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# Quantitative Glucose Release from Softwood after Pretreatment with Low-cost Ionic Liquids

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Softwood is an abundantly available feedstock for the bio-based industry, however, achieving cost-effective sugar release is particularly challenging owing to its guaiacyl-only lignin. Here, we report the highly effective pretreatment of the softwood pine (*Pinus sylvestris*) using ionoSolv pretreatment, a novel ionic liquid-based lignocellulose fractionation technology. Three protic, low-cost ionic liquids, 1-butylimidazolium hydrogen sulfate, triethylammonium hydrogen sulfate and *N,N*-dimethylbutylammonium hydrogen sulfate, were used to fractionate the biomass into a carbohydrate-rich pulp and a lignin. The carbohydrate-rich pulp was hydrolysed into fermentable sugars by enzymatic saccharification. Under the most successful pretreatment conditions, quantitative glucose release from the pulp was achieved, which equates to a projected glucose release of 464 mg per gram of pine wood entering the process. We further intensified the process by increasing the solid to solvent ratio up to 1:2 g/g while maintaining saccharification yields of 75% of the theoretical maximum. We also demonstrate for the first time that *N,N*-dimethylbutylammonium hydrogen sulfate, [DMBA][$HSO_4$] is an excellent low-cost pretreatment solvent, surpassing the pretreatment effectiveness of its symmetrically substituted analogue triethylammonium hydrogen sulfate. This shows that ionoSolv pretreatment with protic hydrogen sulfate ionic liquids is a truly feedstock-independent pretreatment option, further increasing the commercial potential of this pretreatment technology.





## Introduction

Bioenergy and biorefining research has attracted widespread attention in recent years as many countries seek to minimise their reliance on oil imports, reduce their carbon emissions and boost their agricultural and forestry sectors. Petroleum is currently relied upon for the production of the majority of the world's liquid fuels, plastics, solvents and chemicals.[1] A shift towards the use of biomass instead of petroleum will require significant investment in the development of novel process technologies.[2]

Considering the importance of liquid fuels worldwide within the transportation sector, there are increasing opportunities for biofuels to be incorporated into the energy mix. Biofuels can contribute towards energy security, if produced sustainably, however, a number of factors currently limit expansion. These include concerns surrounding competition for land with food production, or with endangered habitats with high carbon-storage capacity.[3] A major barrier is also the economic competitiveness of bio-derived products compared to cheap, highly optimised petroleum-derived fuels and chemicals.[2,4] To minimize unsustainable land use changes associated with feedstock production for biorefining, emphasis has shifted towards utilisation of lignocellulosic biomass, which refers to the woody and thus inedible parts of plants. Fuels and chemicals produced from lignocellulose are referred to as second-generation; promising lignocellulosic crops are dedicated energy crops (*i.e. Miscanthus* and short-rotation coppice willows), agricultural residues (corn stover, sugarcane bagasse and wheat straw) or forestry residues, the latter often being composed of softwoods such as pine and fir.[3] Although lignocellulose is an abundant and relatively inexpensive source of sugars, its greater structural complexity means that the sugars are much harder to isolate than sucrose or starch. In order to release sugars, a pretreatment step is required to increase accessibility of cellulose prior to hydrolysis of the sugars contained within, a process that adds substantial costs.[5] Negative effects on downstream processes by side-products

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generated during pretreatment pose further challenges. These issues currently present a technical and economic obstacle to establishing cellulosic sugar based biorefineries.

Ionic liquid pretreatment is a comparatively new biomass deconstruction approach. Two strategies during the ionic liquid pretreatment of lignocellulosic biomass are being followed: decrystallization of cellulose[6,7] and extraction of lignin (*i.e.* ionoSolv pretreatment).[8,9] The Dissolution pretreatment option relies on ionic liquids with strongly hydrogen-bond basic anions, such as 1-ethyl-3-methylimidazolium acetate [EMim][OAc], which dissolve cellulose in the absence of water.[10] Regenerated cellulose and hemicellulose exhibit a better and faster digestibility (*ca.* 50 times higher enzymatic hydrolysis rate) due to lower crystallinity[11,12] and enlarged surface area, but these ionic liquids suffer from low thermal stability (limiting their recyclability),[13] requirement for dry conditions, and high solvent cost.[14] The ionoSolv pretreatment extracts the majority of lignin from biomass such as *Miscanthus*[8,15,16] sugarcane bagasse[17] and willow[18] in the presence of 10-40 wt% water. Although the cellulose is still crystalline, saccharification is enhanced and accelerated up to 40 times compared to untreated biomass due to increased surface area and the removal of lignin. The ionoSolv pretreatment is therefore in its function similar to Organosolv pretreatment.[8,19,20] The extracted lignin can be recovered *via* addition of water as an anti-solvent, while the cellulose can be hydrolysed to glucose *via* enzymatic saccharification and used in subsequent fermentations or chemical transformations. During ionoSolv pretreatment, β-*O*-4' linkages in the lignin are broken, leading to fragmentation;[21] however, lignin condensation reactions may also occur. It has been discovered that protic ILs, such as 1-butylimidazolium hydrogen sulfate [HBim][$HSO_4$] and triethylammonium hydrogen sulfate [TEA][$HSO_4$][16,22] demonstrate similar efficacy as their dialkylimidazolium counterparts,[23] and the alkylammonium IL [TEA][HSO4] can be recycled multiple times with a slight increase in saccharification yield observed upon recycling.16 This is significant, as protic ionic liquids can be prepared *via* one-step acid-base reactions using readily available amines and mineral acids. A recent study has suggested prices of \$1.24 $kg^{-1}$ for triethylammonium hydrogen sulfate and between \$2.96-\$5.88 $kg^{-1}$ for 1-methylimidazolium hydrogen sulfate, if synthesised on a bulk scale.[24] This compares well to pricing for common organic solvents such as acetone or ethyl acetate, which cost between \$1.30-\$1.40 $kg^{-1}$. Recently, we showed that the pretreatment of *Miscanthus* with [TEA][$HSO_4$] can be carried out at 180°C for 15 min without any yield penalty, leading to commercially relevant process intensification.[22] We have further demonstrated that [TEA][$HSO_4$] can be re-used at least 4 times for pretreatment of *Miscanthus* while maintaining high saccharification yield and no ionic liquid degradation was detected through proton NMR.[16]

Thus far, *Miscanthus*[16,22] sugarcane bagasse[17] and willow[18] have been the main feedstocks investigated for deconstruction and fractionation with ionoSolv ionic liquids, while softwoods have received little attention apart from preliminary experiments.[8] Softwood is produced sustainably for timber and paper production and widely available as a forestry residue, in the form of sawdust at timber and pulp mills, or as construction waste wood.[25] According to NNFCC Bioeconomy Consultants, around 1.3 million tonnes of softwood forest waste arise in the UK annually, which can be removed from forests sustainably at a cost of between £18-50/dry tonne *vs.* around 140 thousand tonnes of *Miscanthus* produced in the UK (including 16% moisture) at a cost of £45-70/tonne.[26] An advantage of softwood is the high content of hexoses (Figure 1), due to the glucomannan dominated hemicellulose.[27] Hexose sugars are readily fermented by conventional yeasts,[28] while many fermentative organisms are not adapted to the conversion of pentose sugars (xylose and arabinose), which make up the majority of hemicellulose sugars in grasses and hardwoods.[27]

Softwoods are known to be more recalcitrant than grasses and hardwoods due to a higher lignin content (up to 30%)[27] and due to the lignin consisting predominantly of guaiacyl (G) units (95%).[29] Guaiacyl units result in a higher content of biphenyls and diphenyl ethers in the lignin,[8] and more easily form condensed structures during processing, therefore presenting a greater challenge to delignify, creating a barrier to quantitative sugar release and requiring high (Costly) enzyme loadings for saccharification.[30] Due to this, low-cost aqueous pretreatment such as steam explosion[31] and dilute acid[32] are largely ineffective in pretreatment of this recalcitrant feedstock. Better results can be obtained with Organosolv[33] and alkaline peroxide[34] pretreatments, however high capital and chemical recovery cost limit their applicability.

A limited number of studies have applied ionic liquid in softwood pretreatment. A study using dissolution pretreatment with the ionic liquid 1-butyl-3-methylimidazolium chloride [BMim]Cl has shown that a highly digestible pulp can be obtained from pine wood chips with a glucose yield of around 78%.[35] Cox *et al.* used the protic ionic liquid 1-methylimidazolium chloride [HMim]Cl, obtaining up to 50% glucose release from pine chips.[36] Both studies, however, employed low, economically unattractive biomass to solvent ratios of 1:20 and ca. 3:100 g/g, respectively.

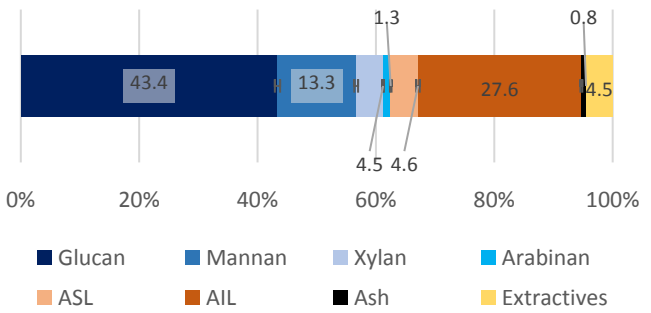


Figure 1 Composition of pine wood used in this study. ASL: acid-soluble lignin, AIL, acid insoluble lignin.

This study investigates the application of protic, hydrogen sulfate based ionic liquids for the fractionation of pine, a

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commonly grown softwood with wide geographical abundance.[37] We optimise pretreatment conditions (temperature and time) according to a bench-scale protocol developed in our laboratory,[15] applying the protic ionic liquid *N*-butylimidazolium hydrogen sulfate. Enzymatic glucose release was used as the key indicator. We also analysed the composition of the cellulose-rich pulp and chemical characteristics of the isolated lignin. We then applied two low-cost alkylammonium hydrogen sulfate ionic liquids at the optimised conditions. For the first time, an asymmetric trialkylammonium hydrogen sulfate ionic liquid was used for ionoSolv fractionation, and its effectiveness was compared with the alkylimidazolium and symmetric tirethylammonium analogues. The ions making up the three ILs used in this study are displayed in Figure 2.

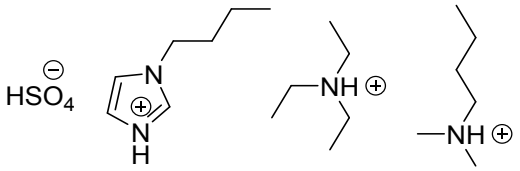


Figure 2 Structures of the ionic liquid ions used in this study: the hydrogen sulfate anion, and the 1-butylimidazolium, $[HBim]^+$, triethylammonium, $[TEA]^+$, and *N,N*-dimethylbutylammonium, $[DMBA]^+$, cations.

## Results and discussion

### Effect of Temperature and Time on pretreatment with [HBim][$HSO_4$]

#### Fractionation effectiveness and enzymatic saccharification yield

A set of experiments was carried out using the protic ionic liquid 1-butylimidazolium hydrogen sulfate [HBim][$HSO_4$] at three different oven temperatures (120, 150 and 170°C) with a 1:10 g/g biomass to solvent ratio. As we discussed in our study on process intensification of *Miscanthus* fractionation using triethylammonium hydrogen sulfate [TEA][$HSO_4$],[22] the designated reaction temperature inside the pretreatment vessel is only reached after around 30 min. This should be borne in mind when interpreting results for pretreatment times of 30 min or less.

Figure 3 shows how a number of indicators for pretreatment and fractionation effectiveness perform over time. The glucan recovery, delignification and (non-glucan) hemicellulose removal were derived by combining compositional analysis data and pulp yields; the precipitated lignin yield was determined gravimetrically. The enzymatic glucose release (saccharification yield) was determined for air-dried pine pulps. The saccharification yield is a key performance indicator, as glucose is a key substrate for fermentations or chemical transformations producing bio-based chemicals and fuels.

For the saccharification yield, we observed an initial decrease compared to untreated pine at all three temperatures. We attribute this to hornification (irreversible pore collapse) occurring during the air-drying of the pulp after washing,[38]

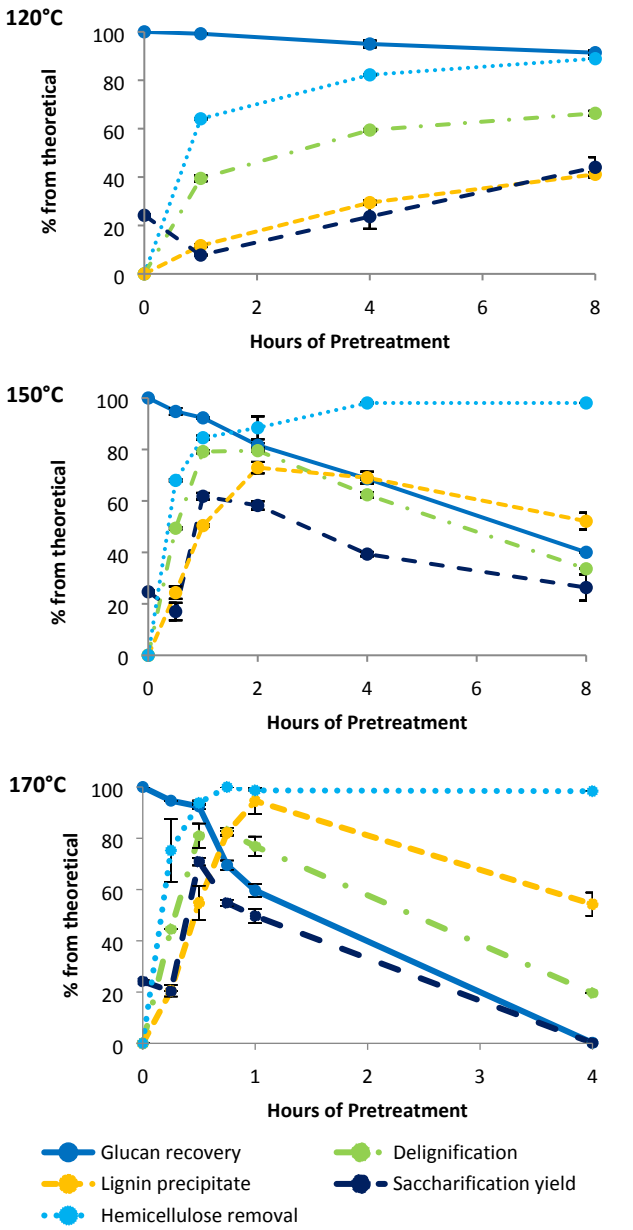


Figure 3 Key indicators of fractionation effectiveness for pine wood pretreated at 120°C, 150°C and 170°C with [$HC_4$im][$HSO_4$] containing 20 wt% water. The biomass to solvent ratio was 1:10 g/g

since we observed high glucose release when performing additional experiments with wet pulp, which are detailed towards the end of the manuscript. After the initial decrease, saccharification yields increased beyond that released from untreated biomass for all investigated temperatures. For 120°C, the saccharification yield did not reach a peak within the timeframe studied, while for experiments at 150 and 170°C, there was a peak saccharification yield at 60 and 30 min, respectively. The highest observed glucose releases were 44, 62 and 71% for 120, 150 and 170°C, respectively, showing that a

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higher pretreatment temperature is essential for effective enzymatic cellulose hydrolysis. The peak saccharification yields occurred at similar pretreatment conditions identified for *Miscanthus,*[22] sugarcane bagasse[17] and willow[18] pretreatment with [TEA][$HSO_4$].

Peak delignification was 79% at 150°C and 81% at 170°C, and it appeared to occur slightly later than peak saccharification. We also observed that saccharification yield and lignin removal correlated strongly, as can be seen from Figure 4. A relationship between delignification and enzymatic saccharification yield was reported previously for *Miscanthus*.[16,22]

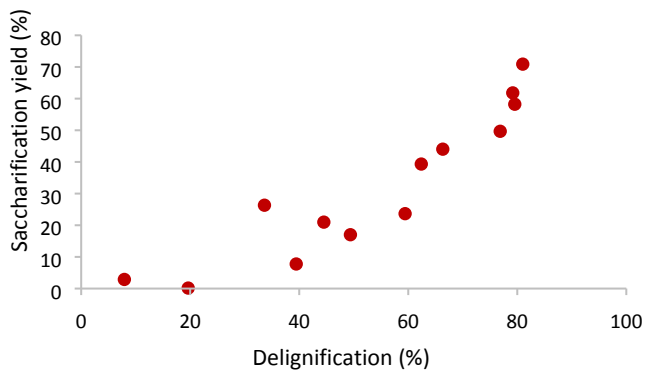


Figure 4 Correlation of enzymatic glucose release and delignification of pine after pretreatment with [$HC_4$im][$HSO_4$] with a water content of 20 wt% and a pine wood to solvent ratio of 1:10 g/g at different temperatures and times. Percentage of the theoretical maximum according to compositional analysis is shown.

At 150 and 170°C, the amount of lignin associated with the pulp increased after reaching a minimum (2 h at 150°C and 30/45 min at 170°C). This has been observed in the past for pretreatment of *Miscanthus* with [TEA][$HSO_4$] at temperatures of 150-180°C.[22] The increase in the amount of lignin present in the pulp at the later stages of pretreatment has been attributed to both condensation reactions within lignin fragments and the formation of pseudo-lignin from hemicellulose which contribute to the formation of high molecular weight substances which can precipitate onto the pulp surface.[39] While the compositional analysis technique fails to distinguish between actual lignin and pseudo-lignin, formation of the latter is strongly suspected under conditions where the lignin precipitate exceeds the measured delignification (*e.g.* after more than 1 h at 170°C). Evidence for condensation reactions was found when analysing the precipitated lignin, which is discussed below.

The re-precipitation of condensed lignin and also that of pseudo-lignin on to the cellulose surface are thought to have a negative effect on saccharification yields and kinetics by making the cellulose substrate less accessible.[40] It should further be noted that the work-up procedure used here involves diluting the IL with ethanol after pretreatment to facilitate its separation from the pulp. Visual observations suggested that ethanol-induced lignin precipitation was responsible for some of the lignin reprecipitation at prolonged pretreatment times. We note that dilution with ethanol is unlikely to be part of an industrial-scale protocol (we anticipate a washing step with recycled ionic liquid instead) and less redeposition is therefore expected under scaled-up conditions. An extensive study of ionoSolv softwood pretreatment up to the 200 L scale has been performed and will be reported in the near future.

We further note that no minimum for the lignin content was found within the studied timeframe at 120°C for pine, while a minimum can be detected for other feedstock types. For pine, delignification and saccharification yield was limited to 66% and 44% after 8 h compared to a 88% delignification and 77% saccharification yield for *Miscanthus* pretreated with [TEA][$HSO_4$], showing that softwood requires higher temperatures in order to achieve good pretreatment results.[16] The reason for this may be that lignin extraction is facilitated above the glass transition temperature of lignin (thought to be in the range 130-150°C).[41] We hypothesise that fast extraction from the cellulose pulp is particularly beneficial for softwood delignification, as guaiacyl-rich lignin is prone to forming stable crosslinks in acidic systems *via* condensation reactions.[42]

Glucan recoveries were fairly constant under mild conditions (*i.e.* at 120°C), while hemicellulose was removed over time. For 120°C, we observed that hemicellulose removal from pine was faster than hemicellulose removal from *Miscanthus* using [TEA][$HSO_4$],[16] with 64% of hemicelluloses removed in the first hour of pretreatment compared to 44% in the case of *Miscanthus*. High temperatures accelerated hemicellulose removal. Near-quantitative hemicellulose removal was achieved after 4 h and 45 min at 150 and 170°C, respectively, which is similar to what was observed for *Miscanthus* pretreated with [TEA][$HSO_4$].[22] The recovery and valorisation of the dissolved hemicellulose portion from ionoSolv solutions remains a challenge. One suggested option is to produce furfural.[16]

For the temperatures above 120°C, we observed that pretreatment effectiveness declined past the optimum point, which we attribute to reduced glucan recovery and deposition of condensed lignin and pseudo-lignin on to the surface of the cellulose fibrils. This was confirmed by compositional analysis of the pulps (numerical values can be found in Table S1 in the ESI). In an extreme case, the glucan recovery dropped to zero after 4 h of pretreatment at 170°C. We note that glucan appeared to be less stable in this study compared to *Miscanthus* pretreatment in [TEA][$HSO_4$], where glucan recovery was nearly 100% at 150°C and 4 h,[22] compared to 70% glucan recovery after 4 h of pretreatment of pine with [HBim][$HSO_4$]. It is currently unclear if the differences in cellulose and hemicellulose stability are due to the nature of the feedstock or the ionic liquid. An initial decrease in glucan recovery in the pulp has been attributed to removal of glucose containing hemicelluloses, such as galactoglucomannans and xyloglucans, rather than degradation of cellulose.[43] Galactoglucomannans constitute up to 15% of softwood.[44]

**Lignin characterisation**

As was shown in the previous section, there is a clear link between residual lignin content in the pulp, and saccharification

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yields (Figure 4). In order to improve the economics of cellulosic biorefineries, not only the cellulose/sugar yield needs to be optimised, but also the characteristics of the lignin. We therefore analysed the isolated lignins for their subunit composition and linkages and the molecular weight to gain insight into the reactions taking place. Pine lignin (and more generally softwood lignin) consists predominantly of guaiacyl (G) units (95%) and around 45-50 β-*O*-4' linkages per 100 aromatic rings.[29] The second most abundant linkage in softwood lignin is the phenylcoumaran (β-5') linkage with around 10-13 linkages per 100 aromatic units. In smaller numbers, resinol (β- β') and diarylpropane (β-1') linkages are also present.[45,46] Figure 5 shows the most important linkages and subunits in pine lignin.

Figure 6 shows the aromatic and the oxygenated regions of a representative HSQC NMR spectrum for one of the isolated pine lignins. Peaks for the three unsubstituted positions of the guaiacyl ring as well as certain linkages were assigned. We note that there are a number of peaks in the aromatic region of the spectrum which are currently unassigned, as they do not match any of the commonly found structures in ball-milled lignin. They probably correspond to new structures formed during the ionic liquid extraction. We also observed a peak at 4.2/67 ppm, which we assign to the plasticiser bis(2-ethylhexyl) phthalate (DEHP). It has been observed in a previous study[16] and is presumed to have been leached from the seals of the pressure tubes used for heating the biomass with the ionic liquid and subsequently precipitated with the lignin fraction during antisolvent addition.

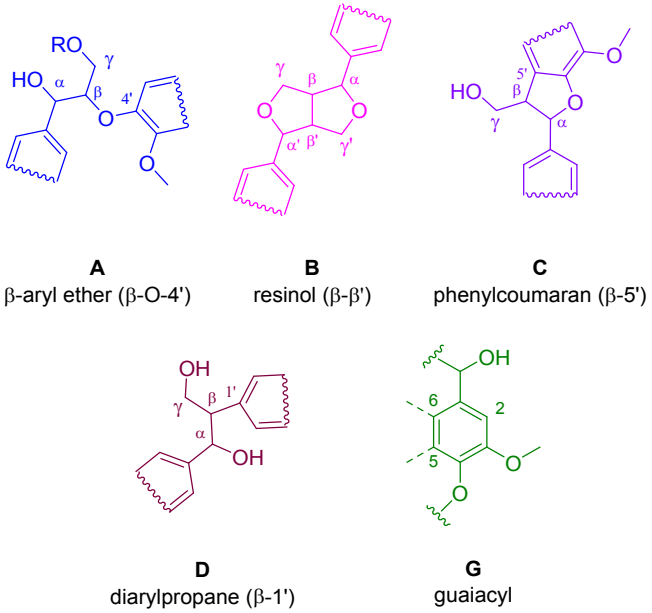


Figure 5 The most prominent ether bonds and lignin subunit in pine lignin.

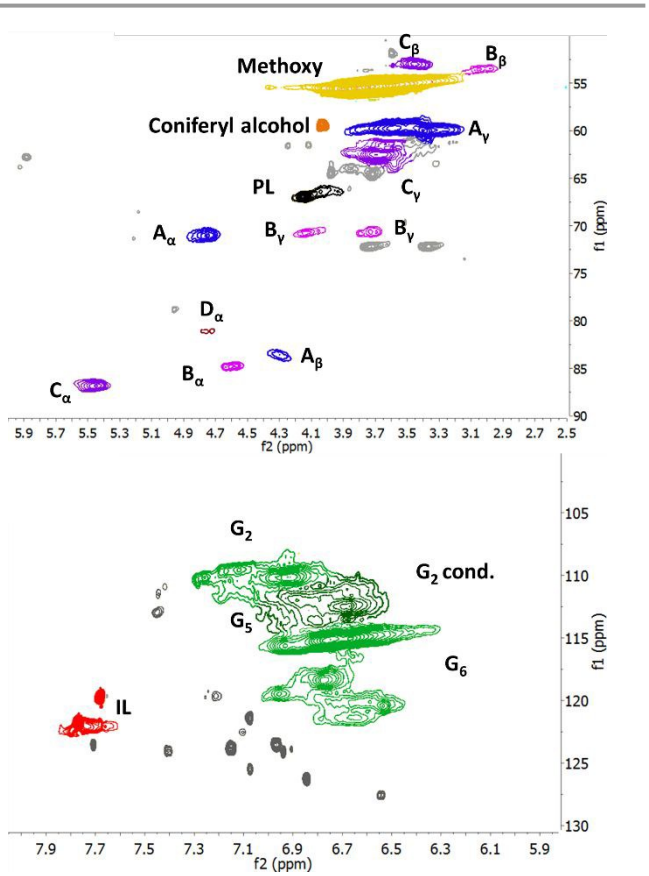


Figure 6 HSQC NMR spectra of pine lignin isolated after extraction with $[HC_4im][HSO_4]$ at 170°C for 30 min with a biomass to solvent ratio 1:10 g/g and a water content of 20 wt%. The side chain region (top) and aromatic region (bottom) are shown. PL is plasticizer. Two signals for residual butylimidazolium are marked in red. Grey peaks could not be assigned.

The pulse sequence used in these experiments only gives semi-quantitative information, since equilibration of the $^{13}C$ excitation is incomplete within the programmed relaxation time (in order to achieve manageable acquisition times), and carbon nuclei will relax at different rates in different chemical environments. However, the observed peak volume is linear with respect to the concentration of the analyte, so time course trends can be established with a suitable internal standard. It has been suggested in previous literature that the $G_2$ signal can be used as such an internal standard in the case of softwoods, as it is not thought to participate in condensation reactions or overlap with signals of degradation products.[45] It should therefore be representative of the total number of aromatic units in the lignin. However, the $G_2$ signal shifts if $G_6$ or $G_5$ sites undergo chemical modification, leading to a $G_{2,cond}$ peak, hence the sum of $G_2$ and $G_{2,cond}$ is used here as a reference integral.

With these assumptions, we compared the signal intensities for the four ether linkages shown in Figure 5 and the different positions of the guaiacyl unit found in the precipitated lignin, in order to discuss characteristics of the isolated lignins, and lignin reactivity more generally.

Figure 7 shows the relative HSQC volume integrals for pine lignin isolated after pretreatment for 1 h at 120, 150 and 170°C. It can be seen that the majority of β-*O*-4' ether linkages (45-

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50%), the most abundant lignin linkages in pine wood,[45] were broken after pretreatment for 1 h. The data suggest that the ether bonds contained in the less abundant β-β' and β-5' linkages break at a slower rate, which has been observed previously.[22] Similarly, the aromatic ring signals provided evidence for substitution reactions occurring on the aromatic rings (lignin condensation). The degree of condensation was higher at high pretreatment temperatures, as evidenced by the lower $G_6$ and $G_2$ signal intensities and the higher $G_{2.cond}$ signal for the 150°C lignin, and even more so for the 170°C lignin. We note that there was an unexpected increase in the $G_5$ signal intensity. We have preliminary evidence that this is due to carbohydrate-derived degradation products which are generated under acidic conditions and precipitate with the lignin. We will provide more data on this topic in a future publication.

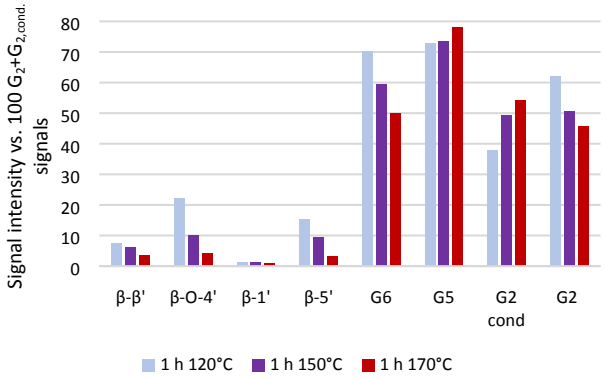


Figure 7 Abundance of ether linkages and C-$H_n$ sites on the guaiacyl ring of isolated pine lignin, obtained after pretreatment with [HBim][$HSO_4$] with 20 wt% water for 1 h.

We also compared the characteristics of the lignin isolated at the optimum pretreatment conditions. Figure 8 shows the relative size of the ether linkage and guaiacyl ring signals for lignins isolated after 0.5 h at 170°C, 1 h at 150°C and 4 h at 120°C, as well as the corresponding lignin precipitate and saccharification yield of the pulp (as a percentage of the maximum possible). The data show that these lignins had a similar degrees of condensation and quantities of unhydrolysed ether linkages, while lignin and saccharification yields were substantially different. We have previously shown that lignin isolated from *Miscanthus* at optimum conditions using [TEA][$HSO_4$] are different in chemical characteristics.[22] Here, we observe that lignin extraction and yield can be increased with temperature without altering the degree of condensation and the degree of hydrolysis in the precipitated lignin. This could be due to the different compositions of grass and softwood lignins.

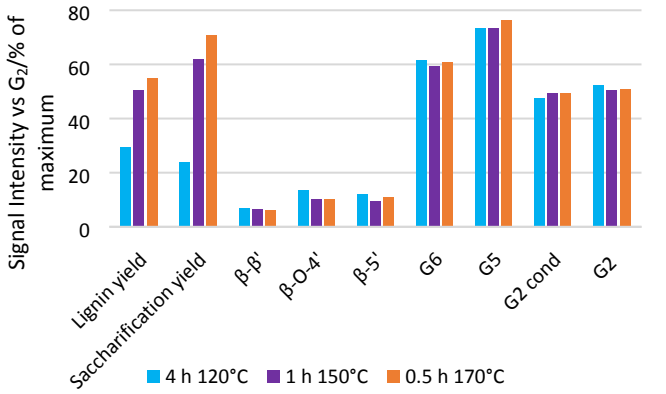


Figure 8 Abundance ether linkages and unsubstituted aromatic ring sites in lignin precipitates obtained after pretreatment of pine wood with [HBim][$HSO_4$] with 20 wt% water. The lignin and saccharification yield are shown for reference. The lignins selected here were similar in composition, while indicators of pretreatment effectiveness were markedly different.

## Effect of Biomass Loading on Pretreatment Effectiveness

Lignocellulose pretreatment is a capital-intensive process, with the pretreatment reactor(s) expected to represent *ca*. 20% of the capital expenditure (CAPEX) in a typical cellulosic ethanol plant.[47,48] Since the pretreatment reactor size and also solvent cost will scale approximately inversely with solids loading (more precisely with total volume), a higher solids loading will intensify the process and decrease CAPEX by approximately 40% when doubled, according to standard power laws (exponent 0.7) for economies of scale.[22] In order to improve the economic viability of ionoSolv pretreatment of pine wood, high solids loadings are therefore important.[49]

We thus assessed the pretreatment efficacy for pine at loadings ranging from 5 to 50 wt%, corresponding to biomass to solvent ratios from 1:20 to 1:2 g/g. These experiments were carried out using the optimal conditions for glucose release from the pulp identified for [HBim][$HSO_4$] (170°C for 30 min). Figure 9 shows the pulp, lignin and saccharification yields (saccharification conducted after air-drying of the pulps) as a function of the biomass loading. Pulp yields increased from around 41% to 56% when increasing the loading from 5 to 50 wt%. Lignin yields remained stable at around 55% for the 5-25% solid loading range, while for the 35 and 50 wt% loadings, a drop in the lignin yield to around 30% was observed. The saccharification yield was highest at the lowest loading, giving 78% of the theoretical yield, decreasing to below 40% for the 50 wt% pine wood loading.

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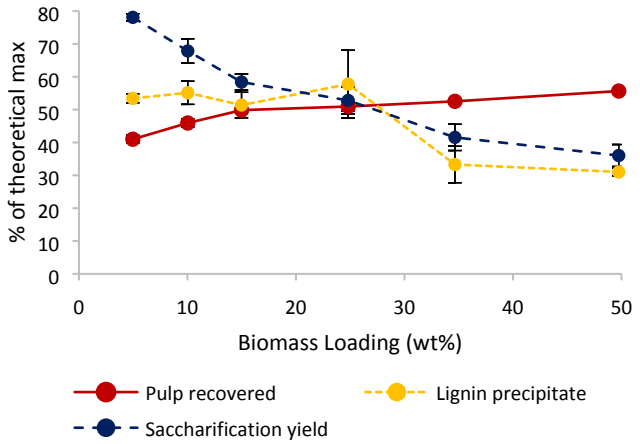


Figure 9 Effect of biomass loading on pulp yield, lignin yield and glucose release from the pulp via enzymatic saccharification after pretreatment of pine with [HBim][$HSO_4$] (20 wt% water) for 30 min at 170°C.

The increase in pulp yield and the decrease in lignin yields with higher loading could be due to limitations of particle wetting in the ionic liquid solution. It is also possible that the lignin was extracted and precipitated during the pulp washing with ethanol, discussed in more detail below.

We also investigated how lignin properties are affected by increasing the biomass loading. Figure 10 shows the HSQC NMR signal intensities, as well as molar mass of the lignins as a function of biomass loading. The HSQC NMR analysis showed only small changes in the chemical functionality of the recovered lignins with increasing biomass loading. It appears that the ether linkage content in the isolated lignin decreased slightly with increased loadings, and the degree of condensation and pseudo-lignin content also rose, which could be due to increased concentration of dissolved lignin fragments and hemicellulose in the ionic liquid solution. The observed differences are small, however, and they may not be significant. Overall, the compositions of the lignins isolated at various loadings were broadly similar.

The GPC data demonstrate a decrease in $M_n$ and $M_w$ with higher loading, although the values found for the 15 and 25 wt% loading experiments were inconsistent with the trend. Furthermore, the PDI of the isolated lignin was lower at higher loadings. As mentioned earlier, the observed differences may be due to the pulp isolation method (employing ethanol washing), resulting in a bias in the fraction recovered rather than the lignin structure being affected by the higher loading directly. This is supported by the fact that sulfate based ionic liquids have been shown to dissolve up to 70 wt% lignin[50] and it is therefore unlikely that we were witnessing saturation even at the highest biomass loadings. However, ethanol has a much lower lignin solubility,[51] and so its addition is likely to precipitate larger fragments out of solution during the washing step. Since the solubility of polymers of equivalent chemical functionality is governed by molecular size, we expect that the cut-off for larger fragments in ethanol would be at slightly lower $M_w$ at higher loadings. This is consistent with the apparent trend towards more ether cleavage with increasing biomass loading. Especially the decrease in the PDI is thought to stem from an upper cut-off for $M_w$ during ethanol dilution at high loadings, resulting in a narrower range of molecular weights in the isolated lignin.

These findings suggest that delignification and the structure of the extracted lignin are not altered in a significant way at higher loadings, but lignin yield and molecular weight of the isolated lignin is. The lignin and saccharification yield, and the pulp recovery, may be improved, and higher loading allowed with an adapted washing protocol that avoids re-precipitation and/or removes re-precipitated lignin from the pulp surface.

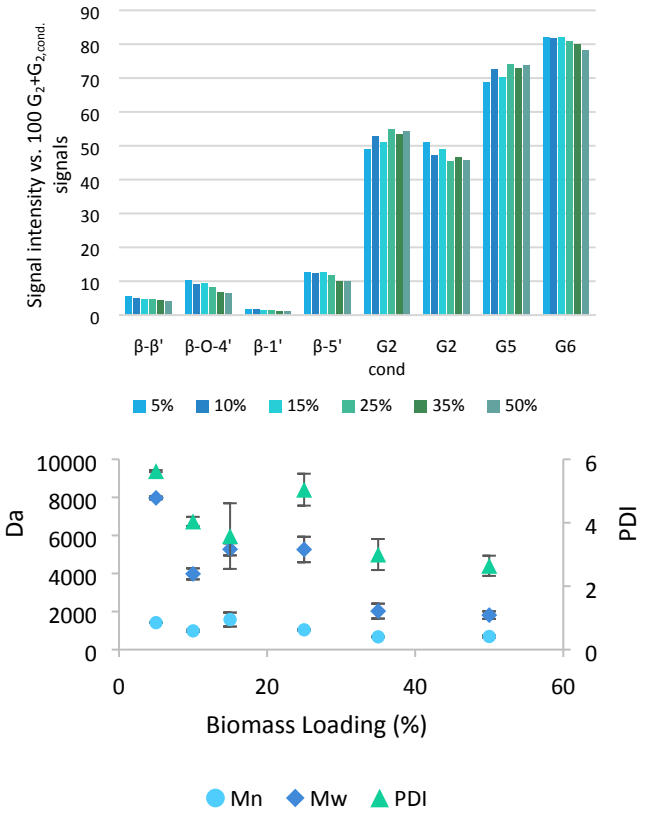


Figure 10 (Top) Abundance of chemical functionalities in isolated pine lignin as a function of biomass loading according to HSQC NMR spectroscopy. Signal intensities are relative to 100 $G_2$+$G_{2,cond}$. (Bottom) Molar masses and polydispersity of recovered lignins as a function of biomass loading. Pine was pretreated with [HBim][$HSO_4$] (20 wt% water) for 30 min at 170°C.

**Effect of pulp drying and hornification on saccharification yields**

In an industrial process, the cellulose-rich pulp is expected to be subjected to enzymatic hydrolysis directly after washing, rather than undergoing an air-drying step (which is part of our laboratory procedure in order to facilitate sample handling). However, we note that there is evidence that air-drying leads to hornification of pretreated lignocellulose which negatively affects enzymatic saccharification yields,[38] hence we investigated whether omitting the air-drying would improve

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glucose release from pine pulps. For this, pulp samples were submerged in water directly after Soxhlet extraction with ethanol. Moisture contents of wet pulps obtained after solvent exchange were around 90 wt%. Wet saccharification was carried out for pulps obtained at 10 or 50 wt% loading (1:10 and 1:2 g/g biomass to solvent ratio), using the optimised pretreatment conditions of 30 min at 170°C for [HBim][$HSO_4$]. The results in Table 1 show that the sugar release from wet pulps was substantially higher. At 10% loading, we observed quantitative glucose release from the wet pulp, while at 50% loading, 75% of the glucose was released, representing an increase of 57 and 120% *vs.* the dried pulps, respectively. This represents a significant improvement. Even with the lower yield at 50% biomass loading, we estimate the reduction in reactor size would reduce CAPEX per tonne of glucose obtained by 57%.

Table 1 Saccharification yield obtained from air-dried and wet pine pulps. Pine was pretreated at 170°C for 30 min with a solid to solvent ratio of 1:10 and 1:2 g/g in [HBim][$HSO_4$].

| | Saccharification yield | |
|---|---|---|
| **Loading** | **with air drying** | **without air drying** |
| 10% | 64.8±3.5% | 102.2±1.9% |
| 50% | 34.4±3.2% | 74.9±2.9% |

## Selection of Ionic Liquid

The imidazolium-based ionic liquid [HBim][$HSO_4$] can be produced at relatively low-cost compared to many imidazolium ILs, due to being a protic IL that can be synthesised from inexpensive sulfuric acid and the imidazole precursor in one step. However, alkylammmonium hydrogen sulfates would be more cost-effective, as the 1-butylimidazole precursor is still fairly expensive. Hence we tested two ILs made from low-cost amines, triethylammonium hydrogen sulfate, [TEA][$HSO_4$] and *N,N*-dimethylbutylammonium hydrogen sulfate, [DMBA][$HSO_4$], under the optimised pretreatment conditions of 30 min at 170°C.

[TEA][$HSO_4$] has been successfully applied for the fractionation of *Miscanthus*,[16,22] sugarcane bagasse,[17] willow[18] and switchgrass,[14] while the asymmetric [DMBA][$HSO_4$] has never been used as a fractionation solvent. We were particularly interested in applying [DMBA][$HSO_4$] due to it being a liquid at room temperature, even in the absence of water, and hence it having a low viscosity, which facilitates handling in the lab and at scale.[52] It has also been shown to be very effective in hydrolysing β-*O*-4 linkages in a lignin model compound.[53] We have previously demonstrated that the optimum pretreatment conditions for 1-butylimidazolium and triethylammonium hydrogen sulfate solutions are the same, if the acid base ratio is the same.[16,23]

The saccharification yields and lignin and pulp recoveries obtained for 10 % solid loadings are shown in Figure 11. We obtained the highest saccharification yields for [DMBA][$HSO_4$] (74%), showing that a lower-cost trialkylammonium hydrogen sulfate IL can achieve similar or an even better pretreatment outcome than a more expensive alkylimdiazolium hydrogen sulfate. Lignin recovery correlated well with saccharification yields. The highest lignin recovery was observed for [DMBA][$HSO_4$](72%), while the lowest lignin recovery and saccharification yield (30 and 36%, respectively) were obtained when using [TEA][$HSO_4$]. This was less than half of the yields achieved by the best-performing [DMBA][$HSO_4$].

[TEA][$HSO_4$] has previously been shown to effectively delignify *Miscanthus,*[16] sugarcane bagasse[17] and willow[18] biomass, resulting in saccharification yields of up to 80% from air-dried pulps,[14,16] although slightly higher yields at optimised conditions have been typically observed for imidazolium based hydrogen sulfate ILs (up to 90%).[23] It is unclear at present why [TEA][$HSO_4$] was so drastically outperformed by [HBim][$HSO_4$] and [DMBA][$HSO_4$] in this case. We note that the order of fractionation performance mirrors how fast the three ionic liquids hydrolyse β-*O*-4 linkages, as measured by Gregorio *et al*.[53] We also note that [DMBA][$HSO_4$] and [TEA][$HSO_4$] have the same molar mass and it is therefore unlikely that the difference in performance is linked to an effect of molar ratio of ionic liquid to water or biomass. Work on correlating the effect of ionic liquid cation substitution pattern with pretreatment efficacy is currently underway and will be presented in due course.

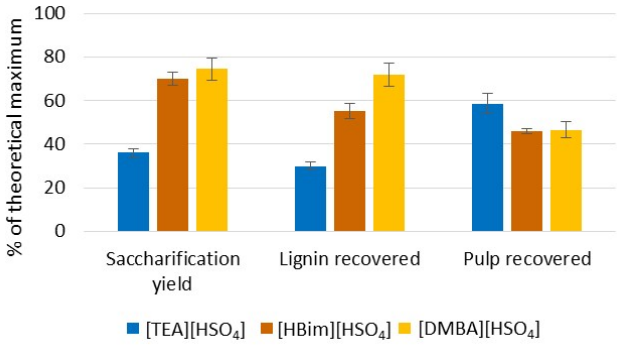


Figure 11 Saccharification yield (from air-dried pulps) and lignin and pulp recovery after pretreatment at 170°C for 30 min with three protic hydrogen sulfate ILs, each containing 20 wt% water. Pine was pretreated at a solid to solvent ratio of 1:10 g/g.

HSQC NMR spectroscopy of the lignins isolated from different ionic liquids showed a decrease in the quantity of ether bonds in the lignin in the order [TEA]>[HBim]>[DMBA], with volume integral sizes for the β-*O*-4' bond of 13.6 *vs* 10.4 and 6.9%, respectively (Figure 12), which again is in line with the

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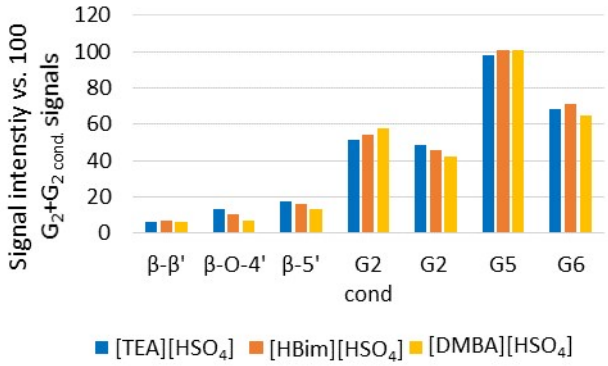
decreasing rate constant observed for the three ILs.




The degree of condensation in the lignin, evidenced by a more intense $G_{2,cond}$ signal, increased in the same order. The lignin isolated using $[DMBA][HSO_4]$ can therefore be regarded as the most modified lignin. Together with the high lignin precipitate yield obtained with this IL, these data suggest that lignin extraction was faster, probably due to a combination of improved mass transfer due to the low viscosity of $[DMBA][HSO_4]$ (giving rise to longer lignin residence times in the acidic ionoSolv solution) and the higher reactivity of lignin in $[DMBA][HSO_4]$. A similar trend was observed for the average molar masses for the isolated lignins, as determined by gel permeation chromatography (Table 2). Pretreatment with $[DMBA][HSO_4]$ yielded a lower $M_n$ and a higher $M_w$ lignin than applying the other two ILs. As a result, a high PDI of 5.1 was found for this IL vs. PDIs of 3.7 and 3.9 for $[TEA][HSO_4]$ and $[HBim][HSO_4]$, respectively. We note that the lignin obtained under optimised conditions with $[DMBA][HSO_4]$ has a notably higher PDI than those observed with the other two ILs, or for *Miscanthus* pretreated with $[TEA][HSO_4]$ at optimised conditions,[22] however, willow pretreatment with $[TEA][HSO_4]$ has resulted in similarly high PDIs of 4.6 up to over 6 for optimised sugar release.[18]

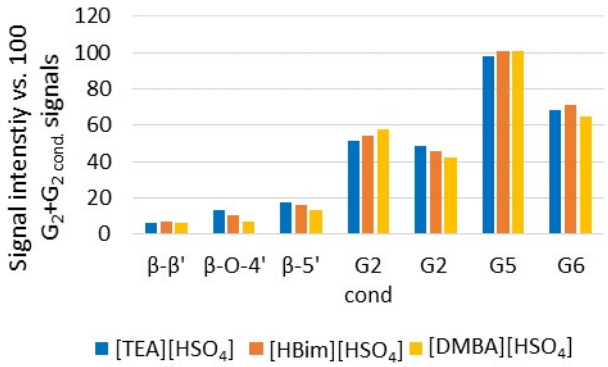



Figure 12 Abundance of ether linkages and C-H motives on the guaiacyl ring in pine lignin recovered after pretreatment at 170°C for 30 min with three protic hydrogen sulfate ILs containing 20 wt% water, according to HSQC NMR spectroscopy. Pine wood was pretreated at a solid to solvent ratio of 1:10 g/g.

Table 2 Molar mass and polydispersity of lignins recovered after pretreatment of pine wood at 170°C for 30 min with three low-cost hydrogen sulfate ILs. A solid to solvent ratio of 1:10 g/g was used.

| IL | $M_n$ | $M_w$ | PDI |
|---|---|---|---|
| $[TEA][HSO_4]$ | 922 | 3452 | 3.7 |
| $[HBim][HSO_4]$ | 967 | 3810 | 3.9 |
| $[DMBA][HSO_4]$ | 775 | 3949 | 5.1 |

$M_w$: Average molar masss, $M_n$: number average molar mass, PDI: Polydispersity index

## Experimental

### Materials

Starting materials for ionic liquid synthesis were purchased from Sigma Aldrich and used as received, unless stated otherwise. $^1$H NMR spectra were recorded on a Bruker 400 MHz spectrometer. Chemical shifts (δ) are reported in ppm, the DMSO signal at 2.500 ($^1$H dimension) and 39.520 ($^{13}$C dimension). Electrospray mass spectrometry experiments were carried out by Dr Lisa Haigh (Imperial College London, Chemistry Department) on a Micromass Premier spectrometer. The Karl-Fisher titrator used in this work was a V20 volumetric Titrator (Mettler-Toledo) and the analytical balance a Sartorius CPA 1003 S balance (±0.001 g).

### Synthesis of 1-hydrogen-3-butylimidazolium hydrogen sulfate [HBim][$HSO_4$].

5 M $H_2SO_4$ (322 mL, 1.61 mol) was cooled with an ice bath. Under stirring, 200 g of freshly distilled *N*-butylimidazole (1.61 mol) was added dropwise. The water was removed under reduced pressure and the product dried in vacuum at 50°C overnight. The ionic liquid was recovered as a clear, pinkish, viscous liquid. $^1$H NMR: δH (400 MHz, DMSO-$d_6$)/ppm: 9.14 (s, 1H, N-C*H*-N), 7.79 (s, 1H, N-C*H*), 7.68 (s, 1H, N-C*H*), 4.26 (br, N-*H*, *H*$SO_4^-$), 4.19 (t, J = 7.2 Hz, 2H, N-C$H_2$), 1.77 (m, 2H, N-$CH_2$-C$H_2$), 1.23 (m, 2H, N-$CH_2$-$CH_2$-C$H_2$), 0.88 (t, J = 7.4 Hz, 3H, N-$CH_2$-$CH_2$-$CH_2$-C$H_3$). $^{13}$C NMR: δC (101 MHz, DMSO-$d_6$)/ppm: 135.72 (N-*C*H-N), 122.46 (N-*C*H), 120.45 (N-*C*H), 48.68 (N-*C*$H_2$), 31.90 (N-$CH_2$-*C*$H_2$), 19.29 (N-$CH_2$-$CH_2$-*C*$H_2$), 13.75 (N-$CH_2$-$CH_2$-$CH_2$-*C*$H_3$).
MS (Magnet FB$^+$) m/z: 125 ([HBim]$^+$, 100%), (Magnet FB$^-$) m/z: 79 ([$HSO_4$]$^-$, 100%).

### Synthesis of triethylammonium hydrogen sulfate [TEA][HSO4].

Triethylamine (75.9 g, 750 mmol) was cooled with an ice bath in a round-bottom flask. 150 mL 5 M $H_2SO_4$ (750 mmol) was added dropwise while stirring. The water was removed using a rotary evaporator and the product dried using the Schlenk line at 40°C overnight. The ionic liquid was recovered as a white, hygroscopic solid.
$^1$H NMR: δH (400 MHz, DMSO-$d_6$)/ppm: 3.39 (s (br), *H*$SO_4^-$, N-*H*$^+$), 3.10 (q, J = 7.3 Hz, 6H, N-C$H_2$), 1.20 (t, J=7.3 Hz, 9H, N-$CH_2$-C$H_3$). $^{13}$C NMR: δC (101 MHz, DMSO-$d_6$)/ppm: 46.21 (N-*C*$H_2$), 9.15 (N-$CH_2$-*C*$H_3$).
MS (Magnet FB$^+$) m/z: 102 ([TEA]$^+$, 100%), (Magnet FB$^-$) m/z: 79 ([$HSO_4$]$^-$, 100%).

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**Synthesis of *N,N*-dimethylbutylammonium hydrogen sulfate [DMBA][$HSO_4$].**

*N,N*-dimethylbutylamine (75.9 g, 750 mmol) was cooled with an ice bath in a round-bottom flask. 150 mL of 5 M $H_2SO_4$ (750 mmol) was added dropwise while stirring. The water was removed using the rotary evaporator and the product dried using the Schlenk line at 70°C overnight. The ionic liquid was recovered as a clear, viscous liquid.
$^1$H NMR: δH (400 MHz, DMSO-$d_6$)/ppm: 9.24 (s, 1H, N-*H*), 3.02 (dt, *J* = 12.9, 5.0 Hz, 2H, N-C$H_2$), 2.76 (d, *J* = 4.3 Hz, 6H, N-(C$H_3$)$_2$), 1.64 – 1.51 (m, 2H, N-CH$_2$-C$H_2$), 1.29 (h, *J* = 7.4 Hz, 2H, N-CH$_2$-CH$_2$-C$H_2$), 0.89 (t, *J* = 7.4 Hz, 3H, N-CH$_2$-CH$_2$-CH$_2$-C$H_3$). $^{13}$C NMR δC (101 MHz, DMSO-$d_6$)/ppm: 56.62 (N-*C*H$_2$), 42.48 (N-*C*H$_3$), 25.82 (N-CH$_2$-*C*H$_2$), 19.40 (N-CH$_2$-CH$_2$-*C*H$_2$), 13.71 (N-CH$_2$-CH$_2$-CH$_2$-*C*H$_3$).
MS (Magnet FB$^+$) m/z: 102 ([DMBA]$^+$, 100%), (Magnet FB$^-$) m/z: 79 ([$HSO_4$]$^-$, 100%).

**Feedstock**

*Pinus sylvestris* was obtained from Metla (Finnish Forest Research Institute). It was air-dried, ground and sieved (180-850 µm, 20 + 80 US mesh scale) prior to use and stored in plastic bags at room temperature in the dark.

**Fractionation of Biomass**

Pretreatments, determinations of oven dried weight and ionic liquid water content measurements were conducted according to the standard operating procedures from our laboratory[15], in triplicate. A biomass to solvent ratio of 1:20 to 1:2 g/g was used (ODW basis). Hydrogen sulfate ionic liquids with 20wt% water were used as the solvent, using between 2 and 10 g of ionic liquid-water mixture and 1 g of biomass, except for the 1:20 g/g experiment, where only 0.5 g of biomass and 10 g of IL-water mixture was used. A detailed description of the procedure can be found in the ESI.
For experiments without air-drying of the pulp, the procedure was followed unchanged until completion of the Soxhlet extraction step. After the extraction, the pulp containing thimbles were transferred from the Soxhlet adapter into 50 mL centrifuge tubes and DI water was added immediately. This was incubated for at least an hour. The thimble was removed from the plastic tube and the pulp transferred back to the centrifuge tube using a spatula. The suspensions were centrifuged for 30 min at 2000*G* and the supernatant decanted. The wet pulps were weighed and their moisture content determined immediately. Thereafter, the pulps were stored in the fridge at 4°C.

**Compositional Analysis**

Compositional analysis was carried out according to a published procedure by the National Renewable Energy Laboratory (NREL).[54] The detailed procedure can be found in the ESI.

**Saccharification Assay.**

Saccharification assays were carried out according to a protocol published by the NREL[55] in triplicates with blanks (also triplicates). All reagents were purchased from Sigma Aldrich. The detailed procedure can be found in the ESI.
Hydrolysis was run for 7 days and Novozymes experimental enzyme mixture NS-22201 was used as the enzymes. Analysis was performed on a Shimadzu HPLC system with RI detector and an Aminex HPX-87P column (BioRad, 300 x 7.8 mm) with purified water as mobile phase (0.6 mL/min).

**Lignin characterisation**

**HSQC NMR spectroscopy**

*Circa* 20 mg of isolated lignin was dissolved in 0.25 mL of DMSO-$d_6$ and the solution transferred to a Shigemi tube. HSQC NMR spectra were recorded on a Bruker 600 MHz spectrometer (pulse sequence hsqcetgpsi2, spectral width of 10 ppm in F2 ($^1$H) with 2048 data points and 160 ppm in F1 ($^{13}$C) with 256 data points, 16 scans and 1 s interscan delay). Spectra were analysed using MestReNova (Version 8.0.0, Mestrelab Research 2012). All spectra were referenced to the DMSO peak at 2.500 ppm ($^1$H) and 39.520 ppm ($^{13}$C). Integrals were obtained for spectra of the same series of experiments simultaneously to ensure that the same areas were integrated. All relevant spectra were copied into one file and selected them while integrating the relevant area in one spectrum. Integration areas were selected visually according to peak assignments found in literature.[21] For ether linkages, the C-$H_\alpha$-signals were integrated. Integral sizes are reported with respect to 100 ($G_2$+$G_{2,cond}$) signals. All spectra can be found in the ESI.

**Gel permeation chromatography**

GPC measurements were performed using an Agilent 1260 Infinity instrument equipped with a Viscotek column set (AGuard, A6000M and A3000M). The Agilent 1260 Infinity RID detector was used for detection. GPC grade DMSO containing LiBr (1 g/L) was used as eluent at a flow rate of 0.4 mL/min at 60°C. Samples were prepared by dissolving 20 mg lignin in 1 ml eluent and filtering through a 0.2 µm syringe filter. Ten pullulan standards (Agilent calibration kit, 180 < Mp < 780,000) were used to calibrate the instrument.

## Conclusions

We have demonstrated the successful application of easily-synthesised protic ionic liquids for the pretreatment of the recalcitrant softwood, pine. A highly digestible cellulose pulp is obtained, giving rise to quantitative glucose yields after only 30 min of pretreatment at 170°C. Quantitative glucose release was achieved at a solid to liquid ratio of 1:10 g/g using 1-butylimidazolium hydrogen sulfate, when the pulp drying step was omitted. High saccharification yields were correlated with extensive lignin removal from the pine wood.
Carbohydrate free lignins were isolated through antisolvent (water) addition and were found to be depolymerised and partially condensed.

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Furthermore, we show that high solids loading of up to 1:2 g/g achieve good pine fractionation, especially if the cellulose-rich pulp is not air-dried. It may be possible to devise an adapted washing protocol that reduces re-precipitation and/or removes re-precipitated lignin from the pulp surface, further improving the performance of ionoSolv pretreatment of softwood at high solids loadings.

The ultra low-cost ionic liquid [DMBA][$HSO_4$] proved to be a more effective pretreatment solvent than [HBim][$HSO_4$] under the conditions studied.

The potential to use low-cost ionic liquids as effective pretreatment media for softwoods is very encouraging, as ionoSolv pretreatment has the potential not only to be economically viable but also to be feedstock independent, which is a unique combination.

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## Notes and references

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