Published in final edited form as:

Plant J. 2013 April ; 74(2): 328–338. doi:10.1111/tpj.12124.

Universal fractionation of lignin–carbohydrate complexes (LCCs) from lignocellulosic biomass: an example using spruce wood

Xueyu Du, Goran Gellerstedt, and Jiebing Li*

Department of Fibre and Polymer Technology, Royal Institute of Technology, Kungliga Tekniska Högskolan, Teknikringen 56-58, SE-10044, Stockholm, Sweden

SUMMARY

It is of both theoretical and practical importance to develop a universally applicable approach for the fractionation and sensitive lignin characterization of lignin–carbohydrate complexes (LCCs) from all types of lignocellulosic biomass, both natively and after various types of processing. In the present study, a previously reported fractionation approach that is applicable for eucalyptus (hardwood) and flax (non-wood) was further improved by introducing an additional step of barium hydroxide precipitation to isolate the mannan-enriched LCC (glucomannan-lignin, GML), in order to suit softwood species as well. Spruce wood was used as the softwood sample. As indicated by the recovery yield and composition analysis, all of the lignin was recovered in three LCC fractions: a glucan-enriched fraction (glucan-lignin, GL), a mannan-enriched fraction (GML) and a xylan-enriched fraction (xylan-lignin, XL). All of the LCCs had high molecular masses and were insoluble or barely soluble in a dioxane/water solution. Carbohydrate and lignin signals were observed in ^1H NMR, ^{13}C CP-MAS NMR and normal- or high-sensitivity 2D HSQC NMR analyses. The carbohydrate and lignin constituents in each LCC fraction are therefore believed to be chemically bonded rather than physically mixed with one another. The three LCC fractions were found to be distinctly different from each other in terms of their lignin structures, as revealed by highly sensitive analyses by thioacidolysis-GC, thioacidolysis-SEC and pyrolysis-GC.

Keywords

lignin–carbohydrate complex (LCC); universal fractionation approach; spruce; NMR; thioacidolysis; pyrolysis-GC

INTRODUCTION

Lignocellulosic biomass is one of the most important raw materials for material, energy and chemicals. Its importance has recently been particularly highlighted due to its inherent renewability and sustainability, as it may be a potential solution for the worldwide demand for alternative energy. Lignin, cellulose and hemicelluloses are the three major polymeric components in lignocellulosic biomass. They have different applications due to their

different structures and different properties. They should be processed differently and/or be handled after selective separation or fractionation. However, separation or fractionation is not an easy task because these components are physically entangled with one another in the raw materials (Salmén and Burgert, 2008). Chemical linkages are also present between these components, and the structure that has chemical bonds/linkages between lignin and carbohydrate is known as the lignin–carbohydrate complex (LCC).

In the theoretical field, there has been on-going debate about the native structure of LCCs. In biomass processing, the inherent presence of LCCs or the formation of LCCs during processing is considered to be the reason for difficulties in chemical or biochemical processing of lignocellulosics, such as delignification during chemical pulping (Gierer and Wannström, 1986; Iverson and Wannström, 1986; Choi *et al.*, 2007), and in the fermentation of lignocellulosic materials for ethanol production (Kim *et al.*, 2003; Aita *et al.*, 2011). However, little evidence has been reported regarding the native presence of LCCs or formation of the LCC structure. Therefore, LCC structures require more thorough investigation to provide more valuable information to truly understand both the complicated bonding relationships among cellulose, hemicelluloses and lignin and the structural changes brought about by biomass processing.

To investigate LCC structure, clear and complete fractionation with proper preservation of the bonding structure between the lignin and carbohydrate is a prerequisite. To separate the lignin or LCC structure, milling has commonly been used to open up the entangled structure with or without a subsequent enzymatic hydrolysis, followed by solvent extraction (Björkman, 1956; Chang *et al.*, 1975; Fiserova *et al.*, 1985; Wu and Argyropoulos, 2003; Anderson *et al.*, 2008). However, solvent extraction may only recover a part of the lignin or LCC, reducing the representative value of the structures obtained. To obtain a higher recovery, harsher milling and/or more severe enzymatic treatment is required, but this may result in damage to the lignin or LCC structure. We have proposed a revolutionary concept for LCC fractionations, under which a biomass sample, native or after processing, is first completely dissolved in a solution so that the various LCCs may freely move in the solution. Further fractionations then separate the various LCCs from one another. All of the lignin is recovered separately and quantitatively, which guarantees the representativeness of the subsequent characterization. The first quantitative fractionation method developed was based on ball milling, endoglucanase enzymatic hydrolysis and alkaline dissolution, by which all of the lignin in softwood was isolated as various LCCs (Lawoko *et al.*, 2003, 2005). The recovery yields of all lignin in the LCCs totalled 100%. However, this method involves many steps and is only applicable for softwoods, which strongly restricts its application. Subsequently, another much simpler fractionation method was established to isolate LCCs based on mild milling and dissolution using dimethyl sulfoxide (DMSO) and tetra-*n*-butylammonium hydroxide (TBAH). The duration of ball milling was optimized at 12 h, which guarantees preservation of the lignin structures in terms of retaining the β -O-4' structure and the extent of lignin condensation (Li *et al.*, 2011). The second method successfully provides clear and complete fractionation of LCCs from hardwood, e.g. eucalyptus (Li *et al.*, 2011), and non-wood species such as soda-anthraquinone (soda-AQ) flax cellulose pulps (Cadena *et al.*, 2011). Two LCC fractions, glucan-lignin (GL) and

xylan-lignin (XL), were obtained and characterized. To develop the approach further and make it universally applicable, it must also be validated for softwood species.

Additionally, systematic and in-depth characterizations of LCCs are necessary. Many characterizations have been reported, e.g. using a combination of a traditional method and a modern analytical technique, such as NMR, to accomplish the task. However, characterization of LCCs is still a challenge because the LCCs are polymeric structures and the content of lignin-carbohydrate bonds is small. The various lignin-carbohydrate linkages have recently been characterized using modern high-sensitivity NMR spectroscopy, with a 950 MHz spectrometer equipped with a cryogenic probe (Balakshin *et al.*, 2011). However, the instrument utilized in that study is not commonly found in an ordinary chemical laboratory.

Therefore, in the present study, the softwood species spruce wood was used, and the second approach mentioned above was modified to include an additional step to obtain a third LCC fraction, i.e. glucomannan-lignin (GML), because glucomannan is known to be the major hemicellulosic component in softwood species. The fractionation was followed by a systematic characterization, and particular effort was devoted to assessing the presence of chemical linkages between lignin and carbohydrates and obtaining structural information on the lignin from thioacidolysis-GC and -SEC and pyrolysis-GC to overcome the limitations of NMR analyses.

RESULTS AND DISCUSSION

Universal LCC fractionation method demonstrated on spruce wood

As shown in Figure 1, spruce wood was first vibrationally ball-milled for 12 h; this time interval was long enough for the milled wood fibres then to be completely dissolved by a solution of DMSO and TBAH as the biomass structure had been sufficiently physically broken down (Li *et al.*, 2011). In solution, all of the components are completely physically separated from one another; there is no more physical entanglement but the chemical bonds are still intact. Enzymatic hydrolysis was avoided because it would have introduced contaminants, e.g. proteins, and may have resulted in other undesirable structural changes. During the 12 h of ball milling, the cellulose was degraded into smaller molecular sizes, but no chemical structural changes resulted with regard to the lignin structure in the LCC (Li *et al.*, 2011). The DMSO + TBAH solution has been reported to be a good cellulose solvent (Lidbrandt *et al.*, 1990), and therefore achieves total dissolution of all of the biomass LCC fractions at room temperature. A mixture of DMSO and TBAF (tetrabutylammonium fluoride, a salt form of TBAH) has been used previously for ball-milled cell-wall dissolution before NMR determination (Lu and Ralph, 2003).

We have previously reported that, after ball milling for less than 12 h and application of DMSO + TBAH, native or processed hardwood, softwood and non-wood biomass, represented by eucalyptus, spruce and flax, respectively, were all completely dissolved (Li *et al.*, 2011). From the investigated eucalyptus fibres and the flax soda-AQ pulps, two types of LCC were fractionated after simple dispersion of the solution into water: a low-solubility

fraction of GL as a precipitate, and a high-solubility fraction of XL as a solute (Cadena *et al.*, 2011; Li *et al.*, 2011).

It is well known that the hemicelluloses in softwood mainly consist of glucomannan. Therefore, in this study, the fractionation approach was modified by introducing a step comprising barium hydroxide addition. It has been reported that barium ions readily form insoluble complexes with mannans by interactions between the ions and the vicinal *cis*-hydroxyl groups on carbons 2 and 3 of the mannose units. Other polysaccharides, e.g. xylan, are not precipitated because they have no such *cis*-hydroxyl group structure (Meier, 1958).

The Ba(OH)₂ precipitation step was introduced after the dispersion for GL fractionation. The complex between the barium ions and the GML was precipitated from the aqueous solution such that the GML was separated from the XL, which was still in solution. The complex was readily reconverted to original GML by soaking and stirring with dilute acid. Thus, three LCCs were fractionated from spruce, rather than the two obtained from hardwood and non-wood species (Figure 1).

Because the biomass from all species tested so far can be completely dissolved (Li *et al.*, 2011), the fractionation approach shown in Figure 1 is considered to be universally applicable for wood, pulp and other original or processed lignocellulosic biomass. In practice, the barium hydroxide step may be skipped if glucomannan is not a major hemicellulose, as in hardwood and non-wood species (Cadena *et al.*, 2011; Li *et al.*, 2011).

LCC fractions from spruce wood

LCC fractionation from spruce wood was very successful using the procedure outlined in Figure 1 because the various carbohydrates were enriched in the various fractions and every fraction contained substantial amounts of lignin (Table 1). GL is glucan-based (85.8% of the sugars were glucose), with 19.3% Klason lignin. GML is mainly composed of glucose and mannose (49.4 and 30.9%, respectively), with 29.2% Klason lignin. XL is xylose-enriched (65.3%), with 42.7% Klason lignin. In all of the isolated LCCs, the lignin and carbohydrate compositions appeared to be constant because repeating the fractionation process (i.e. DMSO + TBAH dissolution, water dispersion and Ba (OH)₂ precipitation) on the isolated LCC fractions did not noticeably alter the compositions. The presence of LCC in spruce has been reported in both wood (Eriksson and Goring, 1980; Lawoko *et al.*, 2003) and kraft pulp (Lawoko *et al.*, 2005; Choi *et al.*, 2007) or sulfite pulp (Lawoko *et al.*, 2006).

All of the isolated LCCs have polymeric structures, as demonstrated by the size-exclusion chromatography (SEC) analysis results shown in Figure 2. The molecular masses of the three LCCs were 4.9×10^5 Da (GL), $1.6 \times 10^5 - 6.3 \times 10^4$ Da (GML) and 1.8×10^4 Da (XL). This finding is in agreement with the common knowledge that the order of molecular size of the carbohydrates is cellulose > glucomannan > xylan. The very high molecular mass of GL is at least partly the rationale for the approach shown in Figure 1, where the GL fraction directly precipitates after water dispersion due to its much higher molecular mass. There were two large fractions in GML, corresponding as expected to galactoglucomannan-lignin and glucomannan-lignin (without galactose), as two hemicelluloses of galactoglucomannan and glucomannan have been reported in the literature (Timell and

Syrancuse, 1967). Compared to GL and GML, XL has a much lower Mp (molar mass at the peak maximum) and is more uniform, with a much smaller molecular mass distribution (Figure 2).

This fractionation was quantitative. Using a mass balance, GL accounted for 49.5% of the sample, GML for 30.9% and XL for only 12.8%, in agreement with the relatively low xylan content in softwoods (Table 1). Based on the Klason lignin contents, all of the lignin, which amounted to approximately 27.1% of the dried weight in the spruce wood, was recovered by 19.3% in GL, 29.2% in GML and 42.7% in XL. According to the proposed ultrastructural model of wood polymers in the secondary cell wall, deduced from investigating the softening behaviour (Salmén and Olsson, 1998), xylan is primarily associated with lignin, whereas glucomannan is more commonly associated with cellulose. The model has been further supported by Fourier transform infrared spectroscopy analyses (Åkerholm and Salmén, 2001). Therefore, it may be expected that the lignin connected with glucan (GL) is always associated with glucomannan, which explains why the isolated GL still contained a certain percentage of mannose and may not be further purified. In addition, as ball milling degrades some portion of the glucan/cellulose into smaller molecules (Li *et al.*, 2011), a portion of the GL complex, especially when it is with a low molecular mass, would not be easily precipitated by water dispersion but would enter the GML fraction by Ba(OH)₂ precipitation due to the presence of mannose in its structure. This outcome explains why the ratio of glucose/mannose in the isolated GML was higher than the 1:3–1:4 ratio reported for typical glucomannans in spruce (Timell and Syrancuse, 1967).

Evidence for chemical linkages between lignin and carbohydrates

It is generally accepted that various types of chemical linkages between lignin and carbohydrate are present in LCC structures, and the most commonly proposed types are benzyl ether, benzyl esters, γ -esters and phenyl glycosides. The work of Balakshin *et al.* (2011) is a representative publication that observed various linkages by high-sensitivity NMR analysis. For the LCCs prepared in this study, the very high molecular masses of the structures were preserved. This made it difficult to detect and assign the NMR signals for the lignin–carbohydrate bonding because there was substantial influence from the high molecular mass, which caused a severe decrease in the signal intensity, especially when ordinary-sensitivity NMR analysis was performed (see below). However, some indirect evidence was collected, demonstrating the presence of lignin–carbohydrate linkages in the polymeric LCC structures.

First, the high molecular masses of the LCCs were revealed by alkaline SEC using UV detection at 280 nm (Figure 2), implying that lignin, which is responsible for the 280 nm adsorptions, is attached to various high-molecular-mass polysaccharides.

Second, LCCs demonstrate a very different solubility to free lignin. In a previous report, a dioxane/water solution was used at a ratio of 96:4 to dissolve the free lignin during milled wood lignin (MWL) preparation (Björkman, 1956), and used at a ratio of 82:18 to isolate lignin via acid hydrolysis from wood or pulp (Gellerstedt *et al.*, 1994). When the solubility of the obtained LCC fractions was investigated in a dioxane/water solution (90:10 v/v), the GL and GML fractions were not soluble, and only a trace amount of XL was soluble (Table

1), possibly because the xylan portion that was attached by the lignin was itself partially dissolved in this solution. For spruce wood itself, only extractives were directly soluble (Table 1).

Third, as mentioned above, although the lignin aromatic signals were clearly observed for the XL fraction using the 2D HSQC NMR technique, with a 400 MHz NMR instrument (Figure 3), no aromatic signals were observed using the same NMR equipment as for GL or GML. NMR assignments of the main polysaccharides in XL were based on previous data (Teleman *et al.*, 2000, 2002; Evtuguin *et al.*, 2003). The cross-peaks at δ 4.76/108.3 (H1/C1), 3.83/82.0 (H2/C2), 3.65/77.7 (H3/C3), 3.97/86.5 (H4/C4) and (3.45; 3.61)/62.0 (H5/C5) are from α -L-(1 $\rightarrow$ 4) arabinosyl units, and those at δ 4.25/102.0 (H1/C1), 3.03/72.9 (H2/C2), 3.23/74.2 (H3/C3), 3.50/75.5 (H4/C4) and (3.16; 3.87)/63.4 (H5/C5) are from β -D-(1 $\rightarrow$ 4) xylosyl units. In the analysis, the XL was directly dissolved in solution as it had the smallest molecular mass of the three LCCs, whereas the GL and GML fractions had larger molecular masses and required acetylation to facilitate the required dissolution. A representative 2D HSQC NMR spectrum for GL is shown in Figure 4, after polysaccharide assignment as described by Lu and Ralph (2003). In this case, the lignin aromatic signals in the ^1H NMR spectrum (Figure 5) were already very weak due to the short spin-spin relaxation time (T_2) caused by the large size of the polymers (Zhang and Gellerstedt, 2007). Solid-state ^{13}C cross polarization-magic angle spinning (CP-MAS) NMR was performed for GML and lignin-free glucomannan (GM) (Figure 6) in order to overcome the solubility difficulty encountered when performing solution-state NMR, and the assignments for GM are based on previous data (Rakhimov *et al.*, 2004). Here, lignin signals (105–160 ppm for aromatic carbons, approximately 87.5 ppm for lignin side-chain carbons and 55.3 for methoxy carbons) were easily observed by comparison of the ^{13}C CP-MAS NMR spectra for GML with those for GM. However, the resolution of the ^{13}C CP-MAS NMR spectra is too low to reveal any more structural details. Using an NMR instrument with a cryo-platform and cryo-probe at 750 MHz, i.e. high-sensitivity solution-state NMR, greatly enhanced 2D NMR spectra were obtained for both original GML and acetylated GML; here, the analysis of GML was performed after acetylation of GML in order to achieve a high enough solubility in CDCl_3 , and thus the lignin aromatic signals from the G units were more clearly observed (Figure 7). Carbohydrate signals, assigned on the basis of previous data (Lu and Ralph, 2003; Qu *et al.*, 2011), were also clearly observed in the spectrum. The cross-peaks at δ 4.41/100.4 (H1/C1), 4.79/71.6 (H2/C2), 5.06/72.3 (H3/C3), 3.71/75.9 (H4/C4), 3.55/72.6 (H5/C5) and (4.05; 4.36)/61.9 (H6/C6) are from β -D-(1 $\rightarrow$ 4) glucosyl units, and those at δ 4.64/97.8 (H1/C1), 5.32/68.5 (H2/C2), 5.03/70.6 (H3/C3), 3.87/72.6 (H4/C4), 3.50/72.6 (H5/C5) and (4.17; 4.28)/62.5 (H6/C6) are from β -D-(1 $\rightarrow$ 4) mannosyl units. The ‘disappearance’ of the lignin signals in the ‘ordinary’-sensitivity NMR (400 MHz) analysis confirmed the high-molecular-mass characteristics of the LCCs, which are the result of chemical linkages between the lignin and the polysaccharides.

Other highly sensitive analyses of lignin structures in LCCs

As discussed above, although lignin signals were observed in various NMR spectra, the information obtained was very limited, and no signals were observed that could be used to unambiguously demonstrate lignin–polysaccharide bonding, even with high-sensitivity

NMR. Therefore, we were unable to effectively distinguish the structural differences in the lignin parts among the three LCCs by NMR analysis. Thus, the NMR analysis was complemented by thioacidolysis and pyrolysis-GC analyses.

It is well known that thioacidolysis completely breaks down arylglycerol- β -aryl ether linkages, the most abundant inter-unit linkage in lignin, by combining a hard Lewis acid, boron trifluoride etherate and a soft nucleophile (ethanethiol at a low pH and high temperature (Rolando *et al.*, 1992). After thioacidolysis, the lignin structures were degraded into monomers and oligomers, the latter being derived from lignin units linked together by non- β -ethers, i.e. bonds that survive the thioacidolysis conditions. A subsequent GC analysis then determined the type and amount of monomeric lignin units that were only linked by β -O-4' bonds, and a subsequent SEC analysis showed the relative amount of oligomers versus monomers after cleavage of all β -O-4' bonds, which revealed the extent of lignin condensation.

In this study, the GC analysis after thioacidolysis showed that GL contains a higher amount of uncondensed guaiacyl (G) units ($312 \mu\text{mol g}^{-1}$) than the other two LCC fractions (approximately $225 \mu\text{mol g}^{-1}$ for both GML and XL; Table 1). After taking into account the lignin content differences in the LCCs, the content of G units in the various LCCs decreases in the order GL > GML > XL (1633 , 781 and $520 \mu\text{mol g}^{-1}$, respectively, on the basis of lignin weight; Table 1). The linearity of lignin structure in the LCCs decreased in the order GL > GML > XL. In addition, the SEC analysis after thioacidolysis revealed that the lignin fragments from all of the LCC fractions were mainly monomers and dimers, and only showed a small amount of condensed structures (Figure 8). This finding is reasonable because all these LCCs were directly isolated from the original wood material without any processing or chemical modification by high temperatures, certain reactive chemicals or the fractionation approach itself. A large amount of condensed lignin structure is not natively present in spruce wood. Notably, the relative amount of the fragments with molecular masses smaller than monomers, shown as peaks of retention times longer than 33 min, was highest in the GL among the three LCCs (Figure 8).

More information about the lignin structure was obtained after performing pyrolysis-gas chromatography/flame ionization detector (pyrolysis-GC/FID) and pyrolysis-gas chromatography/mass spectrometry (pyrolysis-GC/MS) analyses. During pyrolysis, the LCC molecules were broken down by heating, primarily at specific structural points with low chemical bonding energies. The stable and volatile degradation fragments formed were separated and detected by GC/MS, which provided useful information about the entire LCC molecule (Meier and Faix, 1992). For example, when GML was pyrolysed, the degraded products were mainly from glucomannan (data not shown) and lignin (Figure 9), as identified by MS (Meier and Faix, 1992). Table 2 shows that pyrolysis of GML gave rise to a series of substituted monomeric phenols in which the original propanoid side chains had mostly been either split off completely or shortened to one or two carbons. New double bonds were usually created at the side chains after the pyrolytic dehydrogenation/elimination. Notably, the major pyrolytic products formed were generated from the G units. Similar pyrolytic lignin fragments were also found during the pyrolysis of GL and XL, but the abundances of the various fragments were quite different (Table 2). In Table 2, the peak

heights obtained by pyrolysis-GC/FID were used for quantification instead of the peak area because there was a severe interference of the noises for the latter method. Each peak was quantified by taking the peak height as a percentage of the total peak heights of all the identified fragments from lignin G units. The most abundant lignin pyrolytic fragments for GL, GML and XL were vanillin (G-CHO, 15.7%), 4-methyl-guaiacol (Me-G, 18.2%) and 4-vinyl-guaiacol (Vinyl-G, 22.5%). It has been reported that vanillin may be formed from non-phenolic β -O-4' structures (Nakamura *et al.*, 2008) or from β -1'-type model dimers (Kawamoto *et al.*, 2007). Chemically, 4-methyl-guaiacol most likely originates from the cleavage of the C $_{\alpha}$ -C $_{\beta}$ bond in the original propanoid side chains. In addition, there were also differences in the abundance of the carbonyl-containing fragments, such as vanillin, homovanillin and acetoguaiacone, among the GL, GML and XL fractions (Table 2). As suggested by Ohra-aho *et al.* (2005), the differences in carbonyl-containing structures imply that there are differences in the extent of oxidation of the lignin structures present in the sample. Although the exact formation mechanisms of the lignin fragments are very complicated, it may be concluded from the difference observed in pyrolysis-GC analysis that the lignin portions present in different LCCs are structurally different from each other. The differences in fragmentation may also be related to differences in the chemical bonds between the lignin and carbohydrate among these LCCs.

EXPERIMENTAL PROCEDURES

Wood sample and chemicals

Spruce (*Picea abies*) wood chips were supplied by a Swedish sawmill. Dimethyl sulfoxide (DMSO) and tetra-*n*-butylammonium hydroxide (TBAH, 40% w/w in water) were purchased from Fisher Scientific (www.fishersci.com) and Alfa Aesar (www.alfa.com), respectively.

Sample pre-treatments

The wood chips were milled using a Wiley mill (Thomas Scientific, <http://www.thomassci.com/>) to sizes smaller than 40 mesh, and extracted with acetone using a Soxhlet extractor (bought from Fisher Scientific, www.fishersci.com) for 24 h. The extractive-free residues were then dried at room temperature and further ground in a vibratory ball mill (Siebtechnik, <http://www.siebtechnikgmbh.de/>) for 12 h to obtain a ball-milled sample as described by Li *et al.* (2011). Typically, 4 g Wiley-milled wood powders and 2 kg steel balls (diameter approximately 7 mm) were added to the 1 litre steel milling jar for vibratory ball milling. As a reference, lignin-free glucomannan was prepared from holocellulose produced from spruce wood by extraction with NaOH/H₃BO₃, precipitation with Fehling solution, and maceration with an HCl solution as described by Zhang *et al.* (2011).

Lignin-carbohydrate complex (LCC) fractionation

The LCC fractionation approach is shown in Figure 1. A ball-milled sample (2.5 g) was completely dissolved in a mixture of 27 ml DMSO and 27 ml TBAH (40% w/w in water), and then the clear solution was dispersed into 530 ml deionized water to form two phases: precipitate-1 and solution-1. Precipitate-1 was continuously washed with deionized water

until a neutral pH was obtained, and was then freeze dried to obtain glucan-lignin (GL). The two other LCC fractions that remained in solution-1 were fractionated by adding 530 ml saturated Ba(OH)₂ solution (Meier, 1958) into solution-1 to form solution-2 and precipitate-2. Glucomannan-lignin (GML) was recovered by neutralizing precipitate-2 with HCl, followed by dialysis (molecular mass cut-off 1000 Da) and freeze-drying. Xylan-lignin (XL) was obtained from solution-2 by neutralizing the solution with HCl, followed by dialysis and freeze-drying.

Analyses

The various LCC fractions were dissolved in 0.1 M sodium hydroxide and analysed by SEC using three TSK gel columns (3000PW, 4000PW and 3000PW) (Tosoh Bioscience, www.tosohbioscience.com) coupled in series, with 0.1 M sodium hydroxide as the eluent. The flow rate was 1 ml min⁻¹, and a Waters 2487 UV detector (www.waters.com) was used at 280 nm for detection. The columns were calibrated using polyethylene glycol and polyethylene oxide standards, with specific molecular masses ranging from 200 to 250 000 Da.

The carbohydrate composition and Klason lignin content were determined as described by Theander and Westerlund (1986) and Technical Association of the Pulp and Paper Industry (TAPPI) standard T222 om-06 (<http://www.tappi.org/>), respectively.

The ¹H NMR spectra were recorded on a Bruker Avance 400 MHz instrument (<http://www.bruker.com/>) using the standard Bruker pulse program (Zhang and Gellerstedt, 2000, 2007). The 2D HSQC NMR spectra were recorded on either the Bruker Avance 400 MHz instrument using the standard Bruker pulse program (Zhang and Gellerstedt, 2000, 2007) or on a 500 MHz Bruker Biospin instrument equipped with a cryogenically cooled 5 mm Bruker TCI (triple resonance cryoprobe optimized for ¹H and ¹³C observation) gradient probe with inverse geometry (proton coils closest to the sample). In the latter case, the 2D ¹³C–¹H correlation spectra were acquired using an adiabatic HSQC pulse program (Bruker standard pulse sequence ‘hsqcetgpsisp2.2’) and the following parameters: spectra were acquired from 10 to 0 ppm (5000 Hz) in F2 (¹H) using 1000 data points for an acquisition time (AQ) of 200 msec, an inter-scan delay (D1) of 1 sec, and from 200 to 0 ppm (25 154 Hz) in F1 (¹³C) using 400 increments (F1 acquisition time 8 msec) of 40 scans, for a total acquisition time of 5 h 27 min. The ¹J_{CH} used was 145 Hz. Processing utilized typical matched Gaussian apodization in the ¹H dimension and squared cosine-bell apodization in the ¹³C dimension. Prior to Fourier transformation, the data matrices were zero-filled to 1024 points in the ¹³C dimension. The central chloroform solvent peak at δ_{C/H} 77.0/7.26 ppm was used as an internal reference. The samples were acetylated to facilitate sample dissolution using the DMSO-NMI (*N*-methylimidazole) method as described by Lu and Ralph (2003). CDCl₃ was used as the solvent for Ac-GL (acetylated-GL) and Ac-GML, and DMSO-*d*₆ was used for XL analysis. The ¹³C CPMAS NMR spectra were recorded using a Bruker Avance AQS 300 WB instrument operating at 7.04 T after packing the sample uniformly in a zirconium oxide rotor using a double air-bearing probe (from Bruker, www.bruker.com). All measurements were performed at 290 ± 1 K, with a magic angle spinning (MAS) rate of 5 kHz. The acquisition was performed using a CP pulse sequence: a

4.3 μ sec proton 90° pulse, an 800 μ sec ramped (100–50%) falling contact pulse and a 2.5 sec delay between repetitions. A TPPM15 pulse sequence was used for the ^1H decoupling. The Hartman–Hahn matching procedure was performed on glycine. The chemical shift was related to tetramethylsilane $[(\text{CH}_3)_4\text{Si}]$ by assigning a chemical shift of 176.03 ppm to the data point of maximum intensity in the glycine carbonyl line.

To quantify the solubility, an excess amount of each LCC fraction was stirred in 2 ml of a dioxane/water solution (90:10 v/v) at room temperature for 12 h. After centrifuging the mixture (14 100 g for 5 min at room temperature), the dissolved material was quantified by drying the supernatant in an oven at 105°C and then weighing the dried material.

The thioacidolysis reaction was performed as described by Rolando *et al.* (1992). One portion of each thioacidolysis product mixture (approximately 5 mg) was silylated for 2 h at room temperature with 50 μ l pyridine and 50 μ l *N,O*-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) to perform the GC analysis and quantify the monomeric degradation products, i.e. the uncondensed guaiacyl (G) units present in the starting sample. Another portion of the thioacidolysis product mixture was acetylated and analysed using SEC to understand the lignin fragments in terms of the ratios among the monomers, dimers and condensed structures. The runs were performed using a series of three connected ultrastaygel columns (100, 500 and 1000 Å) (Waters, www.waters.com), with a mobile phase of tetrahydrofuran at a flow rate of 0.8 ml min⁻¹ using a Waters 515 HPLC pump and a Waters 2487 UV detector at 280 nm.

Each LCC fraction was pyrolysed using a filament pulse pyrolyser (Pyrola 2000, from Pyrol AB, www.pyrolab.com) at 700°C, and the pyrolysis products were directly analysed by GC/FID and GC/MS. The GC separation was performed using a DB-5MS column (Agilent Technologies, www.agilent.com), with helium as the carrier gas. The temperature was initially 60°C for 3 min, and was then increased at a rate of 10°C min⁻¹ to 200°C. After 2 min at 200°C, the temperature was increased at a rate of 15°C min⁻¹ to 300°C. The injector temperature was 230°C. For pyrolysis-GC/MS, the mass spectra were obtained at 70 eV. The fragmentation peaks were quantified by pyrolysis-GC/FID using the same pyrolyser and similar settings for the GC separation in terms of the column and temperature parameters. The detector temperature was 250°C.

Acknowledgments

This study was financially supported by the EU BIORENEW Project (NMP2-CT-2006-026456). The authors thank Jorge Rencoret Pazo (University of Wisconsin, USA) for the high-sensitivity 2D HSQC NMR analysis of Ac-GML using the National Magnetic Resonance Facility at University of Madison, which is supported by US National Institutes of Health grants P41RR02301 Biomedical Research Training Program of the National Center for Research Resources (BRTP/NCRR) and P41GM66326 National Institute of General Medical Sciences (NIGMS). Additional equipment was purchased at University of Madison with funds from the University of Wisconsin, the US National Institutes of Health (RR02781 and RR08438), the US National Science Foundation (DMB-8415048, OIA-9977486 and BIR-9214394), and the United States Department of Agriculture (USDA). Tomas Larsson (Kungliga Tekniska Högskolan, KTH, Stockholm, Sweden) is thanked for the ^{13}C CP-MAS NMR analysis of GML and lignin-free glucomannan.

References

- Aita GA, Salvi DA, Walker MS. Enzyme hydrolysis and ethanol fermentation of dilute ammonia pretreated energy cane. *Bioresour Technol.* 2011; 102:4444–4448. [PubMed: 21247758]
- Åkerholm M, Salmén L. Interactions between wood polymers studied by dynamic FTIR spectroscopy. *Polymer.* 2001; 42:963–969.
- Anderson G, Lucia LA, Argyropoulos DS. Isolation and characterization of lignins from *Eucalyptus grandis* Hill ex Maiden and *Eucalyptus globulus* Labill. by enzymatic mild acidolysis (EMAL). *Holzforschung.* 2008; 62:24–30.
- Balakshin M, Capanema E, Gracz H, Chang HM, Jameel H. Quantification of lignin–carbohydrate linkages with high-resolution NMR spectroscopy. *Planta.* 2011; 233:1097–1110. [PubMed: 21298285]
- Björkman A. Studies on finely divided wood. Part 1 Extraction of lignin with neutral solvents. *Svensk Papperstidn.* 1956; 59:477–485.
- Cadena EM, Du X, Gellerstedt G, Li J, Fillat A, García-Ubasart J, Vidal T, Colom JF. On hexenuronic acid (HexA) removal and mediator coupling to pulp fiber in the laccase/mediator treatment. *Bioresour Technol.* 2011; 102:3911–3917. [PubMed: 21194930]
- Chang HM, Cowling EB, Brown W, Adler E, Miksche G. Comparative studies on cellulolytic enzyme lignin and milled wood lignin of sweetgum and spruce. *Holzforschung.* 1975; 29:153–159.
- Choi JW, Choi DH, Faix O. Characterization of lignin–carbohydrate linkages in the residual lignins isolated from chemical pulps of spruce (*Picea abies*) and beech wood (*Fagus sylvatica*). *J Wood Sci.* 2007; 53:309–313.
- Eriksson Ö, Goring DAI. Structural studies on the chemical bonds between lignins and carbohydrates in spruce wood. *Wood Sci Technol.* 1980; 14:267–279.
- Evtuguin DV, Tomas JL, Silva AMS, Pascoal Neto C. Characterization of an acetylated heteroxylan from *Eucalyptus globulus* Labill. *Carbohydr Res.* 2003; 338:597–604. [PubMed: 12644372]
- Fiserova M, Polcin J, Opalena E, Masarykova M. The effect of milling on the molecular mass of spruce lignin. *Cell Chem Technol.* 1985; 19:185–196.
- Gellerstedt G, Pranda J, Lindfors EL. Structural and molecular properties of residual birch kraft lignins. *J Wood Chem Technol.* 1994; 14:467–482.
- Gierer J, Wannström S. The formation of ether bonds between lignins and carbohydrates during alkaline pulping processes. *Holzforschung.* 1986; 40:347–352.
- Iverson T, Wannström S. Lignin–carbohydrate bonds in a residual lignin isolated from pine kraft pulp. *Holzforschung.* 1986; 40:19–22.
- Kawamoto H, Horigoshi S, Saka S. Pyrolysis reactions of various lignin model dimers. *J Wood Sci.* 2007; 53:168–174.
- Kim TH, Kim JS, Sunwoo C, Lee YY. Pretreatment of corn stover by aqueous ammonia. *Bioresour Technol.* 2003; 90:39–47. [PubMed: 12835055]
- Lawoko M, Henriksson G, Gellerstedt G. New method for quantitative preparation of lignin–carbohydrate complex from unbleached softwood kraft pulp: lignin–polysaccharide networks I. *Holzforschung.* 2003; 57:69–74.
- Lawoko M, Henriksson G, Gellerstedt G. Structural differences between the lignin–carbohydrate complexes present in wood and in chemical pulps. *Biomacromolecules.* 2005; 6:3467–3473. [PubMed: 16283780]
- Lawoko M, Henriksson G, Gellerstedt G. Characterization of lignin–carbohydrate complexes from spruce sulfite pulp–lignin–polysaccharide networks III. *Holzforschung.* 2006; 60:162–165.
- Li J, Martin-Sampedro R, Pedrazzi C, Gellerstedt G. Fractionation and characterization of lignin–carbohydrate complexes (LCCs) from eucalyptus fibers. *Holzforschung.* 2011; 65:43–50.
- Lidbrandt, O.; Iversen, T.; Kolar, J. Manufacture of cellulose solutions and solvent systems for cellulose Swedish Patent SE, 461277B. 1990. p. 8
- Lu F, Ralph J. Non-degradative dissolution and acetylation of ball-milled plant cell walls: high-resolution solution-state NMR. *Plant J.* 2003; 35:535–544. [PubMed: 12904215]

- Meier H. Barium hydroxide as a selective precipitating agent for hemicelluloses. *Acta Chem Scand.* 1958; 12:144–146.
- Meier, D.; Faix, O. Pyrolysis–gas chromatography–mass spectroscopy. In: Lin, SY.; Dence, CW., editors. *Methods in Lignin Chemistry*. Heidelberg, Germany: Springer-Verlag; 1992. p. 177-199.
- Nakamura T, Kawamoto H, Saka S. Pyrolysis behavior of Japanese cedar wood lignin studied with various model dimers. *J Anal Appl Pyrol.* 2008; 81:173–182.
- Ohra-aho T, Tenkanen M, Tamminen T. Direct analysis of lignin and lignin-like components from softwood kraft pulp by Py-GC/MS techniques. *J Anal Appl Pyrol.* 2005; 74:123–128.
- Qu C, Kishimoto T, Kishino M, Hamada M, Nakajima N. Heteronuclear single-quantum coherence nuclear magnetic resonance (HSQC NMR) characterization of acetylated fir (*Abies sachalinensis* MAST) wood regenerated from ionic liquid. *J Agric Food Chem.* 2011; 59:5382–5389. [PubMed: 21526833]
- Rakhimov DA, Shashkov AS, Zhaunbaeva KS, Malikova MKh, Abdullaev ND. Glucomannan from *Narcissus poeticus* studied by PMR and ^{13}C NMR spectroscopy. *Chem Nat Compd.* 2004; 40:358–361.
- Rolando, C.; Monties, B.; Lapierre, C. Thioacidolysis. In: Lin, SY.; Dence, CW., editors. *Methods in Lignin Chemistry*. Heidelberg, Germany: Springer-Verlag; 1992. p. 334-349.
- Salmén L, Burgert I. Cell wall features with regard to mechanical performance. A review. *Holzforschung.* 2008; 63:121–129.
- Salmén L, Olsson AM. Interactions between hemicelluloses, lignin and cellulose: structure–property relationships. *J Pulp Paper Sci.* 1998; 24:99–103.
- Teleman A, Lundqvist J, Tjerneld F, Ståhlbrand H, Dahlman O. Characterization of acetylated 4-*O*-methylglucuronoxylan isolated from aspen employing ^1H and ^{13}C NMR spectroscopy. *Carbohydr Res.* 2000; 329:807–815. [PubMed: 11125823]
- Teleman A, Tenkanen M, Jacobs A, Dahlman O. Characterization of *O*-acetyl-(4-*O*-methylglucurono) xylan isolated from birch and beech. *Carbohydr Res.* 2002; 337:373–377. [PubMed: 11841818]
- Theander O, Westerlund EA. Studies on dietary fiber. 3 Improved procedures for analysis of dietary fiber. *J Agric Food Chem.* 1986; 34:330–336.
- Timell TE, Syracuse NY. Recent progress in the chemistry of wood hemicelluloses. *Wood Sci Technol.* 1967; 1:45–70.
- Wu S, Argyropoulos DS. An improved method for isolating lignin in high yield and purity. *J Pulp Paper Sci.* 2003; 29:235–240.
- Zhang, L.; Gellerstedt, G. Achieving quantitative assignment of lignin structure by combining ^{13}C and HSQC NMR techniques. *Proceedings of the Sixth European Workshop on Lignocellulosics and Pulp*; Bordeaux, France: University of Bordeaux; 2000. p. 7-10.
- Zhang L, Gellerstedt G. Quantitative 2D HSQC NMR determination of polymer structures by selecting suitable internal standard references. *Magn Reson Chem.* 2007; 45:37–45. [PubMed: 17080511]
- Zhang, Y.; Li, J.; Lindström, ME.; Stepan, A.; Gatenholm, P. In: Wang, L.; Kuang, S.; Hou, Q.; Cao, C.; Si, C.; Zhang, H., editors. *Spruce glucomannan; preparation, purification, characterization and derivation*; *Proceedings of the 16th International Symposium on Wood Fibre and Pulp Chemistry (ISWFPC)*; Tianjin, China: China Light Industry press; 2011. p. 478-483.

Figure 1.
Flow chart of the universally applicable LCC fractionation approach (GL, glucan-lignin; GML, glucomannan-lignin; XL, xylanlignin).

Figure 2. Alkaline SEC analysis of spruce LCC fractions (GL, glucan-lignin; GML, glucomannan-lignin; XL, xylan-lignin).

Figure 3.
2D HSQC NMR spectrum of xylan-lignin (XL).

Figure 4. 2D HSQC NMR spectrum of acetylated glucan-lignin (Ac-GL). A small amount of undeuterated CHCl_3 from the CDCl_3 solvent was also observed, as indicated.

Figure 5.
 ^1H -NMR spectrum of acetylated glucan-lignin (Ac-GL).

Figure 6.
 ^{13}C CP-MAS NMR spectra comparing lignin-free glucomannan and glucomannan-lignin (GML).

Figure 7.
 2D HSQC NMR spectrum of acetylated glucomannan- lignin (Ac-GML).

Figure 8. Thioacidolysis-SEC chromatograms of spruce LCC fractions (GL, glucan-lignin; GML, glucomannan-lignin; XL, xylan-lignin).

Figure 9.
Pyrolysis-GC/MS chromatogram of glucomannan-lignin (GML).

Table 1
The fractionation yield, solubility and composition of spruce wood and its LCC fractions

Sample	Yield (%)	Solubility in dioxane/water (mg ml ⁻¹)	Lignin		Uncondensed G units (μmol g ⁻¹)		Carbohydrates (relative%)					
			Klason lignin (%)		In the fraction	In the original lignin	Ara	Xyl	Man	Gal	Glu	
			In the fraction	In the original wood								
Wood	100	1.2 (extractives)	27.1	27.1	–	100	–	12.4	17.5	3.3	63.2	
GL	49.5	Not detectable	19.3	9.6	312	35.4	1633	2.5	8.6	1.2	85.8	
GML	30.9	Not detectable	29.2	9.0	228	33.2	781	10.6	30.9	4.4	49.4	
XL	12.8	0.14	42.7	5.5	222	20.3	520	65.3	3.2	3.0	15.6	

GL, glucan-lignin; GML, glucomannan-lignin, XL: xylan-lignin.

The substituted monomeric phenols formed during pyrolysis and their relative peak intensities for the LCCs fractionated from spruce wood

Table 2

Monomeric phenol	Abbreviation	RT (min)	Assignment*	Relative peak height (%)		
				GL	GML	XL
Guaiacol	G	7.17	A	7.2	10.0	15.6
4-methyl guaiacol	Me-G	8.88	B	14.0	18.2	11.9
4-ethyl guaiacol	Et-G	10.15	C	3.0	3.4	2.5
4-vinyl guaiacol	Vinyl-G	10.70	D	11.2	16.4	22.5
Eugenol	G-CH ₂ CH=CH ₂	11.27	E	6.2	5.1	4.8
Vanillin	G-CHO	11.90	F	15.7	10.0	11.8
Isoeugenol (<i>cis</i> + <i>trans</i>)	G-CH=CHCH ₃	11.97 (<i>cis</i>)	G (<i>cis</i>)	13.4	13.4	13.1
		12.55 (<i>trans</i>)	H (<i>trans</i>)			
Homovanillin	G-CH ₂ CHO	12.64	I	6.7	5.7	5.4
2-methoxy-4-(propa-1,2-dien-1-yl) phenol	G-CH=C=CH ₂	12.92	J	4.0	2.8	2.6
Acetoguaiacone	G-COCH ₃	13.02	K	5.0	4.7	4.6
Guaiacyl acetone	G-CH ₂ COCH ₃	14.08	L	4.0	3.7	1.4
1-(4-hydroxy-3-methoxyphenyl) prop-2-en-1-one	G-COCH=CH ₂	14.21	M	3.0	2.0	1.0
Coniferyl alcohol (<i>cis</i> + <i>trans</i>)	G-CH=CHCH ₂ OH	15.19 (<i>cis</i>)	N (<i>cis</i>)	6.6	4.6	2.8
		15.97 (<i>trans</i>)	O (<i>trans</i>)			

* See Figure 9.

GL, glucan-lignin; GML, glucomannan-lignin; RT, retention time; XL, xylan-lignin.