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# Effect of pretreatment severity on the cellulose and lignin isolated from *Salix* using ionoSolv pretreatment†

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The ionoSolv pretreatment is a new technique employing protic low-cost ionic liquids and has previously been applied to successfully fractionate switchgrass and the grass *Miscanthus giganteus*. This study investigates the effect of using the protic ionic liquid solution $[N_{2220}][HSO_4]_{80\%}$ with two different acid/base ratios (1.02 and 0.98) at 120, 150 and 170 °C on the pretreatment outcome of the hardwood willow. The ionic liquid solution was able to fractionate willow, and a pulp and lignin fraction were recovered after treatment. The pretreatment success was determined *via* enzymatic hydrolysis of the pulp, which showed that the ionoSolv pretreatment was able to increase enzymatic glucose yields compared to untreated willow biomass. The pretreatment produced a cellulose-rich pulp with high hemicellulose and lignin removal. The pulp composition and glucose yield after saccharification were greatly influenced by the acidity of the ionic liquid solution, temperature and pretreatment time. The extracted lignin was analysed *via* 2-D HSQC NMR spectroscopy and GPC to investigate the changes in the lignin structure induced by the pretreatment severity. The lignin structure (in terms of inter-unit linkages and S/G ratio) and molecular weight varied significantly depending on the pretreatment conditions used.

## Introduction

Using fossil fuels such as oil and coal for the production of electricity and chemicals generates $CO_2$, which is a contributor to climate change and global warming, thus necessitating the search for an alternative carbon source.[1]

Lignocellulosic biomass (composed of cellulose, hemicellulose and lignin) is the only large-scale sustainable source of carbon on the planet, which makes it a promising alternative to fossil resources for the production of fuels and

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chemicals.[1c,2] Three groups of lignocellulosic biomass are important to the bioeconomy: grasses (annual and perennial), hardwoods and softwoods, which display differences in lignin and hemicellulose composition, biopolymer content, cell wall structure as well as hemicellulose and lignin structure.[2]

The content of the biopolymers varies with feedstocks, but typically lignocellulose consists of 35–50 wt% cellulose, 20–25 wt% hemicellulose and 15–35 wt% lignin.[2a]

The lignin content and the lignins' chemical characteristics differ between grasses, softwood and hardwood, with softwoods having the highest lignin content and grasses having the lowest.[3] It is important to note that the differences in the lignin structure and properties greatly affect the delignification chemistry and play a major role in biomass deconstruction,[4] as it leads to differences in recalcitrance towards pretreatment, with grasses being the least resistant and softwoods the most resistant to pretreatment.[2a] Lignin has been identified as a major factor for biomass recalcitrance, hindering enzymatic saccharification of the cellulose to glucose either through non-productive binding with enzymes or through acting as a physical barrier and thus restricting enzyme accessibility.[5] One way to overcome this problem is to introduce a pretreatment step that either redistributes the lignin[6] or removes the lignin from the biomass.[2a]

Ionic liquids are very promising candidates for biomass pretreatment, as they are able to partially (fractionate)[2a] or completely dissolve (disentangle)[7] lignocellulose. The ionic liquid 1-ethyl-3-methylimidazolium acetate $[C_2C_1im][OAc]$ is a prominent candidate for biomass pretreatment and has been widely studied.[2a] However, commercialisation attempts have been prevented so far due to the high cost of many ionic liquids.[8] The inexpensive ionic liquid triethylammonium hydrogen sulfate $[N_{2220}][HSO_4]$ (estimated bulk price of \$1.24 per kg)[9a] was developed as a potentially commercially viable alternative.[9b] The ionic liquid $[N_{2220}][HSO_4]$, used with a minor amount of water, which is needed for pretreatment to occur, is a non-volatile organic solvent with high polarity and acidity.[10] The solvent acidity can be controlled by adjusting the acid/base ratio (a/b) during synthesis, adding an option to tune the pretreatment severity.

The ionoSolv pretreatment selectively extracts lignin and hemicellulose from the biomass and a cellulose-rich pulp and a lignin fraction are recovered.[11] The removal of hemicellulose and lignin leads to an increase in the glucose yield of enzymatic hydrolysis of the pretreated pulp compared to the untreated biomass.[11,12]

The protic ionic liquid solution $[N_{2220}][HSO_4]_{80\%}$ (80 wt% IL and 20 wt% water) with an acid/base ratio of 1 (a/b = 1) was used for the pretreatment of switchgrass[9b] and *Miscanthus giganteus*[12] in the past, but the pretreatment technology has not yet been applied to a hardwood such as willow. The pretreatment severity can be controlled either by the pretreatment time, temperature or acidity of the pretreatment solvent. As mentioned earlier, hardwoods generally show a higher recalcitrance to pretreatment than grasses.[2a] This typically necessitates the use of more severe pretreatment conditions, which can be achieved by using an a/b of greater than 1 (having excess acid present in the ionic liquid). However, little is known about the impact of the acidity of the ionic liquid solution $[N_{2220}][HSO_4]_{80\%}$ on the pretreatment of the hardwood willow.

 

# Results and discussion

### Synthesis of $[N_{2220}][HSO_4]$ and determination of the acid/base ratio

Two ionic liquid solutions with different acid/base ratios were created by carefully mixing the correct amount of the two starting materials, triethylamine and sulfuric acid.

To verify the acid/base ratio, the synthesized ionic liquid solutions with a water content of 20 wt% were characterized by pH and density measurements. The acid/base ratio of the synthesized ionic liquid was calculated according to the method developed by M. S. Y. Jennings,[13] using the following equation:

$$y = 0.0772x + 1.1135$$

where $y$ is the density of the ionic liquid solution and $x$ is the acid/base ratio. Table 1 shows exemplary pH values and densities for $[N_{2220}][HSO_4]$ solutions with different acid/base ratios. The difference in the acid/base ratio of the ionic liquid is clearly reflected by the change in pH and density, with the more acidic ionic liquid solution having a density of 1.1924 g $mL^{-1}$ and a pH of 1.52 compared to 1.1889 g $mL^{-1}$ and a pH of 1.65 for the less acidic one.

### Pulp and lignin recovery after pretreatment with $[N_{2220}][HSO_4]$

In this study, the hardwood willow (variety Endurance) is used to expand the feedstock range for the ionoSolv pretreatment. To overcome the higher recalcitrance of the hardwood cell structure, the ionic liquid solution $[N_{2220}][HSO_4]$ with an excess of 2% of sulfuric acid (a/b = 1.02) was used. However, undesirable acid catalysed cellulose hydrolysis to glucose is well known,[14] which raises the concern that the high acidity of $[N_{2220}][HSO_4]$ with a/b = 1.02 will lead to cellulose degradation in the pulp, which in turn will decrease the glucose yield after enzymatic hydrolysis. To prevent this, pretreatment with $[N_{2220}][HSO_4]$ with a/b = 0.98 was also conducted.

Willow Endurance was pretreated using the following conditions: 0.5 h, 1 h, 2 h, 4 h and 8 h at 120 °C, 0.5 h, 1 h, 2 h and 4 h at 150 °C as well as 0.3 h, 0.5 h, 0.7 h and 1 h at 170 °C. Fig. 1 shows that the physical appearance of the isolated pulp changed significantly with the severity of the pretreatment, which gives a first indication of the success of the pretreatment. It can be seen that harsher pretreatment conditions resulted in a darker pulp, and an almost char-like product for very severe treatments. The pulp and lignin recovery after pretreatment gave a second indication of the pretreatment effectiveness. See Fig. 2 for an overview of the pulp and lignin recovery after pretreatment under various conditions.

Table 1 Examples of the results from pH measurements and density measurements for the synthesized $[N_{2220}][HSO_4]$

| Entry | pH | Density [g $mL^{-1}$] | Acid/base ratio |
|---|---|---|---|
| 1 | 1.52 ± 0.01 | 1.1924 ± 0.0001 | 1.02 |
| 2 | 1.65 ± 0.01 | 1.1889 ± 0.0002 | 0.98 |


Fig. 1 Examples of the physical appearance of the pulp before and after pretreatment at 150 °C for 0.5 h, 1 h, 2 h and 4 h.

Fig. 2 shows that using $[N_{2220}][HSO_4]$ decreased the amount of biomass recovered after pretreatment compared to the starting material, consistent with the ability of $[N_{2220}][HSO_4]$ solutions to remove lignin and hemicellulose from the lignocellulose matrix as reported earlier by Gschwend *et al.*[12a] In general, the pretreatment conditions (time, temperature and acid/base ratio) dictated the amount of pulp and lignin that were recovered, with higher pretreatment severity leading to a lower pulp yield and a higher lignin yield upon precipitation. At 120 °C, an increase in pretreatment time steadily improved the removal of material from the pulp and resulted in a higher lignin recovery. It was found that using $[N_{2220}][HSO_4]$ with a/b = 1.02 overall resulted in a lower pulp yield and higher lignin yield compared to the same ionic liquid solution with a/b = 0.98 (see Fig. 2).

It was reported previously that operating at temperatures above the glass transition temperature of lignin ($T_g$ = 130–150 °C depending on the source of lignin) might improve the delignification of biomass.[15] For more severe pretreatments at 150 °C and 170 °C, an increase in pretreatment effectiveness was discovered and more material was removed from the biomass in a shorter time compared to pretreatment at 120 °C. The most effective pretreatment condition in terms of lignocellulose fractionation was 1 hour at 170 °C ($[N_{2220}][HSO_4]$ with a/b = 1.02) with a pulp recovery of 40%. The highest lignin yield (relative to the biomass input) was 24.7 wt% when willow was pretreated for 40 min at 170 °C

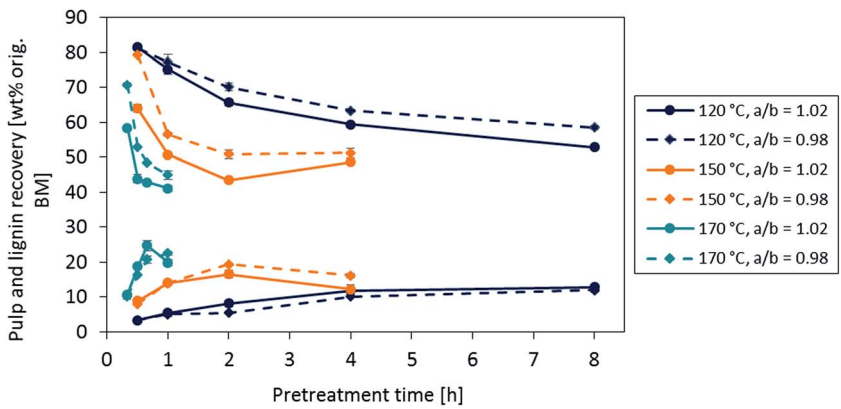


Fig. 2 Pulp and lignin recovery after pretreatment of willow with $[N_{2220}][HSO_4]$ with different acid/base ratios (a/b = 1.02 and 0.98).

 

([$N_{2220}$][$HSO_4$] with a/b = 1.02). This indicates that lignin depolymerisation continued after extraction in the solution phase at high temperatures, increasing the amount of small water-soluble lignin fragments and hence reducing the precipitated lignin yield.

A different trend for pulp and lignin recovery observed for pretreatment at elevated temperatures compared to pretreatment at 120 °C. Initially, an increase of material removal from the biomass was found, but for longer pretreatment times (*e.g.* 4 hours at 150 °C and 1 hour at 170 °C) over-treatment of the pulp was discovered. Signs of overtreatment include the increase in pulp recovery and decrease in lignin recovery, indicating that lignin re-deposition on the pulp has occurred. This means that increasing the temperature did not only accelerate the lignin extraction but could have also increased the formation of pseudo-lignin on the pulp,[16] especially for extended pretreatment times (see section on compositional analysis for detailed discussion). At elevated temperatures, the effect of the acidity on the pretreatment performance was very prominent, with the more acidic ionic liquid solution removing more material from the biomass matrix.

The severity of the pretreatment in terms of acidity seemed to have an interesting effect on the lignin yield, as milder pretreatment conditions (*e.g.* all pretreatments at 120 °C and short pretreatment times at 150 °C and 170 °C) using [$N_{2220}$][$HSO_4$] with a/b = 1.02 consistently gave a higher lignin recovery, but after 2 hours and 4 hours at 150 °C the lignin yield was found to be lower compared to using [$N_{2220}$][$HSO_4$] with a/b = 0.98. This suggests that the lignin in the solution with a/b = 1.02 was more prone to condensation and re-deposition onto the pulp (rather than precipitating during the lignin isolation step) or that the lignin fragments were more water-soluble and not able to precipitate. To verify either hypothesis, compositional analysis of the pulp was performed as well as the chemical characterisation of the isolated lignin, which will be discussed later on in this publication.

### Effect of [$N_{2220}$][$HSO_4$] treatment on the pulp composition

Compositional analysis is a good tool for understanding the changes in biomass composition that occur during pretreatment, such as delignification and hemicellulose removal or glucan degradation. The glucan, hemicellulose and lignin contents of untreated willow and pretreated pulps are shown in Fig. 3. The composition of native willow Endurance was as follows: 42.8% glucan, 18.5% hemicellulose and 27.3% lignin (plus an additional 10.1% of extractives).

It was found that pretreatment of willow with [$N_{2220}$][$HSO_4$] removed lignin and hemicellulose from the biomass matrix, leaving a cellulose-rich pulp. The effectiveness of the pretreatment and the composition of the pulp were strongly dependent on the acid/base ratio of the ionic liquid. In general, using [$N_{2220}$][$HSO_4$] with a/b = 1.02 was better at removing lignin and hemicellulose from the biopolymer matrix (see Fig. 3). However, the composition of the pulp was also greatly influenced by the pretreatment temperature and time.

Pretreatment at 120 °C only partially extracted the lignin from the biomass. The highest delignification and hemicellulose removal were found for the 8 h pretreatment, which led to 44.9% and 56.6% lignin removal and 54.0% and 72.3% hemicellulose removal, using ionic liquid solutions with a/b = 0.98 and a/b = 1.02, respectively. Heteronuclear single quantum correlation (HSQC) NMR

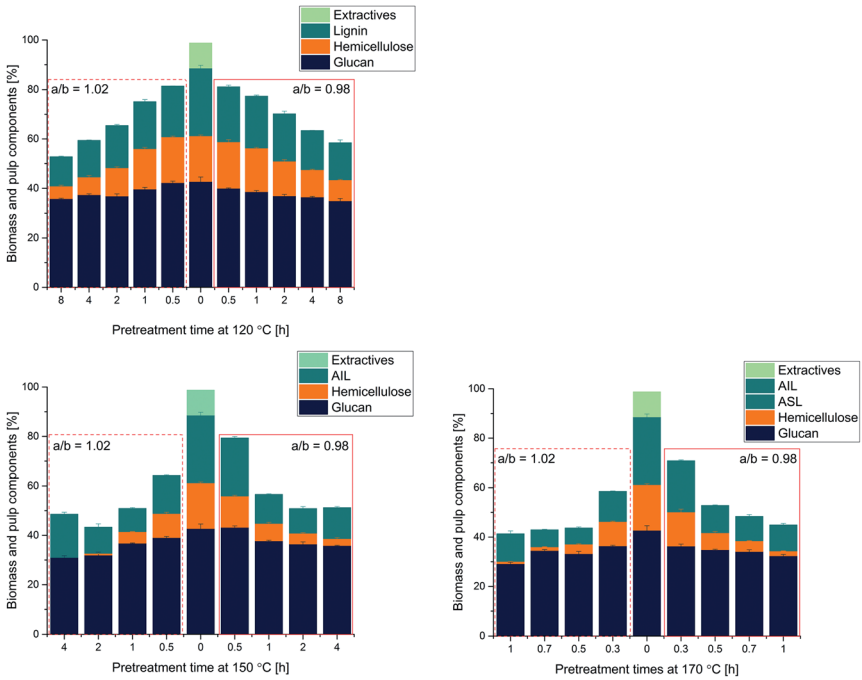


Fig. 3 Glucan, hemicellulose and lignin contents of untreated willow and pulp after pretreatment with $[N_{2220}][HSO_4]$ with two different acid/base ratios a/b = 1.02 (left hand side of graph) and a/b = 0.98 (right hand side of graph).

spectra of the isolated lignin show no carbohydrate cross-peaks, proving that hemicellulose remains in the ionic liquid liquor after pretreatment (see ESI† for HSQC spectra).

The mild pretreatment temperature (120 °C) was not the most effective in terms of delignification and hemicellulose removal but beneficial for preserving a high percentage of the glucan in the pulp, with more than 80% of the glucan remaining in the pulp after 8 hours of pretreatment, for both ionic liquid solutions used. It was also found that for mild pretreatment conditions (0.5 h, 1 h at 120 °C, 0.5 h at 150 °C and 20 min at 170 °C) around 10% of the original glucan was already extracted from the biomass, due to hydrolysis of amorphous cellulose and/or hemicellulose during the pretreatment.

Applying a more severe pretreatment by increasing the temperature to 150 °C significantly changed the composition of the pulp. The influence of the acid/base ratio on the pretreatment effectiveness and thus on the content of the pulp was marked. After only 30 min of treatment, 47.2% and 31.6% of hemicellulose were removed as well as 44.0% and 14.3% of the lignin, for $[N_{2220}][HSO_4]$ with a/b = 1.02 and a/b = 0.98, respectively. However, this significant difference in the delignification of the pulp was not reflected in the saccharification yields, with 55.9% and 44.1% glucose yields (for $[N_{2220}][HSO_4]$ with a/b = 1.02 and a/b = 0.98, respectively), which are similar. This discrepancy could be explained by the amount of glucan preserved in the pulp, namely only 91.4% for the more acidic

ionic liquid compared to 99.2% for the less acidic ionic liquid (compared to the original amount of glucan).

Increasing the pretreatment time at 150 °C from 30 minutes up to 2 hours showed that the more acidic ionic liquid was better at delignifying the biomass and removing the hemicellulose. After treatment for 2 hours, 96.0% of hemicellulose and 57.8% of the lignin were removed from the pulp when $[N_{2220}][HSO_4]$ with a/b = 1.02 was used, whereas treatment with $[N_{2220}][HSO_4]$ with a/b = 0.98 extracted only 76.2% of the hemicellulose but 63.6% of the lignin. The larger amount of acid not only accelerated the lignin and hemicellulose extraction, but also sped up the formation of pseudo-lignin on the pulp. This was especially obvious for pretreatment for 4 hours at 150 °C, where the formation of pseudo-lignin was observed for pulp pretreated with the more acidic ionic liquid, whereas no formation of pseudo-lignin on the pulp was found for the ionic liquid with a lower a/b.

When comparing the delignification for the 2 hours pretreatment to the 1 hour pretreatment, it can be seen that the delignification decreased when $[N_{2220}][HSO_4]$ with a/b = 1.02 was used but increased when $[N_{2220}][HSO_4]$ with a/b = 0.98 was used, indicating that significant condensation of lignin inside the biomass matrix or lignin or humin re-deposition occurred under more acidic pretreatment conditions, with the glucan content in the pulp decreasing to levels as low as 73.1% of the original glucan in the biomass when using the more acidic ionic liquid solution compared to 83.9% for the less acidic ionic liquid solution.

The pulp isolated after pretreatment for 4 hours at 150 °C clearly showed signs of overtreatment and formation of pseudo-lignin (a higher lignin content compared to untreated biomass or pulp pretreated under milder conditions). These effects were more pronounced for the pulp pretreated with the more acidic ionic liquid, where all of the hemicellulose and 27.4% of the glucan were removed, and the delignification decreased even further from 57.8% (150 °C, 2 h) to 35.8%. The less acidic ionic liquid caused less glucan degradation and pseudo-lignin formation, only removing 16.8% of the glucan and a decrease of delignification from 63.6% (150 °C, 2 h) to 54.0%.

Pretreatment at 170 °C accelerated the lignin and hemicellulose extraction and yielded a pulp high in cellulose content after only 30 minutes (using $[N_{2220}][HSO_4]$ with a/b = 1.02). This pretreatment condition exhibited the highest delignification success, with only 23.9% of the lignin left in the pulp. Interestingly, this did not correlate with the largest amount of hemicellulose removal (found to be 96.0% after pretreatment for 2 hours at 150 °C), since only 79.1% was dissolved in the ionic liquid. This shows that, at higher temperatures, the hemicellulose and lignin extractions were not as concerted as seen for pretreatment at 120 °C but lignin removal was faster. The remaining hemicellulose may also have been hydrolysed directly from the pulp.

Elongating the pretreatment time at 170 °C to 1 hour affected the composition of the pulp significantly, which appeared to consist only of glucan, lignin/pseudo-lignin and a negligible amount of hemicellulose. However, glucan degradation was very prominent under these conditions with a glucan loss of 25% into the ionic liquid solution.

### Enzymatic hydrolysis of the pulp and untreated biomass

Enzymatic hydrolysis of the pulp was performed in order to assess whether the pretreatment was successful in increasing glucose release compared to untreated biomass. It is generally believed that the lignin in the biomass/pulp hinders the cellulase enzymes from accessing the cellulose[5] and thus high delignification of the biomass is considered a key aspect of a successful fractionation pretreatment.[2a] The glucose yields after 7 days of enzymatic hydrolysis of the pulp are shown in Fig. 4, demonstrating the influence of the ionic liquid solution acidity and the deconstruction time on the glucose release.

In general, the enzymatic hydrolysis of willow Endurance resulted in a high glucose release even without a pretreatment step. The ionic liquid pretreatment of willow improved the enzymatic digestibility for certain conditions compared to the untreated biomass (see Fig. 4), but this was not universally so. Unsurprisingly, the glucose release was highly dependent on the pretreatment severity applied.

For very mild pretreatments, *e.g.* 0.5, 1 and 2 hours at 120 °C (for both a/b ratios), 0.5 hours at 150 °C (for a/b = 0.98) and 0.3 hours at 170 °C (for a/b = 0.98), the glucose yield was found to be lower than the yield from the raw biomass (46.4%).

This was also previously observed for the mild pretreatment of *Miscanthus giganteus*[12a] and might be due to acid catalysed hydrolysis of parts of the amorphous cellulose followed by dissolution into the ionic liquid. The amorphous part of cellulose is generally hydrolysed faster under acidic conditions than the crystalline part,[17] thus decreasing the glucan recovery for pulp treated with a low severity. The compositional analysis of the pulp revealed a glucan loss of around 10% even for very mild pretreatment conditions, supporting the above statement.

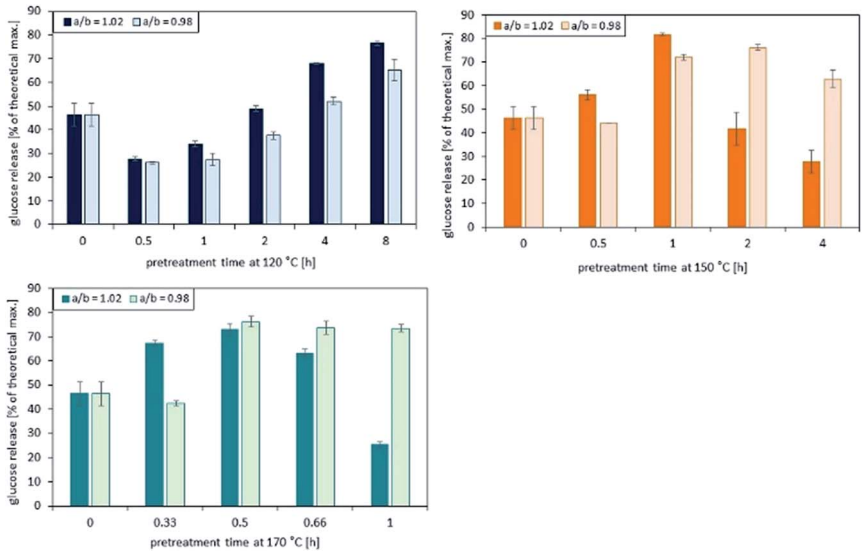


Fig. 4 Glucose yield after enzymatic hydrolysis of the pulp and raw biomass after pretreatment using $[N_{2220}][HSO_4]$ with two different acid/base ratios at 120 °C (top right), 150 °C (top left) and 170 °C (bottom left). BM = biomass.

It was found that the glucose yield after saccharification of the pulp pretreated with the more acidic solution at 120 °C was always higher compared to $[N_{2220}][HSO_4]$ with a/b = 0.98, with the highest yield being 76.5% for a pretreatment time of 8 hours. These results are in contrast to earlier findings reported by P. Verdía *et al.*[18] for the pretreatment of *Miscanthus giganteus* at 120 °C with $[C_4C_1im][HSO_4]$. In this study, the less acidic ionic liquid solution gave slightly higher glucose yields (90% and 84% for a/b = 0.99 and 1.01, respectively) after 96 hours of enzymatic hydrolysis. The contrary conclusions support the hypothesis that hardwoods are more recalcitrant towards pretreatment and harsher conditions are required for the successful break-up of the biopolymer matrix. The higher glucose yields for willow pretreated with $[N_{2220}][HSO_4]$ (a/b = 1.02) can be ascribed to the fact that the ionic liquid with an excess of sulfuric acid yields a purer pulp by removing more lignin and hemicellulose from the pulp and thus increasing the accessibility of the enzymes to the cellulose.[19]

Additionally, lignin acts as a non-active binding side for cellulases,[5] which decreases the efficiency of the enzymatic hydrolysis and explains why a pulp with higher delignification gave higher glucose yields.

The best pretreatment conditions found (in terms of highest glucose yield) were 1 hour at 150 °C with $[N_{2220}][HSO_4]$ with an a/b of 1.02, which resulted in the release of 81.8% of the glucose that was contained in the untreated willow. This shows that the ionic liquid solution $[N_{2220}][HSO_4]$ can be used to successfully pretreat the hardwood willow to improve glucose yields.

However, using very severe pretreatment conditions, such as long pretreatment times at 150 °C and 170 °C, led to overtreatment of the pulp, which dramatically decreased the enzymatic digestibility. Interestingly, using $[N_{2220}][HSO_4]$ with an a/b of 0.98 produced high glucose yields even under harsh pretreatment conditions (2 and 4 hours at 150 °C as well as 1 hour at 170 °C) compared to the same ionic liquid solution with an a/b of 1.02. For example, at 150 °C and 2 hours, a glucose yield of 76.2% (a/b = 0.98) *vs.* 41.9% (a/b = 1.02) was achieved. At 150 °C and 4 hours, the glucose yield was found to be 62.9% (a/b = 0.98) *vs.* 27.8% (a/b = 1.02).

The most significant effect of the acid/base ratio was observed for pretreatment for 1 hour at 170 °C, where the glucose yield was 73.5% (for a/b = 0.98) compared to 25.3% (for a/b = 1.02). This result is in good agreement with the glucan content of the pulps, showing that the less acidic ionic liquid reduces the propensity for severe glucan degradation.

## Lignin structure

**Lignin linkages in the aliphatic region.** It has been shown that isolating lignin from the biomass matrix using acidic conditions, such as in the dilute acid pretreatment[20] or ionoSolv pretreatment, results in the cleavage of aryl ether C–O bonds and decreases the lignin molecular weight.[21] Previous work investigating changes in the structure of *Miscanthus gigantheus* lignin published by A. Brandt *et al.*[21] showed that pretreatment with the acidic ionic liquid 3-butylimidazolium hydrogen sulfate $[HC_4im][HSO_4]$ had a major impact.

Heteronuclear single quantum correlation (HSQC) NMR spectroscopy is a powerful two-dimensional NMR technique that resolves resonances that overlap in one-dimensional spectra and is used for semi-quantitative analysis of lignin

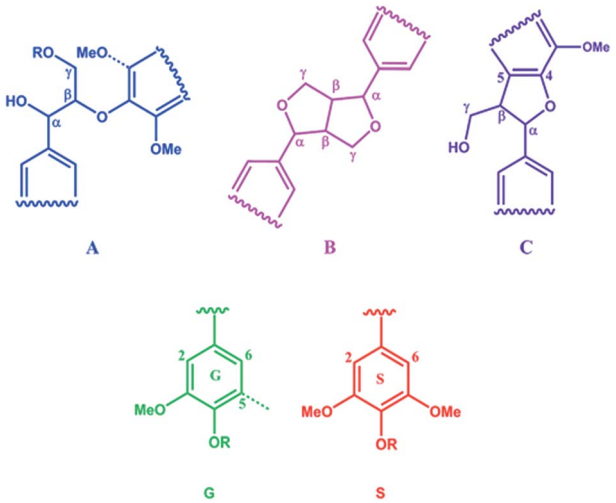


Fig. 5 Typical structural motifs found in hardwood lignin: β-*O*-4 alkyl-aryl ether (A), β-β (resinol) (B), phenylcoumaran (C), guaiacyl unit (G) and syringyl unit (S).

linkages and subunit composition. A selection of common linkages and subunits of hardwoods is shown in Fig. 5.

Several structural units of the complex lignin polymer can be detected by HSQC NMR spectroscopy, which have previously been assigned by comparison with cell wall model compounds in an NMR database.[22] Examples of common subunits and linkages that are resolved by HSQC NMR spectroscopy are β-ether (β-*O*-4, Aα at 4.87/71.8 ppm), β-β (resinol, Bα at 4.62/85.0 ppm) and phenylcoumaran (β-5, Cα at 5.45/86.9 ppm). The aromatic rings of the G ($G_2$ at 6.92/110.2 ppm, $G_5$ at 6.78/114.9 ppm and $G_6$ at 6.75/118.4 ppm) and S units ($S_{2,6}$ at 6.64/103.6 ppm) are also resolved. For calculation of the amount of inter-unit linkages, the α protons of β-*O*-4, β-β and phenylcoumaran were used.

HSQC NMR analysis of the lignin isolated from willow using $[N_{2220}][HSO_4]$ showed that the lignin structure (in terms of lignin linkages) was surprisingly similar for both ionic liquid solutions under mild (all time points at 120 °C and 30 min at 150 °C) or very harsh (1 hour at 170 °C) pretreatment conditions. The acidity of the ionic liquid solution seemed to have little impact on the amount of linkages present in the recovered lignin (see Fig. 6). The most prominent linkage in lignin isolated at 120 °C was the β-*O*-4 aryl ether linkage, as also described earlier by Prado *et al.*[23] It was found that the amount of the β-*O*-4 linkage in the isolated lignin strongly depended on the pretreatment severity applied. The largest amount of β-*O*-4 bonds was found for pretreatment at 120 °C for 30 min (0.66 bonds per $C_9$ unit for both ionic liquid solutions).

With longer pretreatment times, more aryl ether linkages had been broken and the amount of β-*O*-4 bonds dropped to 0.30 and 0.39 bonds (a/b = 1.02 *vs.* a/b = 0.98) per $C_9$ subunit after 8 hours. The lignin isolated at elevated temperatures contained a smaller amount of β-*O*-4 linkages compared to lignin isolated at 120 °C (*e.g.* at 30 min, 0.35–0.41 β-*O*-4 bonds per $C_9$ unit for lignin isolated after 150 °C and 170 °C pretreatments compared to 0.66 β-*O*-4 bonds for lignin isolated

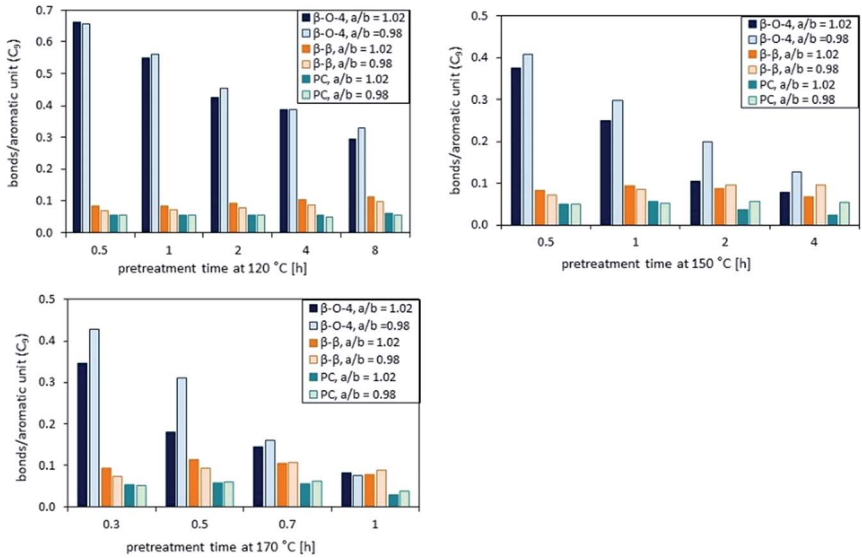


Fig. 6 Typical linkages (β-*O*-4 ether linkage, β-β (resinol) linkage and phenylcoumaran (PC) linkage) in lignin isolated after pretreatment at different temperatures with $[N_{2220}][HSO_4]$ with different acid/base ratios (a/b = 1.02 and 0.98). Top left: 120 °C, top right: 150 °C and bottom left: 170 °C.

after 120 °C pretreatment), meaning that more bonds were hydrolysed at higher pretreatment temperatures.

However, the same trend for the β-*O*-4 bond cleavage was found for lignin isolated at elevated pretreatment temperatures. Interestingly, the more acidic ionic liquid was more effective at breaking the ether bond than the less acidic solution, the phenomenon being more pronounced for pretreatments with higher severity, such as 150 °C and 170 °C. Lignin recovered after pretreatment at 150 °C for 2 hours showed a significant difference in its structure with twice as many β-*O*-4 bonds per $C_9$ (0.20 *vs.* 0.10) when the less acidic ionic liquid was used. A similar ratio was found for lignin isolated after 30 minutes at 170 °C, with 0.18 β-*O*-4 bonds per $C_9$ (a/b = 1.02) compared to 0.31 β-*O*-4 bonds per $C_9$ (for a/b = 0.98).

The abundance of β-β and phenylcoumaran linkages was less than 0.10 per $C_9$ unit for all the lignins, and the number of these linkages was largely unaffected by the pretreatment severity, with only a minor decrease in the number of β-β and phenylcoumaran linkages for very severe pretreatment conditions.

**Lignin subunits in the aromatic region.** The HSQC pulse sequence employed in this study is optimised for resolution and signal strength. It is important to note that this method is not a completely quantitative one, as signal relaxation following each pulse will not be complete for some correlations, especially for end groups such as PCA, which relax more slowly than the bulk.[24] Another limitation of integrating HSQC NMR spectra is the potential for the condensation reactions,[21] which replace aromatic C–H bonds with C–C bonds, particularly at the $G_6$ position. These C–C bonded aromatic positions do not produce cross correlations in HSQC spectra, meaning that the calculated subunit composition may be misrepresented when examining condensed lignins. With these restrictions in

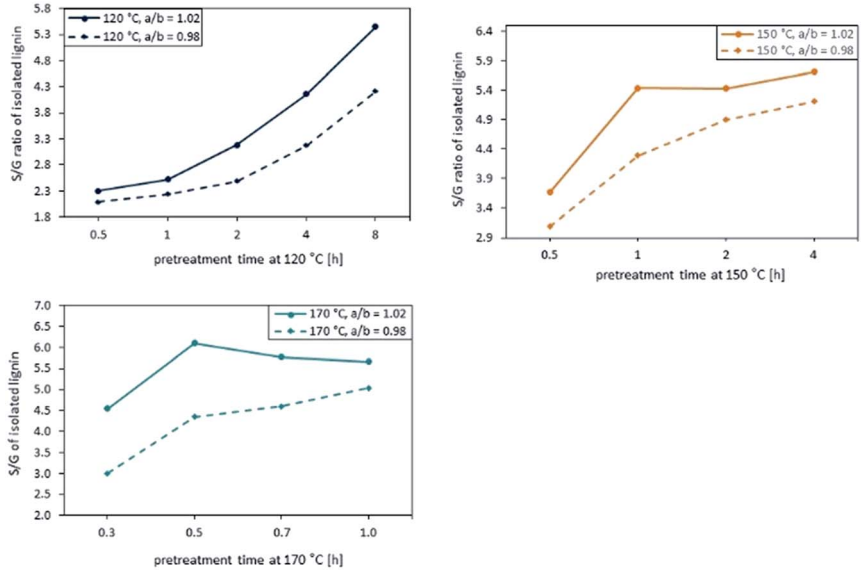


Fig. 7 S/G ratio of lignin isolated after pretreatment at different temperatures with $[N_{2220}][HSO_4]$ with different acid/base ratios (a/b = 1.02 and 0.98). Top left: 120 °C, top right: 150 °C and bottom left: 170 °C.

mind, volume integration was attempted for estimating the subunit composition (see Fig. 7). It should be noted that the $G_5$ and $G_6$ peaks cannot be relied on for quantification of the guaiacyl content because of pronounced participation in condensation reactions. Due to these reasons, the $G_2$ signal as well as the $S_{2,6}$ signal were selected to calculate the S/G ratio.

It was found that the S/G ratio of lignin extracted during pretreatment at 120 °C continually increased from 2.3 to 5.4 (for a/b = 1.02) and from 2.1 to 4.2 (for a/b = 0.98) with longer pretreatment times (see Fig. 7). This is in agreement with the S/G ratios found for another hardwood (poplar) after hydrothermal pretreatment with different severities.[25] As mentioned earlier, the acidity of the ionic liquid solution did not affect the composition of the lignin in terms of linkages at this temperature, but it did affect the S/G ratio. Lignin isolated with the more acidic ionic liquid solution showed a higher S/G ratio, meaning that a greater amount of S subunits was extracted from the cell wall compared to the lignin isolated with the ionic liquid with a/b = 0.98.

The S/G ratio for the lignin extracted after pretreatment at 150 °C was strongly influenced by the acidity of the ionic liquid solution. The subunit ratio for lignin isolated with the more acidic ionic liquid solution initially significantly increased from 3.7 to 5.4 (for the pretreatment times of 0.5 h and 1 h, respectively) and then reached a plateau around 5.5 for longer pretreatment times. This indicates that the pretreatment for 1 hour at 150 °C (with a/b = 1.02) extracted all the available S-lignin from the cell walls and longer pretreatment times did not change the subunit composition. Interestingly, a different trend was observed for pretreatment with the less acidic ionic liquid, where the S/G ratio gradually increased from 3.1 to 5.2, suggesting that the lower acidity of this ionic liquid solution affected the kinetics of the extraction of S-units.

Increasing the pretreatment severity by increasing the temperature to 170 °C showed the same trend for the S/G ratio as that observed for milder pretreatment conditions (when the less acidic ionic liquid was used). The subunit composition of the lignin isolated for pretreatment at 170 °C first increased from 4.5 to 6.1, reaching the highest S/G ratio observed for the lignins isolated for all the conditions studied. Interestingly, the very harsh pretreatment conditions (high temperature and high acidity of the ionic liquid solution) led to a decrease in the S/G ratio from 6.1 (for 30 min) to 5.8 (for 40 min) and 5.7 (for 1 h) of the isolated lignin for pretreatment times of more than 30 minutes. This implies that the extracted lignin got enriched in G-units over time. The data presented here might indicate that S-rich lignin may be extracted under milder reaction conditions, due to it being less cross-linked and thus more easily extracted.[26] Very harsh pretreatment conditions, such as 40 minutes and 1 hour at 170 °C using the IL solution with higher acidity, also extracted the G-rich part of the lignin and thus led to a decrease in the S/G ratio.

**Molecular weight of the isolated lignin.** The extracted lignin was investigated *via* GPC analysis in order to understand the effect of the pretreatment severity on the molecular weight ($M_w$) of the lignin. A summary of the molecular weights of the lignin using different pretreatment conditions is shown in Fig. 8 and Table 2.

The average molecular weight of the lignin changed dramatically with the pretreatment conditions applied. It was found that not only did the pretreatment time and temperature have an influence on the molecular weight, but also the acid/base ratio of the ionic liquid solution.

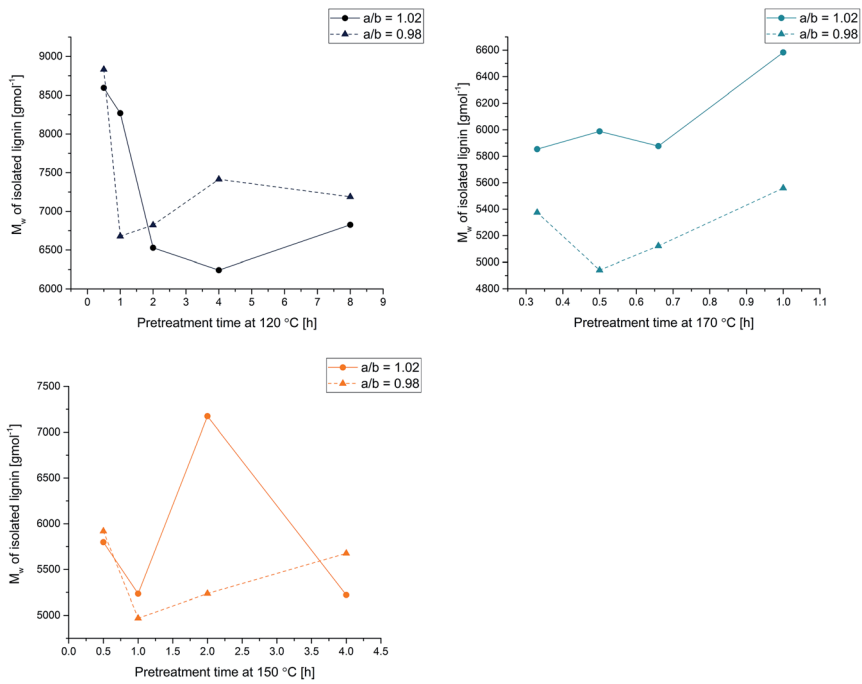


Fig. 8 Molecular weight ($M_w$) of the lignin isolated after pretreatment with $[N_{2220}][HSO_4]$ with different acid/base ratios.

Table 2 Polydispersity index of lignin isolated after pretreatment using $[N_{2220}][HSO_4]$ with a/b = 1.02 and a/b = 0.98

| Entry | Temperature [°C] | Time [h] | PDI (a/b = 1.02) | PDI (a/b = 0.98) |
|---|---|---|---|---|
| 1 | 120 | 0.5 | 7.9 | 7.6 |
| 2 | 120 | 1 | 7.5 | 5.5 |
| 3 | 120 | 2 | 5.8 | 5.0 |
| 4 | 120 | 4 | 5.8 | 5.4 |
| 5 | 120 | 8 | 5.9 | 5.7 |
| 6 | 150 | 0.5 | 5.7 | 4.9 |
| 7 | 150 | 1 | 5.6 | 4.6 |
| 8 | 150 | 2 | 6.9 | 5.2 |
| 9 | 150 | 4 | 5.8 | 5.9 |
| 10 | 170 | 0.3 | 4.9 | 4.6 |
| 11 | 170 | 0.5 | 5.7 | 5.0 |
| 12 | 170 | 0.7 | 5.8 | 5.3 |
| 13 | 170 | 1 | 7.0 | 5.6 |

The $M_w$ of lignin isolated after pretreatment at 120 °C decreased with increasing pretreatment time from *ca.* 8500 g mol$^{-1}$ for a pretreatment time of 30 min to *ca.* 6200 g mol$^{-1}$ for a pretreatment time of 2 hours. This was caused by lignin depolymerisation through cleavage of β-*O*-4 bonds, as discussed in the previous section, which is likely catalysed by the acidic conditions in the ionic liquid solution.[21,23] However, no significant change in molecular weight was found after 2 hours of pretreatment and a plateau at around 6200 g mol$^{-1}$ was reached, indicating that for longer pretreatment times at 120 °C both depolymerisation reactions and cross-linking of the lignin were equally favoured. In addition, the influence of the acidity of the ionic liquid solution appeared of little importance, which is in contrast to its effect on pulp yields, saccharification yields and the S/G ratio of the isolated lignin.

For pretreatment at higher temperatures, a different trend for the molecular weight was observed. At 150 °C and using the less acidic ionic liquid, the molecular weight first decreased from *ca.* 6000 g mol$^{-1}$ after 30 min to *ca.* 5000 g mol$^{-1}$ for lignin isolated after 1 hour, clearly indicating depolymerisation of the lignin. However, a slight increase in molecular weight to 5200 g mol$^{-1}$ and 5700 g mol$^{-1}$ was observed for longer pretreatment times (2 and 4 hours, respectively), indicating lignin condensation reactions were favoured, as suggested earlier by Brandt *et al.*[21] The acidity of the ionic liquid solution greatly impacted the molecular weight of the lignin isolated at this temperature. Using $[N_{2220}][HSO_4]$ with a/b = 1.02 for a pretreatment time of 30 minutes and 1 hour initially led to a decrease in $M_w$ of the isolated lignin from *ca.* 5800 g mol$^{-1}$ to 5200 g mol$^{-1}$, respectively, showing that depolymerisation also occurred. However, this was followed by a significant increase in $M_w$ to *ca.* 7200 g mol$^{-1}$ (for a pretreatment time of 2 hours), showing that lignin cross-condensation had occurred, as seen for the pretreatment at the same temperature with the less acidic ionic liquid solution. However, the molecular weight of lignin isolated after pretreatment with a/b = 1.02 increased by almost 1.5 times compared to only a slight increase for a/b = 0.98, indicating that the greater acidity of this ionic liquid solution supported the cross-condensation of the lignin. Interestingly, the conditions applied for the

pretreatment for 4 hours led to a decrease in lignin molecular weight to *ca.* 5200 g $mol^{-1}$, which might indicate that under these conditions cleavage reactions of the lignin were favoured over cross-condensation due to the high severity of this treatment. This is supported by the HSQC NMR data showing that the amount of β-β and phenylcoumaran bonds decreased for lignin isolated under these conditions compared to shorter pretreatment times at the same temperature.

The general trend for the molecular weight of the lignin extracted after pretreatment at 170 °C showed an increase in $M_w$ over time for both ionic liquid solutions used, indicating that the occurrence of lignin cross-condensation was favoured over lignin depolymerisation at this pretreatment temperature. However, the lignin isolated after pretreatment with the ionic liquid solution with a/b = 0.98 had a smaller molecular weight compared to the lignin extracted with $[N_{2220}][HSO_4]$ with a/b = 1.02 at 170 °C, showing that the lower acidity conditions extracted shorter lignin chains. The molecular weight increased from *ca.* 5400 g $mol^{-1}$ and *ca.* 5900 g $mol^{-1}$ (for a/b = 0.98 and 1.02, respectively) for lignin isolated after 30 min of pretreatment to *ca.* 5600 g $mol^{-1}$ and 6600 g $mol^{-1}$ (for a/b = 0.98 and 1.02, respectively) after 1 hour of pretreatment.

# Experimental

## Materials

Willow chips (variety: Endurance, Plot: CS689) were supplied by Rothamsted Research and the material was air-dried, ground and sieved (180–850 μm, −20 to +80 of US mesh scale) before use at Imperial College London. Triethylamine was purchased from Sigma Aldrich with a purity of >99%, 5 mM sulfuric acid and absolute ethanol were purchased from VWR. All commercial reagents and solvents were used as received unless otherwise stated.

## Synthesis and characterisation of $[N_{2220}][HSO_4]$ with different acid/base ratios

Triethylamine (126.49 g, 1.25 mol) was cooled in an ice bath and sulfuric acid (256 mL, 5 mol $L^{-1}$, 1.28 mol for a/b = 1.02 or 246 mL, 5 mol $L^{-1}$, 1.23 mol for a/b = 0.98) was added drop-wise over 1 hour. The reaction mixture was stirred for 3 hours at 0 °C. Residual water was removed under vacuum from the colourless liquid until a water content of 20 wt% was achieved. The pH and density of the synthesised ionic liquid/water mixtures (containing 20 wt% water) were determined using a Jenway 3510 pH meter and a Metler Toledo DM40 density meter at room temperature. All measurements were performed in triplicates and the average pH and density values are given.

## Ionic liquid pretreatment and biopolymer isolation

The biomass was pretreated following the ionoSolv protocol developed by Brandt *et al.*[12a] In brief, 2.00 g (on an oven-dried weight basis) of ground willow were weighed into a 15 mL Ace pressure tube with a screw-cap and Teflon lining, followed by the addition of 10.00 g of $[N_{2220}][HSO_4]_{80\%}$. The samples were incubated without stirring in an oven at different temperatures (120 °C, 150 °C and 170 °C) for various lengths of time. After pretreatment, the samples were cooled at room temperature and washed four times with absolute ethanol. The pulp was left to air-dry for two days and the ethanol was removed under reduced pressure and

lignin was precipitated using water as the anti-solvent. The lignin was washed three times with water and dried in a vacuum oven at 40 °C over night.

### Enzymatic hydrolysis of untreated biomass and pulp

The enzymatic saccharification of raw willow and pulp was performed according to the protocol "Enzymatic Saccharification of Lignocellulosic Biomass" (NREL/TP-510-42629) published by NREL[27] (National Renewable Energy Laboratory). In brief, 100 mg of untreated or pretreated biomass (oven-dried weight) were mixed with an enzyme master mix consisting of sodium citrate buffer (at pH 4.8), water, antibiotics and a commercial enzyme mixture (*Trichoderma reesei* ATCC 26921 and Cellobiase from *Aspergillus niger*) received from Novozyme. The mixture was incubated for 7 days at 50 °C under shaking at 250 rpm. The samples were analysed for monomeric sugars (glucose, xylose, arabinose, mannose and galactose) using a Shimadzu HPLC with a refractive index detector equipped with an Aminex HPX-87P column (Biorad). The mobile phase was DI water (18.8 mΩ), the flow rate was 0.6 mL $min^{-1}$, the column oven was set to 85 °C and the acquisition time was 20 min. Monomeric sugar yields were calculated based on the glucose content of the untreated biomass.

### Compositional analysis of untreated willow and pulp

For untreated biomass, determination of extractives was included in the protocol, whereas this step was not performed for pretreated pulp due to the assumption that all extractives will be dissolved in the ionic liquid during pretreatment. For determination of the composition of biomass/pulp, the protocols "Preparation of samples for compositional analysis" (NREL/TP-510-42620), "Determination of Structural Carbohydrates and Lignin in Biomass" (NREL/TP-510-42618) and "Extractives in biomass" (NREL/TP-510-42619) published by NREL (National Renewable Energy Laboratory)[27] were followed and all experiments were performed in triplicates. In brief, 300 mg (oven dried weight) of extracted biomass or pulp were used per experiment and analysed for monomeric sugars (glucose, xylose, arabinose, mannose and galactose) using a Shimadzu HPLC with a refractive index detector equipped with an Aminex HPX-87P column (Biorad). The mobile phase was DI water (18.8 mΩ), the flow rate was 0.6 mL $min^{-1}$, the column oven was set to 85 °C and the acquisition time was 20 min.

### NMR spectroscopy

For the NMR measurements, 20–23 mg of air dried lignin were dissolved overnight in 250 μL DMSO-d6, then transferred to a Shigemi NMR microtube. Heteronuclear single quantum correlation (HSQC) measurements were carried out on a Bruker Avance 600 MHz NMR machine at 25 °C. The acquired spectra were analysed using MestReNova and referenced to the solvent peak at 2.50/39.5 ppm ($\delta_H/\delta_C$). The S/G ratio was calculated using the signals for uncondensed and condensed S and G subunits.

### Gel permeation chromatography

For the determination of the molecular weight ($M_w$) and polydispersity (PDI) of the isolated lignin, 20 mg of lignin were dissolved in DMF/LiBr (LiBr

concentration = 1 mg $mL^{-1}$) and filtered using a 0.22 μm syringe filter and plastic syringe. The measurements were performed on an Agilent Technologies 1260 Infinity GPC instrument.

# Conclusions

The study presented here shows that the protic ionic liquid $[N_{2220}][HSO_4]$ can be used to successfully extract lignin and hemicellulose from the hardwood willow. The amount of lignin and hemicellulose extracted were controlled by the pretreatment conditions (time, temperature and acidity of the ionic liquid) and high delignification and hemicellulose removal were achieved for pretreatments at 150 °C and 170 °C.

The pretreatment increased the digestibility of the cellulose-containing pulp during enzymatic hydrolysis. The highest glucose yield of 81.8% was found after pretreatment at 150 °C for 1 hour. However, overtreatment of the pulp (evidenced by the formation and re-deposition of pseudo-lignin on the pulp) was observed for very severe pretreatment conditions, resulting in low saccharification yields. We showed that the pretreatment severity did not only influence the pulp composition and digestibility but also the structure of the extracted lignin and its molecular weight. Lignin isolated after a mild pretreatment contained a large amount of β-*O*-4 ether linkages and a high molecular weight. More severe pretreatments changed the structure of the isolated lignin significantly, with most of the β-*O*-4 ether linkages being cleaved. Depolymerisation and re-condensation (for even more severe pretreatments) took place within the lignin, which led to a decrease in the molecular weight, followed by an increase. The S/G ratio of the isolated lignin was always higher when the more acidic IL solution was used for pretreatment and generally increased over time. An exception was observed at the highest pretreatment temperature (170 °C) using the IL with excess acid, when the S/G ratio decreased slightly at elongated treatment times (30 min or more).

A major factor that influences the price of glucose produced from lignocellulose is the cost of the pretreatment solvent, *i.e.* the ionic liquid solution used. The use of ionic liquids has hence to be weighed against the increase in glucose released from the perspective of the total carbon footprint and process costs before a full justification can be made. The cost of glucose and the environmental impact of the pretreatment can be lowered by near-quantitative recycling of the ionic liquid and reusing it for several pretreatment cycles. Further investigations into this are currently under investigation in our group.

This study demonstrated that ionoSolv pretreatment of willow has the potential to tune the properties of both fractionation products (pulp and lignin) to achieve optimised production of chemicals, materials and biofuels from willow. However, scale-up experiments are required, which are being performed in our group, to explore if the findings reported here also apply for larger scales.

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## Notes and references

1 (*a*) A. J. Ragauskas, C. K. Williams, B. H. Davison, G. Britovsek, J. Cairney, C. A. Eckert, W. J. Frederick, J. P. Hallett, D. J. Leak, C. L. Liotta, J. R. Mielenz, R. Murphy, R. Templer and T. Tschaplinski, *Science*, 2006, **311**, 484; (*b*) J. H. Clark, F. E. I. Deswarte and T. J. Farmer, *Biofuels, Bioprod. Biorefin.*, 2009, **3**, 72; (*c*) J. Pickett, *Sustainable Biofuels: Prospects and Challenge*, The Royal Society, 2008.

2 (*a*) A. Brandt, J. Gräsvik, J. P. Hallett and T. Welton, *Green Chem.*, 2013, **15**, 550; (*b*) R. D. Perlack, L. L. Wright, A. F. Turhollow, R. L. Graham, B. J. Stokes and D. C. Erbach, *Biomass as a Feedstock for a Bioenergy and Bioproducts Industry: the Technical Feasibility of a Billion-ton Annual Supply*, U.S. Department of Energy and U.S. Department of Agriculture, 2005.

3 (*a*) D. V. Evtuguin, C. Pascoal Neto, J. Rocha and J. D. Pedrosa de Jesus, *Appl. Catal., A*, 1998, **167**, 123; (*b*) X. Zhao, L. Zhang and D. Liu, *Biofuels, Bioprod. Biorefin.*, 2012, **6**, 465.

4 F. S. Chakar and A. J. Ragauskas, *Ind. Crops Prod.*, 2004, **20**, 131.

5 W. Boerjan, J. Ralph and M. Baucher, *Annu. Rev. Plant Biol.*, 2003, **54**, 519.

6 M. Li, Y. Pu and A. J. Ragauskas, *Front. Chem.*, 2016, **4**, 45.

7 T. Dutta, J. Shi, J. Sun, X. Zhang, G. Cheng, B. A. Simmons and S. Singh, Ionic Liquid Pretreatment of Lignocellulosic Biomass for Biofuels and Chemicals, in *Ionic Liquids in the Biorefinery Concept: Challenges and Perspectives*, 2016, pp. 65–94.

8 D. Klein-Marcuschamer, B. A. Simmons and H. W. Blanch, *Biofuels, Bioprod. Biorefin.*, 2011, **5**, 562.

9 (*a*) L. Chen, M. Sharifzadeh, N. Mac Dowell, T. Welton, N. Shah and J. P. Hallett, *Green Chem.*, 2014, **16**, 3098; (*b*) A. George, *et al.*, *Green Chem.*, 2015, **17**, 1728.

10 G. F. De Gregorio, C. C. Weber, J. Gräsvik, T. Welton, A. Brandt and J. P. Hallett, *Green Chem.*, 2016, **18**, 5456.

11 A. Brandt, M. J. Ray, T. Q. To, D. J. Leak, R. J. Murphy and T. Welton, *Green Chem.*, 2011, **13**, 2489.

12 (*a*) F. J. V. Gschwend, A. Brandt, C. L. Chambon, W.-C. Tu, L. Weigand and J. P. Hallett, *J. Visualized Exp.*, 2016, **114**, e54246, DOI: 10.3791/54246; (*b*) A. Brandt, J. Hallett and T. Welton, Ammonium cation ionic liquids for biomass fractionation, WO 2014/140643, 2013.

13 M. S. Y. Jennings, *New Characterization Techniques for Acidic Ionic Liquids*, Bachelor Thesis at Imperial College London, 2014.

14 A. M. Orozco, A. H. Al-Muhtaseb, A. B. Albadarin, D. Rooney, G. M. Walker and M. N. M. Ahmad, *RSC Adv.*, 2011, **1**, 839.

15 (*a*) N. J. Nagle, R. T. Elander, M. M. Newman, B. T. Rohrback, R. O. Ruiz and R. W. Torget, *Biotechnol. Prog.*, 2002, **18**, 734; (*b*) E. Kiran and H. Balkan, *J. Supercrit. Fluids*, 1994, **7**, 75.

16 P. Sannigrahi, D. H. Kim, S. Jung and A. J. Ragauskas, *Energy Environ. Sci.*, 2011, **4**, 1306.

17 (*a*) Y. H. P. Zhang and L. R. Lynd, *Biotechnol. Bioeng.*, 2004, **88**, 797; (*b*) S. D. Mansfield, C. Mooney and J. N. Saddler, *Biotechnol. Prog.*, 1999, **15**, 804.

18 P. Verdía, A. Brandt, J. P. Hallett, M. J. Ray and T. Welton, *Green Chem.*, 2014, **16**, 1617.

 

19 (*a*) X. Meng, M. Foston, J. Leisen, J. DeMartini, C. E. Wyman and A. J. Ragauskas, *Bioresour. Technol.*, 2013, **144**, 467; (*b*) T. Jeoh, C. I. Ishizawa, M. F. Davis, M. E. Himmel, W. S. Adney and D. K. Johnson, *Biotechnol. Bioeng.*, 2007, **98**, 112.
20 M. G. S. Chua and M. Wayman, *Can. J. Chem.*, 1979, **57**, 1141.
21 A. Brandt, L. Chen, B. E. van Dongen, T. Welton and J. P. Hallett, *Green Chem.*, 2015, **17**, 5019.
22 S. Ralph, J. Ralph, L. Landucci and L. Landucci, *NMR database of lignin and cell wall model compounds*, US Forest Prod. Lab, Madison, WI, 2004, http://ars.usda.gov/Services/docs.html.
23 R. Prado, X. Erdocia, G. F. DeGregorio, J. Labidi and T. Welton, *ACS Sustainable Chem. Eng.*, 2016, **4**, 5277.
24 S. D. Mansfield, H. Kim, F. Lu and J. Ralph, *Nat. Protoc.*, 2012, **7**, 1579.
25 S. Sun, Y. Huang, R. Sun and M. Tu, *Green Chem.*, 2016, **18**, 4276.
26 (*a*) A. Lourenço, J. Rencoret, C. Chemetova, J. Gominho, A. Gutiérrez, J. C. del Río and H. Pereira, *Front. Plant Sci.*, 2016, **7**, 1612; (*b*) J. L. Ferrer, M. B. Austin, C. Stewart Junior and J. P. Noel, *Plant Physiol. Biochem.*, 2008, **46**, 356.
27 Downloaded from: http://www.nrel.gov/bioenergy/biomass-compositional-analysis.html.