DOI:10.1021/jf901095w

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

In Situ Determination of Lignin Phenolics and Wood Solubility in Imidazolium Chlorides Using ^{31}P NMR

Alistair W. T. King,*,† Luca Zoia,‡ Ilari Filpponen,‡ Anna Olszewska,† Haibo Xie,‡ Ilkka Kilpeläinen,† and Dimitris S. Argyropoulos*,†,‡

†Laboratory of Organic Chemistry, Department of Chemistry, Faculty of Science, University of Helsinki, Helsinki, Finland, and ‡Organic Chemistry of Wood Components Laboratory, Department of Forest Biomaterials, College of Natural Resources, North Carolina State University, Raleigh, North Carolina

Corn stover, Norway spruce, and *Eucalyptus grandis* were pulverized to different degrees. These samples were subjected to quantitative analyses, upon the basis of predissolution into the imidazolium chloride-based ionic liquids [amim]Cl and [bnmim]Cl followed by labeling of hydroxyl groups as phosphite esters and quantitative ^{31}P NMR analysis. Analysis of different pulverization degrees provided semiempirical data to chart the solubility of Norway spruce in these ionic liquids. Further method refinment afforded an optimized method of analysis of the lignin phenolic functionalities, without prior isolation of the lignin from the fiber. The lignin in these samples was further enriched using cellulase and acidolysis treatments, allowing for comparison with the fibrous samples. Analysis of all samples charts the polymerized-monomer availability for each stage of the treatment. Conditions required for adequate signal-to-noise ratios in the ^{31}P NMR analysis were established with a notable improvement observed upon the lignin enrichment steps.

KEYWORDS: ^{31}P NMR; EMAL; ionic liquids; spruce; eucalpytus; corn

INTRODUCTION

Lignin is the major nonpolysaccharide polymeric component of wood (*1*). It is composed of phenylpropanoid monomer units, at varying degrees of oxygenation/substitution on the aromatic ring. Its main function, along with hemicellulose is to act as a composite glue to bind cellulose-based fibers and give the plant strength where needed. A secondary but important function is to provide regions of hydrophobicity to allow for the formation of defined transport channels, such as xylem, which are used for the transport of water or other extracellular fluids. As a result of the complex and elusive nature of the interactions of the various wood components, traditional industries utilizing wood as a renewable resource have resorted to selective degradation of one or more components, usually lignin, for the production of higher value purified materials such as wood pulp (for paper) or cellulose.

As the connectivity (bonding interactions) between the individual plant components (cellulose, hemicelluloses and lignin) is crucial for their structural integrity, development of novel fractionation processes for the complete utilization of woody materials as renewable resources requires detailed structural analysis to thoroughly characterize the material, including revealing details of the connectivity between components. Traditionally, lignin contents have been determined by the standard Klason UV lignin content determination method (*2*). This involves extensive chemical modification of the lignocellulosic substrate and provides no structural information. Further quantitative information about specific lignin structures has been provided by the advent of one-dimensional (1D) ^{1}H NMR, although the limited ppm range for the ^{1}H nuclei allows for significant overlap of resonances (*3*). ^{13}C NMR offers a wider ppm range and resolution between individual polymer backbone resonances (*4*), although the low relative sensitivity of ^{13}C, in comparison to that of ^{1}H, ^{19}F, or ^{31}P, often requires some extent of compromise on acquisition conditions, such as shortening transverse relaxation delay parameters or NOE enhancement due to ^{1}H decoupling, to achieve adequate sensitivity. This reduces the quantitative viability of the analyses, although using the correct acquisition conditions, in combination with the use of internal standards (*5*), we obtain valuable information. Two- (2D) or three-dimensional (3D) ^{1}H and ^{13}C NMR techniques have also been of significant value such as 3D Heteronuclear Multiple Quantum Coherence-Homonuclear Hartman Hahn (HMQC-HOHAHA) NMR spectroscopy to reveal the structural units of lignin (*6*). This provides detailed information about the structure of isolated lignin from wood, primarily the types of bonding patterns observed on the basis of the combination of different monomers. One major drawback of multidimensional techniques, as with ^{13}C NMR, is the reduction in quantitivity. This has been partially resolved with the advent of techniques such as quantitative 2D heteronuclear single quantum coherence (Q-HSQC) (*7*). To date, few publications have provided detailed structural information about native lignin in wood, i.e., without prior isolation from the wood fiber, for the determination of solution phase techniques. The first of these publications has

*To whom correspondence should be addressed. (D.S.A.) E-mail: dsargyro@ncsu.edu. (A.W.T.K.) E-mail: alistair.king@helsinki.fi.

 Published on Web 08/24/2009

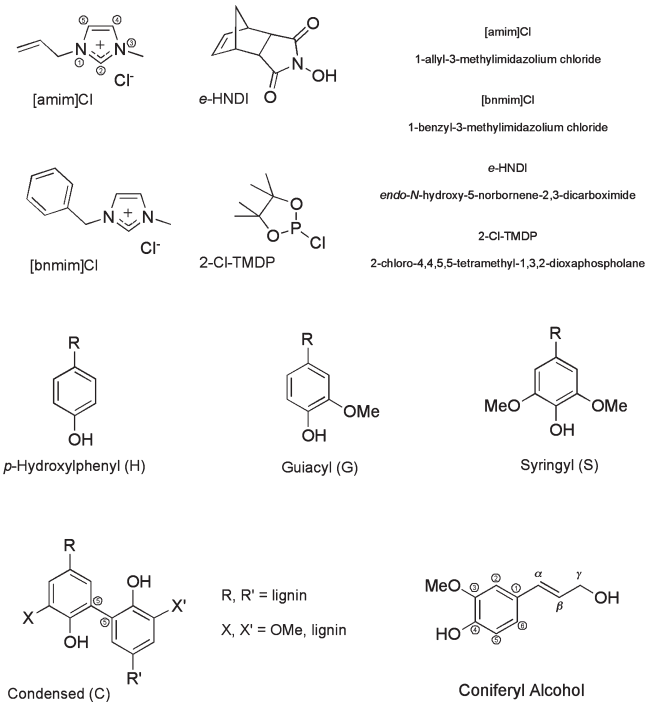


Figure 1. Common reagents used and typical phenolics identified in this study.

been reported by Lu and Ralph (*8*) where they demonstrate the complete dissolution of pulverized wood into a *N*,*N*,*N*,*N*-tetrabutylammonium fluoride−dimethylsulfoxide (TBAF-DMSO) mixture, followed by a combination of chemical modification and 2D NMR analysis techniques.

Lignin preparations such as milled wood lignin (MWL), cellulolytic enzyme lignin (CEL), and most recently enzymatic mild acidolysis lignin (EMAL) (*9*, *10*) have been extensively studied by Argyropoulos et al., using quantitative ^{31}P NMR, as a sensitive technique, for the determination of different phenolic and other lignin functionalities (*9*−*13*). From these cumulative publications, it has been demonstrated that purified lignin preparations can be completely dissolved in traditional organic solvents and phosphitylated, in the presence of the organic base pyridine (Pyr) and 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (2-Cl-TMDP) as a phosphitylating reagent (**Figure 1**), to introduce the ^{31}P label for NMR analysis. 2-Cl-TMDP provides good resolution of the individual syringyl (S, originating from the sinapyl alcohol monomer or ferulate), condensed (C, originating from 5 to 5′ linked coniferyl alcohol monomers), guiacyl (G, originating from coniferyl alcohol monomer and *p*-hydroxyphenyl (H, originating from *p*-coumaryl alcohol monomer or *p*-coumarate), and phenolics (**Figure 1**) over previous labels. The phosphitylated hydroxyls in lignin can then be quantitatively assessed against an internal standard (IS), with *endo*-*N*-hydroxy-5-norbornene-2,3-dicarboximide (*e*-HNDI) IS (**Figure 1**), previously demonstrating adequate stability and satisfactory resolution from other lignocellulosic functionality regions, in the ^{31}P NMR spectra, after phosphitylation. Although this provides an expedient technique for the determination of lignin functionalities in purified lignins, the insolubility of wood in traditional molecular solvents does not allow for quantification of hydroxyl functionalities from fully representative and potentially artifact free native lignin, in minimally treated lignocellulose samples. This requires a solvent capable of solvating the carbohydrate portion of the samples in addition to the lignin.

Ionic liquids (ILs) are typically described to be molten salts below 100 °C. They generally contain anion−cation pairs, one (or both) of which are organic (*14*). ILs such as [amim]Cl and [bnmim]Cl (**Figure 1**), have previously been shown to dissolve wood to such a state that it can be chemically modified (*15*, *16*). This has allowed us to explore the conditions required for quantitative functionalization of microcrystalline cellulose (MCC) and Norway spruce EMAL with 2-Cl-TMDP for ^{31}P NMR analysis (*17*). With the appropriate conditions determined for formation and solubilization of the lignin phosphite esters, this technique can now be applied to wood. In the context of 1D ^{31}P NMR, this will yield information about the quantities and relative ratios of the different phenolic and other functionalities from native lignin in woody materials. This, in comparison to similar analyses of the different wood and lignin preparations, may provide information about the presence or the extent of any additional bonding interactions between lignin and the different wood biopolymer classes, after different treatments. In time, these techniques may be used to identify the occurrence of elusive lignin carbohydrate complexes (LCC's) (*18*, *19*) that may be cleaved or formed during chemical modifications. In this article, we describe our efforts to arrive at a set of conditions for the in situ ^{31}P labeling, solubilization, and acquisition conditions for NMR analysis and determination of lignin functional groups. Additional knowledge about the solubility of pulverized wood in imidazolium chlorides has also been attained upon the basis of semiempirical evidence from a quantitative functionalization and analysis procedure.

MATERIALS AND METHODS

Preparation of Lignocellulose Samples. Two types of pulverized wood samples were prepared. Norway spruce (*Picea abies*) batches were prepared using planetary ball-milling for the generation of 0−96 h planetary milled wood samples for ^{31}P NMR analysis. Rotary ball-milled samples for Norway spruce were also required for the EMAL procedure. The corn stover (*Zea mays* L.) EMAL procedure required the use of planetary milling (10 h total) only. The grinding and planetary milling procedure for Norway spruce was as follows. Norway spruce heartwood was ground to sawdust using a stationary belt sander with grade 60 grit aluminum oxide belts. This was extracted for 72 h with Acetone in a Soxhlet apparatus to remove water and other small organic extractives. The samples (1.0 g) were thoroughly dried in a vacuum oven, and planetary ball-milling was performed using a Fritsch Pulverette planetary ball-miller in a 20 mL zirconium grinding bowl (zirconium dioxide, 95%) in the presence of 4 zirconium balls (10 mm diameter). The milling process was conducted at room temperature at 420 rpm for 30 min with 25 min intervals to allow for cooling of the sample and balls. This cycle was repeated until the total experimental milling time, based on the 30 min milling portion of the cycle, was achieved for each sample. All samples were stored in a desiccator prior to use. The pulverized, cellulase treated, and EMAL samples for Norway spruce, eucalyptus (*Eucalyptus grandis*), and corn stover required for the optimized ^{31}P NMR analysis procedure were all sampled from different stages during their EMAL preparation. The methods used for this were identical to the literature procedure (*10*), with the exception of planetary ball-milling for corn stover rather than rotary ball-milling. The corn stover planetary ball-milling procedure was almost identical to the Norway spruce procedure. In this case, the stover was cut with scissors to as fine a degree as possible before continuing with the Soxhlet extraction. Xylan was purchased as Xylan from oat spelts from Aldrich and planetary milled for 3 h.

^{31}P NMR Analysis Procedure. Full details of the acid free IL preparation and characterization are provided in a previous report (*17*). This report also includes details of the fully quantitative (maximum dilution) analysis procedure, including significant detail about the reaction conditions, solubilization of phosphitylated products, processing, and intepretation of spectra. The following is the typical optimized procedure for the determination of lignin functionalities in lignocellulosic samples: The lignocellulose sample (40.0 mg) was stirred in [amim]Cl (~0.42 mL, 500 mg) for 18 h at 80 °C in a 10 mL screw-top glass sample bottle. Pyridine

(200 μL, 2.50 mmol) was added in one portion and the sample vortexed, at 2500 rpm, using an Janke & Kunkel Vibrofix VF1 Electronic orbital shaker, until visibly homogeneous (~20 s). The sample was allowed to cool to room temperature, whereby 2-Cl-TMDP (300 μL, 1.89 mmol) was added in one portion and vortexed until visibly homogeneous (~30 s) as a cream paste. A preprepared stock solution of $Cr(acac)_3/CDCl_3$ (1 mg/mL, 500 μL) was added in 4× 125 μL portions with vortexing (~30 s) between each addition. *e*-HNDI solution (121.5 mM in 3/2 pyridine/$CDCl_3$, 125 μL) was added in one portion and the solution vortexed (~30 s). ^{31}P NMR spectra (243 MHz) were recorded with 700 μL samples, in a 5 mm o.d. NMR tube, from this preparation and further dilutions upon $Cr(acac)_3/CDCl_3$ solution additions (required optimum dilution of 2000 μL). For each data collection, the sample was allowed to equilibrate in the spectrometer for 5 min before the probe was retuned. Inverse recovery experiments were performed for most samples, and optimum relaxation delay times were determined to range from 4 s at 500 μL $Cr(acac)_3/CDCl_3$ to 8 s at 4000 μL $Cr(acac)_3/CDCl_3$ for a Varian Inova 600 MHz spectrometer equipped with a direct detection probe for broadband nuclei such as ^{31}P and ^{13}C. $CDCl_3$ was used as locking solvent, and standard transients of 256 (lignin enriched samples) to 5000 (wood and grass samples) were collected. Higher quality EMAL spectra were typically run overnight (12 h, 5000 transients). The experiment temperature was maintained at 27 °C for all NMR acquisition experiments. This data was processed routinely, without baseline correction, using VNMRJ version 2.1b by Varian, Inc., running on an openSuse 10.3 linux personal computer. A 3 Hz exponential line-broadening factor was used in all cases. For the determination of Norway spruce solubility in [amim]Cl, the procedure was as described above using 25 mg of lignocellulose sample, 475 mg of [amim]Cl, 150 μL of Pyr, and 200 μL of 2-Cl-TMDP. The solutions were then gradient diluted to the maximum value, required for observation of all phosphite esters, of 4 mL of $Cr(acac)_3/CDCl_3$ solution (1 mg/mL). The full experimental procedure is detailed in a previous publication (*17*). This data was processed using iNMR version 2.6.4 (*20*), by Mestralab Research, running on Mac OSX. Spectra were Fourier transformed with 64 K zero-filling and a 3 Hz exponential line-broadening factor. Phasing was performed manually between the IS region and each region of interest, to be integrated. Baseline correction was performed twice with 0° of correction and a 128 K filter using an interactive algorithm for baseline correction in iNMR, based upon a publication by Cobas et al. (*21*). This provided a baseline free from interference from significantly broadened aliphatic resonances, thus allowing more accurate comparison of guiacyl phenolics.

RESULTS AND DISCUSSION

Semiempirical Norway Spruce Solubility in [amim]Cl under Mild Conditions. As mentioned previously, Lu and Ralph (*8*) have demonstrated the complete dissolution of finely pulverized wood into TBAF-DMSO allowing for solution phase NMR analysis. This solvent in our case was ineffective due to oxo-transfer from DMSO to phosphorus, upon addition of 2-Cl-TMDP, ultimately to form phosphate esters. It has been demonstrated in our laboratories that ionic liquids such as [amim]Cl and [bnmim]Cl can be used as nonderivitizing solvent for lignocellulose dissolution and unreactive media for the phosphitylation reaction to achieve complete phosphitylation and solubilization of MCC or Norway spruce EMAL (*17*). [amim]Cl has also been demonstrated to dissolve Norway spruce sawdust allowing for essentially complete esterification of the hydroxyls (*18*). This was confirmed by IR and NMR, among other analyses. It came as a surprise, however, that when applying our newly developed phosphitylation procedure (*17*), to the phosphitylation of the same sawdust sample from [amim]Cl, that only a fraction of the total hydroxyls (wood hydroxyl-phosphite esters) were observable. A typical intended phosphitylation reaction on lignin hydroxyls is shown in **Scheme 1** where phosphitylation of a terminal guiacyl β-O-4 dimer linkage is shown.

Previously, we have demonstrated that after predissolution EMAL or MCC into imidazolium chloride ILs, phosphitylation

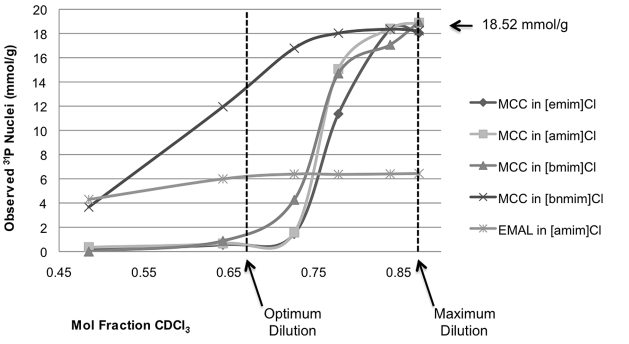
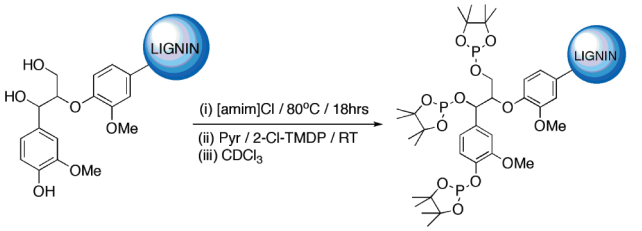


Figure 2. Phosphitylated lignin (EMAL) and cellulose (MCC) solubility based upon integration of all observed phosphite esters (aliphatic, phenolic, and carboxylic), in a range of IL/$CDCl_3$ mixtures.

Scheme 1. Typical Phosphitylation Reaction on a Terminal Lignin Guiacyl β-O-4 Dimer



of the mixture with 2-Cl-TMDP yielded a phase separated mixture, not suitable for quantitative ^{31}P NMR analysis. It was only after gradient dilution with $CDCl_3$ solution, gradually increasing the overall hydrophobicity of the mixture, that it was possible to observe the complete solubilization of the sample, thereby producing a homogeneous mixture suitable for the analyses. Using a $CDCl_3$ mol fraction of 0.88, it was possible for us to completely solubilize fully phosphitylated Norway spruce EMAL and MCC. Using lower mole fractions of $CDCl_3$ in our NMR mixture, it was also possible to fully solubilize the phosphitylated EMAL but leave phosphitylated MCC insoluble (**Figure 2**). The degree of solubilization of phosphitylated MCC at a specific mole fraction of $CDCl_3$ was found to differ for each IL. This phosphitylated MCC solubility profile was demonstrated for [amim]Cl, [bnmim]Cl, 1-butyl-3-methylimidazolium chloride ([bmim]Cl), and 1-ethyl-3-methylimidazolium chloride ([emim]-Cl). To test the limit of the dissolution capabilities of [amim]Cl, the same insoluble Norway spruce wood sawdust sample was pulverized at variable intervals in a planetary mill from 0 to 96 h (0, 24, 48, 72, and 96 h). The same gradient dilution process, whereby the NMR solution was diluted with aliquots of $CDCl_3$ and successive spectra recorded, was carried out as demonstrated in our previous publication (*17*), with the values of observed lignin-phosphite esters plotted as a function of mole fraction of $CDCl_3$ (**Figure 3A**). **Figure 3A** represents the solubilization of the different pulverized and phosphitylated wood samples at each dilution, with the maximum dilution of 4 mL of $CDCl_3$ (mole fraction of 0.88 of $CDCl_3$) furnishing the maximum achievable phosphitylation values. These points were also plotted as a function of milling time (**Figure 3B**) and represent the total available hydroxyls in those samples. These values are indicative of the ability of [amim]Cl to dissolve different pulverized wood samples under mild conditions of dissolution (80 °C for 18 h). It seems that below approximately 48 h of milling time the wood samples are not completely soluble. One could argue that 48 h, or even 96 h, is not a maximum value; however, it is expected that

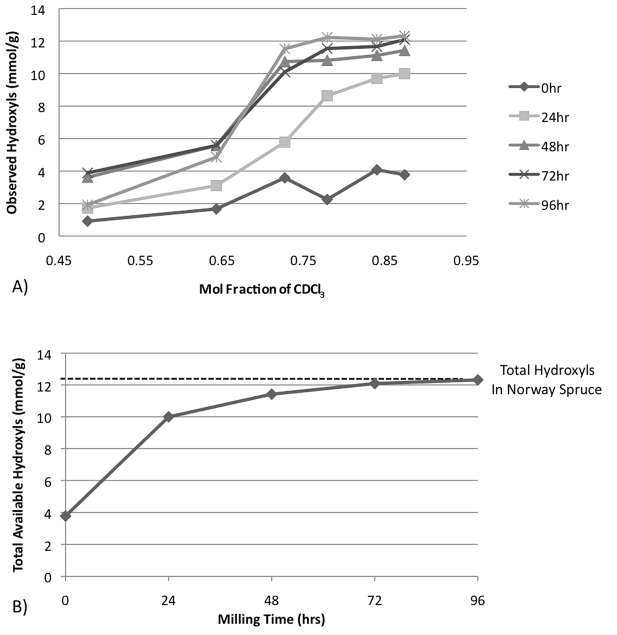


Figure 3. Determination of phosphitylated Norway spruce (**A**) solubility with pulverization in [amim]Cl/$CDCl_3$ mixtures and (**B**) total available hydroxyls.

bond cleavage and formation are occurring throughout the milling, continuously creating reactive sites. One thing that is obvious is that the rate of increase of available hydroxyls between 0−48 h compared to between 48−96 h is much larger indicating massive freeing up of reactive domains beyond 48 h of pulverization. This insolubility <48 h of milling time is corroborated visually by inspection of the hazy ionic liquid mixtures below 48 h but also by visual inspection of the NMR mixtures which display a phase separation for the 0 and 24 h samples, in comparison to no phase separation observed for ≥ 48 h of milling time. This phenomenon is also observed by Argyropoulos' group (*9*) [**Figure 1**] when measuring weight loss, after the cellulase hydrolysis step of lignin purification, as a function of vibratory or rotary ball-milling time. Weight loss is observed to exponentially rise with milling time. This is consistent with our description of freeing up of reactive domains with milling time. With respect to the overall reactive sites in Norway spruce, the maximum available hydroxyl points converge at an approximate value of just over 12 mmol/g of hydroxyls in Norway spruce. These values may be used as reference molarities for future chemistry involving the reactivity of hydroxyls in Norway spruce wood as either the maximum achievable values or values for different degrees of pulverization in [amim]Cl.

Effect of Ionic Liquid Purity on Dissolution of Intact Wood. More important implications, of the increase in available hydroxyls with degree of pulverization, are that imidazolium chloride-based ionic liquids such as [amim]Cl do not have the ability to completely dissolve wood under these mild conditions. This should not be unexpected because of the high molecular weights and complex nature of bonding interactions observed in wood. This insolubility at first seems contrary to our previous report (*18*) and requires further clarification of the definition of the term solution in relation to wood and cellulose chemistry in ionic liquids or other mixtures for that matter. The differences, however, between these two similar studies, are in the quality of ionic liquid and the chemical method used. From the previous dissolution and acetylation of Norway spruce sawdust methodology, this dissolution and reaction is accompanied by a colorization of the hazy dissolving wood−IL media to give a clear dark solution over time which colorized further when reacted with the acetylating mixture, at 80 °C. This is a strong indication of the type of decomposition of wood macromolecules that would be expected from acid catalyzed decomposition. When a small sample of wood sawdust was added to [amim]Cl doped with a catalytic amount of aqueous hydrochloric acid, the same colorization was observed along with rapid dissolution.

Li and Zhao et al. (*22*) have already reported the ability of protic or acid doped ILs to depolymerize wood polysaccharides at moderate temperatures, and it is conceivable that traces of acid are formed in the ILs at the synthesis and purification step. During the development of an efficient procedure for the synthesis, purification, and drying of [amim]Cl on a medium scale (100−500 g), it was noticed that after the charcoal purification step (to remove colored impurities) drying under strong vacuum at high temperature (>100 °C) caused further coloration over time which was accelerated as temperature increased. These batches of IL almost always decomposed wood, indicated by colorization, faster than samples that had been dried at lower temperature. pH analysis of these batches in water showed increased acidity over the samples which had been dried at lower temperatures (<80 °C). It is well known that dialkyl imidazolium halides can thermally disproportionate in a reverse quaternization reaction at elevated temperatures (*14*) giving alkylimidazole and alkyl halide. One could envisage hydrolysis or alcoholysis (in the presence of lignocellulose) of the alkyl halide releasing an inorganic acid decomposition product. In the case of [amim]Cl, this, however, is less likely because of the volatility of chloromethane and allylchloride, under the conditions that acid formation is readily observed (∼120 °C, 0.9 mmHg). Additional mechanisms may be at play here. As regards using a particular IL for lignocellulose chemistry, it is absolutely essential that the pH of an aqueous solution of the IL be measured, whether it be an academic laboratory preparation or a commercial one. It may be that only traces of acid formed via decomposition of only a small portion of the IL preparation, by whichever mechanism, can have a profound effect on the reproducibility of results. Moisture content must also be kept to a minimum to reduce the mobility of dissociated protons in solution, as this may provide a significant alteration in the rate of degradation, with only traces of acid in solution. In this light, it is entirely conceivable that imidazolium chloride-based ionic liquids are capable of dissolving intact wood (with minimal pulverization) with significant industrial ramifications. The extent of degradation to achieve solubilization is unknown and may only require minimal degradation of key linkages or structures to achieve complete solubilization. As such, care must be taken in the dissemination of knowledge regarding the dissolution of lignocellulose in ILs.

Effect of Mechanical Treatment on Lignin Linkages. As the pulverization degree at which we can achieve efficient solubilization in [amim]Cl is already demonstrated, it is possible to assess the validity of the intact wood analysis procedure, in comparison to the traditional lignin analysis procedures. This is carried out over the whole pulverization−solubility regime for comparison. The same maximum dilution data for the pulverized Norway spruce was integrated according to the appropriate integration regions (**Figure 4A**), with the most significant integration regions for Norway spruce lignin being the guiacyl and carboxylic acid regions. The contribution of the unresolvable aliphatic region was removed by baseline correction. This was carried out specifically for the removal of any contribution of the aliphatic peak to the guiacyl region albeit small. In the case of Norway spruce, which contains mostly guiacyl moieties with a very small percentage of *p*-hydroxyphenyl moieties, this is not that necessary because of the increased separation between the guiacyl maximum

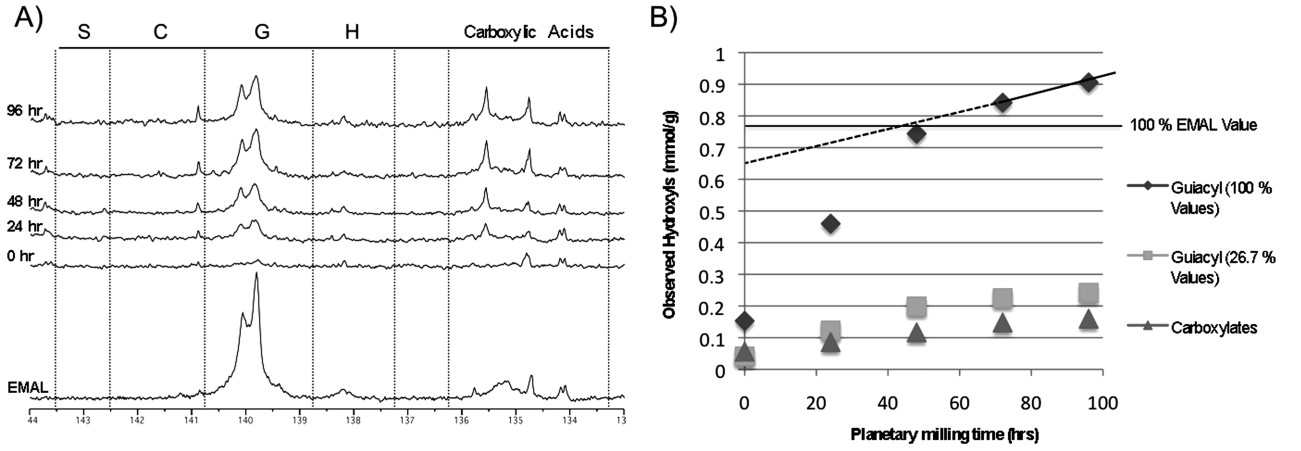


Figure 4. Guiacyl phenolic hydroxyl and carboxylic acid content (from maximum dilution conditions) for planetary milled Norway spruce (0−96 h planetary milling times) and EMAL (from 25 days of rotary ball-milling).

(140 ppm) from the aliphatic maximum (147 ppm); however, in the case of hardwoods such as eucalyptus, the contribution of the aliphatic peak to the syringyl and condensed regions (144−142 ppm) is significant. The integral values, represented in mmol/g of hydroxyls for the guiacyl and carboxylic acid resonances were plotted against milling time (**Figure 4B**). The guiacyl values were plotted, as is, with the addition of the 100% lignin content corrected values, according to a Klason UV lignin content of 26.7% (hydroxyl content values/0.267). The 100% EMAL value (Klason = 92.1%) was also included for comparison. As anticipated, the insolubility of the wood samples below 48 h of planetary milling time is demonstrated by much lower values for the guiacyl integrations. After 48 h, in the pulverization−solubility regime, a steady increase in both guiacyl and carboxylic acid resonances is observed. This concerted increase is most likely due to the cleavage of β-O-4 aryl ether linkages. This has been observed and quantified previously by Guerra and Argyropoulos et al. (*9*) on extracted lignin preparations (MWL, CEL, and EMAL) and is thought to occur through a mechanism involving oxidative fragmentation of phenoxy and aliphatic radical species formed during the mechanical treatment. In this publication, they quote a corresponding decrease in β-aryl ether structures with vibratory milled samples, as quantified by the combination of derivatization followed by reductive cleavage (DFRC) ^{31}P NMR (*23*). They also quote that rotary ball-milling (porcelain balls and milling jar) was a much milder method and had been shown not to decompose lignin via fragmentation in such a manner. In that respect, their vibratory milling method (stainless steel balls and milling jar) visually demonstrated (*9*) (**Figure 5**) similar and perhaps a little faster degradation of β-O-4 aryl ether linkages, in comparison to our planetary ball-milling protocol (zirconium balls and milling bowl) and over the relative time spans quoted for each method. As the time spans were somewhat similar, a coefficient of degradation for the two methods is close to unity, but exact measurment of this should come from one analytical procedure applied to all samples. After calculation of the Klason UV lignin content corrected guiacyl hydroxyl integrations for the 48−96 h milled wood samples and EMAL, it was evident that just above 48 h planetary ball-milling time corresponds well to the values observed from EMAL with 25 days of rotary ball-milling required for its preparation. Qualitative analysis of the carboxylic acid region from the ^{31}P NMR spectra of the different pulverized wood samples (**Figure 4A**) clearly shows the emergence of several new resonances. These are thought not to occur from the cleavage of acetate esters on hemicelluloses because of the broadness of the

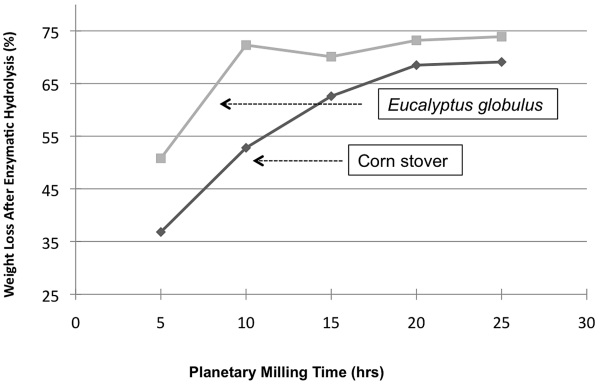


Figure 5. Cellulase digestion of corn stover vs *E. globulus* at different degrees of pulverization.

peaks, indicating attachment of the acid functionalities to high molecular weight species. As mentioned previously, the increase in carboxylic acid resonances correspond well to the formation of guiacyl phenolics and are though to be artifacts of the mechanical treatment, arising from breakage of ether linkages. Two resonances, however, at 134.8 ppm (broad singlet) and 134.1 ppm (broad doublet) can be observed to be present in all of the spectra, including the EMAL spectra. These could be attributed to native carboxylic acids from hemicellulose present in the native sample and with future assignment may offer the possibility to distinguish hemicellulose fractions that may or may not be involved in LCC formation. One additional resonance occurs between the guiacyl and condensed regions at 140.7 ppm. This small but well resolved artifact resonance is observed to occur to a greater extent in the more pulverized samples. It its termed an artifact as it has been determined that this resonance is not attributable to any compound in the native wood sample and is only observed to form over time (>2 h), after preparation of the NMR solution. The most likely explanation for this resonance is that it is due to defragmentation of some portion or specific structures from the lower molecular weight lignin in the pulverized wood. This may be as a result of the chemical contents of the NMR reaction media. The small intensity of this resonance does not affect the phenolic region integration values, and its origin will be investigated in future studies.

The above analysis demonstrates the flexibility of this ^{31}P NMR method in determining lignin functionality contents on the fiber. At these maximum dilution values, however, signal-to-noise does decrease, and the requirement for baseline correction to remove aliphatic signals may introduce error or for some the

Table 1. ^{31}P NMR Analysis Conditions and Phenolic Hydroxyl Values for Corn Stover Samples, Planetary Milled, Cellulase Treated, and EMAL

		phenolic integrations (mmol/g)		
sample (Klason UV lignin content)	dissolution solvent ($CDCl_3$ volume)	S + C	G	H
EMAL (80%)	Pyr/$CDCl_3$	0.60	0.56	1.32
EMAL (80.0%)	[amim]Cl (2000 μL)	0.63	0.55	1.25
cellulase-digested stover (77.0%)	[amim]Cl (2000 μL)	0.55	0.48	1.28
milled stover[a] (18.6%)	[amim]Cl (2000 μL)	0.44	0.21	0.35
milled stover (18.6%)	[bnmim]Cl (2000 μL)	0.60	0.41	0.62

[a] The pulverized stover sample was only partially soluble in [amim]Cl.

Table 2. ^{31}P NMR Analysis Conditions and Phenolic Hydroxyl Values for Norway Spruce Samples, Rotary Ball-Milled, Cellulase Treated, and EMAL

		phenolic integrations (mmol/g)		
sample (Klason UV lignin content)	dissolution solvent ($CDCl_3$ volume)	S + C	G	H
EMAL (88.5%)	Pyr/$CDCl_3$	0.50	0.89	0.08
EMAL (88.5%)	[amim]Cl (2000 μL)	0.56	0.93	0.08
cellulase-digested wood[a] (79.4%)	[amim]Cl (2000 μL)	0.24	0.32	0.07
cellulase-digested wood[a] (79.4%)	[bnmim]Cl (1000 μL)	0.28	0.39	0.09
milled wood (26.1%)	[amim]Cl (2000 μL)	0.51	0.74	0.07
milled wood (26.1%)	[bnmim]Cl (1500 μL)	0.51	0.69	0.11

[a] The cellulase treated sample was only partial solubility in [amim]Cl and [bnmim]Cl.

opportunity for favorable interpretation of data. This is manageable, however, using longer collection times (>4000 transients in our case) and suitable software for baseline correction (e.g., iNMR).

Optimized ^{31}P NMR Wood Analysis Procedure on Three Common Lignocellulose Sources. In light of the potential limitations of ^{31}P NMR wood analysis procedure, at maximum dilution, it would be highly desirable to have more concentrated solutions to increase signal-to-noise ratios and to also offer the possibility to selectivity solubilize the lignin in the sample over the carbohydrate. This would significantly shorten acquisition times. As we have previously demonstrated that by using lower mole fractions of $CDCl_3$ in our NMR mixture it is possible to fully solubilize phosphitylated Norway spruce EMAL but leave phosphitylated MCC insoluble (**Figure 2**), it was decided to use this methodology for the intact wood analysis. This was necessary to increase the signal-to-noise ratios and eliminate part of the aliphatic phosphite ester resonance contributions from cellulose. In addition to using the lower $CDCl_3$ mole fractions of between 0.65−0.80 (1−2 mL of $CDCl_3$) required for optimum dilution, the predissoved lignocellulose samples were also increased from 25 to 50 mg in the same quantity of IL.

To assess this optimized procedure, pulverized softwood species (Norway spruce), hardwood species (*E. grandis*), and a readily available source of grass-based raw material (corn stover) were analyzed. These three samples were subjected to a series of treatments. The first of which was mechanical treatment to form the milled wood (in the case of the wood species) and milled stover (in the case of the grass). This was followed by cellulase treatment to form the cellulase-digested wood in the case of the wood species and cellulase-digested stover in the case of the grass species. This served to remove the partially degraded cellulose, enriching the samples in hemicellulose and lignin. The final treatment was mild acidolysis to cleave potential LCC's and produce fractions enriched in lignin. This is the common order of sequence for all three species, with the final step required for the production of the lignin preparation EMAL. The latter steps in the hardwood and softwood treatments were identical to the different stages required for the production of their EMALs, with the pulverization steps involving rotary ball-milling for 25 days for the two species, to maximize the yield of high purity lignin. The corn stover sample was planetary ball-milled for 10 h as this was the optimum requirement for cellulase digestion to a Klason UV lignin content of 77.0%. This was determined by cellulase digestion of a range of planetary milled samples of corn stover. The weight loss after enzymatic degadation was plotted against milling time (**Figure 5**), including a similar study for another hardwood eucalpytus species (*Eucalyptus globulus*) for comparison. This clearly shows the ability of the cellulase enzymes to digest corn stover at much lower milling times (10 h) than that for the hardwood species, and as such, the EMAL procedure was applied from this material onward. ^{31}P NMR spectra were recorded for the pulverized, cellulase treated, and EMAL samples. The integration values were converted to mmol/g of hydroxyls for their 100% lignin representation (**Table 1−3**). This was achieved by integration against a known quantity of IS and division of the mmol/g values by the Klason UV lignin content fractions for each sample. The EMAL samples were repeated using standard molecular solvent conditions for comparison with the optimized IL method.

From **Tables 1−3**, the first observation is that the EMAL samples for all species correspond very well between the IL and molecular solvent methods.

With the corn stover samples (**Table 1**), there is a notable visual insolubility of milled stover in [amim]Cl as opposed to [bnmim]Cl, which furnishes, after the phosphitylation and optimum dilution with 2 mL of $CDCl_3$ solution, values in good agreement with the cellulase-digested stover and EMAL syringyl (S), condensed (C), and guiacyl (G) values. The most interesting observation for this species is that the *p*-hydroxylphenyl (H) integrations show a sharp increase from 0.62 mmol/g to 1.28 mmol/g on going through the cellulase treatment. This indicates the cleavage of labile linkages attached to H moieties. It has long been known that ferulic or *p*-coumaric acid has been involved in the formation of linkages between lignin and polysaccharides in grass species (*24*). Several publications have attempted to account for the bonding patterns that these nonmonomers display during the formation of LCC's (*25*, *26*). ^{31}P NMR has no way of directly determining the structures involved in these bonding patterns, but from the cleavage of these linkages during the cellulase treatment, a few points can be made. Release of the phenolic portion of ferulic acid (if it is even bound to polysaccharide via the phenolic hydroxyl at all), under these cellulolysis conditions (involves washing the substrate with pH 2 water as the final step) (*10*), is not achieved, because of the lack of any change in the syringyl or condensed (S or C) ^{31}P NMR values between the different treatments, or ferulic acid (or its polysaccharide conjugates) is removed during the washing. As there is only an increase observed for the H values, this could be due to the cleavage of more labile linkages, the origin of which is at present unknown. This is certainly not due to the cleavage of acylated

Table 3. ^{31}P NMR Analysis Conditions and Phenolic Hydroxyl Values for *E. grandis* Samples, Planetary Milled, Cellulase Treated, and EMAL

		phenolic integrations (mmol/g)		
sample (Klason UV lignin content)	dissolution solvent ($CDCl_3$ volume)	S + C	G	H
EMAL (89.6%)	Pyr/$CDCl_3$	0.25	0.46	0.36
EMAL (89.6%)	[amim]Cl (2000 μL)	0.27	0.44	0.34
cellulase-digested wood (74.8%)	[amim]Cl (2000 μL)	0.30	0.48	0.26
milled wood (19.0%)	[amim]Cl (2000 μL)	0.25	0.31	0.23
milled wood [a] (19.0%)	[bnmim]Cl			

[a] The pulverized wood sample was highly insoluble in [bnmim]Cl.

p-coumarate functionalities as publications by Grabber, Ralph, and Jung et al. (*27*−*29*) have conclusively demonstrated that the majority of guiacyl phenolics (potentially originating from *p*-coumaryl alcohol monomers or *p*-coumarates) present in corn are actually from *p*-coumarates, ester linked at the γ-positions on syringyl units. Moreover, all of the *p*-coumarate functionalities are reported to have free phenolics (no substitution). As this method is optimized for Norway spruce lignin observation, no strong conclusions can be made about the formation or breakage of linkages to phenols without further detailed studies of these samples; however, future application of this method for determining the occurrence of LCCs is promising. As regards the development of the analytical procedure relative to grass samples such as corn stover, the more hydrophobic [bnmim]Cl provided suitable media for dissolution, functionalization, and analysis.

With the Norway spruce samples (**Table 2**), there is a notable insolubility of the cellulase-digested wood samples in both the [amim]Cl and [bnmim]Cl ILs. This is visually corroborated and also reflected in the lower integration values after phosphitylation and optimum dilution. [bnmim]Cl, however, does offer slightly higher values for cellulase-digested wood with a lower dilution of $CDCl_3$ solution. The milled wood samples are in good agreement with the EMAL functional group values.

With *E. grandis*, the milled wood sample is quite insoluble in [bnmim]Cl with the formation of a thick gel that we were unable to convert into a homogeneous solution for NMR analysis. [amim]Cl provided a suitable media for the dissolution and optimized phosphitylation procedure with the integration values providing good agreement with both the cellulase-digested wood and EMAL samples.

Hemicelluloses as an Additional Source of Insolubility. All three species showed slightly lower integration values for the milled fiber samples compared to those of the cellulase treated and EMAL samples. However, it is not certain as to what extent the cleavage of potential LCCs increases these values after the acidic treatment steps. One thing that is certain is that there is a selective difference in solubility in some of the underivatized, pulverized fiber samples upon dissolution into the different ILs. This insolubility is very evident for the Norway spruce cellulase-digested wood sample, in comparison to that of the other preparations, where there is a notable visual precipitate in both the IL media and the NMR samples. This can be rationalized on the basis that after the cellulase treatment there is an enrichment of both lignin and hemicellulose fractions. The concentration of these fractions may allow for aggregation of the hemicellulose fractions in these samples. This was corroborated by the attempted dissolution of commercial xylan from oat spelts into

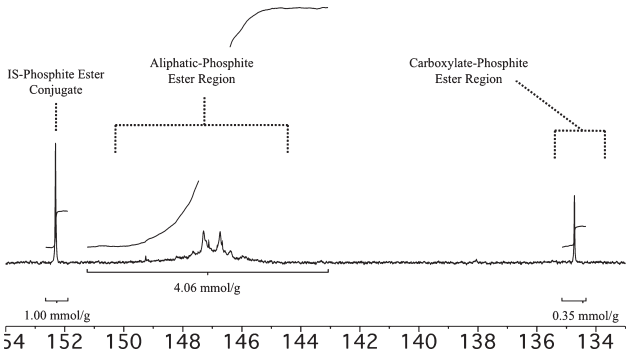


Figure 6. ^{31}P NMR spectra at maximum dilution (4 mL $CDCl_3$) of oat spelt xylan.

[amim]Cl. The same precipitate was phosphitylated and diluted to 4 mL of $CDCl_3$, only to yield again a precipitate and low phosphitylation values of 4.06 mmol/g for aliphatic hydroxyls and 0.35 mmol/g for carboxylic acids (**Figure 6**). Theoretical values calculated from the arrangement of β-D-xylopyranose, 4-*O*-methyl-α-D-glucuronopyranose acid, and α-L-arabinofuranose ratios for arabinoglucuronoxylans, as detailed in a publication by Timell (*30*), give aliphatic hydroxyls at 14.21 mmol/g and carboxylic acids as 0.11 mmol/g. Although these values are dependent on the ratios of the different monomers, the aliphatic hydroxyl value is well above that which is obtained from the ^{31}P NMR labeling and analysis from [amim]Cl, as initial dissolution media. This is a strong indication that some hemicellulose fractions may be insoluble in imidazolium chloride-based ILs, possibly due to increased hydrophobicity or aggregation. This demonstrates the requirement and potential for the design and selection of task-specific ILs for future fractionation and analysis processes based upon partial or complete predissolution of lignocellulosic materials into ILs. Moreover, different species may have different requirements for selective solubilization of their components requiring extension of our knowledge of wood biopolymer solubility in ILs. ^{31}P labeling and NMR analysis has been found to be a reliable semiempirical method to achieve this upon basis of the quantification of total available hydroxyls of a particular lignocellulose sample, after a particular treatment.

These ^{31}P NMR procedures have offered the opportunity to quantify total hydroxyls values (aliphatic, phenolic, and carboxylic combined) of individual phenolic regions in the NMR spectra of the intact wood. This in combination with suitable preparative procedures for enriching or modifying wood components for detailed analysis offers a powerful technique to glean additional information about native wood biopolymer structure and solubility, in anticipation of further refining our utilization of this renewable resource.

LITERATURE CITED

(1) Alén, R.; Holmbom, P.; Steinus, P. In *Forest Products Chemistry*; Fapet Oy: Helsinki, Finland, 2000; Vol. *3*.
(2) Determination of Acid-Insoluble (Klason) Lignin, Tappi standard Method, T222; Technical Association of the Pulp and Paper Industry, August 2nd, 2002.
(3) Lundquist, K. Methods in Lignin Chemistry. In *Proton 1H Spectroscopy*; Lin, S. Y., Dence, C. W., Eds.; Springer: Berlin, Germany, 1992; pp 242−249.
(4) Robert, D. Methods in Lignin Chemistry. In *Carbon-13 Nuclear Magnetic Resonance Spectrometry*; Lin, S. Y., Dence, C. W., Eds.; Springer: Berlin, Germany, 1992; pp 250−273.
(5) Xia, Z.; Akim, L. G.; Argyropoulos, D. S. Quantitative ^{13}C NMR analysis of lignins with internal standards. *J. Agric. Food Chem.* **2001**, *49*, 3573–3578.

(6) Ämmälahti, E.; Brunow, G.; Bardet, M.; Robert, D.; Kilpeläinen, I. Identification of side-chain structures in a poplar lignin using three-dimensional HMQC-HOHAHA NMR spectroscopy. *J. Agric. Food Chem.* **1998**, *46*, 5113–5117.

(7) Heikkinen, S.; Toikka, M. M.; Karhunen, P. T.; Kilpeläinen, I. A. Quantitative 2D HSQC (Q-HSQC) via suppression of J-dependence of polarization transfer in NMR spectroscopy: application to wood lignin. *J. Am. Chem. Soc.* **2003**, *125*, 4362–4367.

(8) Lu, F.; Ralph, J. Non-degradative dissolution and acetylation of ball-milled plant cell walls: high-resolution solution-state NMR. *Plant Journal* **2003**, *35*, 535–544.

(9) Guerra, A.; Filpponen, I.; Lucia, L. A.; Saquing, C.; Baumberger, S.; Argyropoulos, D. S. Toward a better understanding of the lignin isolation process from wood. *J. Agric. Food Chem.* **2006**, *54*, 5939–5947.

(10) Guerra, A.; Filpponen, I.; Lucia, L. A.; Argyropoulos, D. S. Comparative evaluation of three lignin isolation protocols for various wood species. *J. Agric. Food Chem.* **2006**, *54*, 9696–9705.

(11) Argyropoulos, D. S. Quantitative phosphorus-31 NMR analysis of lignins, a new tool for the lignin chemist. *J. Wood Chem. Technol.* **1994**, *14*, 45–63.

(12) Granata, A.; Argyropoulos, D. S. 2-Chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane, a reagent for the accurate determination of the uncondensed and condensed phenolic moieties in lignins. *J. Agric. Food Chem.* **1995**, *43*, 1538–1544.

(13) Crestini, C.; Argyropoulos, D. S. Structural analysis of wheat straw lignin by quantitative ^{31}P and 2D NMR spectroscopy. The occurrence of ester bonds and α-O-4 substructures. *J. Agric. Food Chem.* **1997**, *45*, 1212–1219.

(14) Gordon, C. M.; Muldoon, M. J. Ionic Liquids in Synthesis. In *Synthesis and Purification*, 2nd ed.; Wasserscheid, P., Welton, T., Eds.; Wiley-VCH: Weinheim, Germany, 2007; Vol. *1*, pp 7−53.

(15) Helm, R. F. Lignin: Properties and Materials. In *Lignin-Polysaccharide Interactions in Woody Plants*; Scultz, T. P., Ed.; American Chemical Society: Washington, DC, 2000; Vol. *742*, pp 161−171.

(16) Koshijima, T.; Watanabe, T. In *Association between Lignin and Carbohydrates in Wood and Other Plant Tissues*; Springer Series in Wood Science; Springer: Heidelberg, Germany, 2003; Vol. *1*, pp 298.

(17) King, A. W. T.; Kilpeläinen, I.; Heikkinen, S.; Järvi, P.; Argyropoulos, D. S. Hydrophobic interactions determining functionalized lignocellulose solubility in dialkylimidazolium chlorides, as probed by ^{31}P NMR. *Biomacromolecules* **2009**, *10*, 458–463.

(18) Kilpeläinen, I.; Xie, H.; King, A. W. T.; Granström, M.; Heikkinen, S.; Argyropoulos, D. S. Dissolution of wood in ionic liquids. *J. Agric. Food Chem.* **2007**, *55*, 9142–9148.

(19) Xie, H.; King, A. W. T.; Kilpeläinen, I.; Granström, M.; Argyropoulos, D. S. Thorough chemical modification of wood-based lignocellulosic materials in ionic liquids. *Biomacromolecules* **2007**, *8*, 3740–3748.

(20) http://www.inmr.net/.

(21) Carlos Cobas, J.; Bernstein, M. A.; Martín-Pastor, M.; Tahoces, P. G. A new general-purpose fully automatic baseline-correction procedure for 1D and 2D NMR data. *J. Magn. Reson.* **2006**, *183*, 145–151.

(22) Li, C.; Wang, Q.; Zhao, Z. K. Acid in ionic liquid: An efficient system for hydrolysis of lignocellulose. *Green Chem.* **2008**, *10*, 177–182.

(23) Tohmura, S.; Argyropoulos, D.S. Determination of arylglycerol-*â*-aryl ethers and other linkages in lignins using DFRC/31P NMR. *J. Agric. Food Chem.* **2001**, *49*, 536–542.

(24) Scalbert, A.; Monties, B.; Lallemand, J.; Guittet, E.; Rolando, C. Ether linkage between phenolic acids and lignin fractions from wheat straw. *Phytochemistry* **1985**, *24*, 1359–1362.

(25) Ralph, J.; Helm, R. F.; Quideau, S.; Hatfield, R. D. Lignin−feruloyl ester cross-links in grasses. Part 1. Incorporation of feruloyl esters into coniferyl alcohol dehydrogenation polymers. *J. Chem. Soc., Perkin Trans. 1* **1992**, *21*, 2961–2969.

(26) Lam, T. B. T.; Kadoya, K.; Iiyam, K. Bonding of hydroxycinnamic acids to lignin: Ferulic and p-coumaric acids are predominantly linked at the benzyl position of lignin, not the *β*-position, in grass cell walls. *Phytochemistry* **2001**, *57*, 987–992.

(27) Hatfield, R.; Ralph, J.; Grabber, J. H. A potential role for sinapyl *p*-coumarate as a radical transfer mechanism in grass lignin formation. *Planta* **2008**, *228*, 919–928.

(28) Ralph, J.; Hatfield, R. D.; Quideau, S.; Helm, R. F.; Grabber, J. H.; Jung, H. G. Pathway of *p*-coumaric acid incorporation into maize lignin as revealed by NMR. *J. Am. Chem. Soc.* **1994**, *116*, 9448–9456.

(29) Lu, F.; Ralph, J. Detection and determination of *p*-coumaroylated units in lignins. *J. Agric. Food Chem.* **1999**, *47*, 1988–1992.

(30) Timell, T. E. Recent progress in the chemistry of wood hemicelluloses. *Wood Sci. Technol.* **1967**, *1*, 45–70.

Received May 10, 2009. Revised manuscript received July 31, 2009. Accepted August 3, 2009. The authors would like to gratefully acknowledge the financial contribution of the Finnish Funding Agency for Technology and Innovation (TEKES) for making this project possible.