyama et al. under 70 °C (Iiyama & Wallis, 1990). Interestingly, the incomplete depolymerization of the carbohydrates allows for detection of the lignin-carbohydrate complexes (LCC) in the SEC chromatograms of acetobrominated wood. This issue will be discussed in more detail in Sections 3.2 and 3.5.

The second factor that enables component selectivity is the presence of different UV-active functionalities in the soluble fractions of the samples that allow their selective detection over the UV-inactive components. Selectivity of the UV detection is the result of selecting different wavelengths and by derivatization by either UV active (benzoate) or inactive (acetate) substituents in the benzoylation and the acetobromination procedures, respectively. The poor solubility of the lignin from benzoylated wood excludes majority of the lignin from showing in the resulting chromatograms, while the benzoylated carbohydrates offer a strong signal. Still small quantities of soluble lignin are unavoidably detected in benzoylated samples. In acetobrominated wood only the aromatic backbone in lignin has a UV signal at 285 nm, while the carbohydrate acetate fragments do not have a strong UV signal.

For demonstration purposes, benzoylated and acetobrominated wood samples and model system of cellulose (pulp, CF1 cellulose) and lignin (EMAL) preparations were analysed by UV/VIS spectrometry (see Fig. 1A and B). The negligible absorbance, at 285 nm, of the acetylated cellulose pulp supports selectivity of the methodology. In addition the UV absorbance, at 285 nm, of the isolated, model lignin and the lignin isolated directly from wood are similar. In the case of the benzoylated systems (Fig. 1B) the UV absorbance, at 275 nm, of the benzolyated DMF soluble carbohydrate fraction from pine wood is similar to the benzoylated cellulose pulp. Distinct absorption maxima at 275 nm and low absorption above 290 nm indicate to high cellulose concentration over lignin. Some interference from soluble lignin is observed with the benzoylated wood samples, but these interfering lignin moieties can be usually distinguished in SEC analyses as separate peaks.

3.2. Controlling side reactions during the derivatization procedures

Dissolution of wood using this methodology requires long incubation times, raising concerns about the stability of the targeted fraction. Recent reports highlight the potential for certain ionic liquid systems to cause depolymerization of cellulose under relatively mild (80 °C) dissolution conditions (Gazit & Katz, 2012). In order to use IL solvents in the proposed methodology, the possible depolymerization reactions needed to be eliminated. According to Gazit and Katz (2012) an addition of 1-methylimidazole (NMI) to the IL during the cellulose dissolution can prevent the depolymerization, which offers a solution to the stability issue.

The potential of [amim]Cl to cause unwanted depolymerization of wood components was carefully examined. A significant decrease in the molecular weight of fibrous cellulose was observed in [amim]Cl between 3 and 72 h of dissolution times at 80 °C (Fig. 2A). We examined the addition of NMI to [amim]Cl at 5 and 20 vol% to eliminate hydrolysis of the cellulose. Both concentrations of NMI seemed to prevent the majority of the depolymerization, but the 20 vol% addition offered solutions of significantly lower viscosity of the IL media which is advantageous for performing reactions with dissolved cellulose. The protective action of pyridine (Pyr) was also examined, and found to be better than with NMI (Fig. 2B). As a drawback, the pyridine tended to precipitate out the formed cellulose benzoate early on during the reaction, resulting in an inhomogeneous product. Therefore, as a compromise, we arrived at the use of 20 vol% NMI-Pyr 3:1-mixture since this offered suitable hydrolysis prevention and good solvation characteristics. Addition of a co-solvent also allowed a successful benzoylation of cellulose after a slow (>48 h) dissolution at ambient temperature (Fig. 2C). Still, incubation at 60 °C offered a more practical dissolution rate (~2 h), without cellulose hydrolysis.

While hydrolysis of the carbohydrates can be completely eliminated during the benzoylation, the depolymerization of lignin is acceptable or even beneficial for the analysis.

Based on our observations, the native wood lignin has limited solubility in [amim]Cl under mild conditions, while isolated preparations, such as EMAL, are readily soluble. The insolubility

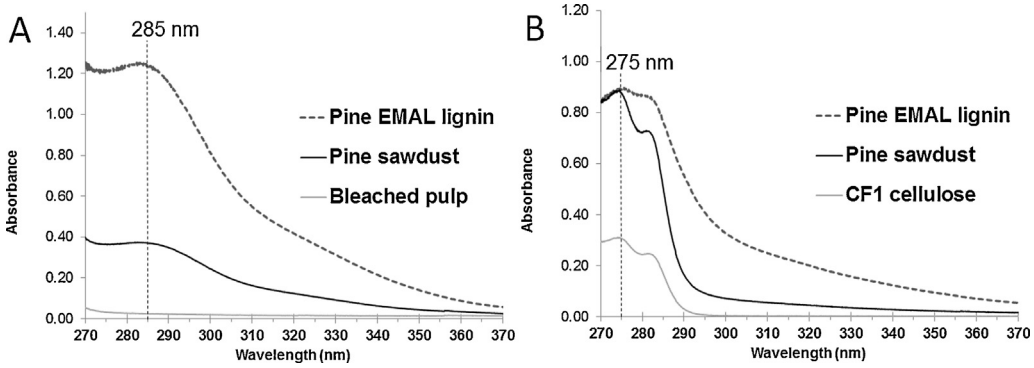

Fig. 1. UV Spectra of derivatized wood and its components in DMF solutions. (A) Acetobrominated materials; derivatized bleached pulp, lignin, and wood were diluted in 1/5 ratio from the solutions used in subsequent SEC analyses; (B) Benzoylated materials. Concentrations for CF1 cellulose and lignin are 0.5 mg/ml DMF. Solutions of benzoylated wood used without further dilution for subsequent SEC analyses.

of the native lignin seems to also hinder the solubility of the carbohydrates from wood in the IL. Thus some limited lignin depolymerization may actually enhance the solubility of the cellulose, while solubility of the hemicelluloses is more dependent on the wood preparation. As a drawback, the use of too drastic dissolution conditons will create soluble low molecular weight lignin fragments, which correspondingly induce SEC signals that interfere with the carbohydrate analysis.

Since several ILs have been reported to depolymerize lignin (George et al., 2011) we examined whether lignin depolymerization can be limited to a beneficial level during a typical benzoylation procedure in [amim]Cl by optimization of the dissolution conditions. In practice this means a limited disintegration, while avoiding formation of low MW fragments that would complicate the SEC profile. Fig. 3A shows a fairly stable MW distribution of Pine EMAL lignin at 60 °C between 2 and 66 h of dissolution, whereas noticeable depolymerization was seen at 80 °C. The data is consistent with the observations of low lignin solubility from wood at 60 °C, whereas the use of 80 °C seemingly enhances the solubility of both lignin and the carbohydrates. The increased solubility of these components, triggered by lignin depolymerization, can be seen as stronger intensities of the detected UV-signals during the SEC analysis of wood.

Analysis of the lignin fraction by acetobromination also includes a risk of unfavorable side reactions. This is because the acetobromination by-product HBr can be detrimental to the alfa and beta ether bonds of lignin (Iiyama & Wallis, 1990). This has been confirmed by Asikkala et al. (2012), who observed partial depolymerization of MWL lignin at high HBr concentrations. During lignin acetobromination, the primary reaction of the HBr is the bromination of benzylic α-carbons of the lignin side chains (Iiyama & Wallis, 1990; Lu & Ralph, 1996). The cleavage of β-*O*-4 may also occur and lead to lignin depolymerization, but this requires more severe conditions compared to the aforementioned α-bromination (Iiyama & Wallis, 1990). According to Lu and Ralph (1996) the β-*O*-4 cleavage does not occur at ambient conditions.

To confirm potential changes to lignin the derivatization conditions used in this study where examined using Pine lignin (EMAL). Noticeable depolymerization took place when the acetobromination reaction for EMAL lignin was prolonged from 2 to 42 h (Fig. 3B).

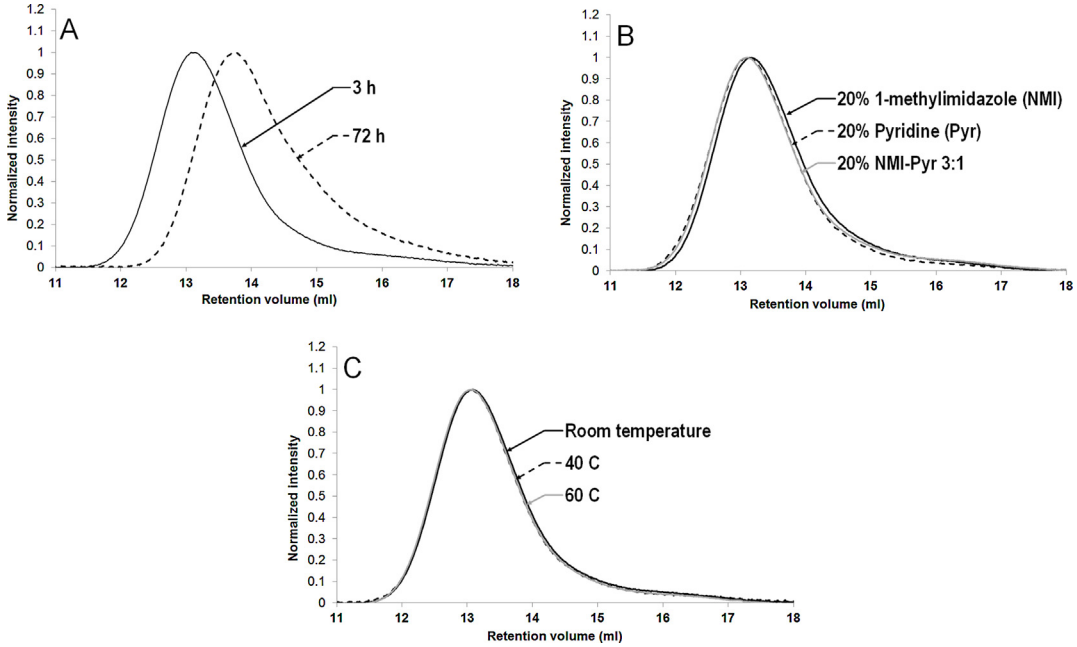

Fig. 2. Chromatograms used to examine the cellulose (Whatman CF1) stability during dissolution in ionic liquid and ionic liquid/co-solvent mixtures. The dissolution step was performed prior the benzoylation reaction. (A) 80 °C dissolution in neat ionic liquid for 3 & 72 h; (B) 72 h dissolution at 80 °C with various co-solvents; (C) 72 h dissolution at various temperatures with 20% NMI-Pyr 3:1 co-solvent. (All samples dissolved completely between 2 and 48 h.).

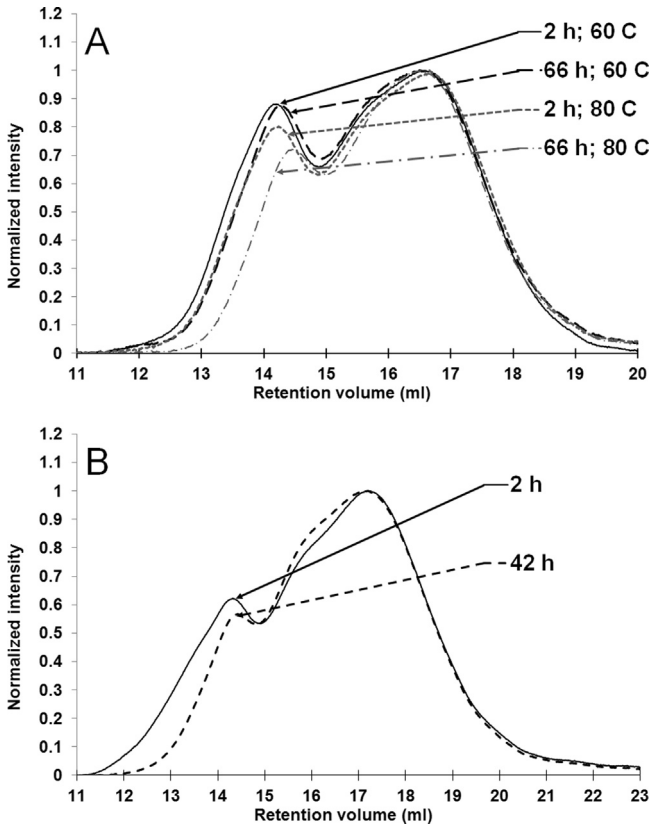

Fig. 3. Observed differences in stability of Pine EMAL lignin during short and long reaction times. (A) Dissolution in IL-NMI-Pyr prior to benzoylation; (B) Acetobromination in acetic acid.

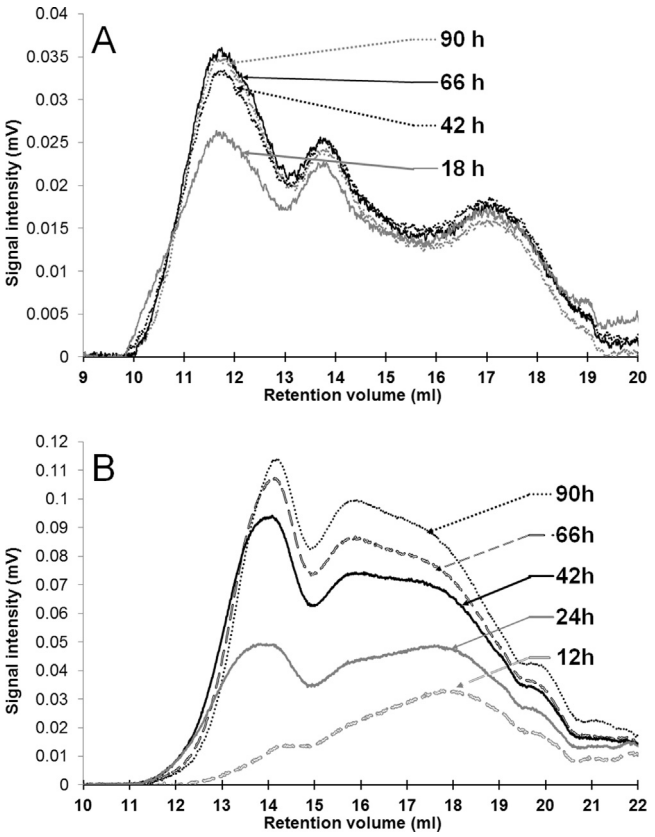

Fig. 4. SEC chromatograms from a series of reaction/dissolution times in order to optimize the benzoylation and acetobromination procedures, using sieved fractions of Pine sawdust. (A) Benzoylation (particle size below 0.25 mm); (B) Acetobromination (particle size 0.85–0.25 mm).

The MW changes observed are limited to the high end of the distribution, without any noticeable signal from low molecular weight fragments. It is expected that a random cleavage of the β-*O*-4 rich backbone should create more low MW fragments than was detected. The observed changes resemble more the degradation patterns seen with branched polymers (Striegel, 2003). This suggests a more severe cleavage of the less frequent non-cyclic α-aryl ethers that represent only around 1% of the bonds between the lignin units (Capanema, Balakshin, & Kadla, 2004), thus creating only relatively larger fragments. Further evidence about the role of α-ethers to these changes in the lignin distributions will be provided in section 3.5.

While the depolymerization of the isolated lignin preparations by α-bromination during the acetobromination cannot be completely ruled out, this degradation process in wood samples seems to be much slower based on the slow initial dissolution of the native lignin and minor changes in the chromatograms between 42 and 90 h reactions (see Fig. 4). This may be due to the different solubility of the native lignin compared to the EMAL preparation and the prominent presence of the wood carbohydrates that are also capable of a competing reaction with the formed HBr (Kartha & Jennings, 1990). Also the presence of the covalently attached carbohydrates and their hydrolysis may affect to the high MW region of the lignin chromatograms (see Section 3.5). Fast hydrolysis of the carbohydrate moieties is expected especially from the readily soluble EMAL lignin preparations directly after the initiation of the reaction, whereas their hydrolysis in the wood samples is at first limited by diffusion and eventually by the complete consumption of the required trace quantities of water from the solvent medium.

3.3. Optimization of reaction times for fibrous wood

Adapting our methodology for wood sawdust required optimization of the original benzoylation and acetobromination procedures in terms of dissolution and reaction times. Conditions for representative analyses were optimized using sieved fractions of Pine sawdust. The resulting chromatograms from a series of applied dissolution/reaction times for both derivatization reactions are shown in Fig. 4A and B.

The optimal dissolution time prior to the benzoylation reaction was estimated to be around 66 h (Fig. 4A). Prolonging the dissolution time to 90 h did not offer noticeable changes in the observed MW distribution. The limited cellulose solubility from native wood indicates the existence of physical barriers such as insoluble moieties or gels of lignin and hemicelluloses rather than simply slow dissolution of the cellulose. The larger sized (0.85–0.25 mm) fraction resulted in a somewhat poor solubility within the same time frame, supporting this conclusion.

The reaction time had a large effect on the data obtained for acetobromination analyses (Fig. 4B), causing the SEC profile to evolve in two ways. First, a significant increase of the high molecular weights (below retention volume of 15 ml) was observed, which slowed down after 42 h. This may be related to the observed incomplete solubility of wood in the reaction mixture during the 12 and 24 h reactions. Second, a new peak appeared around a retention volume of 16 ml with growing intensity as a function of time. This may be due to the cleavage of non-cyclic α-ethers or other minor lignin interunit linkages. Based on these data a 42 h reaction time is suggested as optimum. These conditions provide complete dissolution of the sample, and appear to limit depolymerization reactions.

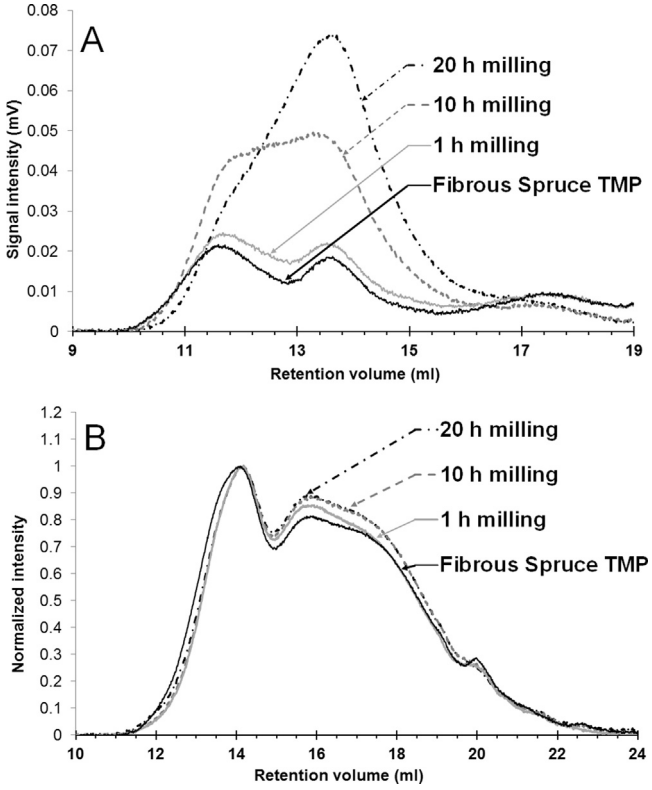

Fig. 5. Effects of ball milling on the molecular weight distribution of Spruce TMP pulp. (A) Benzoylated TMP spruce after 66 h in IL at 60 C. (Chromatograms with the original signal intensity are shown for quantitative comparison); (B) Acetobrominated products after 42 h room temperature reaction (Normalized chromatograms are shown for qualitative comparison).

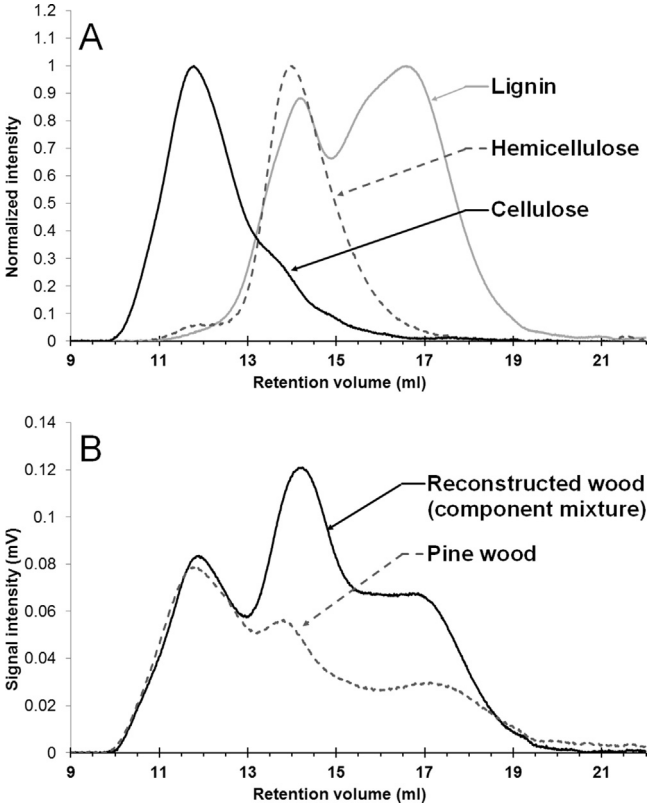

Fig. 6. Resolving the wood chromatogram using model components and physical mixtures. (A) Isolated and benzoylated wood components (bleached softwood pulp, Spruce galactoglucomannan, and Pine EMAL lignin); (B) Model physical mixture of benzoylated components in the ratio of 46:26:28 mass %, and its comparison to the distribution of benzoylated Pine sawdust.

In conclusion, the fibrous wood materials showed significantly slower dissolution and reaction kinetics than ball milled wood or isolated wood component preparations (Zoia et al., 2011; Asikkala et al., 2012). Still, the long reaction times (up to 66 h) needed for the proposed methodology are comparable to the time demands of other SEC methods that have been applied to wood pulps (Sjöholm, Gustafsson, Berthold, & Colmsjö, 2000; Josefsson, Lennholm, & Gellerstedt, 2001).

3.4. Milling as a preparation step to improve wood solubility

Ball milling was examined as a means to enhance the slow and incomplete solubility of wood in the IL in a manner similar to the original procedure (Zoia et al., 2011). The benzoylation and acetobromination reactions were carried out on fibrous Spruce TMP pulp (Wiley milled and sieved to particle size between 0.85 and 0.40 mm) and the same material after 1, 10, and 20 h of planetary ball-milling (micron scale powders). As anticipated, the pulverization gradually enhanced the overall solubility of wood, resulting chromatograms of varying intensities. Chromatograms of the benzoylated products show (Fig. 5A) that the 1 h milling resulted only in minor effects, whereas after 10 h of ball milling a shift of the main peak towards the lower molecular weight was discernible. After 20 h of milling, an unimodal distribution with a peak at the middle range of the whole wood distribution was obtained. This reduction in the proportion of high molecular weight carbohydrates is expected (Howsmon & Marchessault, 1959).

Acetobromination analyses (Fig. 5B) revealed that only a slight decrease in the high MW region of the lignin distribution after ball milling. The mild effects of ball milling to the lignin are somewhat surprising in light of the work of Fujimoto et al. (2005), who saw degradation of the β-*O*-4 as a function of the milling severity. The less apparent lignin degradation in this study may be due to the use of lower milling frequency.

As such, it is concluded that preparative ball milling steps, with the resulting negative impacts on the original sample's MW distribution, should be avoided whenever possible. If the average particle size of coarse particles (e.g. wood chips) needs to be reduced for solubility purposes, initial size reduction by Wiley milling followed by a short ball milling ($\leq$1 h) is a relatively fast and non-destructive alternative step. Further milling reduces the accuracy of the MW determination.

3.5. Resolving the multimodal chromatograms of wood

To further understand the nature of the chromatograms obtained by the proposed methodology, isolated wood components were examined. SEC chromatograms from benzoylated isolated wood components (bleached softwood pulp, softwood galactoglucomannan (GGM), and softwood EMAL lignin) were recorded separately and as a physical mixture containing 46, 26, and 28 mass % respectively.

The data of Fig. 6A shows that the three components are well resolved according to their molecular weights. The physical mixture of the three components (Fig. 6B) showed a trimodal distribution, similar to that observed from wood (Fig. 6B). While the elution times of the isolated components are identical to wood, the differences in the relative intensities of the physical mixture confirms that the cellulose is enriched in the examined DMF soluble fraction of benzoylated wood, which is due to selective dissolution in IL prior to derivatization.

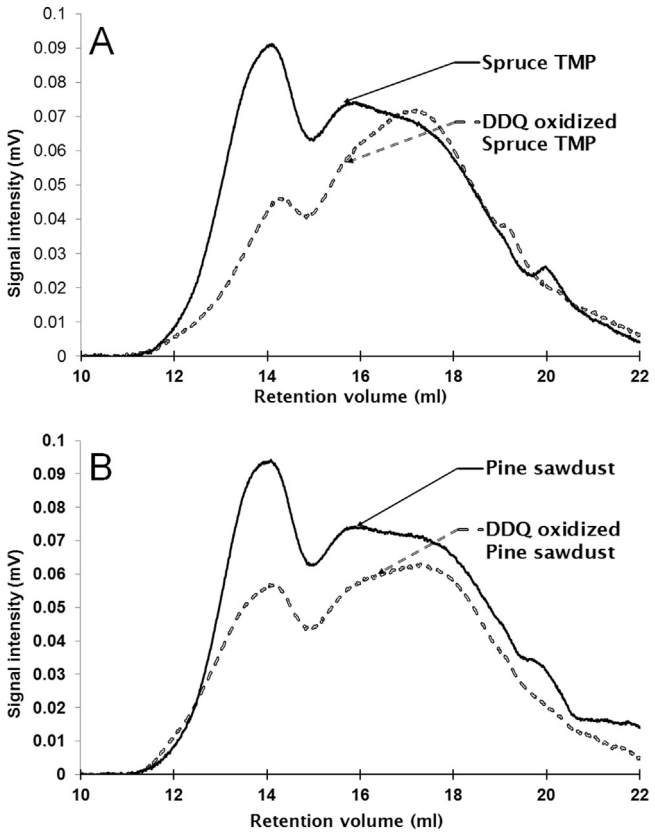

Fig. 7. Elimination of lignin-carbohydrate complexes by DDQ oxidation and the resulting changes to the molecular weight distributions of acetobrominated wood. (A) Spruce TMP pulp, 42 h reaction in dichloromethane-water 50:1 at 40 °C; (B) Pine sawdust, 54 h reaction in dioxane-water 40:1 at 60 °C.

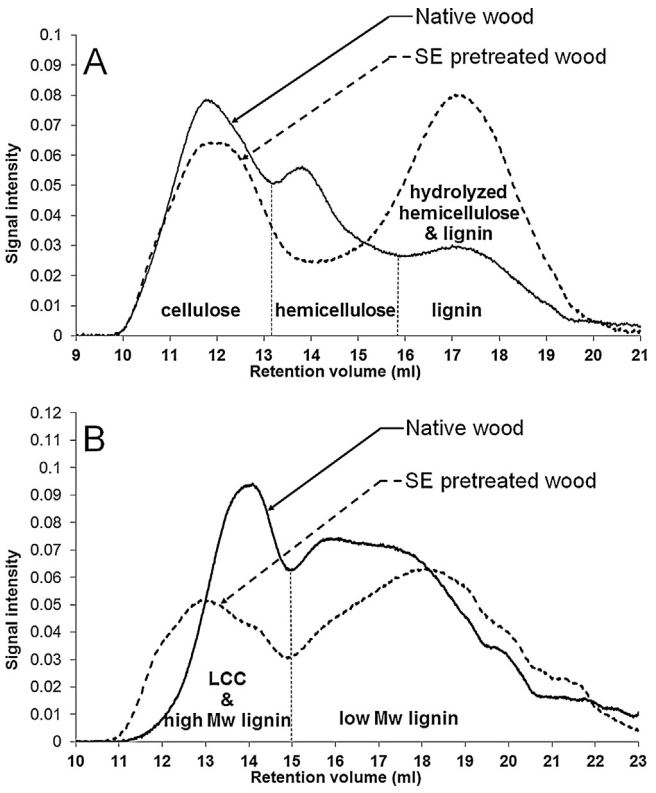

Fig. 8. Molecular weight distributions in the Pine sawdust before and after Steam explosion pretreatment. (A) Benzoylated samples (Varying sample preparations were used for native and SE samples. Reactions after dissolution for 42 h at 80 °C & 66 h at 60 °C, respectively. Dissolution for SEC in 10 & 20 ml of DMF, respectively); (B) Acetobrominated samples.

Further investigations were focused on the bimodal distributions of lignin seen especially in acetobrominated wood, but also to some extent in isolated EMAL preparations. The presence of benzyl ether bonded lignin-carbohydrate complexes (LCC) has been detected in considerable quantities in Pine lignin preparations (Balakshin, Capanema, Gracz, Chang, & Jameel, 2011). The limited quantity of water in the acetobromination system is not expected to facilitate complete hydrolysis of the LCC moieties apart from lignin, leaving them potentially affecting the lignin elution time. Thus, selective oxidation of the benzylic ether bonds using 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) (Watanabe, Ohnishi, Yamasaki, Kaizu & Koshijima, 1989) was applied in order to evaluate the presence of benzyl ether LCC's in the chromatograms of acetobrominated wood.

The DDQ oxidation was examined using two alternative wood sources and reaction solvents, and in both cases this DDQ treatment resulted in a significant reduction in the intensity of the high MW peak (Fig. 7A and B). The lignin distribution in DDQ treated wood resembles somewhat the EMAL lignin from Pine (Fig. 3A). Despite some uncertainty remaining in relation to whether the bimodality arises from the cleavage of the LCC or intra-lignin bonds, the data offers compelling evidence pointing to the significance of the benzyl ether linkages in the molecular architecture of native softwood lignin.

Supporting the preceding conclusion about the presence of LCC, the benzoylated EMAL lignin showed more intense UV-response for the high MW region than observed in the acetobrominated sample (Fig. 3A and B). This difference between the two derivatized lignins may arise from benzoate ester rich moieties such as LCC, having larger MW than the lignin without an attached carbohydrate. Sequential acetobromination and benzoylation reactions for bleached pulp and Pine sawdust offered further insight about the occurrence of LCC in the mid MW region in the wood chromatograms (see the Supporting information Section SI2).

Bimodal elution profiles for lignin are apparent in several reports ascribed to non-covalent lignin association effects (Cathala, Saake, Faix, & Monties, 2003; Connors, Sarkanen, & McCarthy, 1980). The presence of such association phenomena is probably related to the nature of the mobile phase used and its additives (Connors et al., 1980). The SEC acquisition conditions used in this work are selected to eliminate such associations. Therefore, the present data point out to the conclusion that the bimodality of lignin in Pine sawdust is due to covalently linked structures in either the higher MW lignin or the presence of LCC.

3.6. Analysis of wood before and after pretreatments

Finally, the developed benzoylation-acetobromination methodology was applied to examine wood materials resulting from two different pretreatment processes, specifically steam explosion (SE) and green liquor pulping (GLP). As anticipated the SE pretreatment resulted in an increase in the proportion of low molecular weight moieties. The altered MW distribution shown in Fig. 8A is evidently associated with the hydrolytic depolymerization of the hemicelluloses, and plausibly affected also by an increased solubility of the lignin. Lignin distribution was also observed to be significantly altered by the SE pretreatment (Fig. 8B). Increases in the SE lignin polydispersity are consistent with cleavage of LCC bonds, and simultaneous depolymerization and re-polymerization reactions. The depolymerization and re-polymerization of lignin under SE has been reported (Li, Henkriksson, & Gellerstedt, 2007).

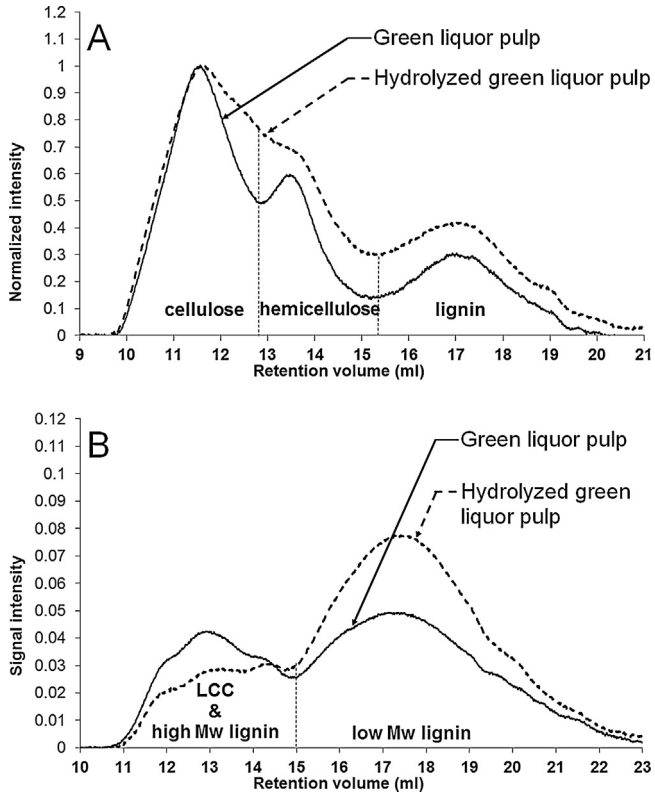

Fig. 9. Molecular weight distributions of carbohydrates and lignin in Pine wood after green liquor pulping to lignin content of 20%, and subsequent enzymatic hydrolysis. (A) Benzoylated products; (B) Acetobrominated products.

The effects of GLP pretreatment and GLP followed by enzymatic hydrolysis are shown in Fig. 9A and B. In Fig. 9A the effects GLP pretreatmenton the SEC profiles of the Pine wood show a loss in lower molecular weight sugars and some associated lignin as reported by Wu et al. (2010). This is followed by further consumption of the cellulose and the hemicelluloses by the enzymatic hydrolysis, which shows as weaker intensity of the carbohydrates in comparison to the lignin signal. The presence of a high MW cellulose component is consistent with the recalcitrant nature of the cellulose in softwoods, leading to incomplete hydrolysis.

The lignin distribution after the GLP pretreatment revealed notably higher MW structures than those present in native wood (Fig. 8B), which points to the occurrence of condensation reactions during the high temperature (170 °C) pretreatment. The lignin distribution of hydrolyzed material showed a reduced high molecular weight region and an intensified low molecular weight region (Fig. 9B). As the cellulolytic enzymes cannot depolymerize lignin, the changes in the detected lignin distribution during the enzymatic hydrolysis can be attributed to cleavage of carbohydrate attached to LCC.

4. Conclusions

Facile analytical methods are essential for the detection of the MWD of the native biomass components, and for observing changes in the MWD of individual components that occur during biomass pretreatments. A novel methodology was developed for the analysis of the MWD of the main wood components in both native and pretreated wood.

Derivatization by benzoylation provided well-resolved chromatograms of the carbohydrate moieties due to the incomplete solubility of wood in the IL. A lignin targeted acetobromination procedure resulted in complete dissolution and evaluation of lignin present in wood samples.

Occurrence of the known side reactions related to the used derivatization systems were investigated, and their effects to the accuracy of the MW analysis was found minimal. Preparative ball milling was not found beneficial as this altered dramatically the observed MW distributions. The use of relatively coarse sawdust for the analyses allowed analysis of the wood components in close to unaltered state.

The three main wood components could be identified from the multimodal chromatograms obtained. Covalent bonds between the lignin and the carbohydrates (LCC) made a noticeable contribution to the observed MWD. DDQ oxidation experiments provided strong evidence for the presence and the role of benzyl ether linkages in the bimodal lignin MWD in softwood. Analysis of enzymatically hydrolyzed pretreated softwood supported the observations related to the role of LCC structures in creating a high MW molecular architecture. The presented results have demonstrated the potential of the current methodology when applied in understanding wood pretreatment and processing.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2014.11.026.

References

Asikkala, J., Tamminen, T., & Argyropoulos, D. S. (2012). Accurate and reproducible determination of lignin molar mass by acetobromination. *Journal of Agricultural and Food Chemistry*, *60*, 8968–8973.

Balakshin, M., Capanema, E., Gracz, H., Chang, H., & Jameel, H. (2011). Quantification of lignin-carbohydrate linkages with high-resolution NMR spectroscopy. *Planta*, *233*(6), 1097–1110. http://dx.doi.org/10.1007/s00425-011-1359-2

Belgacem, M. N., & Gandini, A. (2008). *Monomers, polymers and composites from renewable resources* (1st ed.). UK: Elsevier Science.

Capanema, E. A., Balakshin, M. Y., & Kadla, J. F. (2004). A comprehensive approach for quantitative lignin characterization by NMR spectroscopy. *Journal of Agricultural and Food Chemistry*, *52*(7), 1850–1860. http://dx.doi.org/10.1021/jf035282b

Casas, A., Alonso, M. V., Oliet, M., Santos, T. M., & Rodriguez, F. (2013). Characterization of cellulose regenerated from solutions of pine and eucalyptus woods in 1-allyl-3-methilimidazolium chloride. *Carbohydrate Polymers*, *92*(2), 1946–1952. http://dx.doi.org/10.1016/j.carbpol.2012.11.057

Cathala, B., Saake, B., Faix, O., & Monties, B. (2003). Association behaviour of lignins and lignin model compounds studied by multidetector size-exclusion chromatography. *Journal of Chromatography A*, *1020*(2), 229–239. ⟨http://dx.doi.org/10.1016/j.chroma.2003.08.046⟩.

Connors, W. J., Sarkanen, S., & McCarthy, J. L. (1980). Gel chromatography and association complexes of lignin. *Holzforschung-International Journal of the Biology, Chemistry, Physics and Technology of Wood*, *34*(3), 80–85.

El Seoud, O. A., Koschella, A., Fidale, L. C., Dorn, S., & Heinze, T. (2007). Applications of ionic liquids in carbohydrate chemistry: A window of opportunities. *Biomacromolecules*, *8*(9), 2629–2647. http://dx.doi.org/10.1021/bm070062i

Fujimoto, Y., Matsumoto, Y., Chang, H. M., & Meshitsuka, G. (2005). Quantitative evaluation of milling effects on lignin structure during the isolation process of milled wood lignin. *Journal of Wood Science*, *51*, 89–91.

Gazit, O. M., & Katz, A. (2012). Dialkylimidazolium ionic liquids hydrolyze cellulose under mild conditions. *ChemSusChem*, *5*(8), 1542–1548. http://dx.doi.org/10.1002/cssc.201100803

George, A., Tran, K., Morgan, T. J., Benke, P. I., Berrueco, C., Lorente, E., et al. (2011). The effect of ionic liquid cation and anion combinations on the macromolecular structure of lignins. *Green Chemistry*, *13*(12), 3375–3385. http://dx.doi.org/10.1039/C1GC15543A

Hallac, B. B., & Ragauskas, A. J. (2011). Analyzing cellulose degree of polymerization and its relevancy to cellulosic ethanol. *Biofuels, Bioproducts and Biorefining*, *5*(2), 215–225.

Hortling, B., Turunen, E., & Kokkonen, P. (2003). Molar mass and size distribution of lignins. In C. Wu (Ed.), *Handbook of size exclusion chromatography and related techniques: Revised and expanded* (91) (p. 359). Marcel Dekker Inc.

Howsmon, J. A., & Marchessault, R. H. (1959). The ball-milling of cellulose fibers and recrystallization effects. *Journal of Applied Polymer Science*, *1*(3), 313–322.

Iiyama, K., & Wallis, A. F. A. (1990). Dissolution of wood with acetyl bromide solutions-reactions of lignin model compounds. *Journal of Wood Chemistry and Technology*, *10*(1), 39–58. http://dx.doi.org/10.1080/02773819008050226

Josefsson, T., Lennholm, H., & Gellerstedt, G. (2001). Changes in cellulose supramolecular structure and molecular weight distribution during steam explosion of aspen wood. *Cellulose*, *8*(4), 289–296. http://dx.doi.org/10.1023/A:1015168327450

Kartha, K. P. R., & Jennings, H. J. (1990). A simplified, one-pot preparation of acetobromosugars from reducing sugars. *Journal of Carbohydrate Chemistry*, *9*(5), 777–781. http://dx.doi.org/10.1080/07328309008543873

Kuroda, K., Fukaya, Y., & Ohno, H. (2013). Direct HPILC analysis of cellulose depolymerisation in ionic liquids. *Analytical Methods*, *5*(13), 3172–3176.

Leskinen, T., King, A. W. T., Kilpeläinen, I., & Argyropoulos, D. S. (2011). Fractionation of lignocellulosic materials with ionic liquids. 1. Effect of mechanical treatment. *Industrial and Engineering Chemistry Research*, *50*(22), 12349–12357.

Leskinen, T., King, A. W. T., Kilpeläinen, I., & Argyropoulos, D. S. (2013). Fractionation of lignocellulosic materials using ionic liquids: Part 2. Effect of particle size on the mechanisms of fractionation. *Industrial and Engineering Chemistry Research*, *52*(11), 3958–3966. http://dx.doi.org/10.1021/ie302896n

Li, J., Henriksson, G., & Gellerstedt, G. (2007). Lignin depolymerization/repolymerization and its critical role for delignification of aspen wood by steam explosion. *Bioresource Technology*, *98*(16), 3061–3068. http://dx.doi.org/10.1016/j.biortech.2006.10.018

Lu, F., & Ralph, J. (1996). Reactions of lignin model β-aryl ethers with acetyl bromide. *Holzforschung*, *50*, 360–364.

Naik, S. N., Goud, V. V., Rout, P. K., & Dalai, A. K. (2010). Production of first and second generation biofuels: A comprehensive review. *Renewable and Sustainable Energy Reviews*, *14*(2), 578–597. ⟨http://dx.doi.org/10.1016/j.rser.2009.10.003⟩.

Ragauskas, A. J., Williams, C. K., Davison, B. H., Britovsek, G., Cairney, J., Eckert, C. A., et al. (2006). The path forward for biofuels and biomaterials. *Science*, *311*(5760), 484–489. http://dx.doi.org/10.1126/science.1114736

Salanti, A., Zoia, L., Tolppa, E., & Orlandi, M. (2012). Chromatographic detection of lignin-carbohydrate complexes in annual plants by derivatization in ionic liquid. *Biomacromolecules*, *13*(2), 445–454. http://dx.doi.org/10.1021/bm2014763

Sjöholm, E., Gustafsson, K., Berthold, F., & Colmsjö, A. (2000). Influence of the carbohydrate composition on the molecular weight distribution of kraft pulps. *Carbohydrate Polymers*, *41*, 1–7.

Sjöholm, E. (2003). Size exclusion chromatography of cellulose and cellulose derivatives. In C. Wu (Ed.), *Handbook of size exclusion chromatography and related techniques: Revised and expanded* (91) (p. 311). Marcel Dekker Inc.

Striegel, A. M. (2003). Influence of chain architecture on the mechanochemical degradation of macromolecules. *Journal of Biochemical and Biophysical Methods*, *56*(1), 117–139.

Watanabe, T., Ohnishi, J., Yamasaki, Y., Kaizu, S., & Koshijima, T. (1989). Binding-site analysis of the ether linkages between lignin and hemicelluloses in lignin-carbohydrate complexes by DDQ-oxidation. *Agricultural and Biological Chemistry*, *53*(8), 2233–2252.

Wu, S., & Argyropoulos, D. S. (2003). An improved method for isolating lignin in high yield and purity. *Journal of Pulp and Paper Science*, *29*(7), 235–240.

Wu, S., Chang, H., Jameel, H., & Philips, R. (2010). Novel green liquor pretreatment of loblolly pine chips to facilitate enzymatic hydrolysis into fermentable sugars for ethanol production. *Journal of Wood Chemistry and Technology*, *30*(3), 205–218.

Zoia, L., King, A. W. T., & Argyropoulos, D. S. (2011). Molecular weight distributions and linkages in lignocellulosic materials derivatized from ionic liquid media. *Journal of Agricultural and Food Chemistry*, *59*, 829–838.